‘VALIDATED ANALYTICAL METHOD DEVELOPMENT FOR THE QUANTIFICATION OF MULTICOMPONENT DRUGS WITH SPECIAL EMPHASIS ON HERBAL AND SYNTHETIC DRUG FORMULATIONS’

THESIS

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The Tamil Nadu Dr. M. G. R. Medical University,

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In partial fulfillment of the requirement for the award of the degree of

DOCTOR OF PHILOSOPHY

(FACULTY OF PHARMACY)

Submitted By

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DECLARATION

I hereby declare that the thesis entitled **'Validated Analytical Method Development for the Quantification of Multicomponent Drugs with Special Emphasis on Herbal and Synthetic Drug Formulations'** submitted to The Tamilnadu Dr. M.G.R Medical University, Chennai, in partial fulfillment of the requirements for the award of Degree of Doctor of Philosophy in Pharmacy, is the result of my original and independent research work carried out at Department of Pharmaceutical Chemistry, Ultra College of Pharmacy, Madurai during the period from October 2010 to June 2014 under the supervision of the valuable and efficient guidance of **Dr. K. G. Lalitha, M. Pharm., Ph.D.,** Professor and Head, Department of Pharmaceutical Chemistry, Ultra College of Pharmacy, Madurai. The thesis or any part thereof has not formed the basis for the award of any degree, diploma, associateship, fellowship, or any other similar title, of this or any other University, previously.

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CERTIFICATE

This is to certify that the thesis entitled **'Validated Analytical Method Development for the Quantification of Multicomponent Drugs with Special Emphasis on Herbal and Synthetic Drug Formulations'** is the research work done and submitted in partial fulfillment of the requirement for the award of degree of **Doctor of Philosophy in Pharmacy** of the Tamilnadu Dr. M.G.R Medical University, Chennai. The work was done by **Mr. Nitin Sitaram Jadhav** in the Department of Pharmaceutical Chemistry, Ultra College of Pharmacy, Madurai under my guidance and supervision.

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Thanks a lot

Nitin Jadhav

LIST OF ABBREVIATIONS AND UNITS

RP-HPLC	-	Reverse Phase High Performance Liquid Chromatography
NP-HPLC	-	Normal Phase High Performance Liquid Chromatography
HPTLC	-	High Performance Thin Liquid Chromatography
TLC	-	Thin Layer Chromatography
GC	-	Gas Chromatography
CC	-	Column Chromatography
PC	-	Paper Chromatography
GLC	-	Gas Liquid Chromatography
GSC	-	Gas Solid Chromatography
LC	-	Liquid Chromatography
LLC	-	Liquid-Liquid Chromatography
LSC	-	Liquid-Solid Chromatography
UV	-	Ultra-Violet
IR	-	Infra-Red Spectroscopy
NMR	-	Nuclear Magnetic Resonance
AAS	-	Atomic Absorption Spectroscopy
AES	-	Atomic Emission Spectroscopy
API	-	Active Pharmaceutical reagent
λmax	-	Maximum wavelength
E (1%, 1cm)	-	Absorptivity value of 1% conc in 1cmpathlength
conc	-	Concentration
cm	-	centimeter
µg	-	Micro-gram
µl	-	Micro-liter
µm	-	Micro-meter
nm	-	Nanometer
ml	-	Mili-liter
mm	-	Mili-meter
Fig.	-	Figure
No.	-	Number
ΔA	-	Difference absorbance

cGMP	-	Current Good Manufacturing Practices
FDA	-	Food and Drug Administration
DP	-	Drug Product
C_8	-	Octyl
C_{18}	-	Octadecyl
ODS	-	Octadecysilane
PEI	-	Polyethylamine
v/v	-	Volumn by volumn
R_f	-	Retention Factor/ Retardation Factor
ICH	-	International conference on harminization
pKa	-	Dissociation constant
A^o	-	Angstrom
Rs	-	Resolution
°C	-	Degree centigrade
Sr.No.	-	Serial number
silica gel 60	-	Silica gel with 60 pore size in Ao
silica gel 60F	-	Fluorescent indicator Silica gel with 60 pore size in Ao
F_{254}/F_{366}	-	Excitation wavelength of fluorescence indicator
USP	-	United states of pharmacopoeia
N	-	Theoretical Plates
T	-	Tailing factor
k’	-	Capacity factor
LOD	-	Limit of Detection
LOQ	-	Limit of Quantification
ppm	-	Parts per million
v/w	-	Volumn by weight
DS	-	Drug Substance
DP	-	Drug Products
min	-	Minute
h/hrs	-	hour
LOS	-	Losartan Potassium
CTD	-	Chlorthalidone
ATOV	-	Atovaquone

PROG	-	Proguanil
ACST	-	Acetylcysteine
ACBF	-	Acebrofylline
THF	-	Tetrahydrofuran
ng	-	Nanogram
Hcl	-	Hydrochloric acid
NaoH	-	Sodium hydroxide
NMT	-	Not more than
NLT	-	Not less than
L-dopa	-	Levodopamine
PPD	-	Paraphenylenediamine

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1. INTRODUCTION

The quality is important in every product or service but it is more vital in medicine as it involves life. As a matter of fact, quality is built in from the time of inception of the thought to make a product, to the time it is finally made and sent out. The assurance of the quality together with their careful control are our moral obligations arising from the humanism towards the seek human beings. Analytical chemistry is mainly concerned with determining the qualitative and quantitative composition of material under study. Analytical monitoring of pharmaceutical products, or of specific ingredients within the product, is necessary to ensure its safety and efficacy throughout all phases of its shelf life, including storage, distribution, and use[1,2].

Analytical chemistry, or the art of recognizing different substances and determining their constituents, occupies a prominent position among the applications of science[3]. The development in analytical sciences has been more significant and prominent in recent years than the past and has broadened our vistas to develop new methods of analysis. Pharmaceutical Analysis is the branch of Pharmacy, which is responsible for developing sensitive, reliable and more accurate methods for the estimation of drugs in pharmaceutical dosage forms and in biological system[4,1]. Drug analysis involves identification, characterization and determination of drugs in dosage forms and biological fluids and which play an important role in the different steps of development, manufacture and therapeutic use of drug[5].

Analytical chemistry is basically concerned in the determining the qualitative and quantitative composition of materials under study. The qualitative analysis gives us the information about the presence or absence of various components while quantitative analysis deals with the amount of contents presents in the sample.[6] Modern Pharmaceutical analysis encompasses much more than the analysis of active pharmaceutical ingredients (API), inert ingredients (excipients), or formulated drug product (DP) [7].

1.1 Multicomponent drug formulations

Nowadays, the pharmaceutical dosage forms of combinational drugs are very much useful in multiple therapies rather than the use of single drug formulations because of

- Multiple actions
- Synergistic effect/additive effect
- Economy in productions, distribution and use
- Fewer side effects
- Quicker relief
- Smaller doses of individual drugs avoided
- Easy for storage and carrying
- Convenient for the patients to take single multicomponent tablet instead of two or three different tablets for multitherapies.

Thus, the manufacturing trends have been changed to manufacture more and more complex formulation containing several drugs with very similar chemical behavior. It is a quite easy way for medical practioner to write the prescriptions as well as to give instructions to the patients and simultaneously it is very convenient for the patient to take the medicine without missing their doses. To device an accurate estimation procedure for each ingredient of such multicomponent dosage form containing several therapeutically active drugs is not an easy task, as they are present in widely divergent concentrations and contains additives. The presence of additives, excipients and decomposition products further complicates the development of analytical procedures[8].

1.2 Modern Analytical Methods

Development of the modern analytical methods for the determination of multocomponent drugs in different dosage forms is very essential because of the various reasons like[9]

- New drug combinations are not official in Pharmacopoeias.
- Problems with the interferences of other drug as well as excipients.
- No simple, accurate and precise analytical methods are available for quantifications.
- Limited availability of literature for combination of drugs

- Available methods are quite tedious, costly and time consuming methods for drug separations and their extractions.

Modern analytical methods are generally classified into instrumental and non-instrumental category. By using instrument, measurement of some physical property is made to determine the contents or composition of a substance. It involves chemical colour reactions, solubility, and physical constants like refractive index, melting point, optical rotation, and instrumental techniques like IR, NMR, UV, AAS, AES, and Mass spectroscopy. While in non-instrumental methods, the conventional physicochemical properties are used to analyze the sample. The non-instrumental methods which are primarily based upon the measurement of the mass and volume includes the techniques of volumetric and gravimetric analysis.

The instrumental methods are based upon the measurement of some physical property of substance to determine its chemical composition and structures. Physical properties of substance may be specific or non specific but by converting their signals into units which are used for determination of structure and the concentrations.

Instrumental methods are mostly employed for the estimation of the drug in their formulations includes Spectroscopic method like UV-visible, Fluorimetry, Flame photometry, NMR, IR which are used to measure the electromagnetic radiations which is either absorbed or emitted by the sample as a function of drug concentrations utilized for qualitative and quantitative analysis.

Electro analytical methods include Potentiometry, Amperometry, Voltametry and Conductometry where measurement of current, voltage or resistance as a property of concentration of drugs. Chromatographic methods are widely used for the separation of the individual component from the mixture as well as estimation of drugs in their formulations. Chromatographic methods include gas chromatography (GC), high performance liquid chromatography (HPLC), and high performance thin layer chromatography (HPTLC) where components are separated by differential rate of migration due to adsorption and partition in a system consisting of two phases, one is moving phase and other is stationary phase[6].

Advantages of Instrumental methods

- A small quantity of sample is required
- Estimation of drug is fast
- Complex mixture can be analyzed
- Separation, qualification and quantifications are simultaneously possible in chromatography

1.2.1 Modern Analytical Spectroscopy

A spectrometric technique from the largest and most important single group of techniques used in analytical chemistry provides a wide range of quantitative and qualitative information.

All spectrometric techniques depend on the emission, scattered or absorption of electromagnetic radiation. This absorption, emission or scattered light radiation is associated with changes in the energy states of the interacting chemical species and since each species has characteristic energy states within an atomic or molecular system[9].

The set of energy levels associated with a particular substance is a unique characteristic of that substance and determines the frequencies at which electromagnetic radiation can be absorbed or emitted.

Qualitative information regarding the composition and structure of a sample is obtained through a study of the positions and relative intensities of spectral lines or bands.

Quantitative analysis is possible because of the direct proportionality between the intensity of a particular line or band and the number of atoms or molecules undergoing the transition. The various spectrometric techniques commonly used for analytical purposes and their principle and applications of important methods are given in **Table No. 1.0**

Table No 1.0: Principal and Applications of important Analytical spectrometric technique

Name of technique	Principle	Major applications
Flame photometry, plasma emission spectrometry,	Atomic emission	Qualitative and Quantitative determination of metals, largely as minor or trace constituents
X-ray Fluroscence spectrometry, atomic fluorescence spectrometry	Atomic fluorescence emission	Quantitative determination of metals at minor or trace constituents
Atomic absorption spectrometry	Atomic absorption	Quantitative determination of metals as minor or trace constituents
UV-Visible spectrometry, Infrared spectrometry	Molecular absorption	Quantitative determination of elements and compounds, mainly as trace and minor constituents Identification and structural analysis of organic compounds
NMR spectrometry	Nuclear absorption	Identification and structural analysis of organic compounds
Mass spectrometry	Structural fragmentation or ionization of atoms	Identification and structural analysis of organic compounds

Spectrophotometric Methods For Estimation Of Drugs[6,10]

UV-Visible Spectrophotometry is the absorption spectroscopy in which the organic molecules are analysed by their absorption characteristics of UV or visible radiation resulting in the electronic transition from singlet ground state to singlet excited state accompanied with vibrational and rotational transitions. This transition occurs when the energy of absorption is equal to the difference in the energy between the two states. Most commonly employed spectrophotometric technique includes ultraviolet, visible, infrared and atomic absorption spectrophotometry. There are various methods employed for the single and multicomponent analysis. The basis of single component analysis-

1) Single point or double point standardization method involves the measurement of absorbance of standard and samples are compared at λmax of compound in particular solvents.

2) A standard calibration curve is plotted using series of standard solutions as concentrations against absorbance and the concentration of the sample is determined from the graph.
3) Establishing absorptivity values E (1%, 1cm) for standard at selected wavelength (λmax) in particular solvents.

The basis of multicomponent samples analysis is the property that at all wavelengths is the absorbance of solution is the sum of absorbances of the individual components or the measured absorbance is the difference between the total absorbance of the solution in sample cell and that of the solution in the reference (blank) cell.

Multicomponent analysis is carried out by following methods

1. Simultaneous equation method or Vierodts method
2. Derivative spectrophotometric method
3. Difference spectrophotometric method
4. Geometric correction method
5. Absorbance ratio method
6. Orthogonal polynomial method
7. Absorbance correction method

Simultaneous equation method or Vierodts methods

If sample contains two absorbing drugs (x and y) each of this absorbs at the λmax of the other as shown in **Fig. No. 1.0.** It may be possible to determine both the drugs by the techniques of simultaneous equation methods provided that

- Two absorbing drugs (x and y), each of this absorbs at the λ max of the other
- The λ max of two drugs should be reasonably dissimilar.
- Two components should not interact chemically.

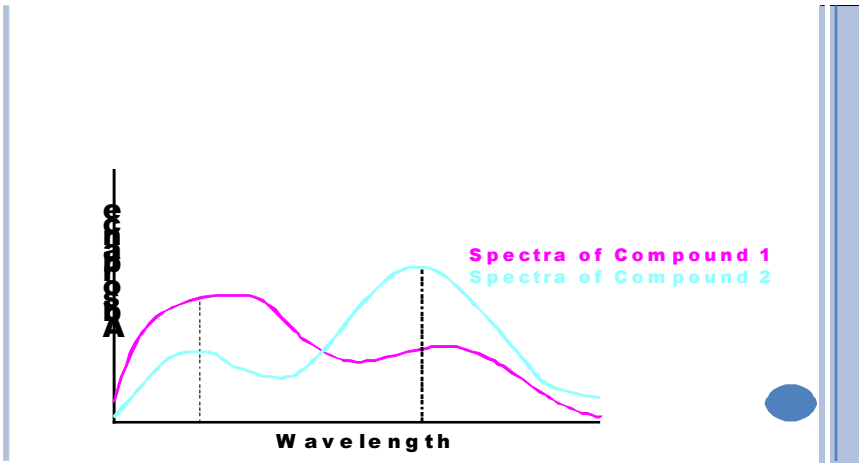


Fig. No.1.0: λmax absorption of two drugs (x and y)

The two equations are constructed based on the fact at λ1 and λ2 the absorbance of mixture is sum of individual absorbances x and y.

At λ_1 $A_1 = ax_1.BC_x + ay_1.bCy$ ---------(1)

At λ_2 $A_2 = ax_2.BCx + ay_2.bCy$ ---------(2)

Rearranging equation (1) and (2) gives,

$$C_x = \frac{A_2.ay_1 - A_1.ay_2}{ax_2.ay_1 - ax_1.ay_2}$$

and

$$C_y = \frac{A_1.ax_2 - A_2.ax_1}{ax_2.ay_1 - ax_1.ay_2}$$

where, C_x and C_y are concentrations of x and y respectively,

ax_1 and ax_2 are absorptivities of x at λ_1 and λ_2 respectively

ay_1 and ay_2 are absorptivities of y at λ_1 and λ_2 respectively

$A_1$and A_2 are absorbances of diluted mixture at λ_1 and λ_2 respectively.

Derivative spectrophotometric method

When the sample of drug shows large irrelevant absorption, then this method is very useful. This method involves conversion of normal spectrum to its first, second and higher derivative spectrum.

Derivative spectra can be used to clarify absorption bands in more complex UV spectra. The technique is used extensively in rapidly developing field of near infrared spectroscopy and can also be applied in the determination of the purity of chromatographic peaks where when they are monitored by diode array detection. The main effect of derivatisation is to remove underlying broad absorption bands where

there is only a gradual change in slope. The first derivative spectrum is obtained by plotting for instance the slopes of 2 nm segments of the spectrum, and this results as shown for a Gaussian band in the below **Fig.No.2.0.** In this spectrum the slope is zero at the maximum of the peak and the slope is maximum at approximately half the peak height. In the second derivative spectrum the slopes of adjacent 2 nm segments are compared and this gives the points of maximum curvature of the spectum. The rate of curvature of a spectrum has its greatest negative value at its maximum and the greatest rates of curvature are observed for narrow absorption bands.

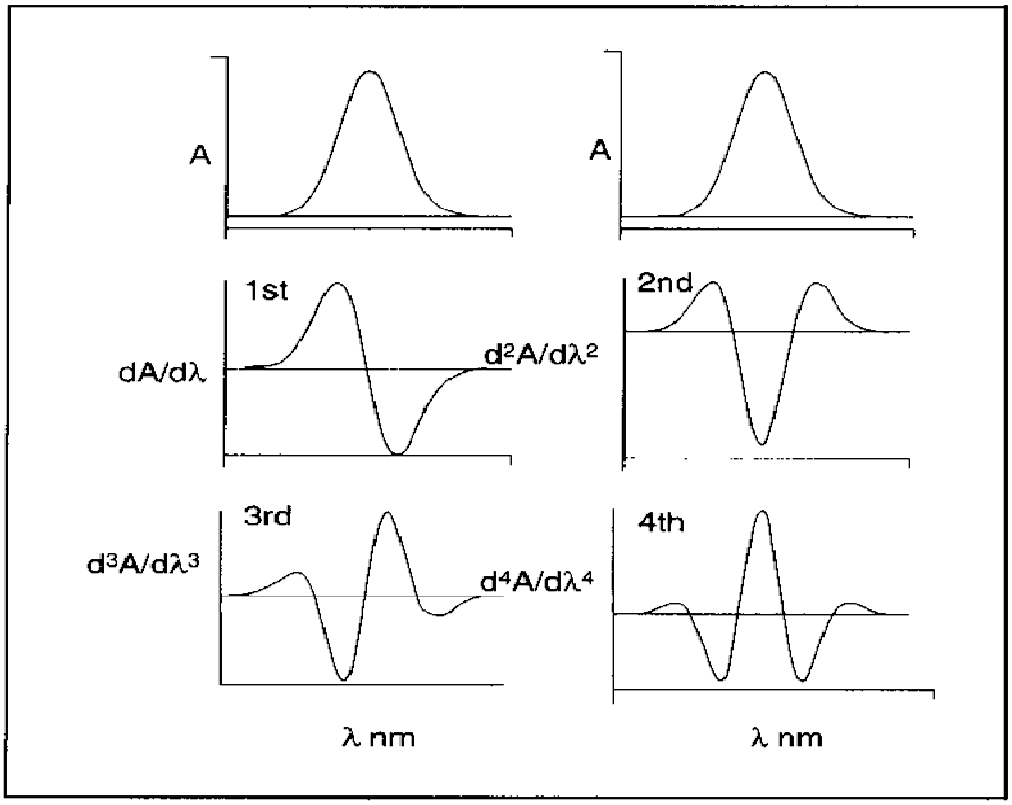


Fig.No. 2.0: First, second, third and fourth derivatives of a Gaussian band.

Difference spectrophotometric method

The essential feature of a difference spectrophotometric assay is that the measured value is the difference absorbance (ΔA) between two equimolar solutions of the analyte in different chemical forms which exhibit different spectral characteristics. The criteria for applying difference spectrophotometry to the assay of a substance in the presence of other absorbing substance are

- Reproducible changes may be induced in the spectrum of the analyte by the addition of one or more reagents.

- The absorbance of the interfering substances is not altered by the reagents.

Geometric correction method

A number of mathematical correction procedures have been developed which reduce or eliminate the background irrelevant absorption that may be present in the sample of biological origin. The simplest of these procedures is the three point's geometric procedure which may be applied if the irrelevant absorption is linear at the three wavelengths selected.

Absorbance ratio method

This method is a modification of the simultaneous equation method. It depends on the property that, for a substance that obeys the beers law at all wavelengths. The ratio of absorbance at any two wavelengths is a constant value independent of concentration or path length.

Orthogonal polynomial method

The technique of orthogonal polynomial method is another mathematical correction procedure which involves complex calculation. The basis of this method is that an absorption spectrum may be represented in terms of orthogonal function as follows:

$$A(\lambda) = P_0P_0 + P_1P_1(\lambda) + P_2P_2(\lambda) + \text{--------}P_nP_n(\lambda)$$

where 'A' denotes the absorbance at wavelength belonging to set of n+1 equally spaced wavelength at which the orthogonal polynomials $P_0(\lambda)$, $P_1(\lambda)$, $P_2(\lambda)$ --------- $P_n(\lambda)$ are each defined.

Absorbance correction method

It is also a modification of simultaneous equation method where quantitative determination of one drug carried out by E (1%, 1cm) value and quantification of other drug carried out by subtracting absorption due to interfering drug using absorption factor. This method includes following steps:

- Selection of wavelength
- Study of beers-lamberts law
- Study of additivity of substance
- Determination of absorptivity E (1%, 1cm) value
- Estimation of drug in laboratory mixture.
- Analysis of marketed formulation.
- Recovery studies.

1.2.2 Chromatography

Chromatography is a very specialized, yet widely applicable technique of separation science by which one can effectively separate chemically similar substances in complex mixtures. There are three distinct physical methods for attaining separation, yet all are characterized by their common application of a stationary and a mobile phase.

The stationary phase provides the chemical surface, which interacts with the components of the mixture to be separated. The difference in the chemistry of these components results in various degrees of molecular bonding or entrapment to the stationary phase. If the mixture is carried across a bed of stationary phase by some mobile phase, the molecules experiencing the least interaction with the stationary phase will tend to advance more quickly than those retained on the bed by stronger molecular interactions. This results in the separation of similar species as influenced by differences in migration rates across the stationary bed. The three distinct separation strategies are-

-**Frontal chromatography**- The mobile phase is the sample mixture, and as the sample migrates down the separation bed, the least retained species are concentrated near the leading edge.

-**Displacement chromatography**- A sample mixture is applied to one end of a stationary phase, and then a mobile phase is introduced. The mobile phase selectively binds to the stationary phase competing for the same binding sites as the sample. Separation is established as a relative function of the intermolecular interactions of the sample and mobile-phase species with the stationary phase.

-**Elution chromatography**- A sample mixture is applied to one end of a stationary phase, which may already contain mobile phase. The mobile phase does not interact significantly with the stationary phase and only carries the sample mixture through the stationary bed. The differences in selective binding of sample mixture components with the stationary phase retard migration of some species so that separation occurs. This is the most analytically useful form of chromatography due to its tremendous power of separation of very similar species[11].

Chromatographic methods can be categorized in two ways. The first classification is based upon the physical means by which the stationary and mobile

phase are brought into contact. In *column chromatography*, the stationary phase is held in a narrow tube through which the mobile phase is forced under pressure. It includes CC, GC, and HPLC. In *planner chromatography*, the stationary phase is supported on a flat plate or in the interstices of a paper; here, the mobile phase moves through the stationary phase by capillary action or under the influence of gravity. It includes TLC, HPTLC and PC. A more fundamental classification of chromatographic method is one based upon the types of mobile and stationary phases as shown in Table No. 2.0

MOBILE PHASE	STATIONARY PHASE
GAS Gas Chromatography (GC)	LIQUID Gas-Liquid Chromatography (GLC)
	SOLID Gas-Solid Chromatography (GSC)
LIQUID Liquid Chromatography (LC)	LIQUID Liquid-Liquid Chromatography (LLC)
	SOLID Liquid-Solid Chromatography (LSC)

Table No. 2.0: Fundamental Classification of chromatographic methods

1.2.3 High Performance Liquid Chromatography[12-13] (HPLC)

High performance liquid chromatography is also named as high pressure liquid chromatography since it provides varieties of features like-

1. High resolving power
2. Speedy separation
3. Continuous monitoring of the column effluent
4. Accurate quantitative measurement
5. Repetitive and reproducible analysis using the same column
6. Automation of the analytical procedure and data handling
7. Suitability for the nonvolatile species or thermally fragile ones
8. Highly sensitive due to availability of various detectors
9. Choice of mobile phases and wider stationary phases

The different types of liquid chromatography are classified based on the mechanism of separation involved. The various mechanism of separation includes adsorption, partition, ion exchange, ion pair, size exclusion and affinity. For solute having molecular weights greater than 10,000, exclusive chromatography and reversed phase partition chromatography are used. For the lower molecular weight ionic species ion- exchange chromatography is widely used. Small polar but non ionic species are best handled by partition method. Adsorption chromatography is often chosen for separating non-polar species, structural isomers, and compound classes such as aliphatic hydrocarbon from aliphatic alcohols.

Adsorption chromatography or Liquid Solid Chromatography (LSC)

Liquid solid chromatography is based on interaction between the solute and fixed active sites on finely divided solid adsorbents used as a stationary phase. The adsorbent generally packed in a column or spread over plate which provides high surface area e.g. Silica gel, Alumina, or charcoal also used but most commonly silica gel is used. Highly active adsorbents may give rise to irreversible solute adsorption so, may not be suitable, silica gel, which is slightly acidic may strongly retain basic compounds, whereas alumina non acid wash is basic and should not be used for the chromatography of base sensitive compounds. Adsorbents of varying partial size generally 5µm are commercially used. The role of the solvent in LSC is vital since a mobile phase molecule competes with solute molecules for polar adsorption sites. The stronger the interaction between the mobile phase and stationary phase, weaker solute adsorption, and vice versa. The classification of solvents according to their strength of adsorption is called an eluotropic series which may be used as a guide to find the optimum solvent strength for a particular separation. In general, the compounds best separated by LSC are those which are soluble in organic solvents and non ionic.

Partition chromatography or Liquid-liquid chromatography (LLC)

Liquid-liquid chromatography is similar in principle to solvent extraction which is based upon the distribution of solute molecules between two immiscible liquid phases according to their relative solubilities. The separating medium consists of a finely divided inert support (e.g. silica gel, kieselguhr) holding a fixed (stationary) liquid phase and separation is achieved by passing a mobile phase over the stationary phase. The stationary phase may be packed in column, a thin layer on glass, or a paper strip. A simple thumb rule operates for partition chromatography; like separate like or like dissolve like where Non polar materials dissolve in and are

separated by non polar phases, and polar materials requires stationary phases that are even molar polar. Hence it is convenient to divide LLC into two categories, based on the relative polarities of the stationary and mobile phases.

- Normal phase HPLC
- Reverse phase HPLC

Normal phase HPLC

In NP-HPLC the Polar stationary phase and non-polar mobile phase is used. In this technique, non-polar compounds travel faster and are eluted first. This is because of the lower affinity between the non-polar 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 take longer time to elute.

Reversed phase HPLC

RP-HPLC 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 non-polar hydrophobic packing with octyl or octadecyl 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 ODS or C18, C8, C4, etc., (in the order of increasing polarity of the stationary phase).

Ion exchange chromatography

Ion exchange chromatography which is often shortened to ion chromatography refers to modern and efficient methods of separating and determining ions based upon ion exchange resin. The ion exchange process are based upon exchange equilibrium between ions in solution and ions of like sign on the surface of an essentially insoluble, high molecular weight solid.

Natural ion exchangers, such as clays and zeolites, have been recognised and used for several decades. Synthetic ion exchange resins were first produced in the mid-1930s for water softening, water deionization, and solution purification. The most common active sites for cation-exchange resins are the sulfonic acid group-SO_3H, a strong acid, and the carboxylic acid group-COOH, a weak acid. Anionic exchanger contains tertiary amine groups-N (CH_3) OH^- or primary amine groups-NH_3OH, the former is strong base and latter is weak one.

Ion pair chromatography

Ion pair chromatography is used for the separation and determination of ionic compounds and this method can also substitute for ion exchange chromatography. The mobile phase in ion phase chromatography consist of an aqueous buffer containing an organic solvents such as methanol or acetonitrile and an ionic compound containing a counter ion of opposite charge to the analyte.

A counter ion is an ion that combines with the analyte ion to form an ion pair which is neutral species that is retained by a reversed-phase packing. Strong acidic and basic compounds may be separated by reversed phase mode by forming ion pairs (columbic 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. Most of the counter ions contain alkyl group to enhance retention of the resulting ion pair on the non popular stationary phase. Elution of the ion pairs is then accomplished with an aqueous solution of methanol or acetonitrile or other water soluble organic solvent.

Size-Exclusion chromatography

Size- exclusion chromatography, which has also been called gel permeation, or gel filtration chromatography, is a powerful technique that is particularly applicable to high molecular weight species. Packing for size-exclusion chromatography consists of small silica or polymer particle containing a network of uniform pores into which solute and solvent molecules can diffuse. While in the pores, molecules are effectively trapped and removed from the flow of the mobile phase. The average residence time in the pores depend upon the effective size of the analyte molecules. Molecules that are larger than the average pore size of the packing are excluded and thus suffer essentially no retention; such species are the first to be eluted.

Molecules having diameter that are significantly smaller than the pore can penetrate or permeate throughout the pore maze and are thus entrapped for the greatest time; these are last to be eluted. Between these two extremes are intermediate size molecules whose average penetration into the pores of the packing depends upon their diameters. Within this group fractionation occurs, which is directly related to molecular size and to some extent molecular shape.

Affinity chromatography

It is the most selective of the mechanisms used in chromatography mostly for the separation of biological molecules. Hence it also named as bioaffinity chromatography. It uses highly specific interaction between the immobile phase and solute molecules. These interactions are usually enzyme-substrate, enzyme inhibitor or antibody-antigen reactions, which are highly selective. For example, it is well known that antibodies are extremely specific in their reactions with antigen and can be used in bio-affinity chromatography where an antibody, immobilised on a stationary phase (by covalently binding to it), can react with one protein (antigen) from a mixture containing even several hundred similar protein, binding it to the column. Once the column has been washed to remove all other proteins, the desired substance can be eluted out of the column by changing the ionic strength of the eluent. The fact that certain organic molecules such as synthetic dyes (used in textile) as well as metal ions exhibit selective affinity for biological molecules has been exploited in the form of dye-ligand chromatography and immobilised metal ion chromatography in biotechnology.

1.2.4 High Performance thin layer chromatography[14-16] (HPTLC)

This is a sophisticated, advanced, versatile and automated version of the thin layer chromatography. It is the fastest growing technique for the analysis of drugs. Of many chromatographic methods presently available TLC/HPTLC provides the rapid analysis of plant drugs leading to semi quantitative or quantitative information on chief constituents of the formulation. It can provide fingerprinting for monitoring the identity and purity of drug and detection of adulteration or substitution.

HPTLC is a type of planar or flat bed chromatography which works on the principle of adsorption in which the components in a sample of complex mixture are separated, identified and quantitated based on their differential adsorption over the stationary phase. Components with greater affinity towards the stationary phase

travels slower and are thus retained and those with lesser affinity towards the stationary phase travels faster and are thus eluted earlier. Its main advantages are

- Disposable Pre-coated plates are available
- Minimum technical training required
- Minimum place for working
- Sample components are stored on plate, allowing repeated analysis
- Quantification at micro, nano and picto-gram levels even in complex formulations.
- Multiple samples can be run simultaneously under identical conditions
- Economic than HPLC technique.

Steps involved in HPTLC

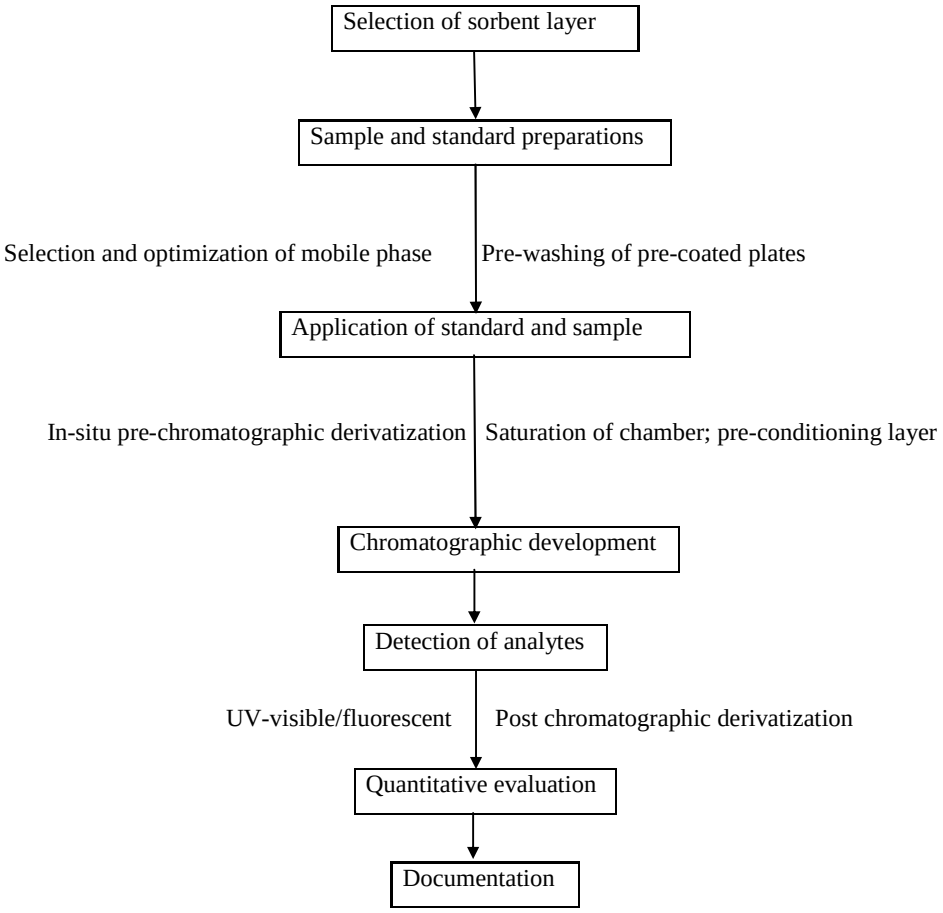


Fig. No 3.0: Steps involved in HPTLC

Selection of chromatographic layer

Sorbents: The most commonly used sorbents in pre-coated plates are silica gel 60, silica gel 60F, silica gel $60F_{254}/F_{366}$ aluminium oxide, high purity silica gel 60, cellulose, PEI impregnated cellulose, chemically modified silica gel including amino, cyano and diol derivative, etc. The pre-coated plates with sorbent thickness of 100-

250 μm are used for qualitative and quantitative analysis. For preparative TLC, sorbent thicknesses of 1-2mm are available in addition to chemical modified layers.

Plates: The plates may be a glass support, polyester (plastic) sheets (0.2mm thick) or aluminum sheet (0.1 mm thick). Pre-coated HPTLC plates in size of 20×20cm with aluminum or polyester support are usually procured mainly for economic reasons. These can be cut to size and shape to suit particular analysis.

Pre-washing of pre-coated plates

Sorbents with large surface area absorb water vapors and other volatile impurities from laboratories atmosphere leading to non-reproducible results thus necessitates pre-washing. Ascending, dipping and continuous mode are the common pre-washing methods. The plates are then dried for a sufficient time to completely remove the washing liquids.

Activation of pre-coated plates (layer pre-conditioning)

Plates exposed to high humidity may have to be activated in oven at 110-120°C for 30 minutes to remove adsorbed water. Over-activation should be avoided as it leads to sample decomposition.

Sample preparation

The sample preparation procedure is to dissolve the dosage form with complete recovery of intact compound(s) of interest and minimum of matrix with a suitable concentration of analyte (s) for direct application on the HPTLC plate. For normal phase chromatography using silica gel pre-coated plates, non-polar, volatile solvents are used for dissolving samples. In contrast, for RP-HPTLC, polar solvents are used. Steps for sample pre-treatment involve sampling, mechanical crushing, extraction, filtration and enrichment of minor compounds. If necessary, sufficient quantity of sample should be initially cleaned up and should be derivatized before chromatographic development (pre-chromatographic derivatization).

Application of sample

The sample should be completely transferred to the layer; however, the application process should never damage the layer, as damaged layer results in unevenly shaped spots. The samples should be applied in small increments with intermediate drying particularly when the sample solution is predominantly aqueous. Sample application volume for HPTLC varies from 0.5-5 μl keeping the size of starting zone(s) ranging from 0.5-1 mm in the concentration range of 0.1-1 μg/ml. The most commonly used applicators are

- Camag Nano-Applicator with micrometer controlled Hamilton syringe which delivers sample volumes in the range of 500-2000 µl.
- Camag Nanomat with a platinum-iridium capillary handles 0.2 µl.

Fig.No. 4.0: Camag Nanomat automatic apllicator

The small diameter of the sample spots applied in HPTLC allows for analysis of 20-30 sample in a single plate, thus reducing the development time of 20 minutes to 1 minute per sample. This certainly places HPTLC in a competitive position with HPLC in terms of speed of analysis.

Mobile phase optimisation

Mobile phase commonly called solvent system is traditionally selected by controlled process of trial and error. It should be chosen taking into consideration chemical properties of analytes and the sorbent layer. Use of mobile phase containing more than three or four components should normally be avoided as it is often difficult to get reproducible ratios of different components. Solvent composition is expressed by volumes (v/v) and usually sum of total volume is 100.

Chamber pre-conditioning (saturation)

The Chamber should be saturated before the chromatographic development to avoid the evaporation of the solvent mainly from the solvent front thus avoiding larger usage of solvents resulting in lower R_f values. Saturation time depends on the nature and composition of mobile phase and increases with increase in the layer thickness.

Chromatographic development and drying

Some of the commonly used development types and the respective chamber used are as follows:

Table No. 3.0: Types of HPTLC Chromatographic Development technique and Drying Chamber

Sr.No.	Types of Development	Chambers used
1.	- Ascending -Descending	Rectangular glass chamber
2.	Two-dimensional	Twin-trough chambers
3.	Horizontal	Horizontal development chamber
4.	Radial (circular)	Circular U chamber
5.	Anti-radial (anti-circular)	Anti-circular U chamber
6.	Multimodal (multi-dimensional	Automated multiple development chambers

The developed plate is then removed from the chamber and the mobile phase is removed completely in a fume cup-board to avoid contamination of laboratories atmosphere. The plates should always be laid horizontally so that the separated components will migrate evenly to the surface where they can be detected easily.

Detection and Visualisation

The zones can be located by various physical, chemical and biological-physiological methods. There is apparently no difficulty in detecting coloured substances or colourless substances absorbing in short wave UV region (254 nm) or with intrinsic fluorescence. Those which do not have the above properties should be derivatised as detectable substances by means of chromogenic or fluorogenic reagents (Post-chromatographic derivatisation). Detection under UV light is the first choice and is non-destructive in most of the cases and is commonly employed for densitometric scanning. The derivatising reagents are applied both by spraying or dipping technique. While resorting to in situ pre- or post-chromatographic derivatisation followed by quantitative analysis, it is absolutely essential to ensure that reaction on the plate is complete or atleast stoichiometric and reproducible.

Stabilisation of developed zones

The derivatised color or fluorescent chromatographic zones should be stable at least for 30 minutes for carrying out various steps involved in quantitative analysis.

Therefore, the chromatographs should be stabilized and intensified by adopting suitable conditions.

Quantitation (evaluation)

Quantitation basically involves two types of methods:

- The first method involves some type of measurement on the layer such as visual comparison, area measurement or in-situ densitometry on the layer.
- The second method involves removal of the analyte from the plate followed by quantitation.

Visual Comparison

This method can be used to determine if impurities are within a certain limit and to determine the number and approximate quantities of impurities that may be present. Since spots with low R_f values are too concentrated to be evaluated accurately and since the spots with high R_f values may be too diffuse to estimate accurately, visual comparison should be used for spots with R_f values between 0.3 and 0.7.

Area Measurement

The use of area measurement after separation of the spot reduces the errors in quantitation to 5-15%. Several methods for area measurements used are planimetry, tracing spots on writing paper followed by the traditional cutting and weighing approach and photographing the spots for cutting and weighing. The area can also be measured by counting squares occupied by the trace of the spot on millimetre graph paper and photocopying the spot prior to cutting and weighing. The quantity of material in the sample spot can be determined by interpolation after construction of a standard curve. The area of the spot is linearly related to the log of the sample weight.

In-Situ Densitometry

Densitometry is the in-situ instrumental measurement of visible, UV absorbance, fluorescence or fluorescence quenching directly on the layer without resorting to scrapping and elution. The measurements are usually made by reflection from the plate using single beam, double beam or single beam-dual wavelength operation of scanning instruments at the wavelength of maximum absorbance to achieve sensitivity. The purpose of the scanner is to convert the spot or band in the layer into chromatogram consisting of peaks whose position on the recorder chart are related to R_f values of the spots on the layer and peak height or area is related to the concentration of the substances on the spot. The signals which are measured represent

the absorption of transmitted or reflected light that passes through the spot compared to blank portion of the sorbent layer. The HPTLC densitometric analysis, three or four standard and purified samples are applied on the same plate. A calibration curve consisting of scan area of standard versus amount of analyte is constructed and amount of analyte in the sample represented by scan area is interpolated from the standard curve. Concentration of the analyte in the sample is calculated by considering the weight of the sample initially taken and dilution factors.

Scrapping and Elution

This involves the following steps:

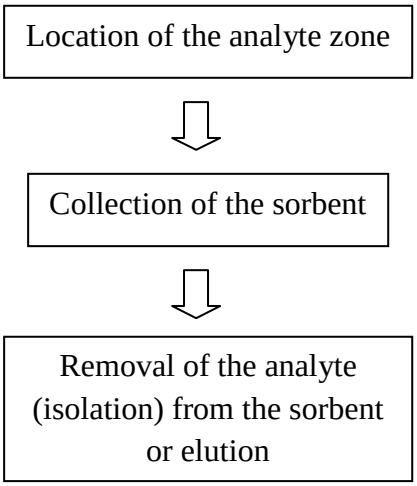


Fig. No 5.0: Steps involved in scrapping and elution

Location of the Analyte

The analyte zones are located by any of the detection methods like exposure to UV light or Iodine vapours or by the formation of coloured / fluorescent derivatives using corresponding derivatising reagents.

Collection of the Sorbent

Once the zone has been located, the sorbent can be removed quantitatively by the following procedures:

- Simple scrapping of the sorbent as a circular ring along with the analyte
- Drawing the sorbent through a glass tip of a vacuum device and depositing it on a glass frit / glass wool.

Removal of Analyte (Isolation) From the Sorbent (Elution)

The elution chamber is sealed over the analyte on the circular sorbent ring and the eluent is pumped into the chamber.

- The analyte is eluted using the solvent with high solvent strength for the analyte. If the analyte has high R_f value (~ 0.8), the mobile phase is used as the eluent.

1.3 Introduction to Herbal drug formulation[17-21]

Owing to the curiosity of present generation, ayurvedic and siddha systems of medicines have been regaining their importance over their synthetic counterpart owing to the expected adverse effects of the latter and the superiority of the former. So there is resurgence of use of herbs both as drugs and cosmetics[17]. In developed country, there is great demand for herbal products as a mainstream of medicine or as an alternative medicine. These products are increasingly being sought out as medicinal products, nutraceuticals and cosmetics. There are around 6000 herbal manufacturers in India. More than 4000 units are producing Ayurveda medicines[18-20].

Herbal formulations are not only therapeutically effective but also establish their part as exclusively important in improving the aesthetic sense of humans in the form of cosmetics[17]. Cosmetics have been enjoying renaissance among the youngsters throughout the world. Hence the quality control of a herbal formulation is a general requirement to be fulfilled if the formulation is to be released into the market.

In the past, approach to analysis was technically not as sound as it is today. Due to the introduction of new analytical techniques in this world of research and development, analysts have performed difficult tasks with ease. Modern methods of analytical techniques likes UV spectroscopy, HPLC and HPTLC are extremely sensitive, providing precise and detailed information from small samples of material. These are most rapidly applied and in general are readily amenable to automation. For these reasons, these are now in widespread use in product development, in the control of manufacture and formulation, as a check on the stability during storage, and in monitoring the use of drugs and medicines[21].

The current good manufacturing practices (cGMP), ICH and Food Drug Administration (FDA) guidelines insist for adoption of sound methods of analysis with greater sensitivity and reproducibility. Therefore, the complexity of problems encountered in pharmaceutical analysis with the importance of achieving the selectivity, speed, low cost, simplicity, precision and accuracy in estimation of drugs.

New methods are now being developed with a great deal of consideration to worldwide harmonization. As a result, new products can be assured to have comparable quality and can be brought to international markets faster.

1.4 Introduction to Analytical Method Development[22-24]

A good method development strategy should require only as many experimental runs as are necessary to achieve the desired final result. Finally method development should be as simple as possible and it should allow the use of sophisticated tools such as computer modeling. The important factors, which are to be taken into account to obtain reliable quantitative analysis, are:

- Careful sampling and sample preparation.
- Appropriate choice of the column.
- Choice of the operating conditions (system suitability) to obtain the adequate resolution of the mixture.
- Reliable performance of the recording and data handling systems.
- Suitable integration/peak height measurement technique.
- The mode of calculation best suited for the purpose.
- Validation of the developed method.

Steps in HPLC Method Development

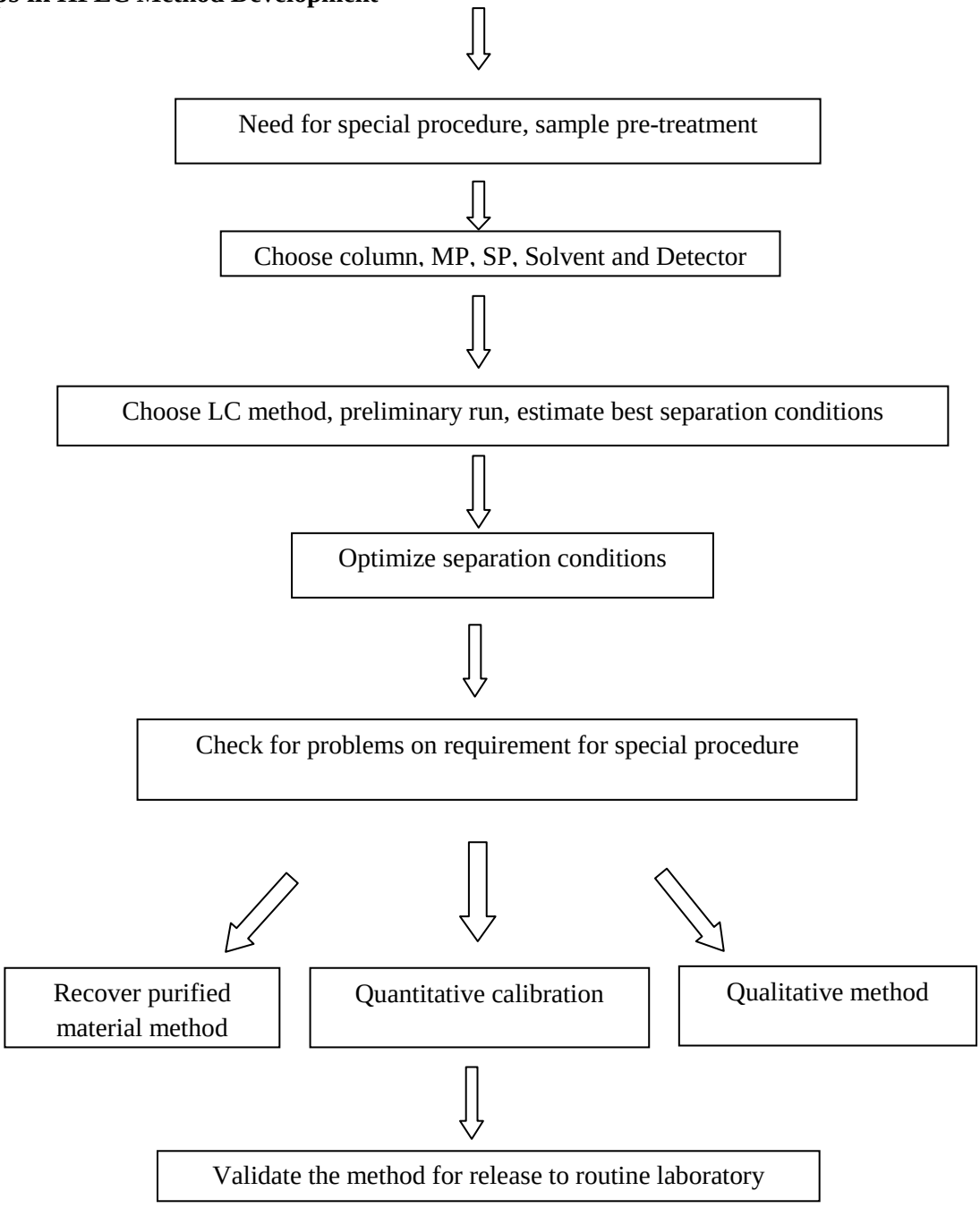


Fig. No.6.0: A Flow Chart of HPLC Method Development

Careful Sampling and Sample Preparation

Before beginning method development it is need to review what is known about the sample in order to define the goals of separation. The sample related information that is important is summarized in following.

The sample related summarized relation:

- Number of compounds present, chemical structures
- Molecular weights of compounds

- pK_a values of compounds, UV spectra of compounds
- Concentration range of compounds in samples of interest and Sample solubility

The chemical composition of the sample can provide valuable clues for the best choice of initial conditions for an HPLC separation. Generally sample comes in various forms,

- solutions ready for injections
- solution that require dilution, buffering, addition of internal standard
- solids that may be first dissolved and extracted
- sample that require sample pretreatment to remove interferences and or to protect column

Selection of the Column

The selection of the column in HPLC is depends upon the different modes like **adsorption** and **partition** and also the separation mechanism is based on **inductive forces, dipole-dipole interactions** and **hydrogen bond formation**. In case of ion-exchange chromatography, the separation is based on the differences in the charge, size of the ions generated by the sample molecules and the nature of ionisable group on the stationary phase. In the case of size-exclusion chromatography the selection of the column is based on the molecular weight and size of the sample components.

Selection of Operating Conditions to Obtain the Adequate Resolution Of the mixture:

Most of the drugs come under the category of regular samples. Regular samples mean typical mixtures of small molecules (<2000Da) that can be separated using more or less standardized starting conditions. Regular samples can be further classified as neutral or ionic. Samples classified as ionic include acids, bases, amphoteric compounds and organic salts. If the sample is neutral buffers or additives are generally not required in the mobile phase. **Acids or bases** usually require the addition of a buffer to the mobile phase. For basic or cationic samples, less acidic reverse phase columns are recommended. Based on recommendations of the conditions, the first exploratory run is carried and then improved systematically. On the basis of the initial exploratory run isocratic or gradient elution can be selected as most suitable. If typical reverse-phase conditions provided inadequate sample retention it suggests the use of either ion-pair or normal phase HPLC. Alternatively the sample may be strongly retained with 100% acetonitrile as mobile phase

suggesting the use of non-aqueous reverse phase chromatography or normal phase HPLC.

Selection of solvent system:

Basically it is better to use an isocratic mobile phase of some average organic solvent strength (50%). It is usually not recommended to begin method development with an intermediate strength mobile phase. A better alternative is to use a very strong mobile phase first (80-100%) then reduce % of organic solvent as necessary. The initial separation with 100% results in rapid elution of the entire sample but few groups will separate. Separation or resolution is a primary requirement in quantitative HPLC analysis which is depends on the solvent system used. Usually, a sample containing two or more components, baseline resolution (Rs >1.5) can be obtained easily for the band of interest. Resolution usually decreases along with the life of the column and can be vary from day to day. Therefore, the values of Rs =2 or more should be the goal during method development for sample mixture. Goals that are to be achieved in method development are briefly summarized in Table No. 5.0.

Table No. 4.0 Goals to be achieved in method development

Goal	Comment
Separation time	<5-10 min is desirable for routine procedures.
Quantitation	≤2% for assays; ≤5% for less-demanding analyses ≤15% for trace analyses.
Pressure	<150 bars is desirable, <200 bars is usually essential (new column assumed).
Peak height	Narrow peaks are desirable for large signal/noise ratios.
Solvent consumption	Minimum mobile-phase use per run is desirable.

Resolution with Rs >2 favor both improved assay precision and greater method ruggedness. Some HPLC assays do not require base line separation of the compounds of interest (qualitative analysis). In such cases only enough separation of individual components is required to provide characteristic retention times for peak identification. The time required for a separation (runtime = retention time for base band) should be as short as possible and the total time spent on method development is reasonable (runtimes 5 to 10 minutes are desirable). Generally column equilibration is achieved after passage of 10 to 20 volumes of the new mobile phase through the column. However this should be confirmed by repeating the experiment under the same conditions. When constant retention times are observed in two such back-to-

back repeat experiments (± 0.5% or better), it can be assumed that the column is equilibrated and the experiments are repeatable.

Optimization of HPLC method

During the optimization stage, the initial sets of conditions that have evolved from the first stages of development are improved or maximized in terms of resolution and peak shape, plate counts, asymmetry, capacity factor, elution time, detection limits, limit of quantitation and overall ability to quantify the specific analyte of interest. The various parameters that include to be optimized during method development are Selection of mode of separation, Selection of stationary phase, Selection of mobile phase and Selection of detector.

Selection of Mode of Separation

In reverse phase mode, the mobile phase is comparatively more polar than the stationary phase. For the separation of polar or moderately polar compounds the most preferred mode is reverse phase. The nature of the analyte is the primary factor in the selection of the mode of separation. A useful and practical measurement of peak shape is peak asymmetry factor and peak tailing factor. Peak asymmetry is measured at 10% of full peak height and peak tailing factor at 5%. Reproducibility of retention times and capacity factor is important for developing a rugged and repeatable method.

Buffers and buffer capacity

Buffer and its strength play an important role in deciding the peak symmetries and separations. Some of the most commonly employed buffers are Phosphate buffers, citrate and borate etc.

Mobile Phase Composition

Most chromatographic separations can be achieved by choosing the optimum mobile phase composition. This is due to the fact that fairly large amount of selectivity can be achieved by choosing the qualitative and quantitative composition of aqueous and organic portions. Most widely used solvents in reverse phase chromatography are Methanol and Acetonitrile. Experiments should be conducted with mobile phases having buffers with different pH and different organic phases to check for the best separations of analyte peak.

Selection of Detector

The detector was chosen depending upon some characteristic property of the analyte like UV absorbance, florescence, conductance, oxidation, reduction etc. The characteristics that are to be fulfilled by a detector to be used in HPLC determination are,

- High sensitivity facilitating trace analysis
- Negligible baseline noise to facilitate lower detection, Low dead volume.

Pharmaceutical ingredients do not absorb all UV light equally, so that selection of detection wavelength is important. An understanding of the UV light absorptive properties of the organic impurities and the active pharmaceutical ingredient is very helpful. For the greatest sensitivity λmax should be used. Ultra violet wavelengths below 200nm should be avoided because detector noise increases in this region. Higher wavelengths give greater selectivity to the detector.

System parameter optimization

This is done to get a desired balance between resolution & analysis time. The parameters optimized here are the column dimensions, column packing particle size and flow rate. These parameters may be changed without selectivity.

Column length

Longer column gives increased resolution while shorter column require shorter analysis time but lower back pressure gives Fast equilibrium & less solvent consumption.

Column internal diameter

Wider for greater sample loading and narrow for more sensitivity as well reduced mobile phase consumption.

Particle shape

Spherical particles lower back pressure & column stability thus Greater efficiency and Irregular particles-when high surface area and high capacity needed.

Particle size

Particle size 3-4 micron for complex mixtures with similar components, 5-10 micron for sample with structurally different compounds (especially multicomponents), 10-50 micron-fast, high resolution separations, 15-20micron-preparative separations.

Pore size

Pore size of 150A^{o} or less for samples with mol. wt<2000 and 300A^{o} or greater for samples with mol. wt>2000.

Surface area

High surface area provides more capacity, Greater resolution, and longer retention while Low surface area gives quick equilibration time.

Carbon load

High carbon load gives greater column capabilities, Resolution and Low carbon load fast analysis times.

End capping

End capped packing used to eliminate unpredictable secondary interactions with the base material while Non-end capped packing for selectivity differences for polar compounds by controlling secondary interactions.

Flow rate

Increase in flow rate decreases the retention time and increases the peak sharpness, decreases the resolution while Decrease in flow rate increase the retention time, decreases the peak sharpness and increases the resolution. Generally optimized parameter for different compounds given in Table No. 5.0

Table No. 5.0 Optimization Parameters in HPLC Method Development

Analytes	HPLC method	Optimize
Neutral	Reverse phase	Solvent strength, solvent type
Weak acids and/or weak bases	Ion suppression	pH, solvent strength, solvent type
Strong acid and/or strong bases	Ion pairing	Ion pairing reagent concentration, pH solvent strength , solvent type
Inorganic anions/cations	Ion exchange	Eluting ion concentration

1.5 Introduction to Analytical Method Validation[25-29]

Doing a thorough method validation can be tedious, but the consequences of no doing it right are wasted Time, Money and Resources

Validation is establishing documented evidence that provides a high degree of assurance that a specific process will consistently produce a product or results meeting its predetermined specifications and quality attributes.

Validation is required for introducing a new method in routine use, whenever there is change in the synthesis of drug substance and whenever there is change in the composition of finished product. Analytical method validation is completed to ensure that analytical methodology is accurate, specific, reproducible and robust over the specified range that an analyte will be analyzed. It provides an assurance of reliability during normal use, and is referred to as the process of providing documented evidence that the method is acceptable for its intended purpose. Although a thorough validation cannot rule out all potential problems, the process of method development and validation find-out the common problem associated with method likes analytical recovery, matrix interferences with the analyte determination, inadequate sample preparation procedures, sampling errors, stability of materials, and general robustness. For pharmaceutical methods, guidelines from United States Pharmacopoeia (USP), International Conference on Harmonization (ICH) and Food and Drug Administration (FDA) provide a framework for performing such validation.

Parameters used for the validation of analytical method are:

- System Suitability
- Specificity
- Linearity
- Range
- Accuracy
- Precision
- Limit of Detection (LOD)
- Limit of Quantitation (LOQ)
- Ruggedness
- Robustness.

Fig. No.7.0: Parameters used for the validation of analytical method

System Suitability

System suitability is the checking the system performance before or during the analysis of unknowns. It is providing data of acceptable quality and used to verify that resolution and repeatability of the system are adequate for the analysis to be performed. It is based on the concept that equipment, electronics, analytical operations and sample constitute an integral system that can be evaluated as a whole. As per USP, following parameters and recommendation are used to determine system suitability before analysis.

Table No. 6.0: System suitability parameter and recommendations

Sr. No.	PARAMETERS	RECOMMENDATIONS
1.	Theoretical plates (N)	Should be > 2000
2.	Tailing factor (T)	T of ≤ 2
3.	Resolution (R_S)	R_S of >2 between the peak of interest & the closest eluting potential interference.
4.	Repeatability	RSD ≤ 1% for N ≥ 5 is desirable.
5.	Capacity factor (k')	K' > 2.0 (well resolved from other peak & void volumes)

Specificity

Specificity is the ability of the method to accurately measure the analyte response in presence of all potential sample components. The response of the analyte in the test mixture containing the analyte and all potential sample components (placebo formulation, synthesis intermediates, excipients, degradation products, process impurities, etc) is compared with the response of the standard solution of the analyte.

Linearity

Linearity study verifies that the sample solutions are in a concentration range where analyte response is linearly proportional to the concentration. Five concentration levels from 50-150% of the standard solutions of the analyte are required to allow detection of curvature in the plotted data. Generally, five concentrations from 80-120% of standard concentration should be used in three replicates of each concentration.

Acceptability of linearity is often judged by correlation co-efficient and y-intercept of linear regression line for the response versus concentration plot. A correlation co-efficient of >0.999 is generally considered as evidence of acceptable fit of the data to the regression line. The y-intercept should be less than a few percent of the response obtained for the analyte at the target level.

Range

Range is the interval between the upper and lower levels of analyte that have been demonstrated to be determined with precision, accuracy and linearity. Range of an analytical method is the concentration interval over which acceptable accuracy, linearity and precision are obtained and it is expressed in terms of ppm or percentage.

Table No.7.0: Recommended validation ranges for Linearity studies

SL.NO	ANALYSIS CATEGORIES	TYPICAL RANGE (%)	RECOMMENDED VALIDATION RANGE (%)
1.	Assay specifications for release	95-105	80-120
2.	Assay specifications for check	90-110	80-120
3.	Content uniformity test	75-125	70-130
4.	Assay of a preservative in a stability study	50-110	40-120
5.	Determination of a degradant in a stability study	0-10	0-20

Accuracy

Accuracy of a method is the closeness of the measured value to the true value for the sample. Accuracy is usually determined in one of the four approaches:

a) By analyzing sample of known concentration and comparing the measured value to the true value.
b) By comparing test result from the new method with the result from an existing alternate method that is known to be accurate.
c) By spiking analyte in blank matrices which is mostly used for recovery studies.
d) The fourth approach is the technique of standard addition, which can also be used to determine the recovery of the spiked analyte. This approach is used if it is not possible to prepare blank sample matrix without the presence of the analyte.

Accuracy criteria for an assay method is that the mean recovery will be 100±2% at each concentration over the range of 80-120 at the target concentration. For impurity method, the mean recovery will be within 0.1% absolute of the

theoretical concentration or 10% relative, whichever is greater, for impurities in the range of 0.1-2.5% v/w.

Precision

Precision is the measure of the degree of repeatability of analytical method under normal operation and is normally expressed as %RSD for the statistically significant number of samples. The precision of an analytical procedure is usually expressed as the variance, % relative standard deviation or coefficient of variation of a series of measurements. ICH documents recommend that precision should be assessed using a minimum of nine determinations covering the specified range of the procedure (i.e., three replicates of three concentrations) or using a minimum of six determinations at 100% of the test concentration. For an assay method, the instrument precision (intermediate precision) will be 1% and the intra-assay precision (Repeatability) will be 2% and for an impurity method, the instrument precision will be 5% and intra-assay precision will be 10%.

Ruggedness

Ruggedness is the degree of reproducibility. If test results obtained by the analysis of same sample under variety of normal test conditions such as different laboratories, different analyst, different instruments, different lots of reagent, different elapse assay times, different assay temperatures, different days, etc. It is normally expressed as lack of influence on test results of operational and environmental variables of the analytical method.

Robustness

The robustness of a method is its ability to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal use. These method parameters may be evaluated one factor at a time or simultaneously as part of a factorial experiment. These typical variations are includes Variation in wavelength (λmax) for detection**,** variation in mobile phase composition, Variation in column temperature**,** variation in flow rate / injection volume.

Limit of detection (LOD)

It is the lowest concentration of an analyte in the sample that can be detected but not necessarily quantitated under the stated experimental condition. It is a parameter of limit tests. It is expressed as a concentration at a specified signal to noise ratio usually 3:1.

Limit of quantitation (LOQ)

It is the lowest concentration of the analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational and experimental conditions of the method. It is a parameter of the quantitative determination of low levels of compounds in sample mixtures, such as degradation in finished products. It is expressed as a concentration at a specified signal to noise ratio usually 10:1.

1.6 Introduction to Forced Degradation Studies[30-37]

The development of forced degradation and stability indicating studies of drugs is very important as it affects the safety and efficacy of the drug substance and drug product. Forced degradation studies are carried out at more severe than accelerated conditions so as to design to generate product-related variants and develop analytical methods to determine the degradation products formed. Forced degradation studies are also designed to specificity of stability indicating methods and also provide an insight into degradation pathways and degradation products of the drug substance and helps in elucidation of the structure of the degradation products and also to increase the rate of chemical degradation or physical change of an active drug substance or drug product using exaggerated storage condition. Any significant degradation product should be evaluated for potential hazard and the need for characterization and quantization. According to the guidelines by the FDA and ICH, these stability data are quite essential for the quality of the drug substance and drug product to know how quality changes with time under the influence of the various environmental factors and which helps in the designing the proper formulation and packing system along with proper storage and shelf life of the drug substance and drug products. As per ICH guidelines, the stress testing is design to identify the degraded product and to validate the stability indicating procedure.

But these guidelines do not provide details about how to conduct forced degradation studies even though forced degradation studies are scientifically very essential and are the regulatory requirements during the drug developments. Now, it has become mandatory to conduct stability studies of new formulations before filling approaching to the registration dossier. Generally a stability study includes long term for 12 month and accelerated studies for 6 month. But as we compare to stability studies, a forced degradation studies helps in generating possible degradants in much

shorter period of time i.e. within a few weeks. Generally these studies are designed to measure any drug condition subject to change during storage.

A degradation level of 10 to 15% is considered adequate for validation of a chromatographic purity assay. It is not necessary that forced degradation would results in a degradation product. In the Testing of stressed samples is required to demonstrate the following abilities of analytical techniques to achieve following purposes like:

- Evaluate stability of DS and DP in solution
- Generate stability indicating method.
- Determine structural transformations of the drug substance and drug product
- Detect low concentrations of potential degradation products
- Detect unrelated impurities in the presence of the desired product and product-related degradants
- Separate product-related degradants from those derived from excipients and intact placebo.
- Understanding chemical behavior of DS and DP
- Reveal degradation mechanism such as hydrolysis, oxidation, thermolysis, or photolysis of the DS and DP.
- Generating more stable formulations.
- Design shelf life for the new DS and DP.

Forced degradation is normally carried out under more severe conditions than those used for accelerated studies. A minimal list of stress factors **(Fig. No.8.0**) and conditions suggested for forced degradation studies must include acid and base hydrolysis, thermal degradation, photolysis, and oxidation shown in **Table No. 8.0**

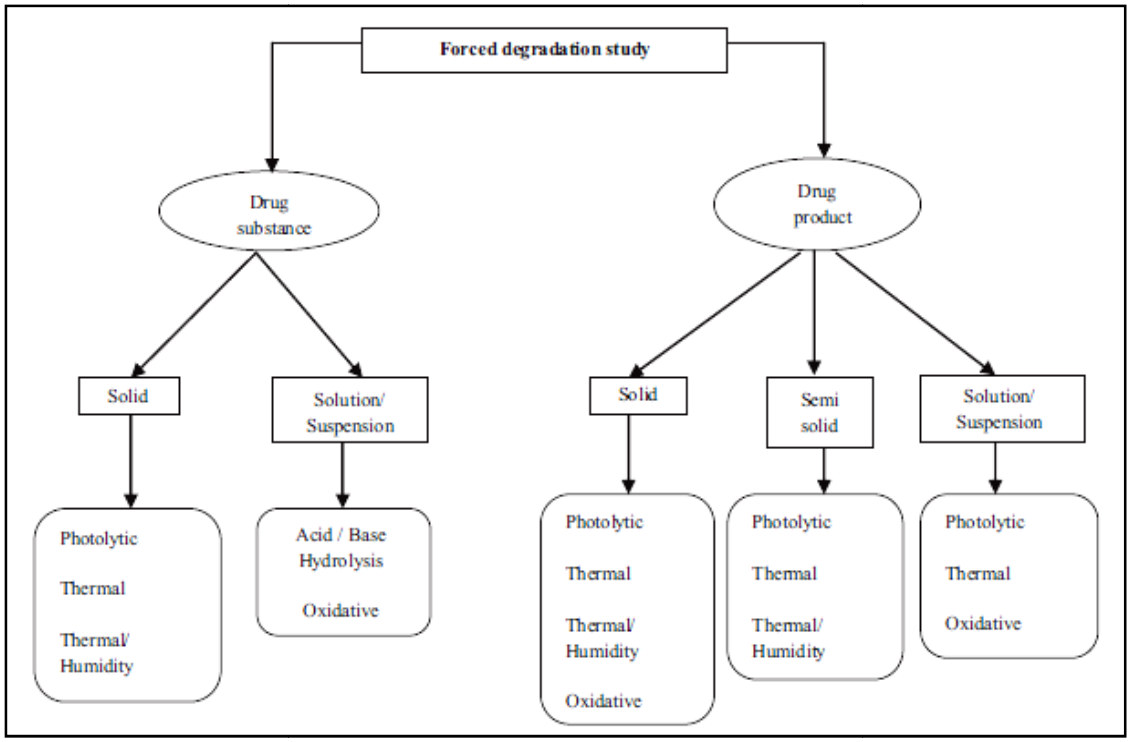


Fig.No.8.0: Flow chart for different stress conditions used for the degradation of drug substance and drug products

Table No. 8.0: Commonly used condition for Forced degradation study

Degradation type	Experimental conditions	Storage conditions	Sampling time (days)
Hydrolysis	Control API (no acid or base)	40 °C, 60 °C	1,3,5
	0.1 M HCl	40 °C, 60 °C	1,3,5
	0.1 M NaOH	40 °C, 60 °C	1,3,5
	Acid control (no API)	40 °C, 60 °C	1,3,5
	Base control (no API)	40 °C, 60 °C	1,3,5
	pH: 2,4,6,8	40 °C, 60 °C	1,3,5
Oxidation	3% H_2O_2	25 °C, 60 °C	1,3,5
	Peroxide control	25 °C, 60 °C	1,3,5
	Azobisisobutyronitrile (AIBN)	40 °C, 60 °C	1,3,5
	AIBN control	40 °C, 60 °C	1,3,5
Photolytic	Light 1 × ICH	NA	1,3,5
	Light 3 × ICH	NA	1,3,5
	Light control	NA	1,3,5
Thermal	Heat chamber	60 °C	1,3,5
	Heat chamber	60 °C/75% RH	1,3,5
	Heat chamber	80 °C	1,3,5
	Heat chamber	80 °C/75% RH	1,3,5
	Heat control	Room temp.	1,3,5

The objectives of forced degradation study is to provide evidence on how the drug quality of a drug substance or drug product varies with time under influence of a different conditions like

- Acid degradation
- Base degradation
- Peroxide degradation
- Photo degradation
- Neutral Degradation
- Thermal degradation

The samples data generated from forced degradation studies can be used to develop the stability indicating method which can be applied latter for the analysis of samples generated from accelerated and long term stability studies.

2. REVIEW OF LITERATURE

A literature survey was carried out to identify multicomponent formulations for which analytical method development was justified. The information for the drugs selected for the research work is available on the world wide web[38-45] and also the monographs for individual drugs is availalble in Pharmacopoeias[46-53], but combinations of these formulations were not official in any pharmacopoeia *viz.* Indian Pharmacopoeia (IP), British Pharmacopoeia (BP) and United states of Pharmacopoeia (USP), European Pharmacopoeia (EU), The official method of analysis for Losartan, Chlorthalidone, and Proguanil as single components is reported in IP. Likewise, the official BP method is available for individual analysis of Chlorthalidone, Proguanil and Acetylcysteine formulation and the official USP method is available for analysis of Chlorthalidone and Acetylcysteine individually.

Losartan Potassium and Chlorthalidone

A plethora of methods, using various analytical techniques have been reported for losartan potassium and chlorthalidone alone and in combination. A few are metntionedbelow:

Sathe SR et al[54] have reported simultaneous analysis of losartan potassium, atenolol, and hydrochlorothiazide in bulk and in tablets by high-performance thin-layer chromatography (HPTLC) with UV absorption densitometry. The drug was extracted by using methanol and then sample and standard solutions were applied to prewashed silica gel plates and developed with toluene–methanol– triethylamine 6.5:4:0.5 (v/v) as mobile phase. Zones were scanned densitometrically at 274 nm. The Rf values of losartan potassium, atenolol, and hydrochlorothiazide were 0.60, 0.43, and 0.29 respectively. Calibration plots were linear in the ranges 1000–5000ng/band for losartan potassium and atenolol and 250–1250ng/band for hydrochlorothiazide; the correlation coefficients (r), were 0.9994, 0.9993, and 0.9994, respectively. The suitability of this method for quantitative determination of these compounds was proved by validation in accordance with the requirements of pharmaceutical

regulatory standards. The method was used for routine analysis of these drugs in bulk and in a formulation.

G. Carlucci et al[55] have developed method for the simultaneous determination of losartan and hydrochlorothiazide in tablets by high-performance liquid chromatography (HPLC). The procedure, based on the use of reversed phase high performance liquid chromatography, is linear in the concentration range 3.0-7.0μg/ml for losartan and 0.5-2.0μg/ml for hydrochlorothiazide, is simple and rapid and allows accurate and precise results. The limit of detection was 0.08μg/ml for losartan and 0.05μg/ml for hydrochlorothiazide.

Mhaske RA. et al[56] have developed RP-HPLC method for the simultaneous estimation of irbesartan, ilosartan , hydrochlorthaizide, and Chlorthalidone applications to commercially available Drug product. The separation was achieved on Hypersil BDS (250 mm × Diameter 4.6 mm Particle size 5 μm) column with gradient flow. The mobile phase at a flow rate of 1.0 ml/min consisted of 0.05M sodium dihydrogen phosphate buffer and acetonitrile (Gradient ratio). The UV detection was carried out at 220 nm. The method was successfully validated in accordance to ICH guidelines. Further, the validated method was applied for commercially available pharmaceutical dosage form.

Sibel. A. Ozkan[57] has developed method for simultaneous determination of losartan potassium and hydrochlorothiazide from tablets and human serum by RP-HPLC. Chromatography was carried out on a C_{18} reversed-phase column using a mixture of 0.01 M KH_2PO_4: acetonitrile (65:35; v/v) adjusted to pH 3.1 with H_3PO_4 at a flow rate 1.0 ml/min. Detection was realized at 232 nm using a UV detector. Linearity was obtained in the concentration range of 25–10000 ng/ml and 50–10000 ng/ml for losartan potassium and hydrochlorothiazide, respectively. The limit of detection and the limit of quantification of the procedure were found to be 1.02 ng/ml and 3.39 ng/ml for losartan potassium; 4.49 ng/ml and 14.96 ng/ml for hydrochlorothiazide, respectively.

Krishna KC. et al[58] have reported RP-HPLC method development and validation of amlodipine and losartan in binary mixture. The chromatographic system consisted of a LC-20AT VP series model chromatograph equipped spin chrome

software. The separation was achieved from a Inertsil ODS 3V C_{18} (150 X 4.6 mm, 5µm) at ambient temperature with a mobile phase containing a mixture mobile Phase-A with 70% v/v of buffer pH-3.7 and 30%v/v of acetonitrile and mobile phase–B containing 70% v/v of acetonitrile and 30% v/v of buffer pH-3.7. The samples were monitored at 237 nm for detection at a flow rate of 1.0 ml/min and the retention time was about 5.13 and 11.11 mins. for Amlodipine and Losartan respectively. The calibration curve was linear over the concentration range 1.25-7.5µg/ml and 12.5-75µg/ml for Amlodipine and Losartan respectively. The proposed method is accurate in the range of 99.95% - 100.133% recovery and precise.

Shaikh Anis[59] performed the development of stability indicating method for validation of losartan potassium and hydrochlorothiazide drugs in solid dosage form by RP-HPLC chromatography was carried out on a C_{18} reversed-phase column using a gradient system mixture of buffer and acetonitrile in different concentration with time at a flow rate 1.0 ml/min. Detection was realized at 270nm using a UV detector. Linearity was obtained in the concentration range of 35-65µg/ml and 8.75-16.25µg/ml for losartan potassium and hydrochlorothiazide, respectively. tailing factor is not more than 2.0%. The Correlation coefficient for Losartan potassium is 0.99937 and for Hydrochlorothiazide is 0.99967.

Srinivasa KR. et al[60] have developed reverse phase liquid chromatographic method for the simultaneous determination of losartan potassium and ramipril in tablet dosage forms. A hypersil ODS C18, (4.6×250 mm, 5 µm) column in Isocratic mode, with mobile phase acetonitrile: methanol: 10mM tetra butyl ammonium hydrogen sulphate in water in the ratio of 30:30:40% v/v/v was used. The flow rate was 1.0 ml/min and effluent was monitored at 210 nm. The retention times of losartan potassium and ramipril were 4.7 and 3.3 min, respectively. The linearity range for losartan potassium and ramipril were in the range of 0.04-100 µg/ml and 0.2-300 µg/ml, respectively. The proposed method was also validated and successfully applied to the estimation of losartan potassium and ramipril in combined tablet formulations.

Kumar GS et al[61] have developed and validated RP-HPLC method for simultaneous estimation of atenolol and chlorthalidone in marketed formulations. This determination was carried out on an Xterra RP8 (150 X 4.6 mm, 5µm) column using a mobile phase of KH_2PO_4 -methanol (50:50, pH 3.6) with flow rate 0.5 ml/min and UV

detection at 240 nm. The retention time for Atenolol was 3.2min and chlorthalidone is 5.0 min. The linearity response at range of 50-150 µg/ml. The Correlation coefficient for Atenolol and Chlorthalidone was 0.9996. The RSD for intra-day and inter-day was found to be less than 2% and the % recoveries obtained from the 100.54-103.32% and 98.03-102.77% respectively.

Tengli AR et al[62] has developed for the simultaneous estimation of losartan, atenelol and hydrochlorothiazide in tablet dosage form and telmisartan as an internal standard. Separation was achieved with an phenomenex luna (CN 100R, 250×4.60 mm 5µ) size column, ambient temperature with a low pressure gradient mode with mobile phase containing acetonitril, and 0.2% of diammonium hydrogen orthophosphate buffer pH 2.8 adjusted with orthophosphoric acid (50:50). The flow rate was 1 ml/min and eluent was monitored at 230 nm. The selected chromatographic conditions were found to effectively separate losartan, atenolol and hydrochlorothiazide with retention time of 6.0, 5.0 and 4.0 min respectively. The proposed method was found to be rectilinear over the range of 25-150 µg/ml, 25-150 µg/ml and 6.25-37.50 µg/ml for losartan, atenolol and hydrochlorothiazide respectively. The proposed method was found to be accurate, precise, reproducible and specific and it can also be used for routine quality control analysis of these drugs in biological samples either alone or in combined pharmaceutical dosage forms.

Manoela RB. et al[63] have reported a method for the determination of losartan associated with chlorthalidone or hydrochlorothiazide in capsules by capillary zone electrophoresis using 50 mmol/l of sodium carbonate buffer with detection at 226 nm. The experiments were carried out in a capillary electrophoresis system (model HP3d CE, Agilent Technologies, Palo Alto, USA) equipped with a diode array detector set at 226 nm, a temperature control device (maintained at 25 °C) and acquisition and treatment data software (HP ChemStation, rev A.06.01). Samples were hydro dynamically injected (12.5 mbar, 5 s) and the electrophoretic system was operated under normal polarity and constant voltage conditions of +20 kV. Fused-silica capillaries coated with polyacrylate (Microtube, Araraquara, São Paulo, Brazil) 48.5 cm (40 cm effective length) ´ 75 µm I.D. ´ 375 µm O.D. were used.

Bhimashankar HS. et al[64] have developed method for the simultaneous determination of telmisartan and clorthalidone from pharmaceutical formulation by

reversed-phase high performance liquid chromatography. The separation was carried out on C18 column using mobile phase consisting of a mixture of acetonitrile: methanol and pH adjusted to 3.4 with orthophosphoric acid in the ratio (80:20 v/v). The flow rate was maintained at 1 ml/min. The UV detection was carried out at a wavelength of 225 nm. The retention time for telmisartan and clorthalidone was found to be 3.1 min and 4.6 min respectively. Linear response obtained for telmisartan was in the concentration range 10-60 μg/ml (r2 = 0.999) and clorthalidone in the range 10-50 μg/ml (r2 = 0.999). The relative standard deviation in the tablets was found less than 2% for six replicates. The method was validated according to the ICH guidelines with respect to linearity, precision, accuracy, ruggedness and robustness.

Lastra OC et al[65] have reported development and validation of an UV derivative spectrophotometric determination of Losartan potassium in tablets. A study was carried out of all the parameters established by USP XXIV to validate an analytical method and the spectrophotometric characteristics of Losartan potassium, a signal at 234 nm of the first derivative spectrum (1D234) was found adequate for quantification. The linearity between signal 1D234 and concentration of Losartan potassium in the range of 4.00-6.00 mg/ml in aqueous solutions presents a square correlation coefficient (r2) of 0.9938. The mean recovery percentage was 100.7+/-1.1% and the precision expressed as relative standard deviation RSD is 0.88%. In addition, the proposed method is simple, easy to apply, low-cost, does not use polluting reagents and requires relatively inexpensive instruments.

Kavitha J. et al[66]**.** developed and validated for the analysis of Atenolol, Hydrochlorothiazide and Losartan potassium in tablet formulation. Best chromatographic resolution was achieved on a reverse-phase phenomenex C18 column using acetonitrile: 50mM potassium dihydrogen ortho phosphate (pH 3.5) ratio 50:50 as mobile phase with a flow rate of 1ml/min and isocratic elution with a total run time of 14 minutes. Sulphadoxine was selected as internal standard. The retention time of Atenolol, Hydrochlorothiazide, Losartan potassium and Internal Standard was found to be 5.550, 3.280, 7.370 and 12.397 respectively. Detection of the multicompounds was carried out at 270nm.

Maggio RM. **et al**[67] have reported multivariate approach for the simultaneous determination of losartan potassium and hydrochlorothiazide in a combined

pharmaceutical tablet formulation The procedure, based on the multivariate analysis of spectral data in the 220-274 nm region by the partial least squares algorithm, is linear in the concentration range 1.06-5.70 mg/L for hydrochlorothiazide and 4.0-22.2 mg/L for losartan. It is simple, rapid and robust, allowing accurate and precise results, with drug recovery rates of 99.3 and 100.4% and relative standard deviations of 1.7 and 1.0% obtained for hydrochlorothiazide and losartan, respectively. The method was applied to the simultaneous determination of both analytes in tablets.

Baing MM. et al[68] have developed method for the simultaneous determination of losartan potassium, ramipril, and hydrochlorothiazide. The three drugs were separated on a 150 mm × 4.6 mm, 5 μm particle, Cosmosil C_{18} column. The mobile phase was 0.025 M sodium perchlorate–acetonitrile, 62:38 (*v*/*v*), containing 0.1% heptanesulphonic acid, pH adjusted to 2.85 with orthophosphoric acid, at a flow rate of 1.0 ml/min. UV detection was performed at 215 nm. The method was validated for linearity, accuracy, precision, and limit of quantitation, linearity, accuracy, and precision were acceptable in the ranges 35–65 μg/ml for losartan, 1.75–3.25 μg /ml for ramipril, and 8.75–16.25 μg/ml for hydrochlorothiazide.

Ramya Gavini et al[69] have reported stability indicating RP-HPLC method for the simultaneous estimation of amlodipine and losartan in bulk drug and tablet dosage formulations. The separation was achieved on the Enable C_{18} G 150 mm × 4.6 mm, 5 μm analytical column with mobile phase comprising of 0.005M KH_2PO_4:Acetonitrile pH 3(50:50v/v) at isocratic flow of 1.0 ml /min with UV detection at 230nm. The retention time for amlodipine and losartan was found to be 4.3 and 6.7 min respectively. The linearity range for amlodipine was 0.125-0.75 μg/ml and 1.25- 7.5 μg/ml for losartan potassium.

Gandhimathi M. et al[70] have developed method for simultaneous estimation of losartan potassium and hydrochlorothiazide in combination. The method employs simultaneous equations to estimate these drugs in methanol, losartan potassium and hydrochlorothiazide showed maximum absorbance at 236 and 270nm respectively. Losartan potassium and hydrochlorothiazide obeyed Beer Lambert's law in the concentration range from 2-20 μg/ml and 1-50mug/ml, respectively. The results of analysis have been validated statistically and by recovery studies.

Mohammed AS. et al[71] have reported the validation of an isocratic HPLC method for the assay of Losartan Potassium tablets and the evaluation of the stability of drug substance after stress test by photodiode array detection. The HPLC separation was achieved on a liquid chromatogram is equipped with a 254 nm detector and L1 (Hypersil BDS C18 4.6´ 250 mm, 5 mm) column was used. A mixture of ammonium dihydrogen phosphate buffer pH 3.0 and acetonitrile (65:35) as a mobile phase, the flow rate is about 1.5 ml/min. Chromatograph the Standard preparation, and record the peak response as directed under procedure: the tailing factor for the analyte peak is not more than 3.0 and the relative standard deviation of replicated injections is not more than 2%.

Lakshmi KS, et al[72] have developed high performance thin layer chromatography method for the simultaneous determination of losartan and perindopril in tablet and Separation was carried out on precoated TLC plates, coated with silica gel 60 F 254. The separation was done using a mobile phase toluene: acetonitrile: formic acid (5:5:0.3 v/v/v). After development, the chromatoplates were scanned at 215 nm. The Rf value of losartan and perindopril was found to be 0.55 and 0.27 respectively. The results of the analysis have been validated statistically and by recovery studies.

Parmar KE. et al[73] have developed a densitometric method for the simultaneous estimation of telmisartan and chlorthalidone in bulk and pharmaceutical dosage form. In this method separation of drugs was carried out using acetonitrile: toluene: glacial acetic acid (7.5: 2.5: 0.05 v/v/v) as mobile phase on precoated silica gel 60F254 plates. The densitometric evaluation of spots was carried out at 242 nm. The Rf value for telmisartan and chlorthalidone were found to be 0.26 ± 0.02 and 0.67 ± 0.02, respectively. The method was validated as per the ICH guidelines. The drug response was linear over the concentration range 400-2400 ng/spot (n=6) and 125-750 ng/spot (n=6) for telmisartan and chlorthalidone respectively. The limit of detection and limit of quantitation were found to be 9.05 ng/spot and 27.42 ng/spot for Telmisartan and 5.15 ng/spot and 15.6 ng/spot for chlorthalidone. The percentage recovery of Telmisartan and Chlorthalidone was found to be 100.03 and 100.026 respectively. The % RSD values for intra-day precision study and inter-day precision study were <1.0%, confirming that the method was sufficiently precise.

Bhaumik C Patel[74] has developed and validated stability indicating RP-HPLC method for quantitative determination of enalapril maleate and losartan potassium in tablet formulation. Here both peaks are well separated from its excipient peaks and with total run time of 10 min, The RP-HPLC analysis is carried out using buffer-acetonitrile(60:40 v/v) pH4.5 adjusted With O-Phosphoric acid as a mobile phase and Hyperchrom phase C-18 BDS Hypersil column (250mm × 4.6 mm id 5µm) as stationary phase at 235nm and 1 ml/min flow rate. The retention time of enalapril maleate and losartan lotassium was found to be 3.150 and 5.420 minutes respectively. Linearity was obtained in the concentration range of 5-15 µg/ml and 25-75 µg/ml with % recoveries were found to be 98.47% – 100.68% and 98.49% –100.61% for enalapril maleate and losartan potassium respectively. LOD were found to be 0.120µg/ml and 0.606µg/ml at 235 nm for enalapril maleate and losartan potassium respectively. LOD and LOQ for enalapril maleate was found to be 0.365 µg/ml and 0.120 and for losartan potassium 1.839 µg/ml and 0.606 respectively. Stability method shows that in stress conditions i.e. acidic, basic, oxidation, thermal and photolytic, comparison of % degradation of tablet dosage form of drug and its API is satisfactory and less. As per ICH guidelines HPLC method for enalapril maleate and losartan potassium was developed and validated.

Patel AI. et al[75] have reported RP-HPLC method for the simultaneous estimation of losartan potassium and perindopril erbumine in its tablet formulation. by using HiQSil-C-18W ODS, (250 mm × 4.5 mm i.d.), 5 µm column and mobile phase was ACN:water in proportion of 50:50 v/v, pH adjusted to 3.2 ± 0.1 with 1% o-phosphoric acid. The flow rate was 1.0 Ml/min and effluent was monitored at 210 nm. The retention time of losartan potassium and perindopril erbumine were eluted at 6.7 min and 4.5 min respectively. The method was found to be linear in the range of 2-18 µg/ml for both the drug. The coefficient of variance for both the drug was more than 0.999. The mean percentage recovery was found to be 98.40 % for losartan potassium and 97.50 % for perindopril erbumine. The limits of quantification of losartan potassium and perindopril erbumine were found to be 0.109µg/ml and 0.041µg/ml.

Venkata MR. et al[76] have developed RP-HPLC method for the simultaneous estimation of losartan potassium and perindopril erbumine in pharmaceutical dosage form. The mobile phase consisted of Acetonitrile: pot.dihydrogen phosphate buffer

(0.1% trifluoroacetic acid is used as a peak sharpener) in the ratio of 40:60 delivered at a flow rate of 0.8 ml/min and wavelength of detection at 217 nm. The retention times of were Losartan Potassium and Perindopril erbumine 5.87 min and 3.61 min respectively. The method was found to be linear in the range of 25-150µg/ml and 1-6 µg/ml for losartan potassium and perindopril erbumine respectively. The coefficient of variance for both the drug was more than 0.999. The proposed method can be used for determination of these drugs in combined dosage forms.

Mandal S. and coworker[77] have develop and validate RP-HPLC method for the estimation of losartan and chlorthalidone in combined dosage form by using Phenomenex C18 column (250mm × 4.6 mm id 5µm), mobile phase is acetonitrile:0.1% orthophosphoric acid in water pH 2.5(50:50v/v) with flow rate of 1.2 ml/min at 220nm. The retention time for chlorthalidone and losartan was 2.790 and 3.957 minutes respectively. The linearity was calculated in range of 1-6µg/ml and 4-24µg/ml.

Acebrofylline and Acetylcysteine

Several analytical methods are reported individually for acebrofylline and acetylcysteine hydrochloride or for their combination with other drugs, a few of which are described below:

Bhagavati S. et al[78] have developed and validated a derivative spectroscopic method for estimation of acebrophylline in bulk and its dosage form & in presence of impurity, ambroxol Hcl. The developed spectrophotometric method is simple, rapid, precise, accurate, reliable and economical. Method is specific in presence of ambroxol which is the impurity of acebrophylline. The method is accurate, precise and linear over a range of 5-50µg/ml for acebrophylline. The limit of detection was observed as 0.178µg /ml, the limit of quantification was observed as 0.598µg/ml. The %RSD is less than 2% in methanol. Method accurately estimates acebrophylline in presence of ambroxol with 99-100% recovery. The method can be applied for acebrophylline tablet formulation with 99-100% recovery.

Ramanjaneyulu S. et al[79] have reported a simple, rapid, precise and accurate isocratic RP- HPLC method for the determination of acebrophylline in commercial tablets. The method has shown adequate separation of acebrofylline. Separation was achieved on Agilent XDB C18, 5µm, 150 x 4.6 mm i.d. column using a mobile phase

consisting of buffer (pH 3.0) and methanol in the ratio 70:30v/v at a flow rate of 0.8ml/min and UV detection at 273nm. The retention time for acebrofylline was 2.788 and the run time was 8 minutes. Linearity of the proposed method was investigated in the range of 10-150 µg/ml (R2 =0.998) for acebrofylline. The limit of detection (LOD) was 0.31µg/ml and the limit of quantification was 0.96µg/ml. The %Recovery values in between 98.0%-102.0% for accuracy studies indicate that the method can be used for the estimation of acebrophylline in tablets.

Jignesh M. et al[80] have developed and validated a method for the simultaneous estimation of acebrophylline and montelukast sodium by solving of simultaneous equations (vierodt's method). Acebrophylline and montelukast Sodium were found to have absorbance maxima at 313 and 344.5nm respectively in methanol. The method obeys Beer's law in the concentration range of 20-140 µg/ml (R2 = 0.9997) for acebrophylline and 1-7 µg/ml (R2 = 0.9993) for montelukast sodium. The LOD and LOQ were found to be 2.46µg/ml and 7.45µg/ml for acebrophylline and 0.22µg/ml and 0.68µg/ml for montelukast sodium respectively. The recoveries of acebrophylline and montelukast sodium were found to be 99.57% and 100.78% respectively showing accuracy of the method.

Acelino Cardoso de Sá et al[81] have reported determination of N-acetylcysteine by cyclic voltammetry using modified carbon paste electrode with copper nitroprusside adsorbed on the 3–aminopropylsilica. The cyclic voltammogram of CuNPSD were found to exhibit two redox couples with (Eθ')1 = 0.34 V; (Eθ')2 = 0.76 V *vs*. Ag/AgCl, KCl(sat) (KCl = 1.0 mol L-1; v = 20 mV s-1) attributed to the redox processes Cu(I)/Cu(II) and Fe(II)(CN)5NO/Fe(III)(CN)5NO respectively. The linear range for the determination of N-acetylcysteine was found between 9.9 × 10-5 and 8.9 × 10-4 mol/L showing a detection limit of 4.18 × 10-5 mol/L and an amperometric sensitivity of 3.02 × 10-2 A / mol/L.

Rim Haggag et al [82] have reported derivatization with 4-chloro-7-nitro-2,1,3-benzoxadiazole for the spectrophotometric and differential pulse polarographic determination of acetylcysteine and captopril; the methods were based on reacting the drugs with 4-chloro-7-nitro-2,1,3-benzoxadiazole (nbd-cl) in the presence of sodium tetraborate in absolute methanol. the yellow colored products obtained were measured spectrophotometrically at 417 and 420 nm for (i) and (ii), respectively and by

differential pulse polarography at -872 and -1007 mv (vs. ag/agcl electrode) for compounds (i) and (ii) after getting rid of excess unused reagent by extraction with ether. the different experimental parameters were studied and optimized spectrophotometrically.

Sravani T. et al [83] have reported RP-HPLC method for the determination of acebrophylline in bulk and capsule dosage form. The λmax of acebrophyllline was found to be 274 nm. An enable C18 (250x4.6mm, 5µm) column was used as a stationary phase. Mobile phase consisting of a mixture of acetonitrile and double distilled water (70:30) pumped isocratically at a flow rate of 1mL/min at ambient temperature. The retention time of acebrophylline was found to be 1.75 min. The calibration curve was linear over a concentration rangeof 5 to 50µg/mL with coefficient regression (r^2) =0.9986. Percentage relative standard deviation value is below 2.0 for intraday (n=3) and interday (n=3) precision. The percentage recovery value (average 98.47 %) indicated the accuracy of the method. The proposed method has a short retention time of 1.75 min, which makes the method suitable for the routine analysis of acebrophylline in bulk and capsule dosage form.

Kotkar TA. et al[84] have developed and validated for the simultaneous estimation of Acebrophylline and Montelukast Sodium in bulk and tablet dosage form by Absorbance ratio method. The method involved Q-absorption analysis based on the measurement of absorbance at two wavelengths, i.e λmax of Acebrophylline (310.6 nm) and Iso-absorptive point of both drugs (328.6 nm) using methanol: water (50:50v/v) as a solvent. The method obeys Beer's law in the concentration range of 20-90 µg/ml (r2 = 0.999) for Acebrophylline and 2-18 µg/ml (r2 = 0.999) for Montelukast sodium. The LOD and LOQ were found to be 0.57µg/ml and 1.75µg/ml for Acebrophylline and 1.77µg/ml and 5.37µg/ml for Montelukast Sodium respectively. The recovery of Acebrophylline and Montelukast Sodium were found to be 99.61% and 99.48% respectively showing accuracy of the method. The assay of marketed tablet formulation (Montek- AB) was found to be 99.45% and 99.50% for Acebrophylline and Montelukast Sodium respectively. The method was validated statistically as per ICH guidelines. The method showed good reproducibility and recovery with % RSD less than 2.

Heyden YV. et al[85] has reported RP-HPLC method for the determination of both low endogenous and high therapeutic concentrations of N-acetylcysteine (NAC) in plasma. The compound was detected fluorimetrically after derivatisation with ortho-phthalaldehyde in the presence of a primary amine. Validation of the method revealed injection and method repeatability were good. The linear range was adequate and the limit of quantification was between 0.4 and 0.6 μm. Recovery of N-acetylcysteine from plasma samples was also acceptable. This method was applied to plasma samples from patients with a clinical septic shock who had received very high doses of N-acetylcysteine. Six samples were taken at different times after administration of N-acetylcysteine. The blood-concentration profiles obtained indicate the method is suitable for following the evolution of NAC in plasma under these conditions and can therefore be used for pharmacokinetic profiling.

Aline FO. **et al**[86] have reported reversed-phase liquid chromatography (LC) method with UV detection was developed and validated for the assay of *N*-acetylcysteine (NAC) in granules and effervescent tablets. The method involved the use of the same chromatographic conditions and avoids any derivatization step. The separation was done using a reversed-phase column LC-18, as the stationary phase, and a mixture of 0.05 M KH_2PO_4 and acetonitrile (95 : 5 v/v) containing 0.095% (v/v) of phosphoric acid, as the mobile phase. UV detection was performed at 214 nm and a linear response was observed over the concentration range between 10 and 50 μg mL^{-1} of NAC. The method was revealed to be suitable and was successfully validated in accordance with the official guidelines for specificity, linearity (r = 1), detection and quantification limits (0.70 and 2.11 μg mL^{-1}), precision (RSD < 2%), accuracy (recovery > 97%), and robustness (RSD < 4%). Therefore, NAC can be assayed in granules and effervescent tablets using the same chromatographic conditions without derivatization.

Toussaint B. et al[87] have developed method for the simultaneous determination of N-acetylcysteine and its pharmacopeial impurities, cysteine, cystine, N,N'-diacetylcystine and N,S-diacetylcysteine in an effervescent tablet has been developed. The method was based on on-line LC-UV-MS using a pneumatically-assisted electrospray interface (ionspray). The stability of the thiol moieties of the analytes was ensured by the acidic pH of the LC mobile phase. Quantitation of N-

acetylcysteine was performed with UV detection to avoid ion-source overloading effect due to its higher concentration, whereas the impurities could be easily separated and quantified in MS. The method was validated in terms of stability, linearity, precision and accuracy.

Lu C. et al[88] have reported LC-MS/MS method to determine total N-acetylcysteine in human plasma. Mass spectrometric detection was achieved in positive electro spray ionization and multiple reaction monitoring mode. The mass transition pairs of N-acetylcysteine and the isotope-labeled internal standard d3-N-acetylcysteine were 164 → 122 and 167 → 123, respectively. The method was linear over the range of 10-5000 ng/mL in human plasma. The adoption of trichloro acetic acid significantly enhanced the extraction recovery. The blank matrix was screened to minimize the influence of endogenous N-acetylcysteine. After being fully validated, the method was successfully applied to the pharmacokinetic and bioequivalent study of N-acetylcysteine after oral administration of 600 mg tablets to 24 healthy Chinese volunteers.

Shaikh S. et al[89] have reported the RP HPLC method for the analysis of Acetylcysteine in wet cough syrup dosage form. Gradient system was used with mobile phase consisting of Acetonitrile and 0.05 M phosphate buffer (pH was adjusted to 3.0 + 0.05 by using ortho phosphoric acid), at a flow rate of 0.8 ml/min at ambient temperature. Detection was carried out at 214nm. The retention time was found to be at 4.6 minutes. Force degradation studies were carried out for acidic, alkaline, oxidative, reductive and photolytic exposure of the drug substance drug product. The method was found to be specific for Acetylcysteine and was able to resolve the NAC peak from formulation excipients. The calibration curve was linear over the concentration range of 400-600 μg/ml(R=0.999). The proposed method was applicable to routine analysis of Acetylcysteine in wet cough syrup dosage form.

Tyree HK. et al[90] carried out the physical and chemical stabil ity of repackaged acetylcysteine 600 mg/3 mL solution in oral syringes stored under refrigeration or at room temperature for six months and concluded that Repackaging the solution in syringes in bulk rather than in single doses demonstrated a measurable cost saving.

Ercal N. et al[91] have reported sensitive, rapid method for determining reduced N-acetylcysteine (NAC) concentration in biological samples, which uses a modified reversed-phase high-performance liquid chromatography (HPLC) technique in conjunction with the derivatizing agent N-(1-pyrenyl)maleimide (NPM). The NAC-NPM adduct was analyzed by HPLC with fluorescence detection. The calibration curve for NAC was linear over the range 8-2500 nM and the coefficient of variation obtained for the within-run precision and the between-run precision for 0.5 mM NAC was 1.5% and 2.7%, respectively. Relative recovery of NAC from biological materials ranged between 86% and 96% and the limit of quantitation from biological samples was 32 nM. These results suggest practical advantages relative to other widely-accepted methods of NAC measurement.

Wei W. et al[92] have developed RP-HPLC method for simultaneous detection and quantitation of total cysteine, glutathione, homocysteine and cysteinylglycine in human plasma. The two key steps in the analysis are reduction of disulfides and treatment with 1-benzyl-2-chloropyridinium bromide, which rapidly and quantitatively reacts with thiol groups to form stable S-pyridinium derivatives with intense UV absorption. The derivatives are well separated on a Zorbax SB C(18) column using reversed-phase high-performance liquid chromatography and monitored at 315 nm. The calibration graphs were linear over concentration ranges covering most experimental and clinical cases with a regression coefficients better than 0.999. The detection and quantitation limits for all analytes were 0.2 and 0.5 micromol/L, respectively. The recoveries were 99.25-101.68%. The intra- and inter assay precisions were 0.88-4.24 and 1.68-5.14%, respectively. The method was applied for plasma samples donated by apparently healthy volunteers.

Baeyens W. et al[93] have developed HPLC method by derivatization of acetylcysteine with fluorescent reagent thiolyte monoBromine, here determination of acetylcysteine is done by using aqueous acetic acid-acetone mixtures with a fluorescence detector installed at 380 nm and 470 nm as excitation and emission wavelengths, respectively. Penicillamine is used as an internal standard, and cysteine and homocysteine can be determined with the same system.

Głowacki R. et al [94] reported the new, sensitive, repeatable, and robust high performance liquid chromatography assay method for the determination of total *N*-

acetylcysteine, cysteine, cysteinylglycine, glutathione, and homocysteine in human plasma. The thiol concentrations were measured using precolumn specific derivatization with 2-chloro-1-methylquinolinium tetrafluoroborate, followed by high performance liquid chromatographic reversed phase separation with spectrophotometric detection. The oxidized and protein bound forms of thiols were converted into their reduced forms employing a reductive agent tris(2-carboxyethyl)phosphine. The chromatographic separation was accomplished in 12.5 min; the within run and between run imprecision were all less than 10%. The elution profile was as follows: 0–4 min, 11% B; 4–8 min, 11–30% B; 8–12 min, 30–11% B. The calibration graphs, obtained with the use of normal plasma spiked with growing amounts of analytes, were linear over the concentration ranges, covering most experimental and clinical cases (1–32 μM for homocysteine and glutathione, 1–320 μM for N-acetylcysteine and cysteine, and 1–64 μM for cysteinylglycine). For all analytes, recoveries between 91.2 and 108.6%, were observed

Paraskevas D. et al[95] proposed HPLC method for the determination of N-acetylcysteine (NAC) in pharmaceutical formulations, where analyte was separated from the samples matrix using a 100% aqueous mobile phase [0.05% v/v CH_3COOH+1 mmol/L ethylenediaminetetraacetic acid (EDTA) in water] and a suitable analytical column (Prevail reversed phase column). Detection was carried out at 285nm after on-line post-column derivatization (PCD) with methyl-propiolate (MP) in alkaline medium. Method development included both chromatographic and reaction parameters, while validation was based on international recommendations. The developed analytical scheme excludes the use of organic modifiers from all steps of pretreatment/analysis and offers adequate figures of merit for the quality control (assay and content uniformity) of NAC-containing formulations.

Ogwu V. et al[96] have developed simple colorimetric method for the simultaneous determination and to distinguish between N-acetylcysteine and cysteine. This method is based on the use of the enzyme acylase, which hydrolyzes N-acetylcysteine to cysteine. Cysteine is subsequently measured with a specific colorimetric procedure. Unhydrolyzed N-acetylcysteine gives only a weak colorimetric response (11.5% that for cysteine); after hydrolysis, however, the two are

equivalent. Hence, N-acetylcysteine can be distinguished by the enhanced response after hydrolysis.

Saraswathi D. et al[97] has developed two methods for the estimation of acebrofylline in bulk and pharmaceutical formulations. In first method A is based on formation of red colored chromogen with ferric chloride in the presence of 1,10-phenanthroline and it obeys beer's law in the concentration ranging from 10–60µg/ml and exhibiting maximum absorption at 510nm. While in second method B is based on formation of bluish green colored chromogen with ferric chloride in the presence of potassium ferricyanide and it obeys beer's law in the concentration ranging from 5–40µg/ml and exhibiting maximum absorption at 765nm. These methods have been statistically validated and are found to be precise and accurate.

Mohit DB. et al[98] have reported a method for the Determination of acebrophylline in capsule by using RP-HPLC method with UV-Spectrophotometric detector at 240nm. Standards for calibration graph ranging from 200 to 600 µg/ml from stock solution. The method was accurate with 98.7% recovery value and précised with RSD 0.902. A reverse phase HPLC method with C18 column with ammonium ortho-phosphate (0.01M) acetonitrile-methanol (65:25:10) (pH-7) mobile phase was used and UV-detector was set to 240nm.

Sam Solomon WD. et al [99] have developed HPTLC method for the estimation of Acebrophylline in Pharmaceutical formulation. In this method standard and sample solutions of Acebrophylline were applied on pre-coated 6 x 10 silica gel 60F254 TLC plate, and developed using chloroform: isopropanol: toluene (8: 1: 1 v/v), as mobile phase. A Camag HPTLC system comprising of Camag Linomat -5-applicator, Camag twin trough chamber, Camag TLC-3 scanner was used for the analysis. The drugs on the plate were scanned at 254 nm. The dynamic linearity range was 1Î¼l, 2Î¼l, 3Î¼l, 4Î¼l & 5Î¼l (1000-5000ng/spot) for Acebrophylline. The method was validated for precision, accuracy and recovery.

Atovaquone and Proguanil

Literature survey revealed a number of methods; using various analytical techniques for atovaquone and proguanil. A few of them are mentioned below:

Vijay SV. et al[100] have developed method for the Simultaneous Determination of Atovaquone and Proguanil Hydrochloride in Tablet Dosage Form by High Performance Liquid Chromatography. The chromatographic separation was performed on Supelcosil C8, DB 250 x 4.6 mm, 5 μm particle size. Mobile phase consisted of a mixture of ammonium formate buffer (10 mM HCOONH4, pH adjusted to 3.5 using formic acid) and acetonitrile-methanol (90:10 v/v) in the ratio of 30:70 v/v at a flow rate of 1.0 mL/min. The wavelength was set at 254 nm. The proposed method was validated for linearity, accuracy, precision, LOD and LOQ. The calibration was linear over the range of 125-375 μg/mL for atovaquone and 50-150 μg/mL for proguanil hydrochloride. The retention times were found as 4.2 min for proguanil hydrochloride and 8.3 min for atovaquone. This method can be successfully employed for simultaneous quantitative analysis of atovaquone and proguanil hydrochloride in bulk drugs and formulations.

Adewuyi GO. et al[101] have reported derivatized HPLC method for determination of proguanil after derivatisation with sodium benzoxazole-2 sulphonate. Proguanil was derivatised to its corresponding derivative [(N1-(4-chlorophenyl)-N5-(1-methyl ethyl) imidocarbonimideamide-N-benzoxazole]. The derivatisation reaction was conducted in methanol at 60°C using sodium benzoxazole-2-sulphonate under alkaline conditions. The resulting derivative was extracted with chloroform after which the extract was observed under UV lamp at 254 nm before TLC and HPLC analysis.

Viplava K. et al[102] have developed and validated a method for determination of Atovaquone in API. Atovaquone was found to be degraded under different set of conditions as followed according to ICH guidelines and the degradants so formed along with Atovaquone are separated by using Thermo Hypersil BDS C18, 250mm×4.6mm×5μm column using Buffer: Acetonitrile (20:80) as mobile phase, with a flow rate of 1.5ml/min with a detection wavelength of 283nm with a injection volume of 20μl. The method was validated for specificity, linearity, accuracy, robustness, and precision.

Sahoo S. et al[103] have developed RP-HPLC procedure for the estimation of proguanil and atovaquone in tablet dosage form. Gradient separation was achieved using YMC Pack Pro, C8, 150"4.6mm, 5μ column with a flow rate of 2.0 ml/min

using UV detection at 254nm. The mobile phase A consisted of water (pH 2.0) and mobile phase B consisted of acetonitrile: methanol in the ratio 50:50 v/v. The retention times of proguanil and atovaquone were about 6.0 and 12.0 min. respectively. The method was statistically validated for linearity, accuracy and precision. The linearity of proguanil and atovaquone shows a correlation coefficient of 0.999 and 0.999. The method was reproducible with intra and inter-day variations.

Varsha HC. et al[104] have reported A very simple and selective UV method was developed and validated for the estimation of atovaquone in pure form and the nanosuspension. The adequate drug solubility and maximum assay sensitivity was found in 8.00pH IPA Phosphate Buffer in 40:60 ratios v/v. The absorbances were measured at the λmax. of 494 nm in the wavelength range of 200-800 nm. The linear calibration range was found to be 20-140 μg/ml. The same method was applied and validated for the determination of atovaquone in nanosuspensions.

Kalpesh NP. et al[105] have developed UV spectrophotometric method for the estimation of atovaquone in bulk and tablet dosage form. A solution of drug in methanol showed maximum absorbance at a wavelength of 251nm. The Beer's law is obeyed in the concentration range of 1-10 μg/mL of the drug. The slope and intercept values are 0.111 and 0.012, respectively. Results of analysis of this method have been validated statically and by recovery studies. The method was applied to the marketed tablet formulation. A result of the analysis of tablet formulation, given as a percentage of label claim ± standard deviation, is 99.14 ± 0.66. The precision and accuracy has been examined by performing recovery studies and found to be 100.09 ± 1.14.

Benjamin UE. et al[106] have developed new simple, sensitive, cost-effective and reproducible high performance liquid chromatographic (HPLC) method for the determination of proguanil (PG) and its metabolites, cycloguanil (CG) and 4-chlorophenylbiguanide (4-CPB) in urine and plasma is described. The extraction procedure was a simple three-step process that has eliminated the need for costly extraction and evaporation equipment. The mobile phase consisted largely of buffer, making the method cheap to run. The calibration plots were linear over the concentration range up to 3.0 μg/ml PG, CG and 4-CPB in urine and concentration range up to 1000 ng/ml in plasma. The correlation coefficients (r) were of the order of 0.99 and above for PG and 4-CPB and 0.98 for CG. The ion pair method was carried

out on a 5 μ reversed-phase C-18 column, using perchlorate ion as the counter ion and ultra violet detection at 254 nm. The method was reproducible with coefficient of variation for PG, CG and 4-CPB, being less than 10% in urine and plasma. PG was well resolved from its metabolites, CG and 4-CPB, and the internal standard, pyrimethamine. The limit of detection of PG was 10 ng/ml and the recovery was greater than 90% in urine and plasma.

Bergqvist Y. et al[107] have developed an improved and validated method for the determination of proguanil, cycloguanil, and 4-chlorophenylbiguanide in plasma, whole blood, and urine using solid-phase extraction (SPE) technique and reversed-phase high-performance liquid chromatography (HPLC). The HPLC method uses isocratic elution with acetonitrile:phosphate buffer 0.1 mol/l, pH 2.6 (21.5:78.5 vol/vol) at a flow rate of 1.0 ml/min for the separation. The recovery of proguanil and metabolites ranged from 82% to 104%. The limit of determination was 20 nmol/l for proguanil and its metabolites in plasma and approximately 50 nmol/l for proguanil and metabolites in whole blood.

Lindegardh N. et al[108] have reported bioanalytical method for the simultaneous quantitative analysis of the highly lipophilic atovaquone and the strong basic proguanil with metabolites in plasma. The drugs were extracted from protein precipitated plasma samples on a novel mixed-mode solid-phase extraction (SPE) column containing carboxypropyl and octyl silica as functional groups. The analytes were further separated and quantitated using a steep gradient liquid chromatographic method on a Zorbax SB-CN column with UV detection at 245 nm.

Prasada Rao. M. et al[109] have reported UPLC-UV method used for a quantitative estimation of Atovaquone (AQ) in Rat plasma using 2,3-diphenyl-1-indenone (AQIS) as an internal standard (IS). Chromatographic separation was performed on Waters Acquity UPLC BEH C18, 2.1 x 50 mm, 1.7 μm column with an isocratic mobile phase composed of 0.1% formic acid : methanol (20:80 v/v), at a flow-rate of 0.2 mL/min AQ and AQIS were detected with waters TUV detector at UV wave length 277 (nm). Liquid-liquid extraction method was used and validated over a linear concentration range of 50.0-10000.0 ng/ mL with a correlation coefficient of (r2) ≥ 0.9981. The lower limit of quantification (LOQ), limit of detection (LOD) was found as 50.0ng/ml and 100ng/ml respectively. Intra and inter-

day precision within 1.0 to 8.5 and 1.9 to 4.3 % and accuracy within 101.0 to 107.5 and 103.9 to 104.7 % for AQ. Drug found to be stable throughout three freeze-thaw cycles. This method was successfully applied into a pharmacokinetic study of rats through i.v administration.

Silpa Kala E. et al [110] have developed UV spectrophotometric methods for the determination of atovaquone in suspension. Method A is simple and direct UV spectrophotometric method and is based on determination of atovaquone in methanol and acetate buffer in the ratio 70:30 at 277nm. Method B is first order derivative spectrophotometric method and involved in the estimation of atovaquone in same solvent mixture using the first-order derivative technique at 267nm as maxima and 297nm as minima. Calibration curve was prepared by plotting the absorbance difference between maxima and minima versus concentration. Linearity was obtained in the concentration range of 2-10µg/ml for both the methods. These methods were successfully applied to pharmaceutical formulations because no interferences from formulation excipients were found. The suitability of these methods for the quantitative determination of Atovaquone was proved by validation.

Goswami D. et al[111] have developed LC—APCI mass spectrometric method for human plasma determination of atovaquone using lapachol internal standard. A single-step protein precipitation technique for plasma extraction of atovaquone achieving mean recovery of 94.17% (CV 8%) without compromising sensitivity (limit of quantitation 50.3 ng/mL) or linearity (50.3 ng/mL—23924.6 ng/mL) is delineated in this paper. Heated nebulizer in negative multiple reaction monitoring mode was employed with transitions *m/z* 365.2 → *m/z* 337.1 and *m/z* 240.9 → *m/z* 185.7 for atovaquone and lapachol respectively in this liquid chromatographic–tandem mass spectrometric method. Excellent chromatographic separation on a synergi 4µ Polar-RP 80A (150 × 2.0 mm) column, using 100 µL of plasma extraction volume along with 10 µL of injection load, completing analysis run-time within 2.5 min, highlights this simple yet unique bioanalytical method.

Nandini RP. et al [112] has reported RP-HPLC method for determination of proguanil hydrochloride1 in tablet dosage formulation. Proguanil hydrochloride was found to be degraded under different set of conditions as followed according to ICH guidelines and the degradants so formed along with proguanil hydrochloride were

separated using Kromasil C18, 150 mm × 4.6 mm × 5 μm column using buffer:methanol (45 : 55) as mobile phase, with a flow rate of 1.2 ml/min with a detection wavelength of 254 nm with injection volume of 20 μl. The method was validated for specificity, linearity, accuracy, robustness, and precision.

Julius OS. et al [113]., have reported RP-HPLC method to quantitate plasma levels of proguanil(PGN) and its active metabolite, cycloguanil (CGN) in order to conduct single dose pharmacokinetic studies. The drug and the internal standard were added to plasma samples, vortexed and rendered alkaline with 2 M NaoH and the samples extracted with ether, evaporated to dryness and the residue was reconstituted in methanol, where mixed before injecting an aliquot onto the HPLC system. The calibration plots were linear over the concentration range up to 4.0 μg /ml. The correlation coefficients (r) were of the order of 0.99 and above for both PGN and CGN. The ion pair method was carried out on a 5 μ reverse phase C-18 column, using perchlorate ion as the counter ion and ultra violet detection at 254nm. The method was reproducible with coefficient of variation for PGN and CGN, being less than 4.0 %. PGN was well resolved from its active metabolite, CGN, and the internal standard, pyrimethamine. The limit of detection of PGN was 10 ng /ml and the recovery was greater than 95% in plasma. The analytical method therefore, exhibits good precision and sensitivity in detecting and quantifying PGN and CGN and has been demonstrated to be suitable for the pharmacokinetic studies of proguanil. The clinical applicability of the method was assessed by the preliminary pharmacokinetic study of PGN and CGN, in fifteen healthy Volunteers. The in vivo study was carried out according to a single dose randomized design.

Lindegardh N. et al [114] reported bioanalytical method for the determination of atovaquone in 100 μl blood-spots by solid-phase extraction and high-performance liquid chromatography. Atovaquone was extracted from the sampling paper in 0.2M phosphoric acid and a structurally similar internal standard was added with acetonitrile before being loaded onto a C8 end-capped solid-phase extraction column. Atovaquone and internal standard were analysed by high-performance liquid chromatography on a C_{18} J'Sphere ODS-M80 (150×4.0 mm) column with mobile phase acetonitrile–phosphate buffer, 0.01M, pH 7.0 (65:35, v/v) and UV detection at 277 nm. The intra-assay precision was 2.7% at 12.00 μm and 13.5% at 1.00 μm. The

inter-assay precision was 3.3% at 12.00 μm and 15.6% at 1.00 μm. The lower limit of quantification was 1.00 μm. The limit of detection was 0.50 μm

Khagga BV. et al [115] have reported RP-UPLC method for the simultaneous determination of proguanil and Atovaquone in combination. The separation was carried out using a mobile phase consisting of 0.05% perchloric acid and Acetonitrile. The column used is extended C18 (50 × 3.0 mm, 1.8 μm). using flow rate of 0.8 ml/min using UV detection at 257nm. The total run time is 6 min and the retention time of proguanil and Atovaquone is 1.6 min and 3.0 min respectively. The described method is linear for the assay of proguanil and Atovaquone over a concentration range of 2.5-20 μg/ml and 6.25-50 μg/ml respectively. Results of the analysis have been validated statistically and by recovery studies. The limit of quantitation for proguanil and Atovaquone has been found to be 0.0357μg/ml and 0.126 μg/ml respectively. The results of the studies showed that the proposed RP-UPLC method is simple, rapid, precise, and accurate, which is useful for the routine determination of proguanil and Atovaquone in bulk drug and its pharmaceutical dosage form.

Patil SD. et al[116] has developed RP-HPLC method for the estimation of atovaquone and proguanil in tablet dosage form. Separation was achived on an Inertsil (ODS) 250×4.6mm, particle size 5μm, C18 column using isocratic composition of methanol as mobile phase A and 0.02M phosphate buffer as mobile phase B in ratio of 80:20 at a flow rate of 1ml/min with detection at 260 nm. The retention times of the ATV and PRO was about 10.62 and 3.83 min. respectively. The detector response is linear from 50-350μg/ml and 20-120μg/ml of test concentration for atovaquone and proguanil respectively. The method was validated by determining its linearity, accuracy and precision. The proposed method is simple, fast, sensitive, linear, accurate, rugged and precise hence can be applied for routine quality control in bulk and in tablet dosage form.

Lawsone and p-phenylenediamine, L-dopa

Many analytical methods are reported for the individual estimation of lawsone or p-phemylenediamine or L-dopa, or their combination with other herbal actives. From the literature survey it was observed that HPLC was the most common method employed for analysis and very few reports are available for the estimation of

lawsone, p-phenylenediamine L-dopa using HPTLC. A few of the reported methods are mentioned below:

M. S. Karawya et al[117]., isolated lawsone from the species ***Lawsonia inermis*** by maceration and percolation with saturated sodium bicarbonate solution, acidification with dilute hydrochloric acid and successive extraction with chloroform and estimated the percentage content of lawsone by direct colorimetric method and colorimetric TLC method with

Stationary phase : Silica gel G-Aluminium oxide G plates

Mobile phase : Ethyl acetate: Methanol: 5N Ammonium hydroxide

60 : 15 : 5

Lawsone content was found to be 0.6% by this method**.**

Johan G. van Damme et al[118]., reported three methods for the fluorescence assay of hydroxynaphthoquinones.

Method 1**:** It is based on the prior sodium dithionite reduction and determination of the fluorophore in butyl acetate and is applicable to all hydroxy naphthoquinones.

Method 2**:** It involves warm reduction with stannous chloride in an acid medium and determination of the fluorophore in chloroform and is specific for 5-hydroxy naphthoquinones (Eg. Juglone).

Method 3**:** It is based on the reaction of Guilbault and Kramer and is applicable for hydroxynaphthoquinones having both free quinonoid positions like 5-hydroxy naphthoquinones and 5,8-dihydroxynaphthoquinones.

A. Lobstein et al[119]., developed and validated an RP-HPLC method for the quantitative determination of 2-hydroxy 1,4-naphthoquinone (lawsone) and its methylated derivative in the flowers of Impatients glandulifere (Balsamaceae) collected between July and August and found out that I. glandulifera has the maximal content of 0.8-1.1% which is 2.5-37 times higher level that the three other species compared with.

Renee Jelly et al[120]., used lawsone (2-hydroxy 1,4-naphthoquinone) as a novel reagent for the detection of latent finger marks on paper surfaces. The fingermarks yield purple-brown impressions which are also photoluminescent.

Lawsone represents the first in a completely new class of fingermark detection reagent.

Jallad K. N. et al[121]., evaluated a number of spectroscopic techniques used in identifying and quantifying the presence of lead in twelve commercial and traditional henna samples. The lead levels found in henna were low with concentration ranging from 2.29 ppm to 65.98 ppm.

Hao Wu et al[122]., conducted spectrophotometric determination of anilines base on charge-transfer reaction and the molecular interactions between aniline, p-toluidines, benzidine and p-phenylenediamine as electron donors and 7,7,8,8-tetracyano quinodimethane as acceptor have been investigated. These methods were applied successfully for the determination of the studied compounds in waste water and relative standard deviation of the methods were 0.8-30 % and percentage recoveries ranged from 97.22 to 102.78%.

M. T. Galceran et al[123]., carried out the determination of hydroxy polycyclic aromatic hydrocarbons by LC-MS and compared the Atmospheric Pressure Chemical Ionization and Electrospray. LC-MS detection limits (signal to noise ratio 3:1) were determined for APCI and ESP in both ionization modes, ranging from 1.5- 35 µg/ml for ESP and from 0.3 -50 µg/ml for APCI.

H. E. Cox[124] worked on the chemistry and anlaysis of henna of hair dyes and reported about the chemical constituents of henna and chemical reaction of Lawson. He also determined lawsone in crude drug and in henna hair dyes by titrimetry and colorimetry. The percentage content of lawsone in these of henna powders were found to be 1.21, 1.23 &1.08.

Petr Babula et al[125]., worked on the simulataneous analysis of 1,4-naphthoquinone, lawsone, juglone and plumbagin in *Dionaea muscipula* by HPLC-DAD technique using 0.1 mol l^{-1} acetic acid : methanol in the ratio 35:65 as the mobile phase with the flow rate of 0.8 ml/min and temperature of 40ºC and auto sampler injection volume of 0.5 µl. It was reported that the recoveries of determined naphthoquinones were from 96-104 %. Linearity is obtained in the concentration ranging from 1.7 to 25 µg/ml.

El-Shaer NS et al[126]., quantitatively evaluated the lawsone content in the phenolic-chloroform soluble fraction of eight commercial henna powders and two collected henna leaves by HPTLC. The examined samples showed considerable variation in lawsone concentration ranging from 0.004-0.608 wt% along with other pigments indicating that some samples were almost devoid of lawsone.

B. M. Badri et al[127]., worked on the dyeing of wool and nylon 6.6 with henna extract and lawsone and employed HPLC technique to determine the percentage content of lawsone and reported it to be 0.5 % along with a high proportion of another colourant.

Lin Zhang et al[128]., analysed the five monocyclic aromatic amines like p-toluidine, aniline, 3,4-dimethyl aniline, N-ethyl-p-toluidine and p-phenylenediamine using HPLC with pre-column fluorescence derivatisation by a novel reagent 2(9-Carbazole)-ethyl-chloroformate. This results in improved peak shapes without the addition of any competition amines, good quantitative precision with high tolerance of the matrix of samples.

Brancaccio RR et al[129]., performed HPLC for the identification and quantification of p-phenylenediamine(which develop contact allergy) in various commercially available henna powders, hair dye products and black henna tattoo mixtures. This study demonstrated that p-phenylenediamine was present in the black henna tattoo mixture at a concentration of 15.7 % which is significantly higher than the commercial hair dye preparations.

Yashiaki Ikarashi et al[130]., determined p-phenylenediamine and related anti-oxidants in rubber boots by HPLC with UV-VIS detector using methanol : water (85:15) as the mobile phase. The adopted flow rate is 1.0 ml/min with column temperature of 35^{o}C and detection at 290 nm.

B. H. Shao et al[131]., employed RP-HPLC method for the quantitative determination of p-phenylenediamine, p-aminophenol, m-aminophenol, resorcinol & 2,6-diamino pyridine in two types of commercial oxidation hair dyes using µ-Bondapak C_{18} column with methanol : aqueous solution containing 0.1 %

triethylamine and 0.02 mol/litre ammonium acetate(pH = 5.20) in the ratio of 10:90 v/v as the mobile phase.

Maria Luisa Di Gioiaa et al[132]., performed GC-MS technique for the determination of p-phenylenediamine in hair dyes after converting it into an imine derivative by treatment with benzaldehyde in SIM mode. It involves the use of N-benzylidene-4-methylbenzeneamine as the internal standard. The calibration curves for p-phenylenediamine in hair dyes are linear within 0.1±25 mg/ml with 0.99 as correlation co-efficient. This allows the estimation of concentration levels of p-PD upto 0.05 mg/ml.

Natalia A. Penner et al[133]., carried out the simulataneous determination of dihydroxy benzenes (pyrocatechol, resorcinol, hydroquinone), aminophenols (o-, m- & p-aminophenols) and p-phenylenediamines in three different hair dyes by HPLC on hypercross-linked polystyrene. The influence of separation parameters such as concentration of acetonitrile, buffer(citrate, phosphate) concentration, ionic strength and pH of the eluent on their retention were also investigated. The detection limits on these compounds are in the range in 0.05 – 0.16 µg/ml.

L J Marton et al[134]., developed an automated micromethod for the quantitative analysis of diamines and polyamines in biological fluids utilising a sensitive high pressure liquid chromatographic procedure.

Kang IJ et al[135]., quantified p-phenylenediamine and heavy metals such nicket, cobalt, chromium, lead and mercury in fifteen henna dye samples available in Korea by HPLC, AAS, mercury analyzer and ICP emission spectroscopy. p-PD, nickel and cobalt were detected in 3, 11 & 4 samples respectively.

N Seiler et al[136]., carried out the determination of diamines and polyamines by HPLC separation of their 5-dimethyl aminonaphthalene-1-sulfonyl derivatives using Lichrosorb RP-8 reverse phase column and a methanol : water gradient elution program. The separation can be achieved within 40 minutes and it can determine routinely even in picomole quantities of the natural di- and polyamines in tissues and body fluids.

M. C. Garrigos et al[137]., employed HPLC method with UV detection for the determination of aromatic amines released after reductive cleavage of the azo colourants mostly used in toys. Sodium dithionite is used as the reducing agent. The expected aromatic amines forming azo colourants were detected, and in the presence of a nitro group, a further reduction was observed. The yield of total reduction process was determined by using standard addition of different quantities of amines to the colourants.

V. Anridsano et al[138]., analysed the basic hair dyes by HPLC with on-line post-column photochemical derivatisation and this enabled the unambiguous identification of both commonly used, approved and banned basic hair dyes. This method was found to have high sensitivity so that it can determine the analytes present even in low concentration of 0.03 % in complex commercial formulations.

Rodrigues SV et al[139]., determined the solubility of 1,4-naphthoquinone, plumbagin, lawsone and juglone in supercritical carbon dioxide by UV/Vis spectroscopy at a temperature of 40C and a pressure range of 8-18 MPa. The solubilities at 12 MPa were between 0.3 and 10 gms/litre.

Carlos Frontana et al[140]., worked on the effects of molecular structure on the electrochemical properties of naturally occurring hydroxyquinones (2-hydroxy 1,4-naphthoquinone, Perezone, Horminone & 7-o-methyl Conacytone). The electrochemical study reveals the reduction mechanism occurring at the first volatmmetric reduction signal which is determined by the presence of self-protonation reactions. As during the self-protonation process an important amount of deprotonated quinine is produced, the spectroelectrochemical study at a potential region at the second reduction process showed well resolved ESR spectra, corresponding to the dianion radical structures originated from the reduction of the deprotonated anions.

Archana PR. et al[141] have reported HPTLC method for the quantification of L-DOPA obtained from the seeds of muccna. L-DOPA separated on precoated silica gel 60 GF_{254} HPTLC plates using a solvent system of n-butanol-acetic-acid-water (4:1:1, v/v) as the mobile phase at 280 nm. Linearity was found in the concentration range of 100 to 1000 ng/spot with the correlation coefficient value of 0.9980. The method was validated for accuracy, precision and repeatability. Mean recovery was

100.89%. The LOD and LOQ for L-DOPA determination were found to be 3.41 ng/spot and 10.35 ng/spot respectively.

Gulendem G. et al[142] have developed a new TLC method for separation and quantitation method for L-tyrosine and L-Dopa mixture. The minimum tyrosine and Dopa quantities which can be measured by this technique are 0.7 and 1.5 µg respectively. The method may be used to measure the kinetic parameters of polyphenoloxidase as well as to trace the enzyme catalyzed conversion of L-tyrosine into L-Dopa. For a set of 20 measurements the maximum difference between any two measurements of spot areas was found to be 3 mm2 (4.5%) for L-Dopa (4 µg) and 2mm2 (5.6%) for L-tyrosine (1µg).

Raman M. et al[143] have developed HPLC method for the quantification of levodopa in methanolic extract of *Mucuna utilis*. The method separation was carried out on a Eurosphere C18, 250 X 4.0 mm, 5 ˝m particle size column, using methanol and 0.5% v/v acetic acid in the ratio of 70:30 v/v as the mobile phase. Detection wavelength selected was 284 nm. The method was validated in terms of linearity, precision (inter and intra day), accuracy, Limit of Detection (LOD) and Limit of Quantitation (LOQ). The proposed HPLC method was found to be precise, specific, accurate and can be used for the identification and quantitative determination of levodopa in herbal extracts.

Mohan MR., et al[144] have reported quantification of L-dopa by TLC. The amount of L-DOPA, lupeol and β-sitosterol quantified from the leaves of *C phlomidis* were 0.06806, 0.01733 and 0.06324 % w/w, respectively. TLC procedure used effectively for identity, quality evaluation as well as quantitative determination L DOPA and its derived products.

Paresh BS. et al[145] have developed a spectrofluorimetric analysis of L-dopa in Mucuna pruriens seed extract and its formulations. The excitation and emission wavelength were found to be 282 and 630 nm respectively in 0.1 N formic acid. The relationship between the concentration (ng/ml) of L-dopa and corresponding fluorescence intensity (FI) was found to be linear in the range of 30-800 ng/ml. The method was validated for precision (inter and intraday), repeatability, and accuracy. Mean recovery was 99.94%. The relative standard deviation (RSD) values of the

precision were found to be in the range 0.58-0.95%. The proposed method was found to be precise, specific and accurate and can be used for identification and quantitative determination of L-dopa in herbal extract and its formulations.

3. AIMS & OBJECTIVE

The concept of drug treatment, which was earlier *"right drug for right person"* is now changing from *"right dose for the right person"* to *"right time of the dose for the right person"*.

As with time the concept of drug treatment has been changed continuously and nowadays similarly a drug treatment is also changed from the mono drug therapy to the multi component drug therapy and hence today's market is flooded with the combination of drugs in the various dosage forms.

The multicomponent drug therapy has gained a lot of importance nowadays, due to greater patient acceptability, increased potency, multiple actions, fewer side effects and quicker relief.

The contributions of herbal products to the field of medicines after 1950 have been outstanding. The herbal plants have enormous commercial potentials throughout the world. In addition, they are also the source of chemical intermediate needed for the production of some drugs. Hence there is great demand for the plant derived product in the developed country.

It is fact that analysis of single drug formulations is quite easy as compared to multicomponent drug formulations having more than one drug components either by UV-visible spectrophotometry as well as HPLC and HPTLC method. The analysis of such multicomponent formulation is generally time consuming and tedious due to interferences from the excipients and also due to incomplete extractions. Therefore it is very essential to develop simple, rapid and accurate instrumental methods like UV spectrophotometry and very sensitive HPLC and HPTLC methods for the multicomponent drug formulations in the markets.

Drug combinations recently introduced into the market, were selected for the analytical method development and validation by UV Spectrophotometric, HPLC and HPTLC methods. A thorough survey of literature revealed that the selected combinations were not official in any of the pharmacopoeias and so far, no efficient

analytical methods are available for the simultaneous determination in their combinations. There are only few methods available for single drug and combination with other drugs but no method available for the estimation of the selected combination dosage forms by RP-HPLC, HPTLC and UV Spectrophotometry; moreover, many of them suffer from one disadvantage or the other, such as low sensitivity, lack of selectivity and simplicity.

So, an attempt is made in this research to develop a rapid, economical method for the simultaneous estimation of the selected combinations and also validation of new analytical methods according to ICH norms.

Selected combinations are

- Losartan potassium 50mg &chlorthalidone 6.25mg
- Atovaquone 250mg &Proguanil 100mg
- Acebrophylline 100mg &Acetylcysteine 600mg
- Lawsone&para-phenylenediamine
- L-dopa

For above selected formulations an attempt is made in this research to develop a rapid, economical method for the simultaneous estimation include

1. To develop the method for the simultaneous estimation by HPLC
2. To establish the stability of the formulations by performing forced degradation studies as per ICH guidelines by HPLC.
3. To develop the method for the simultaneous estimation by HPTLC
4. To develop the method for the simultaneous estimation by UV Spectrophotometry
5. To validate all developed methods as per ICH and USP norms for the following parameters:
   - ✓ Accuracy
   - ✓ Precision (Repeatability And Reproducibility),
   - ✓ Specificity,
   - ✓ Linearity and Range,
   - ✓ Limit of Detection (LOD)/Limit of Quantitation (LOQ),
   - ✓ Selectivity/Specificity,
   - ✓ Ruggedness/Robustness,
   - ✓ Stability
   - ✓ System Suitability.

4. SCOPE AND PLAN OF WORK

The quality is important in every product or service but it is more vital in medicine as it involves life. Nowadays, manufacturing trends have been change to manufacture more or more complex formulation containing several drugs with very similar chemical behavior. To device an accurate estimation procedure for each ingredient of such multicomponant dosage form containing several therapeutically active drugs is not an easy task, as they are present in widely divergent concentrations and contains additives. The presence of additives, excepients and decomposition products further complicates the development of analytical procedures.

The conventional analytical (titrations, gravimetry) methods were sufferes to get desired intended specifications as per regulatory guidelines during the analysis of the formulation. Moreover, they lack sensitivity, selectivity, specificity, accuracy and repeatability.

Hence, in the present research work, we selected modern analytical methods like, HPLC, HPTLC, and UV Spectroscopic method which are found to be more reproducible, sensitive and specific and rapid. These methods have wide scope to overcome with all the drawbacks and insufficiency of conventional analytical methods.

Therefore the method to be developed will have wide scope in the estimation of the drug in the selected multicomponent formulations.

Literature surely revealed that assay methods by using simple spectroscopic and chromatography techniques like UV, HPLC, HPTLC are available for losartan potassium and chorthalidone alone or with other formulations involving these two drugs with other drugs but no chromatographic method is reported for simultaneous estimation of losartan potassium and chlorthalidone from tablet formulation.

Similarly number of methods have been reported either for estimation of acebrofylline, acetylcysteine, atovaquone and proguanil separately and as such no

method of estimation using chromatographic techniques such as HPLC and HPTLC and UV spectroscopic are reported for simultaneous estimation of acebrofylline and acetylcysteine or for atovaquone and proguanil from capsule and tablet formulation respectively.

Compounds from natural sources have been gaining importance in recent years due to the enormous chemical diversity that they offer. This has led to a tremendous increase in the demand for herbal medicines. One of the methods for quality assessment is phytochemical evaluation which includes preliminary screening, chemo-profiling and analysis of marker compound using modern analytical techniques like HPLC, HPTLC, SFC and so on. High-performance thin layer chromatography (HPTLC) has emerged as a simple, reliable, and efficient method for simultaneous analysis of two or more components.

Literature survey revealed that there were several reports for individual estimation of the phytoconstituents lawsone and p-phenylenediamine however no analytical method was reported for simultaneous estimation of these phytoconstituents in combination using HPTLC.

In view of above, rapid, reliable, economical and accurate chromatographic methods (HPLC and HPTLC) were needed to be developed for the synthetic and herbal multicomponent formulations in there dosage forms:

i. Losartan potassium and chlorthalidone (Tablet Dosage form)
ii. Acebrofylline and acetylcysteine (Capsule Dosage form)
iii. Atovaquone and proguanil (Tablet Dosage form)

And for the phytochemical evaluation of the herbal formulation containing

iv. Lawsone and p-phenylenediamine (herbal hair dye)
v. L-dopa in Zandopa (herbal powder)

Thus, the objective of work was to develop and validate chromatographic and spectroscopic methods for the simultaneous estimation of above listed combinations from the respective tablet or herbal formulations

Plan of work

The project was carried out in the following stages:

Stage I: Considerations of flowing parameter for HPLC method Development

- Solubility studies and physical and chemical properties of drug
- Selection of solvents/
- Selection of type of chromatographic separation
- Selection of detector based upon the property of the drug

Stage II: Optimization of the method

- Selection of wavelength
- Selection of initial separation conditions
- Nature and selection of stationary phase
- Nature of mobile phase (pH, peak modifier, solvent strength, ratio & flow rate)
- Sensitivity and Specificity of method

Stage III: Optimized HPLC method validated for the ICH parameter

Stage IV: Forced degradation study for the selected developed methods by using stress conditions like acid, alkali, peroxide, heating, and uv light exposure.

Stage V: Development of UV spectrophotometric methods like

- Selection of solvent
- Determination of λ max
- Absorbance of different concentration obeying beer's law

Stage VI: UV method validated for the ICH parameter

Stage VII: Development of HPTLC method for drugs in herbal formulations

Stage VIII: HPTLC developed method validated for ICH parameter

The various validation parameters are

- Accuracy.
- Precision (repeatability and reproducibility),
- Linearity and range,
- Limit of detection (LOD)/ Limit of quantitation (LOQ),
- Selectivity / Specificity,
- Robustness/ Ruggedness,
- Stability and system suitability.
- Robustness and
- Stability and system suitability studies

5. MATERIALS AND METHODS

A. Materials

I. Instruments

i. Single pan digital balance (M500P) - Sartorious

ii. pH meter - Thermoscientific

iii. Ultra sonicator - Systronics

iv. UV Double Beam Spectrophotometer with following specifications

- PG Instruments T60 model
- UV win Software 5.1.1 version.

v. Waters 2695 HPLC with following configurations

- quaternary pump,
- Auto sampler,
- Auto injector,
- PDA 2996 Photo diode array Detector
- Empower V.1.2 software was used for data processing

vi. Analytical column used following specifications

- Agilent XDB C_{18} (150×4.6mm×5µ)
- Hypersil BDS C_{18} (150×4.6mm×5µ)
- Hypersil BDS C_{18} (100×4.6mm×5µ)

vii. Camag HPTLC with following configurations

- Linomat 5 sample applicator
- CamagTwin trough Chamber
- Camag TLC Scanner 3 (Densitometry)
- CamagWinCATS software version 1.3

II. Drug Standards

Drug Standard	Procured from
Atovaquone (99.95%)	Glenmark Pharmaceuticals Ltd.Mumbai.
Proguanil (99.97%)	Glenmark Pharmaceuticals Ltd.Mumbai.
Losartan (100.2%)	SL Drugs & Pharmaceuticals, Hyderabad.
Chlorthalidone (100.3%)	SL Drugs & Pharmaceuticals, Hyderabad
Acebrophylline (99.67%)	SL Drugs & Pharmaceuticals, Hyderabad.

Acetylcysteine (99.69%)	SL Drugs & Pharmaceuticals, Hyderabad.
Lawsone 97% (2hydroxy,1,4 naphoquinone)	Icon Biosystem equipments and fine chemicals, Hyderabad.
Para phenylenediamine	Icon Biosystem equipments and fine chemicals, Hyderabad.
L-dopa	Sigma Aldrich.

III. Reagents and Chemicals

Chemicals	Manufacturer
Methanol (HPLC Grade)	Merck specialities Pvt. Ltd, Mumbai
Acetonitrile (HPLC Grade)	Rankem India limited, Ankleshwar
Orthophosphoric acid (AR Grade)	RFC limited, New Delhi
Triethylamine (AR Grade)	SDFCL Limited, Mumbai
Potassium dihydrogen orthophosphate (AR Grade)	Merck specialities Pvt. Ltd, Mumbai
Sodium hydroxide (AR Grade)	SDFC Limited, Mumbai
Hydrochloric acid (AR Grade)	Merck specialities private limited, Mumbai
Concentrated Sulfuric Acid (AR Grade)	Merck specialities private limited, Mumbai
Milli Q water (HPLC Grade)	

IV. Marketed Formulations

Commercial preparation	Component	Manufacturer
CTD-L tablets	Losartan potassium 50mg & chlorthalidone 6.25mg	IPCA Pharmaceuticals ltd.,
MALARONE tablets	Atovaquone250mg& Proguanil 100mg	GlaxoSmithKline Pharmaceutica ltd.,
PULMOCLEAR capsules	Acebrophylline 100mg&&Acetylcysteine 600mg	Fourrts(India) Laboratories Pvt. Ltd.,
Indica herbal hair dye	Lawsone& para-phenylenediamine	ClavinKare Pvt. Ltd.
Zandopa	L-dopa	Zandu Pharmaceuticals Pvt. Ltd

B. Methods

The analytical method is developed and validated for the quantification of individual drug in the selected herbal and synthetic multicomponent drug formulations. The synthetic multicomponent drug formulations includes CTD-L tablets (contains Losartan Potassium 50mg and Chlorthalidone 6.25mg Manufactured by IPCA Pharmaceuticals ltd.,) MALARONE tablets (contains Atovaquone 250mg and Proguanil 100mg Manufactured by GlaxoSmithKline Pharmaceuticals ltd.,) PULMOCLEAR capsules (contains Acebrophylline 100mg and Acetylcysteine 600mg Manufactured by Fourrts (India) Laboratories Pvt. Ltd.,) were used for HPLC, Forced degradation studies by HPLC, HPTLC and UV spectrophotometric method development and validation as per ICH and USP guidelines. The herbal marketed product of Henna mehandi power and Indica herbal hair dye are used for estimation of Lawsone and para-phenylenediamine by HPTLC and validated as per ICH guidelines. The Zandopa used for estimation of L-dopa by HPTLC and validated as per ICH guidelines.

The validated analytical method development was divided into following parts

Part 1: Development of HPLC methods for Seleted multocomponents

Part 2: Developed HPLC methods are validated as per ICH/USP guidelines for different validation parameter

Part 3: Forced degradation study carried out for different stress conditions likes

- Acid degradation
- Alkali degradation
- Peroxide degradation
- Thermal degradation
- Photo degradation

Part 4: Development of UV Spectrophotometric methods Seleted multocomponents

Part 5: Developed UV Spectrophotometric methods are validated as per ICH/USP guidelines for different validation parameter

Part 6: Development of HPTLC methods Seleted multocomponents

Part 7: Developed HPTLC methods are validated as per ICH guidelines for Validation parameter

Part8: Development of HPTLC method for Harbal formulations

5.1 Part 1.0: RP-HPLC Method Development

During the optimization stage, the initial sets of conditions that have evolved from the first stages of development are improved or maximized by selection of wavelength, selection of initial separation conditions, selection of column i.e. stationary phase nature, selection mobile phase include organic modifier ratio, solvent strength, and flow rate etc. in terms of resolution and peak shape, also plate counts, asymmetry, capacity factor, elution time, detection limits, limit of quantitation and overall ability to quantify the specific analyte of interest.

General Consideration for developing RP-HPLC Method

Following are the common steps should be considered during RP-HPLC method development for the selected multicomponent drug formulations.

Selection of Chromatographic mode of separation:

Selection of chromatographic method in general is done by taking into consideration the several parameters like the nature of the drugs, molecular weight and solubility. Since most of the drugs selected are polar in nature, so the reversed phase chromatography has been used for the development and validation of the selected multicomponent formulations.

Selection of mobile phase

The mobile phase composition, buffer mixture and diluents solvents were selected based on the solubility test results, stability and suitability in order to achieved separation and well resolution with sharp peaks. The solubility tests were performed using the common solvents like water, methanol, acetonitrile, THF, 0.1N HCl, 0.1N NaOH, The sample retention time were adjusted by varying the solvent strength. As a strong solvent reduced retention time and weak solvent increases retention. By changing the proportion acetonitrile, methanol, THF, temperature and pH gives change in the method and desired separation. Hence mixture of solvents were selected for selected combination of drug in different ratios like 70:30, 90:10, 50:50 and 70:30 in isocratic and gradient mode used.

Selection of Stationary phase

The selection of stationary phase always depends upon the sample nature and composition of the mobile phase in order to achieve optimization in method development process. Systematic reproducible changes in selectivity were achieved by changing the column from cyno or phenyl to C_{18} columns and also by changing the source of the

column i.e column manufacturer. In this Studies, Agilent XDB and Hypersil BDS C_{18} columns were used for the selected combinations of drugs.

Selection of detector and wavelength:

Before selecting the wavelength, it is important to select the right detector and the sensitivity of the developed method is depend upon the selected wavelength. Hence, 2996 Photo diode array Detector was selected to detect the anlytes ate ideal wavelength. The ideal wavelength gives good response for the analytes presents in the formulation. In order to ascertain the wavelength of maximum absorption (λ_{max}) of the drug, 10µg/ml solution of the drugs in different solvent were scanned for different selected combinations using UV spectrophotometer within the wavelength region of 190–400nm.

5.1.1 Method Development: Losartan and Chlorthalidone

From the knowledge of properties of the selected drugs and above consideration the following initial conditions were set and then were optimized by making deliberate changes in the parameters like flow rate, injection volume, working concentration and varying mobile phase composition. Before fixing the final optimized chromatographic conditions the numbers of trials were run, following are the few trials run were shown in different trials with **Fig. No 9.0 to Fig. No.13.0**

Trial -1

Chromatographic conditions

Column	:	Agilent XDB, C18, 150 x 4.6 mm, 5µ.
Mobile phase	:	Buffer : Acetonitrile (90:10 % v/v pH 3.5)
Detector	:	PDA detector
Flow rate	:	1.0ml/min
Detection wavelength	:	254nm
Injection volume	:	10µl
Temperature	:	30^0c
Run time	:	10min

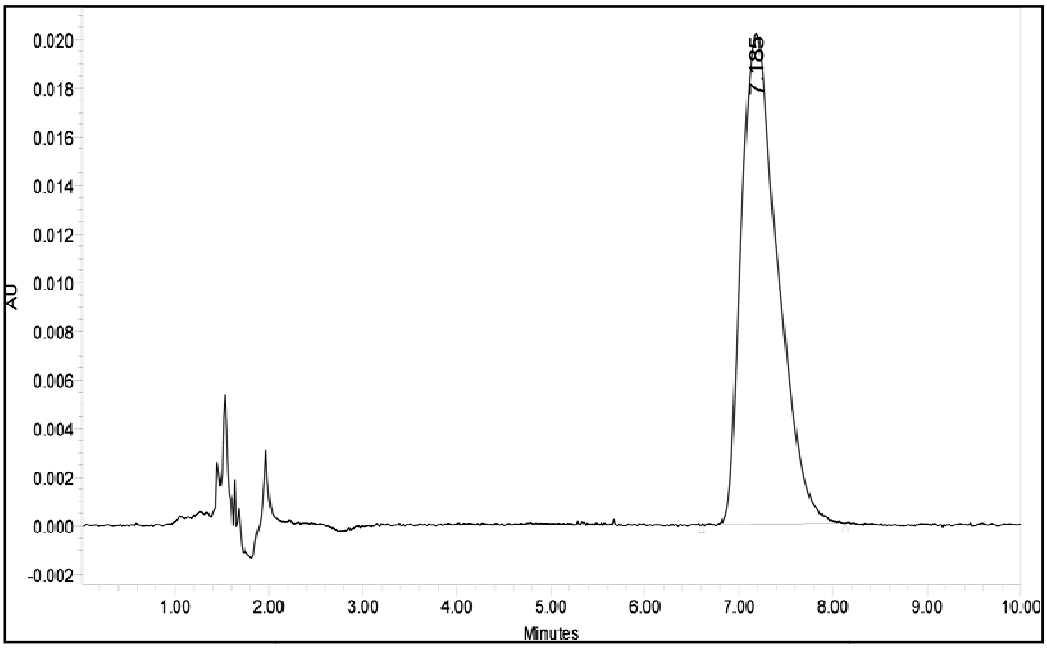


Fig. No. 9.0: Trial 1 chromatograms for losartan and chlorthalidone

Sr.No	Peak Name	RT	Area	% Area	USP Plate count	Tailing
1.		3.191	515943	100	1784	1.61
2.		-	-	-	-	-

Only one peaks were observed in the chromatogram. Plate count reduced.

Hence this method was not suitable for the simultaneous estimation of Losartan and Chlorthalidone.

Trial -2

Chromatographic conditions

Column	:	Agilent XDB, C18, 150 x 4.6 mm, 5µ.
Mobile phase	:	Buffer : Acetonitrile (80:20 % v/v pH 3.5)
Detector	:	PDA detector
Flow rate	:	1.0ml/min
Detection wavelength	:	254nm
Injection volume	:	10µl
Temperature	:	30^0c
Run time	:	15min

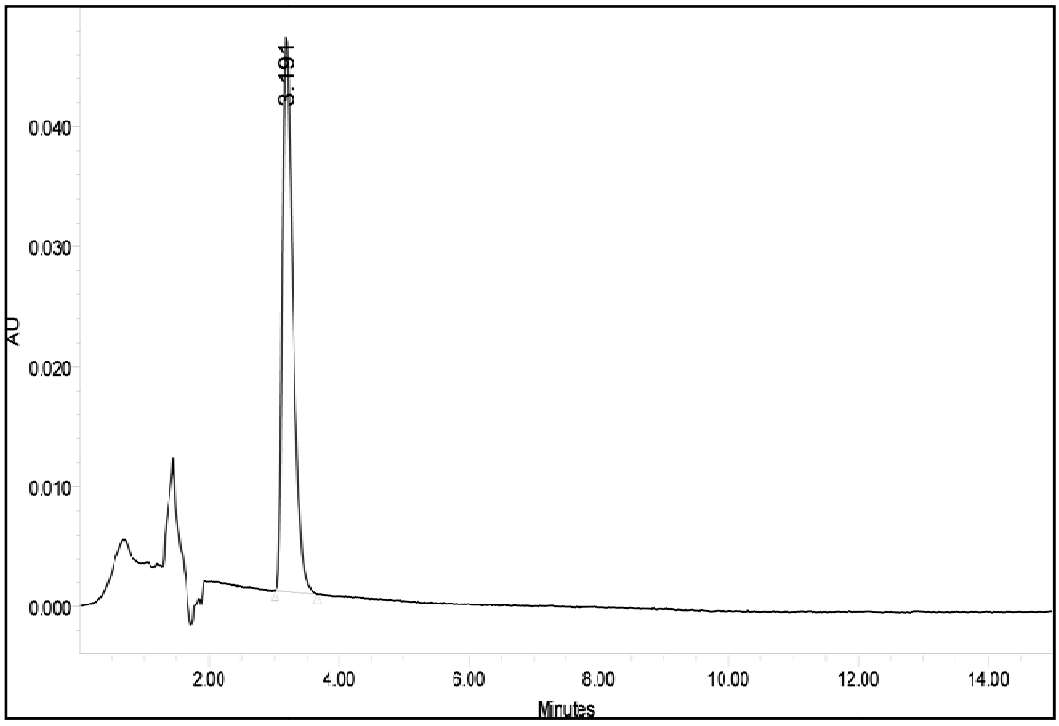


Fig. No. 10.0: Trial 2 chromatograms for losartan and chlorthalidone

Sr.No	Peak Name	RT	Area	% Area	USP Plate count	Tailing
1.		3.191	515943	100	1784	1.61
2.		-	-	-	-	-

Only one peaks was observed in the chromatogram, No separation of two components was observed.

Hence this method was not suitable for the simultaneous estimation of Losartan and Chlorthalidone.

Trial -3

Chromatographic conditions

Column	:	Agilent XDB, C18, 150 x 4.6 mm, 5µ.
Mobile phase	:	Buffer : Acetonitrile (75:25 % v/v pH 3.5)
Detector	:	PDA detector
Flow rate	:	1.0ml/min
Detection wavelength	:	254nm
Injection volume	:	10µl
Temperature	:	30^0c
Run time	:	15min

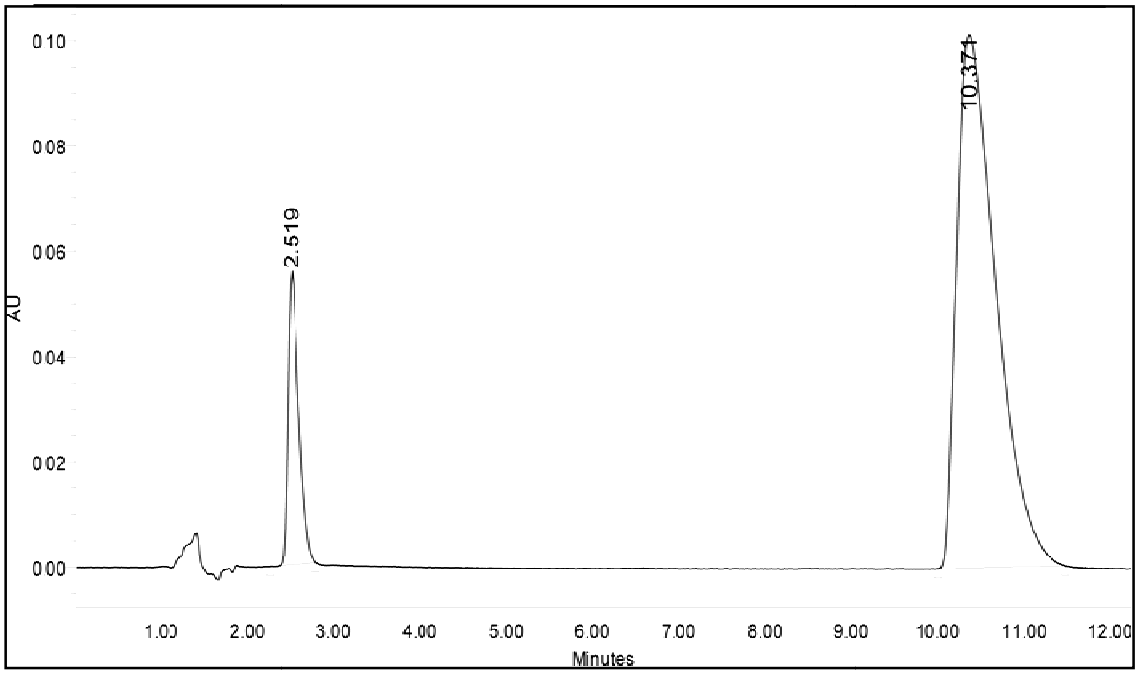


Fig. No. 11.0: Trial 3 chromatograms for losartan and chlorthalidone

Sr.No	Peak Name	RT	Area	% Area	USP Plate count	Tailing
1.	Chlorthalidone	2.519	503878	13.81	1674	1.55
2.	Losartan	10.371	3145698	86.19	2452	1.95

The two peaks were well resolved with good resolution but Peak Retention times were very long so run time is more with increase in tailing.

Hence this method was not suitable for the simultaneous estimation of Losartan and Chlorthalidone.

Trial -4

Chromatographic conditions

Column	:	Agilent XDB, C18, 150 x 4.6 mm, 5µ.
Mobile phase	:	Buffer : Acetonitrile (65:35 % v/v pH 3.5)
Detector	:	PDA detector
Flow rate	:	1.0ml/min
Detection wavelength	:	254nm
Injection volume	:	10µl
Temperature	:	30^0c
Run time	:	10min

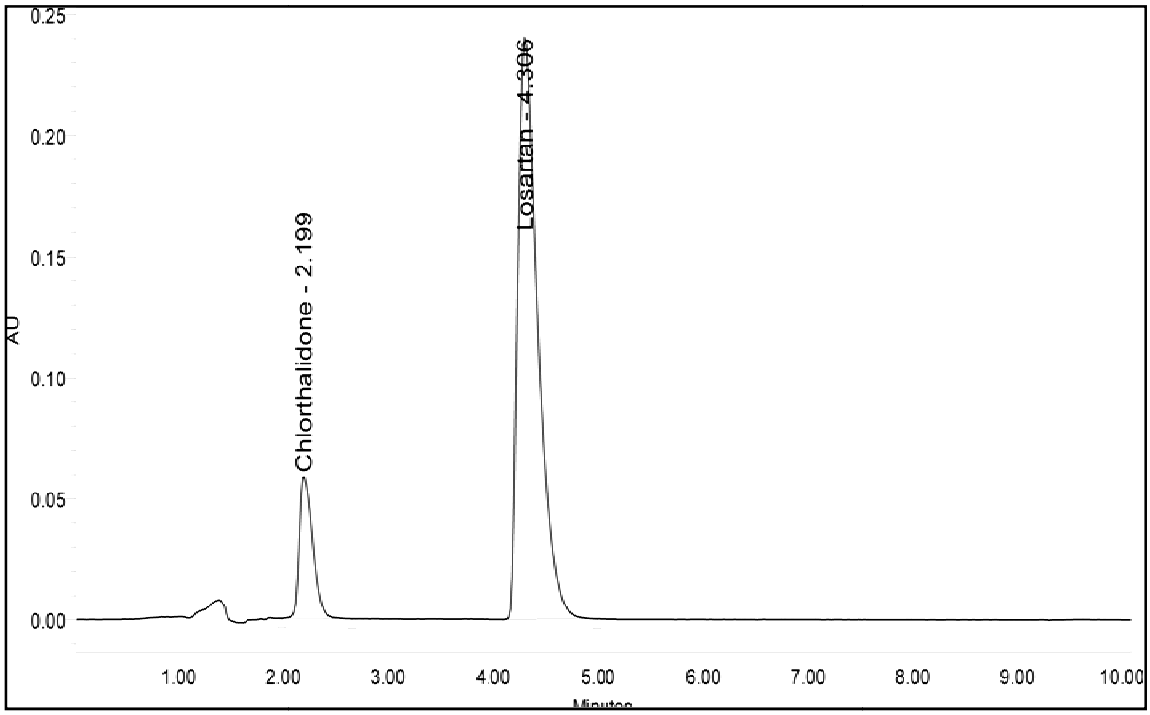


Fig. No. 12.0: Trial 4 chromatograms for losartan and chlorthalidone

Sr.No	Peak Name	RT	Area	% Area	USP Plate count	Tailing
1.	Chlorthalidone	2.199	516172	14.02	1488	1.50
2.	Losartan	4.306	3164350	85.98	2371	1.76

In this Trial Peaks were well separated but USP Plate count were less below the acceptance criteria. So this Chromatographic conditions are Not suitablefor Estimation of selected formulation.

Trial -5

Chromatographic conditions

Column	:	Agilent XDB, C18, 150 x 4.6 mm, 5µ.
Mobile phase	:	Buffer : Acetonitrile (70:30 % v/v pH 3.5)
Detector	:	PDA detector
Flow rate	:	1.0ml/min
Detection wavelength	:	254nm
Injection volume	:	10µl
Temperature	:	30^0c
Run time	:	8min

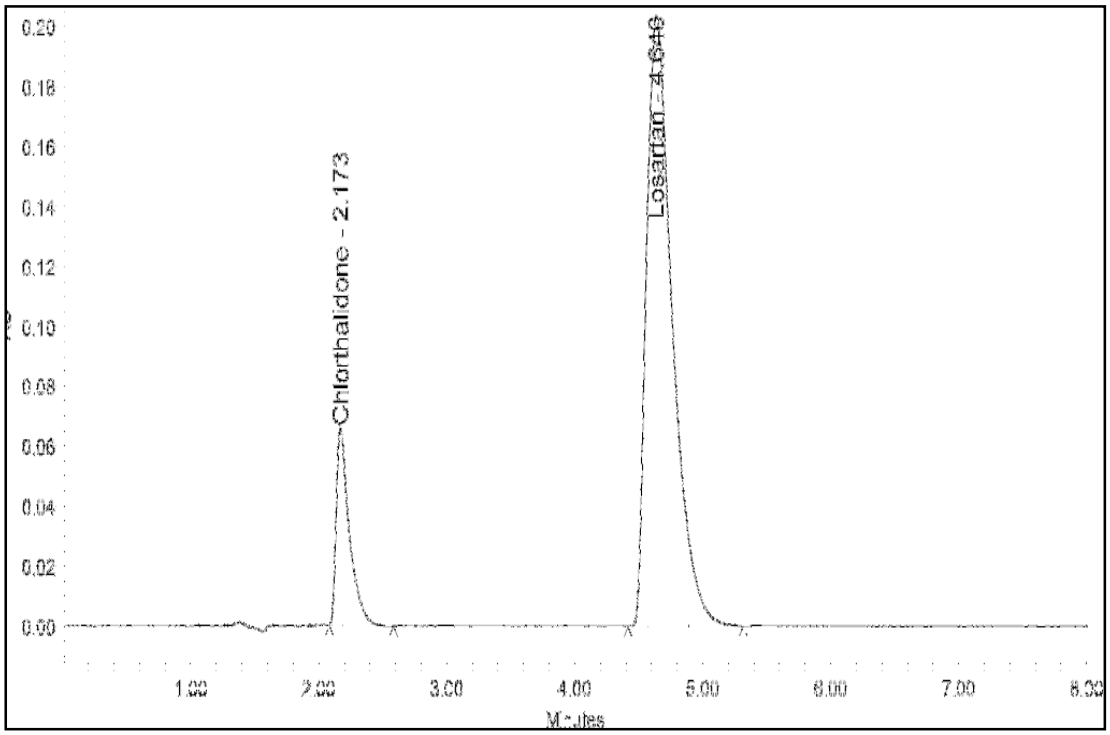


Fig. No. 13.0: Trial 5 chromatograms for losartan and chlorthalidone

	Peak Name	RT	Area	% Area	USP Plate count	Tailing
1.	Chlorthalidone	2.173	462220	13.91	2329	1.93
2.	Losartan	4.640	2860680	86.09	2488	1.66

The two peaks were well resolved with good resolution and efficiency and Peak Retention times were good.

Hence this method was finalized as optimized method for the simultaneous estimation of Losartan and Chlorthalidone.

Procedures for Losartan and Chlorthalidone

Preparation of Diluent:

The diluent was prepared by mixing 50ml of HPLC water and 50ml of Acetonitrile and the resulting solution was sonicated for 15min.

Preparation of Buffer

2.72 gm of Potassium dihydrogen orthophosphate (KH_2PO_4) was accurately weighed and transferred into a 1000 ml volumetric flask, about 900ml of milli-Q water was added and 1ml of triethylamine was added then made up to the final

volume using milli-Q water, the resulting solution was sonicated. Dilute ortho Phosphoric acid solution was added to adjust the solution to pH-3.5

Preparation of Standard Stock Solution

Accurately weighed 50mg of Losartan and 6.25 mg of Chlorthalidone working Standards into a 25 ml clean dry volumetric flask, 15ml of diluent was added and sonicated for 5 min and made up to the final volume with diluents.

Preparation of Sample Solution

Twenty tablets were weighed and crushed. Powder equivalent to 50mg of Losartan and 6.25mg of Chlorthalidone were transferred into separate 500 ml volumetric flasks, 200ml of diluents was added to dissolve drug and sonicated for 10 min, further the volume made up with diluent and the solution is filtered through 0.45µm filter and this solution was further diluted with diluents to get the same concentration as that of final standard solutions.

Analysis of Tablet Formulation

Twenty tablets were weighed and crushed. Powder equivalent to 50mg of Losartan and 6.25mg of Chlorthalidone were transferred into separate 500 ml volumetric flasks, 200ml of diluents was added to dissolve drug and sonicated for 10 min, further the volume made up with diluent and the solution is filtered through 0.45µm filter and this solution was further diluted with diluents to get the same concentration as that of final standard solutions. The sample solution of $10\,\mu g/ml$ was Injectied to the column and peak ared was recorded. Then by comparing with peak area of standard was calculated to find out content of losartan and chloarthalidone in tablet formulations.

5.1.2 Method Development: Atovaquone and Proguanil

By studing the physical property and soullbility of the drug the number of trials were carried out and then optimized Chromatographic Condition were fixed for the estimation of Atovaquone and Proguanil Hydrochloride in selected multicomponent formulations. Some of the trials chromatogram and chromatographic condition are shown in **Fig. No.14.0 to Fig. No. 19.0**

Trial -1

Chromatographic conditions

Column	:	Inertsil ODS, 150 x 4.6 mm, 5µ
Mobile phase	:	Buffer : Acetonitrile (70:30)
Detector	:	PDA detector
Flow rate	:	1.0ml/min
Detection wavelength	:	258nm
Injection volume	:	10µl
Temperature	:	30^0c
Run time	:	13min

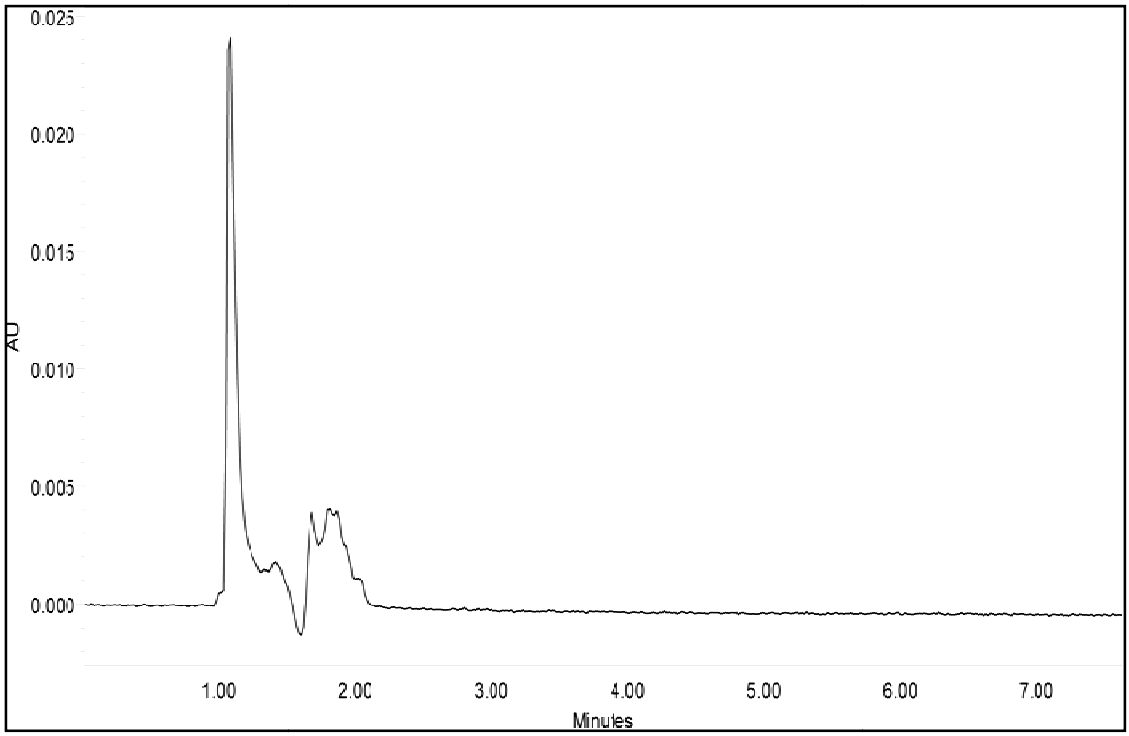


Fig. No.14.0: Trial 1 chromatogram for Proguanil and Atovaquone

	Peak Name	RT	Area	% Area	USP Plate count	Tailing
1.	Proguail	2.100	-	-	-	-
2.	Atovaquone	-	-	-	-	-

The two peaks were not well resolved at all. So this trial was considered as not suitable for the estimation.

Trial -2

Chromatographic conditions

Column	:	Inertsil ODS, 150 x 4.6 mm, 5µ
Mobile phase	:	Buffer : Acetonitrile (70:30)
Detector	:	PDA detector
Flow rate	:	1.0ml/min
Detection wavelength	:	258nm
Injection volume	:	10µl
Temperature	:	30^0c
Run time	:	13min

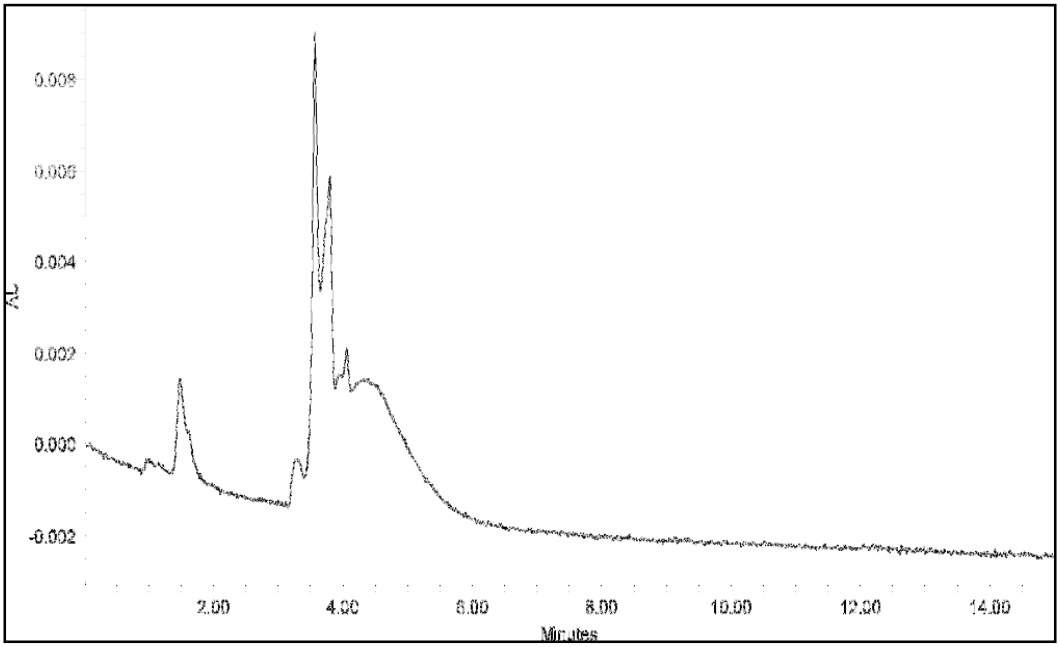


Fig. No.15.0: Trial 2 chromatogram for Proguanil and Atovaquone

	Peak Name	RT	Area	% Area	USP Plate count	Tailing
1.	Proguail	2.100	-	-	-	-
2.	Atovaquone	-	-	-	-	-

In this Chromatographic conditions the two peaks were not well resolved and it is not suitable for the estimation.

Trial -3

Chromatographic conditions

Column	:	Inertsil ODS, C18, 150 x 4.6 mm, 5µ
Mobile phase	:	Buffer : Methanol (65:35)
Detector	:	PDA detector
Flow rate	:	1.0ml/min
Detection wavelength	:	258nm
Injection volume	:	10µl
Temperature	:	30^0c
Run time	:	13min

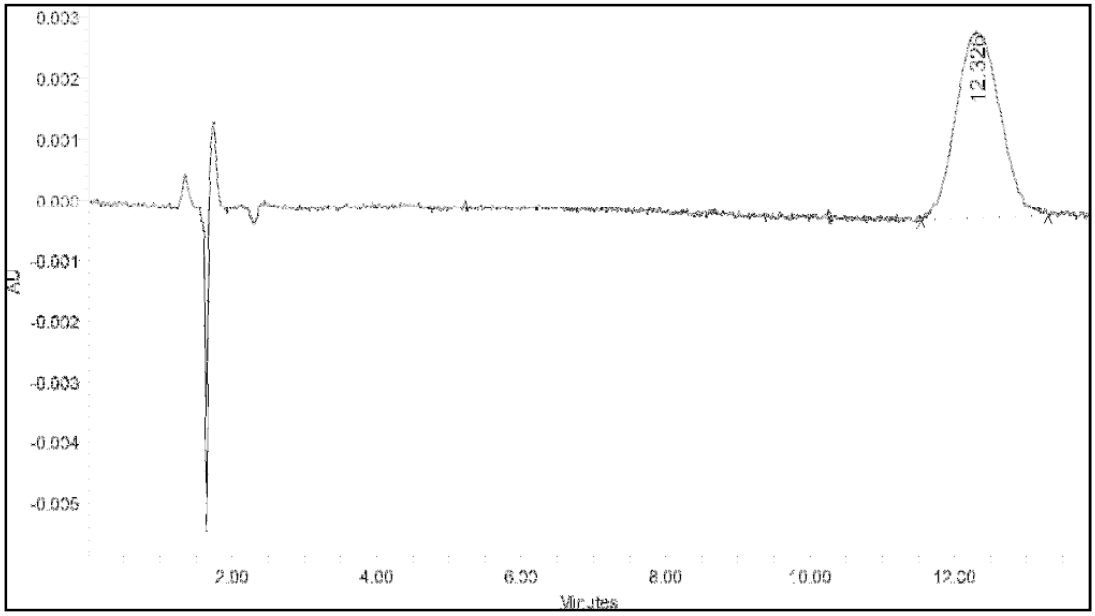


Fig. No.16.0: Trial 3 chromatogram for Proguanil and Atovaquone

	Peak Name	RT	Area	% Area	USP Plate count	Tailing
1.	Proguail	-	-	-	-	-
2.	Atovaquone	12.36	131568	100	1705	1.02

The two peaks were not well resolved at all, So this trial was considered as not suitable for the estimation.

Trial -4

Chromatographic conditions

Column : Inertsil ODS, C18, 150 x 4.6 mm, 5µ

Mobile phase : Buffer : Acetonitrile (65:35)

Detector : PDA detector

Flow rate : 1.0ml/min

Detection wavelength : 258nm

Injection volume : 10µl

Temperature : 30^0c

Run time : 13min

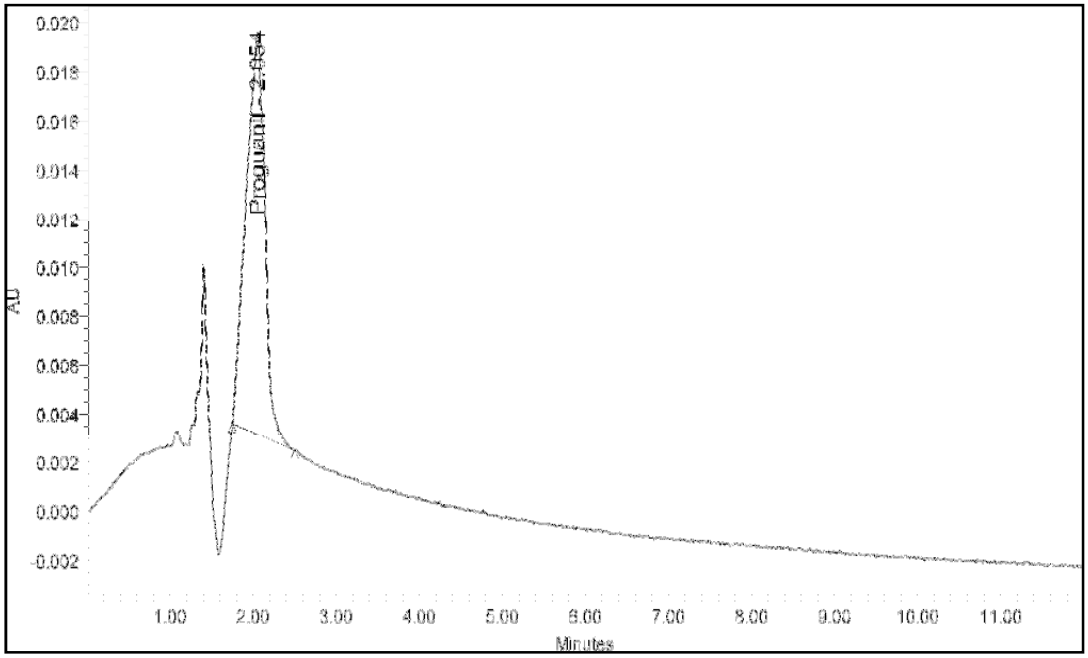


Fig. No.17.0: Trial 4 chromatogram for Proguanil and Atovaquone

	Peak Name	RT	Area	% Area	USP Plate count	Tailing
1.	Proguail	2.054	258293	100.00	364	0.95
2.	Atovaquone	9.600	-	-	-	-

The two peaks were not well resolved at all, So this trial was considered as not suitable for the estimation.

Trial -5

Chromatographic conditions

Column : Inertsil BDS, C18, 250 x 4.6 mm, 5µ

Mobile phase : Buffer : Acetonitrile (50:50)

Detector : PDA detector

Flow rate : 1.0ml/min

Detection wavelength : 258nm

Injection volume : 10µl

Temperature : 30^0c

Run time : 13min

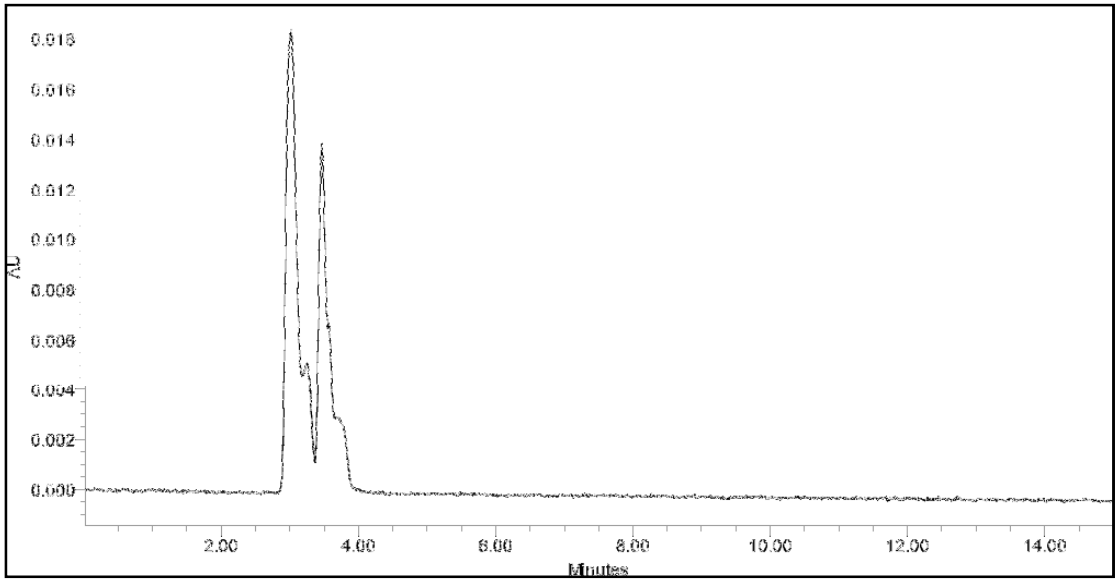


Fig. No.18.0: Trial 5 chromatogram for Proguanil and Atovaquone

	Peak Name	RT	Area	% Area	USP Plate count	Tailing
1.	Proguail	2.164	-	-	-	-
2.	Atovaquone	9.600	-	-	-	-

The two peaks were not well resolved at all, So this trial was considered as not suitable for the estimation.

Trial -6

Chromatographic conditions

Column	:	Hypersil BDS, C18, 150 x 4.6 mm, 5µ
Mobile phase	:	Buffer : Acetonitrile (Gradient Programme)
Detector	:	PDA detector
Flow rate	:	1.1ml/min
Detection wavelength	:	258nm
Injection volume	:	10µl
Temperature	:	30^0c
Run time	:	13min
Diluent	:	Acetonitrile (for Preparation of stock Solution)
		Remaining Dilutions or Further diluted with Methanol

Tables No 9.0: Mobile phase gradient programme

Time	Flow Rate	% Buffer	% Acetonitrile
0	1.1	50	50
3	1.1	50	50
4	1.1	20	80
9	1.1	20	80
9.1	1.1	50	50
12.0	1.1	50	50

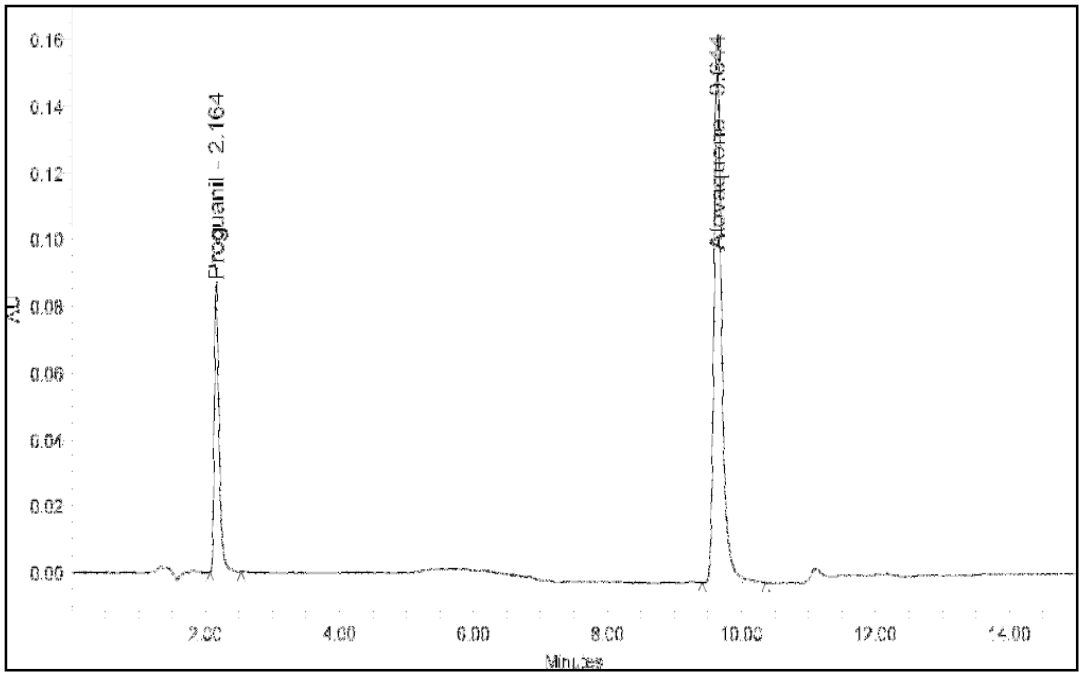


Fig. No.19.0: Trial 6 chromatogram for Proguanil and Atovaquone

Sr. No.	Peak Name	RT	Area	% Area	USP Plate count	Tailing
1.	Proguail	2.164	430266	24.69	4743	1.43
2.	Atovaquone	9.644	1312144	75.31	39260	1.38

The two peaks were well resolved with good resolution also plate count and tailing was good.

Hence this gradient elution method was optimized for the simultaneous estimation of Atovaquone and Proguanil

Procedures for Atovaquone and Proguanil Hydrochloride

Preparation of Diluent

The HPLC grade Acetonitrile was used for the preparations of stock solutions and further dilutions is done by using HPLC grade Methanol.

Preparation of Buffer solutions

Weigh accurately about 2.72 gm of Potassium dihydrogen orthophosphate (KH_2PO_4) into a 1000 ml volumetric flask , add about 900ml of milli-Q water and add 1ml of triethylamine then make up to the final volume using milli-Q water, sonicate the resulting solution and then adjust the solution to pH-3.5 with dilute ortho Phosphoric acid solution.

Preparation of Standard Solution

Accurately weighed and transferred 250mg of Atovaquone and 100mg of Proguanil working Standards into a 25 ml clean, dry volumetric flask, add 15ml of diluent and sonicated for 30 minutes and make up to the final volume with diluents.

Preparation of Sample Solution

20 tablets were weighed and calculate the average weight of each tablet then the weight equivalent to 1 tablets was transferred into a 100 mL volumetric flask, 60mL of diluent added and sonicated for 30 min, further the volume made up with diluent and filtered. From the filtered solution 0.4ml was pipeted out into a 10 ml volumetric flask and made upto 10ml with diluent.

Analysis of Tablet Formulation

20 tablets were weighed and calculate the average weight of each tablet then the weight equivalent to 1 tablets was transferred into a 100 mL volumetric flask,

60mL of diluent added and sonicated for 30 min, further the volume made up with diluent and filtered. From the filtered solution 0.4ml was pipeted out into a 10 ml volumetric flask and made upto 10ml with diluent. The sample solution of $10\,\mu g/ml$ was Injectied to the column and peak ared was recorded. Then by comparing with peak area of standard was calculated to find out content of losartan and chloarthalidone in tablet formulations.

5.1.3 Method Development: Acebrophylline and Acetylcysteine

Like above optimized method, few trials were carried out for optimization of method used for estimation of acebrophylline and acetylcysteine in capsule formulations shown in **Fig. No. 20.0 to Fig. No. 25.0**

Trial -1

Chromatographic conditions

Column	:	Hypersil BDS, 100 x 4.6 mm, 5µ
Mobile phase	:	Buffer : Acetonitrile (70:30)
Detector	:	PDA detector
Flow rate	:	1.0ml/min
Detection wavelength	:	260nm
Injection volume	:	5µl
Temperature	:	30^0c
Run time	:	10min

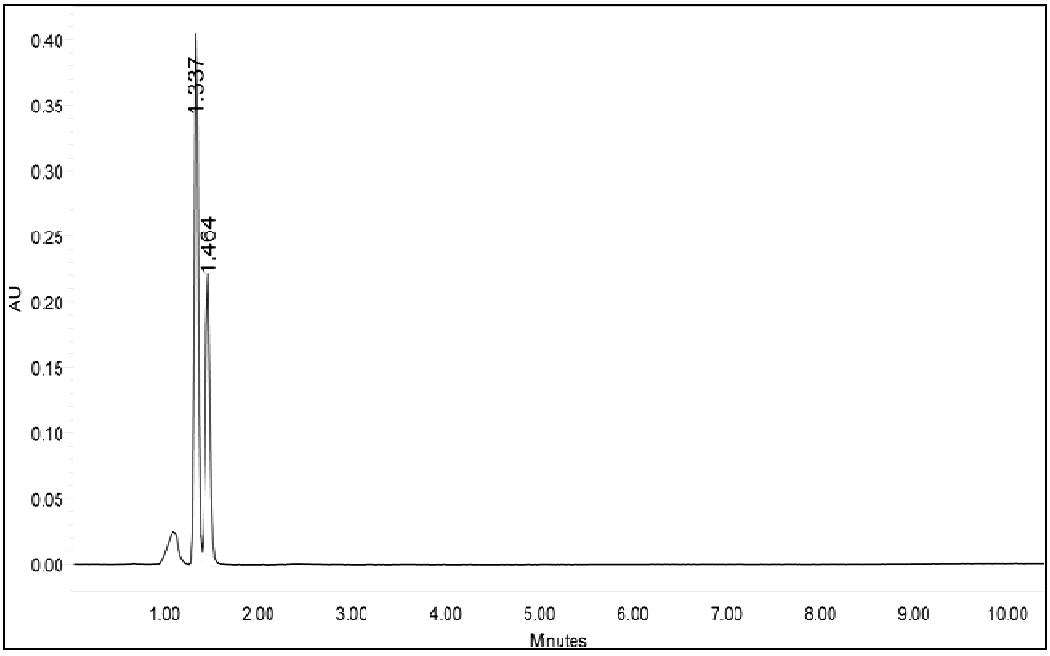


Fig.No. 20.0 : Trial 1 chromatogram for Acebrophylline and Acetylcysteine

Sr. No.	Peak Name	RT	Area	% Area	USP Plate count	Tailing
1.	Acetylcysteine	1.337	995180	60.05	5782	1.06
2.	Acebrofylline	1.464	662108	39.95	4515	1.01

The two peaks were not well resolved at all. So the chromatiographic conditions was not suitable for estimation.

Trial -2

Chromatographic conditions

Column : Hypersil BDS, 100 x 4.6 mm, 5µ
Mobile phase : Buffer : Acetonitrile (80:20)
Detector : PDA detector
Flow rate : 0.8ml/min
Detection wavelength : 260nm
Injection volume : 5µl
Temperature : 30^0c
Run time : 10min

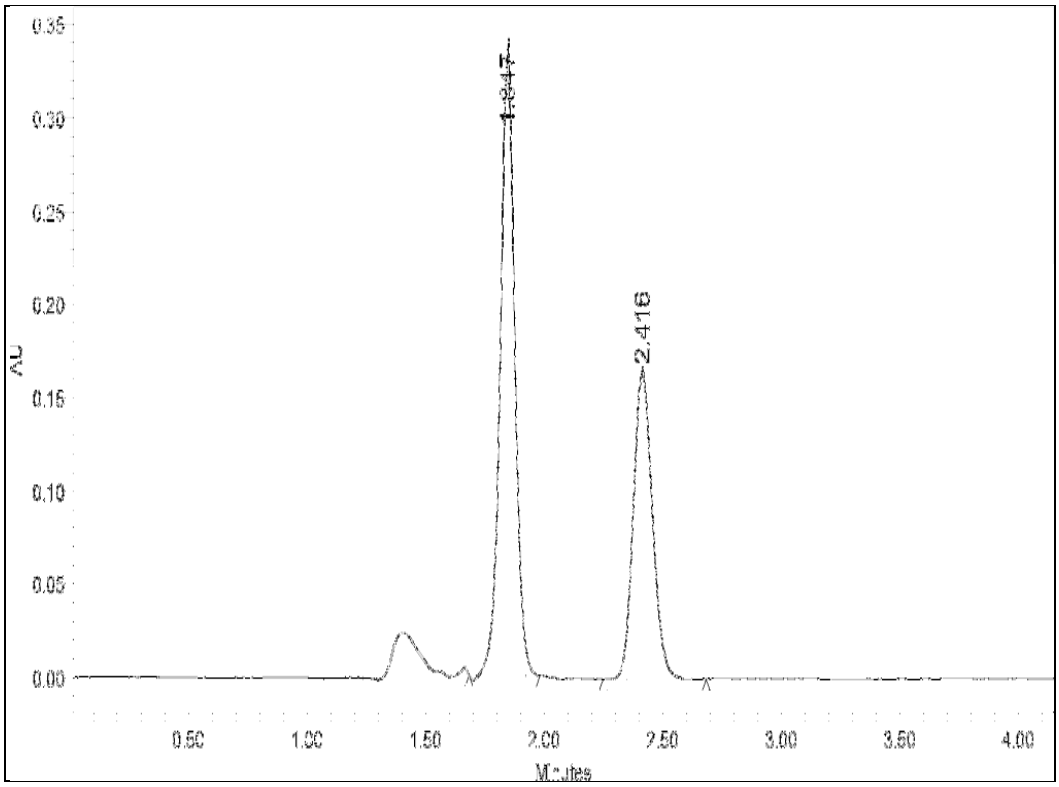


Fig.No. 21.0 : Trial 2 chromatogram for Acebrophylline and Acetylcysteine

Sr. No.	Peak Name	RT	Area	% Area	USP Plate count	Tailing
1.	Acetylcysteine	1.847	1433301	60.39	4572	0.98
2.	Acebrofylline	2.416	920457	39.11	4267	1.11

The resolution is less So the chromatiographic conditions was not suitable for estimation

Trial -3

Chromatographic conditions

Column	:	Hypersil BDS, 100 x 4.6 mm, 5µ
Mobile phase	:	Buffer : Acetonitrile (85:15)
Detector	:	PDA detector
Flow rate	:	0.8ml/min
Detection wavelength	:	260nm
Injection volume	:	5µl
Temperature	:	30^0c
Run time	:	10min

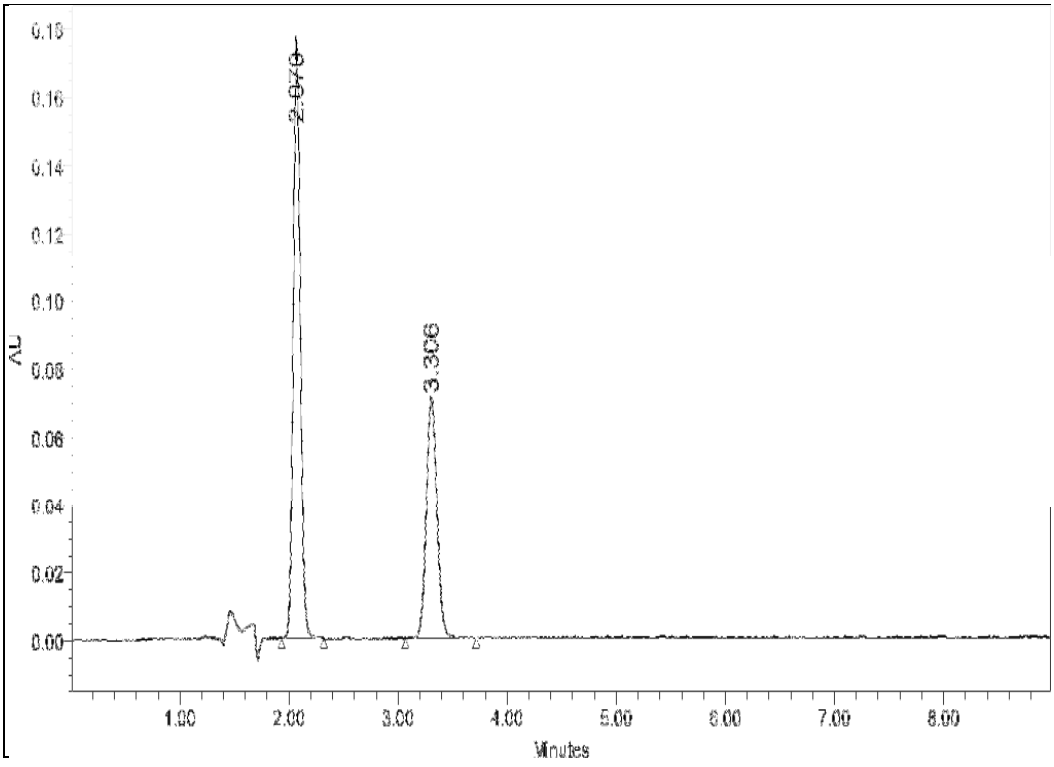


Fig.No. 22.0 : Trial 3 chromatogram for Acebrophylline and Acetylcysteine

Sr. No.	Peak Name	RT	Area	% Area	USP Plate count	Tailing
1.	Acetylcysteine	2.070	782517	61.94	4841	1.11
2.	Acebrofylline	3.306	480776	38.06	5453	1.07

The two peaks were not well resolved at all, So the chromatiographic conditions was not suitable for estimation

Trial -4

Chromatographic conditions

Column : Hypersil BDS, 100 x 4.6 mm, 5µ

Mobile phase : Buffer : Acetonitrile (85:15)

Detector : PDA detector

Flow rate : 0.9ml/min

Detection wavelength : 260nm

Injection volume : 5µl

Temperature : 30^0c

Run time : 10min

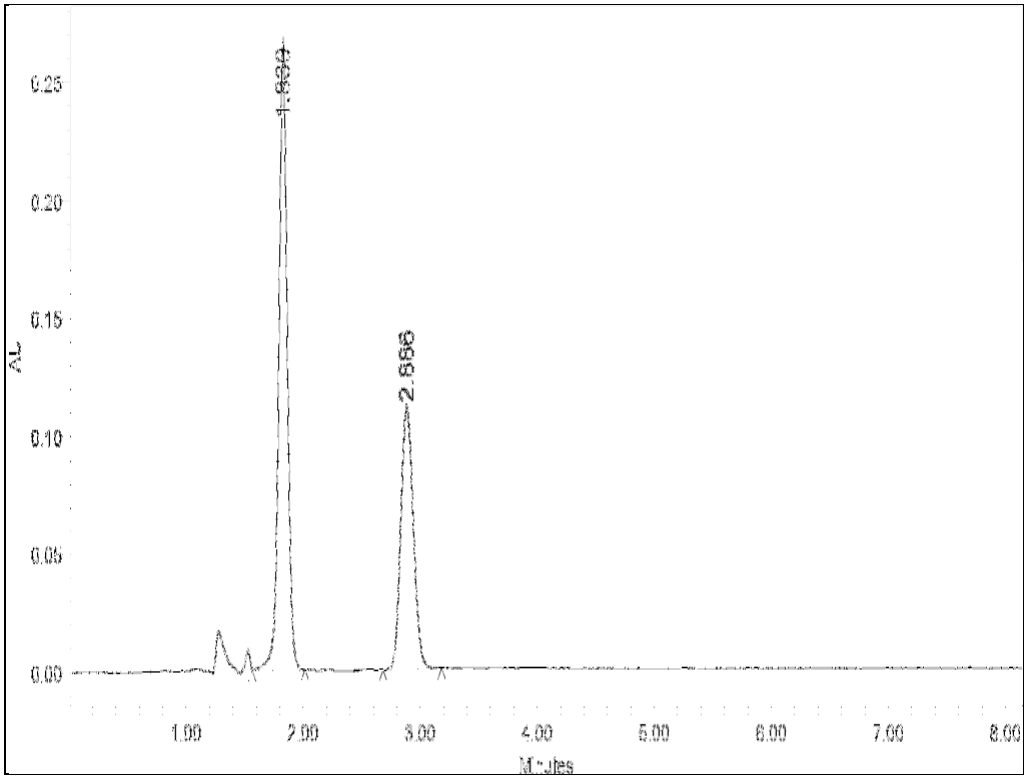


Fig.No. 23.0 : Trial 4 chromatogram for Acebrophylline and Acetylcysteine

Sr. No.	Peak Name	RT	Area	% Area	USP Plate count	Tailing
1.	Acetylcysteine	1.830	1336651	61.75	3260	0.99
2.	Acebrofylline	2.886	827850	38.35	3416	1.09

The two peaks were not well resolved at all, So the chromatiographic conditions was not suitable for estimation

Trial -5

Chromatographic conditions

Column : Hypersil BDS, 100 x 4.6 mm, 5µ

Mobile phase : Buffer : Acetonitrile (90:10)

Detector : PDA detector

Flow rate : 0.9ml/min

Detection wavelength : 260nm

Injection volume : 5µl

Temperature : 30^0c

Run time : 10min

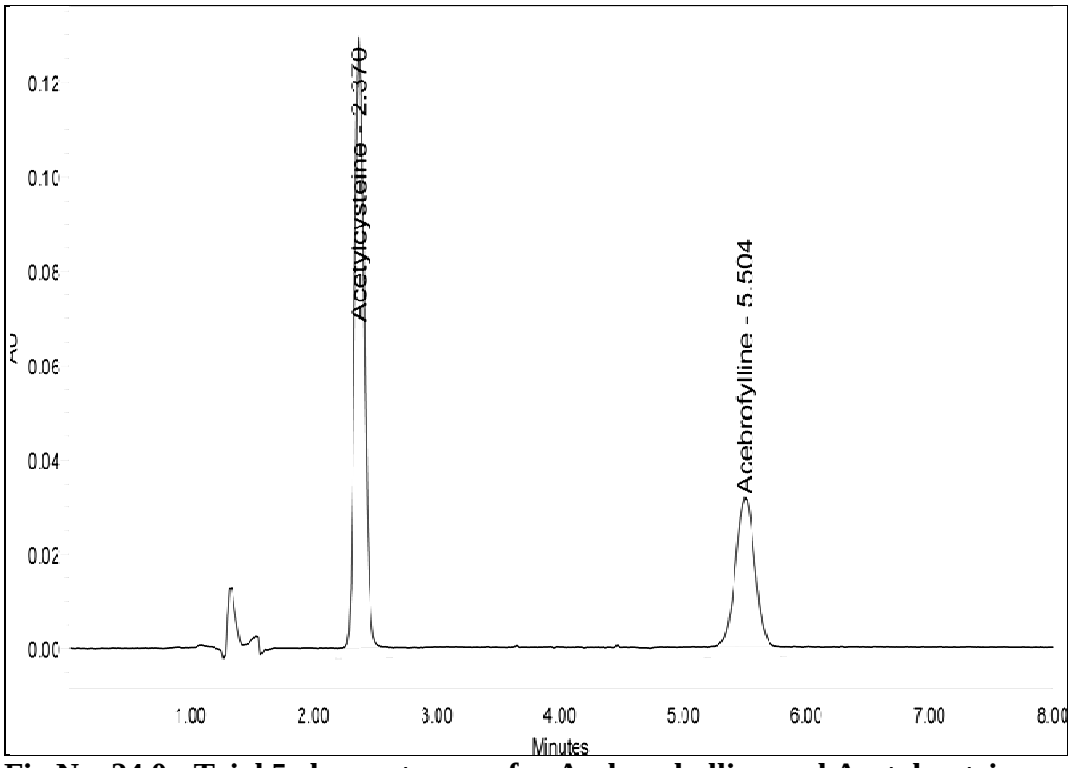


Fig.No. 24.0 : Trial 5 chromatogram for Acebrophylline and Acetylcysteine

Sr. No.	Peak Name	RT	Area	% Area	USP Plate count	Tailing
1.	Acetylcysteine	2.370	686033	66.71	4376	1.09
2.	Acebrofylline	5.504	342289	33.29	5843	1.00

The two peaks were well resolved with good peak symmetry

Hence this method was selected for the simultaneous estimation of Acetylcystein and Acebrofylline

Procedure for Acerbrofylline and Acetylcysteine

Preparation of Diluent

The diluent was HPLC grade methanol.

Preparation of Buffer

Accurately weighed 2.72gm of potassium dihydrogen orthophosphate was transferred in a 1000ml of volumetric flask and about 900ml of milli-Q water was added.1ml of triethylamine was added then sonicated and finally made up the volume with water. Then pH was adjusted to 3.2 with dilute ortho phosphoric acid solution.

Preparation of Standard Stock Solution

Accurately weighed 10mg of Acebrophylline and 12.5mg of Acetylcysteine working Standards were transferred into separate 10 ml clean and dry volumetric flasks, 7ml of diluents was added and sonicated for 30 min and made up to the final volume with diluents.

Preparation of Sample Solution

20 capasules were weighed and the average weight of each capasules was calculated. Then the weight equivalent to 1 capsule was transferred into a 100 ml volumetric flask, 50ml of diluent was added and sonicated for 30 min, further the volume made up with diluent and filtered. From the filtered solution 0.2ml was pipetted out into a 10 ml volumetric flask and made up to 10ml with diluent. The sample solution of $10\,\mu g/ml$ was injectied to the column and peak ared was recorded. Then by comparing with peak area of standard was calculated to find out content of losartan and chloarthalidone in tablet formulations.

5.2 Part 2: RP-HPLC Method Validation

Developed methods should be validated in order to demonstrate the analytical procedure is suitable for its intended use and also shows that the results generated by the particular analytical procedure are reliable and accurate. All the three developed methods of selected multicomponent formulations Losartan+Chlorthalidone, Atovaquone+ Proguanil and Acebrofylline+Acetylcysteine were validated as per ICH/USP guidelines for the following parameters like Accuracy – Precision – Specificity – Selectivity – Detection limit - Quantitation limit – Linearity – Range – Ruggedness - Robustness.

Common Validation Parameters for HPLC and Spectroscopic method

The common validation parameters for the developed HPLC and Spectroscopic methods were thoroughly described under chapter introduction in detail earlier. So, here we describe the procedure followed for the validation of spectroscopic and HPLC methods developed.

ASSAY

Procedure

The solutions of 100% level solutions containing respective concentration which were previously prepared in duplicate were injected at the optimized method conditions and the chromatograms were recorded and the percentage drug content was calculated and reported in chapter Results in for selected drug combination

5.2.1. System suitability tests

From the std. stock solution of 10 $\mu g/ml$ was injected sixtimes and std peak area was Recorded and the system suitability parmeter were checked to ensure the system performance (resolution and reproducibility) before or during analysis of unknown sample. Data from six injections of 10 µl of the working standard solutions of the selected multi component formulations were used for the evaluation of the system suitability parameters like tailing factor, the number of theoretical plates, retention time and resolution factor. As per USP guidelines for validation out of this parameter only two parameters are required. The standard solution was prepared as per the proposed assay method and was injected into the HPLC system. The system was suitable for analysis if % relative standard deviation (%RSD) of area should be not more than 2.0%, and USP tailing factor should be not more than 2 and theoretical plate count should not be

less than 2000. The respective data for system suitability were shown under chapter Results

5.2.2. Linearity

The linearity of the method was evaluated by analyzing different concentration (80% to 120%) of the drugs. According to ICH recommendations, at least five concentrations must be used. In the present study six concentrations were chosen & injected. The peak areas of the chromatograms were plotted against the concentration of drug to obtain the calibration curve. From this corresponding data co-relation coefficient, slope and y-intercept were calculated and shown under chapter Results and the respective calibration curve shown under chapter Ideally co-relation coefficient should be not less than 0.999 and calibration curve should be Liniear.

5.2.3. Accuracy

The accuracy of the method was determined by recovery study. The accuracy of an analytical method should be established across its range. The solutions were injected in triplicate in 50%, 100% and 150% concentrations by spiking the previously analyzed formulation with standard drug and reanalyzing the content by the developed spectrophotometric and HPLC methods and percentage recovery was calculated and shown under chapter Results. Average % recovery at each spike level not less than 98.0% and not more than 102.0%.

5.2.4. Precision

Precision of the developed method was determined by studying intra-day and inter-day variation by injecting the sample solution six times and calculating the mean of the amount present, percentage label claim and percentage RSD were calculated and shown under chapter Results. While in case of HPLC % RSD of the peak area obtained for the drug in multicomponent formulation were should not be more than 2.0

5.2.5. Limits of Detection (LOD) and Limit of Quantitation (LOQ):

In accordance with ICH recommendations, the method based on the standard deviation of the response and the slope of the calibration plots was used to determine

detection and quantification limits. LOD and LOQ values were estimated [(standard deviation of repeatability)/ (Slope of the regression equation)] by multiplying with 3.3 and 10 respectively. The corresponding LOD and LOQ data for all selected combinations were shown under chapter Results.

5.2.6. Specificity

The Specificity of the method was evaluated by assessing whether excipients present in the pharmaceutical formulations interfered with the analysis. Excipients for each tablet were mixed in order to prepare a placebo, and solutions were prepared by following the procedure described in the section on sample preparation. The excipients did not interfere with the method.

$$amount\ of\ placebo\ to\ be\ taken = avg\ wt\ of\ tablet - label\ claim\ of\ the\ drugs$$

There should not peak at the retention time of drug of the formulations.

5.2.7. Robustness

Robustness is a measure of capacity of analytical methods to remain unaffected by small but deliberate variation of the operating conditions. This was tested by studying the effect of changing column temperature ±5^{o}C, the mobile phase composition by 2%, and flow rate by ±0.1ml. The corresponding Robustness data for all selected combinations were shown under chapter Results for different varying conditions.

All the Validation Parameter the injection volumn was injected in triplicate for the linearity accusary for precision

5.3 Part 3: RP-HPLC Forced Degradation Study

Losartan-Chlorthalidone, Atovaquone-Proguanil and Acebrophylline-Acetylcysteine samples are prepared accordingly following their procedure of HPLC method.

Degradation studies

Generaly there is no specific guideline available for the conducting forced degradation study by the regulatory but the most recommended stress condition were used for the forced degradation study include acid degradation, alkali degradation, oxidation, dry heat and photolysis.

5.3.1 Acid Degradation Studies

To 1ml of stock solution of Losartan and Chlorthalidone, 1ml of 2N Hydrochloric acid was added and refluxed for 30min at 60°C. The resultant solution was diluted to obtain 50µg/ml & 6.25µg/ml solution and 10µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample.

5.3.2 Alkali Degradation Studies

To 1 ml of stock solution of Losartan and Chlorthalidone, 1 ml of2N sodium hydroxide was added and refluxed for 30min at 60°C. The resultant solution was diluted to obtain 50µg/ml & 6.25µg/ml of Losartan and Chlorthalidone and 10µl was injected into the system and the chromatograms were recorded to assess the stability of sample.

5.3.3. Oxidation Studies

To 1 ml of stock solution of Losartan and Chlorthalidone, 1 ml of 20% hydrogen peroxide (H_2O_2)was added. The solution was kept for 30 min at 60°C. For HPLC study, the resultant solution was diluted to obtain 50µg/ml & 6.25µg/ml of Losartan and Chlorthalidone and 10µl was injected into the system and the chromatogram was recorded to assess the stability of sample.

5.3.4 Dry Heat (Thermal) Degradation Studies

The standard drug solution was placed in oven at105°C for 6h to study dry heat degradation. For HPLC study, the resultant solution was diluted to 50µg/ml & 6.25µg/ml of Losartan and Chlorthalidone and 10µl were injected into the system and the chromatogram was recorded to assess the stability of the sample**.**

5.3.4 Photo degradation

The standard drug solution was exposed to the ultra violet light radiation for 7 days. For HPLC study the resultant solution was diluted to 50 ug/ml %6.25µg/ml of Losartan and Chlorthalidone and 10µl were injected into the system and the chromatogram was recorded to assess the stability of the sample**.**

Similarly standard sample were prepared for the atovaqoune and proguanil combination and acetylcysteine and acebrofylline combination.

5.4 Part 4: UV Spectrophotometric Method Development

Proper selection of the spectrophotometric method depends upon the nature of the sample (ionic or neutral molecule) and solubility. In the present study the selected drugs are polar in nature and hence spectrophotometric methods are considered to be more suitable because they are economic, specific, linear, precise, accurate, sensitive and rapid methods. Spectroscopic optimization conditions for UV spectrophotometer are,

5.4 A. Selection of solvent

The solubility of drugs was determined in a variety of solvents as per Pharmacopoeial standards. Solubility was carried out in polar to non-polar solvents. The common solvent was found to be distilled water and methanol. Atovaquone and Proguanil combination drug and Losartan and Chlorthalidone combination drug are freely soluble in water/ methanol and Acetylcystein and Acebrophylline combination drug is freely soluble in methanol/ethanol.

5.4.B. Selection of wavelength

From the stock solution of losartan and chlorthalidone 8µg/ml and 1µg/ml concentration solutions were prepared respectively. From the stock solutions of Atovaquone and Proguanil, 10 µg/ml and 4 µg/ml concœntration solutions were prepared respectively. While from the stock solutions of Acetylcysteine and acebrophylline 4µg/ml and 24µg/ml concentration weœ prepared respectively, and the solutions were scanned separately between the wavelength ranges from 200 - 400 nm and the corresponding spectra were recorded.

From the overlain spectra of Atovaquone and Proguanil 279nm and 254 nm were selected for the estimation by Simultaneous equation method respectively. From the overlain spectra of losartan and chlorthalidone 235nm and 215nm were selected for the estimation by Simultaneous equation method respectively. From the overlain spectra of Acetylcysteine and acebrophylline 273nm and 220nm were selected for the estimation by Simultaneous equation method respectively.

5.4. C. Preparation of standard stock solution

a) 50mg of Losartan and 6.26 mg of chlorthalidone standards were weighed and transferred into 100 ml volumetric flasks separately and dissolved in distilled water and made up to the volume with water. Further dilutions were made by diluting 1 ml to 10ml volumetric flask, further diluted 1.6 ml to 10 ml to obtain 8 µg/ml of Losartan and 1µg/ml of Chlorthalidone.

b) 250 mg of Atovaquone and 100mg of Proguanil Standards were weighed and transferred into 100 ml volumetric flasks separately and dissolved in distilled water and made up to the volume with water. These solutions were observed to contain 2500 and 1000 µg/ml. And further dilution was made to get concentration 10 µg /ml Atovaquone and 4 µg/ml Proguanil.

c) 100mg of Acebrophylline and 600mg of Acetylcysteine Standards were weighed and transferred into 100 ml volumetric flasks separately and dissolved in methanol and made up to the volume with methanol. These solutions were observed to contain 1000 and 6000 µg/ml concentration respectively. From the above stock solution 0.5ml was pipetted out into a 10 ml volumetric flask and made upto 10ml with diluent. From the above stock solution 0.8ml was pipetted out into a 10 ml volumetric flask and made upto 10ml with diluent to get concentration 4µg/mlAcebrophylline and 24 µg/ml Acetylcysteine.

5.4. 1Spectrophotometry assay procedure for Losartan and Chlorthalidone:

Twenty tablets of formulation (Losartan 50 mg and 6.25 mg of Chlorthalidone) were weighed and powdered. The tablet powder equivalent to Losartan 50 mg and 6.25 mg of Chlorthalidone was weighed and transferred into 100 ml volumetric flask and a minimum quantity of distilled water was added to dissolve the substance by using ultra sonication for 15 min and made up to the volume with the same diluents. The content was filtered through Whattman filter paper No. 41. From the cleared solution, further dilutions were made by diluting 2 ml to 100ml volumetric flask, further diluted 1.6 ml to 10 ml to obtain 8 µg /ml of Losartan and 1µg /ml of Chlorthalidone theoretically.

The absorbance measurements were made 6 times for the formulation at 235nm, 215nm. From the absorptivity values of Losartan and Chlorthalidone at

235nm, 215nm and the amount of Losartan and Chlorthalidone in tablet formulation were determined by using Simultaneous equation method.

Simultaneous Equation Method

The simultaneous equations formed were,

At λ_1 $A_1 = ax_1bCx + ay_1bCy$ ------ (1)

At λ_2 $A_2 = ax_2bCx + ay_2bCy$ ------ (2)

where A_1 and A_2 are the absorbance of sample solution at 235 nm, 215 nm respectively. Cx and Cy are the concentrations of Losartan and Chlorthalidone respectively (µg /ml) in sample solution.

The absorbance (A_1& A_2) of the sample solution were recorded at 235 nm & 215 nm respectively and concentration of both the drugs were calculated using above simultaneous equation.

5.4. 2 UV Spectrophotometry assay procedure for Atovaquone and Proguanil

Twenty tablets of formulation (Atovaquone 250 mg and 100 mg of Proguanil) were weighed accurately and powdered. The tablet powder equivalent to Atovaquone 250 mg and 100 mg of Proguanil was weighed and transferred into 100 ml volumetric flask and a minimum quantity of distilled water was added to dissolve the substance by using ultra sonication for 15 min and made up to the volume with the same(1000 µg/ml).

The content was filtered through Whattman filter paper No. 41. From the cleared solution, further dilutions were made by diluting 1 ml to 25ml volumetric flask, further diluted 2 ml to 100 ml to obtain 10 µg/ml of Atovaquone and 4µg/ml of Proguanil theoretically.

The absorbance measurements were made 6 times for the formulation at 279 nm, 254 nm. From the absorptivity values of Atovaquone and Progaunil at 279 nm, 254 nm, the amount of Atovaquone and Progaunil were determined by using Simultaneous equation method.

5.4. 3 UV Spectrophotometry assay procedure for Acebrophylline and Acetylcysteine:

Twenty capsule of formulation (Acebrophylline 100 mg and 600 mg of Acetylcysteine) were weighed accurately and powdered. The capsule powder equivalent to Acebrophylline 100 mg and 600 mg of Acetylcysteine was weighed and transferred into 100 ml volumetric flask and a minimum quantity of distilled water was added to dissolve the substance by using ultra sonication for 15 min and made up to the volume with the same diluent (5000 µg/ml). The content was filtered through Whattman filter paper No. 41. From the cleared solution, further dilutions were made by diluting 2 ml to 100ml volumetric flask, further diluted 0.4 ml to 10 ml to obtain 4 µg/ml of Acebrophylline and 24µg/ml of Acetylcysteine theoretically.

The absorbance measurements were made 6 times for the formulation at 273 nm, 220 nm. From the absorptivity values of Acebrophylline and Acetylcysteine at 273 nm, 220 nm, the amount of Acebrophylline and Acetylcysteine were determined by using Simultaneous equation method.

5.5 Part 5: UV Spectrophotometric Method Validation

All the developed UV Spectroscopic methods were validated as per ICH guidelines for all the selected multicomponent formulation.

5.5.1 Linearity

A calibration curve was plotted as concentration*Vs* absorbance and the corresponding linearity range was given for the selected absorption maxima.

1) Losartan was found to be linear in the concentration range of 6 to 16 µg/ ml at 235nm and chlorthalidone was found to be linear in the concentration range of 0.75 to 2 µg/ml at 215 nm.
2) Atovaquone was found to be linear in the concentration range of 7.5 to 20 µg/ ml at 279 nm andProguanil was found to be linear in the concentration range of 3 to 8 µg/ml at 254 nm.
3) Acebrophylline was found to be linear in the concentration range of 1 to 6 µg/ ml at 273 nm andAcetylcysteine was found to be linear in the concentration range of 6 to 36 µg/ml at 220 nm.

Acceptance Criteria : - Coerrelation Coefficient (r) = 0.999

5.5.2 Precision

The repeatability of the method was confirmed by the formulation analysis, repeated for six times with the same concentration. The amount of each drug present in the tablet formulation was calculated. The percentage RSD was calculated. The intermediate precision of the method was confirmed by intra-day and inter-day analysis i.e. the analysis of formulation was repeated three times in the same day and on three successive days, respectively. The amount of drugs was determined and % RSD was calculated.

Acceptance Criteria : - % RSD = NMT2

5.5.3 Accuracy

Accuracy of the method was studied by recovery experiments. The recovery experiments were performed by adding known amounts of standard drug to formulation samples. The recovery was performed at three different concentrationslevels (i.e. 50%, 100% and 150%). This procedure was repeated for three times for each concentration. The results of recovery studies were calculated for %RSD.

Acceptance Criteria : - % RSD = NMT2

5.5.4 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The limit of detection (LOD) and the limit of quantitation (LOQ) of all selected combination of drugs were derived by calculating the signal to-noise ratio using the following equations as per the ICH guidelines. LOD=3.3xσ/S;LOQ=10xσ/S,where σ-standard deviation of the response and S- slope of calibration curve.

5.6 Part 6: HPTLC Method Development

As we already disccess about the selection at the solvent and wavelength during the development study of the HPLC and UV method so here we have optimized HPTLC method by selecting following parameter.

High Performance Thin Layer Chromatography works on the principle based on the adsorption and partition co-efficient of the sample and the chromatographic layer. In the present study for the selected combined drugs, HPTLC is considered to

be more suitable because they are economic, specific, linear, precise, accurate, sensitive and rapid method.

5.6.1 HPTLC method development for Chlorthalidone and Losartan

Standard stock solution of chlorthalidone and losartan

100 mg of chlorthalidone and 100 mg of losartan were accurately weighed and transfer in to 100ml volumetric flask separately. 50ml of methanol was added and sonicated for 30min and made up to the volume with methanol. 6.25ml of chlorthalidone and 50ml of losartan was pipette out and transfer in to a 100ml of volumetric flask and made up to the volume with methanol. From this 2.5ml of the solution was further diluted to 10ml with methanol to contain This gave 15.62µg/ml (15.62ng/µl) of chlorthalidone and 125µg/ml (125ng/µl)of losartan.

Preparation of sample stock solution

20 tablets (CTD-L tablets; Lable claim – 6.25mg of chlorthalidone and 50mg of losartan; IPCA Ltd) were accurately weighed and the average weight of each tablet was calculated and powdered. Weight equalent to one tablet 6.25mg of chlorthalidone and 50mg of losartan was weighed and transferred in to 100ml of volumetric flask and 50ml of methanol was added and sonicated for 30 min and made up to the volume with methanol. The content was filtered through whatmann filter paper no.41. From this 1ml of the solution was further diluted to 10ml with methanol to contain 62.5µg/ml of chlorthalidone and 500µg/ml of losartan.

Optimization of Solvent System and Chromatographic Conditions

Chromatographic separation studies were carried out on the stock solution of chlorthalidone and losartan. The solvent system optimization experiment was carried out by the method suggested by Poole et al[118]. Initially the plates spotted with 2µl, 62.5µg/ml of chlorthalidone and 50µg/ml of losartan were developed by linear ascending development using neat solvents like toluene, hexane, methanol, chloroform, dichloromethane, ethyl acetate, acetone, acetonitrile, etc. without chamber saturation. Based on the results of these initial chromatograms binary and ternary mixtures of solvents were tried to achieve optimum resolution between chlorthalidone and losartan. Other chromatographic conditions like chamber saturation time, run length, sample application rate and volume, sample application positions, distance between tracks, detection wavelength, were optimized to give

reproducible R_f values, better resolution, and symmetrical peak shape for the two drugs.

When chlorthalidone and losartan were applied on plate and the plates were developed using neat solvents, the results obtained were-

Solvents		Chlorthalidone	Losartan
Benzene	:	spot at R_f 0.46	At initial spot itself
Chloroform	:	R_f 0.78	R_f 0.47 (tailing & no clear separation)
Toluene	:	R_f 0.74	R_f 0.51 (tailing & no clear separation)
Ethyl Acetate	:	a big spot at R_f 0.24	(no separation)
Methanol	:	(no separation)	(no separation)

Calibration Experiments

Increasing volumes from the mixed standard stock solution 1 to 6 µl for chlorthalidone and 2 to 12 µl losartan solution were applied on a 20 X 20 cm TLC plate to obtain the final concentration 300-1800 ng/spot for chlorthalidone and 50-300 ng/spot for losartan. Each concentration was applied to the TLC plates. The plate was then developed using the previously described mobile phase and peak areas were plotted against the corresponding concentrations to obtain the calibration curves.

Table No 10.0 : Calibration of standards volume and concentration standards applied/spot on plate Losartan and Chlorthalidone

Standard	Volume of chlorthalidone stock solution applied (µl)	Volume of losartan stock solution (µl)	Amount of chlorthalidone applied (ng)	Amount of losartan applied (ng)
1	2	2	31.25	250
2	4	4	62.5	500
3	6	6	93.75	750
4	8	8	135.0	1000
5	10	10	156.25	1250
6	12	12	187.50	1500

After completion of chromatographic analysis, peak areas of chlorthalidone and losartan were noted for each track. Chlorthalidone peak areas (ordinates) were

plotted against concentration (abscissa) of chlorthalidone in the mixed standards and the data of X,Y pairs were subjected to least square linear regression. The calibration equation for chlorthalidone was thus obtained.

Similarly, peak areas of losartan were plotted against corresponding concentrations and least square regression analysis was performed to generate the calibration equation for losartan.

Analysis of a marketed formulation

To determine the content of Chlorthalidone and losartan in a conventional tablets (CTD-L tablets; Lable claim – 6.25mg of chlorthalidone and 50mg of losartan;) 20 tablets were weighed their mean weight determined and finally powdered. The weight of the powder equivalent to 6.25 mg of chlorthalidone and 50 mg of losartan was transferred in to a 100 ml volumetric flask containing 50ml of methanol and sonicated for 30 min and diluted to 100ml with methanol and filtered to produce the concentration of 6.25µg/ml of chlorthalidone and 50µg/ml of losartan and 1µl the working standard was applied. The final concentration obtained was 6.25 ng/spot for chlorthalidone and 50 ng/spot for losartan which was developed in an optimized mobile phase. The analysis was repeated in triplicate. The possibility of excipient interference with the analysis was examined. The area of the peak corresponding to the R_f value of chlorthalidone and losartan standard was recorded and the amount present was calculated.

5.7. HPTLC Method Validation

The developed method was validated as per the ICH Q2 (R1) guidelines. The validation parameters studied were accuracy, precision, linearity and range, selectivity and robustness.

5.7.1 HPTLC Method Validation for Losartan and Chlorthalidone

Linearity and range

Increasing volumes from the mixed standard stock solution 2 to 12 µl for chlorthalidone and 2 to 12 µl losartan solution were applied on a 20 X 20 cm TLC plate to obtain the final concentration 31.25-187.50 ng/spot for chlorthalidone and 50-300 ng/spot for losartan. Each concentration was applied to the TLC plates. The plate

was then developed using the previously described mobile phase and peak areas were plotted against the corresponding concentrations to obtain the calibration curves.

Precision

The precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were performed by analysis of three different concentrations 93.75, 135.0, 156.2ng/spot for chlorthalidone 750, 1000, 1250 ng/spot losartan, were spotted to a TLC plate from the drug solutions in six replicates and developed. The intra-day precision (RSD %) was assessed by analyzing standard drug solutions within the calibration range, six times on the same day. Inter-day precision (RSD %) was assessed by analyzing drug solutions within the calibration range 93.75, 135.0, 156.2 ng/spot for chlorthalidone 750,1000,1250 ng/spot losartan, from the drug solution in six replicates, on three different days over a period of a week.

Accuracy

Twenty blank tablets were weighed, powdered and mixed thoroughly. Quantity equivalent to average weight of the tablets was spiked with 80%, 100% and 120% of the label claim of each drug. 80% of label claim corresponded to 5 mg of chlorthalidone and 40 mg of losartan, 100% of label claim corresponded to 6.25 mg of chlorthalidone and 50 mg of losartan while 120% corresponded to 7.5 mg of chlorthalidone and 60 mg of losartan. The powder mixtures containing 80, 100 and 120% of label claim were dissolved in about 80 ml of methanol, the resulting solutions were sonicated for about 30 min and volume was made up to 100 ml with methanol. The solutions were filtered and 2 µl of the filtrate from each solution was applied on TLC plates for analysis. The analysis was performed in three replicates over three days. **Accuracy was inferred from % relative error while the % RSD indicated the precision of the method.**

Selectivity

Twenty blank tablets were weighed and triturated and quantity equal to the average tablet weight was analyzed. The chromatogram was studied for interference at the retention times of chlorthalidone and losartan. Lack of interfering peaks in the blank at the retention times of the two drugs was taken as indication of the selectivity of the method.

Specificity

The specificity of the method was ascertained by analysis of drug standards and samples.

The identities of the bands for chlorthalidone and losartan were determined by comparing the R_f and spectra of the each bands with those of standards. 254nm was selected for densitometric scanning. Peak purity for chlorthalidone and losartan was assessed by comparing the spectra of standards with those acquired at three different points of spectra obtained from the sample, i.e. the peak start (S), peak apex (M) and peak end (E) positions.

Limit of detection and limit of quantification

The detection limit of assay is the lowest concentration that can be detected but necessarily quantified; the quantitation limit is the lowest concentration that can be quantified with acceptable precision. The quantitation limit is the lowest level of analyte that can be reported. The ICH guidelines suggest three different methods for determining the detection and quantitation limits. They were determined from the slope of the calibration (S) curve and SD of the blank sample using following equations:

LOD is calculated by, LOD = 3 X SD/slope of calibration curve. LOQ can be calculated as follows, LOQ=10 X SD/slope of calibration curve.

Robustness of the method

Robustness of the proposed TLC densitometric method was determined to evaluate the influence of small deliberate changes in the chromatographic conditions during determination of chlorthalidone and losartan. Robustness was determined by changing detection wavelength, duration of saturation and development distance.

5.6.2 HPTLC Method for Atovaquone and Progunil

Standard stock solution of and proguanil

100 mg of Atovaqvone and 100 mg of proguanil were accurately weighed and transfer in to 100ml volumetric flask separately. 50ml of methanol was added and sonicated for 30min and made up to the volume with methanol. 12.5 ml of Atovaqvone and 5 ml of proguanil was pipette out and transfer in to a 100ml of volumetric flask and made up to the volume with methanol. This gave 125µg/ml (125ng/µl) of Atovaqvone and 50µg/ml (50ng/µl)of proguanil.

Preparation of sample stock solution

20 tablets (MALARONE tablets; Lable claim – 250mg of Atovaqvone and 100mg of proguanil; Glaxo Smithlinekline Pharmaceuticals Ltd) were accurately weighed and the average weight of each tablet was calculated and powdered. Weight equalent to one tablet 250mg of Atovaqvone and 100mg of proguanil was weighed and transferred in to 100ml of volumetric flask and 50ml of methanol was added and sonicated for 30 min and made up to the volume with methanol. From this solution 1ml was diluted to 10 ml with methanol. The content was filtered through whatmann filter paper no.41. From this 1ml of the solution was further diluted to 10ml with methanol to contain 250µg/ml of Atovaqvone and 100µg/ml of proguanil.

Optimization of Solvent System and Chromatographic Conditions

Chromatographic separation studies were carried out on the stock solution of Atovaqvone and proguanil. The solvent system optimization experiment was carried out by the method suggested by Poole et al. Initially the plates spotted with 4µl, 125µg/ml of Atovaqvone and 50µg/ml of proguanil were developed by linear ascending development using neat solvents like toluene, hexane, methanol, chloroform, dichloromethane, ethyl acetate, acetone, acetonitrile, etc. without chamber saturation. Based on the results of these initial chromatograms binary and ternary mixtures of solvents were tried to achieve optimum resolution between Atovaquone and proguanil. Other chromatographic conditions like chamber saturation time, run length, sample application rate and volume, sample application positions, distance between tracks, detection wavelength, were optimized to give reproducible R_f values, better resolution, and symmetrical peak shape for the two drugs.

Calibration Experiments

Increasing volumes from the mixed standard stock solution 4 to 24 µl for Atovaquone and proguanil solution were applied on a 20 X 20 cm TLC plate to obtain the final concentration 500-3000 ng/spot for Atovaqvone and 200-1200 ng/spot for proguanil. Each concentration was applied to the TLC plates. The plate was then developed using the previously described mobile phase and peak areas were plotted against the corresponding concentrations to obtain the calibration curves.

Table No 11.0: Calibration of standards volume and concentration standards applied/spot on plate Proguanil and Atovaquone

Standard	Volume of Atovaquone stock solution applied (µl)	Volume of proguanil stock solution (µl)	Amount of Atovaquone applied (ng)	Amount of proguanil applied (ng)
1	4	4	500	200
2	8	8	1000	400
3	12	12	1500	600
4	16	16	2000	800
5	20	20	2500	1000
6	24	24	3000	1200

After completion of chromatographic analysis, peak areas of Atovaqvone and proguanil were noted for each track. Atovaqvone peak areas (ordinates) were plotted against concentration (abscissa) of Atovaqvone in the mixed standards and the data of X,Y pairs were subjected to least square linear regression. The calibration equation for Atovaqvone was thus obtained.

Similarly, peak areas of proguanil were plotted against corresponding concentrations and least square regression analysis was performed to generate the calibration equation for proguanil.

Analysis of a marketed formulation

To determine the content of Atovaqvone and proguanil in a conventional tablets ((MALARONE tablets; Lable claim – 250mg of Atovaqvone and 100mg of proguanil) 20 tablets were weighted their mean weight determined and finally powdered. The weight of the powder equivalent to 250 mg of Atovaqvone and 100 mg of proguanil was transferred in to a 100 ml volumetric flask containing 50ml of methanol and sonicated for 30 min and diluted to 100ml with methanol and filtered. Then 1ml of filtered solution was diluted to produce the concentration of 250µg/ml of Atovaqvone and 100µg/ml of proguanil and 6µl the working standard was applied. The final concentration obtained was 1500 ng/spot for Atovaqvone and 600 ng/spot for proguanil which was developed in an optimized mobile phase. The analysis was repeated in triplicate. The possibility of excipient interference with the analysis was

examined. The area of the peak corresponding to the R_f value of Atovaqvone and proguanil standard was recorded and the amount present was calculated.

5.7.2 HPTLC Method Validation for Atovaquone and Proguanil

The developed method was validated as per the ICH Q2 (R1) guidelines. The validation parameters studied were accuracy, precision, linearity and range, selectivity and robustness.

Linearity and range

Increasing volumes from the mixed standard stock solution 4 to 24 µl for Atovaqvone and proguanil solution were applied on a 20 X 20 cm TLC plate to obtain the final concentration 500-3000 ng/spot for Atovaqvone and 200-1200 ng/spot for proguanil. Each concentration was applied to the TLC plates. The plate was then developed using the previously described mobile phase and peak areas were plotted against the corresponding concentrations to obtain the calibration curves.

Precision

The precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were performed by analysis of three different concentrations 1000,1500,2000 ng/spot for Atovaqvone 400, 600, 800 ng/spot proguanil, were spotted to a TLC plate from the drug solutions in six replicates and developed. The intra-day precision (RSD%) assessed by analyzing standard drug solutions within the calibration range, six times on the same day. Inter-day precision (RSD %) was assessed by analyzing drug solutions within the calibration range 1000,1500,2000 ng/spot for Atovaqvone 400, 600, 800 ng/spot proguanil, from the drug solution in six replicates, on three different days over a period of a week.

Accuracy

Twenty blank tablets were weighed, powdered and mixed thoroughly. Quantity equivalent to average weight of the tablets was spiked with 80%, 100% and 120% of the label claim of each drug. 80% of label claim corresponded to 200 mg of Atovaqvone and 80 mg of proguanil, 100% of label claim corresponded to 250 mg of Atovaqvone and 100 mg of proguanil while 120% corresponded to 300 mg of Atovaqvone and 120 mg of proguanil. The powder mixtures containing 80, 100 and 120% of label claim were dissolved in about 80 ml of methanol, the resulting solutions were sonicated for about 30 min and volume was made up to 100 ml with methanol. The solutions were filtered and 6 µl of the filtrate from each solution was

applied on TLC plates for analysis. The analysis was performed in three replicates over three days. **Accuracy was inferred from % relative error while the % RSD indicated the precision of the method.**

Selectivity

Twenty blank tablets were weighed and triturated and quantity equal to the average tablet weight was analyzed. The chromatogram was studied for interference at the retention times of Atovaqvone and proguanil. Lack of interfering peaks in the blank at the retention times of the two drugs was taken as indication of the selectivity of the method.

Specificity

The specificity of the method was ascertained by analysis of drug standards and samples.

The identities of the bands for Atovaqvone and proguanil were determined by comparing the R_f and spectra of the each bands with those of standards. 260nm was selected for densitometric scanning. Peak purity for Atovaqvone and proguanil was assessed by comparing the spectra of standards with those acquired at three different points of spectra obtained from the sample, i.e. the peak start (S), peak apex (M) and peak end (E) positions.

Limit of detection and limit of quantification

The detection limit of assay is the lowest concentration that can be detected but necessarily quantified; the quantitation limit is the lowest concentration that can be quantified with acceptable precision. The quantitation limit is the lowest level of analyte that can be reported. The ICH guidelines suggest three different methods for determining the detection and quantitation limits. They were determined from the slope of the calibration (S) curve and SD of the blank sample using following equations:

LOD is calculated by, LOD = 3 X SD/slope of calibration curve. LOQ can be calculated as follows, LOQ=10 X SD/slope of calibration curve.

Robustness of the method

Robustness of the proposed TLC densitometric method was determined to evaluate the influence of small deliberate changes in the chromatographic conditions during determination of Atovaqvone and proguanil. Robustness was determined by changing in detection wavelength and with different analyst.

5.6.3 HPTLC Method Development for Acetylcysteine and Acebrofylline

Standard stock solution of acetylcysteine and acebrofylline:

100 mg of acetylcysteine (ASCT) and 100 mg of acebrofylline (ASBF) were accurately weighed and transfer in to 100ml volumetric flask separately. 50ml of methanol was added and sonicated for 30min and made up to the volume with methanol. 15ml of acetylcysteine and 2.5ml of acebrofylline was pipette out and transfer in to a 100ml of volumetric flask and made up to the volume with methanol. This gave 150µg/ml of acetylcysteine and 25µg/ml of acebrofylline.

Preparation of sample stock solution

20 capsules (PULMOCLEAR capsules; Lable claim - 600mg of acetylcysteine and 100mg of acebrofylline; Fourrts (India) Laboratories Pvt Ltd) were accurately weighed and the average weight of each capsule was calculated and powdered. Weight equalent to one capsule 600mg of acetylcysteine and 100mg of acebrofylline was weighed and transferred in to 100ml of volumetric flask and 50ml of methanol was added and sonicated for 30 min and made up to the volume with methanol. The content was filtered through whatmann filter paper no.41. From this 1ml of the solution was further diluted to 10ml and 5ml of the solution diluted to 10 ml with methanol to contain 300µg/ml of acetylcysteine and 50g/ml of acebrofylline.

Optimization of Solvent System and Chromatographic Conditions

Chromatographic separation studies were carried out on the stock solution of acetylcysteine and acebrofylline. The solvent system optimization experiment was carried out by the method suggested by Poole et al[118]. Initially the plates spotted with 2µl (150µg/ml) of acetylcysteine and (25µg/ml) of acebrofylline were developed by linear ascending development using neat solvents like toluene, hexane, methanol, chloroform, dichloromethane, ethyl acetate, acetone, acetonitrile, etc. without chamber saturation. Based on the results of these initial chromatograms binary and ternary mixtures of solvents were tried to achieve optimum resolution between acetylcysteine and acebrofylline. Other chromatographic conditions like chamber saturation time, run length, sample application rate and volume, sample application positions, distance between tracks, detection wavelength, were optimized to give reproducible R_f values, better resolution, and symmetrical peak shape for the two drugs.

When acetylcysteine and acebrofylline were applied on plate and the plates were developed using neat solvents, the results obtained were-

Solvent		**Acetylcysteine**	**Acebrofylline**
Benzene	:	a big spot at R_f 0.63	(no separation)
Chloroform	:	R_f 0.64R_f 0.75	(tailing & no clear separation)
Toluene	:	R_f 0.72R_f 0.84	(tailing & no clear separation)
Ethyl Acetate	:	a big spot at R_f 0.28	(no separation)
Methanol	:	R_f 0.12	(no separation)

It was concluded that no single solvent was able to resolve the two drugs. Even in the strongest solvent i.e. methanol, the movement of both drugs was very less. Hence, mixtures of solvents were used.

Thus, the solvent system, chloroform: toluene: methanol (6:2:2 v/v/v) was selected.

Calibration Experiments

Increasing volumes from the mixed standard stock solution 2 to 12 µl for acetylcysteine and 2 to 12 µl acebrofylline solution were applied on a 20 X 20 cm TLC plate to obtain the final concentration 300-1800 ng/spot for acetylcysteine and 50-300 ng/spot for acebrofylline. Each concentration was applied to the TLC plates. The plate was then developed using the previously described mobile phase and peak areas were plotted against the corresponding concentrations to obtain the calibration curves

Table No 14.0:Calibration of standards volume and concentration standards applied/spot on plate Acetylcysteine andAcebrofylline

Standard	**Volume of acetylcysteine stock solution applied (µl)**	**Volume of acebrofylline stock solution (µl)**	**Amount of acetylcysteine applied (ng)**	**Amount of acebrofylline applied (ng)**
1	2	2	300	50
2	4	4	600	100
3	6	6	900	150
4	8	8	1200	200
5	10	10	1500	250
6	12	12	1800	300

After completion of chromatographic analysis, peak areas of acetylcysteine and acebrofylline were noted for each track. Acetylcysteine peak areas (ordinates) were plotted against concentration (abscissa) of acetylcysteine in the mixed standards and the data of X,Y pairs were subjected to least square linear regression. The calibration equation for acetylcysteine was thus obtained.Similarly, peak areas of acebrofylline were plotted against corresponding concentrations and least square regression analysis was performed to generate the calibration equation for acebrofylline.

Analysis of a marketed formulation

To determine the content of Acetylcysteine and acebrofylline in a conventional capsule (PULMOCLEAR capsules; Lable claim - 600mg of acetylcysteine and 100mg of acebrofylline) 20 capsules were weighted their mean weight determined and finally powdered. The weight of the powder equivalent to 600 mg of acetylcysteine and 100 mg of acebrofylline was transferred in to a 100 ml volumetric flask containing 50ml of methanol and sonicated for 30 min and diluted to 100ml with methanol and filtered. Then 1ml of filtered solution was diluted to produce the concentration of 300µg/ml of acetylcysteine and 50µg/ml of acebrofylline and 2µl the working standard was applied. The final concentration obtained was 600 ng/spot for acetylcysteine and 100 ng/spot for acebrofylline which was developed in an optimized mobile phase. The analysis was repeated in triplicate. The possibility of excipient interference with the analysis was examined. The area of the peak corresponding to the R_f value of acetylcysteine and acebrofylline standard was recorded. Then amount present was calculated.

5.7.3 HPTLC Method Validation for Acetylcysteine and Acebrofylline

The developed method was validated as per the ICH Q2 (R1) guidelines. The validation parameters studied were accuracy, precision, linearity and range, selectivity and robustness.

Linearity and range

Increasing volumes from the mixed standard stock solution 2 to 12 µl for acetylcysteine and 2 to 12 µl acebrofylline solution were applied on a 20 X 20 cm TLC plate to obtain the final concentration 300-1800 ng/spot for acetylcysteine and 50-300 ng/spot for acebrofylline. Each concentration was applied to the TLC plates.

The plate was then developed using the previously described mobile phase and peak areas were plotted against the corresponding concentrations to obtain the calibration curves.

Precision

The precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were performed by analysis of three different concentrations 600,900,1200 ng/spot for acetylcysteine 100, 150, 200 ng/spot acebrofylline, were spotted to a TLC plate from the drug solutions in six replicates and developed. The intra-day precision (RSD%) assessed by analyzing standard drug solutions within the calibration range, six times on the same day. Inter-day precision (RSD %) was assessed by analyzing drug solutions within the calibration range 600,900,1200 ng/spot for acetylcysteine 100, 150, 200 ng/spot acebrofylline, from the drug solution in six replicates, on three different days over a period of a week.

Accuracy

Twenty blank tablets were weighed, powdered and mixed thoroughly. Quantity equivalent to average weight of the tablets was spiked with 80%, 100% and 120% of the label claim of each drug. 80% of label claim corresponded to 480 mg of acetylcysteine and 80 mg of acebrofylline, 100% of label claim corresponded to 600 mg of acetylcysteine and 100 mg of acebrofylline while 120% corresponded to 720 mg of acetylcysteine and 120 mg of acebrofylline. The powder mixtures containing 80, 100 and 120% of label claim were dissolved in about 80 ml of methanol, the resulting solutions were sonicated for about 30 min and volume was made up to 100 ml with methanol. The solutions were filtered and 2 µl of the filtrate from each solution was applied on TLC plates for analysis. The analysis was performed in three replicates over three days. **Accuracy was inferred from % relative error while the % RSD indicated the precision of the method.**

Selectivity

Twenty blank tablets were weighed and triturated and quantity equal to the average tablet weight was analyzed. The chromatogram was studied for interference at the Rf of acetylcysteine and acebrofylline. Lack of interfering peaks in the blank at the Rf of the two drugs was taken as indication of the selectivity of the method.

Specificity

The specificity of the method was ascertained by analysis of drug standards and samples.

The identities of the bands for acetylcysteine and acebrofylline were determined by comparing the R_f and spectra of the each bands with those of standards. 215nm was selected for densitometric scanning. Peak purity for acetylcysteine and acebrofylline was assessed by comparing the spectra of standards with those acquired at three different points of spectra obtained from the sample, i.e. the peak start (S), peak apex (M) and peak end (E) positions.

Limit of detection and limit of quantification

The detection limit of assay is the lowest concentration that can be detected but necessarily quantified; the quantitation limit is the lowest concentration that can be quantified with acceptable precision. The quantitation limit is the lowest level of analyte that can be reported. The ICH guidelines suggest three different methods for determining the detection and quantitation limits. They were determined from the slope of the calibration (S) curve and SD of the blank sample using following equations:

LOD is calculated by, LOD = 3 X SD/slope of calibration curve. LOQ can be calculated as follows, LOQ=10 X SD/slope of calibration curve.

Robustness of the method

Robustness of the proposed TLC densitometric method was determined to evaluate the influence of small deliberate changes in the chromatographic conditions during determination of acetylcysteine and acebrofylline. Robustness was determined by changing in detection wavelength,mobile phase saturation time.

5.8 : HPTLC Method Development and Validation for Herbal Drug Analysis

5.8.1 HPTLC Quantification of L-Dopa in Zandopa powder

Quantification of L-Dopa in Zandopa powder

Standard stock solution of L-Dopa

10 mg of L-Dopa was weighed and dissolved in 10 methanol and made upto 100 ml with 0.1NHCl. This gave 100µg/ml of L-Dopa.

Sample Preparation : 7. 5 gm of Zandopa powder was refluxed with 25ml methanol, filtered and concentrated to 5ml and mixed with 5 ml of 0.1NHCl

Mobile Phase : n-Butanol: Water: Acetic acid (8:2:2)

Detection : @520nm in Densitometry TLC Scanner

Derivatization : Ninhydrine reagent-heat the plate in 105^0C at 5 min

Calibration standards and sample applied on plate

Table No 13.0:Calibration of standards volume and concentration standards applied/spot on plate for L-Dopa

Standard	Volume of standard solution of L-Dopa applied (µl)	Amount of L-Dopa applied (ng)
1	0.5	50
2	1	100
3	2	200
4	3	300
5	4	400
6	5	500

Optimized Chromatographic conditions

The samples were applied in form of bands of width 6 mm on precoated silica gel aluminum sheets 60 $F_{254.}$ A constant application rate of 150nl/s was selected according to the recommendations in-built in the Wincats software. The application position (X) and (Y) were kept at 10mm to avoid edge effect. Linear ascending development was carried out in a twin trough glass chamber (20cmx10 cm, 10X10 cm), without using filter paper and without saturation. 20 ml mobile phase for 20X10 cm tank and 10 ml for 10X10 cm tank was used per chromatographic development; the length of chromatogram run was 80 mm. The developed plates were dried in the flow of nitrogen. A visualizing agent, Ninhydrine reagent was sprayed on plate and dried at room temperature. Before densitometric scanning, plates were heated for 5 min in oven at 105°C. Densitometric evaluation of the plates was performed at λ = 520 nm using a Camag TLC scanner-3 equipped with wincats software, using a Tungsten light source. The slit dimension was kept at 6 X 0.45 mm and 100 nm/s scanning speed was employed.

5.8.2 HPTLC Method Validation for L-dopa

The developed method was validated as per the ICH Q2 (R1) guidelines. The validation parameters studied were accuracy, precision, linearity and range, selectivity and robustness.

Linearity and range

Increasing volumes from the mixed standard stock solution 0.5 to 5 µl of L-dopa solution was applied on a 20 X 20 cm TLC plate to obtain the final concentration 50-500 ng/spot L-dopa. Each concentration was applied to the TLC plates. The plate was then developed using the previously described mobile phase and peak areas were plotted against the corresponding concentrations to obtain the calibration curves.

Precision

The precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were performed by analysis of three different concentrations 200,300,400 ng/spot for L -dopa, was spotted to a TLC plate from the drug solutions in six replicates and developed. The intra-day precision (RSD %) was assessed by analyzing standard drug solutions within the calibration range, six times on the same day. Inter-day precision (RSD %) was assessed by analyzing drug solutions within the calibration range 200,300,400 ng/spot for L -dopa from the sample solution in six replicates, on three different days over a period of a week.

Accuracy

Pre quantified herbal formulation was spiked with 80%, 100% and 120% of the label claim of each drug. The powder mixtures containing 80% of constituent (of quantified sample) (corresponded to 4 mg of L-dopa), 100% (corresponded to 5 mg of L-dopa), 120% (corresponded to 6 mg of L-dopa) were dissolved in about 8 ml of methanol, the resulting solutions were sonicated for about 30 min and volume was made up to 10 ml with methanol. The solutions were filtered and 2 µl of the filtrate from each solution was applied on TLC plates for analysis. The analysis was performed in three replicates over three days. Accuracy was inferred from % relative error while the % RSD indicated the precision of the method.

Selectivity

Twenty blank tablets were weighed and triturated and quantity equal to the average tablet weight was analyzed. The chromatogram was studied for interference at the retention time of L-dopa. Lack of interfering peaks in the blank at the retention times of the two drugs was taken as indication of the selectivity of the method.

Specificity

The specificity of the method was ascertained by analysis of drug standards and samples.

The identities of the bands of L-dopa were determined by comparing the R_f and spectra of the each bands with those of standards. 520nm was selected for densitometric scanning. Peak purity for L-dopa was assessed by comparing the spectra of standards with those acquired at three different points of spectra obtained from the sample, i.e. the peak start (S), peak apex (M) and peak end (E) positions.

Limit of detection and limit of quantification

The detection limit of assay is the lowest concentration that can be detected but necessarily quantified; the quantitation limit is the lowest concentration that can be quantified with acceptable precision. The quantitation limit is the lowest level of analyte that can be reported. The ICH guidelines suggest three different methods for determining the detection and quantitation limits. They were determined from the slope of the calibration (S) curve and SD of the blank sample using following equations:

LOD is calculated by, LOD = 3 X SD/slope of calibration curve. LOQ can be calculated as follows, LOQ=10 X SD/slope of calibration curve.

Analysis of a marketed herbal formulation

Methanolic extract of L-Dopa was applied and developed according to method described above. Using the calibration data, the regression analysis was performed and from the equation for regression line obtained, the amount of L-Dopa in Zandopa powder was determined.

5.8.3 HPTLC estimation for the lawsone and PPD in Indica herbal hair dye

Standard stock solution of lawsone and p-phenylenediamine:

10 mg of lawsone and 10 mg of p-phenylenediamine was accurately weighed and dissolved in methanol to make 10 ml. This gave 1000µg/ml of lawsone and p-phenylenediamine respectively. The solutions were further diluted with methanol to yield working standard solutions having concentration of 5µg/ml for lawsone and 50µg/ml for p-phenylenediamine.

Optimization of Solvent System and Chromatographic Conditions

Chromatographic separation studies were carried out on the stock solution of lawsone and p-phenylenediamine. The solvent system optimization experiment was carried out by the method suggested by Poole et al. Initially the plates spotted with 10µl of stock solution prepared as per section 1.1 were developed by linear ascending development using neat solvents like butanol, hexane, methanol, chloroform, dichloromethane, ethyl acetate, acetone, acetonitrile, etc. Based on the results of these initial chromatograms binary and ternary mixtures of solvents were tried to achieve optimum resolution between lawsone and p-phenylenediamine. Other chromatographic conditions like chamber saturation time, run length, sample application rate and volume, sample application positions, distance between tracks, detection wavelength, were optimized to give reproducible R_f values, better resolution, and symmetrical peak shape for the two drugs.

Calibration Experiments

Increasing volumes of the stock solution prepared as per section 1.1 were applied on a 20 X 20 cm plate to get applied quantities as per table 1.

Table No 14.0:Calibration of standards volume and concentration standards applied/spot on plate for lawsone and p-phenylenediamine

Standard	Volume of lawsone stock solution applied (µl)	Volume of p-phenylenediamine stock solution (µl)	Amount of lawsone applied (ng)	Amount of p-phenylenediamine applied (ng)
1	2	2	10	100
2	4	4	20	200
3	6	6	30	300
4	8	8	40	400
5	10	10	50	500
6	12	12	60	600
7	14	14	70	700

After completion of chromatographic analysis, peak areas of lawsone and p-phenylenediamine were noted for each track. Lawsone peak areas (ordinates) were plotted against concentration (abscissa) of lawsone in the mixed standards and the data of X,Y pairs were subjected to least square linear regression. The calibration equation for lawsone was thus obtained.

Similarly, peak areas of p-phenylenediaminewere plotted against corresponding concentrations and least square regression analysis was performed to generate the calibration equation for p-phenylenediamine.

Analysis of Marketed Herbal Formulation

To determine the content of lawsone and p-phenylenediamine in a commercial herbal formulation (Indica herbal hair dye) , 1000mg of commercial herbal formulation was accurately weighed and transferred in 50 ml volumetric flask. Lawsone and p-phenylenediamine were extracted and made up to mark with methanol and then filtered. 0.5ml of the above solution was pipetted out in a 10 ml volumetric flask and made up to the mark with methanol to give a final concentration falling within the calibration curve.

The peak areas of lawsone and p-phenylenediaminewere referred to the respective calibration equations to get the amount of the two constituents on the sample track. The analysis was repeated in triplicate. The content of lawsone and p-phenylenediamine per in herbal formulation was thus calculated.

5.8.3 HPTLC Validation for the Lawsone and PPD in Indica herbal hair dye

The developed method was validated as per the ICH Q2 (R1) guidelines. The validation parameters studied were accuracy, precision, linearity and range, selectivity and robustness.

Linearity and range

Increasing volumes from the mixed standard stock solution 2 to 14 µl for lawsone and p-phenylenediamine solution were applied on a 20 X 20 cm TLC plate to obtain the final concentration 10-70 ng/spot for lawsone and 100-700 ng/spot for p-phenylenediamine Each concentration was applied to the TLC plates. The plate was then developed using the previously described mobile phase and peak areas were plotted against the corresponding concentrations to obtain the calibration curves.

Precision

The precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were performed by analysis of three different concentrations 20,30,40 ng/spot for lawsone and 200, 300 , 400ng/spot p-phenylenediamine, were spotted to a TLC plate from the drug solutions in six replicates and developed. The intra-day precision (RSD%) assessed by analyzing

standard drug solutions within the calibration range, six times on the same day. Inter-day precision (RSD %) was assessed by analyzing drug solutions within the calibration range 20,30,40 ng/spot for lawsone and 200, 300 , 400ng/spot p-phenylenediamine from the drug solution in six replicates, on three different days over a period of a week.

Accuracy

Pre quantified herbal formulation was spiked with 80%, 100% and 120% of the label claim of each drug. The powder mixtures containing 80% of constituent (of quantified sample) (corresponded to 4 mg of lawsone and 20 mg of p-phenylenediamine,100% (corresponded to 5 mg of lawsone and 25 mg of p-phenylenediamine), 120% (corresponded to 6 mg of lawsone and 30 mg of p-phenylenediamine) were dissolved in about 8 ml of methanol, the resulting solutions were sonicated for about 30 min and volume was made up to 10 ml with methanol. The solutions were filtered and 2 µl of the filtrate from each solution was applied on TLC plates for analysis. The analysis was performed in three replicates over three days. Accuracy was inferred from % relative error while the % RSD indicated the precision of the method.

Selectivity

Twenty blank tablets were weighed and triturated and quantity equal to the average tablet weight was analyzed. The chromatogram was studied for interference at the retention times of lawsone and p-phenylenediamine. Lack of interfering peaks in the blank at the retention times of the two drugs was taken as indication of the selectivity of the method.

Specificity

The specificity of the method was ascertained by analysis of drug standards and samples.

The identities of the bands for lawsone and p-phenylenediamine were determined by comparing the R_f and spectra of the each bands with those of standards. 254nm was selected for densitometric scanning. Peak purity for lawsone and p-phenylenediamine was assessed by comparing the spectra of standards with those acquired at three different points of spectra obtained from the sample, i.e. the peak start (S), peak apex (M) and peak end (E) positions.

Limit of detection and limit of quantification

The detection limit of assay is the lowest concentration that can be detected but necessarily quantified; the quantitation limit is the lowest concentration that can be quantified with acceptable precision. The quantitation limit is the lowest level of analyte that can be reported. The ICH guidelines suggest three different methods for determining the detection and quantitation limits. They were determined from the slope of the calibration (S) curve and SD of the blank sample using following equations:

LOD is calculated by, LOD = 3 X SD/slope of calibration curve. LOQ can be calculated as follows, LOQ=10 X SD/slope of calibration curve.

6. RESULTS AND DISCUSSION

6.1: PART – 1 RESULT FOR HPLC METHOD DEVELOPMENT

In this Part-1 of Results, three newer multicomponents methods are developed by RP-HPLC method for the simultaneous estimation of three drugs combination in the marketed formulations.

1. HPLC Method Development of Losartan and chlorthalidone in Tablet formulation.
2. HPLC Method Development Atovaquone and Proguanil in Tablet formulation.
3. HPLC Method Development Acetylcysteine and Acebrofylline in Capsule formulation.

6.1.1: HPLC Method Development of Losartan and Chlorthalidone

The literature suvey reveals that there are number of methods were available for Losartan and chlorthalidone alone or in combination with other drug but no single method is available for the estimation of this recently developed novel multicomponet drug formulation of Losartan and chlorthalidone in tablet dosage form so, we have taken initiative for the development of novel drugs combination of losartan and chlorthalidone useful in the treatment of hypertension but, recently we came across with one method published by Surjyanarayan Mandal et al.[77] in Jan 22 of 2014.

For the estimation of Losartan and Chlorthalidone in tablet formulation and as we compared with recented reported method[77] our method is quite superior because it is simple and economical while existing method has less resolution and require more mobile phase as flow rate is more and not economical since organic phase acetonitrile in used in 50:50 ratio with buffer solution.

For the development of the method the optimized chromatographic condition are fixed after several trails, from that some of the trails were shown with there respective chromatograms in Fig.No 9.0 to Fig.No 13.0 and the optimized chromatographic condition for the losartan and chlorthalidone are given in below in Table No. 15.0 and corrousponding typical chromatogram is given in Fig.No 25.0 and from this developed method marketed formulation of Losartan and Chlorthalidone are

successfully analyzed by propased method for Tablet brand name CTD-L manufactured by IPCA Pharmaceutical ltd., containing losartan potassium 50mg and Chlorthalidone 6.25mg. HPLC assay details are given in Table No 15.0

Table No. 15.0: Optimized Chromatographic condition for Losartan Chlorthalidone

Flow rate	1.0 ml/min
Column	Agilent XDB, C18, 150 x 4.6 mm, 5µ.
Detector wave length	254 nm
Column temperature	30°C
Injection volume	10µl
Run time	8 min
Diluent	Water: Acetonitrile (50:50)
Mobile phase	Buffer : acetonitrile (70:30 % v/v pH 3.5)

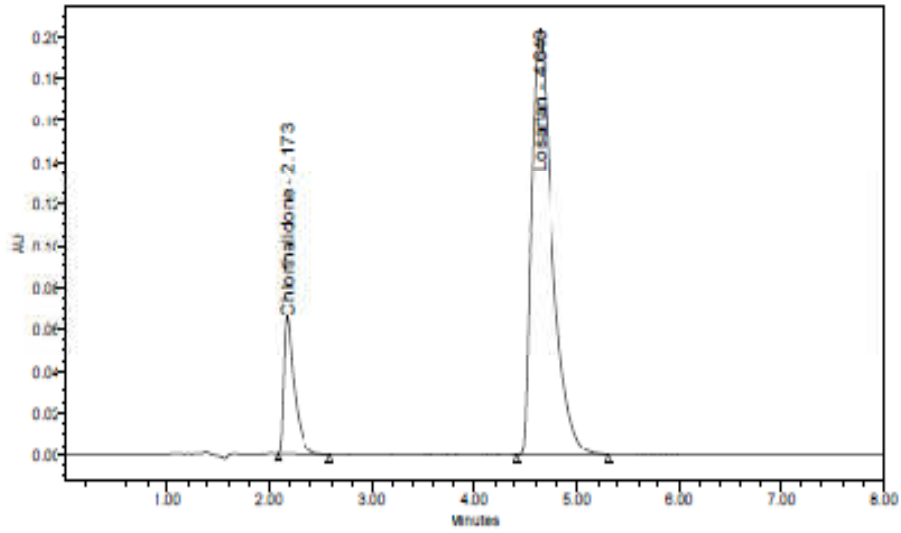


Fig. No. 25.0: HPLC Chromatogram for Losartan Chlorthalidon

Table No 16.0 HPLC Assay details for Losartan and Chlorthalidone

Parameters	Losartan	Chlorthalidone
Average weight	200mg	
Label claim	50mg	6.25mg
Standard peak area (avg)	2857887	463580
Test peak area average	2861997	465415
Standard % purity	100.2%	100.3 %
% drug content	100.3 %	100.3 %
Amount obtained	50.04 mg	6.25 mg
Dilution factor	40	40

6.1.2: HPLC Method Development of Atovaquone and Proguanil:

Atovaquone and Proguanil is very new formulation recently manufactured by the first company GLAXO *Smith Kline* ltd. In the brand name of tablet malarone containing Atovaquone 250mg and Proguanil 100mg for treatment and the prevention of malaria. During the method development both drug are not separated by number of trials (shown in Fig.No14.0 toFig. No 19.0) performed by changing flow rate, column change and mobile phase composition. But two peaks were not at all separated by the isocratic mode of separation then we selected gradient method by changing the mobile phase buffer concentration with acetonitrile composition with time for the separation of two drugs. The mobile phase gradient programme used for this separation and optimized chromatographic condition are given in Table No.18.0 and Table No.17 respectively.

The marketed formulation (Tab. Malarone) of this combination is assayed by this developed method and the assay detailes are given in Table No.19.0 and respective typical chromatograme is given in Fig. No 26.0.

Tables No 17.0: Optimized chromatographic condition for Atovaquone and Proguanil

Flow rate	1.1 ml/min
Column	Hypersil BDS, C18, 150 x 4.6 mm, 5µ
Detector wave length	258 nm
Column temperature	30°C
Injection volume	10µL
Run time	13 min
Diluent	Acetonitrile (For Stock Solution) Methanol (for Further Dilution)
Mobile phase	Buffer : Acetonitrile (Gradient Programmeas below)

Tables No 18.0: Mobile phase gradient programme for Atovaquone and Proguanil

Time	Flow Rate	% Buffer	% Acetonitrile
0	1.1	50	50
3	1.1	50	50
4	1.1	20	80
9	1.1	20	80
9.1	1.1	50	50
12.0	1.1	50	50

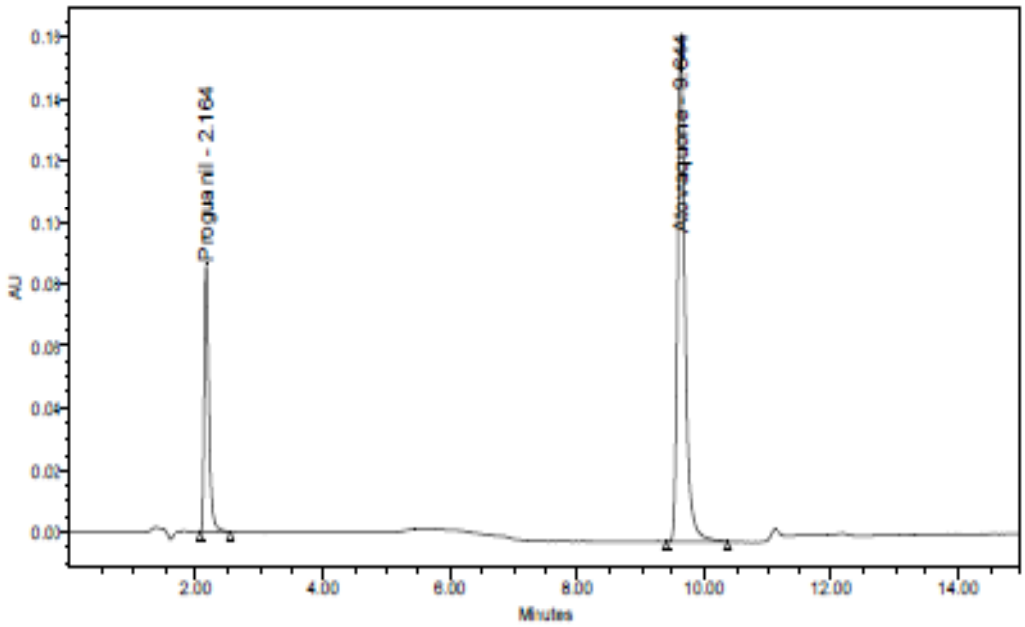


Fig. No. 26.0: HPLC Chromatogram for Atovaquone and Proguanil

Tables No 19.0: HPLC Assay details for Atovaquone and Proguanil

Parameters	Atovaquone	Proguanil
Average weight	500mg	
Lable claim	250mg	100mg
Standard peak area (avg)	1297420	437014
Test peak area average	1302301	434215
Standard % purity	99.9	100
% drug content	99.11	99.95
Amount obtained	247.7mg	99.9mg
Dilution factor	100	100

6.1.3: HPLC Method Development of Acetylcysteine and Acebrophylline

Acetylcysteine and acebrofylline is another newer combination of multicomponent formulation widely used to treat the asthmatic patient. Ther were no method reported for simultaneous estimation of acetylcysteine and acebrofylline in capsule formulation by using RP-HPLC so, we developed very novel, simple, economic method for the estimation of the acetylcysteine and acebrofylline in marketed formulation of capsule dosage form available in Capsule formulation with brand name of Cap.Pulmoclear manufactured by the fourrts India. Ltd. The number of trails was carried out for the optimization of the chromatographic condition, the respective chromatograms for five trails were shown in Fig.No 20.0 to Fig.No 26.0 The optimized chromatographic

condition are given in Table No. 20.0 and HPLC assay details is given in Table No.21.0 with typical chromatograme in Fig.No27.0.

Table No 20.0: Optimized chromatographic condition for Acebrophylline & Acetylcysteine

Flow rate	0.9 ml/min
Column	Hypersil BDS, C18, 100 x 4.6 mm, 5µ.
Detector wave length	260 nm
Column temperature	30°C
Injection volume	5µL
Run time	8 min
Diluent	Methanol
Mobile phase	Buffer : Acetonitrile (90:10 % v/v pH 3.2)

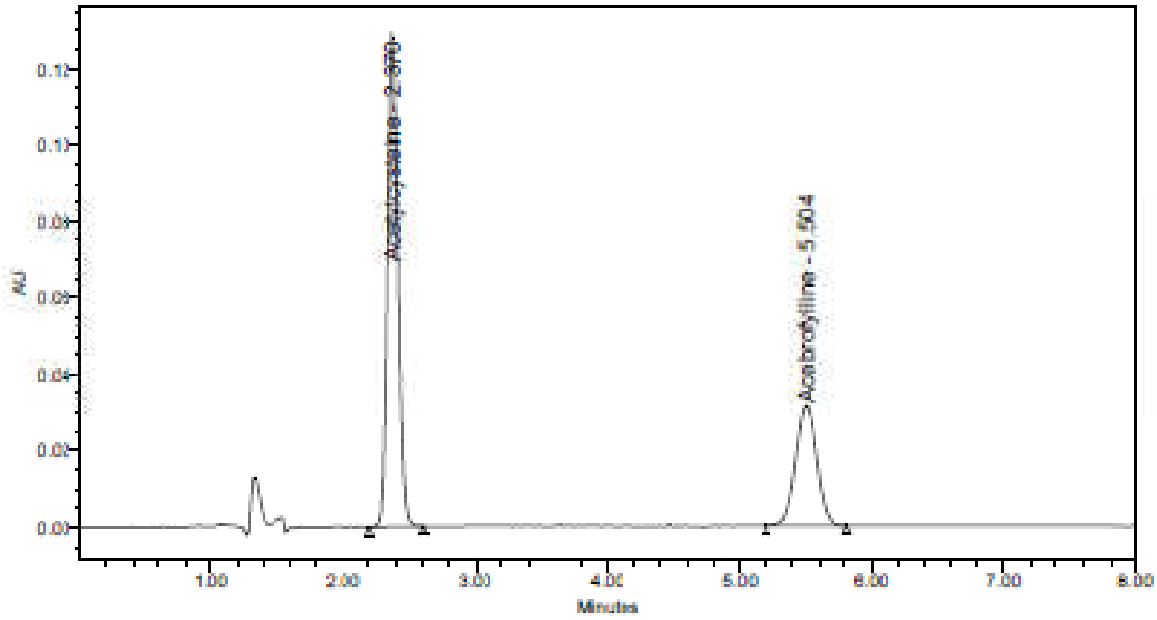


Fig. No. 27.0: HPLC Chromatogram for Acetylcysteine and Acebrofylline

Table No 21.0: HPLC Assay details for Acetylcysteine and Acebrophylline

Parameters	**Acetylcysteine**	**Acebrophylline**
Average weight	1000 mg	
Label claim	600mg	100mg
Standard peak area (avg)	686120	341164
Test peak area average	686157	344065
Standard % purity	99.61	100.4
% drug content	100.3	100.8
Amount obtained	604.1mg	100.39mg
Dilution factor	10	10

6.2: Part – 2 Results for HPLC Method Validation

The RP-HPLC developed methods were validated as per ICH[26] Q2A and USP guideline and for the different validation parameter of Liniarity, Specificity, accuracy, precision, and system suitability, Robustness, LOD and LOQ. The respected data for each parameter for selected method shown in the followings Losartan and chlorthalidone shown in Table No. 22.0 and

a. System Suitability

The system suitability of method is an important parameter to ensure the optimized method is valid or not. The system suitability is established by the various instruments parameters such as retention time (R_t), theoretical plates, taling factors and peak area % RSD. The system suitability all parameters pass the acceptance criteria given in Table No 6.0 during the entire period of validation for all the selected combination of drug formulations.

6.2.1a: System suitability for Chlorthalidone and Losartan

The % RSD, theoretical plates, resolution and tailing factors passes the test for the suitability, provide that system is suitable for the conducting the analytical method validation work. The respective data for Losartan and chlorthalidone is given in Table No22.0 and 23.0 respectively, for Atovaquone and Proguanil is given in Table No 24.0 and 25.0 respectively and for Acetylcysteine and Acebrofylline in Table No 26.0 and Table No 27.0 respectively.

Table No 22.0: System Suitability data of Chlorthalidone

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	2.170	462535	2858	1.90
2	2.173	462220	2329	1.93
3	2.175	463198	2496	1.93
4	2.181	465141	2635	1.92
5	2.183	464407	2768	1.92
6	2.190	463980	2756	1.72
Mean	-	463580	-	-
STD DEV	-	1128.5	-	-
%RSD	-	0.243	-	-

SD= Standard deviation, RSD=Relative standard deviation

Rt, % RSD, USP plate count, USP Tailing found within Acceptance criteria

Table No 23.0: System Suitability data of Losartan

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	4.537	2858879	2820	1.72
2	4.593	2832002	2589	1.70
3	4.615	2857237	2510	1.66
4	4.640	2860680	1.72	1.70
5	4.698	2869028	1.66	1.66
6	4.756	2869497	1.67	1.71
Mean	-	2857887	-	-
SD	-	13697.84	-	-
%RSD	-	0.4793	-	-

SD= Standard deviation, RSD=Relative standard deviation

Rt, % RSD, USP plate count, USP Tailing found within Acceptance criteria

6.2.2a: System suitability for Atovaquone and Proguanil

Table No 24.0: System Suitability data of Proguanil

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	2.154	440804	4230	1.45
2	2.156	441237	4205	1.47
3	2.168	440863	4181	1.46
4	2.17	431599	4534	1.46
5	2.17	435597	4347	1.45
6	2.18	431983	4449	1.48
Mean	-	437014	-	-
SD	-	4553	-	-
%RSD	-	1.04	-	-

SD= Standard deviation, RSD=Relative standard deviation

Rt, % RSD, USP plate count, USP Tailing found within Acceptance criteria

Table No 25.0: System Suitability data of Atovaquone

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	9.629	1300879	38384	1.39
2	9.632	1310021	37831	1.39
3	9.657	1309999	38362	1.40
4	9.708	1289375	37426	1.42
5	9.708	1292521	38378	1.40
6	9.779	1281722	38209	1.39
Mean	-	1297420	-	-
SD	-	11524.9	-	-
%RSD	-	0.9	-	-

SD= Standard deviation, RSD=Relative standard deviation

Rt, % RSD, USP plate count, USP Tailing found within Acceptance criteria

6.2.3a: System suitability for Acetylcysteine and Acebrofylline

Table No 26.0: System Suitability data of Acetylcysteine

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	2.36	686974	4492	1.09
2	2.362	685310	4513	1.1
3	2.363	686086	4502	1.1
4	2.367	683964	4290	1.1
5	2.37	686033	4376	1.09
6	2.371	688352	4430	1.09
Mean	-	686120	-	-
STD DEV	-	1485	-	-
%RSD	-	0.2	-	-

SD= Standard deviation, RSD=Relative standard deviation

Rt, % RSD, USP plate count, USP Tailing found within Acceptance criteria

Table No 27.0: System Suitability data of Acebrofylline

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	5.484	340354	5842	0.99
2	5.485	340140	5830	1.0
3	5.504	342289	5843	1.0
4	5.507	338474	5877	1.0
5	5.518	344216	5970	0.99
6	5.533	341513	5715	1.0
Mean	-	341164	-	-
STD DEV	-	1982.1	-	-
%RSD	-	0.6	-	-

SD= Standard deviation, RSD=Relative standard deviation

Rt, % RSD, USP plate count, USP Tailing found within Acceptance criteria

b. Linearity

The method is said to be liniear, when the calibration curve is constructed by the plotting concentration versus peak area of the sample in the selected range of the different increasing concentrations.

In the determination of the linearity, slope, y-intercept, correlation coefficient and regression coefficient should be calculated and correlation coefficient should be 0.999. The HPLC method is linear for the all 3 selected drug formulations and their data were given in below tables and figures.

The linearity data for the losartan and chlorthalidone shown in Table No 28.0 and respective calibration is given in Fig. No. 28.0 and 29.0 respectively, data for the Proguanil and Atovaquone shown in Table No 29.0 and respectively calibration is given in Fig. No. 30.0 and 31.0 respectively and data for the Acetylcysteine and acebrofylline shown in Table No 30.0 and respective calibration is given in Fig. No. 32.0 and 33.0 respectively.

6.2.1b: Linearity data for Chlorthalidone and Losartan

Table No 28.0: Linearity data for Chlorthalidone and Losartan

Sr.No	Concentration in ppm (LOS)	Peak area (LOS)	Concntration in ppm (CTD)	Peak area (CTD)
1	12.5	753820	1.5625	131737
2	25	1535317	3.125	253128
3	37.5	2255254	4.6875	367882
4	50	2956937	6.25	485151
5	62.5	3778461	7.8125	606678
6	75	4495215	9.375	713679
Slope		59841.67	Slope	75953.33
Intercept		9060.333	Intercept	9435
Correlation Coefficient		0.999	Correlation Coefficient	0.999

*Mean of three determinations

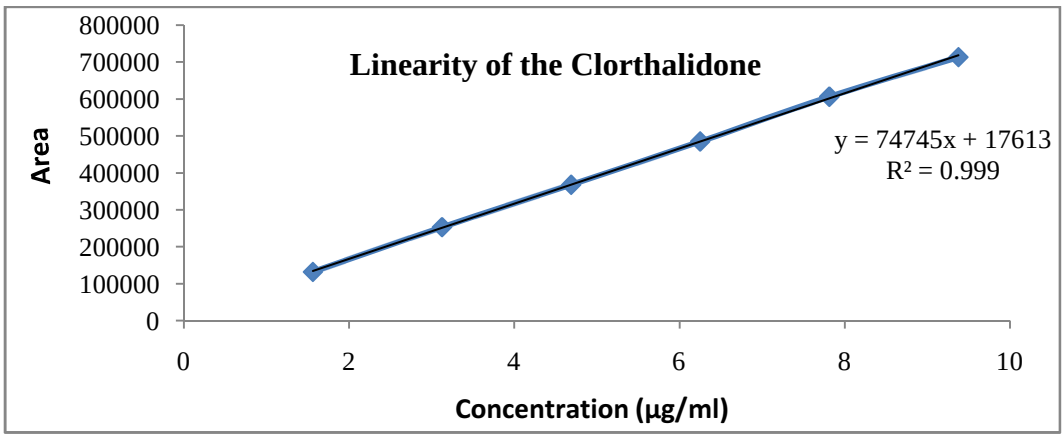


Fig. No. 28.0: HPLC Calibration Curve for Chlorthalidone

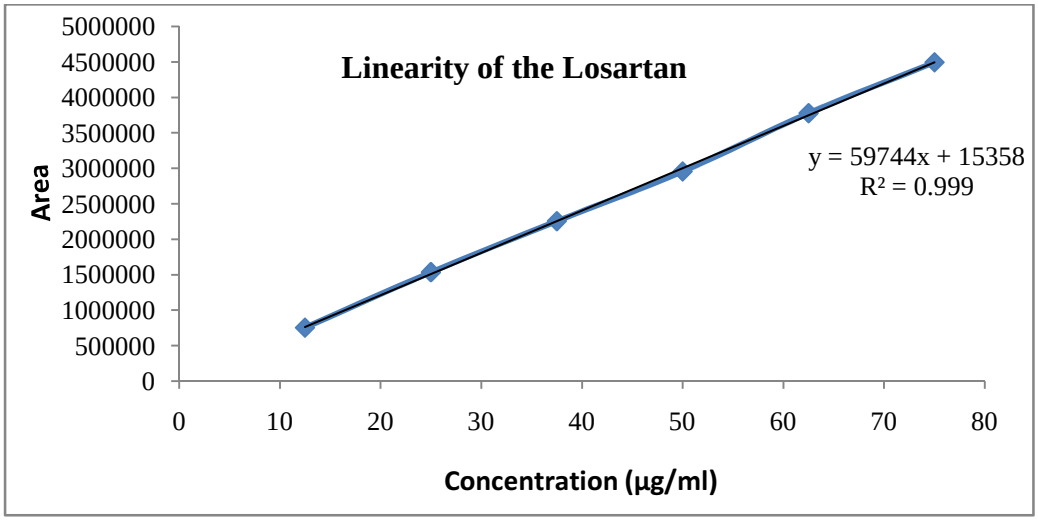


Fig. No. 29.0: HPLC Calibration Curve for Losartan

6.2.2b: Linearity data for Proguanil and Atovaquone

Table No 29.0: Linearity data for Proguanil and Atovaquone

Sr.No	Concentration in ppm (PROG)	Peak area (PROG)	Concntration in ppm (ATOV)	Peak area (ATOV)
1	40	92608	100	283458
2	100	225067	250	665259
3	140	311260	350	934259
4	200	430416	500	1315641
5	240	516706	600	1564832
6	300	645799	750	1930235
Slope		2135.333	Slope	2570.667
Intercept		6208.667	Intercept	19607
Correlation Coefficient		0.999	Correlation Coefficient	0.999

*Mean of three determinations

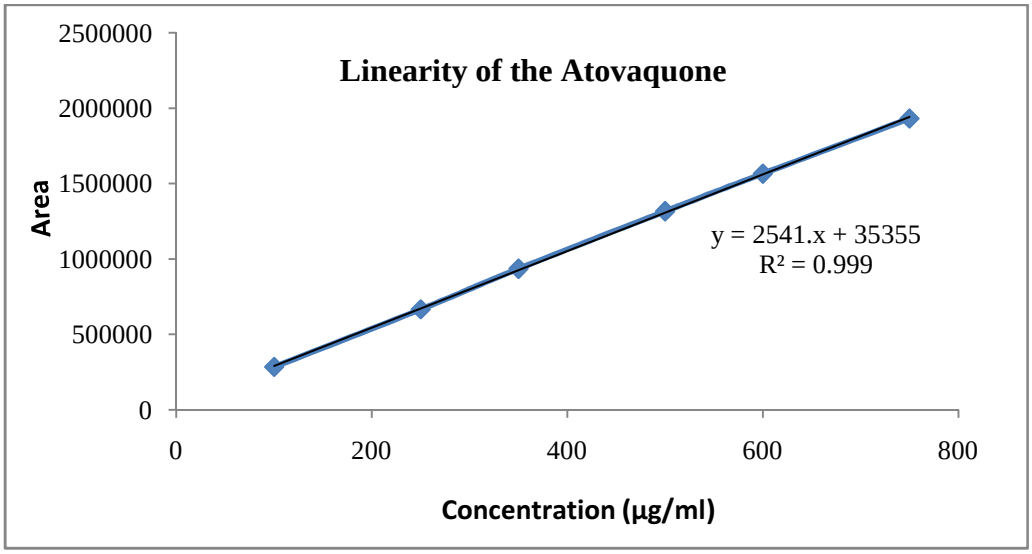


Fig. No. 30.0: HPLC Calibration Curve for Atovaquone

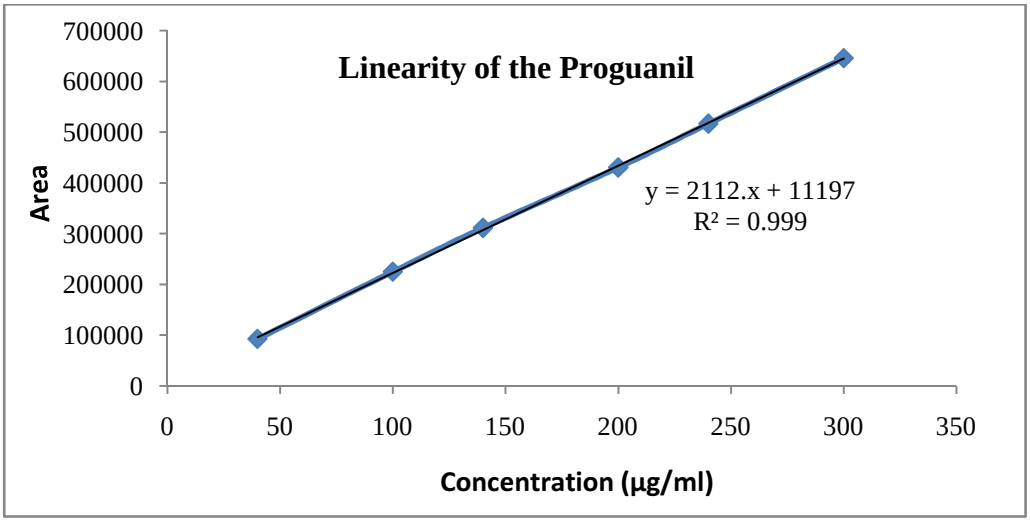


Fig. No. 31.0: HPLC Calibration Curve for Proguanil

6.2.3b: Linearity data for Acetylcysteine and Acebrofylline

Table No. 30.0: Linearity data for Acetylcysteine and Acebrofylline

Sr.No	Concentration in ppm (ACST)	Peak area (ACST)	Concntration in ppm (ACBF)	Peak area (ACBF)
1	150	174619	25	88736
2	300	355507	50	175892
3	450	514460	75	257873
4	600	690463	100	345418
5	750	855800	125	431317
6	900	1039137	150	515247
Slope		1145	Slope	3428.7
Intercept		2102.667	Intercept	2022.333
Correlation Coefficient		0.999	Correlation Coefficient	0.999

*Mean of three determinations

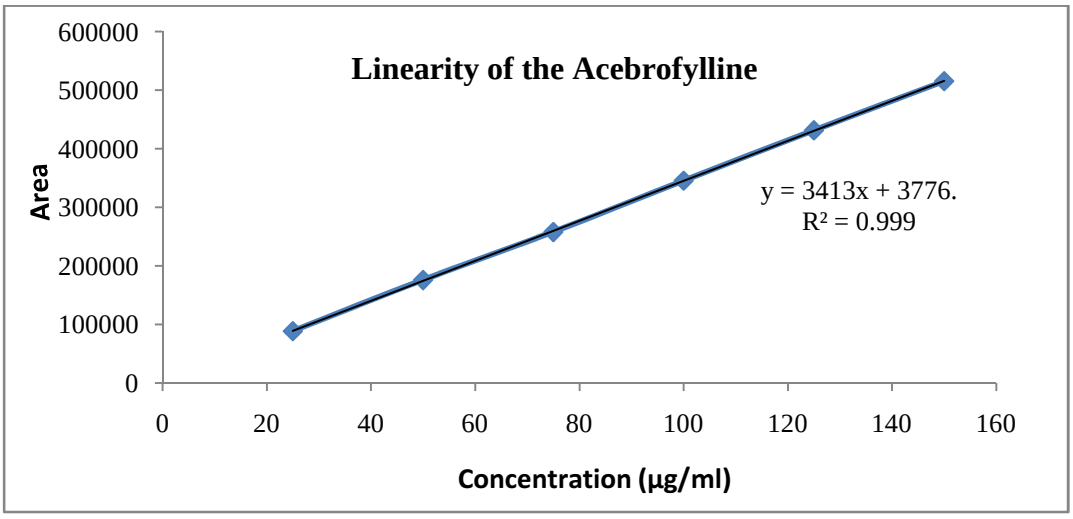


Fig. No. 32.0: HPLC Calibration Curve for Acebrofylline

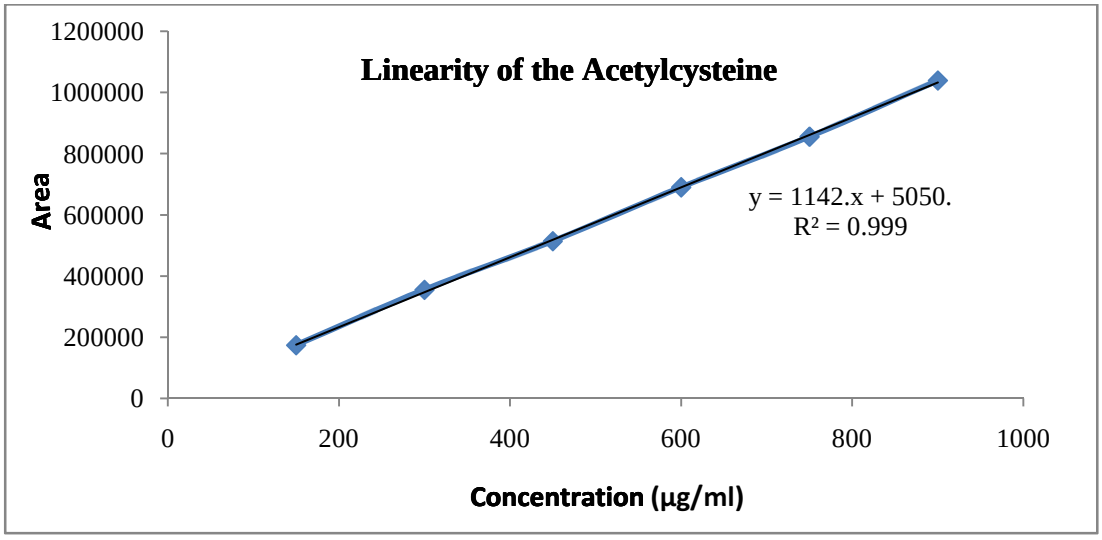


Fig. No. 33.0: HPLC Calibration Curve for Acetylcysteine

c. Accuracy

The accuracy of the method was calculated by the recovery studies at the three concentration of 50%, 100% and 150% levels by the standard addition method and the mean % recovery was calculated and it should be in between 98%-102% and the % RSD should be less than 2.

The developed HPLC method are found to be accurate, as all the 3 methods were passes the %RSD and % mean. The accuracy data for the chlorthalidone and losartan given in Table No. 31.0 and 32.0, Proguanil and Atovaquone in Table No. 33.0 and 34.0 and Acetylcysteine and acebrofylline in Table No. 35.0 and 36.0 respectively.

6.2.1c: Accuracy for Chlorthalidone and Losartan

Table No. 31.0: Accuracy data for Chlorthalidone

Level	Amount added	Amount recovered	%Recovery	% Mean	% RSD
50%	1.5625	1.569243	100.4316	100.3108	0.869991
50%	1.5625	1.562081	99.97319		
50%	1.5625	1.566084	100.2294		
100%	6.25	6.293178	100.6908		
100%	6.25	6.347817	101.5651		
100%	6.25	6.329055	101.2649		
150%	9.375	9.287201	99.06347		
150%	9.375	9.281737	99.00519		
150%	9.375	9.428801	100.5739		

**RSD=Relative Standard Deviation*

% RSD, % Assay Content found within Acceptance criteria

Table No. 32.0: Accuracy data for Losartan

Level	Amount added	Amount recovered	%Recovery	% Mean	% RSD
50%	25	25.31801	101.272	100.84	0.379
50%	25	25.19355	100.7742		
50%	25	25.38314	101.5326		
100%	50	50.34144	100.6829		
100%	50	50.28357	100.5671		
100%	50	50.53741	101.0748		
150%	75	75.35674	100.4756		
150%	75	75.61834	100.8245		
150%	75	75.28347	100.378		

**RSD=Relative Standard Deviation*

% RSD, % Assay Content found within Acceptance criteria

6.2.2c: Accuracy for Proguanil and Atovaquone

Table No. 33.0: Accuracy data for Proguanil

Level	Amount added	Amount recovered	%Recovery	% Mean	% RSD
50%	100	99.92131	99.92131	100.0003	0.445455
50%	100	99.78361	99.78361		
50%	100	100.785	100.785		
100%	200	199.3789	99.68946		
100%	200	199.0684	99.53419		
100%	200	198.9415	99.47073		
150%	300	301.2529	100.4176		
150%	300	300.0398	100.0133		
150%	300	301.1621	100.3874		

**RSD=Relative Standard Deviation*

% RSD, % Assay Content found within Acceptance criteria

Table No. 34.0: Accuracy data for Atovaquone

Level	Amount added	Amount recovered	%Recovery	% Mean	% RSD
50%	250	247.7958	99.11832	100.231	0.839797
50%	250	250.0898	100.0359		
50%	250	249.1408	99.65632		
100%	500	504.3664	100.8733		
100%	500	508.3652	101.673		
100%	500	505.0766	101.0153		
150%	750	754.1513	100.5535		
150%	750	746.8775	99.58366		
150%	750	746.8051	99.57402		

**RSD=Relative Standard Deviation*

% RSD, % Assay Content found within Acceptance criteria

6.2.3c: Accuracy for Acetylcysteine and Acebrofylline

Table No. 35.0: Accuracy data for Acetylcysteine

Level	Amount added	Amount recovered	%Recovery	% Mean	% RSD
50%	300	303.0838	101.0279	100.317	0.72
50%	300	302.724	100.908		
50%	300	299.1572	99.71907		
100%	600	595.2000	99.20000		
100%	600	600.9755	100.1626		
100%	600	597.3328	99.55546		
150%	900	909.5546	101.0616		
150%	900	901.2856	100.1428		
150%	900	909.683	101.0759		

**RSD=Relative Standard Deviation*

Table No. 36.0: Accuracy data for Acebrofylline

Level	Amount added	Amount recovered	%Recovery	% Mean	% RSD
50%	50	49.93	99.85302	100.864	0.78
50%	50	50.09	100.1831		
50%	50	50.17	100.3348		
100%	100	100.67	100.6713		
100%	100	102.15	102.1525		
100%	100	101.73	101.7314		
150%	150	152.02	101.3483		
150%	150	150.40	100.2697		
150%	150	151.9	101.2353		

**RSD=Relative Standard Deviation*

d.Precision

It measures the closeness of the agreement between the series of measurements obtained from the multiple samples of the same homogenious sample under the prescribed conditions. The method is said to be precise when the % RSD obtained under different conditions was less than 2%. The relative standard deviation (RSD) was found to be less than 2% for selected drug combinations. The Precision data for Chlorthalidone and Losartan, Proguanil and Atovaquone and Acetylcysteine and Acebrofylline was given in Table No. 37.0, Table No. 38.0 and Table No. 39.0 respectively.

6.2.1d: Precision of Chlorthalidone and Losartan

Table No. 37.0: Precision data for Chlorthalidone and Losartan

Assay No.	Peak Area (LOS)	% Assay (LOS)	Peak Area (CTD)	% Assay (CTD)
01	2875455	100.6147	465373	100.3868
02	2876656	100.6567	465290	100.3689
03	2881896	100.8401	466007	100.5235
04	2859022	100.0397	465226	100.3551
05	2849123	99.69334	463956	100.0811
06	2843289	99.4892	466640	100.6601
Mean		100.2223	Mean	100.3959
% RSD		0.55	% RSD	0.19

*Mean of six determinations

6.2.2d: Precision of Proguanil and Atovaquone

Table No. 38.0: Precision data for Proguanil and Atovaquone

Assay No.	Peak Area (PROG)	% Assay (PROG)	Peak Area (ATOV)	% Assay (ATOV)
01	434205	99.25	1302301	100.27
02	441463	100.92	1288242	99.19
03	439401	100.40	1323567	101.91
04	440291	100.64	1295495	99.75
05	436846	99.86	1292213	99.49
06	435304	99.50	1298902	100.01
Mean		100.09	Mean	100.11
% RSD		0.66	% RSD	0.96

*Mean of six determinations

6.2.3d: Precision of Acetylcysteine and Acebrofylline

Table No. 39.0: Precision data for Acetylcysteine and Acebrofylline

Assay No.	Peak Area (ACBF)	% Assay (ACBF)	Peak Area (ACST)	% Assay (ACST)
01	344629	100.6116	687668	99.82
02	341064	99.57081	686777	99.70
03	344819	100.667	690885	100.29
04	342999	100.1357	684256	99.33
05	346857	101.262	686946	99.72
06	344019	100.4335	680410	98.77
Mean		100.44	Mean	99.61
% RSD		0.56	% RSD	0.51

*Mean of three determinations

e.Specificity

According to the Pharmacopoeial forum and USP guideline, absence of peak at the retention time of analyte (API) from the placebo formulations demonstrates the specificity of the present method.

As there were no peaks found at the retention times of selected multicomponents placebo formulation

1) Chlorthalidone and Losartan
2) Proguanil and Atovaquone
3) Acetylcysteine and Acebrofylline

Hence, the proposed method was specific for the estimation of the above drug specifically in their formulations. The respective chromatograme for Chlorthalidone and Losartan (Fig. 34) Proguanil and Atovaquone (Fig.35), and Acetylcysteine and Acebrofylline (Fig.36), given in Fig A, B and C, shows that ther were

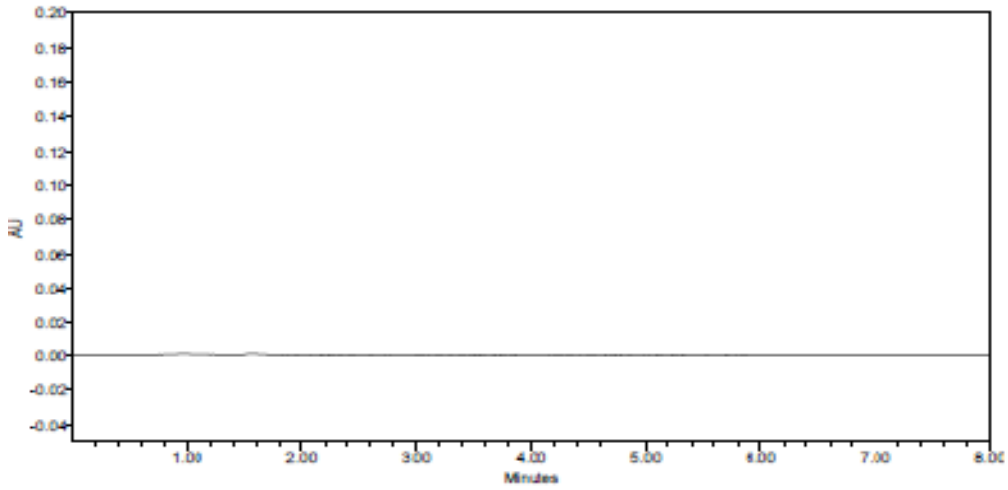


Fig No.34 : Specificity for Chlorthalidone and Losartan

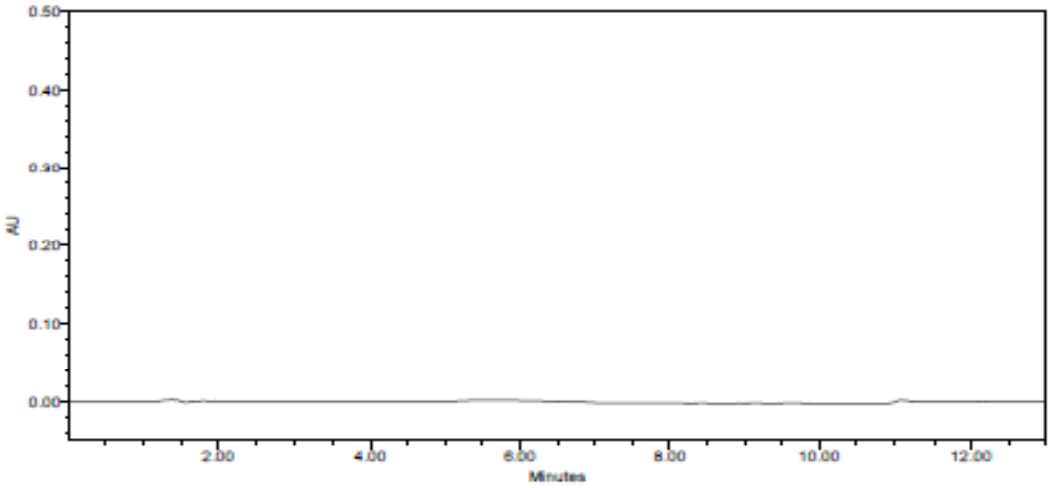


Fig No.35 : Specificity for Proguanil and Atovaquone

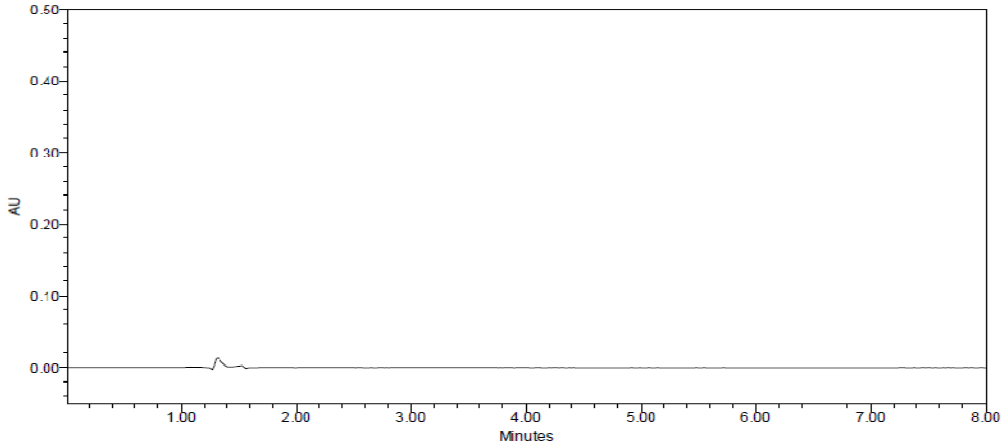


Fig No.36 : Specificity for Acetylcysteine and Acebrofylline

f. Limits of detection (LOD) and limit of quantitation (LOQ)

The limit of detection and limit of quantification is the lowest amount of the analyte in the sample that can be detected and determined with acceptable precision and accuracy under the stated experimental conditions. The quantification limit is expressed as aconcentration of the analyte in the sample. The LOD and LOQ data for the three HPLC method are calculated by using folloing formula.

LOD and LOQ values were estimated by using below formula

LOD = standard deviation / Slope of the regression equation × 3.3

LOQ = standard deviation / Slope of the regression equation × 10

The limit for detection and quantification for the multicomponents drugs given in the Table No. 40.0 for losartan and chlorthalidone, Table No. 41.0 for Atovaquone and Proguanil, Table No. 42.0 for Acetylcysteine and Acebrofylline respectively.

6.2.1f: LOD and LOQ for Chlorthalidone and Losartan

Table No. 40.0: LOD and LOQ data for Chlorthalidone and Losartan

Sr. No	Statistical Parameter	Chlorthalidone	Losartan
01	Standard deviation	387.439	2209.54
02	Slope of the regression equation	75953	59841
	LOD	0.016	0.121
	LOQ	0.051	0.369

6.2.2f: LOD and LOQ for Proguanil and Atovaquone

Table No. 41.0: LOD and LOQ data for Proguanil and Atovaquone

Sr. No	Statistical Parameter	Proguanil	Atovaquone
01	Standard deviation	236.21	2004.55
02	Slope of the regression equation	2135.33	2570.66
	LOD	0.365	2.573
	LOQ	1.106	7.797

6.2.3f: LOD and LOQ for Acetylcysteine and Acebrofylline

Table No. 42.0: LOD and LOQ data for Acetylcysteine and Acebrofylline

Sr. No	Statistical Parameter	Acetylcysteine	Acebrofylline
01	Standard deviation	521.93	194.78
02	Slope of the regression equation	1145	3429
	LOD	1.504	0.187
	LOQ	4.558	0.568

g. Robustness

Robustness studies was carried out by deliberately making changes in the wavelength, column temperature, mobile composition and flow rate in order to affect the method by means of peak symmentary, resolution and retention time.

Here, robustness study was calculated by the changing flow rate by (+/- 0.10ml), by changing mobile phase composition by (+/-2%) and column temperature by (+/- 5º)

In order to demonstrate the robustness of the method, system suitability parameter were evaluated and reported in Table No 43.0 to Table No 78.0 for all individual drug which shows that the developed methods were robust as the % RSD is calculated for the peak area which is >2 but only in case of acetylcysteine and acebrofylline % RSD is 2>

6.2.1g1 : A) Change in Flow rate for Chlorthalidone and Losartan

Table No. 43.0: Flow rate Change (-0.10ml) data for Chlorthalidone

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	2.411	513734	2330	2.08
2	2.418	517817	2519	1.96
3	2.421	521530	2430	1.93
4	2.424	516313	2492	1.97
5	2.424	516242	2456	1.95
6	2.426	527019	2478	2.02
Mean	-	518776	-	-
SD	-	4782.5	-	-
%RSD	-	0.92	-	-

Interpretation- Method was found to be stable for changing flow rate %RSD2<

Table No. 44.0: Flow rate Change (-0.10ml) data for Losartan

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	5.499	3234056	2514	1.58
2	5.528	3226686	2612	1.59
3	5.566	3229433	2286	1.63
4	5.571	3236188	2587	1.71
5	5.593	3302380	2379	1.71
6	5.642	3261566	2645	1.61
Mean	-	3248385	-	-
SD	-	29234.7	-	-
%RSD	-	0.89	-	-

Interpretation- Method was found to be stable for changing flow rate %RSD2<

Table No. 45.0: Flow rate Change (+0.1ml) data for Chlorthalidone

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	1.961	418569	2402	2.01
2	1.967	418521	2316	1.99
3	1.969	418185	2270	1.93
4	1.971	419027	2442	1.89
5	1.982	419351	2608	1.92
6	1.989	419528	2630	1.88
Mean	-	418863	-	-
SD	-	523.5	-	-
%RSD	-	0.12	-	-

Interpretation- Method was found to be stable for changing flow rate %RSD2<

Table No. 46.0: Flow rate Change (+0.10ml) data for Losartan

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	4.395	2626251	2444	1.67
2	4.423	2625742	2445	1.61
3	4.464	2625363	2440	1.65
4	4.486	2621211	2647	1.60
5	4.556	2617474	2704	1.58
6	4.565	2626618	2558	1.57
Mean	-	2623776	-	-
SD	-	3657.2	-	-
%RSD	-	0.1	-	-

Interpretation- Method was found to be stable for changing Flow rate %RSD2<

6.2.1g2: B) Change in Mobile Phase Composition for Chlorthalidone and Losartan

Table No. 47.0: Mobile Phase composition Change (-2%) data for Chlorthalidone

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	2.101	483960	2486	2.13
2	2.103	483577	2619	2.16
3	2.103	483782	2398	2.12
4	2.105	482278	2662	2.11
5	2.107	487405	2390	2.01
6	2.111	488522	2462	2.16
Mean	-	484921	-	-
SD	-	2455.6	-	-
%RSD	-	0.50	-	-

Interpretation- Method was found to be stable for changing Mobile Phase composition %RSD2<

Table No. 48.0: Mobile Phase composition Change (-2%) data for Losartan

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	4.216	2968253	2399	1.64
2	4.225	2952161	2433	1.66
3	4.231	2903801	2648	1.59
4	4.231	2916514	2490	1.68
5	4.248	2906424	2476	1.69
6	4.269	2901646	2563	1.63
Mean	-	2924800	-	-
SD	-	28355.5	-	-
%RSD	-	0.96	-	-

Interpretation- Method was found to be stable for changing Mobile Phase composition %RSD2<

Table No. 49.0: Mobile Phase composition Change (+2%) data for Chlorthalidone

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	2.209	481406	2391	2.07
2	2.211	460854	2515	2.13
3	2.216	468083	2636	2.08
4	2.223	472902	2350	2.07
5	2.224	463263	2571	2.13
6	2.225	469136	2500	2.11
Mean	-	469274	-	-
SD	-	7332.4	-	-
%RSD	-	1.56	-	-

Interpretation- Method was found to be stable for changing Mobile Phase composition %RSD2<

Table No. 50.0: Mobile Phase composition Change (+2%) data for Losartan

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	5.681	2900566	2636	1.69
2	5.719	2895347	2505	1.61
3	5.742	3024038	2621	1.67
4	5.753	2936759	2538	1.64
5	5.768	2930303	2571	1.69
6	5.798	2969486	2539	1.64
Mean		2942750		
SD		48041.4		
%RSD		1.6		

Interpretation- Method was found to be stable for changing Mobile Phase composition %RSD2<

6.2.1g3: C) Change in Column Temperature for Chlorthalidone and Losartan

Table No. 51.0: Column Temperature Change (-5º) data for Chlorthalidone

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	2.116	478638	2371	1.96
2	2.148	483015	2315	1.97
3	2.153	462809	2115	2.05
4	2.154	468837	2163	2.03
5	2.158	474117	2261	2.03
6	2.166	468564	2193	2.07
Mean	-	472663	-	-
SD	-	7396.3	-	-
%RSD	-	1.56	-	-

Interpretation- Method was found to be stable for changing column temperature %RSD2<

Table No. 52.0: Column Temperature Change (-5º) data for Losartan

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	5.049	2938142	2340	1.67
2	5.097	2922899	2375	1.61
3	5.114	3028595	2255	1.71
4	5.134	2958899	2340	1.54
5	5.150	3034194	2357	1.60
6	5.197	2985120	2408	1.56
Mean	-	2977975	-	-
SD	-	46385.1	-	-
%RSD	-	1.55	-	-

Interpretation- Method was found to be stable for changing column temperature %RSD2<

Table No. 53.0: Column Temperature Change (+5º) data for Chlorthalidone

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	2.069	459312	2525	1.90
2	2.076	459445	2484	1.98
3	2.077	460149	2829	1.96
4	2.084	456605	2758	1.94
5	2.087	462166	2675	1.87
6	2.088	460251	2760	1.88
Mean	-	459655	-	-
SD	-	1809.3	-	-
%RSD	-	0.39	-	-

Interpretation- Method was found to be stable for changing column temperature %RSD2<

Table No. 54.0: Column Temperature Change (+5º) data for Losartan

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	4.785	2890396	2744	1.50
2	4.821	2882212	2817	1.56
3	4.831	2892717	2576	1.60
4	4.866	2908988	2863	1.53
5	4.904	2896057	2827	1.55
6	4.927	2898343	2985	1.59
Mean	-	2894785	-	-
SD	-	8917.6	-	-
%RSD	-	0.30	-	-

Interpretation- Method was found to be stable for changing column temperature %RSD2<

6.2.2g1A) Change in Flow rate for Proguanil and Atovaquone

Table No. 55.0: Flow rate Change (-0.10ml) data for Proguanil

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	2.405	511553	4476	1.56
2	2.410	505066	4485	1.58
Mean	-	508310	-	-
SD	-	4587.6	-	-
%RSD	-	0.90	-	-

Interpretation- Method was found to be stable for changing flow rate %RSD2<

Table No. 56.0: Flow rate Change (-0.10ml) data for Atovaquone

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	10.500	1487441	34496	1.40
2	10.552	1457424	34927	1.37
Mean	-	1472432	-	-
SD	-	21225.5	-	-
%RSD	-	1.44	-	-

Interpretation- Method was found to be stable for changing flow rate %RSD2<

Table No. 57.0: Flow rate Change (+0.10ml) data for Proguanil

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	1.988	399146	4006	1.52
2	1.999	397772	4294	1.46
Mean	-	398459	-	-
STD DEV	-	971.6	-	-
%RSD	-	0.24	-	-

Interpretation- Method was found to be stable for changing flow rate %RSD2<

Table No. 58.0: Flow rate Change (+0.10ml) data for Atovaquone

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	9.202	1187276	37553	1.44
2	9.258	1180557	37004	1.43
Mean	-	1183917	-	-
STD DEV	-	4750.9	-	-
%RSD	-	0.40	-	-

Interpretation- Method was found to be stable for changing flow rate %RSD2<

6.2.2g2: B) Change in Mobile Phase Composition for Proguanil and Atovaquone

Table No. 59.0: Mobile Phase Composition change (-2%) data for Proguanil

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	2.113	467216	3848	1.61
2	2.119	463287	3843	1.58
Mean	-	465252	-	-
STD DEV	-	2778.0	-	-
%RSD	-	0.59	-	-

Interpretation- Method was found to be stable for changing mobile phase composition %RSD2<

Table No. 60.0: Mobile Phase Composition change (-2%) data for Atovaquone

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	9.524	1377647	3637	1.56
2	9.549	1362107	3708	1.54
Mean	-	1369877	-	-
STD DEV	-	10988.2	-	-
%RSD	-	0.80	-	-

Interpretation- Method was found to be stable for changing mobile phase composition %RSD2<

Table No. 61.0: Mobile Phase Composition change (+2%) data for Proguanil

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	2.187	477395	3941	1.61
2	2.196	479495	4214	1.63
Mean	-	478445	-	-
STD DEV	-	1485.0	-	-
%RSD	-	0.31	-	-

Interpretation- Method was found to be stable for changing mobile phase composition %RSD2<

Table No. 62.0: Mobile Phase Composition change (+2%) data for Atovaquone

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	9.653	1429591	3735	1.55
2	9.688	1419772	3695	1.54
Mean	-	1424681	-	-
STD DEV	-	6943.1	-	-
%RSD	-	0.48	-	-

Interpretation- Method was found to be stable for changing mobile phase composition %RSD2<

6.2.2g3:C) Change in Column Temperature for Proguanil and Atovaquone

Table No. 63.0: Column Temperature change (-5º) data for Proguanil

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	2.148	481215	3574	1.63
2	2.158	478841	4083	1.69
Mean	-	480028	-	-
SD	-	1678.5	-	-
%RSD	-	0.34	-	-

Interpretation- Method was found to be stable for changing column temperature %RSD2<

Table No. 64.0: Column Temperature change (-5º) data for Atovaquone

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	9.686	1419222	3457	1.56
2	9.826	1411916	3470	1.56
Mean	-	1415569	-	-
SD	-	5166.4	-	-
%RSD	-	0.36	-	-

Interpretation- Method was found to be stable for changing column temperature %RSD2<

Table No. 65.0: Column Temperature change (+5º) data for Proguanil

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	2.153	460728	4286	1.50
2	2.169	466669	4545	1.49
Mean	-	463698	-	-
SD	-	4200.8	-	-
%RSD	-	0.90	-	-

Interpretation- Method was found to be stable for changing column temperature %RSD2<

Table No. 66.0: Column Temperature change (+5º) data for Atovaquone

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	9.474	1391335	4026	1.43
2	9.487	1386154	3995	1.45
Mean	-	1388744	-	-
SD	-	3663.6	-	-
%RSD	-	0.26	-	-

Interpretation- Method was found to be stable for changing column temperature %RSD2<

6.2.3g1: A) Change in Flow Rate for Acetylcysteine and Acebrofylline

Table No. 67.0: Flow Rate change (-0.1ml) data for Acetylcysteine

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	2.622	774842	4544	1.11
2	2.625	782195	4472	1.11
3	2.630	777897	4516	1.08
4	2.630	775340	4536	1.08
5	2.631	779703	4523	1.09
6	2.631	772235	4550	1.09
Mean	-	777035	-	-
SD	-	3611.2	-	-
%RSD	-	0.46	-	-

Interpretation- Method was found to be stable for changing flow rate %RSD2<

Table No. 68.0: Flow Rate change (-0.10ml) data for Acebrofylline

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	6.012	383697	6059	0.99
2	6.048	387015	6150	1.00
3	6.064	381578	6011	1.00
4	6.071	384265	6083	0.99
5	6.074	385533	5978	0.99
6	6.082	386273	5966	0.99
Mean	-	384727	-	-
SD	-	1972.9	-	-
%RSD	-	0.51	-	-

Table No. 69.0: Flow Rate change (+0.10ml) data for Acetylcysteine

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	2.334	697975	4251	1.09
2	2.335	699011	4285	1.09
3	2.340	695904	4491	1.09
4	2.341	694966	4495	1.10
5	2.348	694073	4374	1.09
6	2.352	693331	4321	1.09
Mean	-	695877	-	-
STD DEV	-	2226.7	-	-
%RSD	-	0.31	-	-

Interpretation- Method was found to be stable for changing flow rate %RSD2<

Table No. 70.0: Flow Rate change (+0.10ml) data for Acebrofylline

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	5.376	347473	5831	0.99
2	5.379	345439	5876	1.00
3	5.384	346244	5725	0.99
4	5.419	344648	5804	0.99
5	5.430	343814	5858	0.99
6	5.434	343739	5911	0.99
Mean	-	345226	-	-
STD DEV	-	1460.8	-	-
%RSD	-	0.42	-	-

Interpretation- Method was found to be stable for changing flow rate %RSD2<

6.2.3g2: B) Change in Mobile Phase Composition for Acetylcysteine and Acebrofylline

Table No. 71.0: Mobile Phase Composition change (-2%) data for Acetylcysteine

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	2.212	763472	4569	1.08
2	2.212	748796	4607	1.08
3	2.215	766401	4452	1.08
4	2.217	763566	4404	1.08
5	2.218	755764	4389	1.09
6	2.229	763390	4712	1.07
Mean	-	760231	-	-
STD DEV	-	6639.1	-	-
%RSD	-	0.87	-	-

Interpretation- Method was found to be stable for changing Mobile Phase Composition %RSD2<

Table No. 72.0: Mobile Phase Composition change (-2%) data for Acebrofylline

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	4.716	365795	5829	1.00
2	4.722	373145	5719	1.00
3	4.735	374013	5847	1.00
4	4.738	373303	5826	0.99
5	4.745	371388	5772	0.99
6	4.754	372022	5779	1.00
Mean	-	371611	-	-
STD DEV	-	3001.0	-	-
%RSD	-	0.80	-	-

Interpretation- Method was found to be stable for changing Mobile Phase Composition %RSD2<

Table No. 73.0: Mobile Phase Composition change (+2%) data for Acetylcysteine

No of Injection	**Retention Time R_t (min)**	**Peak Area**	**USP Plate Count**	**USP Tailing**
1	2.544	746464	4402	1.07
2	2.550	749432	4451	1.08
3	2.554	745956	4371	1.10
4	2.558	745236	4421	1.09
5	2.562	744338	4448	1.07
6	2.563	751269	4440	1.07
Mean	-	747116	-	-
STD DEV	-	2669.6	-	-
%RSD	-	0.35	-	-

Interpretation- Method was found to be stable for changing Mobile Phase Composition %RSD2<

Table No. 74.0: Mobile Phase Composition change (+2%) data for Acebrofylline

No of Injection	**Retention Time R_t (min)**	**Peak Area**	**USP Plate Count**	**USP Tailing**
1	6.561	364312	5956	0.97
2	6.570	364747	6036	0.98
3	6.581	366602	5966	0.97
4	6.589	365120	5869	0.98
5	6.592	364710	6038	0.97
6	6.599	367003	6016	0.97
Mean	-	365416	-	-
STD DEV	-	1111.6	-	-
%RSD	-	0.30	-	-

Interpretation- Method was found to be stable for changing Mobile Phase Composition %RSD2<

6.2.3g3: C) Change in Column Temperature for Acetylcysteine and Acebrofylline

Table No. 75.0: Column Temperature change (-5º) data for Acetylcysteine

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	2.287	752464	4482	1.09
2	2.312	804424	4535	1.09
3	2.337	775656	4500	1.09
4	2.345	776062	4576	1.09
5	2.355	777579	4549	1.09
6	2.361	780106	4625	1.09
Mean	-	777715	-	-
STD DEV	-	16514.8	-	-
%RSD	-	2.12	-	-

Interpretation- Method was found to be quite unstable for changing column temperature %RSD<2

Table No. 76.0: Column Temperature change (-5º) data for Acebrofylline

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	5.187	366248	5905	0.99
2	5.284	395634	5798	0.99
3	5.365	379941	5928	0.99
4	5.395	379578	5945	0.99
5	5.436	381667	5994	0.99
6	5.441	383067	5888	0.98
Mean	-	381022	-	-
STD DEV	-	9377.1	-	-
%RSD	-	2.46	-	-

Interpretation- Method was found to be quite unstable for changing column temperature %RSD<2

Table No. 77.0: Column Temperature change (+5º) data for Acetylcysteine

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	2.251	702056	4482	1.09
2	2.256	699473	4451	1.10
3	2.261	704747	4448	1.09
4	2.262	704874	4449	1.09
5	2.269	699151	4262	1.09
6	2.270	697754	4428	1.09
Mean	-	701343	-	-
STD DEV	-	3024.6	-	-
%RSD	-	0.43	-	-

Interpretation- Method was found to be stable for changing column temperature %RSD2<

Table No. 78.0: Column Temperature change (+5º) data for Acebrofylline

No of Injection	Retention Time R_t (min)	Peak Area	USP Plate Count	USP Tailing
1	4.980	349081	5630	0.99
2	5.046	347306	5827	0.99
3	5.052	349272	6009	0.99
4	5.063	345338	5921	0.99
5	5.064	349022	5879	0.99
6	5.078	346715	5910	0.98
Mean	-	347789	-	-
STD DEV	-	1599.0	-	-
%RSD	-	0.45	-	-

Interpretation- Method was found to be stable for changing column temperature %RSD2<

From the above studies it was found that the developed are successfully validated as per ICH guideline and all 3 methods combination were found to be stable, accurate, precise linear, and robust and also found to be very simple economic, and time saving methods and can be successfully adopted for analysis of the marketed formulations in the respective dosage forms.

6.3: Part – 3 Results for Forced Degradation Study by RP-HPLC

The forced degradation studies are carried out at more severe conditions than accelerated condition in order to demonstrate specificity of the stability indicating method[30]. As there were no specification in the regulatory guidelines about the conditions of Ph, temperature and specific oxidizing agents to be used for the study. Here, forced degradation studies[33,34] were carried out for Losartan and chlorthalidone, atovaquone and proguanil and acetylcysteine and acebrofylline marketed formulation by using RP-HPLC method by expoxing sample to stressed conditions like acid, base peroxide, temperature, and uv or fluorescence light for photo-stability[32].

Here sample (selected multicomponents) is easily hydrolysed by acid and base treatment with the formulation here in acid degradation we used 2N of the concentration of acid for acid degradation and 2N Conc. Of base was used for the hydrolyzing the drug compounds. In oxide degradation, mostly suggested hydrogen peroxide 2% was used, for thermal degradation sample is exposed at 105º for 6h and photolysis is done by exposing sample under the uv light for 7 days and the % of drug degradation is given in Table No.79.0 for Losartan and chlorthalidone and the degraded chromatograms with acid, base, peroxide, temperature and uv light were shown in Fig. No37.0 to 41.0 respectively and for atovaquone proguanil it is given in Table No.80.0 and degraded chromatograms in Fig.No42.0 to 46.0 respectivly and for actylcysteine and acebrofylline is given in Table No.81.0 and degraded chromatograms were shown in Fig.No47.0 to 51.0 respectivly.

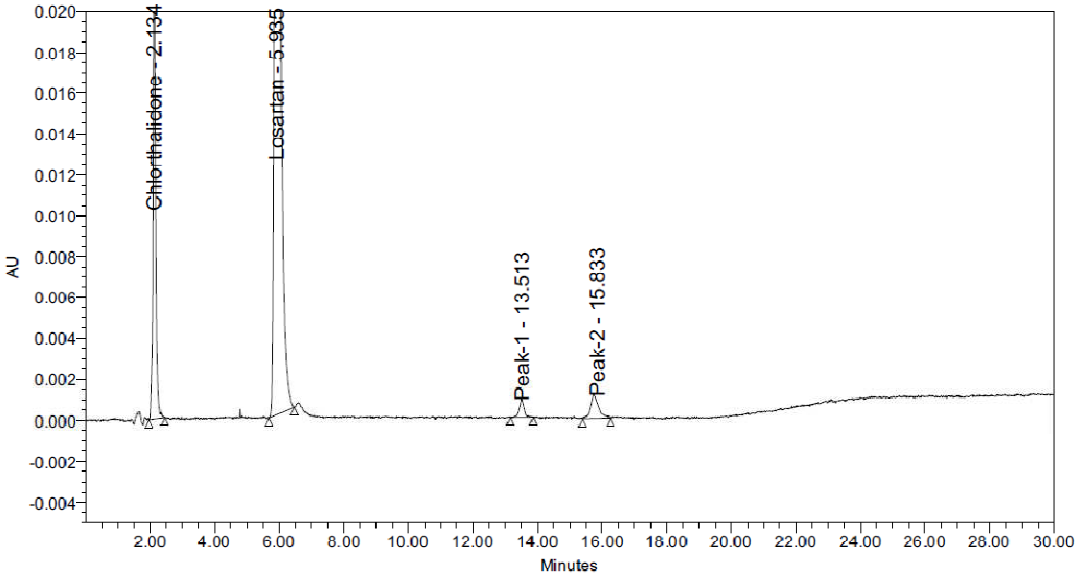
6.3.1: Chlorthalidone and Losartan

From the literature survey shows the presence of stability indicating method of losartan with hydrochlorthiazide by sheikh Anis[59] and Losartan with enalapril maleate by bhaumik patel[74] So, here we are developing new stability indicating method for the simultaneous estimation of the losartan and chlorthalidone in tablet formulation.

In the degradation study of losartan and shows that both the drugs were quite stable in the both acid and the base as the degradation % was below the 10% for the both losartan and chlorthalidone while the combination is more suseptible to the peroxide degradation where losartan is degraded by 11% and chlorthalidone is degraded by 9.35% while both the drug where stable in the both thermal and the photolysis degradation. The data for forced degradation study for Chlorthalidone and Losartan is given in Table No. 79.0

Table No. 79.0: Data of forced degradation study for Chlorthalidone and Losartan

Stress condition	Degradation time	Losartan			Chlorthalidone		
		Peak Area	% degradation	% Active drug remain	Peak Area	% degradation	% Active drug remain
Standard Drug	NA	1179776	NA	NA	126663	NA	NA
Acid	30 min	1084312	8.10	91.90	121237	4.29	95.71
Base	30 min	1062350	9.96	90.04	118246	6.65	93.35
Peroxide	30 min	1084221	11.16	88.84	114823	9.35	90.65
Thermal	6hrs	1119041	5.15	94.85	118331	6.58	93.42
UV-Light	7days	1149282	2.59	97.41	122566	3.24	96.76



% Degradation is within Acceptance Criteria (NMT 10%)

Fig. No. 37.0: Acid degraded chromatogram for Losartan and Chlorthalidone

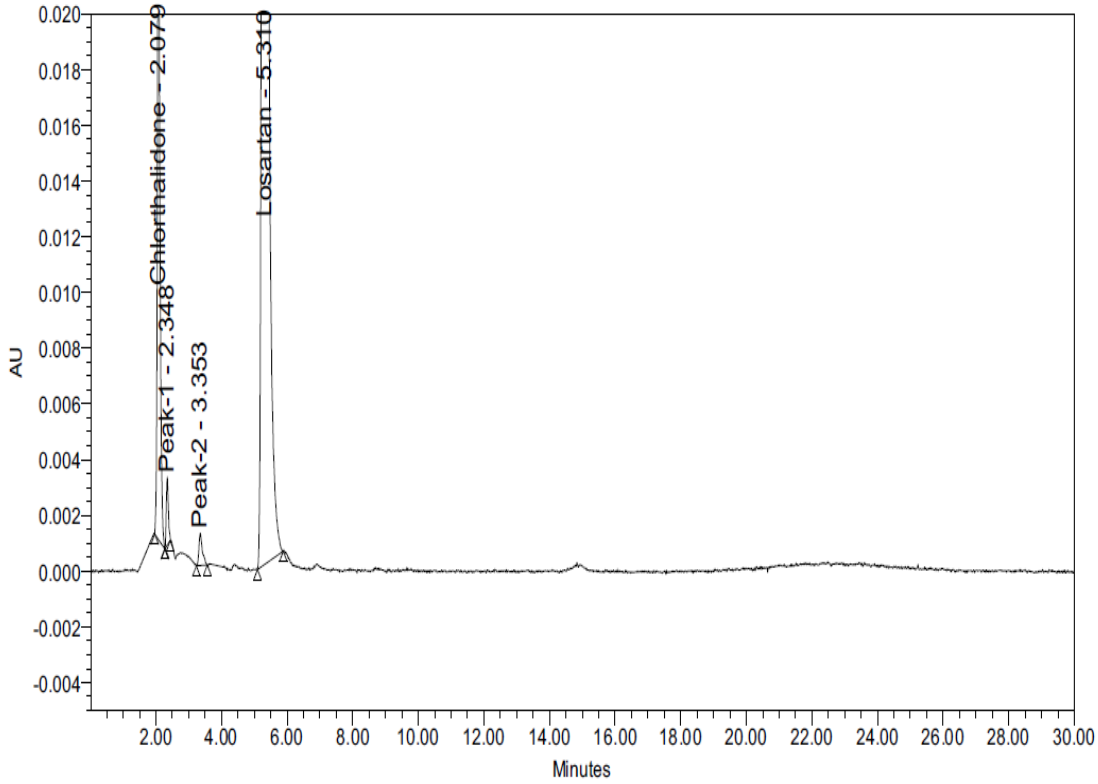


Fig. No. 38.0: Base degraded chromatogram for Losartan and Chlorthalidone

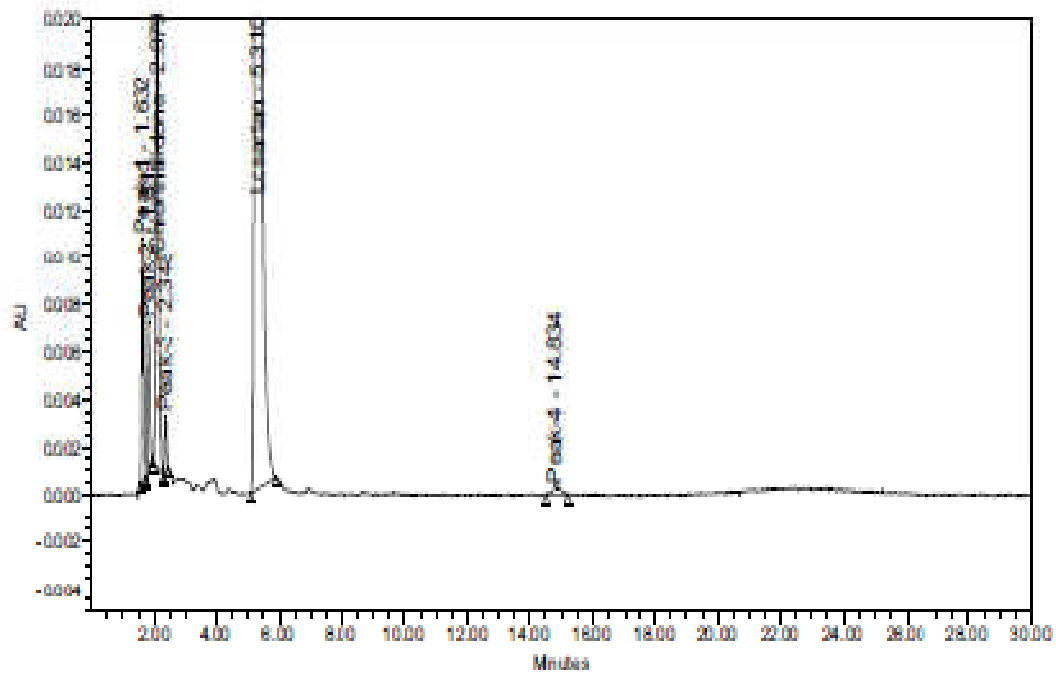
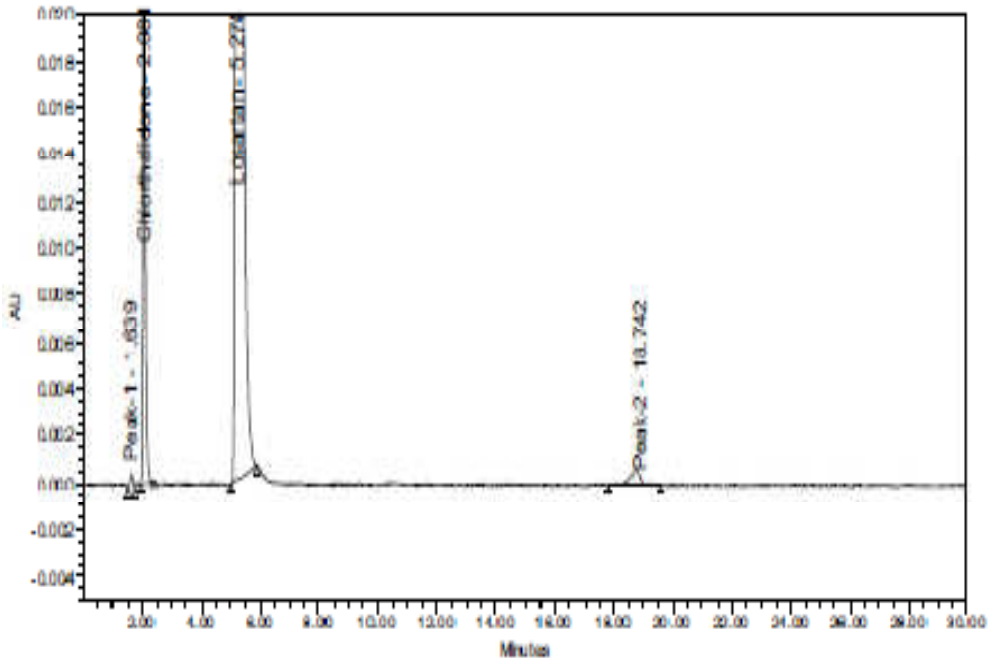
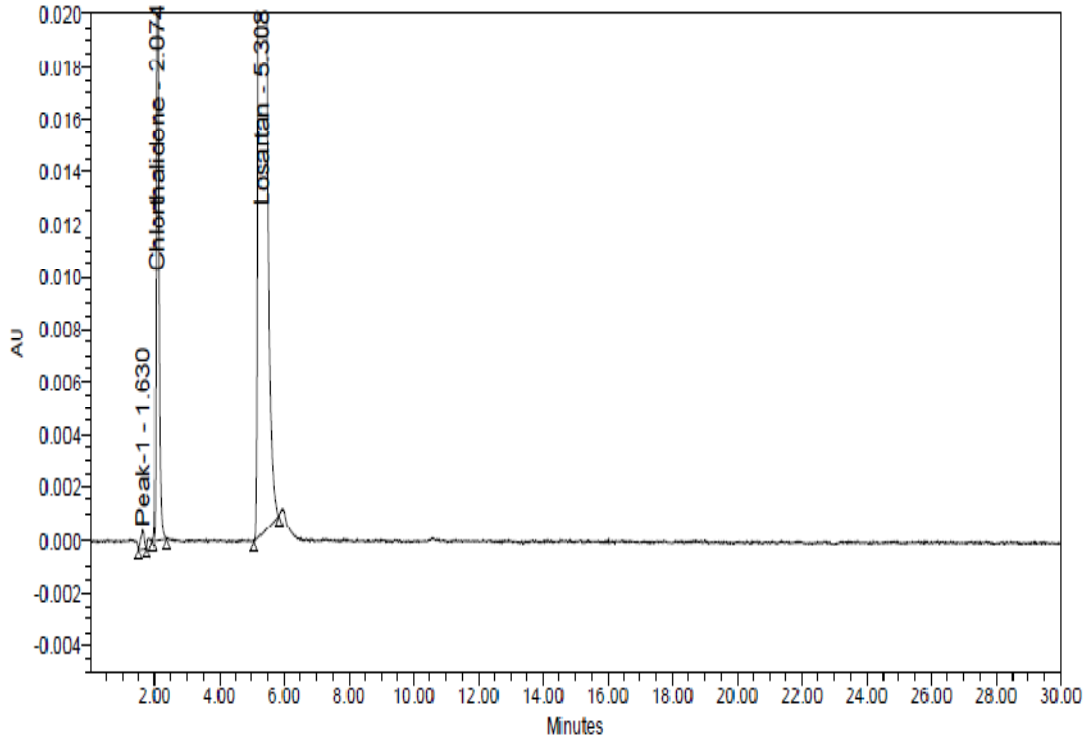


Fig. No. 39.0: Peroxide degraded chromatogram for Losartan and Chlorthalidone



% Degradation is within Acceptance Criteria (NMT 10%)

Fig. No. 40.0: Thermal degraded chromatogram for Losartan and Chlorthalidone



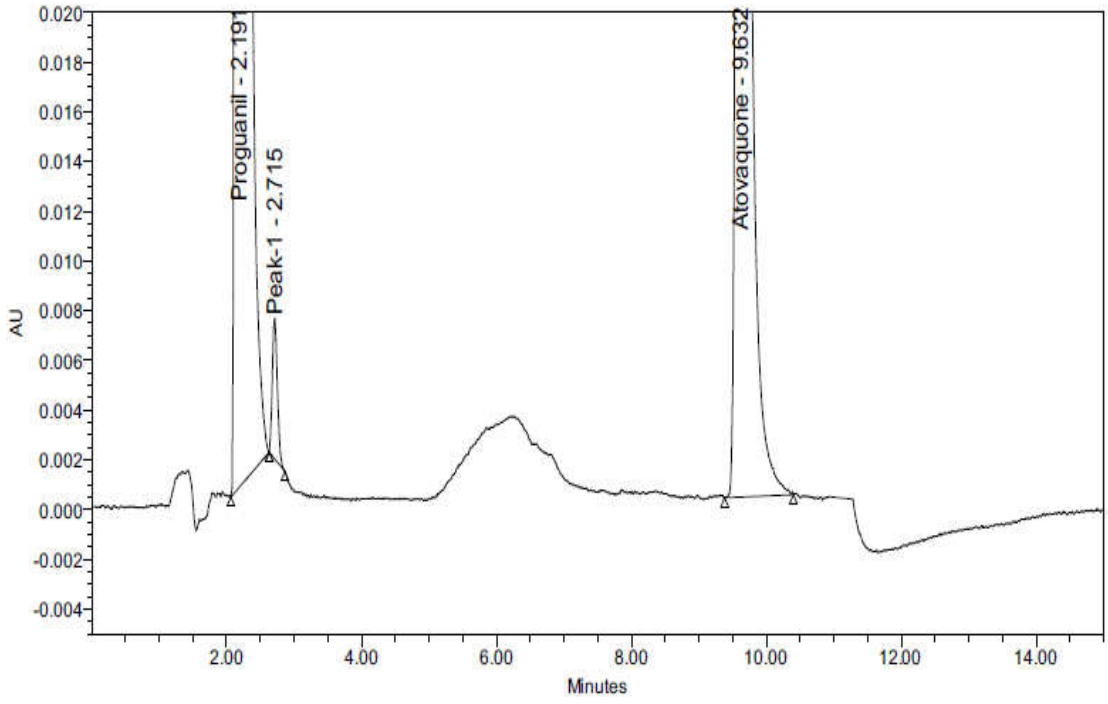
% Degradation is within Acceptance Criteria (NMT 10%)

Fig. No. 41.0: Photo degraded chromatogram for Losartan and Chlorthalidone

6.3.2: Atovaquone and Proguanil

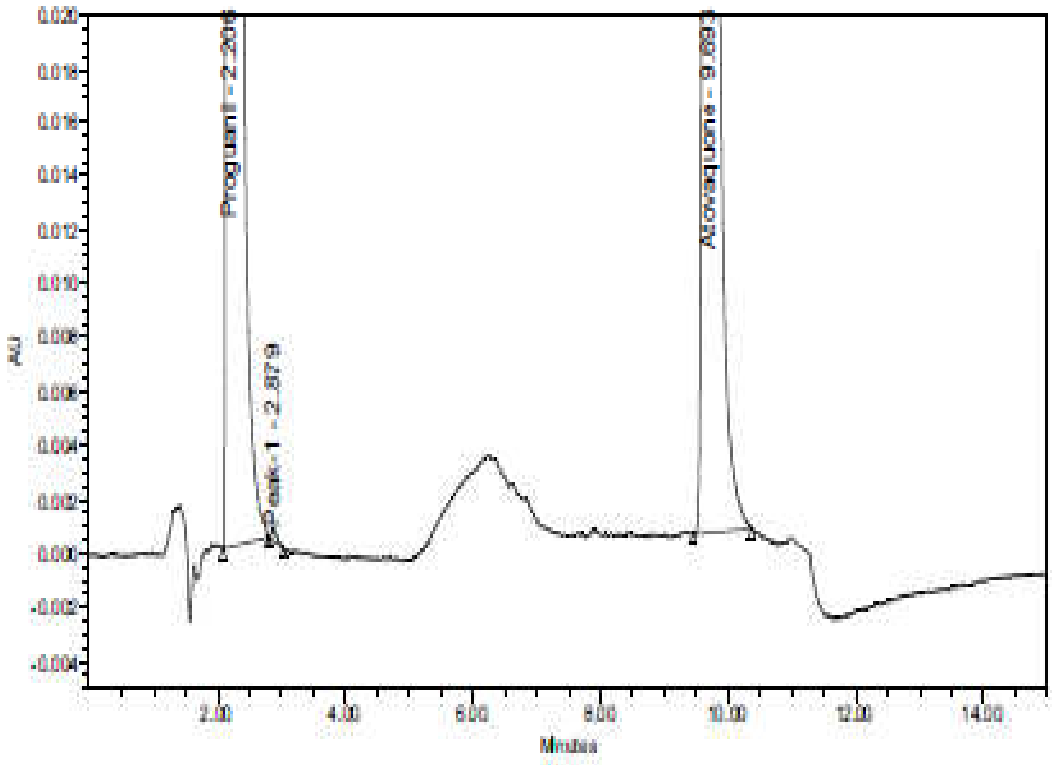
Table No. 80.0: Data of forced degradation study for Atovaquone and Proguanil

Stress condition	Degradation time	Atovaquone			Proguanil		
		Peak Area	% degradation	% Active drug remain	Peak Area	% degradation	% Active drug remain
Standard Drug	NA	2351105	NA	NA	498868	NA	NA
Acid	30 min	2207658	6.11	93.89	466716	6.45	93.55
Base	30 min	2154349	8.37	91.63	461752	7.45	92.55
Peroxide	30 min	2085578	11.3	88.7	455398	8.72	91.28
Thermal	6hrs	2152938	8.43	91.57	477317	4.32	95.68
UV-Light	7days	2261265	3.83	96.17	488728	2.04	97.96



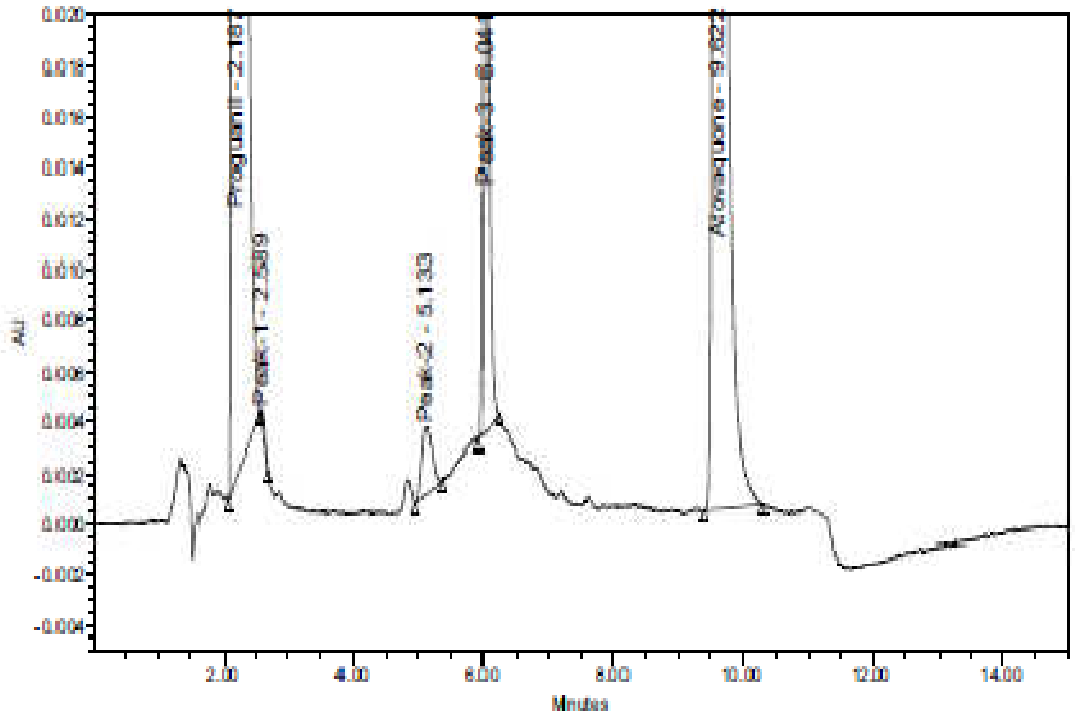
% Degradation is within Acceptance Criteria (NMT 10%)

Fig. No. 42.0: Acid degraded chromatogram for Atovaquone and Proguanil



% Degradation is within Acceptance Criteria (NMT 10%)

Fig. No. 43.0: Base degraded chromatogram for Atovaquone and Proguanil



% Degradation is more not in Acceptance Criteria (NMT 10%)

Fig. No. 44.0: Peroxide degraded chromatogram for Atovaquone and Proguanil

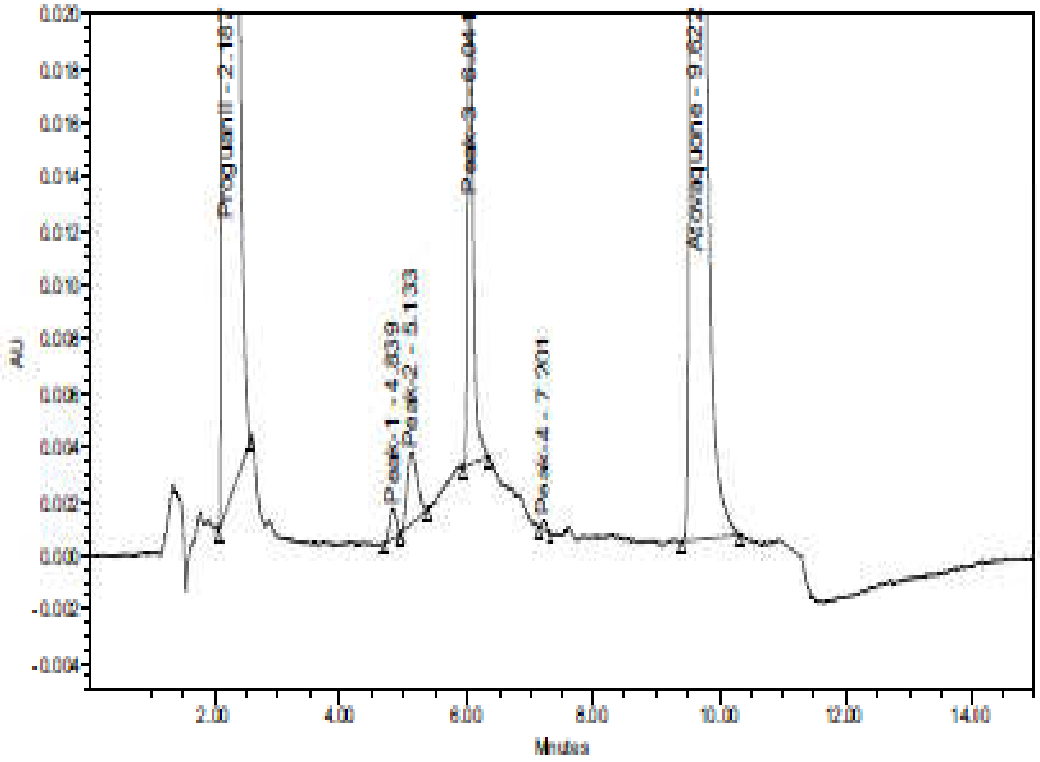
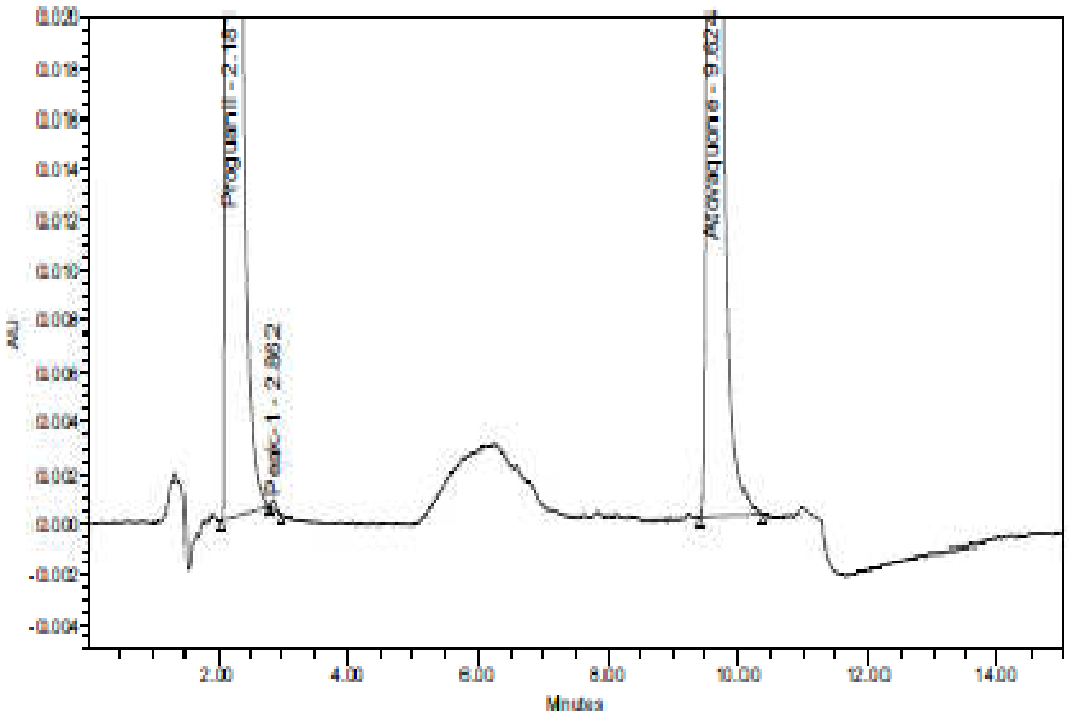


Fig. No. 45.0: Thermal degraded chromatogram for Atovaquone and Proguanil



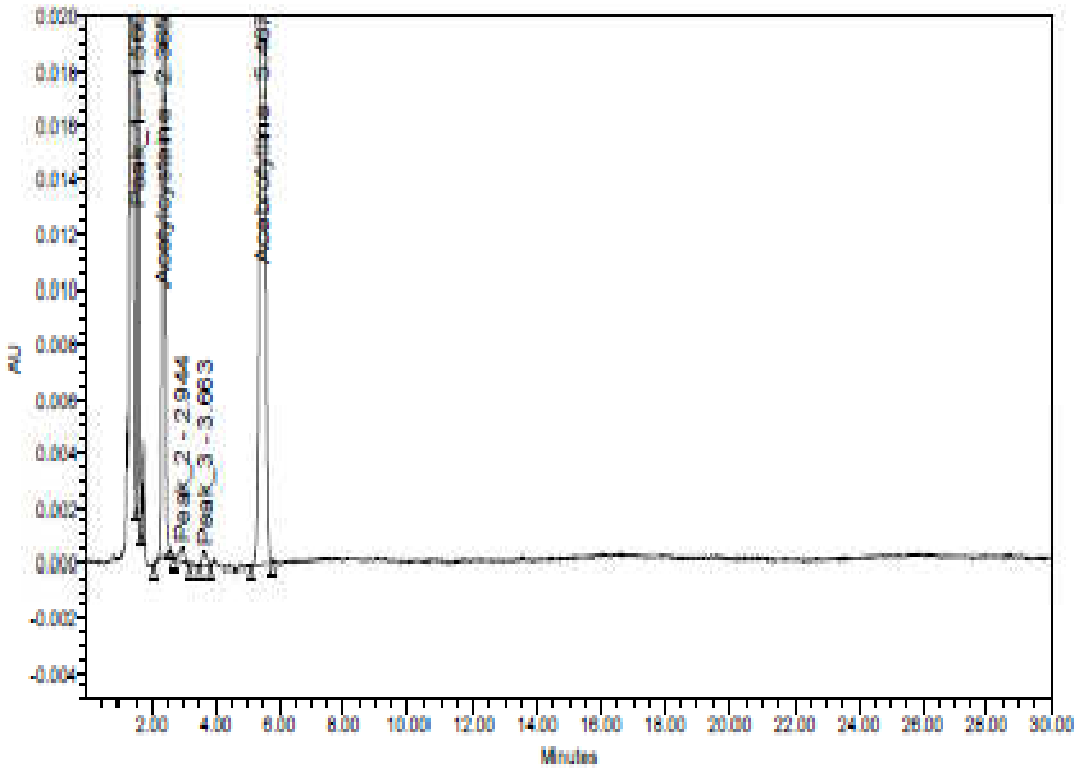
% Degradation is within Acceptance Criteria (NMT 10%)

Fig. No. 46.0: Photo degraded chromatogram for Atovaquone and Proguanil

6.3.3: Acetylcysteine and Acebrophylline

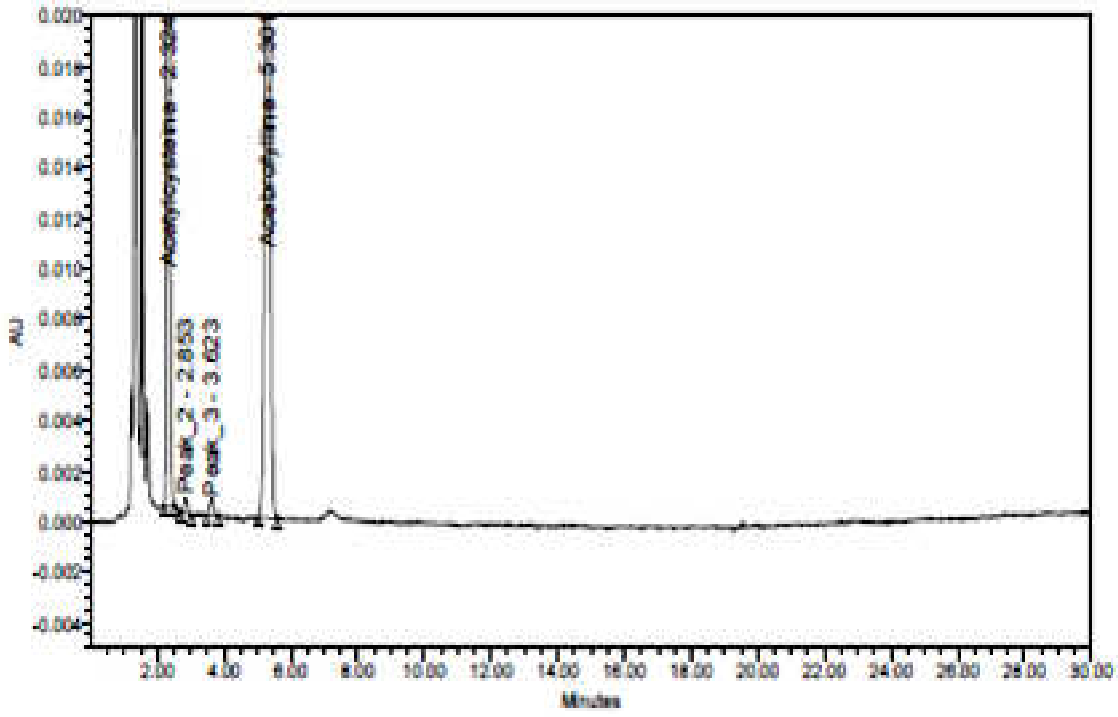
Table No. 81.0: Data of forced degradation study for Acetylcysteine and Acebrophylline

Stress condition	Degradation time	Acetylcysteine			Acebrophylline		
		Peak Area	% degradation	% Active drug remain	Peak Area	% degradation	% Active drug remain
Standard Drug	NA	677921	NA	NA	342377	NA	NA
Acid	30min	607589	10.74	89.26	322819	6.28	93.72
Base	30min	617323	9.31	90.69	314732	8.63	91.37
Peroxide	30min	613425	9.88	90.12	316865	8.01	91.99
Thermal	6hrs	641366	5.78	94.22	331037	3.9	96.1
Photo	7 days	645223	5.21	94.79	329141	4.45	95.55



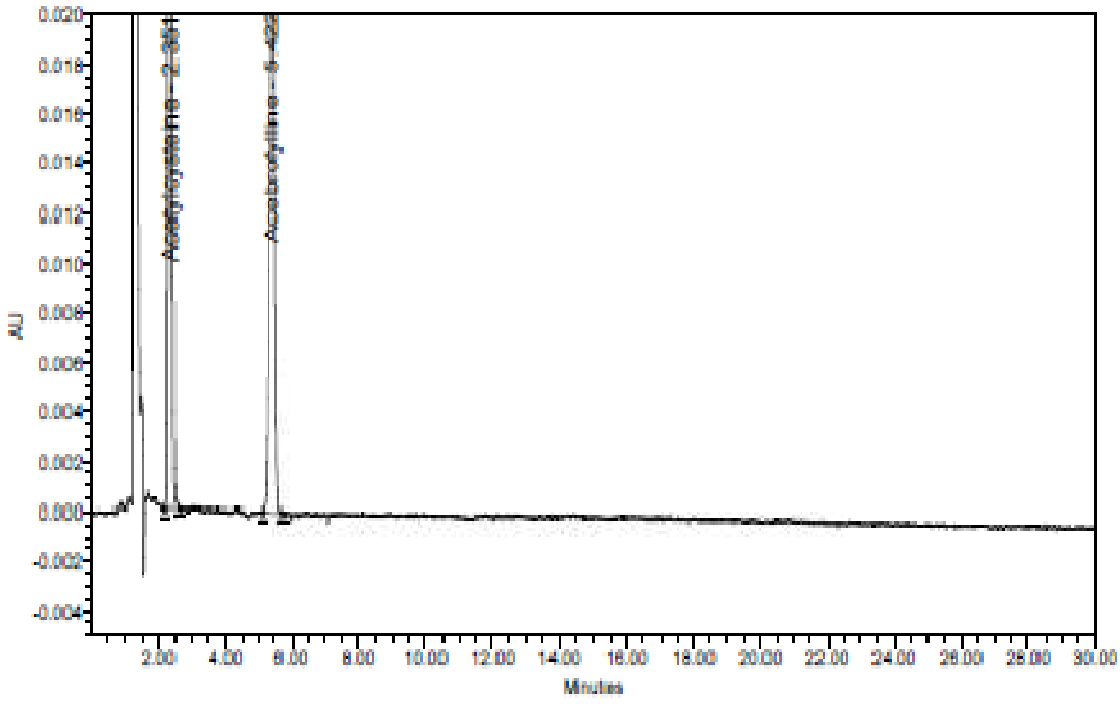
% Degradation is within Acceptance Criteria (NMT 10%)

Fig. No. 47.0: Acid degraded chromatogram for Acetylcysteine and Acebrophylline



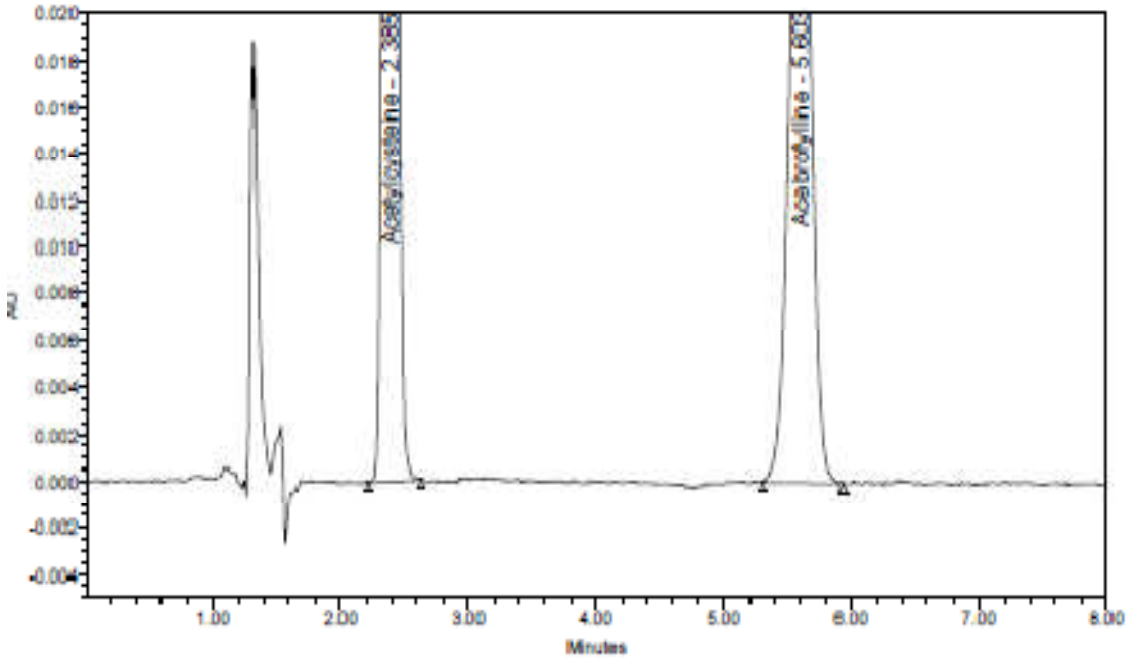
% Degradation is within Acceptance Criteria (NMT 10%)

Fig. No. 48.0: Base degraded chromatogram for Acetylcysteine and Acebrophylline



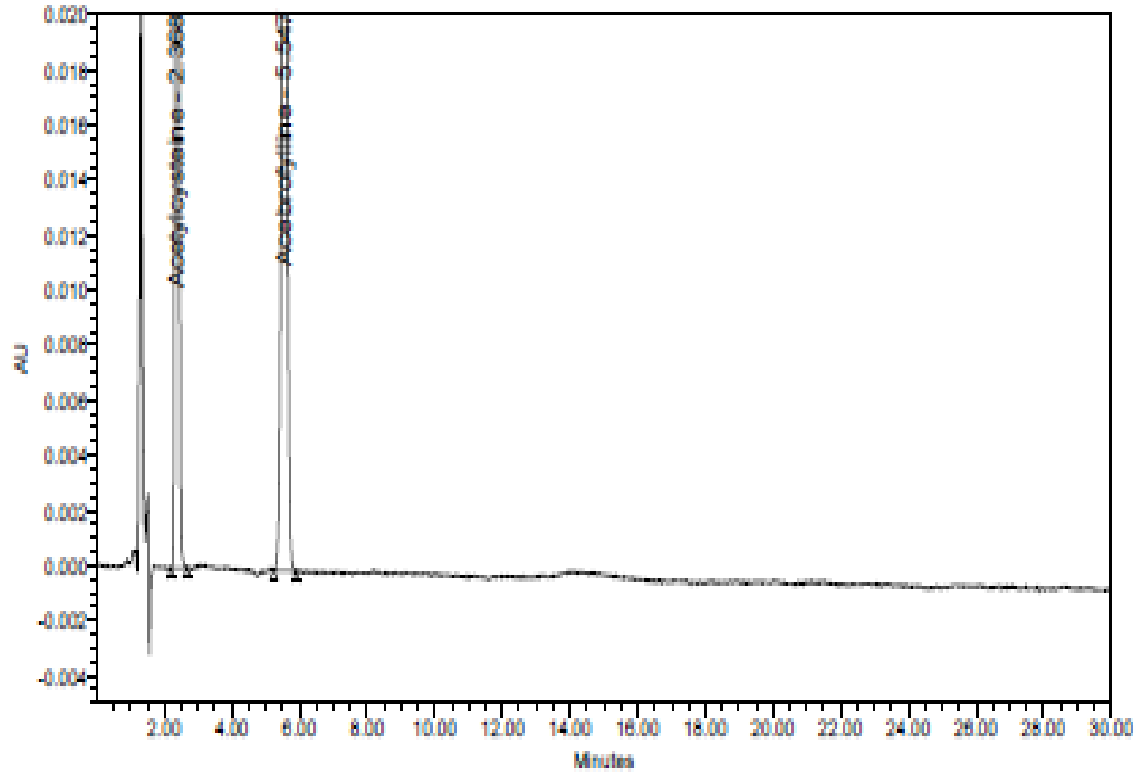
% Degradation is within Acceptance Criteria (NMT 10%)

Fig. No. 49.0: Peroxide degraded chromatogram for Acetylcysteine and Acebrophylline



% Degradation is within Acceptance Criteria (NMT 10%)

Fig. No. 50.0: Thermal degraded chromatogram for Acetylcysteine and Acebrophylline



% Degradation is within Acceptance Criteria (NMT 10%)

Fig. No. 51.0: Photo degraded chromatogram for Acetylcysteine and Acebrophylline

6.4: Part – 4 Results For Uv Spectrophotometric Method Development

6.4.1: UV Spectroscopic Method Development of Losartan and chlorthalidone:

The literature suvey reveals that very few methods[65] are available spectrophometrically for the estimation of the single drug but there were no single spectroscopic method is available for the simultaneous estimation of Losartan and chlorthalidone in tablet dosage form so that we developed spectroscopic method for the estimation of Losartan and chlorthalidone in tablet dosage form. In developed uv method of losartan and chlorthalidone water is selected as solvent since both drugs are soluble in water and all other polar solvent, then From the stock solution of losartan and chlorthalidone 8µg/ml and 1µg/ml concentration solutions were prepared respectively and scanned in uv range from 200 to 400nm, then from Overlay spectra we selected absorption maxima (λ max) 235nm and 215nm for losartan and chlorthalidone respectively the overlay spectra is shown in **Fig. No. 52.0.** The method is applied for the assay of marketed formulation by simultaneous equation method. The respective data are given in **Table No. 82.0**

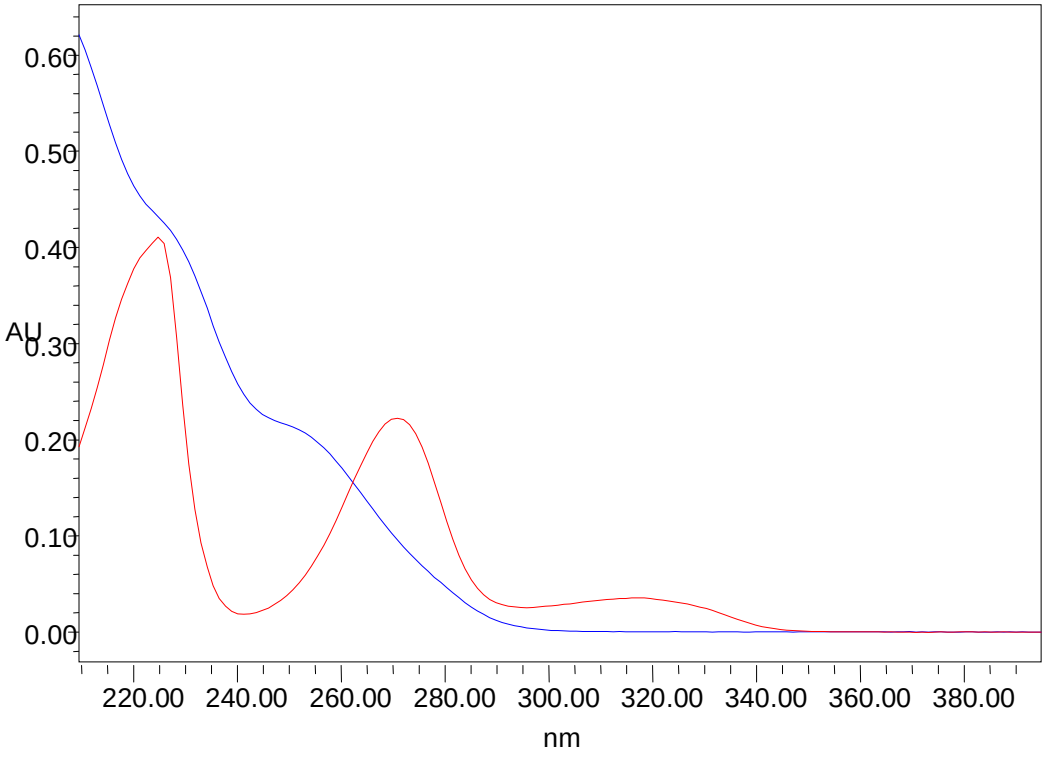


Fig. No. 52.0: Overlay spectra of Losartan and Chlorthalidone

Table No. 82.0: UV Spectrophotometric assay Results for Losartan and Chlorthalidone

Drug name	Label claim (mg/tab)	Estimated Amount (mg/tab)	% of lable claim S.D (n=6)
Losartan	50	51.135	102.27
Chlorthalidone	6.25	6.280625	100.49

6.4.2: UV Method Development of Atovaquone and Proguanil

The literature suvey reveals that very few methods[104,105] are available spectrophometrically for the estimation of the single drug atovaquone in bulk and tablet dosage forms but there were no single spectroscopic method is available for the simultaneous estimation of Atovaquone and Proguanil in tablet dosage form so that we developed spectroscopic method for the estimation of Atovaquone and Proguanil in tablet dosage form. During the development of uv method of Atovaquone and Proguanil water is selected as solvent since both drugs are soluble in water and all other polar solvent, then From the stock solution of Atovaquone and Proguanil 10µg/ml and 4µg/ml concentration solutions were prepared respectively and scanned in uv range from 200 to 400nm, then from Overlay spectra we selected absorption maxima (λ max) 279nm and 254nm for Atovaquone and Proguanil respectively for the estimation by Simultaneous equation method the overlay spectra is shown in **Fig. No. 53.0.** The method is applied for the assay of marketed formulation by simultaneous equation method. The respective data are given in **Table No. 83.0**

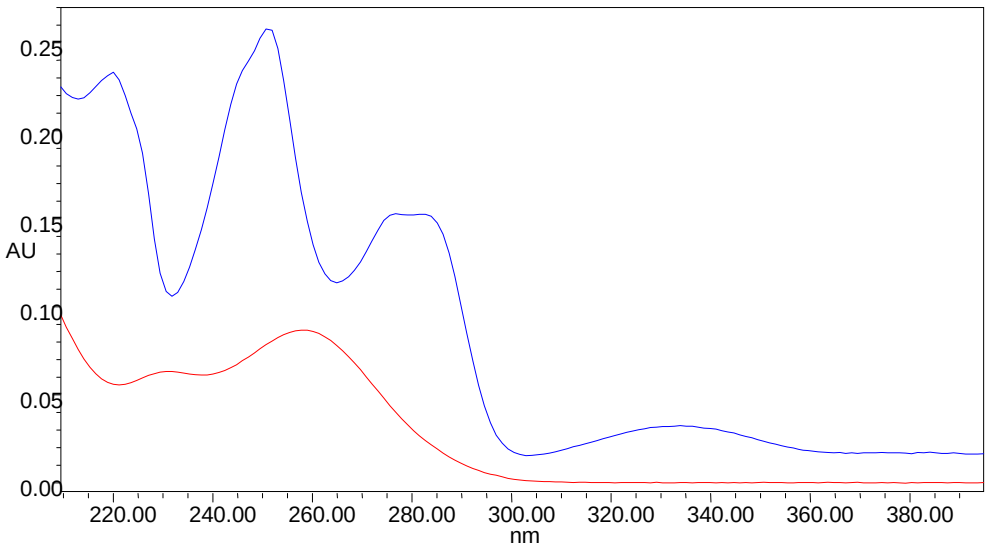


Fig. No. 53.0: Overlay spectra of Atovaquone and Proguanil

Table No. 83.0: UV Spectrophotometric assay Results for Atovaquone and Proguanil

Drug name	Label claim (mg/tab)	Estimated Amount (mg/tab)	% of lable claim S.D (n=6)
Atovaquone	250	251.175	100.47
Proguanil	100	99.24	99.24

6.4.3 UVMethod Development of Losartan and chlorthalidone:

The literature suvey reveals that number of spectroscopic methods are available for the estimation of the single drug acebrofylline[78,80,97] and single acetylcysteine[85,96] in bulk and tablet dosage forms and with other drug combination[82,84] but there were no single spectroscopic method is available for the simultaneous estimation of Acetylcysteine and acebrofylline in capsule dosage form so that we developed spectroscopic method for the estimation of Acetylcysteine and acebrofylline in capsule dosage form. During the development of uv method of Acetylcysteine and acebrofylline methanol is selected as solvent since both drugs are soluble in methanol and all other polar solvent, then From the stock solution of Acetylcysteine and acebrofylline 4µg/ml and 24µg/ml concentration solutions were prepared respectively and scanned in uv range from 200 to 400nm, then from Overlay spectra we selected absorption maxima (λ max) 273nm and 220nm for Acetylcysteine and acebrofylline respectively for the estimation by Simultaneous equation method the overlay spectra is shown in **Fig. No. 54.0.** The method is applied for the assay of marketed formulation by simultaneous equation method. The respective data are given in **Table No. 84.0**

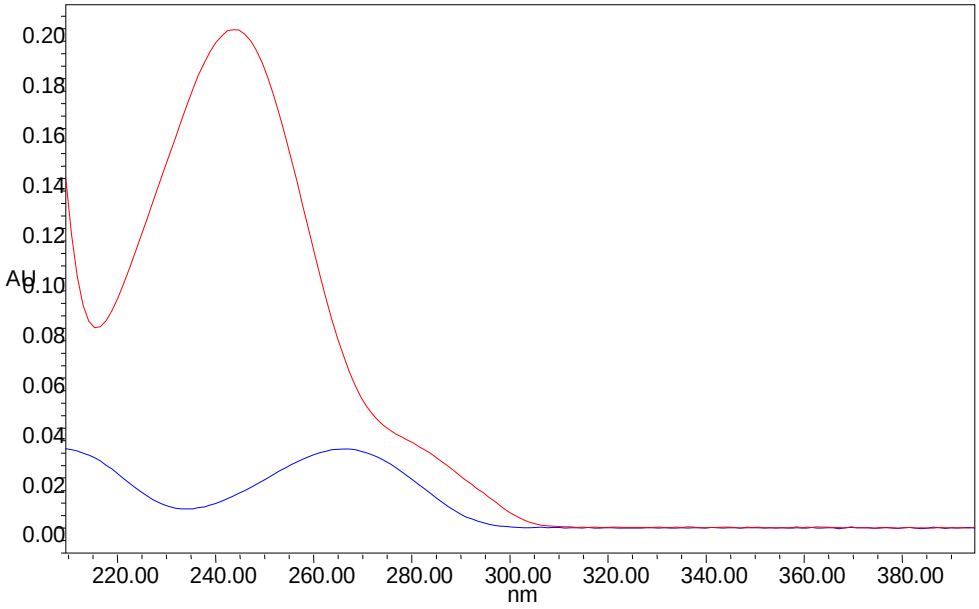


Fig. No. 54.0: Overlay spectra of Acebrophylline and Acetylcysteine

Table No. 84.0: UV Spectrophotometric assay Results for Acebrophylline and Acetylcysteine

Drug name	Label claim (mg/tab)	Estimated Amount (mg/tab)	% of lable claim S.D (n=6)
Acebrophylline	100	101.81	101.81
Acetylcysteine	600	598.968	99.828

6.5: Part – 5 UV Spectrophotometric Method Validations

The developed UV Spectroscopic methods for selected combination of multicomponent formulation were successfully validated for different validation parameters per ICH/USP guidelines and respected data for each parameter of the selected method shown in following tables.

6.5.1a: Losartan and Chlorthalidone

a. Linearity

The method is said to be liniear, when the calibration curve is constructed by the plotting concentration versus Absorbance of the sample in the selected range of the different increasing concentrations. In the determination of the linearity, slope, y-intercept, correlation coefficient and regression coefficient should be calculated and correlation coefficient should be 0.999. here, Losartan and Chlorthalidone was found to be liniear in range of 6 - 16 µg/ml and 0.75 – 2 µg/ml and coerrelation coefficient was

0.9999 and 0.9998 respectively. The Linearity Data for Losartan and Chlorthalidone was given in **Table No. 85.0,** the calibration curve for the losartan and chlorthalidone was given in **Fig. No. 55 and 56.0 respectively.**

Table No. 85.0: UV Spectroscopy Data for Losartan and Chlorthalidone

Sr.No	Concentration in ppm (LOS)	Absorbance AT 235nm (LOS)	Concentration in ppm (CTD)	Absorbance AT 215nm (CTD)
1	6	0.267	0.75	0.092
2	8	0.362	1	0.121
3	10	0.457	1.25	0.151
4	12	0.563	1.5	0.178
5	14	0.658	1.75	0.209
6	16	0.759	2	0.237
Slope		0.0493	**Slope**	0.1161
Intercept		-0.0317	**Intercept**	0.0050
Correlation Coefficient		0.9998	**Correlation Coefficient**	0.9999

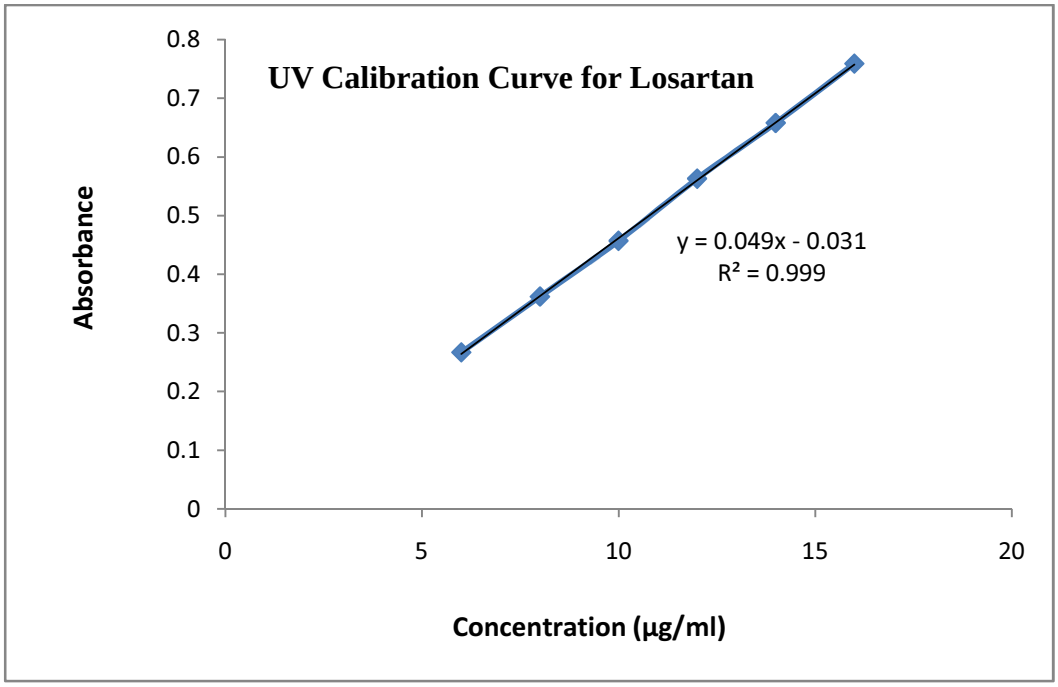


Fig. No. 55.0: UV Calibration Curve for Losartan

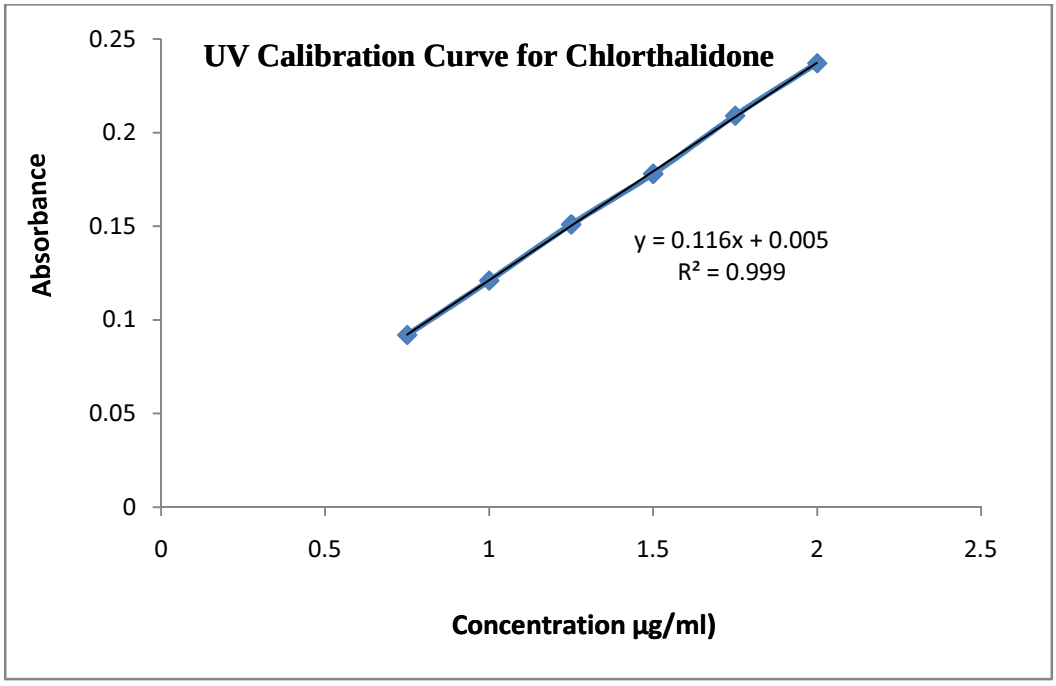


Fig. No. 56.0: UV Calibration Curve for Chlorthalidone

6.5.2a: Atovaquone and Proguanil

Atovaquone and Proguanil spectroscopic method was found to be liniear in range of 3 - 8 µg/ml and 7.5 – 20 µg/ml and coerrelation coefficient was 0.9994 and 0.9997 respectively. The Linearity Data for Atovaquone and Proguanil was given in **Table No. 86.0,** the calibration curve for the Atovaquone and Proguanil was given in **Fig. No. 57.0 and 58.0 respectively.**

Table No. 86.0: UV Spectroscopy Data for Atovaquone and Proguanil

Sr.No	Concentration in ppm (PROG)	Absorbance AT 254nm (PROG)	Concentration in ppm (ATOV)	Absorbance AT 279nm (ATOV)
1	3	0.154	7.5	0.365
2	4	0.199	10	0.479
3	5	0.255	12.5	0.593
4	6	0.313	15	0.699
5	7	0.366	17.5	0.824
6	8	0.417	20	0.944
Slope		0.0535	**Slope**	0.0461
Intercept		-0.0104	**Intercept**	0.0164
Correlation Coefficient		0.9994	**Correlation Coefficient**	0.9997

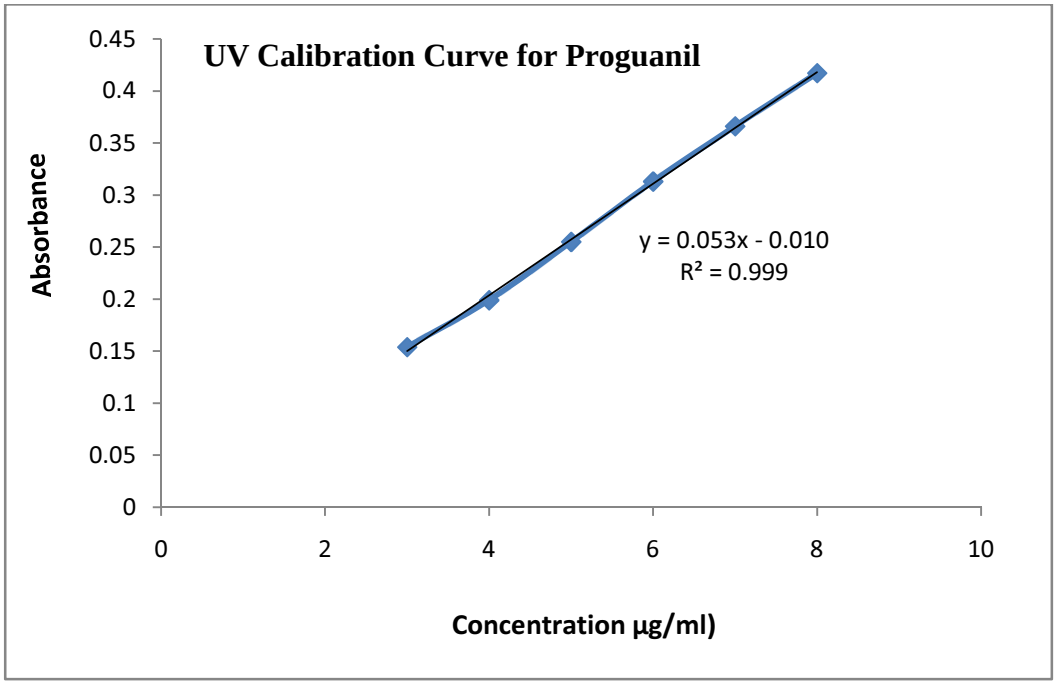


Fig. No. 57.0: UV Calibration Curve for Proguanil

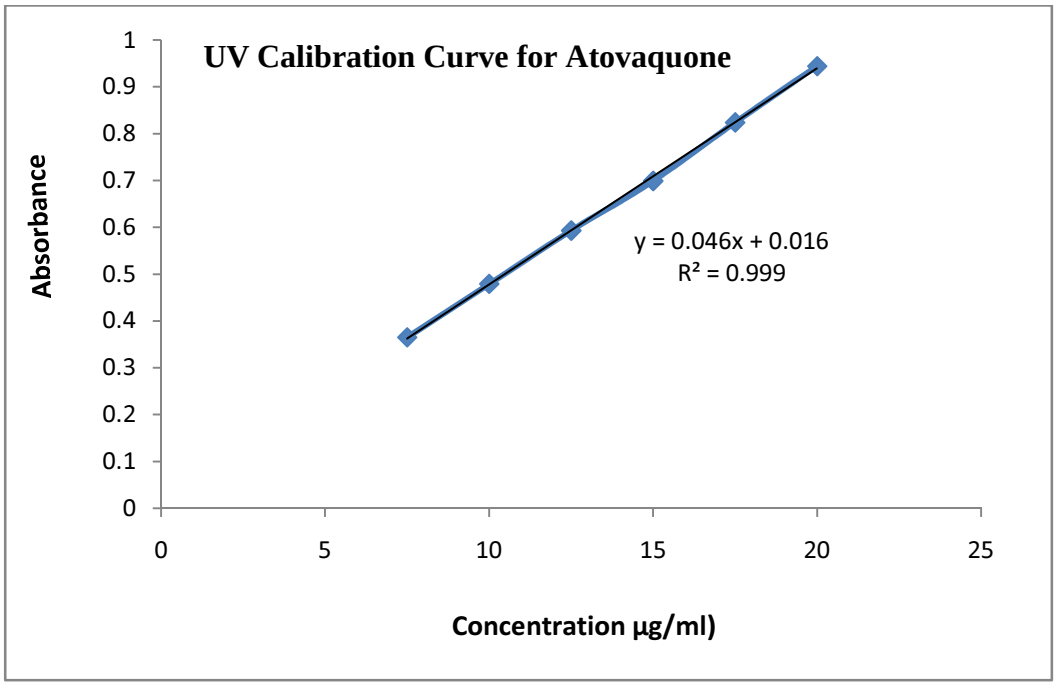


Fig. No. 58.0: UV Calibration Curve for Atovaquone

6.5.3a: Acetylcysteine and Acebrofylline

Acetylcysteine and Acebrofylline spectroscopic method was found to be liniear in range of 6 - 36 µg/ml and 1 – 6 µg/ml and coerrelation coefficient was 0.9994 and 0.9997 respectively. The Linearity Data for Acetylcysteine and Acebrofylline was given

in **Table No. 87.0,** the calibration curve for the Acetylcysteine and Acebrofylline was given in **Fig. No. 59 and 60.0 respectively.**

Table No. 87.0: UV Spectroscopy Data for Acetylcysteine and Acebrofylline

Sr.No	Concentration in ppm (ACST)	Absorbance AT 220nm (ACST)	Concentration in ppm (ACBF)	Absorbance AT 273nm (ACBF)
1	6	0.167	1	0.04
2	12	0.317	2	0.082
3	18	0.456	3	0.129
4	24	0.628	4	0.167
5	30	0.755	5	0.21
6	36	0.919	6	0.256
Slope		0.0249	**Slope**	0.0429
Intercept		0.0157	**Intercept**	-0.0028
Correlation Coefficient		0.9995	**Correlation Coefficient**	0.9997

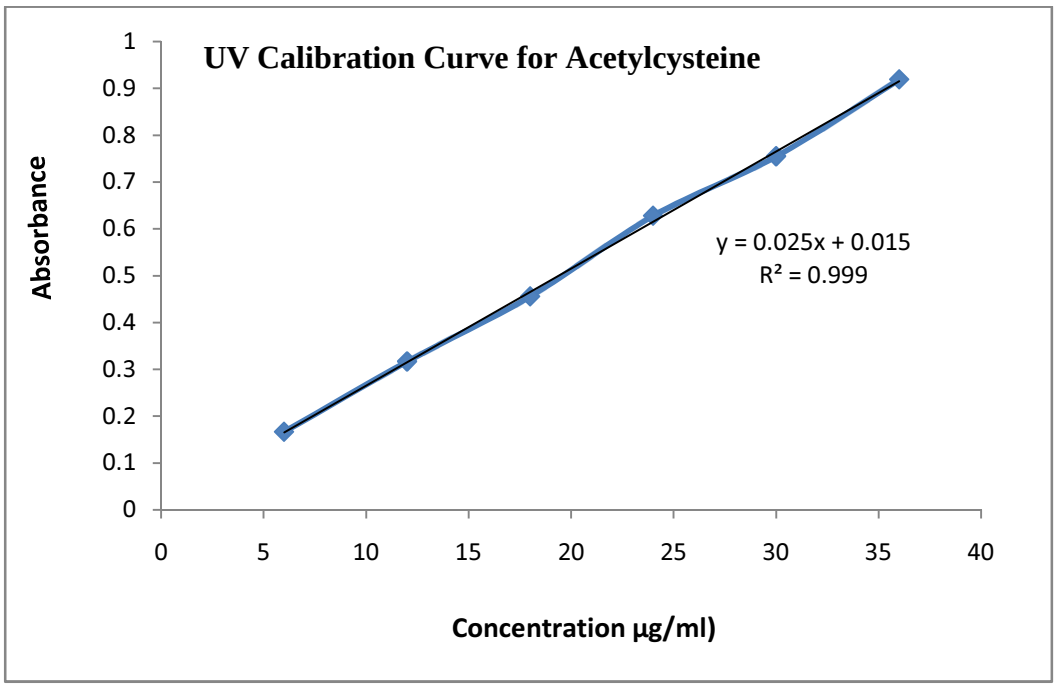


Fig. No. 59.0: UV Calibration Curve for Acetylcysteine

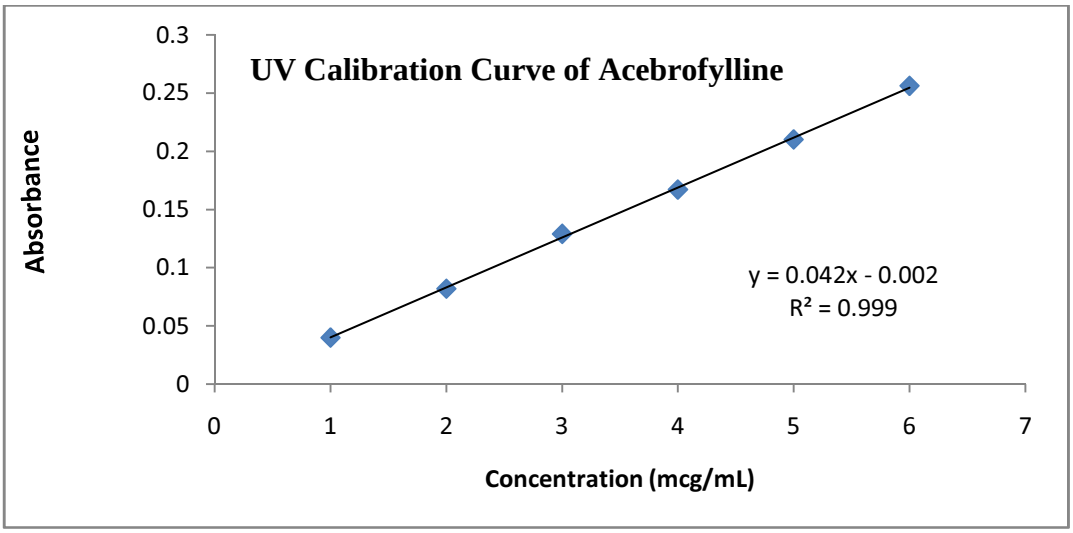


Fig. No. 60.0: UV Calibration Curve for Acebrofylline

b. Accuracy

The accuracy of the method was determined by recovery study. The accuracy of an analytical method should be established across its range. The accuracy of the method was calculated by the recovery studies at the three concentration of 50%, 100% and 150% levels by the standard addition method and the mean % recovery was calculated and it should be in between 98%-102% and the % RSD should be less than 2.

6.5.1b: Chlorthalidone and Losartan

The developed UV method are found to be accurate, as all the 3 methods were passes the %RSD and % mean. The accuracy data for the chlorthalidone and losartan given in **Table No. 88.0 and 89.0** respectively**.**

Table No. 88.0: UV Spectroscopy Accuracy Data for Chorthalidone

% level	Spiked amount	Amount found	% recovery
50%	0.5	0.48	96.54
100%	1	0.99	98.67
150%	1.5	1.49	99.65
% Mean			98.29
Standard Deviation			1.59
% RSD			1.61

Table No. 89.0: UV Spectroscopy Accuracy Data for Losartan

% level	Spiked amount	Amount found	% recovery
50%	4	4.07	101.74
100%	8	8.09	101.11
150%	12	11.79	98.28
% Mean			100.38
Standard Deviation			1.84
% RSD			1.83

6.5.2b: Atovaquone and Proguanil

The developed UV method are found to be accurate, as all the 3 methods were passes the %RSD and % mean. The accuracy data for the Proguanil and Atovaquone is given in **Table No. 90.0 and 91.0** respectively**,**

Table No. 90.0: UV Spectroscopy Accuracy Data for Proguanil

% level	Spiked amount	Amount found	% recovery
50%	2	1.98	98.98
100%	4	3.99	99.67
150%	6	5.92	98.69
% Mean			99.11
Standard Deviation			0.50
% RSD			0.50

Table No. 91.0: UV Spectroscopy Accuracy Data for Atovaquone

% level	Spiked amount	Amount found	% recovery
50%	5	4.98	99.64
100%	10	9.82	98.21
150%	15	14.91	99.38
% Mean			99.08
Standard Deviation			0.76
% RSD			0.77

6.5.3b: Acetylcysteine and Acebrofylline

The developed UV method are found to be accurate, as all the 3 methods were passes the %RSD and % mean. The accuracy data for the Acetylcysteine and Acebrofylline is given in **Table No. 91.0 and 92.0** respectively**,**

Table No. 92.0: UV Spectroscopy Accuracy Data for Acetylcysteine

% level	Spiked amount	Amount found	% recovery
50%	12	11.98	99.83
100%	24	24.01	100.04
150%	36	35.93	99.80
% Mean			99.89
Standard Deviation			0.13
% RSD			0.13

Table No. 93.0: UV Spectroscopy Accuracy Data for Acebrofylline

% level	Spiked amount	Amount found	% recovery
50%	2	2.00	99.93
100%	4	4.00	100.01
150%	6	5.99	99.89
% Mean			99.94
Standard Deviation			0.06
% RSD			0.06

c. Precision

It measures the closeness of the agreement between the series of measurements obtained from the multiple samples of the same homogenious sample under the prescribed conditions. The method is said to be precise when the % RSD obtained under different conditions was less than 2%. The relative standard deviation (RSD) was found to be less than 2% for selected drug combinations.

6.5.1c: Losartan and Chlorthalidone

The developed method was found to be Precise as the %RSD was less than 2

Table No. 94.0: UV Spectroscopy Precision Data for Losartan and Chlorthalidone

Sr.No	Intra-Day Precision		Inter-Day Precision	
	CTD	LOS	CTD	LOS
1	103.54	102.30	97.67	102.59
2	100.61	102.44	97.67	102.59
3	103.54	102.30	100.61	102.44
4	100.61	102.44	100.61	102.44
5	100.61	102.44	100.61	102.44
6	100.61	102.44	97.67	102.59
Mean	101.59	102.40	99.14	102.52
SD	1.52	0.07	1.61	0.08
%RSD	**1.49**	**0.07**	**1.62**	**0.07**

6.5.2c: Proguanil and Atovaquone

The developed method was found to be Precise as the %RSD was less than 2

Table No. 95.0: UV Spectroscopy Precision Data for Proguanil and Atovaquone

Sr.No	Intra-Day Precision		Inter-Day Precision	
	PROG	ATOV	PROG	ATOV
1	101.32	99.51	102.84	99.07
2	98.70	100.65	98.06	100.72
3	98.47	100.96	97.83	101.03
4	97.41	100.79	99.35	100.58
5	97.18	101.10	101.09	99.82
6	101.09	99.82	99.57	100.27
Mean	99.03	100.47	99.79	100.25
SD	1.79	0.65	1.90	0.71
%RSD	**1.80**	**0.64**	**1.90**	**0.70**

6.5.3c: Acetylcysteine and Acebrofylline

The developed method was found to be Precise as the %RSD was less than 2

Table No. 96.0: UV Spectroscopy Precision Data for Acetylcysteine and Acebrofylline

Sr.No	Intra-Day Precision		Inter-Day Precision	
	ACST	ACBF	ACST	ACBF
1	99.75	101.98	99.04	101.68
2	99.34	102.01	99.69	101.95
3	99.40	101.19	99.81	102.10
4	99.10	101.86	99.34	102.13
5	99.69	101.77	99.75	101.98
6	99.87	102.04	99.75	101.70
Mean	99.52	101.81	99.56	101.92
SD	0.29	0.32	0.31	0.19
%RSD	**0.29**	**0.31**	**0.30**	**0.19**

d. LOD and LOQ

6.5.1d: Losartan & Chlorthalidone

The lower limit of detection and lower limit of quanification is calculated in **Table No. 96.0**

Table No. 97.0: UV Spectroscopy LOD and LOQ Data for Losartan & Chlorthalidone

Drug Name	Parameter Used		LOD	LOQ
Losartan	STEYX(SD)	0.0032	**0.21**	**0.64**
	SLOPE	0.0493		
Chorthalidone	STEYX(SD)	0.0008	**0.024**	**0.07**
	SLOPE	0.1161		

6.5.2d: Proguanil and Atovaquone

The lower limit of detection and lower limit of quanification is calculated in **Table No. 97.0**

Table No. 98.0: UV Spectroscopy LOD and LOQ Data for Proguanil and Atovaquone

Drug Name	Parameter Used		LOD	LOQ
Proguanil	STEYX(SD)	0.0035	**0.22**	**0.66**
	SLOPE	0.0535		
Atovaquone	STEYX(SD)	0.0055	**0.39**	**1.19**
	SLOPE	0.0461		

6.5.3d: Acetylcysteine and Acebrofylline

The lower limit of detection and lower limit of quanification is calculated in **Table No. 98.0**

Table No. 99.0: UV Spectroscopy LOD and LOQ Data for Acetylcysteine and Acebrofylline

Drug Name	Parameter Used		LOD	LOQ
Acetylcysteine	STEYX(SD)	0.0096	**1.28**	**3.87**
	SLOPE	0.0249		
Acebrofylline	STEYX(SD)	0.0021	**0.17**	**0.50**
	SLOPE	0.0429		

Table No. 100.0: UV Summary of Results for selected Combinations

Parameter	Losartan	Chlorthalidone	Proguanil	Atovaquone	Acetylcysteine	Acebrofylline
Absorption maxima (λ_{max}) in nm	235nm	215nm	279	254	220	273
Beers law limit (µg/ml)	6 to 16	0.75 to 2	3-8	7.5-20	6-36	1-6
Correlation coefficient(r)	0.9998	0.9999	0.9994	0.9997	0.9995	0.9997
Regression Coefficient(r^2)	0.9996	0.9998	0.9988	0.9994	0.9990	0.9994
Regression equation Slope	0.0493	0.1161	0.0535	0.0461	0.0249	0.0429
Intercept	-0.0317	0.0050	-0.0104	0.0164	0.0157	-0.0028
STEYX	0.0032	0.0008	0.0035	0.0055	0.0096	0.0021
LOD(µg/ml)	0.21	0.024	0.22	0.39	1.28	0.17
LOQ(µg/ml)	0.64	0.07	0.66	1.19	3.87	0.50
Recovery(102 - 98%)&(%RSD)	100.38 (1.83)	98.29 (1.61)	99.11 (0.50)	99.08 (0.77)	99.89 (0.13)	99.94 (0.06)
Intraday precision(%RSD)	0.07	1.49	1.79	0.65	0.29	0.31
Interday precision(%RSD)	0.0770	1.623	1.90	0.71	0.30	0.19

6.6: Part – 6 Results for HPTLC Method Development

Losartan and Chlorthalidone

6.6.1: Optimization of Solvent System and Chromatographic Conditions for Losartan and Chlorthalidone

When chlorthalidone and losartan were applied on plate and the plates were developed using neat solvents, the results obtained were-

Solvent		**Chlorthalidone**	**Losartan**
Benzene	:	spot at R_f 0.46	At initial spot itself
Chloroform	:	R_f 0.78	R_f 0.47 (tailing & no clear separation)
Toluene	:	R_f 0.74	R_f 0.51 (tailing & no clear separation)
Ethyl Acetate	:	a big spot at R_f 0.24	(no separation)
Methanol	:	(no separation)	(no separation)

It was concluded that no single solvent was able to resolve the two drugs. Even in the strongest solvent i.e. methanol, the movement of both drugs was very less. Hence, mixtures of solvents were used.

Thus, the solvent system, Toluene: chloroform:methanol:ammonia (2.3:5.0:2.0:0.2v/v/v/v) was selected.

The samples were applied in form of bands of width 8 mm on precoated silica gel aluminum sheets 60 $F_{254.}$ As the samples were prepared in methanol, spreading of bands was avoided. A constant application rate of 150 nl/s was selected according to the recommendations in-built in the Wincats software. The application position (X) and (Y) were kept at 10mm to avoid edge effect. Linear ascending development was carried out in a twin trough glass chamber (20cmx10 cm, 10X10 cm), without using filter paper and without saturation. 20 ml mobile phase for 20X10 cm tank and 10 ml for 10X10 cm tank was used per chromatographic development; the length of chromatogram run was 80 mm. The developed plates were dried in the flow of nitrogen Densitometric scanning was performed in the reflectance- absorbance mode at 254 nm. The slit dimension was kept at 6 X 0.45 mm and 100 nm/s scanning speed was employed. The source of radiation was deuterium lamp emitting continuous UV spectrum between 190 and 400nm.

Table No. 101.0: HPTLC Optimized Chromatographic Condition for chlorthalidone and losartan

Stationary phase	:	Pre coated silica gel $60F_{254}$ on aluminium sheets (Merck)
Mobile phase (2.3:5.0:2.0:0.2v/v/v/v)	: Toluene: chloroform:methanol:ammonia	
Chamber saturation	:	20 min
Migration distance	:	80 mm
Band width	:	8mm
Slit dimensions	:	6× 0.45 mm
Source of radiation	:	Deuterium lamp
Scanning wavelength	:	254 nm

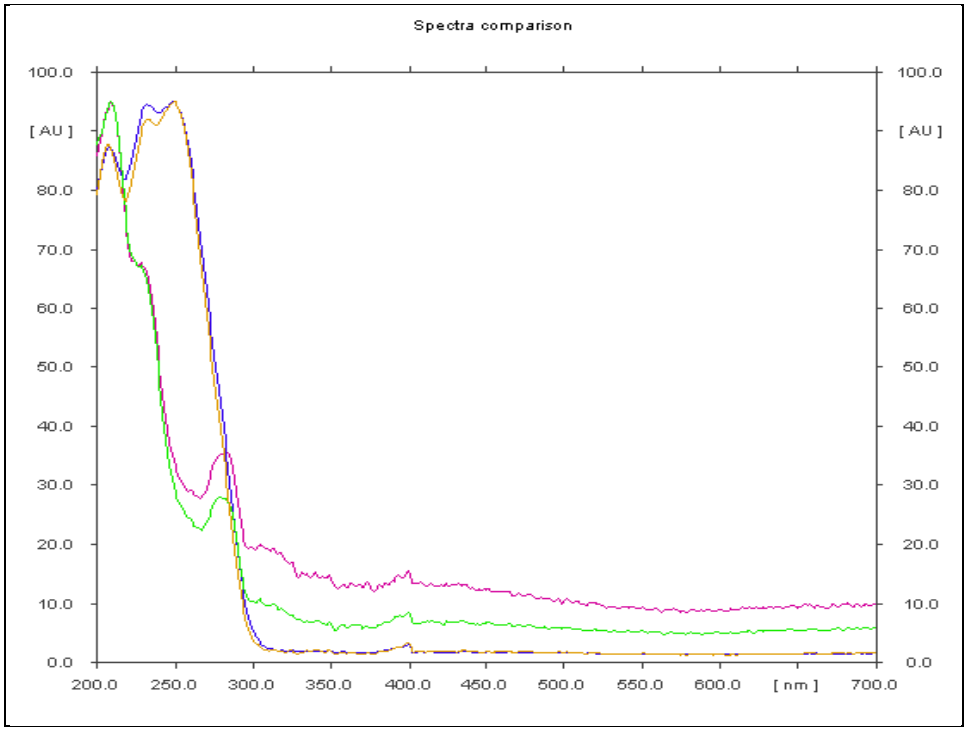


Fig No 61.0: HPTLC Overlay spectra of chlorthalidone and losartan

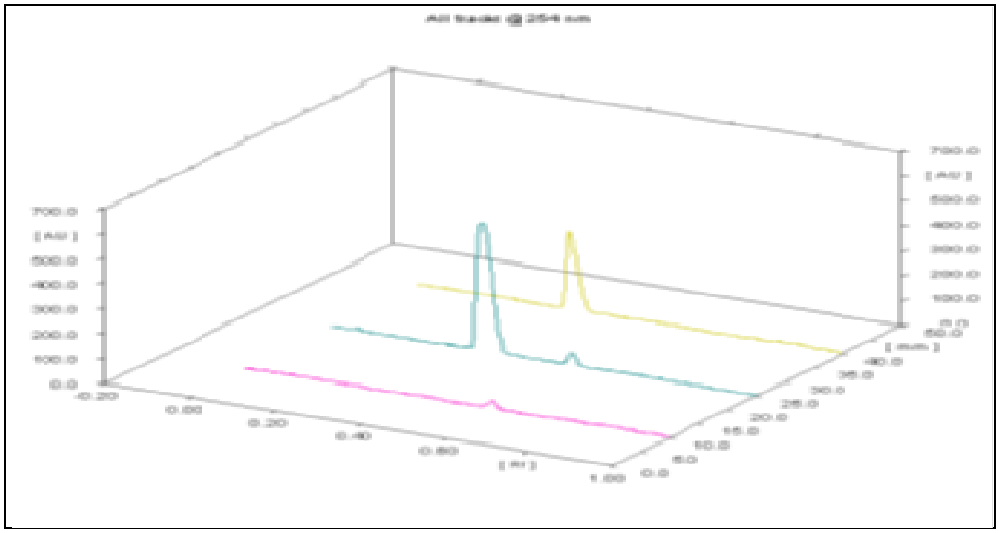


Fig. No 62.0 chromatogram of mixed calibration standards of chlorthalidone and losartan.

Table No102.0: HPTLC Assay Results for LOS &CTD

S.No	Drug	Label claim (mg/tablets)	Amount found	% content	%RSD
1.	Chlorthalidone	6.25	6.23±0.02	100.01±0.14	1.20
2.	Losartan	50	49.80±0.10	99.67±0.1	1.14

*Mean of 6 determinations

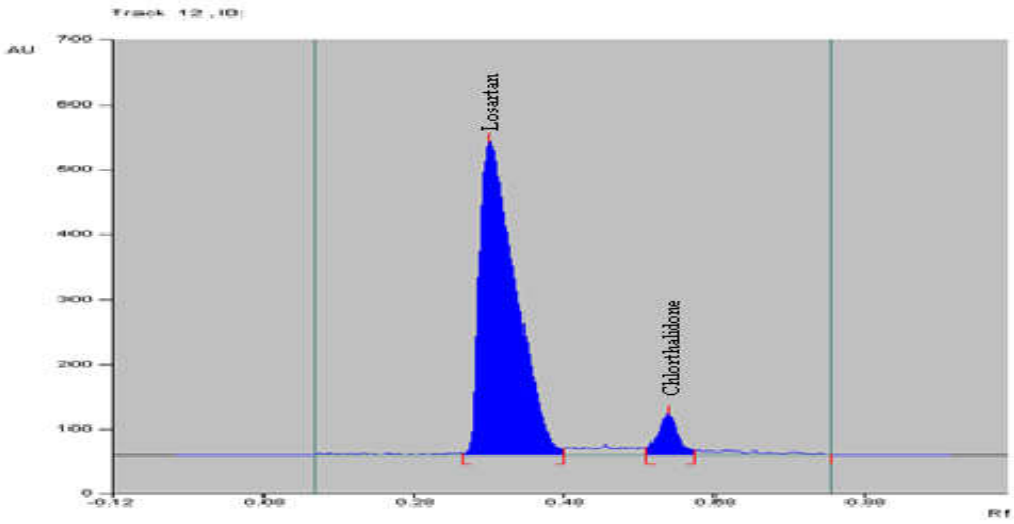


Fig No 63.0 : Representative chromatogram of chlorthalidone (R_f 0.26) and losartan (R_f 0.37)

Atovaquone and Proguanil

6.6.2: HPTLC Optimization of Solvent System and Chromatographic Conditions

When proguanil hydrochloride and atovaquone were applied on plate and the plates were developed using neat solvents, the results obtained were-

Solvent		**Proguanil hydrochloride**	**Atovaquone**
Benzene	:	No movement	No movement
Chloroform	:	No movement	(no separation)
Toluene	:	R_f 0.52 R_f 0.65	(tailing & no clear separation)
Ethyl Acetate	:	R_f 0.34 R_f 0.48	(tailing & no clear separation)
Methanol	:	R_f 0.1R_f 0.21	(tailing & no clear separation)
THF	:	R_f 0.89	Spot goes to solvent front

It was concluded that no single solvent was able to resolve the two drugs. Even in the strongest solvent i.e. methanol, the movement of both drugs was very less. Hence, mixtures of solvents were used.

Thus, the solvent system, THF: water (6:4 v/v) was selected.

The samples were applied in form of bands of width 10 mm on precoated silica gel aluminum sheets 60 $F_{254.}$ As the samples were prepared in methanol, spreading of bands was avoided. A constant application rate of 150 nl/s was selected according to the recommendations in-built in the Wincats software. The application position (X) and (Y) were kept at 10mm to avoid edge effect. Linear ascending development was carried out in a twin trough glass chamber (20cmx10 cm, 10X10 cm), without using filter paper and without saturation. 20 ml mobile phase for 20X10 cm tank and 10 ml for 20X10 cm tank was used per chromatographic development; the length of chromatogram run was 80 mm. The developed plates were dried in the flow of dry air with the help of an air drier. Densitometric scanning was performed in the reflectance- absorbance mode at 260 nm. The slit dimension was kept at 6 X 0.45 mm and 100 nm/s scanning speed was employed. The source of radiation was deuterium lamp emitting continuous UV spectrum between 190 and 400nm.

Table No.103.0 : HPTLC Optimized Chromatographic Condition for Atovaqvone and proguanil

Stationary phase	:	Pre coated silica gel $60F_{254}$ on aluminium sheets (Merck)
Mobile phase	:	THF: water (6:4 v/v)
Chamber saturation	:	20 min
Migration distance	:	80 mm
Band width	:	10mm
Slit dimensions	:	6× 0.45 mm
Source of radiation	:	Deuterium lamp
Scanning wavelength	:	260 nm

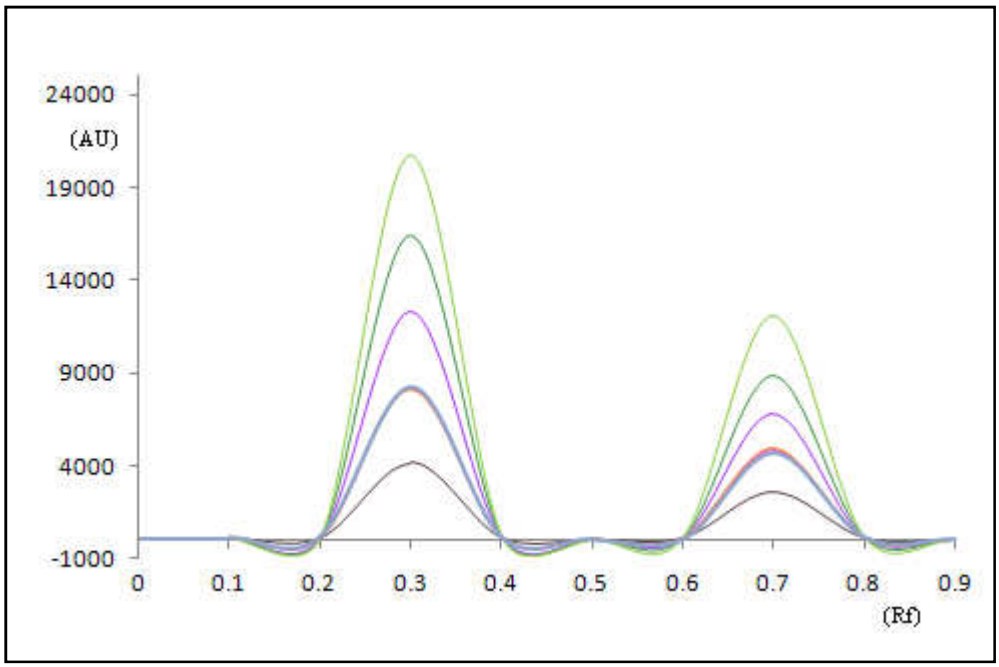


Fig.No 64.0: Chromatogram of mixed calibration standards of Atovaqvone and proguanil

Table No 104.0: HPTLC Assay Results for ATOV and PROG

S.No	Drug	Label claim (mg/tablets)	Amount found	% content	%RSD	R_f
1.	Atovaquone	250	251.39±0.03	100.60±0.03	0.61	0.74
2.	Proguanil	100	100.46±0.01	100.46±0.01	0.39	0.32

*Mean of 6 determinations

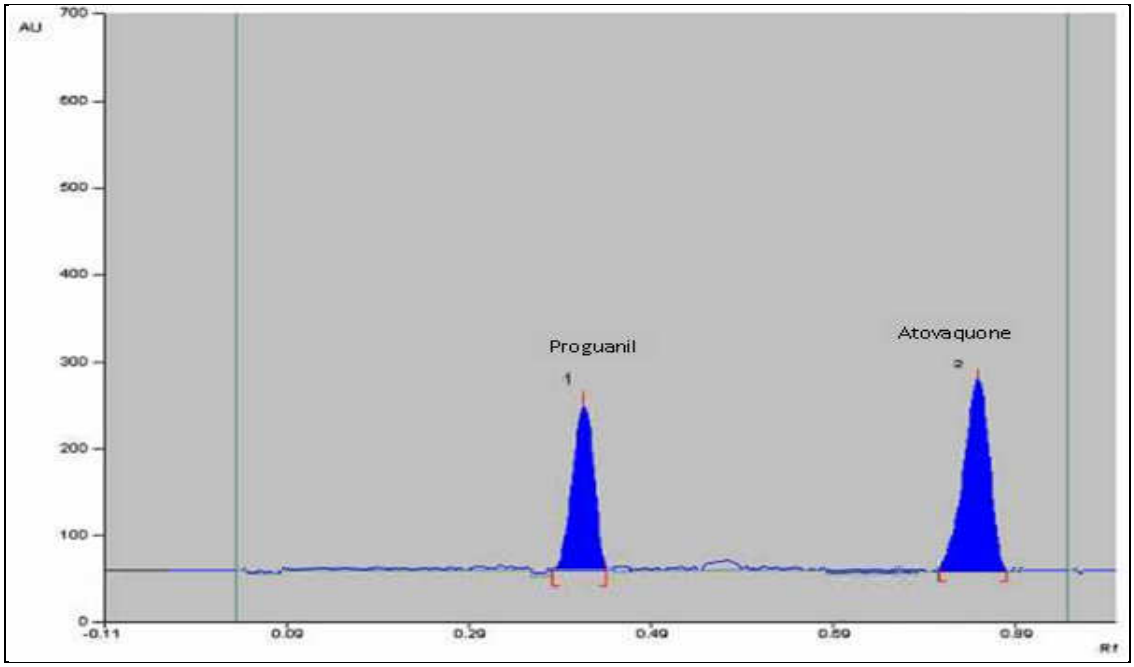


Fig. No 65.0: Representative chromatogram of Atovaqvone (Rf 0.78) and proguanil (Rf 0.36) Acetylcysteine and Acebrofylline

6.6.3: HPTLC Optimized Chromatographic Condition for Acetylcysteine and Acebrofylline.

The samples were applied in form of bands of width 8 mm on precoated silica gel aluminum sheets 60 $F_{254.}$ As the samples were prepared in methanol, spreading of bands was avoided. A constant application rate of 150 nl/s was selected according to the recommendations in-built in the Wincats software. The application position (X) and (Y) were kept at 10mm to avoid edge effect. Linear ascending development was carried out in a twin trough glass chamber (20cmx10 cm, 10X10 cm), without using filter paper and without saturation. 20 ml mobile phase for 20X10 cm tank and 10 ml

for 10X10 cm tank was used per chromatographic development; the length of chromatogram run was 80 mm. The developed plates were dried in the flow of nitrogen Densitometric scanning was performed in the reflectance- absorbance mode at 215 nm. The slit dimension was kept at 6 X 0.45 mm and 100 nm/s scanning speed was employed. The source of radiation was deuterium lamp emitting continuous UV spectrum between 190 and 400nm.

Table No. 105.0: HPTLC Optimized Chromatographic Condition for acetylcysteine and acebrofylline.

Stationary phase	:	Pre coated silica gel $60F_{254}$ on aluminium sheets (Merck)
Mobile phase	:	chloroform: toluene: methanol (6:2:2 v/v/v)
Chamber saturation	:	20 min
Migration distance	:	80 mm
Band width	:	8mm
Slit dimensions	:	6× 0.45 mm
Source of radiation	:	Deuterium lamp
Scanning wavelength	:	215 nm

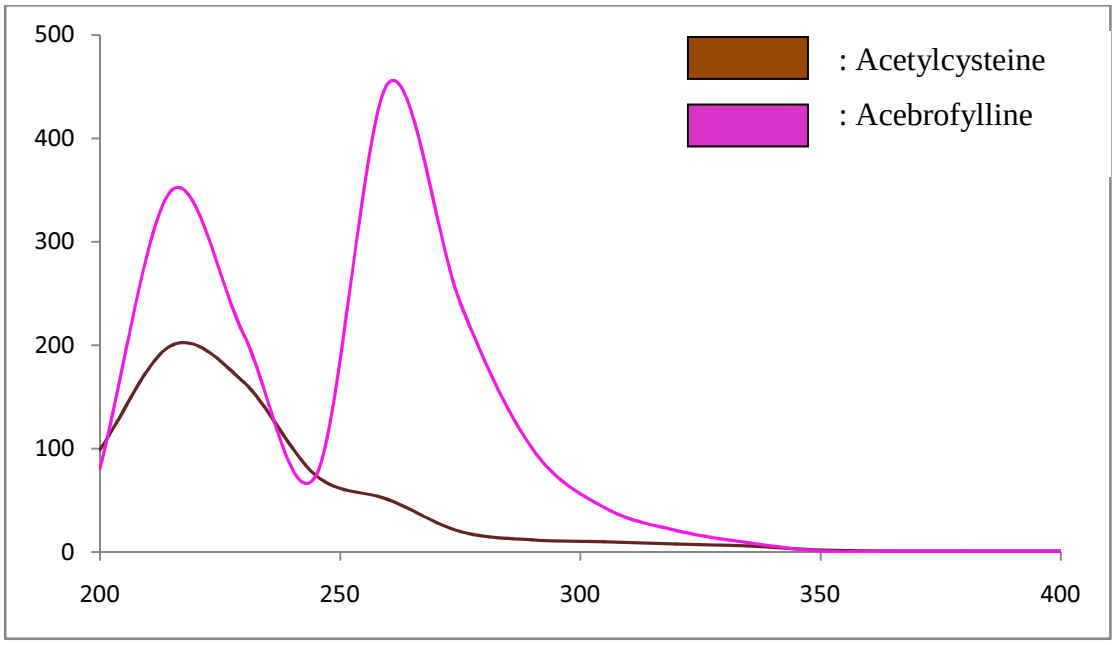


Fig.No 66.0 : HPTLC Overlay spectra of Acetylcysteine and Acebrofylline

Table No106.0: HPTLC Assay Results for ATOV and PROG

S.No	Drug	Label claim (mg/capsule)	Amount found	% content	%RSD
1.	Acetylcysteine	600	600.02±2.02	100.01±1.10	1.10
2.	Acebrofylline	100	99.66±1.10	99.66±1.34	1.34

*Mean of 6 determinations

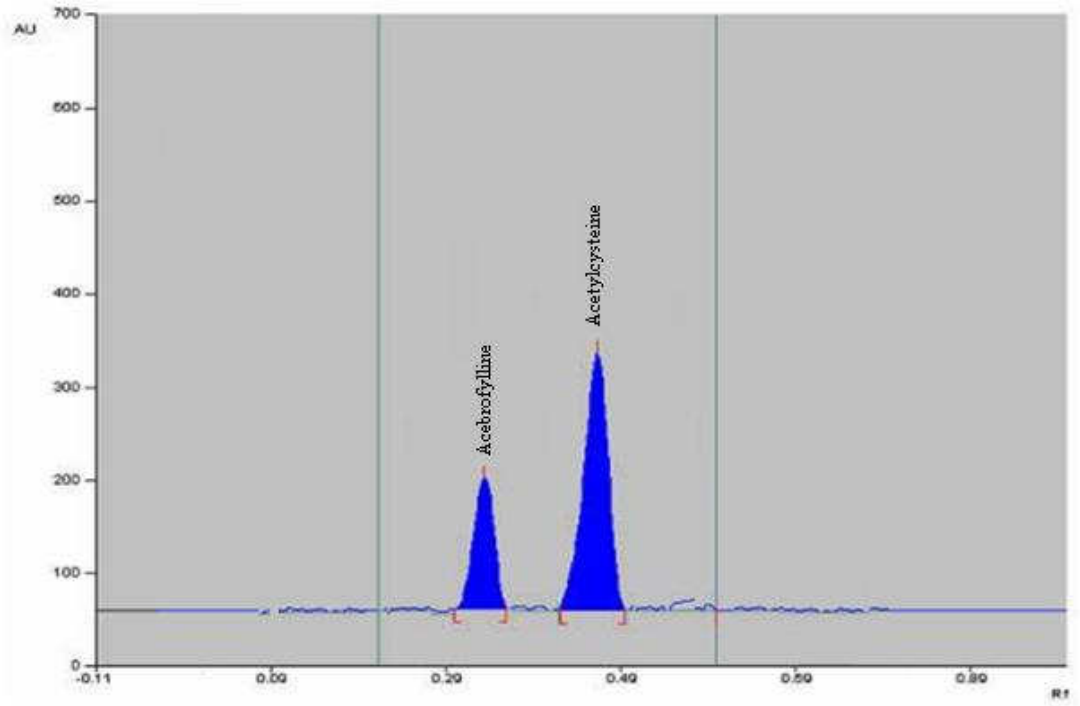


Fig. No 67: Representative chromatogram of acetylcysteine (Rf 0.37) and acebrofylline (Rf 0.26)

6.7: HPTLC METHOD VALIDATION

6.7.1 : Losartan and Chlothalidone

6.7.1a: Linearity (Calibration Experiments)

Linearity was confirmed by the calibration experiments. Linear regression data for the calibration plots revealed good linear relationships between area and concentration over the ranges 31.25-187.5 ng/spot for chlorthalidone and 250-1500

ng/spot for losartan. The linear regression equations were Y=1.1513 X + 681.25 and Y=1.9872 X +13.623 for chlorthalidone and losartan respectively. The calibration plots show regression (r^2) being 0.9955 and 0.9800 for chlorthalidone and losartan respectively.

Table No 107.0: HPTLC Calibration data for Chlorthalidone

Standard	**Amount applied (ng)**	**Peak Area of replicate**			**Mean peak area**	**Standard deviation of peak area**
		1	2	3		
1	31.25	730.23	732.29	732.12	731.54	1.14
2	62.5	754.61	758.91	756.23	756.58	2.17
3	93.75	770.32	772.41	771.68	771.47	7.06
4	125	805.96	812.21	804.98	807.71	3.92
5	156.2	865.34	862.33	864.62	864.09	1.57
6	187.5	910.69	912.54	911.56	911.59	0.92
Equation	y = 1.1513x + 681.25					
R^2	0.9955					

Table No 108.0 : HPTLC Calibration data for Losartan

Standard	Amount applied (ng)	Peak Area of replicate			Mean peak area	Standard deviation of peak area
		1	2	3		
1	250	14100.26	14146.57	14124.43	14123.75	23.16
2	500	14785.30	14776.22	14775.12	14778.88	5.58
3	750	14998.41	14997.58	14996.68	14997.55	0.86
4	1000	15540.63	15544.49	15532.45	15539.17	6.14
5	1250	15994.71	15997.69	15982.78	15991.72	7.89
6	1500	16762.42	16759.98	16761.36	16761.25	1.12
Equation	y = 1.9872x + 13623					
R^2	0.9800					

The calibration curves for chlorthalidone and losartan are given in **Fig 68** and **Fig 69** respectively.

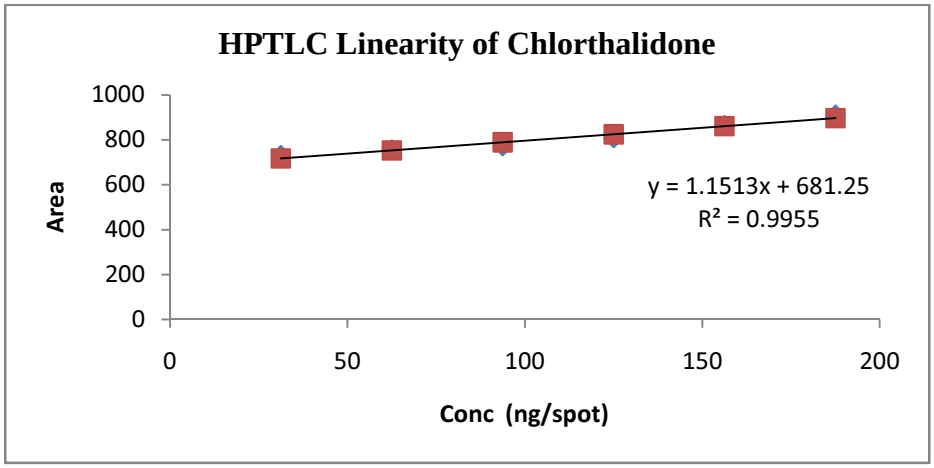


Figure No 68.0: HPTLC Calibration curve for Chlorthalidone

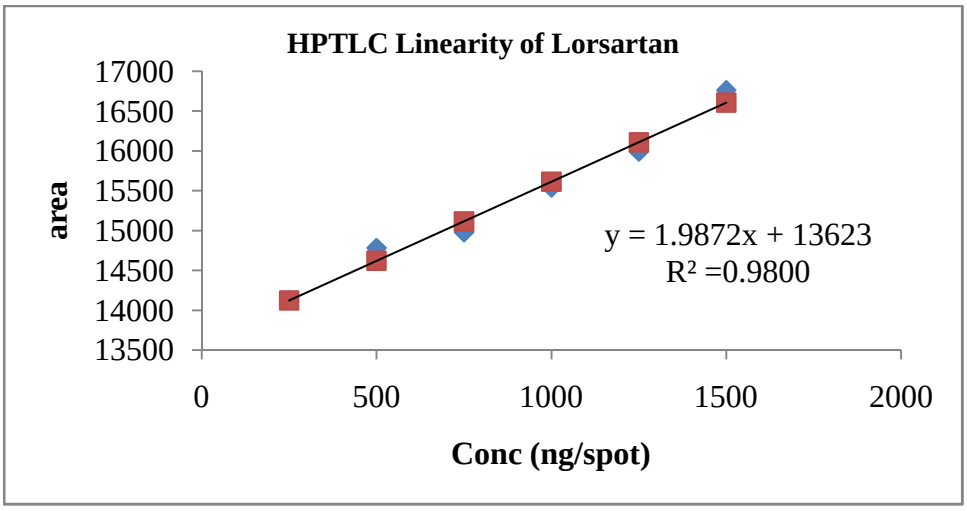


Figure No 69.0: HPTLC Calibration curve for Losartan

6.7.2a: Linearity Atovaquone and Proguanil

Linearity was confirmed by the calibration experiments. Linear regression data for the calibration plots revealed good linear relationships between area and concentration over the ranges 500-3000 ng/spot for Atovaquone and 200-1200 ng/spot for Proguanil The linear regression equations were y =8.1639x + 79.147and y = 9.536x + 956.88 for Atovaquone and Proguanil respectively. The calibration plots show regression (r^2) being 0.9998 and 0.9958 for chlorthalidone and losartan respectively Table No. 109.0, 110.0 and Fig 70.0,71.0

Table No 109.0: HPTLC Calibration data for Atovaquone

Standard	Amount applied (ng)	Peak Area of replicate			Mean peak area	Standard deviation of peak area
		1	2	3		
1	500	4144.13	4128.04	4159.11	4143.76	15.538
2	1000	8200.47	8262.68	8186.36	8216.50	40.61
3	1500	12304.48	12378.02	12292.95	12325.15	46.15
4	2000	16450.75	16410.82	16380.23	16413.93	35.36
5	2500	20524.14	20496.81	20981.44	20667.46	272.25
6	3000	24434.34	24401.28	24451.69	24429.10	25.60
Equation	y =8.1639x + 79.147					
R^2	0.9998					

Table No 110.0: HPTLC Calibration data for proguanil

Standard	Amount applied (ng)	Peak Area of replicate			Mean peak area	Standard deviation of peak area
		1	2	3		
1	200	2602.12	2584.14	2562.47	2582.91	19.85
2	400	4256.14	4298.68	4210.11	4854.98	44.30
3	600	6946.43	5980.34	6040.54	6822.44	541.23
4	800	8654.51	8612.89	8694.74	8854.05	40.93
5	1000	10646.47	10597.34	10541.47	10595.09	52.53
6	1200	12346.03	12290.02	12212.57	12082.87	67.02
Equation	y = 9.536x + 956.88					
R^2	0.9954					

The calibration curves for Atovaquone and proguanil are given in **Fig 70** and **Fig 71** respectively.

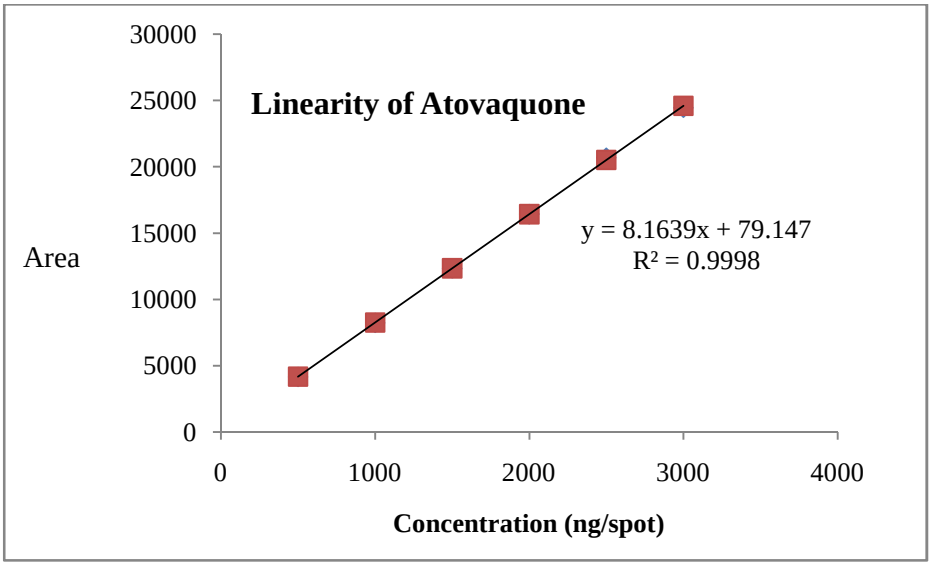


Figure No. 70.0: HPTLC Calibration curve for Atovaquone

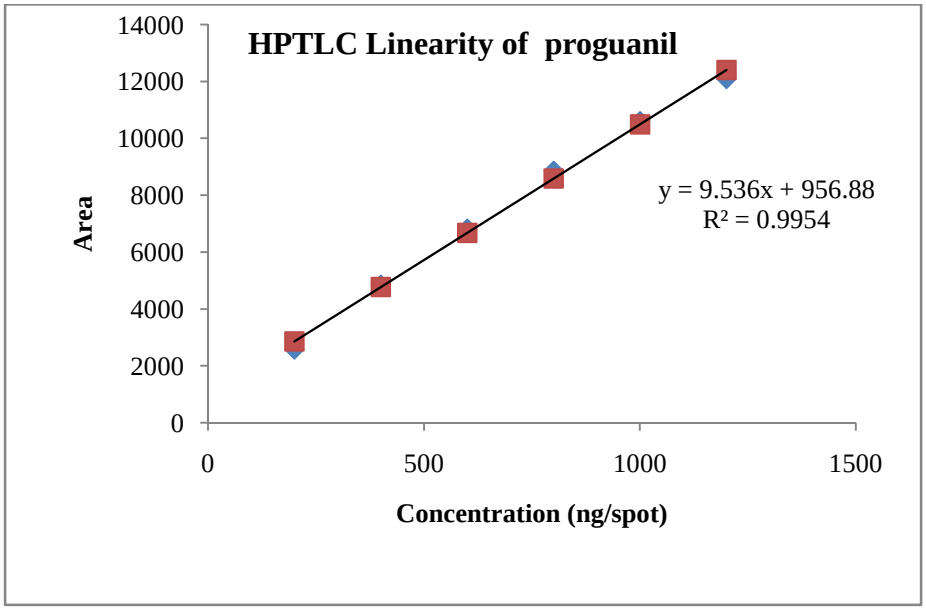


Figure No71.0: HPTLC Calibration curve for Proguanil

6.7.3a: Linearity (Calibration Experiments) Acetylcysteine and Acebrofylline Linearity

Linearity was confirmed by the calibration experiments. Linear regression data for the calibration plots revealed good linear relationships between area and concentration over the ranges 600-3600 ng/spot for acetylcysteine and 100-600 ng/spot for acebrofylline. The linear regression equations were Y=0.151 X + 9.4224 and Y=0.1628 X – 7.0707 for acetylcysteine and acebrofylline respectively. The calibration plots show regression (r^2) being 0.9998 and 0.999 for acetylcysteine and acebrofylline respectively (Table 111,112 Fig 73 and 74).

TableNo 111.0: HPTLC Calibration data for Acetylcysteine

Standard	Amount applied (ng)	Peak Area of Triplicate			Mean peak area
		1	2	3	
1	300	814	746	851	804±53.25
2	600	1637	1621	1584	1614±27.18
3	900	2453	2408	2495	2452±43.50
4	1200	3172	3239	3272	3228±50.95
5	1500	4315	4016	3986	4106±181.90
6	1800	4825	4761	4883	4823±61.024
Equation	y = 1.352x+1722.5				
Slope	1.352				
Intercept	1722.5				
Correlation coefficient (r)	0.9901				
R^2	0.9901				

Table No 112.0: HPTLC Calibration data for Acebrofylline

Standard	Amount applied (ng)	Peak Area of replicate			Mean peak area±SD
		1	2	3	
1	50	600	612	608	607±6.11
2	100	1243	1197	1204	1215±24.78
3	150	1864	1825	1813	1834±26.66
4	200	2389	2468	2457	2438±42.79
5	250	3036	3054	2943	3011±59.57
6	300	3642	3587	3679	3636±46.29
Equation	y = 12.078x + 9.8				
Slope	12.078				
Intercept	9.8				
Correlation coefficient (r)	0.9998				
R^2	0.9995				

The calibration curves for acetylcysteine and acebrofylline are given in **Fig 75** and **Fig 74** respectively.

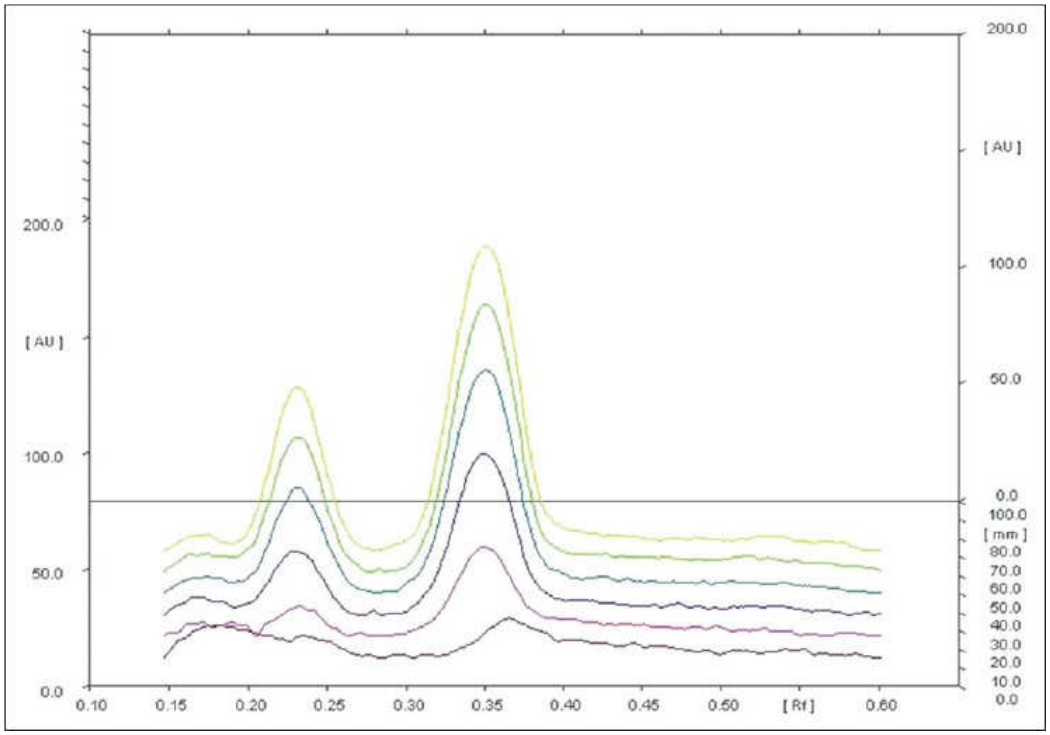


Fig. No 72.0: Representative chromatogram of mixed calibration standards

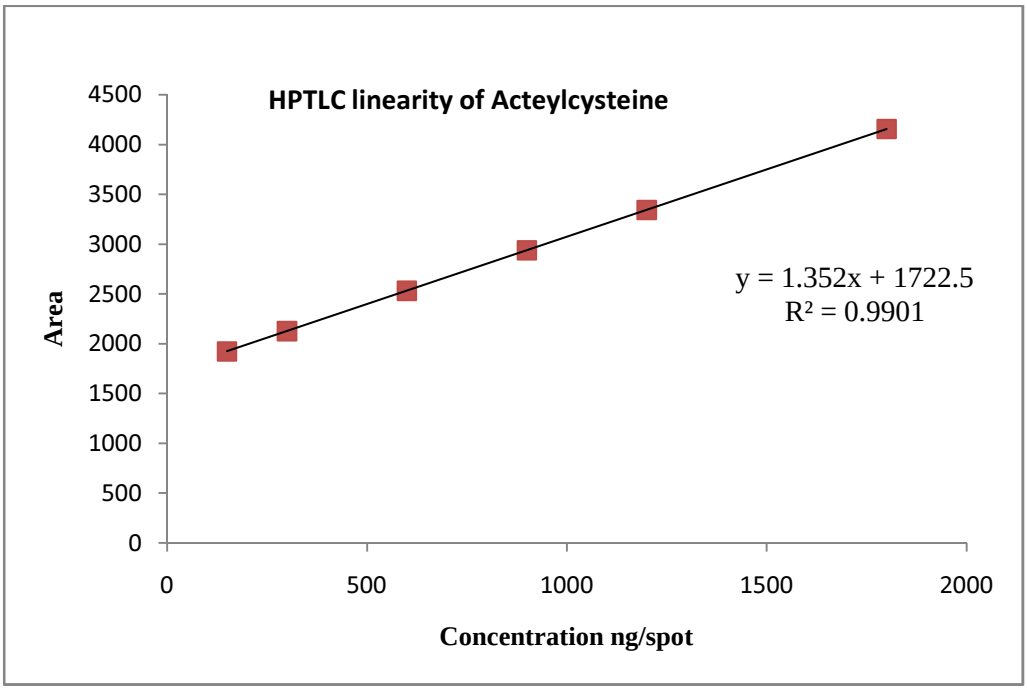


Fig.No 73.0: HPTLC Calibration curve for Acetylcysteine

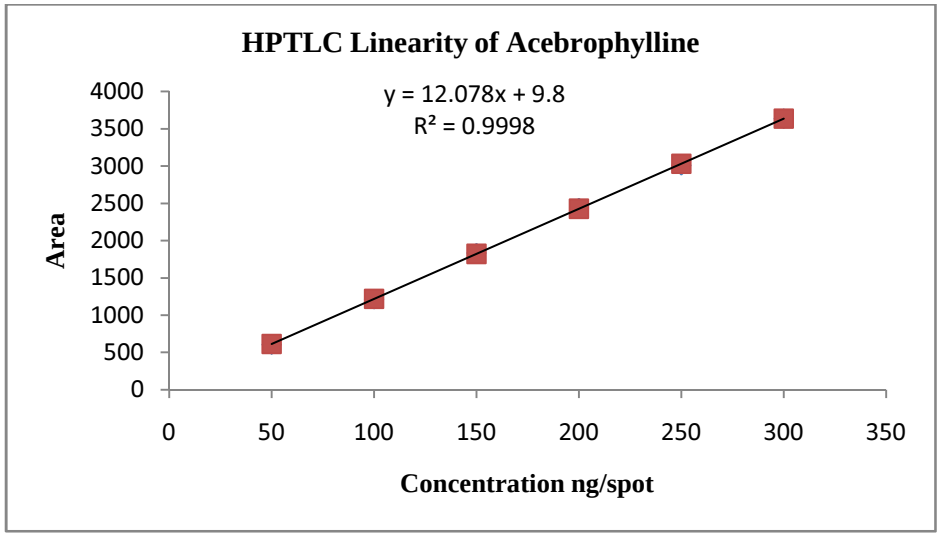


Fig.No 74.0: HPTLC Calibration curve for acebrofylline

6.7.1c: Accuracy

The data indicates that the maximum % RSD chlorthalidone at 80%, 100% and 120% was 0.03, 0.02 and 0.04 respectively indicating that the method has acceptable accuracy. For losartan the maximum % RSD at 80%, 100% and 120% was 0.17, 0.13 and 0.27 respectively indicating that the method has acceptable accuracy.

Accuracy data in terms of area is presented in **Table 113.**

Table No 113.0: Results from recovery studies of Losartan and Chlorthalidone

Drug	Label claim	Amount added (%)	Total amount(mg)	Amount recovered(mg)	Recovery (%)	(%)RSD
Chlor-thalidone	6.25 mg/tab	80	5	5.05	101.00±1.93	1.91
		100	6.25	6.26	100.11±0.24	0.24
		120	7.5	7.51	100.09±0.20	0.20
Losartan	50 mg/tab	80	40	39.82	99.55±1.26	1.26
		100	50	49.99	99.97±1.09	1.09
		120	60	60.01	100.01±0.97	0.97

Table No 114.0: Results from recovery studies of Atovaquone and Proguanil

Drug	Label claim	Amount added (%)	Total amount(mg)	Amount recovered(mg)	Recovery (%)	(%)RSD
Atovaquone	250 mg/tab	80	200	200.25	100.12±0.05	0.05
		100	250	250.45	100.18±0.21	0.21
		120	300	300.30	100.24±0.31	0.31
Proguanil	100 mg/tab	80	80	80.23	100.28±0.23	0.23
		100	100	100.43	100.43±0.17	0.17
		120	120	120.57	100.47±0.41	0.40

Table No 115.0: Results from recovery studies of Acetylcysteine and Acebrofylline

Drug	Label claim	Amount added (%)	Total amount(mg)	Amount recovered(mg)	Recovery (%)	(%)RSD
Acetylcysteine	600 mg/tab	80	480	480.21	100.04±0.03	0.03
		100	600	600.23	100.04±0.02	0.02
		120	720	720.37	100.05±0.04	0.04
Acebrofylline	100 mg/tab	80	80	80.34	100.43±0.17	0.17
		100	100	100.31	100.31±0.13	0.13
		120	120	120.46	100.38±0.27	0.27

Table No 116.0: Results from recovery studies of L Dopa

Drug	Estimated content	Amount added (%)	Total amount(mg)	Amount recovered(mg)	Recovery (%)	(%)RSD
L Dopa	5.47%	80	4.38	4.37	99.77±0.23	0.23
		100	5.47	5.48	100.12±0.10	0.10
		120	6.56	6.57	100.15±0.15	0.15

Table No 117.0: Results from recovery studies of Lawsone and p-phenylenediamine

Drug	Estimated content	Amount added (%)	Total amount(mg)	Amount recovered(mg)	Recovery (%)	(%)RSD
Lawsone	5%	80	4	3.95	98.75±0.90	0.91
		100	5	4.95	98.93±0.61	0.62
		120	6	5.92	98.70±0.26	0.26
p-phenylenediamine	25%	80	20	20.08	100.41±1.20	1.20
		100	25	24.92	99.68±0.16	0.16
		120	30	28.81	99.38±0.54	0.54

6.7.1b: Precision

Results from determination of Intraday and Interday precision, were expressed as SD and relative standard deviation (RSD %) are shown in Table: 118. The precision of the proposed method were < 2%, confirming that the method was sufficiently precise.

Table No 118.0: HPTLC Intra-Day and Inter-Day data of chlorthalidone

Concentration (ng/spot)	Intra-Day area				Inter-Day area			
	Day	Area	Mean (n=3)±SD	%RSD	Day	Area	Mean (n=3)±SD	%RSD
9.375	1	774.32	780.13 ±5.07	0.65	1	774.32	780.13 ±5.07	0.65
	1	782.42			2	782.42		
	1	783.66			3	783.66		
12.5	1	808.56	809.24 ±3.2	0.396	1	808.56	809.24 ±3.2	0.396
	1	812.74			2	812.74		
	1	806.43			3	806.43		
15.62	1	870.21	876.31 ±6.09	0.695	1	870.21	876.31 ±6.09	0.695
	1	882.40			2	882.40		
	1	876.32			3	876.32		

Table No 119.0: HPTLC Intra-Day and Inter-Day data of losartan

Concentration (ng/spot)	Intra-Day area				Inter-Day area			
	Day	Area	Mean (n=3)±SD	%RSD	Day	Area	Mean (n=3)±SD	%RSD
75	1	14987.97	14962.65 ±22.91	0.15	1	14994.38	14955.95 ±38.78	0.259
	1	14956.64			2	14916.82		
	1	14943.34			3	14956.66		
100	1	15588.57	15589.75± 3.37	0.02	1	15524.60	15564.60 ±35.81	0.230
	1	15587.48			2	15574.32		
	1	15593.56			3	15594.12		
125	1	15934.87	15954.15 ±29.19	0.18	1	15924.32	15963.01 ±37.11	0. 232
	1	15939.85			2	15966.40		
	1	15987.75			3	15998.32		

6.7.2: Atovaquone and Proguanil

6.7.2b: Precision

Results from determination of Intraday and Interday precision, were expressed as SD and relative standard deviation (RSD %) are shown in Table: 120 and 121. The precision of the proposed method were < 2%, confirming that the method was sufficiently precise.

Table No 120.0: HPTLC Intra-Day and Inter-Day study of Atovaquone

Concentration (ng/spot)	Intra-Day area				Inter-Day area			
	Day	Area	Mean (n=3)±SD	%RSD	Day	Area	Mean (n=3)±SD	%RSD
1000	1	8264.42	8236.50 ±35.59	0.43	1	8197.25	8206.66 ±28.26	0.34
	1	8248.68			2	8184.28		
	1	8196.42			3	8238.42		
1500	1	12343.41	12369.55 ±22.63	0.18	1	12384.72	12385.49 ±8.92	0.072
	1	12401.86			2	12394.78		
	1	12382.62			3	12376.98		
2000	1	16550.98	16485.87 ±59.52	0.36	1	16470.98	16437.82 ±29.55	0.17
	1	16472.41			2	16428.24		
	1	16434.24			3	16414.24		

Table No 121.0 : HPTLC Intra-Day and Inter-Day study of proguanil

Concentration (ng/spot)	Intra-Day area				Inter-Day area			
	Day	Area	Mean (n=3)±SD	%RSD	Day	Area	Mean (n=3)±SD	%RSD
400	1	4362.84	4323.73 ±35.29	0.81	1	4297.82	4300.17 ±23.02	0.53
	1	4294.24			2	4278.42		
	1	4314.12			3	4324.28		
600	1	6984.24	6943.16 ±48.85	0.70	1	6894.26	6924.58 ±30.19	0.43
	1	6889.14			2	6924.84		
	1	6956.12			3	6954.64		
800	1	10657.96	10640.37 ±16.69	0.15	1	10674.96	10668.42 ±18.16	0. 17
	1	10638.41			2	10682.42		
	1	10624.74			3	10647.89		

6.7.3: Acetylcysteine and Acebrofylline

6.7.3b: Precision

Results from determination of Intraday and Interday precision, were expressed as SD and relative standard deviation (RSD %) are shown in Table: 122 and 123. The precision of the proposed method were < 2%, confirming that the method was sufficiently precise.

Table No 122.0: HPTLC Intra-Day and Inter-Day data of acetylcysteine

Concentration (ng/spot)	Intra-Day area				Inter-Day area			
	Day	Area	Mean (n=3)±SD	%RSD	Day	Area	Mean (n=3)±SD	%RSD
600	1	1627.12	1626.62 ±16.00	0.98	1	1697.42	1718.70 ±26.31	1.53
	1	1610.38			2	1710.56		
	1	1642.38			3	1748.12		
900	1	2564.24	2679.58 ±15.97	0.61	1	2686.73	2664.18 ±19.80	0.74
	1	2596.12			2	2649.18		
	1	2578.38			3	2656.63		
1200	1	3180.17	3161.09 ±17.34	0.54	1	3290.18	3265.51 ±22.31	0.68
	1	3156.84			2	3246.73		
	1	3146.26			3	3259.63		

Table No 123.0: HPTLC Intra-Day and Inter-Day data of acebrofylline

Concentration (ng/spot)	Intra-Day area				Inter-Day area			
	Day	Area	Mean (n=3)±SD	%RSD	Day	Area	Mean (n=3)±SD	%RSD
100	1	1206.44	1188.63 ±16.24	1.36	1	1176.47	1164.39 ±39.61	1.40
	1	1174.62			2	1196.56		
	1	1184.84			3	1120.14		
200	1	1876.20	1839.06 ±32.36	1.75	1	1883.40	1890.81 ±7.70	0.40
	1	1824.16			2	1890.26		
	1	1816.84			3	1898.78		
300	1	2328.12	2366.31 ±34.34	1.45	1	2416.83	2449.21 ±24.02	0.98
	1	2376.18			2	2456.47		
	1	2394.64			3	2474.33		

6.7.1f: Robustness of the method

The results of robustness are shown in Table: 124. Low values of relative standard deviation of peak areas were less than 2%. These percent RSD indicates the robustness of the method.

Table No 124.0: HPTLC Robustness data of Chlorthalidone and Losartan

Experimental conditions	Chlorthalidone		Losartan	
	SD	%RSD	SD	%RSD
Nanometer change				
+2nm (256nm)	18.78	0.74	34.32	0.35
254nm	12.68	1.92	42.14	0.31
-2nm (254nm)	18.28	0.42	24.12	0.44
Development distance				
7.5cm	5.60	0.83	34.34	0.25
8.0 cm	3.99	0.54	43.10	0.31
8.5 cm	4.04	0.47	50.35	0.35
Duration of saturation				
20 min	2.01	0.26	5.08	0.10
25 min	1.67	0.32	10.11	0.07
30 min	1.60	0.22	14.88	0.12

6.7.2f: Robustness of the method

The results of robustness are shown in Table: 125. Low values of relative standard deviation of peak areas were less than 2%. These percent RSD indicates the robustness of the method.

Table No 125.0: HPTLC Robustness data of Atovaquone and Proguanil

Experimental conditions	Atovaquone		Proguanil	
	SD	%RSD	SD	%RSD
Nanometer change				
+2nm (262nm)	22.62	0.16	14.10	0.26
260nm	20.69	1.15	31.80	0.54
-2nm (258nm)	20.86	1.33	33.23	0.50
Development distance				
7 .5cm	25.67	0.45	15.97	1.10
8.0 cm	10.04	0.19	25.44	0.54
8.5 cm	17.21	1.05	45.24	0.75
Duration of saturation				
20 min	19.72	0.49	30.65	0.48
25 min	15.12	1.72	31.65	0.49
30 min	37.21	0.61	16.30	0.28

6.7.3f: Robustness of the method

The results of robustness are shown in Table: 126. Low values of relative standard deviation of peak areas were less than 2%. These percent RSD indicates the robustness of the method.

Table No 126.0: HPTLC Robustness data of Acetylcysteine and Acebrofylline

Experimental conditions	Acetylcysteine		Acebrofylline	
	SD	%RSD	SD	%RSD
Nanometer change				
+2nm (217nm)	20.85	0.71	48.25	0.80
215nm	9.01	1.09	19.54	0.30
-2nm (213nm)	17.84	0.29	26.16	0.45
Development distance				
7 .5cm	23.69	1.17	28.82	0.49
8.0 cm	25.01	1.13	45.09	0.74
8.5 cm	18.41	1.15	46.28	0.64
Duration of saturation				
20 min	20.72	0.49	31.65	0.49
25 min	16.07	1.85	31.59	0.48
30 min	38.79	0.57	17.30	0.26

6.7.1d: Selectivity

When blank tablet powder was subjected to chromatographic analysis, it was observed that there were no interfering peaks at the retention times of chlorthalidone and losartan, atovaquone and Proguanil and Acetylcysteine and Aacebrofylline. This indicated the selectivity of the method.

6.7.2d: Selectivity

When blank tablet powder was subjected to chromatographic analysis, it was observed that there were no interfering peaks at the retention times of Atovaqvone and proguanil. This indicated the selectivity of the method.

6.7.3d: Selectivity

When blank tablet powder was subjected to chromatographic analysis, it was observed that there were no interfering peaks at the retention times of acetylcysteine and acebrofylline. This indicated the selectivity of the method.

6.7.1e: Limit of detection and limit of quantification

By standard deviation and slope method. The detection limit of assay is the lowest concentration that can be detected 19.38 and 100.79 ng/ spot and necessarily quantified was determined as 58.74 and 305.43 ng/spot for chlorthalidone and losartan respectively.

6.7.2e: Limit of detection and limit of quantification

By standard deviation and slope method. The detection limit of assay is the lowest concentration that can be detected 137.11 and 72.20 ng/ spot and necessarily quantified was determined as 417.19 and 218.78 ng/spot for Atovaqvone and proguanil respectively.

6.7.3e: Limit of detection and limit of quantification

By standard deviation and slope method. The detection limit of assay is the lowest concentration that can be detected 137.11 and 72.20 ng/ spot and necessarily quantified was determined as 417.19 and 218.78 ng/spot for acetylcysteine and acebrofylline respectively.

6.7.1g: Specificity

The mobile phase resolved both the drugs very efficiently, as shown in Fig 61.0. Typical overlain absorption spectra of chlorthalidone and losartan are shown in Fig 63.0 . The peak purity of chlorthalidone and losartan were assessed by comparing their respective spectra at the peak start, apex, and peak end positions of the spot. A good correlation was also obtained between the standard and sample spectra of chlorthalidone and losartan Also excipients from formulation were not interfering with the assay.

6.7.2g: Specificity

The mobile phase resolved both the drugs very efficiently as shown in Fig 65.0, Typical overlain absorption spectra of Atovaqvone and proguanil are shown in Fig 64.0. The peak purity of Atovaqvone and proguanil were assessed by comparing their respective spectra at the peak start, apex, and peak end positions of the spot. A good correlation was also obtained between the standard and sample spectra of Atovaqvone and proguanil Also excipients from formulation were not interfering with the assay.

6.7.3g: Specificity

The mobile phase resolved both the drugs very efficiently, Typical overlain absorption spectra of acetylcysteine and acebrofylline are shown in Fig No 66.0. A good correlation was also obtained between the standard and sample spectra of acetylcysteine and acebrofylline Also excipients from formulation were not interfering with the assay.

6.8 : HPTLC result for the Quantification of L-dopa

L-Dopa

Chromatographic conditions

The samples were applied in form of bands of width 6 mm on precoated silica gel aluminum sheets 60 $F_{254.}$ A constant application rate of 150nl/s was selected according to the recommendations in-built in the Wincats software. The application position (X) and (Y) were kept at 10mm to avoid edge effect. Linear ascending development was carried out in a twin trough glass chamber (20cmx10 cm, 10X10 cm), without using filter paper and without saturation. 20 ml mobile phase for 20X10

cm tank and 10 ml for 10X10 cm tank was used per chromatographic development; the length of chromatogram run was 80 mm. The developed plates were dried in the flow of nitrogen. A visualizing agent, Ninhydrine reagent was sprayed on plate and dried at room temperature. Before densitometric scanning, plates were heated for 5 min in oven at 105°C. Densitometric evaluation of the plates was performed at λ = 520 nm using a Camag TLC scanner-3 equipped with wincats software, using a Tungsten light source. The slit dimension was kept at 6 X 0.45 mm and 100 nm/s scanning speed was employed.

Table No 127.0:HPTLC Calibration data for L-dopa

Standard	Amount applied (ng)	Peak Area of replicate			Mean peak area	Standard deviation of peak area
		1	2	3		
1	100	102.46	112.68	106.46	107.20	5.15
2	200	198.27	189.26	188.23	191.92	5.52
3	300	310.06	312.16	310.76	310.99	1.07
4	400	404.89	405.83	403.89	404.87	0.97
5	500	512.63	514.43	516.63	514.56	2.00
Equation	y = 77.02+ 1.071x					
r	0.9910					

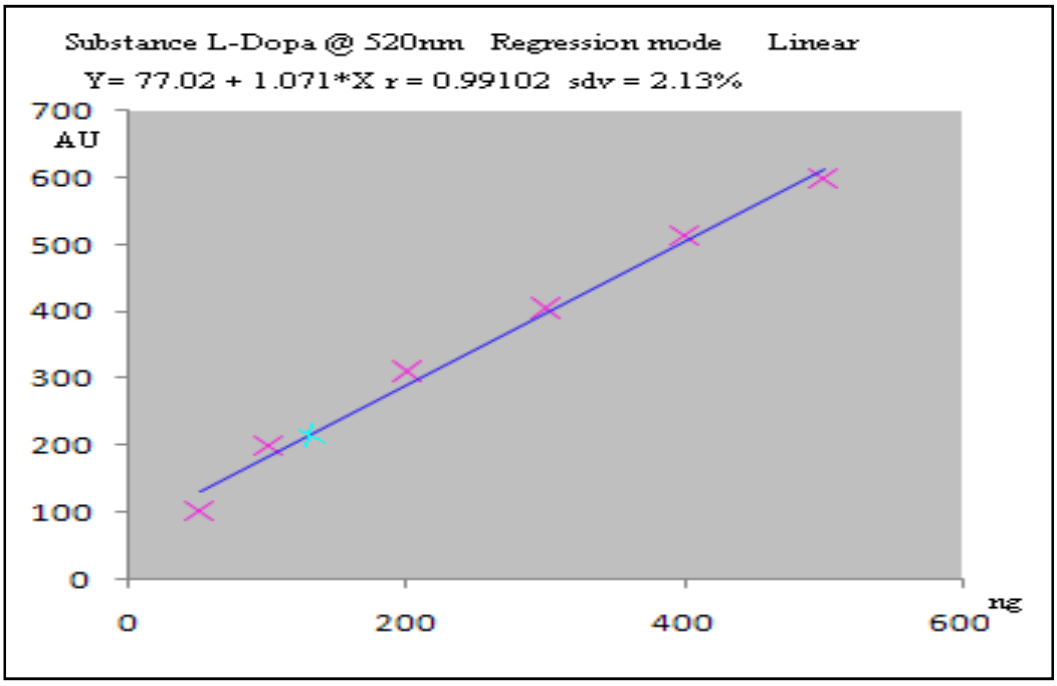


Fig No 75.0: HPTLC Calibration plot for L-Dopa

Standard and sample area after development

Table No 128.0: HPTLC Peak Area of L- Dopa for standard and sample

Name	Peak Area for L-Dopa(AU)
Std 1	102.46
Std 2	198.27
Std 3	310.06
Std 4	404.89
Std 5	512.63
Std 6	597.01
Sample	210.32

Calculation for content of L-Dopa

From the equation for regression line, amount of L-Dopa was found to be 124.46ng. So applying the dilution factor, if 3µl of the sample extract applied contains 124.46ng of L-Dopa, the total volume of extract i.e. 10ml contains 0.41gm of L-Dopa. So the % content of L-Dopa in the given sampleofZandopa powder is 0.41 *100/7.5 = 5.47%

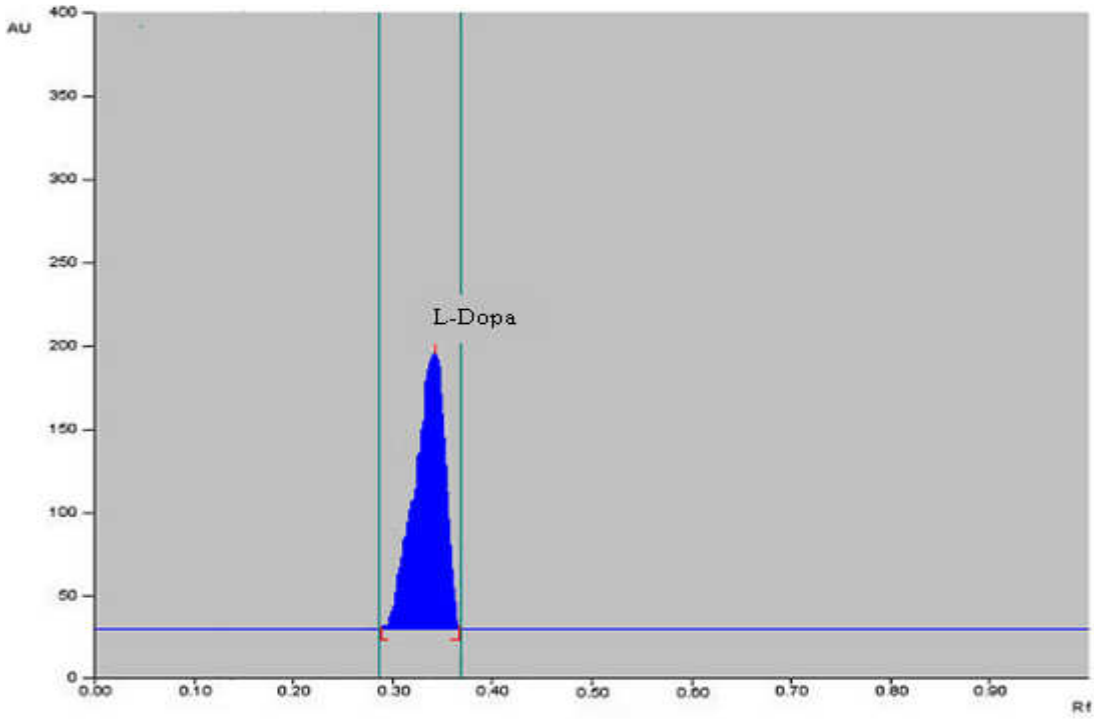


Fig. No 76.0 : Typical HPTLC chromatogram of L dopa in standard

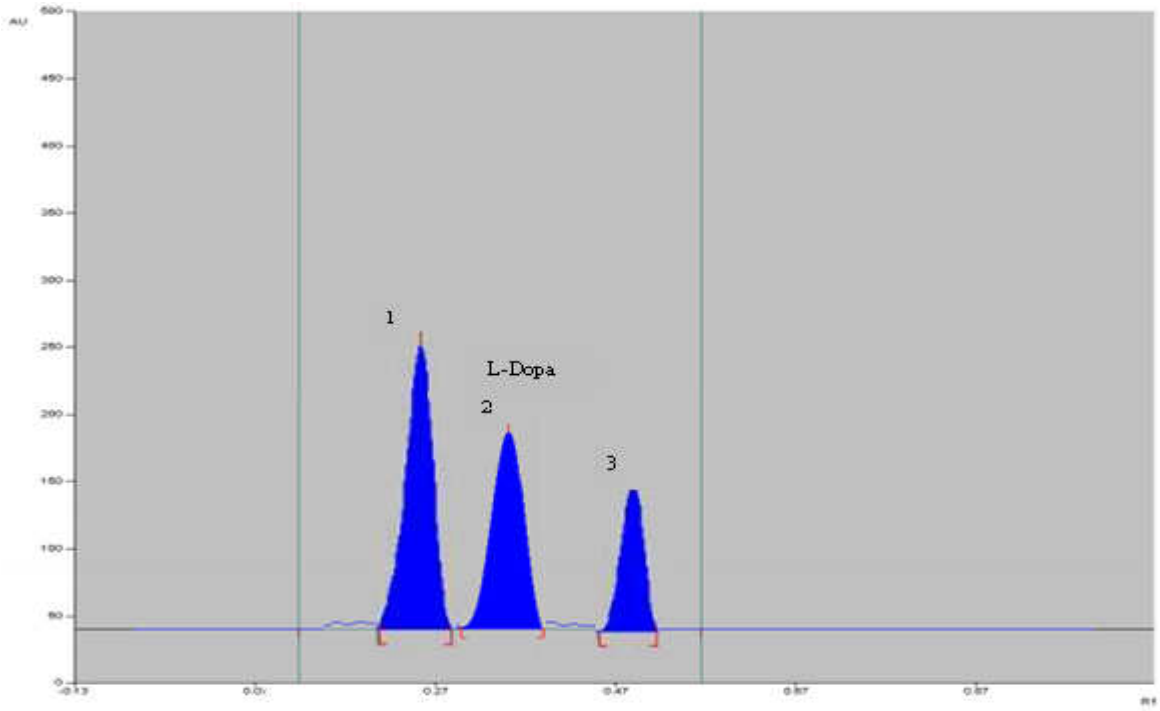


Fig No 77.0 : Typical HPTLC chromatogram of L dopain sample (methanolic extract of Zandopa powder

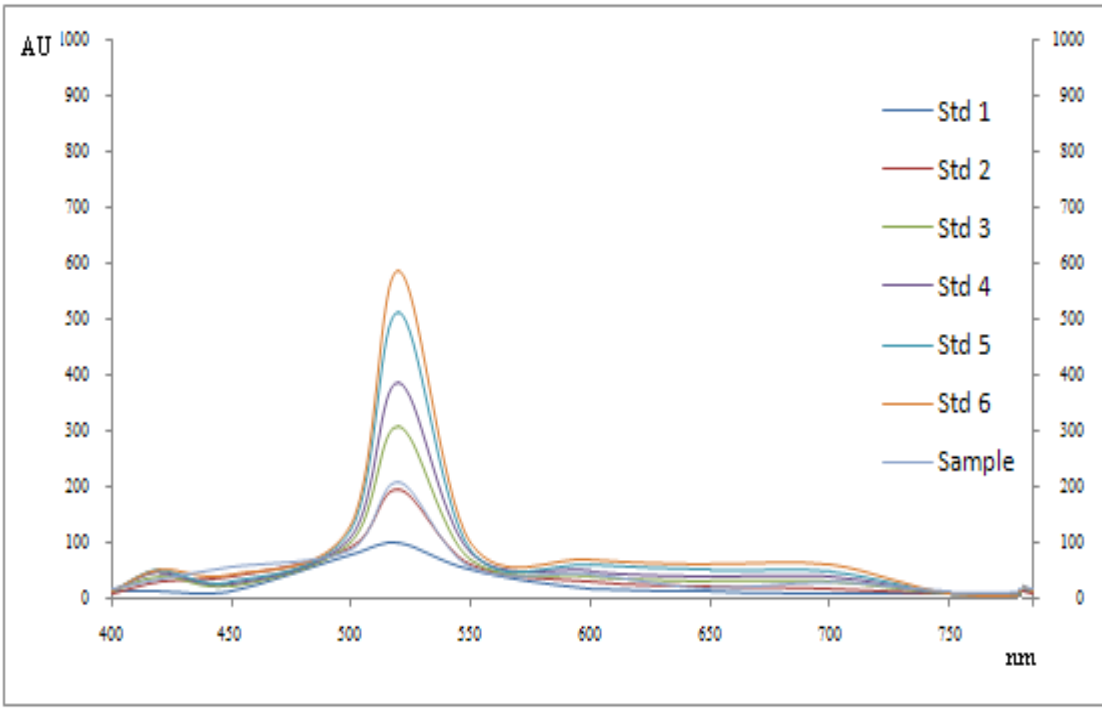


Fig No 78.0 : HPTLC Overlay spectral display for STD and sample

Table No 129.0 : HPTLC Intra-Day and Inter-Day Data of L Dopa

Concentration (ng/spot)	Intra-Day area				Inter-Day area			
	Day	Area	Mean (n=3)±SD	%RSD	Day	Area	Mean (n=3)±SD	%RSD
200	1	310.42	315.88 ±5.06	0.11	1	314.41	317.08 ±3.79	1.19
	1	316.82			2	315.42		
	1	320.42			3	321.42		
300	1	410.52	410.48 ±2.05	0.49	1	412.41	414.14 ±2.19	0.52
	1	412.52			2	413.41		
	1	408.41			3	416.61		
400	1	512.63	517.22 ±6.31	1.21	1	514.21	517.40 ±6.25	1.20
	1	514.62			2	513.40		
	1	524.42			3	524.61		

1.3 METHOD VALIDATION

Precision

Results from determination of Intraday and Interday precision, were expressed as SD and relative standard deviation (RSD %) are shown in Table: 133 and 134 . The precision of the proposed method were < 2%, confirming that the method was sufficiently precise.

Accuracy

The data indicates that the maximum % RSD for L-dopa at 80%, 100% and 120% was0.23,0.10 and0.15 respectively indicating that the method has acceptable accuracy. Accuracy data in terms of area is presented in **Table 117.**

Selectivity

When blank tablet powder was subjected to chromatographic analysis, it was observed that there was no interfering peaks at the retention times of L-dopa. This indicated the selectivity of the method.

Linearity

Linearity was confirmed by the calibration experiments. Linear regression data for the calibration plots revealed good linear relationships between area and concentration over the ranges 50 to 500ng per spotng/spot for L-Dopa . The linear regression equations were y = 77.02+ 1.071x .Table .

Limit of detection and limit of quantification

By standard deviation and slope method. The detection limit of assay is the lowest concentration that can be detected 27.65ng/ spot and necessarily quantified was determined as 83.80 ng/spot for L-dopa respectively.

Specificity

Fig 127.0 and 128.0 is the representative chromatogram showing the resolution L-Dopa. The mobile phase resolved both the drugs very efficiently, Typical overlain absorption spectra of L-Dopa are shown in Fig 127. The peak purity of L-Dopa was assessed by comparing their respective spectra at the peak start, apex, and peak end positions of the spot. A good correlation was also obtained between the standard and sample spectra of L-Dopa. Also excipients and other constituents of formulation were not interfering with the assay.

6.8.2 : HPTLC results for the Lawsone and PPD in Indica herbal hair dye

6.8.2A: Optimization of Solvent System and Chromatographic Conditions

When lawsone and p-phenylenediaminewere applied on plate and the plates were developed, it was concluded that no single solvent was able to resolve the two constituents. Hence, mixtures of solvents were used.

Thus, the solvent system, n-butanol: acetic acid: water (7:0.1:3 v/v/v) was selected.

The samples were applied in form of bands of width 6 mm on precoated silica gel aluminum sheets 60 $F_{254.}$ As the samples were prepared in methanol, spreading of bands was avoided. A constant application rate of 150 nl/s was selected according to the recommendations in-built in the Wincats software. The application position (X) and (Y) were kept at 10mm to avoid edge effect. Linear ascending development was carried out in a twin trough glass chamber (20cmx10 cm, 10X10 cm), 20 ml mobile phase for 20X10 cm tank and 10 ml for 20X10 cm tank was used per chromatographic development; the length of chromatogram run was 80 mm. The developed plates were dried in the flow of dry air with the help of an air drier. Densitometric scanning was performed in the reflectance- absorbance mode at 254 nm. The slit dimension was kept at 6 X 0.45 mm and 100 nm/s scanning speed was

employed. The source of radiation was deuterium lamp emitting continuous UV spectrum between 190 and 400nm.

6.8.2B: Calibration Experiments

When the standards in **table 130** were analyzed in three replicates, the results presented in **table 131**

Table No 130.0: HPTLC Calibration data for lawsone

Standard	Amount applied (ng)	Peak Area of replicate			Mean peak area	Standard deviation of peak area
		1	2	3		
1	10	35.07	34.12	33.48	34.22	0.80
2	20	64.03	61.64	63.85	63.17	1.33
3	30	98.43	99.17	97.25	98.28	0.97
4	40	127.1	128.6	126.39	127.34	1.12
5	50	152.4	151	154.67	152.71	1.84
6	60	187.7	188.2	189.34	188.42	0.84
7	70	221.4	223.4	222.65	222.49	1.00
Equation	y = 3.213x – 3.301					
R^2	0.997					

Table No 131.0 : HPTLC Calibration data for p-phenylenediamine

Standard	Amount applied (ng)	Peak Area of replicate			Mean peak area	Standard deviation of peak area
		1	2	3		
1	100	61.04	62.14	60.64	61.27	0.78
2	200	121.5	124	122.08	122.51	1.30
3	300	169.3	171	168.44	169.6	1.31
4	400	208.8	210	212.31	210.37	1.79
5	500	247.4	248.7	247.06	247.69	0.85
6	600	288.1	288.2	289.34	288.57	0.67
7	700	321.4	323.4	322.65	322.49	1.01
Equation	y = 0.426x + 32.66					
R^2	0.9991					

Figure 79.0 and **Figure 80.0** are the calibration curves for lawsone and p-phenylenediaminerespectively.

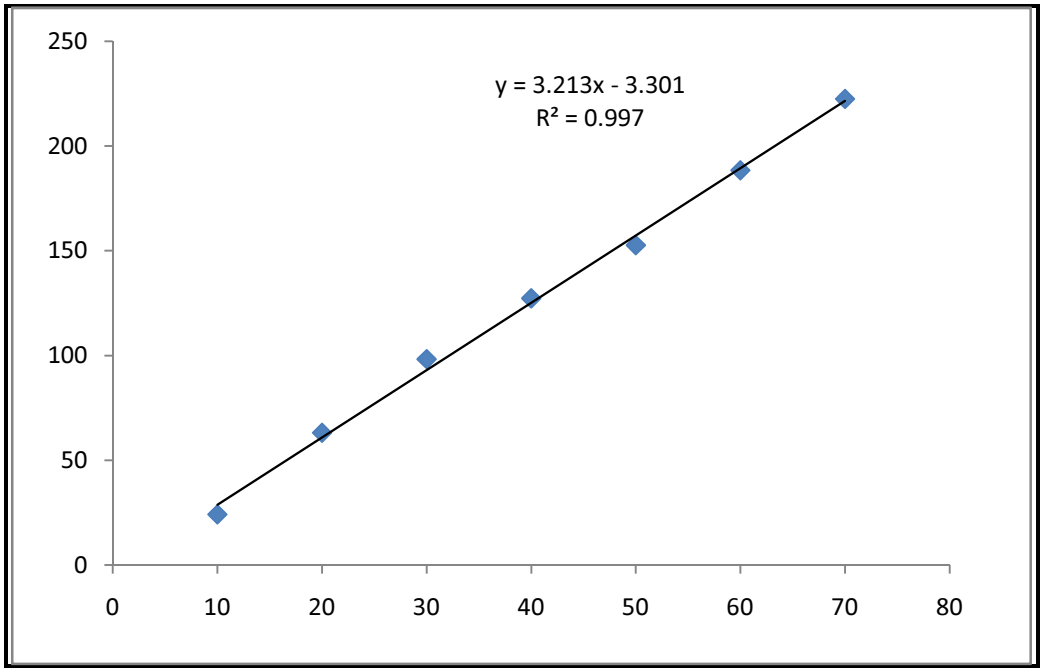


Fig.No 79.0: HPTLC Calibration curve for lawsone

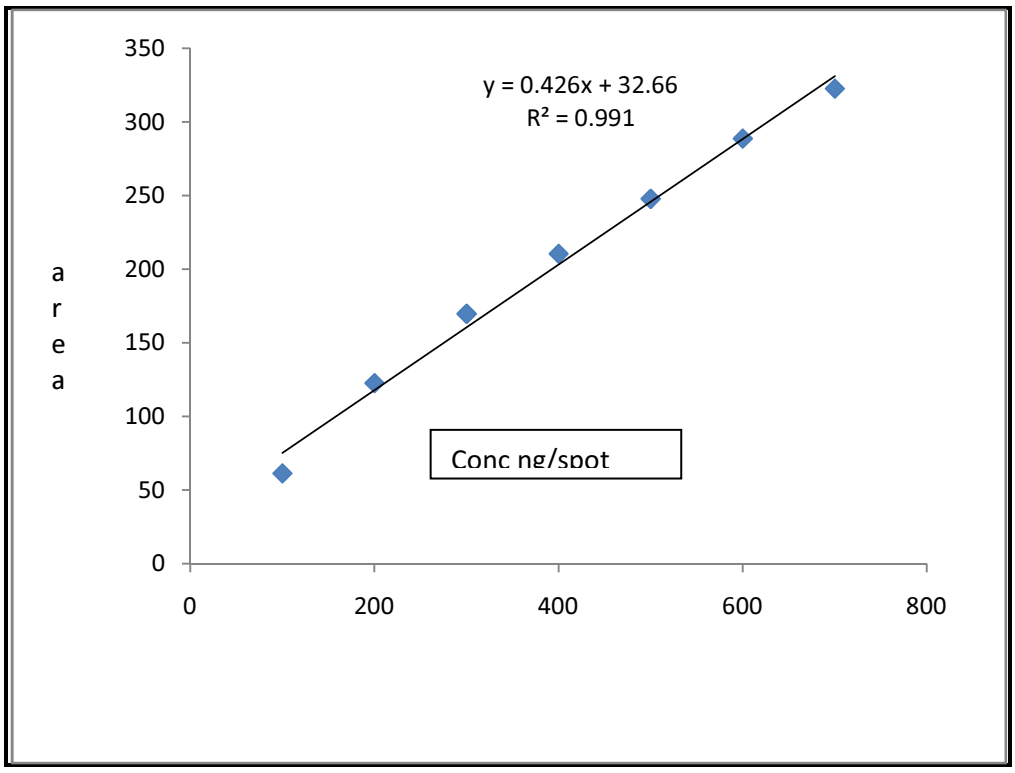


Fig.No 80.0: HPTLC Calibration curve for p-phenylenediamine

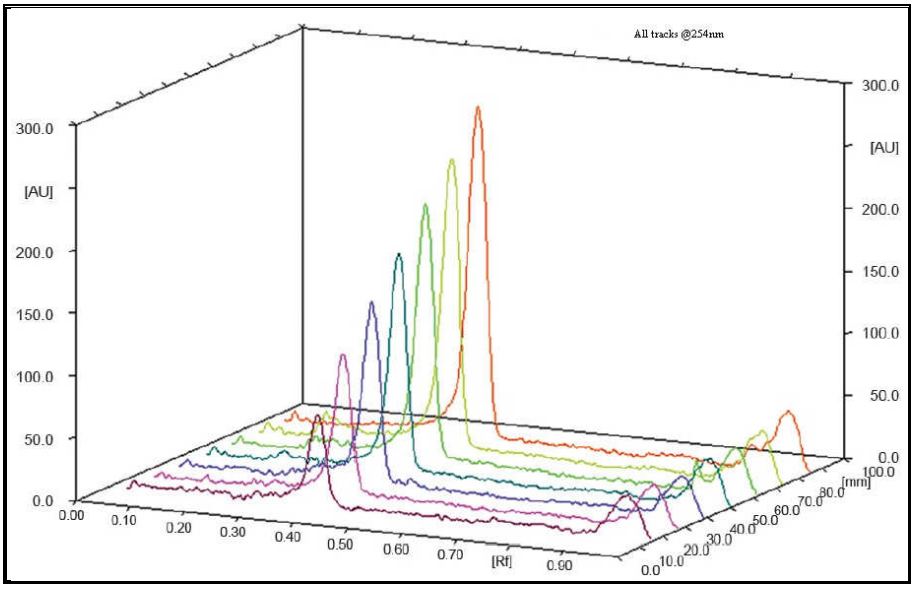


Fig.No 81.0: HPTLC Chromatogram for calibration plot of lawsone and PPD

Table No 132.0: HPTLC Assay of lawsone and p-phenylenediamine in herbal formulation

S.No.	Area	
	Lawsone	p-phenylenediamine
Standard 1	34.22	61.27
Standard 2	63.17	122.51
Standard 3	98.28	169.6
Standard 4	127.34	210.37
Standard 5	152.71	247.69
Standard 6	188.42	288.57
Standard 7	222.49	322.49
Sample 1 (2µl)	35.12	251.06
Sample 1 (2µl)	34.34	249.74

Fig.No 81.0 is the chromatogram for sample of herbal formulation showing resolution of lawsone and p-phenylenediamine and in mixed standard solution.

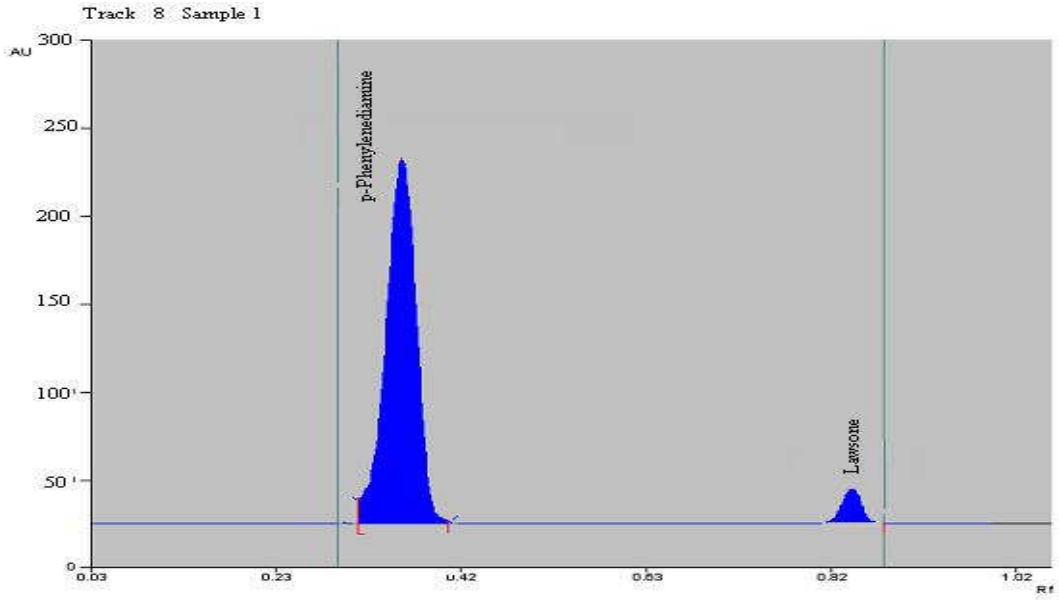


Fig.No 82.0: Representative chromatogram of lawsone (R_f 0.82) and p-phenylenediamine(R_f 0.34)

The average % content of lawsone and p-phenylenediamine in 1000mg of herbal forumulation was found to be 0.592% and 25.55 respectively%

6.8.2C: Precision

Results from determination of Intraday and Interday precision, were expressed as SD and relative standard deviation (RSD %) are shown in Table: 4 and 5. The precision of the proposed method were < 2%, confirming that the method was sufficiently precise.

Table No 133.0: HPTLC Intra-Day and Inter-Day data of lawsone

Concentration (ng/spot)	Intra-Day area				Inter-Day area			
	Day	Area	Mean (n=3)±SD	%RSD	Day	Area	Mean (n=3)±SD	%RSD
20	1	64.03	64.85 ±3.24	0.57	1	69.42	68.08 ±1.16	1.16
	1	68.43			2	67.34		
	1	62.11			3	67.48		
30	1	97.24	97.24 ±1.13	1.16	1	98.73	98.51 ±0.25	0.45
	1	96.12			2	98.18		
	1	98.38			3	98.63		
40	1	126.42	125.90 ±1.20	0.95	1	125.68	126.34 ±0.57	0.57
	1	126.77			2	126.73		
	1	124.53			3	126.63		

Table No 134.0: HPTLC Intra-Day and Inter-Day data of p-phenylenediamine (PPD)

Concentration (ng/spot)	Intra-Day area				Inter-Day area			
	Day	Area	Mean (n=3)±SD	%RSD	Day	Area	Mean (n=3)±SD	%RSD
200	1	121.4	120.53 ±0.80	0.67	1	125.7	126.00 ±0.70	0.55
	1	120.4			2	126.8		
	1	119.8			3	125.5		
300	1	169.3	170.03 ±o.64	0.38	1	169.6	169.4 ±1.11	0.65
	1	170.3			2	168.2		
	1	170.5			3	170.4		
400	1	208.8	208.73 ±0.64	0.37	1	205.9	205.03 ±0.75	0.36
	1	207.8			2	204.7		
	1	207.6			3	204.5		

Selectivity

When the herbal formulation was subjected to chromatographic analysis, it was observed that there were no interfering peaks at the retention times of lawsone and p-phenylenediamine. This indicated the selectivity of the method.

Linearity and range

Linearity was confirmed in the calibration experiments from the randomness of residuals plot and homogenous % relative error values over the working range.

7. SUMMARY AND CONCLUSION

The present work involved the development of accurate, precise, simple and rapid Spectroscopic (UV) and Chromatographic (HPLC, HPTLC) methods for simultaneous estimation of the drugs in synthetic and herbal multicomponent formulations containing

- Losartan potassium and Chlorthalidone
- Acebrofylline and Acetylcysteine
- Atovaquone and Proguanil
- Lawsone and p-phenylenediamine
- L- dopa in Zandopa herbal powder

This thesis invoves different chapter providing valuable information about the related topic studied detailed in this thesis. Chapter first deals with the brief introduction about modern analytical techniques like hplc, hptlc, and uv-spectroscopic method and gives brief information about the methods and techniques for the estimation of the multicomponent drug analysis. In this chapter also involves detailed about analytical method developments, analytical method validation, herbal analysis and forced degradation study.

The Introduction of present work covers optimum information about the project name entitled here. The objectives of the study were to develop and validate methods for the estimation of drugs in multicomponents drug formulations by using HPLC, HPTLC, and UV Spectrophotometry was explained.

In next chapter of this thesis deals with review of literature for the analytical methods available for the estimation of the selected drug formulation either present in single or in the combination with the other drug products.

In fourth chapter discuss about the scope and planning for achieving the objectives of the study explain here.

The material and methods chapter described with step by step procedure performed for the optimizing condition for the development of method on HPLC, HPTLC and UV Spectrophotometry methods and validation of the developed method were performed in accordance with ICH guideline and the methods also described the stability indicating assay method carried out by subjecting sample to the stress condition like acid, alkali, peroxide, neutral, thermal and UV light. The optimized chromatographic and spectrophotometric conditions are presented along with data table, graphs,and typical chromatogram were disscuss.

In the next chapter of this thesis deals with result and discussion are presented in series of tables and figures which includes all the trials chromatograms, the calibration curve, validation data , degradation study data, and herbal analysis of drugs by using modern analytical instroments like HPLC, HPTLC and UV Spectroscopic methods.

The over all of the developed methods were summarized below.

In the HPLC method developed for losartan potassium and chlorthalidone in tablet (CTD-L) formulations, isocratic mobile phase was found to be unsuitable. Thus gradient conditions were chosen which brought about the elution of losartan potassium and chlorthalidone.

The optimized chromatographic conditions were-

Injection volume	:	10µl
Analytical column	:	Agilent XDB, C_{18}, 150 × 4.6 mm, 5µ
Detection	:	254
Mobile phase	:	Buffer: acetonitrile in (70:30), pH3.5

The adopted Isocratic system and chromatographic conditions resolved the losartan potassium and chlorthalidone with retention times of 4.63 and 2.17 min respectively.

The method was linear in the range of 12.5 – 75 µg/ml for losartan potassium and 1.56 – 9.37 µg/ml for chlorthalidone.

The method was validated for system suitability, accuracy, precision, linearity, range, limit of detection (LOD), limit of quantification (LOQ), specificity and robustness The accuracy and precision studies gave very low values of % relative error and % relative standard deviation.

Likewise, the HPLC method developed for atovaquone and proguanil in tablet (MALARONE) formulations, isocratic mobile phase was found to be unsuitable. Thus gradient conditions were chosen which brought about the elution of atovaquone and proguanil. The following gradient system was optimized-

Mention the mobile phase for gradient elution

The chromatographic conditions were-

Injection volume : 10µl
Analytical column : Hypersil BDS, C_{18}, 150 × 4.6 mm, 5µ
Detection : 258 nm
Mobile phase : Buffer: acetonitrile in Gradient programme

Time	**Flow Rate**	**% Buffer**	**% Acetonitrile**
0	1.1	50	50
3	1.1	50	50
4	1.1	20	80
9	1.1	20	80
9.1	1.1	50	50
12.0	1.1	50	50

The adopted gradient system and chromatographic conditions resolved the atovaquone and proguanil with retention times of 2.16 and 9.68 min respectively.

The method was linear in the range of 40–300 µg/ml for Proguanil and 100 – 750 µg/ml for Atovaquone.

The method was validated for system suitability, accuracy, precision, linearity, range, limit of detection (LOD), limit of quantification (LOQ), specificity and robustness The accuracy and precision studies gave very low values of % relative error and % relative standard deviation.

The HPLC method developed for acebrofylline and acetylcysteine used the following chromatographic conditions with isocratic elution:

Injection volume	:	5µl
Analytical column	:	Hypersil BDS, C_{18}, 150 × 4.6 mm, 5µ
Mobile phase	:	Buffer: Acetonitrile in (90:10), pH3.2

The above conditions gave excellent resolution for acebrofylline and acetylcysteine with acebrofylline being eluted at 5.5 min and acetylcysteine at 2.36 min.

The HPLC method was linear from 25 – 150 µg/ml for acebrofylline and 150 – 900 µg/ml for acetylcysteine.

The method was validated for system suitability, accuracy, precision, linearity, range, limit of detection(LOD), limit of quantification (LOQ), specificity and robustness with good results.

Stability indicating assay is a method that is employed for the analysis of stability samples in pharmaceutical industry. When developing SIMs, forced degradation studies are undertaken particularly when little information is available about potential degradation products. These studies also provide information about the degradation pathway and degradation products that could form during storage. Forced degradation studies help facilitate pharmaceutical development as well as in cases such as formulation development, manufacturing and packaging in which knowledge of chemical behavior can be used to improve a drug product.

With this view, forced degradation studies were carried out for the multicomponent combinations of Losartan-Chlorthalidone, Atovaquone-Proguanil and

Acebrophylline-Acetylcysteine. The degradation studies were carried out under acidic, alkaline, oxidative, and dry heat (thermal) and photolytic conditions. The degraded samples and standard drug were subjected to chromatographic separation and the conditions required for resolving the drug peak from any potential degradation products were optimized.

During forced degradation studies, Losartan (11.16%)and Atovaquone (11.3%) was found to degrade under peroxide degradation conditions and other drug shows % degradation below 10% which is acceptable range.

It can be concluded that the stability indicating HPLC method developed for the multicomponent combinations of Losartan-Chlorthalidone, Atovaquone-Proguanil and Acebrophylline-Acetylcysteine is capable of discriminating between the drug and the degradation products.

Simultaneous equation method for estimation of drugs in combination is one of the effective methods of multicomponent.

Hence UV spectrophotometric methods were developed and validated for simultaneous estimation of Losartan-Chlorthalidone, Atovaquone-Proguanil and Acebrophylline-Acetylcysteine from multicomponent formulations.

The method developed for simultaneous estimation of losartan potassium and chlorthalidone showed absorbance maxima at 235nm and 215nm for losartan potassium and chlorthalidone respectively. The developed method was validated as per ICH Guidelines. The method was found to be accurate, precise, specific, robust and linear in the range of 6-16 for losartan and 0.75 - 2 μg /ml for chlorthalidone.

The method developed for simultaneous estimation of Atovaquone and Proguanil showed absorbance maxima at 279nm and 254nm for Atovaquone and Proguanil respectively. The developed method was validated as per ICH Guidelines. The method was found to be accurate, precise, specific, robust and linear in the range of 7.5- 20 for Atovaquone and 3 - 8 μg /ml for Proguanil.

The method developed for simultaneous estimation of Acetylcysteine and Acebrofylline showed absorbance maxima at 220nm and 273nm for Acetylcysteine and Acebrofylline respectively. The developed method was validated as per ICH Guidelines. The method was found to be accurate, precise, specific, robust and linear in the range of 6 - 36 for Acetylcysteine and 1 - 6 μg /ml for Acebrofylline.

The HPTLC method for estimation of Acetylcysteine and acebrofylline made use of unmodified silica plates and solvent system of chloroform : tolune: methanol (6:2:2 V/V/V). The HPTLC method gave linearity for 300 to 1800 ng/spot of the applied quantity for Acetylcysteine and 50 to 300 ng/spot for Acebrofylline.

This method gave good results in the validations studies.

The HPTLC method for estimation of Atovaquone and Proguanil made use of unmodified silica plates and solvent system of THF : water (6 : 4 V/V). The HPTLC method gave linearity for 500 to 3000 ng/spot of the applied quantity for Atovaquone and 200 to 1200 ng/spot for Proguanil.

This method also gave good results in the validations studies.

The HPTLC method for estimation of Losartan and Chlorthalidone made use of unmodified silica plates and solvent system of Tolune :chloroform: methanol: ammonia (2.3: 5.0: 2.0: 0.2 V/V/V/V). The HPTLC method gave linearity for 200 to 1500 ng/spot of the applied quantity for Losartan and 31.25 to 187.5 ng/spot for Chlorthalidone.

This method also gave good results in the validations studies.

The HPTLC method for estimation of Lawsone and Paraphenylenediamine made use of unmodified silica plates and solvent system of n-butanol: acetic acid: water (7: 0.1: 3 V/V/V). The HPTLC method gave linearity for 10 to 70 ng/spot of the applied quantity for Lawsone and 100 to 700 ng/spot for Paraphenylenediamine.

The HPTLC method for estimation of L-dopa made use of unmodified silica plates and solvent system of n-butanol n-butanol:water:acetic acid(4:1:1v/v/v). The

HPTLC method gave linearity for 100-500ng/spot for L-dopa. This method also gave good results in validation.

All analytical methods summarized above were successfully developed and validated HPLC, HPTLC, and UV Spectrophotometric methods are better, improved and innovative, less expensive and time saving as well as sensitive, specific, linear, precise, and accurate, for the quantification of both the herbal and synthetic multicomponent formulations can be used in the routine quality control of the above mentioned multicomponent formulations.

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RESEARCH ARTICLE

VALIDATED RP-HPLC METHOD DEVELOPMENT FOR THE SIMULTANEOUS ESTIMATION OF ACETYLCYSTEINE AND ACEBROFYLLINE IN CAPSULE FORMULATION

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ABSTRACT

A new simple, precise, rapid and accurate reverse phase high performance liquid chromatographic method had been developed for the simultaneous estimation of Acetylcysteine (ACST) and Acebrofylline (ACBF) in capsule dosage form. The chromatographic separation was achieved on a Hypersil BDS, C18, 100 x 4.6 mm, 5µm particle size column was used with PDA detector by using mobile phase containing mixture of 0.02M Potassium dihydrogen orthophosphate (KH_2PO_4) buffer : acetonitrile (90:10 % v/v pH 3.2) was used. . The flow rate was 0.9 ml / min and effluents were monitored at 260 nm. Chromatogram showed two main peaks corresponding to Acetylcysteine and Acebrofylline at retention times 2.365 and 5.505 min respectively. The method was liniear over the concentration range of 150-900µg/ml for Acetylcysteine and 25-150 µg/ml for Acebrofylline respectively. The developed method was validated in accordance to ICH guidelines.

Key words: Acetylcysteine, Acebrofylline, RP-HPLC, Validation, ICH, Acetonitrile

INTRODUCTION:

The present research work deals with the development and validation of a simple, specific, accurate, and precise reverse phase high performance liquid chromatographic (RP-HPLC) method for the estimation of Acetylcysteine and Acebrofylline in capsule formulations. Chemically Acetylcysteine[1] is the N-acetyl derivative of the amino acid L-cysteine and a precursor in the formation of antioxidant glutathione in the body. The thiol (sulfahydryl) group confers antioxidants effects and is able to reduce free radicals. Acetylcysteine[1,2] IUPAC name is a (2R)-2-acetamido-3-sulfanylpropanic acid [Figure - 1], represents mucolytic drug which decreases the viscosity of secretions by splitting of disulphide bonds in mucoproteins and it also promotes the detoxification of an intermediate paracetamol metabolite which is used in the management of paracetamol overdose.

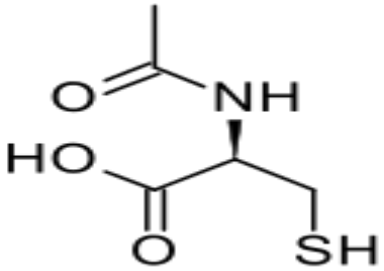

Figure 1: Structure of Acebrofylline

Acebrofylline[3] IUPAC name is 4-[(2-amino-3,5-dibromophenyl) methylamino] cyclohexan-1-ol; 2-(1,3-dimethyl-2,6-dioxopurin-7-yl)acetic acid. Acebrofylline is the salt obtained by reaction of equimolar amounts of theophylline-7-acetic acid, a xanthine derivative with specific bronchodilator activity and ambroxol, a mucolytic and expectorant with molecular formula $C_{22}H_{28}Br_2N_6O_5$ and molecular weight 616.302 g/mol as shown in Figure 2.0. It is a novel drug with bronchodilating, anti-inflammatory and mucus regulating effect due to inhibition of phospholipase A, and phosphatidylcholine. Literature survey[4-13] reveals that some methods have been reported for the estimation of single and very few methods for the combinations, but still there is no RP-HPLC method developed for the simultaneous determination of of Acebrofylline and Acetylcysteine in capsule formulations. So the present method developed is relatively simple, rapid and highly sensitive and validated as per ICH guidelines[14] in the analysis of multicomponent of interest and it can be used for routine quality control analysis in laboratories.

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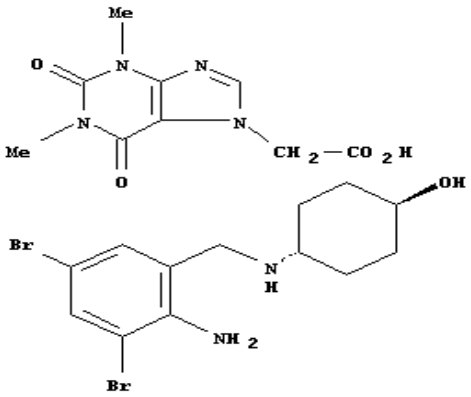

Figure 2: Structure of Acetylcysteine

Chemicals and reagents:

Acebrofylline and Acetylcysteine pure samples were obtained from SL Drugs & Pharmaceuticals, Hyderbad, India and all other chemicals were of analytical grade. The commercial capsule Acebrofylline and Acetylcysteine formulations of combined of brand Caps. Pulmoclear Manufactured by Fourrts (India) Laboratories Pvt. Ltd were obtained from local retail pharmacy.

Chromatographic conditions:

The HPLC water system was equipped with empower software for data processing. The optimize chromatographic conditioned were shown in Table No. 1.0

MATERIALS AND METHODS:

Table No. 1.0: The optimize chromatographic conditioned

Flow rate	0.9 ml/min
Column	Hypersil BDS, C18, 100 x 4.6 mm, 5µ.
Detector wave length	260 nm
Column temperature	30°C
Injection volume	5µL
Run time	8 min
Diluent	Methanol
Mobile phase	Buffer : Acetonitrile (90:10 % v/v pH 3.2)

Preparation of diluent:

The diluent was HPLC grade Methanol alone.

Preparation of buffer:

Accurately weighed 2.72gm of potassium dihydrogenorthophosphate was transferred in a 1000ml ofvolumetric flask and about 900ml of milli-Q water was added.1ml of triethylamine was added and sonicated and finally made up the volume with water. Then pH was adjusted to 3.2 with dilute ortho phosphoric acid solution.

Preparation of standard stock solution:

Accurately weighed 10mg of Acebrophylline and 12.5mg of Acetylcysteine working Standards were transferred into separate 10 ml clean and dry volumetric flasks, 7ml of diluents was added and sonicated for 30 minutes and made up to the final volume with diluents.

Preparation of sample solution:

Twenty Tablets were weighed and the average weight of each tablet was calculated. Then the weight equivalent to twenty tablets was transferred into a 100 ml volumetric flask, 50mL of diluent was added and sonicated for 30 min, further the volume made up with diluent and filtered. From the filtered solution 0.2ml was pipetted out into a 10 ml volumetric flask and made up to 10ml with diluent.

Method validation[15]:

The developed method was validated as per the ICH guidelines with respect to system suitability, specificity, linearity, accuracy, precision, LOD and LOQ.

System suitability:

To ensure the resolution and reproducibility of the HPLC system was adequate for the analysis, a system suitability test was established. Data from six injections of 10 µL of the working standard solutions were used for the evaluation of the system suitability parameters like tailing factor, the number of theoretical plates and retention time. The system suitability results obtained for Acetylcysteine and Acebrofylline is summarized in Table No. 2.0 and Table No.3.0 respectively

Table 2, 3: The results obtained for system suitability of Acetylcysteine and Acebrofylline is summarized in Table No. 2.0 and 3.0 respectively.

Sr. No.	Retention Time	Peak Area	Theoretical plates	Tailing factor
1	2.36	686974	4492	1.09
2	2.362	685310	4513	1.1
3	2.363	686086	4502	1.1
4	2.367	683964	4290	1.1
5	2.37	686033	4376	1.09
6	2.371	688352	4430	1.09
	Mean	686120		
	Std. Dev.	1485		
	%RSD	0.2		

Table No 2.0:

Sr. No	Retention Time	Peak Area	Theoretical plates	Tailing factor
1	5.484	340354	5842	0.99
2	5.485	340140	5830	1.0
3	5.504	342289	5843	1.0
4	5.507	338474	5877	1.0
5	5.518	344216	5970	0.99
6	5.533	341513	5715	1.0
	Mean	341164		
	Std. Dev.	1982.1		
	%RSD	0.6		

Table No 3.0:

LINIARITY:

The linearity of the method was evaluated by analyzing different concentration of the drugs. According to ICH recommendations, at least six concentrations must be used. In the present study six concentrations were chosen & injected. The peak areas of the chromatograms were plotted against the concentration of drug to obtain the calibration curve and the corresponding calibration curve data and graph for ACST and ACBF shown in Table No.4.0 and Graph in Figure – 3 and Figure – 4 respectively.

Table 4: The corresponding Linearity (calibration curve) data

Sr. No	Concentration in ppm (ACST)	Peak area (ACST)	Concentration in ppm (ACBF)	Peak area (ACBF)
1	150	174619	25	88736
2	300	355507	50	175892
3	450	514460	75	257873
4	600	690463	100	345418
5	750	855800	125	431317
6	900	1039137	150	515247
SLOPE		1145	**SLOPE**	3428.7
INTERCEPT		2102.667	**INTERCEPT**	2022.333
CORRELATION COEFFICIENT		0.999	**CORRELATION COEFFICIENT**	0.999

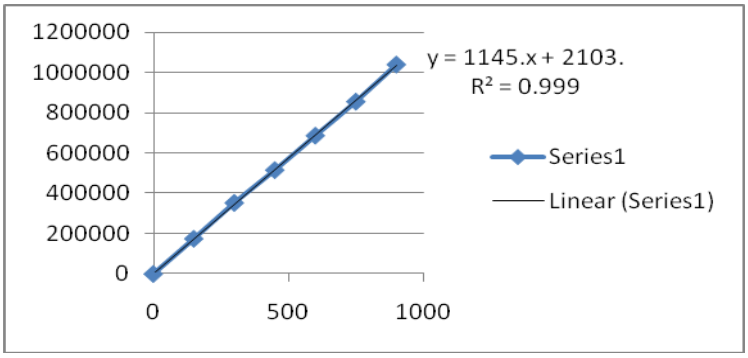

Figure 3: Calibration curve for Acetylcysteine

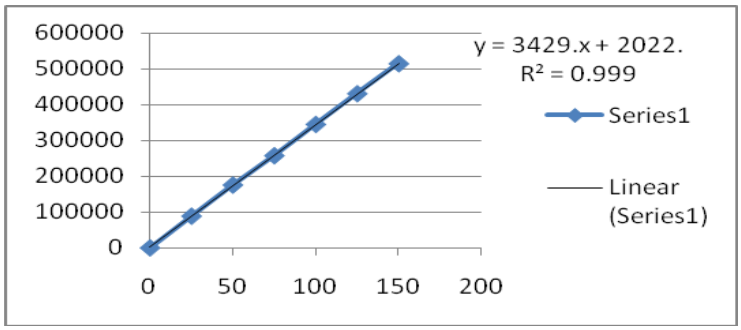

Figure 4: Calibration curve for Acebrofylline

ACCURACY:

The accuracy of the method was determined by recovery experiments. The solutions were injected in triplicate in 50%, 100% and 150% concentrations and percentage Recovery was calculated separately for ACBF and ACST and summarized in Table No. 5.0 and Table No. 6.0 Respectively.

Table 5, 6: The accuracy data (recovery study) for ACBF and ACST were summarized here

LEVEL IN %	Amount added	Amount recovered	%Recovery	% Mean	% RSD
50%	50	49.93	99.85302	100.864	0.78
50%	50	50.09	100.1831		
50%	50	50.17	100.3348		
100%	100	100.67	100.6713		
100%	100	102.15	102.1525		
100%	100	101.73	101.7314		
150%	150	152.02	101.3483		
150%	150	150.40	100.2697		
150%	150	151.9	101.2353		

Table 5.0:

LEVEL IN %	Amount added	Amount recovered	%Recovery	% Mean	% RSD
50%	300	303.0838	101.0279	100.317	0.72
50%	300	302.724	100.908		
50%	300	299.1572	99.71907		
100%	600	595.2	99.2		
100%	600	600.9755	100.1626		
100%	600	597.3328	99.55546		
150%	900	909.5546	101.0616		
150%	900	901.2856	100.1428		
150%	900	909.683	101.0759		

Table 6.0:

Precision:

Precision of the method was determined by studying intra-day and inter-day variation. In the intra-day studies, standard and sample solutions were analyzed on the same day and percentage RSD was calculated. In the inter day studies, standard and sample solutions were analyzed on consecutive days and percentage RSD were calculated and individual data for ACST and ACBF summarized in Table No 7.0

Table 7: The Precise individual data for ACST and ACBF summarized in

Assay No.	Peak Area ACBF	% Assay ACBF	Peak Area ACST	% Assay ACST
01	344629	100.6116	687668	99.82
02	341064	99.57081	686777	99.70
03	344819	100.667	690885	100.29
04	342999	100.1357	684256	99.33
05	346857	101.262	686946	99.72
06	344019	100.4335	680410	98.77
Mean		100.4468		99.61
% RSD		0.56		0.51

Specificity:

The Specificity of the method was evaluated by assessing whether excipients present in the pharmaceutical formulations interfered with the analysis. Excipients for each capsule were mixed in order to prepare a placebo, and solutions were prepared by following the procedure described in the section on sample preparation. The capsule excipients did not interfere with the method.

Limits of detection(LOD) and Limit of quantitation(LOQ):

In accordance with ICH recommendations, the method based on the standard deviation of the response and the slope of the calibration plots was used to determine detection and quantification limits. LOD and LOQ values were estimated [(standard deviation of repeatability)/ (Slope of the regression equation)] by multiplying with 3.3 and 10 respectively. And corresponding results given in Table No. 8.0

Table 8: The results and summary for the developed and validated method of Acetylcysteine(ACST) and Acebrofylline (ACBF) was given below

Sr. No.	Parameter	Acetylcysteine	Acebrophylline
1.	Peak area (%RSD)	686120(0.2)	341164(0.6)
2.	Retention Time	2.365	5.505
3.	USP Theoretical Plate	4434	5846
4.	USP Tailing	1.09	0.99
5.	Specificity	No peak	No peak
6.	Linearity (µg/ml)	150-900	25-150
7.	Slope	1145	3429
8.	Y-Intercept	2103	2022
9.	Correlation coefficient	0.999	0.999
10.	Accuracy	0.72	0.78
11.	Precision	0.51	0.6
12.	LOD	1.5042	0.1874
13.	LOQ	4.558	0.568
14.	Ruggedness	0.48	0.76
15.	Flow rate(+0.1)	695877(0.3)	345226(0.4)
16.	Flow rate(-0.1)	0.46	0.51
17.	Mobile phase (+2%)	0.51	0.56
18.	Mobile phase(-2%)	0.87	0.81
19.	Column temp(+5)	0.43	0.46
20.	Column temp(-5)	1.53	1.56

Robustness:

Robustness is a measure of capacity of analytical methods to remain unaffected by small but deliberate variation of the operating conditions. This was tested by studying the effect of changing column temperature ±5°C, the mobile phase composition by 2%, and flow rate by ±0.1ml. And corresponding results given in Table No. 8.0

RESULTS AND DISCUSSION:

The results and summary for the developed and validated method of Acetylcysteine(ACST) and Acebrofylline (ACBF) was given below Table No- 8.0

CONCLUSION:

The RP-HPLC assay method was developed and validated for simultaneous determination of Acetylcysteine (ACST) and Acebrofylline (ACBF) in capsule dosage forms. The method was found to be simple, specific, Precise and Robust and can be applied for the routine and stability analysis for commercially available formulation.

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SPECTROSCOPIC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF ATOVAQUONE AND APROGUANIL IN TABLET DOSAGE FORM

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ABSTRACT

A novel combination of Atovaquone and Proguanil is used in the treatment of Pneumocystis Pneumonia, toxoplasmosis and for chemoprophylaxis of malaria. A simple, economic, sensitive, accurate and reproducible spectroscopic method has been developed and validated for the simultaneous estimation of Atovaquone and Proguanil in tablet dosage form by Simultaneous equation method. Atovaquone and Proguanil were found to have absorbance maxima at 279 and 254 nm respectively in distilled water. Atovaquone was found to be linear in the concentration range of 7.5 to 20 µg/ ml at 279 nm and Proguanil was found to be linear in the concentration range of 3 to 8 µg/ml at 254 nm. The LOD and LOQ were found to be 15.20 µg/ml and 46.08µg/ml (at 279nm) for Atovaquone and 6.31µg/ml and 19.12µg/ml at (254nm) for Proguanil respectively. The recovery of Atovaquone and Proguanil were found to be 100.36% and 99.41% respectively showing accuracy of the method. The method was validated statistically as per ICH guidelines. The method showed good reproducibility and recovery with % RSD less than 2. So, the proposed method was found to be simple, specific, precise, accuracy, linear, and economical. Hence it can be applied for routine analysis of Atovaquone and Proguanil in pharmaceutical formulations.

KEYWORDS: Atovaquone and Proguanil, Simultaneous euation method, Validation, ICH.

INTRODUCTION

The present research work deals with the development and validation of a simple, economic, sensitive, accurate and reproducible spectroscopic method for the novel combinations of

Atovaquone and Proguanil in tablet dosage form by Simultaneous equation method. Chemically Proguanil hydrochloride [1] is 1-(4-chlorophenyl)-5-isopropyl-biguanide hydrochloride (Figure-1). Proguanil hydrochloride is a synthetic buguanide derivative of pyrimidine.

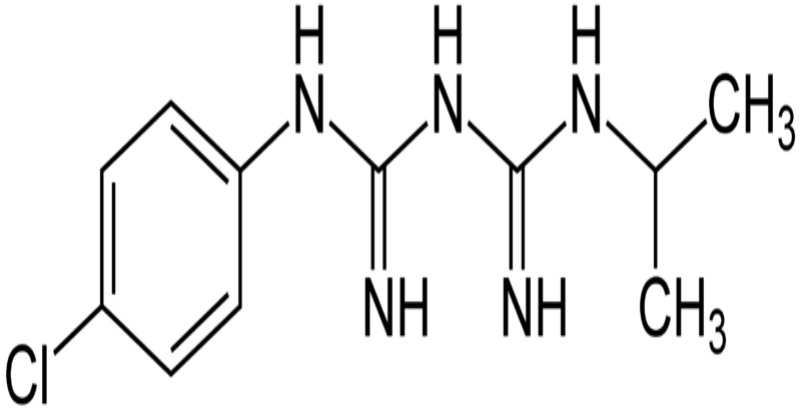

Figure No 1.0

It is widely used in the chemoprophylaxis of malaria. And Atovaquone[2] is chemically trans 2-[4-(4-chlorophenyl)cyclohexyl]-3-hydroxy-1,4 naphthalene-dione.

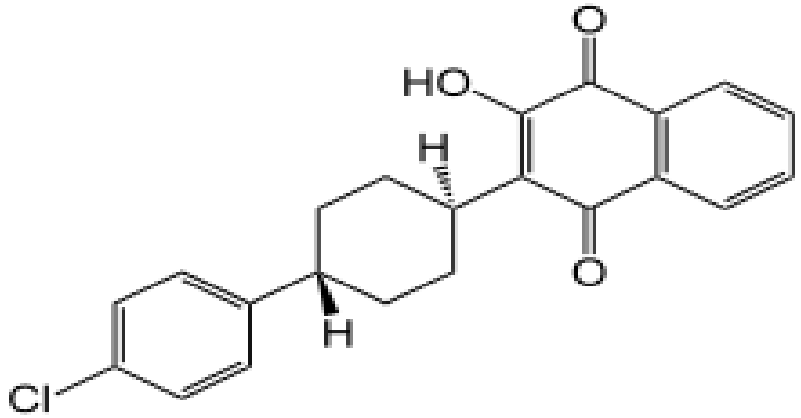

Figure No 2.0

It is used to treat the Pneumocystis Pneumonia, toxoplasmosis and malaria As shown in Figure 2.0. Literature survey[3-16] reveals that no spectroscopic simultaneous equation methods have been reported for this combination of drug while estimation of single and very few methods for the combinations with other drugs was developed for the simultaneous determination of of Proguanil and Atovaquone in tablet formulations. So the present spectroscopic method developed is relatively simple, rapid and highly economic , sensitive and validated as per ICH guidelines[17] in the analysis of multicomponant of interest and it can be used for routine quality control analysis in laboratories.

MATERIALS AND METHODS

Chemicals And Reagents

Proguanil hydrochloride and Atovaquone pure samples were obtained from Glenmark Pharmaceuticals Ltd. Mumbai, India and all other chemicals were of analytical grade. The commercial Proguanil hydrochloride and Atovaquone formulations of combined of brand MALARONE tablets (contain Atovaquone 250mg and Proguanil 100mg Manufactured by GlaxoSmithKline Pharmaceuticals Ltd. were obtained from local retail pharmacy.

Instrumentation

A PG Instruments double beam UV–visible spectrophotometer, Model: T-60, with a UV win Software 5.1.1 version. It has 1cm quartz cell used for the spectral and absorbance measurements.

Determination of λmax:

By appropriate Aliquots of drugs with solvent containing 10µg/ml of Atovaquone and 4 µg/ml of Proguanil were scanned separately in the UV range of 400-200 nm to determine the λ max of Atovaquone (279nm) and Proguanil (254nm) .The overlain spectra indicating λmax of both drugs as shown in Figure No. 3.0

Fig. No. 3.0 Overlay spectra of Atovaquone and Proguanil

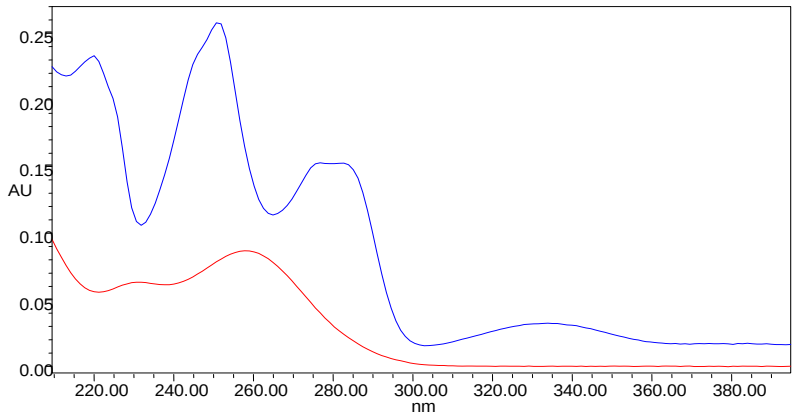

Figure No 3.0

Preparation of standard stock solution

250 mg of Atovaquone and 100mg of Proguanil Standards were weighed and transferred into 100 ml volumetric flasks separately and dissolved in distilled water and made up to the

volume with water. These solutions were observed to contain 2500 and 1000 µg/ml. And further dilution was made to get concentration 10 µg /ml Atovaquone and 4 µg/ml Proguanil.

Preparation of sample solution and formulation Analysis

Twenty tablets of formulation (Atovaquone 250 mg and 100 mg of Proguanil) were weighed accurately and powdered. The tablet powder equivalent to Atovaquone 250 mg and 100 mg of Proguanil was weighed and transferred into 100 ml volumetric flask and a minimum quantity of distilled water was added to dissolve the substance by using ultra sonication for 15 min and made up to the volume with the same(1000 µg/ml). The content was filtered through Whattman filter paper No. 41. From the cleared solution, further dilutions were made by diluting 1 ml to 25ml volumetric flask, further diluted 2 ml to 100 ml to obtain 10 µg/ml of Atovaquone and 4µg/ml of Proguanil theoretically. The absorbance measurements were made for the formulation at 279 nm, 254 nm. From the absorptivity values of Atovaquone and Proguanil at 279 nm, 254 nm, the amount of Atovaquone and Proguanil were determined by using Simultaneous equation method Results of formulation analysis shown in Table No 1.0

Simultaneous Equation Method

From the standard preparation, various dilutions were made at concentration range from 7.5-20µg/ml and 3-8µg/ml.

The simultaneous equations formed were,

At λ1 A1 = ax1bCx + ay1bCy ------ (1)

At λ2 A2 = ax2bCx + ay2bCy ------- (2)

Where A1 and A2 are the absorbance of sample solution at 279 and 254 nm respectively. Cx and CY are the concentration of Atovaquone and Proguanil respectively (µg /ml) in sample solution. The absorbance's (A1& A2) of the sample solution were recorded at 279 and 254nm respectively and concentration of both the drugs were calculated using above mentioned equation.

Table No. 1: UV Spectrophotometric assay Results for Atovaquone and Proguanil

Drug name	Label claim (mg/tab)	Estimated Amount (mg/tab)	% of lable claim S.D (n=6)
Atovaquone	250	251.175	100.47
Proguanil	100	99.24	99.24

Linearity

A calibration curve was plotted as concentration*Vs* absorbance and the corresponding linearity range was given for the selected absorption maxima.

Atovaquone was found to be linear in the concentration range of 7.5 to 20 µg/ml at 279 nm and Proguanil was found to be linear in the concentration range of 3 to 8 µg/ml at 254 nm. The data for the absorbance of Atovaquone and Proguanil drugs are given in Table No. 2.0 & Table No.3.0 as well as respective calibration curve shown in figure No.4.0 and figure No.5.0 respectively.

Table No. 2.0 Linearity data for Atovaquone

Sr.No	Concentration Range	Absorbance
1.	7.5	0.365
2.	10	0.479
3.	12.5	0.593
4.	15	0.699
5.	17.5	0.824
6.	20	0.944

Table No. 3.0 Linearity data for Proguanil

Sr.No	Concentration Range	Absorbance
1.	3	0.154
2.	4	0.199
3.	5	0.255
4.	6	0.313
5.	7	0.366
6.	8	0.417

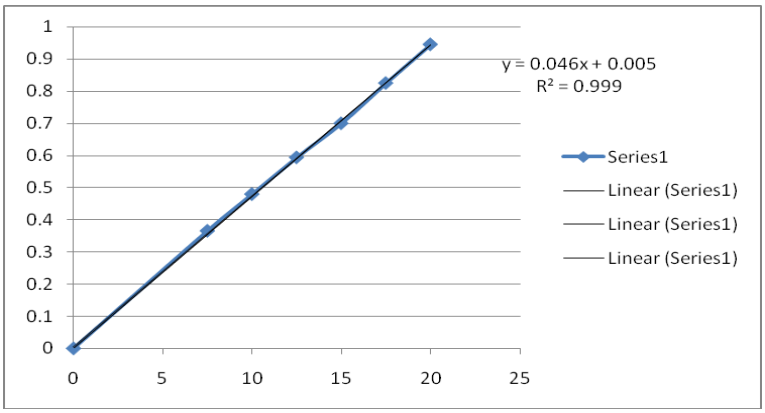

Fig. No.4.0 Calibration Curve for Atovaquone

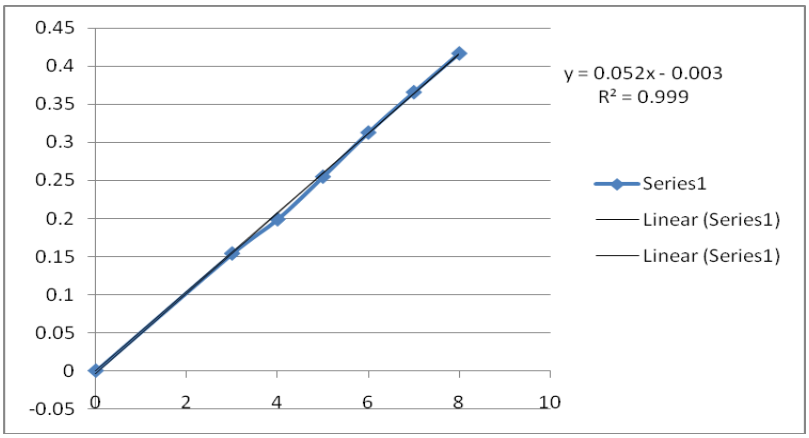

Fig. No.5.0 Calibration Curve for Proguanil

Precision

The repeatability of the method was confirmed by the formulation analysis, repeated for six times with the same concentration. The amount of each drug present in the tablet formulation was calculated. The percentage RSD was calculated. The intermediate precision of the method was confirmed by intra-day and inter-day analysis i.e. the analysis of formulation was repeated three times in the same day and on three successive days, respectively. The amount of drugs was determined and % RSD was calculated and reported in Results and Discussion Table No. 3.0.

Accuracy

Accuracy of the method was studied by recovery experiments. The recovery experiments were performed by adding known amounts of standard drug to formulation samples. The recovery was performed at three different concentrations levels (i.e. 50%, 100% and 150%). This procedure was repeated for three times for each concentration. The results of recovery studies were calculated for %RSD and shown in Results and Discussion Table No. 3.0.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The limit of detection (LOD) and the limit of quantitation (LOQ) of all selected combination of drugs were derived by calculating the signal to-noise ratio using the following equations as per the ICH guidelines. LOD=3.3xσ/S;LOQ=10xσ/S,where σ-standard deviation of the response and S- slope of calibration curve LOD and LOQ values shown in Results and Discussion Table No. 3.0.

RESULTS AND DISCUSSION

Overlay spectra of Atovaquone and Proguanil (Fig.No.3.0) shows that the estimation of both the drug can be possible using simultaneous equation method at the wavelength of 279nm

and 254nm. Using appropriate dilutions of standard stock solution, the two solutions were scanned separately. A critical evaluation of proposed method was performed by statistical analysis of data where slope, intercept, correlation coefficient were studied. Beer's law is obeyed in the concentration range 7.5-20µg/ml and 3-8µg/ml and correlation coefficient of 0.999 and 0.999 for Atovaquone and Proguanil respectively. The results and Optical parameters for the developed and validated method of Atovaquone (ATOV) and Proguanil (PROG) was given below Table No. 3.0

Table No. 3.0 Atovaquone and Proguanil data of validation parameter

Parameter	Atovaquone	Proguanil
Absorption maxima (λ_{max})	279nm	254nm
Beers law limit (µg/ml)	7.5-20 µg/ml	3-8 µg/ml
Correlation coefficient(r)	0.999	0.998
Slope	0.04683(at 279nm)	0.05405(at 254nm)
	0.0405(at 254nm)	0.01288(at279nm)
Intercept	0.00567(at 279nm)	-0.0362(at 254nm)
	-0.0504(at 254nm)	-0.0129(at279nm)
LOD(µg/ml)	15.20(at 279nm)	6.31(at 254nm)
	18.03(at 254nm)	8.19(at279nm)
LOQ(µg/ml)	46.08(at 279nm)	19.12(at 254nm)
	54.63(at 254nm)	24.84(at279nm)
Recovery study	100.36±0.67	99.41±1.85
Interday precision	0.70	1.90
Intraday precision	0.64	1.80

CONCLUSION

The Spectroscopic method was developed and validated for simultaneous determination of Atovaquone (ATOV) and Proguanil (PROG) in tablet dosage forms. The method was found to be simple, specific, Precise and economical and can be applied for the routine and stability analysis for commercially available formulation.

ACKNOWLEDGEMENTS

The authors are thankful to ultra college of pharmacy, Madurai for providing necessary facilities for carrying out this work.

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Research Article

DEVELOPMENT AND VALIDATION OF SPECTROSCOPIC METHOD FOR SIMULTANEOUS ESTIMATION OF ACEBROPHYLLINE AND ACETYLCYSTEINE IN CAPSULE DOSAGE FORM

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ABSTRACT

A novel combination of Acebrophylline and Acetylcysteine is used in the treatment of chronic obstructive pulmonary disease (COPD) and bronchial asthma. A simple, economic, sensitive, accurate and reproducible spectroscopic method has been developed and validated for the simultaneous estimation of Acebrophylline and Acetylcysteine in capsule dosage form by Simultaneous equation method. Acebrophylline and acetylcysteine were found to have absorbance maxima at 273 and 220 nm respectively in distilled water. Acebrophylline was found to be linear in the concentration range of 1 to 6 µg/ ml at 273 nm and Acetylcysteine was found to be linear in the concentration range of 6 to 36µg/ml at 220 nm. The LOD and LOQ were found to be 6.22 µg/ml and 18.84µg/ml (at 273nm) for Acebrophylline and 36.64µg/ml and 111.04µg/ml at (220nm) for Acetylcysteine respectively. The recovery of Acebrophylline and Acetylcysteine were found to be 101.86% and 99.54% respectively showing accuracy of the method. The assay of marketed tablet formulation (Pulmoclear Capsules) was found to be 101.81% and 99.82% for Acebrophylline and Acetylcysteine respectively. The method was validated statistically as per ICH guidelines. The method showed good reproducibility and recovery with % RSD less than 2. So, the proposed method was found to be simple, specific, precise, accurate and linear. Hence it can be applied for routine analysis of Acebrophylline and Acetylcysteine in pharmaceutical formulations.

KEYWORDS: Acetylcysteine, Acebrofylline, Simultaneous euation method, Validation, ICH.

1. INTRODUCTION

Chemically Acetylcysteine[1] is the N-acetyl derivative of the amino acid L-cysteine and a precursor in the formation of antioxidant glutathione in the body. The thiol (sulfahydryl) group confers antioxidants effects and is able to reduce free radicals. Acetylcysteine[1,2] IUPAC name is a (2R)-2-acetamido-3-sulfanylpropanic acid [Figure 1], represents mucolytic drug which decreases the viscosity of secretions by splitting of disulphide bonds in mucoproteins and it also promotes the detoxification of an intermediate paracetamol metabolite which is used in the management of paracetamol overdose.

Acebrofylline[3] IUPAC name is 4-[(2-amino-3,5-dibromophenyl) methylamino] cyclohexan-1-ol; 2-(1,3-dimethyl-2,6-dioxopurin-7-yl)acetic acid. Acebrofylline is the salt obtained by reaction of equimolar amounts of theophylline-7-acetic acid, a xanthine derivative with specific bronchodilator activity and ambroxol, a mucolytic and expectorant with molecular formula $C_{22}H_{28}Br_2N_6O_5$ and molecular weight 616.302 g/mol as shown in Figure 2. It is a novel drug with bronchodilating, anti-inflammatory and mucuregulating effect due to inhibition of phospholipase A, and phosphatidylcholine. Literature survey[4-14] reveals that some methods have been reported for the estimation of single and very few methods for the combinations, but still there is no UV-Visible simultaneous equation method developed for the determination of Acebrofylline and Acetylcysteine in capsule formulations. So the present method developed is relatively simple, rapid and highly sensitive and validated as per ICH guidelines[15] in the analysis of multicomponent of interest and it can be used for routine quality control analysis in laboratories.

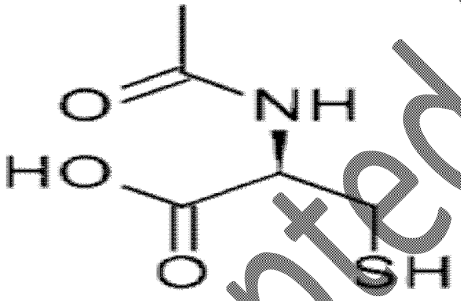

Figure 1: Chemical structure of Acetylcysteine

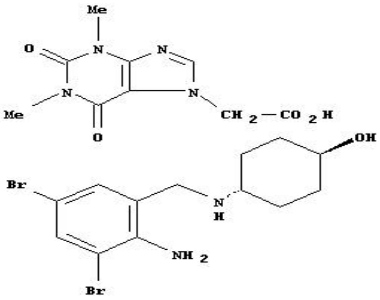

Figure 2: Chemical structure of Acebrofylline

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Acebrofylline (99.67%) and Acetylcysteine (99.69%) are samples were obtained from SL Drugs and Pharmaceuticals, Hyderbad, India and all other chemicals were of analytical grade. The commercial Pulmoclear (brand name) capsule formulation contain Acebrofylline 100mg and Acetylcysteine 600mg Manufactured by Fourrts(India) Laboratories Pvt. Ltd were obtained from local retail pharmacy.

2.2 Instrumentation

A PG Instruments double beam UV–visible spectrophotometer, Model: T-60, with a UV win Software 5.1.1 version. It has 1cm quartz cell used for the spectral and absorbance measurements.

2.3 Determination of λmax

By appropriate Aliquots of drugs with solvent containing 4µg/ml of Acebrofylline and 24 µg/ml of Acetylcysteine were scanned separately in the UV range of 400-200 nm to determine the λ max of Acebrophylline (273nm) and Acetylcysteine (220nm).

2.4 Preparation of standard stock solution

100mg of Acebrophylline and 600mg of Acetylcysteine Standards were weighed and transferred into 100 ml volumetric flasks separately and dissolved in distilled water and made up to the volume with same. These solutions were observed to contain 1000 and 6000 µg/ml concentration respectively. From the above stock solution 0.5ml was pipetted out into a 10 ml volumetric flask and made upto 10ml with diluent. From the above stock solution 0.8ml was pipetted out into a 10 ml volumetric flask and made upto 10ml with diluent to get concentration 4µg/mlAcebrophylline and 24µg/ml Acetylcysteine.

2.5 Preparation of sample solution and formulation Analysis

Twenty tablets of formulation (Acebrophylline 100 mg and 600 mg of Acetylcysteine) were weighed accurately and powdered. The tablet powder equivalent to Acebrophylline 100 mg and 600 mg of Acetylcysteine was weighed and transferred into 100 ml volumetric flask and a minimum quantity of distilled water was added to dissolve the substance by using ultra sonication for 15 min and made up to the volume with the same diluent (5000 µg/ml). The content was filtered through Whattman filter paper No. 41. From the cleared solution, further dilutions were made by diluting 2 ml to 100ml volumetric flask, further diluted 0.4 ml to 10 ml to obtain 4 µg/ml of Acebrophylline and 24µg/ml of Acetylcysteine theoretically.

The absorbance measurements were made for the formulation at 273 nm, 220 nm. From the absorptivity values of Acebrophylline and Acetylcysteine at 273 nm, 220 nm, the amount of Acebrophylline and Acetylcysteine were determined by using Simultaneous equation method.

2.6 Simultaneous Equation Method

From the standard preparation, various dilutions were made at concentration range from 1-6µg/ml and 6-36µg/ml.

The simultaneous equations formed were,

At λ1 $A1 = ax1bCx + ay1bCy$ ------ (1)

At λ2 $A2 = ax2bCx + ay2bCy$ ------- (2)

Where A1 and A2 are the absorbance of sample solution at 273 and 220 nm respectively. Cx and CY are the concentration of Acebrofylline and Acetylcysteine respectively (µg /ml) in sample solution. The absorbance's (A1and A2) of the sample solution were recorded at 273 and 220nm respectively and concentration of both the drugs were calculated using above mentioned equation.

2.7 Linearity
Linearity of the concentrations was determined in the range of 1-6µg/ml at 273nm and 6-36µg/ml at 220nm for acebrofylline and acetylcysteine respectively. And Slope, intercept and correlation coefficient (R^2) was calculated from the calibration curve.

2.8 Precision
The repeatability of the method was confirmed by the formulation analysis, repeated for six times with the same concentration. The amount of each drug present in the tablet formulation was calculated. The percentage RSD was calculated. The intermediate precision of the method was confirmed by intra-day and inter-day analysis i.e. the analysis of formulation was repeated three times in the same day and on three successive days, respectively. The amount of drugs was determined and % RSD was calculated.

2.9 Accuracy
Accuracy of the method was studied by recovery experiments. The recovery experiments were performed by adding known amounts of standard drug to formulation samples. The recovery was performed at three different concentrations levels (i.e. 50%, 100% and 150%). This procedure was repeated for three times for each concentration. The results of recovery studies were calculated for %RSD.

2.10 Specificity
Specificity is the ability of the method to measure the analyte in the presence of other relevant components. The evaluation of specificity of the method was determined against placebo.

2.11 Selectivity
It is confirmed by determining the Limit of Detection (LOD) and Limit of Quantitation (LOQ).

2.12 Limit of Detection (LOD) and Limit of Quantitation (LOQ)
The limit of detection (LOD) and the limit of quantitation (LOQ) of all selected combination of drugs were derived by calculating the signal to-noise ratio using the following equations as per the ICH guidelines. LOD=3.3xσ/S, LOQ=10xσ/S,where σ-standard deviation of the response and S- slope of calibration curve.

3. RESULTS AND DISCUSSION

Overlay spectra of Acebrofylline and Acetylcysteine (Figure 3) shows that the estimation of both the drug can be possible using simultaneous equation method at the wavelength of 273nm and 220nm. Using appropriate dilutions of standard stock solution, the two solutions were scanned separately. A critical evaluation of proposed method was performed by statistical analysis of data where slope, intercept, correlation coefficient were studied. Beer's law is obeyed in the concentration range 1-6 µg/ml and 6-36µg/ml and correlation coefficient of 0.9997 and 0.9995 for Acebrofylline and Acetylcysteine respectively. The results and Optical parameters for the developed and validated method of Acetylcysteine (ACST) and Acebrofylline (ACBF) was given below Table 4, The Linearity data for the absorbance of Acebrofylline and Acetylcysteine drugs are given in Table 2 and Table 3 as well as respective calibration curve shown in figure 4 and figure 5 respectively. The recovery was performed at three different concentrations levels

(i.e. 50%, 100% and 150%). The results of recovery studies were calculated for %RSD and shown in Table 3.

3.1 Specificity

The method was found to be specific in presence of Ambroxol which is the impurity of the drug.

3.2 Sensitivity

The limit of detection value for Acebrophylline and acetylcysteine was calculated and reported in Table 4. It was found to be selective method for this combination of drug.

3.3 Linearity and Range

The developed method was found to be Linear in it range and the slope, intercept and correlation coefficient was reported in Table 4.

3.4 Precision

It is expressed as the percentage coefficient of variation (%CV)/%RSD which is calculated as per the following expression:

% CV= (standard deviation /mean)*100

It was found to be less than 2%. The results for interday and intraday precision were reported in Table 4.

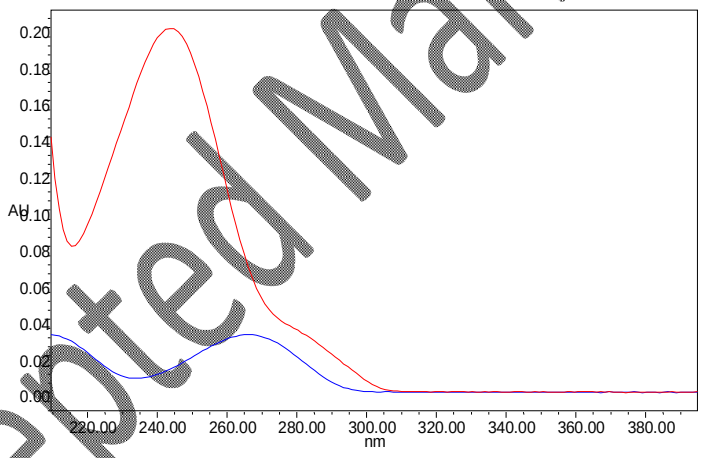

Fig. 3: Overlay spectra of Acebrophylline and Acetylcysteine

Table 1: UV Spectrophotometric assay Results for Acebrophylline and Acetylcysteine

Drug name	Label claim (mg/tab)	Estimated Amount (mg/tab)	% of Lable claim S.D (n=6)
Acebrophylline	100	101.81	101.81
Acetylcysteine	600	598.968	99.828

Table 2: Linearity data for Acebrofylline

Sr. No	Concentration in (µg/ml)	Absorbance
1.	1	0.04
2.	2	0.082
3.	3	0.129
4.	4	0.167
5.	5	0.21
6.	6	0.256

Table 3: Linearity data for Acetylcysteine

Sr.No	Concentration in (µg/ml)	Absorbance
1.	6	0.167
2.	12	0.317
3.	18	0.456
4.	24	0.628
5.	30	0.755
6.	36	0.919

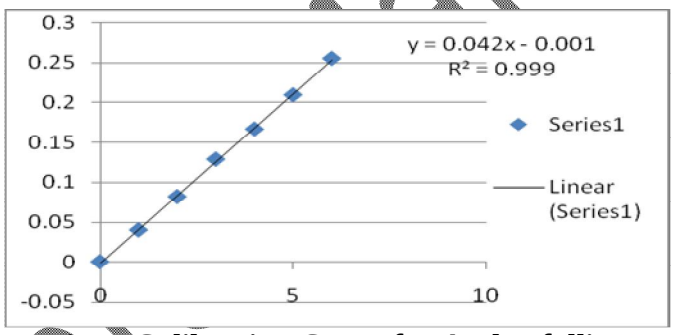

Fig.4: Calibration Curve for Acebrofylline

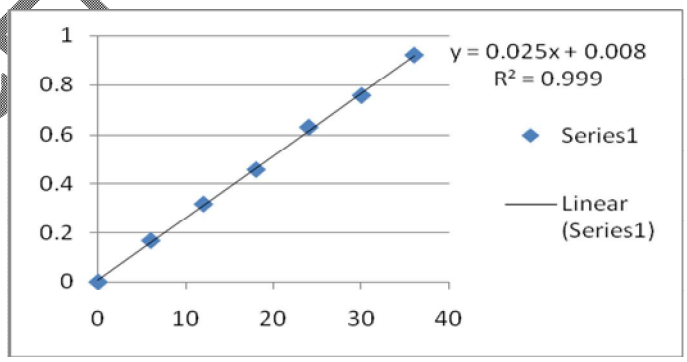

Fig. 5: Calibration Curve for Acetylcysteine

Table 4 : Acetylcysteine and Acebrophylline data of validation parameter

Parameter	Acebrophylline	Acetylcysteine
Absorption maxima (λ_{max})	273	220
Beers law limit (µg/ml)	1-6 µg/ml	6-36 µg/ml
Correlation coefficient(r^2)	0.9997	0.9995
Slope	0.0429 (at 273nm)	0.0249(at 220nm)
	0.1300(at 220nm)	0.00706(at 273nm)
Intercept	-0.002866(at 273nm)	0.01573(at 220nm)
	-0.000133(at 220nm)	0.00786(at 273nm)
LOD(µg/ml)	0.166 (at 273nm)	1.275 (at 220nm)
	0.166(at 220nm)	1.275(at 273nm)
LOQ (µg/ml)	0.503(at 273nm)	3.866(at 220nm)
	0.5037(at 220nm)	3.866(at 273nm)
Recovery study	101.86±0.252	99.54±0.299
Interday precision	0.190	0.306
Intraday precision	0.315	0.293

4. CONCLUSION

The Spectroscopic method was developed and validated for simultaneous determination of Acebrofylline (ACBF) and Acetylcysteine(ACST) in capsule dosage forms. The method was found to be simple, specific, Precise and economical and can be applied for the routine quality control analysis for commercially available formulation.

5. ACKNOWLEDGEMENTS

The authors are thankful to ultra college of pharmacy, Madurai for providing necessary facilities for carrying out this work.

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