QUANTITATIVE
ANALYSIS OF DRUGS

Dr. MADHUKAR A. BADGUJAR

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Dedicated to

My Family

PREFACE

I have decided to write this book because in a simple manner I would like to represent the different analytical methods for the analysis of drugs in combined dosage form. This book has been designed to incorporate the latest analytical techniques for the analysis of pharmaceutical drugs.

- This book represents different methods for the quantitative analysis of drugs like high performance liquid chromatography (HPLC), high performance thin layer chromatography (HPTLC) and UV spectrophotometry.
- It also includes the development of methods and its validation. With the help of statistical parameters like mean, mode, median, standard deviation, correlation coefficient the proper results are obtained.
- In this book all the essential parameters of method development are discussed with the results. There are four pharmaceutical combinations are analyzed with their results.
- This book also contain the drug profile of the anti-inflammatory and antibacterial drugs.
- In the introduction part all the basics of the HPLC, HPTLC and UV spectrophotometry are included.
- At the end of the chapter the references are mentioned.
- This book describes the applications of various analytical techniques for the analysis of pharmaceutical formulations.

- The entire subject matter has been presented with proper subheadings, tables and figures to facilitated the reader to understand the text in a systematic manner.
- This book is helpful for the quantitative analysis of pharmaceutical formulations.

I wish to extend my appreciation to the teachers and colleague for the constructive suggestions to write this book.

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In this whole process of my work, I would like to mention the name, last but not least, of my wife Mrs. Uma Badgujar for her constant co-operation, without any hesitation.

Dr. Madhukar A. Badgujar

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CHAPTER- I
INTRODUCTION

Analytical chemistry is a school of science consisting of a set of powerful ideas and methods that are useful in all fields of science and medicine. Analytical chemistry is the study of the separation, identification, & quantification of the chemical components of natural and artificial materials. Analytical chemistry is the science of chemical characterization and is concerned with the methods used in chemical analysis, while chemical analysis is concerned with qualitative and quantitative composition of matter. The primary interest of the analytical chemist is to develop experimental methods of measurement to obtain information about the qualitative and quantitative composition of matter. In the development of methods of chemical analysis, every possible chemical reaction and physicochemical property is of interest to the analytical chemist. The chemical analyst uses the methods developed by the analytical chemist.

Modern Analytical Chemistry

Modern analytical chemistry is dominated by instrumental analysis. There are so many different types of instruments today that it can seem like a confusing array of acronyms rather than a unified field of study. Many analytical chemists focus on a single type of instrument. Academics tend to either focus on new applications and discoveries or on new methods of analysis. The discovery

of a chemical present in blood that increases the risk of cancer would be a discovery that an analytical chemist might be involved in. An effort to develop a new method might involve the use of a tunable laser to increase the specificity and sensitivity of a spectrometric method. Many methods, once developed, are kept purposely static so that data can be compared over long periods of time. This is particularly true in industrial quality assurance (QA) and forensic and environmental applications. Analytical chemistry plays an increasingly important role in the pharmaceutical industry where, aside from QA, it is used in discovery of new drug candidates and in clinical applications where understanding the interactions between the drug and the patient are critical.

Analytical chemistry has made phenomenal progress during the last twenty or thirty years. Accuracy, reproducibility and reliability are the basis of analytical method today. The degree of sophistication, that is now available, is so high that impurities of the order of parts per billion in food products and ultrapure material can be routinely determined. Sophisticated research in chemistry, biology, physics, biochemistry and the rapid technological advancement created analytical problems which the analytical chemist had to tackle with high degree of skill and accuracy. The extensive use of known physicochemical principle and the discovery of new techniques began only recently. Polarography, spectrophotometry, gas-liquid chromatography, high performance thin layer chromatography, mass spectrometry to mention only a few, are analytical methods developed during the last few years.

Analytical methods:

Information about chemical properties of a given substance is obtained by measuring the physical or physic-

chemical properties of the substance before, during and following a chemical reaction involving the substance. On the basis of property measured, analytical method has been classified into volumetric, gravimetric, optical and electrochemical methods. Titrimetric and gravimetric methods based on chemical reactions and hence the two together are called chemical methods.

The volumetric method involves measurements of volumes of the reacting substances. The weight of the desired constituent is indirectly obtained by measuring the volume of solution of known composition required to react quantitatively with a known volume of solution containing an unknown weight of the desired constituent.

Gravimetric analysis involves determining the amount of material present by weighing the sample before and/or after some transformation. The method consists of converting the desired constituent into a compound of known composition, separation of the compound thus precipitated, by filtration and weighing the dry compound.

Optical and electrochemical methods of analysis are based on measurement of physical properties of sample by an instrument. These methods are therefore called physical or instrumental methods of analysis. Optical methods of analysis are based on the interaction of the desired constituent with electromagnetic radiations. This may involve absorption (spectrophotometry), rotation (polarimetry), or scattering of electromagnetic radiation (nephelometry, turbidimetry, Raman spectroscopy)

Electrochemical methods of analysis are based on relationship between electrical properties and chemical reactions. The methods involve the measurements of one

or more of the fundamental electrical quantities: voltage, current or resistance. The magnitude of the property thus measured can then be related, directly or indirectly, to the concentration of the chemical species subjected to analysis. It included conductometry, potentiometry, polarography, voltametry, amperometry and coulometry. Apart from these methods the separation and estimation of the analytes are possible by instrumental methods.

Instrumental methods in pharmaceutical chemistry comprise mainly spectrophotometry, refractometry, polarimetry etc. electrochemical methods are also some times used. While colorimetry and its sophisticated counterpart spectrophotometry are the most used ones, they suffer from the fact that they can be used only for single component and not normally for mixtures. Where mixtures are concerned chromatography comes in. This technique is a separation technique and is versatile as it can separate complex mixtures into its components. All these mentioned analytical methods have been extensively used in pharmaceutical industry.

Importance of Analytical chemistry in pharmaceutical Industry

Pharmaceutical industry is another example, where analytical chemistry plays a crucial role from drug development to the quality control of the drug. A number of drug formulations are marketed everyday by different pharmaceutical industries. As each formulation differs from another, it needs a separate and specific method of analysis. That is why innumerable methods of analysis are reported in the literature to satisfy the quest arising due to numerous formulations available in the market.

The process of developing methods of analysis like titrimetry, gravimetry etc. are being replaced by instrumental methods, which provide specific, sensitive, accurate and fast means of analysis. The strict control on all drugs for their quality also necessitates development a method, which would be essay to carry out and which would give accurate, specific and reproducible results of formulation with the availability of modern analytical instruments to the analytical chemist, developing such methods has become easier. Even in the official methods (I.P, B.P and U.S.P) for assay of most of the drugs, the instrumental methods like spectrophotometry, gas chromatography, liquid chromatography have been adopted. At the same time no country can afford to keep away of itself from the recent development in drug analysis.

This has resulted in the availability of indigenous instruments like spectrophotometer, high performance liquid chromatography, gas chromatography, high performance thin layer chromatography etc. in almost all analytical laboratories and pharmaceutical companies. Due to advent automation, small sample size and high sensitivity of the instrument, very accurate and precise assay methods can be developed on chromatographic instruments.

The analysis of complex mixtures particularly requires the combination of both separation techniques and mass spectrometry. The first step in this direction was made by gas chromatography-mass spectrometry coupling (GC-MS), and soon, GC-MS became a routine method. The desire to realize a liquid chromatography-mass spectrometry coupling (LC-MS) was the driving force for the

development of API methods Coupling of other liquid phase separation techniques to mass spectrometry followed: capillary zone electrophoresis- mass spectrometry (CZE-MS), and supercritical fluid chromatography- mass spectrometry (SFC-MS)Whatever the separation technique, it adds an additional dimension to the analytical measurement. The hyphen used to indicate the coupling of a separation technique to mass spectrometry led to the term hyphe-nated methods. However, mass spectrometry itself offers two additional “degrees of freedom”.

The combination of high performance liquid chromatography and mass spectrometry (LC/MS) has had a significant impact on drug development over the past decade. Instrumentation and techniques related to the automated analysis of biomolecules and new drugs now account for a large percentage of the research and publications in this field. In comparison, gas chromatography /mass spectrometry (GC/MS) and electron ionization (EI) mass spectra of ‘‘small’’ molecules play a less important role than they once did. But GC/MS is far from dead, and EIMS continues to be the ionization method of choice for many laboratories that routinely analyze volatilizable low molecular mass compounds such as drugs, flavor and odor components, pesticides, and petroleum products. This situation seems unlikely to change in the near future.

New technologies have created a situation where the rate of sample generation far exceeds the rate of sample analysis. The growth in LC/MS applications has been extensive, with a retention time and molecular weight emerging as essential analytical features from drug target to product.

In the present research work following modern analytical techniques are used for qualitative and quantitative analysis.

CHROMATOGRAPHY

The dynamic and thermodynamic effects that result in a chromatographic separation are logical and easy to understand. It is wise to always bear in mind the comment made by Einstein: **"first order effects are simple".** In other words, in any physical chemical process, the phenomenon that accounts for the major effect will be elementary in nature and easy to understand. Only when second order effects are considered, and dealt with quantitatively, does the theory and accompanying mathematics become more complex.

There are two essential theories needed to explain the processes involved in a chromatographic separation. There have been a number of contributions to chromatography theory over the intervening 40 years, but most of these have been largely involved in the extension and confirmation of existing concepts and a more detailed examination of second order effects. The more recent and original contributions to chromatography theory have come largely from developments in the field of preparative chromatography and electro-chromatography. Chromatography theory is not merely an abstract study of the separation process; rather it is directly related to the practice of the technique. In fact, the remarkable advances and improvements that have taken place in chromatographic performance over the years have been directly predicted and pre-empted from theoretical studies. In addition, without fully understanding the physical

processes involved in a chromatographic separation, the optimum use of a chromatograph would be virtually impossible or, at the very least, depend heavily on serendipity.

Chromatography has been defined in the classical manner as,

"A separation process that is achieved by the distribution of the substances to be separated between two phases, a stationary phase and a mobile phase". Those solutes, distributed preferentially in the mobile phase, will move more rapidly through the system than those distributed preferentially in the stationary phase. Thus, the solutes will elute in order of their increasing distribution coefficients with respect to the stationary phase.

It follows that during the development of a chromatographic separation, two processes will occur simultaneously and to large extent, independently. Firstly, the individual solutes in the sample are moved apart in the distribution system as a result of their different affinities for the stationary phase. Secondly, as the bands are moved apart, their tendency to spread or disperse is constrained to ensure that the separation that has been achieved is maintained. Thus, the phase system must be chosen to provide the necessary relative retention of the solutes, and the distribution system must be appropriately designed to minimize this dispersion and permit the components of the mixture to be eluted discretely. Chromatography theory provides a basis for these choices; it discloses the mechanisms that control retention, it explains the different processes that can cause band dispersion, and it shows how solute retention and band dispersion are controlled by the

operating variables of the chromatographic system. In addition, chromatography theory reveals how the separation is affected by the properties of those parts of the chromatographic system those are not directly associated with solute distribution (i.e., sampling devices, detector sensors, etc.).

The Chromatographic Process

Chromatography is a separation process in which the sample mixture is distributed between two phases in the chromatographic bed (column or plane). One phase is stationary whilst the other passes through the chromatographic bed. The stationary phase is either a solid, porous, surface-active material in small-particle form or a thin film of liquid coated on a solid support or column wall. The mobile phase is a gas or liquid. If a gas is used, the process is known as gas chromatography; the mobile phase is always liquid in all types of liquid chromatography, including the thin-layer variety.

High Performance Liquid Chromatography

A powerful separation method must be able to resolve mixtures with a large number of similar analytes. High-performance liquid chromatography (HPLC) is very efficient, i.e. it yields excellent separations in a short time. The 'inventors' of modern chromatography, Martin and Synge were aware as far back as 1941 that, in theory, the stationary phase requires very small particles and hence a high pressure is essential for forcing the mobile phase through the column. As a result, HPLC is sometimes referred to as high-pressure liquid chromatography

LIQUID CHROMATOGRAPHIC SEPARATION MODES

Adsorption Chromatography

The principle of adsorption chromatography is known from classical column and thin-layer chromatography. A relatively polar material with a high specific surface area is used as the stationary phase, silica being the most popular, but alumina and magnesium oxide are also often used. The mobile phase is relatively non-polar (heptane to tetrahydrofuran). The different extents to which the various types of molecules in the mixture are adsorbed on the stationary phase provide the separation effect. A non-polar solvent such as hexane elutes more slowly than a medium-polar solvent such as ether.

Rule of thumb: polar compounds are eluted later than non-polar compounds.

Chromatography with Chemically Bonded Phases

The stationary phase is covalently bonded to its support by chemical reaction. A large number of stationary phases can be produced by careful choice of suitable reaction partners. The reversed-phase method described above is the most important special case of chemically bonded-phase chromatography.

Ion-Exchange Chromatography

The stationary phase contains ionic groups (e.g. NR_3^+ or SO_3^- which interact with the ionic groups of the sample molecules. The method is suitable for separating, e.g. amino acids, ionic metabolic products and organic ions.

Ion-Pair Chromatography

Ion-pair chromatography may also be used for the separation of ionic compounds and overcomes certain

problems inherent in the ion-exchange method. Ionic sample molecules are 'masked' by a suitable counter ion. The main advantages are, firstly, that the widely available reversed-phase system can be used, so no ion exchanger is needed, and, secondly, acids, bases and neutral products can be analysed simultaneously.

Ion Chromatography

Ion chromatography was developed as a means of separating the ions of strong

acids and bases (e.g. Cl^-, NO_3^-, Na^+, K^+). It is a special case of ion-exchange chromatography but the equipment used is different.

Size-Exclusion Chromatography

This mode can be subdivided into gel permeation chromatography (with organic solvents) and gel filtration chromatography (with aqueous solutions). Size-exclusion chromatography separates molecules by size, i.e. according to molecular mass. The largest molecules are eluted first and the smallest molecules last. This is the best method to choose when a mixture contains compounds with a molecular mass difference of at least 10%.

Affinity Chromatography

In this case, highly specific biochemical interactions provide the means of separation. The stationary phase contains specific groups of molecules which can only adsorb the sample if certain steric and charge-related conditions are satisfied (cf. interaction between antigens and antibodies). Affinity chromatography can be used to isolate proteins (enzymes as well as structural proteins), lipids, etc., from complex mixtures without involving any great expenditure.

In the present research work Reverse – Phase Chromatography technique was used for the retention and separation of the analytes.

Reversed-Phase Chromatography

- The stationary phase is very non-polar.

  The mobile phase is relatively polar (water to tetrahydrofuran).

- A polar solvent such as water elutes more slowly than a less polar solvent

  such as acetonitrile.

Rule of thumb: non-polar compounds are eluted later than polar compounds.

Reversed-phase chromatography is the term used to describe the state in which the stationary phase is less polar than the mobile phase. Chemically bonded octadecyl silane (ODS), an n-alkane with 18 carbon atoms, is the most frequently used stationary phase. C_8 and shorter alkyl chains and also cyclohexyl and phenyl groups provide other alternatives. Phenyl groups are more polar than alkyl groups.

Water is often described as the strongest elution medium for chromatography, but in fact this is only true for adsorption processes. Water may interact strongly with the active centers in silica and alumina, so that adsorption of sample molecules becomes highly restricted and they are rapidly eluted as a result. Exactly the opposite applies in reversed-phase systems: water cannot wet the non-polar (hydrophobic = water-repellent) alkyl groups and does not interact with them in any way. Hence it is the weakest mobile phase of all and gives the slowest sample elution

rate. The greater the amount of water in the eluent, the longer is the retention time.

Sample compounds are better retained by the reversed-phase surface the less water soluble (i.e. the more non-polar) they are. The retention decreases in the following order:

Aliphatics > induced dipoles (e.g. CCl_4) > permanent dipoles (e.g. $CHCl_3$) > weak Lewis bases(ethers, aldehydes, ketones) > strong Lewis bases (amines) > weak Lewis acids (alcohols, phenols) > strong Lewis acids (carboxylic acids).

Also, the retention time increases as the number of carbon atoms increases, as a

general rule the retention increases with increasing contact area between sample molecule and stationary phase, i.e. with increasing number of water molecules which are released during the 'adsorption' of a compound. Branched-chain compounds are eluted more rapidly than their corresponding normal isomers. Yet the retention mechanisms on reversed-phase are complex and not easy to understand.

Mobile phases in reversed-phase chromatography

The mobile phase generally consist of mixtures of water or aqueous buffer solutions with various water-miscible solvents, e.g. methanol, acetonitrile, ethanol decreasing polarity, isopropanol, dimethylformamide increasing elution power, propan-1-ol dioxane, tetrahydrofuran. However, non-aqueous eluents are needed for the reversed-phase chromatography of highly non-polar analytes.

Stationary phases

As a general rule, retention times are longer the more C atoms the bonded stationary phase contains. (The

reason is that the volume taken up by the bonded non-polar groups, i.e. that required by the actual stationary phase, is greater in long chains than it is in shorter chains; retention is directly proportional to the volume ratio between the stationary and mobile phases This means that retention is stronger the longer is the alkyl moiety (C_{18} is more retentive than C_8), the higher is the bonding density of the alkyl chains (in groups per nm of the surface), the higher is the degree of end-capping, the thicker is the organic stationary phase (polymeric layers are more retentive than monomeric ones), or, as a summary, the higher is the carbon content of the material, determined by elemental analysis.

METHOD DEVELOPMENT IN REVERSED- PHASE CHROMATOGRAPHY

In reversed-phase separations, the first step in method development is usually a solvent gradient from 10 to 100% solvent B. What follows here are proposals with a recommended sequence of changes of the various parameters. It is, e.g., more convenient to try another B solvent before changing the stationary phase

Proposal for non-ionic samples

Use a C_8 or C_{18} stationary phase with unbuffered water-acetonitrile at ambient temperature.

1) Adjust %B or gradient range for retention factors between 1 and 10 (or 1 and 20 for difficult separations). If the separation is inadequate, adjust selectivity in the following order:

2) Change the organic B solvent.

3) Use a mixture of organic B solvents.

4) Change the stationary phase (preferably to a type which has markedly

different properties, It is probably necessary to start at step 1 again.

5) Change the temperature.

6) Optimize the physical parameters such as column dimensions, particle size or flow rate.

Proposal for ionic samples

Use C_8 or C_{18} stationary phase which is suitable for basic analytes with buffer pH 2.5-methanol at ambient temperature if possible.

1 Adjust %B or gradient range. If the separation is inadequate:

2 (a) Change pH or (b) use ion-pair chromatography.

3 Adjust %B.

4 Change the organic B solvent.

5 change pH or (b) change pH and ion-pair reagent.

6 Change temperature.

7 Change the stationary phase to phenyl or cyano.

8 Optimize the physical parameters.

THE HPLC INSTRUMENT

An HPLC instrument can be a set of individual modules or elements, but it can be designed as a single apparatus as well. The module concept is more flexible in the case of the failure of a single component; moreover,

the individual parts need not be from the same manufacturer. If you do not like to do minor repairs by yourself you will prefer a compact instrument. This, however, does not need less bench space than a modular set. An HPLC instrument has at least the elements which are shown in Fig. 1.1 solvent reservoir, transfer line with

frit, high-pressure pump, sample injection device, column, detector, and data recorder, usually together with data evaluation. Although the column is the most important part, it is usually the smallest one. For temperature-controlled separations it is enclosed in a thermostat. It is quite common to work with more than one solvent, thus a mixer and controller are needed. If the data acquisition is done by a computer it can also be used for the control of the whole system.

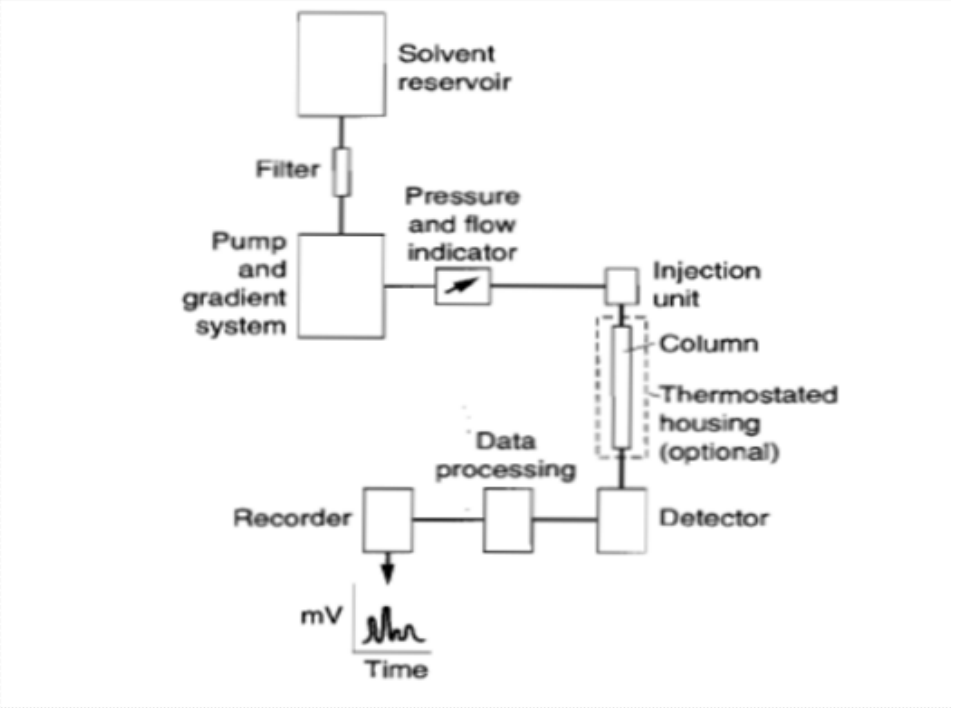


Fig. 1.1 Schematic diagram of an HPLC unit

The solvent reservoir system consists of one or more glass or stainless vessels which can contain 1 to 2 liters of the solvent. Dissolved oxygen and nitrogen in the solvent are removed by degasers. These gases forms

bubbles in the analytical column and the resolution of peak is affected. If single solvent is used, the separation is called isocratic elution. Separation efficiency can be increased by using two or more solvents. Such an elution is called gradient elution.

The column is the heart of the HPLC instrument because the separation occurs here. The analytical column consists of stainless steel tube about 5 to 25 cm in length and 3 to 9mm inner diameter. The selection of the column packing depends upon the chemical nature of the sample component and the mobile phase to be used. The sample gets separated on the column due to the partition between the mobile phase and stationary phase and the components register on a detector. The signal from the detector goes to the recorder and the chromatogram of the sample is obtained. The most commonly used detector is UV detector.

Application of HPLC in Pharmaceutical analysis

High performance liquid chromatography is an efficient, highly selective method of separation. HPLC is a form chromatography used frequently in biochemistry and analytical chemistry to separate, identify, and quantify compounds. HPLC is currently most preferred instrument in the pharmaceutical industry especially for the simultaneous determination of all the actives used in the combination drug theory. HPLC is probably the most universal type of analytical procedure; its application areas include quality control, process control, forensic analysis, environmental monitoring and clinical testing. In addition HPLC also ranks as one of the most sensitive analytical procedures and is unique in that it easily copes with multi-

component mixtures. It has achieved this position as a result of the constant evolution of the equipment used in LC to provide higher and higher efficiencies at faster and faster analysis times with constant incorporation of highly selective column packing.

High performance thin layer Chromatography

Introduction to Thin-layer Chromatography

TLC was originally developed (1951) by J.G. Kirchner and colleagues and later on standardized in 1956 by E. Stahl and colleagues. TLC/ HPTLC is one of the simplest separation techniques available today. The basic TLC procedure has largely remained unchanged over the last fifty years. It involves the use of a thin, even sorbent layer, usually about 0.10 to 0.25 mm thick, applied to a firm backing of glass, aluminium or plastic sheet to act as a support. Of the three, glass has always proved the most popular, although aluminium and plastic offer the advantage that they are flexible and can more easily be cut to any size with minimal disruption to the sorbent layer. Numerous sorbents have been used, some more successfully than others, including silica gel, cellulose, aluminium oxide, polyamide and chemically bonded silica gels. The sample is dissolved in an appropriate solvent and applied as spots or bands along one side of the sorbent layer approximately 1cm from the edge. An eluent (single solvent or solvent mixture) is allowed to flow by capillary action through the sorbent starting at a point just below the applied samples. Most commonly this is achieved by using a glass rectangular tank in which the eluent is poured to give a depth of about 5 mm. The plate is placed in the tank or chromatography chamber and the whole covered with a lid. As the eluent

front migrates through the sorbent, the components of the sample also migrate, but at different rates, resulting in separation. When the solvent front has reached a point near the top of the sorbent layer, the plate or sheet is removed and dried. The spots or bands on the developed layer are visualized, if required, under UV light or by chemical treatment or derivatisation.

For quantitative determinations, zones can be removed or eluted from the layer, or the plate can be scanned at pre-determined wavelengths without disturbing the layer surface. The modern use of TLC has seen a strong move in the direction of plate scanning and video imaging as a means of providing sensitive and reliably accurate results and a more permanent record of the chromatogram. This is in addition to its obvious labour saving aspect and chemically ''clean'' approach.

Although TLC is an analytical method in its own right, it is also complimentary to other chromatographic techniques and spectroscopic procedures. Results obtained with TLC can often be transferred to HPLC or vice versa with some adjustment in eluting solvent conditions. For multi-component samples (e.g. pesticides in water), fractions of interest from an HPLC separation can be collected and subsequent re-chromatography of these on HPTLC can give a ''fine tuned''separation of the components of the fractions.1–3 Thin-layer chromato-graphy has been successfully hyphenated with high performance liquid chromatography (HPLC), mass spectroscopy (MS), Fourier transform infra-red (FTIR), and Raman spectroscopy, to give far more detailed analytical data on separated compounds. Even the UV/visible diode array technique has been utilized in TLC to determine peak purity or the presence of

unresolved analytes. Undoubtedly TLC is a modern analytical separation method with extensive versatility, much already utilized, but still with great potential for future development into areas where research apparently is only just beginning.

Application of HPTLC in Pharmaceutical analysis

The analysis of combination drugs is challenging because these are complex mixtures of compounds which exhibit natural variability. Identification methods must be specific to distinguish the presence of wrong species.

HPTLC is a valuable tool for reliable identification it can provide chromatographic fingerprints that can be visualized and stored as inherent to HPTLC, reproducible results and images must be ensured. Compared with conventional TLC, HPTLC is considerably faster and can be standardized much better. After a great deal of scientific investigation this technique has become invaluable tool for the daily routine in analytical laboratories of the pharmaceutical industry, foodstuff chemistry and clinical chemistry. All phyto-constituents can be detected visually or optically. TLC/ HPTLC provides unique phytochemical fingerprint of plants. TLC/ HPTLC is an offline technique in which various stages are carried out independently. TLC/ HPTLC is also useful in plant analysis because it allows the use of crude extracts. It is suitable for confirming the identity and purity of plants, and for detecting adulterations and substitutions. HPTLC can be used to conform or support label claims of formulations

Nowadays, HPTLC has become a routine analytical technique due its advantages of reliability in quantitation of analytes at micro and even in nanogram levels and cost

effectiveness. The major advantage of HPTLC is that several samples can be analyzed simultaneously using a small quantity of mobile phase unlike HPLC. This reduces the time and cost of analysis and possibilities of pollution of the environment. Simultaneous assay of several component formulation is possible.

UV-Visible Spectrophotometry:

Measurement based on light and other forms of electromagnetic radiation are widely used throughout analytical chemistry. The interactions of radiation and matter are the subject of the science called spectroscopy. Spectroscopic analytical methods are based on measuring the amount of radiation produced or absorbed by molecular atomic specific of interest. Spectroscopy has played a vital role in the development of modern atomic theory. In addition, spectrochemical methods have provided perhaps the most widely use tools for the elucidation of the molecular structure as well as the quantitative and qualitative determination of organic compounds.

Application of UV spectrophotometer in pharmaceutical analysis

UV spectrophotometry is useful analytical technique for the qualitative and quantitative determination of the drugs combination from their formulations.

UV spectra of the analyte have given the valuable information about the functional groups present in the molecule. The technique provides high sensitivity upto concentration as low as 10^{-6}M. Further, if a solution contains more than one component, each absorbing at a different wavelength, then by measuring the absorbance at different wavelength, the concentration of the different components

can be determined. The spectrophotometer is now an essential equipment in industries and laboratories for the continuous monitoring and process control. Spectrophotometry is effectively used in simultaneous determination of the analytes using various methods in pharmaceutical formulations.

Pharmaceutical Analysis

Pharmaceutical analysis is traditionally defined as analytical chemistry dealing with drugs both as bulk drug substances and as pharmaceutical products (formulations). However in academia, as well as in pharmaceutical industry, other branches of chemistry are also involved viz. Bioanalytical chemistry, drug metabolism studies and analytical biotechnology.

Drug analysis means identification, characterization and determination of drugs. Drug assay refers to determination of drugs in mixtures such as dosage forms and biological fluids. Drugs may be gases liquids or solids. Drugs used in formulations such as syrups, tablets and aerosol preparations are referred to as bulk drugs. Prior to the formulation and manufacture of dosage forms, bulk drugs must be properly identified (qualitative analysis) and analyzed for drug content (quantitative analysis). Quantitative and qualitative determination of drugs related to compounds (metabolites) in biological fluids is also necessary once drugs are used in animals and humans during experiment development and treatment of patients.

Analysis of bulk drugs and pharmaceutical products

Identity testing

Identity testing is used to verify that the drug substance is what it is related to be or that the formulation

contains the correct compounds. Tests such as melting point, colour reactions, optical rotation values, and ultra violet or infrared spectra are used. Today chromatographic data are also used to support identity tests.

Impurities

Impurities or degrades require separation methods and are usually studied from the level from 0.1 to 2% (purity patterns) or 0.1 to 5% (stability profiles). This means that the analytes have to be quantified in up to a 1000-fold excess of major compound. In practice quantitative work is performed still at lower levels. This sometimes creates problems in the chromatographic methods as minute amounts of related substances may be hidden under the peak of the drug itself. This is the background for interest in peak purity tests. With the advent of diode-array detection in liquid chromatography compounds with different chromophores may be differentiated, either through spectral comparison or by absorbance rationing at selected wavelengths. However, a peak impurity present at below 1% may be difficult to detect. The use of mass spectral data for the verification of peak purity is still better and has been practiced in gas chromatography-mass spectrometry (GC-MS). However the peak purity tests are inferior to the use of complimentary separation systems.

Physicochemical Characterization

Physicochemical characterization yields a number of important parameters that can be used in the control of quality of an analyte. Typical properties are melting point and other thermal data, acid base behavior with pKa values, redox potentials, polymorphism, solubility and spectral information.

Purity Tests

Purity tests are in particular focused on related substances such as homologues, analogues, and by-products from the synthesis or degradants. Enantiomeric purity has been a more common test since the early 1980s. Chromatography has revolutionized our ability to determine the analyte purity and is currently the most important check, giving essentially the fingerprint of a synthesis. For qualitative studies LC-MS is beginning to establish its role as the primary online analytical tool for the elucidation of unknown structures among the impurities. From a toxicological point of view the impurity profile of the analyte batch used in safety studies should form a reference for the full scale production material. This means that in batches, impurities in amounts that deviate from those found in batches used for toxicology should be avoided. The high standard and good reproducibility required for the purity profile is clearly evident from that perspective. There are some other tests that also contribute to the general impression of the quality of a analyte, i.e. tests for protolytic impurities, content of chloride, sulphated ash or residue upon ignition that gives the inorganic content. This test reflects the performance of the purification process in general.

Heavy metals

Heavy metals are routinely determined, often with one or other forms of sulphide precipitation. These tests are performed from the viewpoint of safety and general limits (1-30ìg/ml) are now more often related to the dose. For metals such as mercury, lead, cadmium or nickel atomic absorption spectrometry or other instrumental methods

are often prescribed. Copper and other transition metals can act as catalysts in certain degradation reaction and thus require special attention.

Potency Assay

Assay is the estimation of potency of an active principle in a unit quantity of preparation. Common for all bulk drugs is an assay of potency. This can be aqueous or non aqueous titration based on protolytic properties or on some other property. Ideally assays should not only be specific for chemical entity under examination, but also be stability determining.

Many compounds lack functional groups suitable for titration and here chromatographic methods (LC in particular are often used). However, titrations are preferable as their precision is, in general superior.

Content Uniformity

Consistency of dosage units is important. All divided dosage forms have strict requirements for uniformity of contents, i.e. a statistical sampling of a batch should show a uniform distribution of the active component. This involves individual dosage form assay. This requirement is especially important for units with very small amounts of active components i.e. from a few μg per dose to 50mg per dose.

Dissolution Testing

Dissolution is defined as the process by which a solid substance enters in a solvent to yield a solution. The pharmacokinetic performance of a drug influences the construction of the formulation. The characteristic properties of the formula are evaluated in in-vivo tests

where blood samples are analyzed often by extremely sensitive bioanalytical methods. However for routine quality control it is usual to rely on in-vitro models which obviously have to be correlated with the in-vivo data.

ANALYTICAL APPROACH

In order to investigate problem in the field of both natural and social sciences, an analytical approach is generally used first. That is to say, the problem is first broken down into simple units, each of which is studied, and then after the individual pieces of information are combined, the problem as a whole is finally understood.

If the analytical scientist is to be effective in solving the problems of the society, there is another dimension to his/her role, which must be recognized. A successful analytical chemist must be adept to number of techniques and must be at the forefront of chemical knowledge. The function of an analytical chemist is to provide information of sufficiently valid nature that is of the requisite statistical significance so that meaningful decisions can be made about materials or problems. The emphasis here again is that an analytical chemist is essentially a 'problem solver' and to do the job most effectively, he/she should use a complete analytical approach for solving the problems.

Development of New Analytical method

One of the major decision to be made by analytical chemist is the choice of the most effective procedure for a given analysis. In order to arrive at the correct decision, he must be familiar with the practical details of the various techniques and of theoretical principle upon which they are based. The techniques have differing degrees of sophistication, of sensitivity, of selectivity of cost and also

of time requirement. This will require the careful consideration of the following criteria.

- The type of the analysis required : elemental or molecular, routine or occasional.
- Problem arising from the nature of the materials to be investigated.
- Possible interferences from components of the materials other than those of the interest.
- The concentration range that need to be investigated.
- The accuracy and precision required.
- The facilities available ; this will refer particularly to all the kinds of instrumentation, which are at hand.
- The time required for the completing the analysis ; this will be particularly relevant when the analytical results are required quickly for the control of manufacturing processes.
- The number of analysis of similar type , which have to be performed.
- Cost per test.

To develop a sensitive and specific method of analysis one should the related information about the sample;

- Number of components present.
- Chemical structure of compounds.
- Molecular weight of the compounds.
- PKa values, melting point and boiling points of the compounds.
- UV spectra of compounds.

- Concentration range of compounds in the sample.
- Sample solubility.

Method Validation

Analytical monitoring of a pharmaceutical product, 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. Ideally this monitoring should be conducted in accordance in specification elaborated and validated during product development. The principle of analytical validation is to ensure that the selected analytical procedure will give reproducible and reliable results that are adequate for the intended purpose. It is thus necessary to define properly, both the conditions in which the procedure is to be used and the purpose for which it is intended. The USP has published specific guidelines for method validation for compound evaluation. USP defines eight steps for validation.

- Accuracy
- Precision
- Specificity
- Limit of detection
- Limit of quantitation
- Linearity and range
- Ruggedness
- Robustness
- Solution stability

The FDA has also published guidance for the validation of bioanalytical methods. The most comprehensive

document is the conference report of the 1990 Washington conference.

Strategy for the validation of the methods

The validity of the specific method should be demonstrated in laboratory experiments using samples or standards that are similar to unknown samples analysed routinely. The preparation and execution should follow a validation protocol, preferably written in a step-by-step instruction format. This proposed procedure assumes that the instrument has been selected and the method has been developed. It meets criteria such as ease of use ; ability to be automated and to be controlled by computer systems; cost per analysis; sample throughput; turnaround time; and environmental, health and safety requirements.

- Develop a validation protocol, an operating procedure or a validation master plan for the validation
- For specific validation project define owners and responsibilities
- Develop a validation project plan
- Define the application, purpose and scope of the method.
- Define the performance parameters and acceptance criteria
- Define validation experiments
- Verify relevant performance characteristics of equipment
- Quality materials, e.g. standards and reagents for purity, accurate amounts and sufficient stability
- Perform pre-validation experiments
- Adjust method parameters or/ and acceptance criteria if necessary

- Perform full internal and external validation experiments
- Develop SOPs for executing the method in the routine
- Define criteria for revalidation
- Document validation experiments and results in the validation report.

Steps in Method Validation

Successful acceptance of the validation parameters and performance criteria, by all parties involved, requires the cooperative efforts of several departments, including analytical development, QC, regulatory affairs and the individuals requiring the analytical data. The operating procedure or the validation Master plan (VMP) should clearly define the roles and responsibilities of each department involved in the validation of analytical methods.

The scope of the method and its validation criteria should be defined early in the process. These include the following questions:

- What analytes should be detected? What are the expected concentration levels?
- What are the sample matrices?
- Are there interfering substances expected, and, if so, should they be detected and quantified.
- Are there any specific legislative or regulatory requirements?
- Should information be qualitative and quantitative?
- What are the required detection and quantitation limits?
- What is expected concentration range?
- What precision and accuracy is expected?

- How robust should the method be?
- Which type of equipment should be used? Is the method for one specific instrument, or should it be used by all instrument of the same type?
- Will the method be used in one specific laboratory or should it be applicable in all laboratories at one side or around the globe?
- What skills do the anticipated users of the method have?

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

Validation Parameters for specific Tasks

The Validation experiments should be carried out by an experienced analyst to avoid errors due to inexperience. The analyst should be very well versed in the technique and operation of the instrument. Before an instrument is used to validate a method, its performance specification should be verified using generic chemical standards. Satisfactory results for a method can be obtained only with equipment that is performing well. Special attention should

be paid to those equipment characteristics that are critical for the method.

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

1. available in sufficient quantities
2. accuracy identified
3. sufficiently stable
4. checked for exact composition and purity

There are no official guidelines on the correct sequence of validation experiments, and the optimal sequence may depend on the method itself. Based on the author's experience, for a liquid chromatographic method, the following sequence has proven to be useful:

1. Selectivity of standards (optimizing separation and detection of standard

   mixtures if selectivity is insufficient)
2. Linearity, limit of quantitation, limit of detection, range
3. Repeatability (short-term precision) of retention times and peak areas
4. Intermediate precision
5. Selectivity with real samples
6. Trueness/accuracy at different concentrations
7. Ruggedness (interlaboratory studies)

During method validation, the parameters, acceptance limits and frequency of ongoing system suitability tests or QC checks should be defined. Once the method has been developed and validated, a validation report should be prepared.

REFERENCES

1. Lioyd R. Snyder, Joseph J. Kirkland, Joseph J. Glajch. "Practical HPLC Method Development", 2nd Edition published by John Wiley & Sons, 1997.
2. S.M. Khopkar, Basic Concept of Analytical Chemistry, Wiley Eastern Limited, New Delhi
3. Johan Lindholm, "Development and Validation of HPLC Methods for Analytical and Preparative Purposes" Acta Universities Upsaliensis 2004
4. L.R. Snyder & J.J. Kirkland, "Introduction to Modern Liquid Chromatography", 2nd Edition, Published by John Wiley & Sons.
5. "Merck Index" 14th Edition.
6. B. K. Sharma, Instrumental methods of Chemical Analysis, Goel Publishing House, Meerat, 1998
7. "Martindale-The Complete Drug Reference", 32nd Edition.
8. Robert L. Grob, Eugene F. Barry, "Modern Practice of Gas Chromatography", 4th Edition published by John Wiley & Sons.
9. Raymond P.W Scott, "Liquid Chromatography", Chrom Ed Book Series.
10. D.A Skoog, D.M West, F.J Holler, S.R Crouch, "Fundamentals of Analytical Chemistry", 8th Edition, Thompson Brooks/Cole.
11. John Kenkel, "Analytical Chemistry for Technicians", 3rd Edition.
12. A. Braithwaite and F.J Smith, "Chromatographic Methods", 5th Edition, Kluwer Academic Publishers.

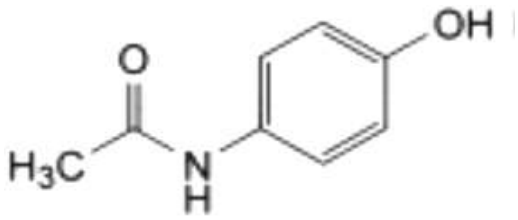
13. Elena Katz, Roy Eksteen, Peter Schoemakers, Neil Miller, "Chromatography Handbook of HPLC", Edited by Chromatographic Science Series, Volume 79.
14. "Indian Pharmacopoeia" Volume I and II, New Delhi, 1996.
15. "The United States Pharmacopoeia", United States Pharmacopoeial Convention Inc, USA, 2006.
16. "British Pharmacopoeia" Volume I and II, Her Majesty's Stationary office, London, 2009.
17. Ian A. Fowlis, "Gas Chromatography", Analytical Chemistry by open Learning, 2nd edition, Published by John Wiley and Sons.
18. W.J Lough, I.W Wainer, "High Performance Liquid Chromatography", Edited by Blackie Academic and Professional.
19. P.D Sethi, Volume I, "HPLC : Quantitative Analysis of Pharmaceutical Formulations, CBS Publishers and Distributors.
20. A. Pryde, M.T. Gilest, "Applications of HPLC", Chapman and Hall, London, (1979).
21. International Conference on Harmonization, "Validation of Analytical Procedures: Methodology," ICH Steering Committee, (1996).

CHAPTER - II

Drug Profile

DRUG PROFILE

1. PARACETAMOL



CAS Name

Action and use

Analgesic; antipyretic..

Molecular Formula: $C_8H_9NO_2$

Molecular Weight: 151.2

Melting point : 168 °C to 172 °C.

Appearance

White or almost white, crystalline powder.

Solubility

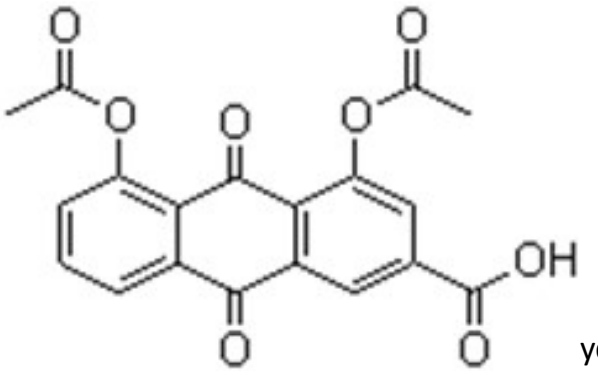
Sparingly soluble in water, freely soluble in alcohol, very slightly soluble in methylene chloride.

Dosage: Tablet

Pharmacology:

Paracetamol has a narrow therapeutic index – the therapeutic dose is close to the toxic dose. Additionally, paracetamol is contained in many preparations. This means that, despite being one of the safest analgesics available at recommended doses, there is large dosage for overdose and toxicity. Paracetamol is mostly converted to inactive compounds via Phase II metabolism by conjugation with sulfate and glucuronide, with a small portion being oxidized via the Cytochrome P450 enzyme system. Cytochrome P4502E1(CYP2E1) converts paracetamol to a highly reactive intermediary metabolite, N-acetyl –p- benzo-quinone imine (NAPQI)

2. DIACEREIN



CAS Name ydro-9,10-dioxo-

anthracene-2-carboxylic acid];

Action and use

It is found to be effective in the treatment of osteoarthritis as a synthetic chemical and also in native form from many plants.

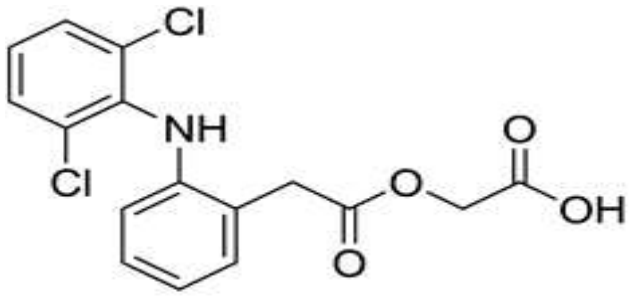
Molecular Formula : $C_{19}H_{12}O_8$

Molecular Weight : 368.294

Pharmacology:

Diacerein is an Anthraquinone derivative 9, 10-dihydro-4, 5 bis (acetyl)-9, 10-dioxo-2- anthracene carboxylic acid, mainly used in osteoarthritis. From the anthronoid, Diacerein and its metabolites Rhine break both the production of zylokine (IL-1, IL-6, IL-16 TNFa) at the point of the inflammation cascade and proteolytic enzyme. Additionally Diacerein stimulates the synthesis of cartilage components such as proteoglycane, glykosaminoglycane and hyaluronsaure1-2. It is not official in any pharma-copoeia. The solubility of a poorly soluble drug can be altered in many ways, such as modification of drug crystal forms, addition of co-solvents, addition of surfactant, and complexation with cyclodextrin etc.

3. ACECLOFENAC



CAS Nam ıyl acetoxy acetic acid,

Action and use

It has pronounced anti-inflammatory, antipyretic, antirheumatoid and analgesic effect and an improved gastro-intestinal tolerance. It is use in various pain

conditions like rheumatoid arthritis, osteoarthritis and ankylosing spondylatis.

Molecular Formula : $C_{16}H_{13}Cl_2NO_4$

Molecular Weight : 354.19

Appearance:

White to almost white crystalline powder

Solubility:

Practically insoluble in water, freely soluble in acetone, soluble in ethanol (96 per cent).

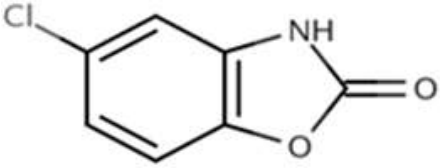
Indication:

It is a highly effective anti-inflammatory drug and used for the treatment of arthritis.

Pharmacology:

Aceclofenac belongs to a group of medicines called non-steroidal anti-inflammatory drugs (NSAIDs). It works by blocking the action of a substance in the body called cyclo-oxygenase. Cyclo-oxygenase is involved in the production of various chemicals in the body. Some of which are known as prostaglandins. Prostaglandins are produced in response to injury or certain diseases and would otherwise go on to cause pain, swelling and inflammation. Aceclofenac is used to relieve pain and inflammation in arthritic conditions.

4. CHLORZOXAZONE



CAS Name: 5-chloro

Action and use

A centrally acting central muscle relaxant with sedative properties. It is claimed to inhibit muscle spasm by exerting an effect primarily at the level of the spinal cord and subcortical areas of the brain

Molecular Formula : $C_7H_4ClNO_2$

Molecular Weight : 169.56

Melting point : 191.5°C

Appearance

It is a white to off white crystalline powder

Solubility

Soluble in water, but freely soluble in alcohols especially methanol.

Indication:

For the relief of discomfort associated with acute painful musculoskeletal conditions.

Pharmacology:

Chlorzoxazone, a synthetic compound, inhibits antigen-induced bronchospasms and, hence, is used to treat asthma and allergic rhinitis. Chlorzoxazone is used as an ophthalmic solution to treat conjunctivitis and is taken orally to treat systemic mastocytosis and ulcerative colitis. Chlorzoxazone is also a centrally-acting agent for painful musculoskeletal conditions. Data available from animal experiments as well as human study indicate that chlorzoxazone acts primarily at the level of the spinal cord and subcortical areas of the brain where it inhibits multisynaptic reflex a.c. involved in producing and maintaining skeletal muscle spasm of varied etiology. The

clinical result is a reduction of the skeletal muscle spasm with relief of pain and increased mobility of the involved muscles.

Mechanism of Action:

Chlorzoxazone inhibits degranulation of mast cells, subsequently preventing the release of histamine and slow-reacting substance of anaphylaxis (SRS-A), mediators of type I allergic reactions. Chlorzoxazone also may reduce the release of inflammatory leukotrienes. Chlorzoxazone may act by inhibiting calcium influx

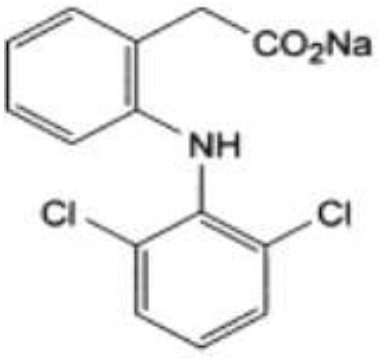
Toxicity:

Oral, mouse: LD_{50} = 440 mg/kg; Oral, rat: LD_{50} = 763 mg/kg; Symptoms of overdose include diarrhoea, dizziness, drowsiness, headache, light-headedness, nausea, and vomiting.

Biotransformation:

Chlorzoxazone is rapidly metabolized in the liver and is excreted in the urine, primarily in a conjugated form as the glucuronide.

5. DICLOFENAC SODIUM



CAS Name: Sodium 2-[(2,6-dichlorophenyl) amino]phenyl] acetate.

Action and use

Cyclo-oxygenase inhibitor; analgesic; anti-inflammatory.

Molecular Formula : $C_{14}H_{10}Cl_2NNaO_2$

Molecular Weight : 318.1

Melting point : 283-285°C

Appearance

White or slightly yellowish, slightly hygroscopic, crystalline powder.

Solubility

Sparingly soluble in water, freely soluble in methanol, soluble in ethanol (96 per cent), slightly soluble in acetone.

Indication:

For the acute and chronic treatment of signs and symptoms of osteoarthritis and rheumatoid arthritis.

Pharmacology:

Diclofenac is an acetic acid nonsteroidal anti-inflammatory drug (NSAID) with analgesic and antipyretic properties. Diclofenac is used to treat pain, dysmenorrhea, ocular inflammation, osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, and actinic keratosis

Mechanism of Action

The anti-inflammatory effects of diclofenac are believed to be due to inhibition of both leukocyte migration and the enzyme cylooxygenase (COX-1 and COX-2), leading to the peripheral inhibition of prostaglandin synthesis. As prostaglandins sensitize pain receptors, inhibition of their

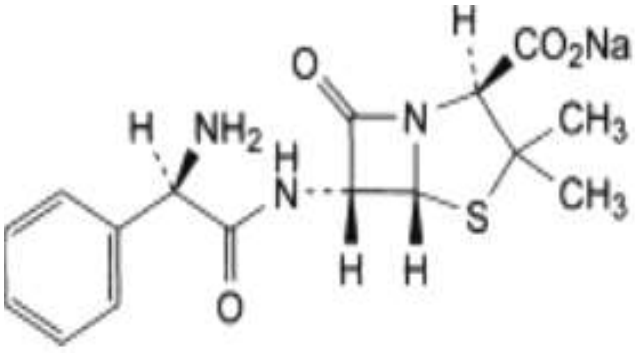
synthesis is responsible for the analgesic effects of Diclofenac. Antipyretic effects may be due to action on the hypothalamus, resulting in peripheral dilation, increased cutaneous blood flow, and subsequent heat dissipation.

Toxicity:

Symptoms of overdose include loss of consciousness, increased intracranial pressure, and aspiration pneumonitis. LD_{50}=390mg/kg (orally in mice)

Biotransformation: Hepatic.

6. AMPICILLIN SODIUM



CAS Name: Sodium (2*S*,5*R*,6*R*)-6-[[(2*R*)-2-amino-2-phenylacetyl]amino]-3,3-

dimethyl-7-oxo-4-thia-1- azabicyclo[3.2.0]heptane-2-carboxylate.

Action and use

Penicillin antibacterial.

Molecular Formula : $C_{16}H_{18}N_3NaO_4S$

Molecular Weight : 371.4

Melting point : 215^0C

Appearance

White or almost white powder, hygroscopic.

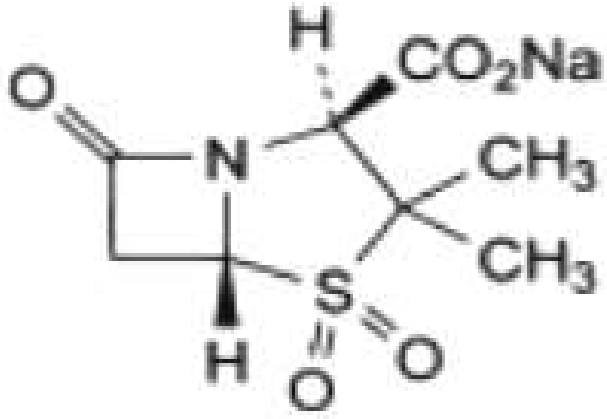
Solubility

Freely soluble in water, sparingly soluble in acetone, practically insoluble in fatty oils and in liquid paraffin.

Pharmacology:

Ampicillin a derivative of 6-aminopenicillanic acid has a wide therapeutic use. But it is ineffective against organisms producing â-lactamase. Hence it needs to be administered with a â-lactamase inhibitor such as Sulbactam.

7. SULBACTAM SODIUM



CAS Name : (2*S*,5*R*)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo [3.2.0]heptane-2- carboxylic acid 4,4-dioxide

Action and use

Beta-lactam antibacterial.

Molecular Formula : C8H10NNaO5S

Molecular Weight : 255.2

Appearance

White or almost white, hygroscopic, crystalline powder.

Solubility

Freely soluble in water, sparingly soluble in ethyl acetate, very slightly soluble in ethanol (96 per cent). It is freely soluble in dilute acids.

Pharmacology:

Sulbactam is a molecule that is given in combination with beta-lactam antibiotics to inhibit beta-lactamase, an enzyme produced by bacteria that destroys the antibiotics. Sulbactam is an irreversible inhibitor of beta-lactamase; it binds the enzyme and does not allow it to interact with the antibiotic Sulbactam is able to inhibit the most common forms of beta-lactamase but is not able to interact with the ampC cephalosporinase. Thus, it confers little protection against bacteria such as Pseudomonas aeruginosa, Citrobacter, Enterobacter, and Serratia, which often express this gene.

CHAPTER- III

Simultaneous determination of Diacerein and Aceclofenac in tablet dosage form by high performance liquid chromatography

METHOD DEVELOPMENT:

Method development in chromatography is the setting up of an analytical procedure that will be appropriate for the analysis of particular sample. In liquid chromatography the choice of the stationary phase and selection of the mobile phase plays a vital role in the separation of analytes. The choice of the stationary phase and mobile phase depends upon the nature of the analyte.

Selection of chromatographic mode:

Reverse phase chromatography is the first choice of separation for most regular samples, due to its simplicity and best column performances. Development of the method depends upon the nature of the analyte (ionic/ nonionic), its molecular weight and solubility. In present research work the drugs selected are less ionic in nature. The hydrophobicity of Diacerein is 1.9 and its molecular weight is 368.29. Its molecular formula is $C_{19}H_{12}O_8$. The molecular weight of Aceclofenac is 354.2. The hydrophobicity of Aceclofenac is greater than Diacerein and hence in reverse phase liquid chromatography it retains for a longer time as compared to Diacerein. Hence, a reverse phase mode of separation was employed taking into account the semi polar nature of the drug molecules.

Selection of stationary phase:

The availability of a stable high performance column is essential in developing a rugged and a reproducible method. When selecting an HPLC column it is necessary to consider column to column reproducibility. The selection of the column depends upon the nature of the analyte. Many columns have been tried in the separation of analytes.

Finally the Inertsil C18 column with 250mm × 4.6mm i.d with a 5 µm particle size Inertsil C18 column was employed as the stationary phase in the separation of the analytes

Selection of mobile phase:

Acetonitrile and Methanol are the most popularly used organic solvents in HPLC. Initially Acetonitrile – Water mixture was tried for the development of the method because it has lower UV cut off (185-210) and low viscosity leading to higher plate numbers and lower column back pressures. However methanol is also a reasonable alternative in place of acetonitrile. In this present research work many compositions of the mobile phases have been tried. Finally a mixture of buffer, acetonitrile and methanol was selected for the separation of analytes. For better separation and resolution the different buffers were tried. It has been found that potassium dihydrogen phosphate buffer, pH 5.9 adjusted with 0.1% triethylamine gave better peak shape than other buffers. The different compositions of mobile phase were tried for getting better separation of analytes. Thus the mobile phase composed of the mixture of methanol, acetonitrile and buffer (0.02MKH_2PO_4, pH 5.9

adjusted with 0.1% triethylamine) in the ratio of (50:20:30 v/v/v) was finalized.

Selection of Detector and Detection wavelength:

The detector is chosen depending upon some characteristic properties of the analyte like UV absorbance, fluorescence, conductance, oxidation, reduction etc. Diacerein and Aceclofenac shows the absorbance in the UV region of the electromagnetic spectrum. Hence a UV- Visible detector was chosen for the detection of the eluent in the chromatographic process. The ëmax of Diacerein and Aceclofenac is 258nm and 276nm respectively. But both the drugs have shown maximum absorbance at 265nm wavelength. Hence it is the most suitable wavelength for the analysis of the analytes.

Chromatographic conditions:

The chromatographic system consist of a Waters HPLC system having Waters 501 isocratic pump equipped with Waters ™ 717plus autosampler and a Waters 486 tunable absorbance UV-detector. The data was recorded using Millenium[32] chromatographic software. Separation was performed on a 250mm × 4.6mm i.d., 5 µ particle size Inertsil C18 column.

Mobile phase consisted of a mixture of methanol: acetonitrile: buffer (50:20:30v/v/v), pH 5.9 adjusted with 0.1% triethylamine. Flow rate was kept at 1.0mL/min. Wavelength was set at 265nm.

Table 3.1 Optimized Chromatographic conditions for Diacerein and Aceclofenac

Chromatographic mode	Chromatographic condition
Standard solution	100 µg/mL of Paracetamol , 100
Equipment system	µg/mL of Chlorzoxazone and 10
Pump	µg/mL of Diclofenac Na
Detector	Waters HPLC
Data processor	Waters 501 isocratic pump
Injector	Waters 486 tunable absorbance UV-
Stationary phase/Column	detector
Buffer solution	Millenium^{32} chromatographic
Mobile phase	software
	Waters ^{TM} 717plus autosampler
Detection wavelength	Inertsil C_{18} column (250 X 4.6 mm ,
Flow rate	i.d 5µm)
Injection Volume	Aqueous buffer solution of 0.02M
Column temperature	KH_2PO_4
Pump Mode	Buffer(0.02M KH_2PO_4, pH 5.9
	adjusted with 0.1% triethylamine) :
	Acetonitrile : Methanol(30 : 20 :
	50,v/v/v)
	265 nm
	1.0mL/min
	20µl
	Ambient
	Isocratic

EXPERIMENTAL CONDITIONS:

Instruments:

Waters HPLC system having Waters 501 isocratic pump equipped with ™ 717plus auto sampler and a Waters 486 tunable absorbance UV-detector. Millenium^{32} software was used for data acquisition.

Reagents

HPLC grade Acetonitrile, AR grade potassium dihydrogen phosphate, Triethylamine and double distilled water was used throughout the work.

Preparation of standard solutions:

50 mg of Diacerein USP working standard and 100 mg of Aceclofenac USP working standard was weighed and transferred to a 100mL volumetric flask along with 10.0 mL of Dimethyl sulfoxide and then sonicated to dissolve. The solution was cooled to room temperature and diluted to 100mL volume with diluents. 5 mL of the above solution was diluted to 50 mL with diluent to give 50 µg/mL of Diacerein and 100 µg/mL of Aceclofenac.

Preparation of Sample solution:

Twenty tablets (Dyacerin-A, Glenmark) were weighed and the average weight was calculated. These tablets were powdered and weight equivalent to one tablet was taken in a 100 mL volumetric flask, 10mL of Dimethyl sulfoxide was added and sonicated for 20minutes and shaken by mechanical means for 20minutes at 250rpm. Further the solution was diluted with diluents. The solution was mixed and allowed to settle for 5 minutes and then filtered through 0.45µ syringe filter. 5 mL of the filtrate was diluted to 50 mL with diluents and mixed. The concentrations obtained were 50 µg/mL of Diacerein and 100 µg/mL of Aceclofenac. 20 µL of standard and sample solutions were injected in triplicate under optimized chromatographic conditions.

METHOD IN BRIEF:

A simple, precise, fast and selective stability indicating reverse phase High performance liquid chromatography

(HPLC) method has been developed for the simultaneous determination of Diacerein and Aceclofenac from tablets. The separation of Diacerein and Aceclofenac was carried out on an Inertsil ODS column using Methanol, Acetonitrile and Potassium dihydrogen phosphate buffer as the mobile phase. The mobile phase consisted of methanol, acetonitrile and 0.02M KH_2PO_4 buffer (pH=5.9adjusted with 0.1% triethylamine) in the ratio (50:20:30, v/v/v). Separation was carried out isocratically at ambient temperature and flow rate of 1.0mL/min. The detection of analytes was carried out at 265nm using UV-Visible detector at the wavelength of maximum absorbance of the analytes. Both the analyte shows maximum absorbance at 265nm wavelength. The proposed HPLC method was validated and applied for the simultaneous determination of Diacerein and Aceclofenac from its pharmaceutical dosage preparation and bulk drugs. All the analytical data was subjected to statistical analysis.

METHOD VALIDATION OF HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF DIACEREIN AND ACECLOFENAC IN TABLET DOSAGE FORM BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The following experiment was carried out to determine the working concentration range for Diacerein and Aceclofenac. The proposed developed method was subjected to method validation process to determine its suitability for intended use. With reference to the following parameters the method was validated.

1) Selectivity and Specificity 2) Degradation studies

3) System suitability 4) Linearity

5) Precision (Repeatability) 6) Accuracy (% Recovery)

7) LOD and LOQ 8) Solution stability
9) Intermediate precision (Ruggedness) 10) Robustness

SELECTIVITY AND SPECIFICITY:

Specificity is the measure of the degree of the interference in the analysis of the complex sample mixtures such as analyte present in the matrix containing endogenous substances and related chemical compounds, etc. To validate specificity of the method following samples were prepared and injected into the HPLC system and chromatograms were recorded.

Blank solution i.e. diluent

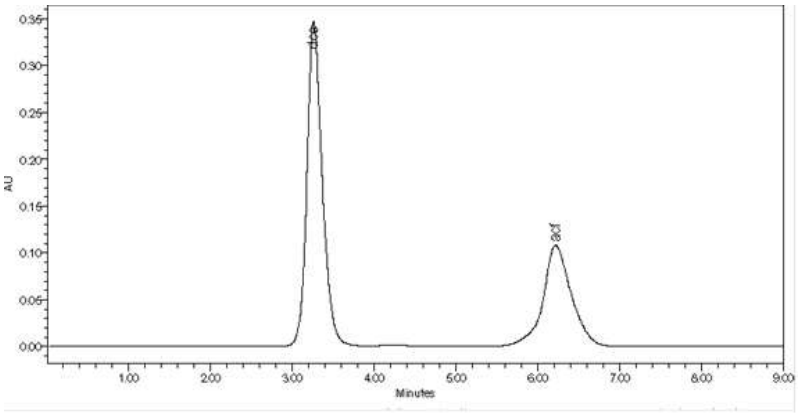
Blank + individual drug having assay concentration of individual drug

Blank + all drugs having assay concentration of individual drug

By performing this exercise it has been found that there were no interferences at the retention time of the individual drugs due to other components of the sample solution.

Chromatogram of standard preparation

Figure 3.1 Chromatogram of mixed standard solution containing Diacerein and Aceclofenac



Chromatogram of Sample preparation

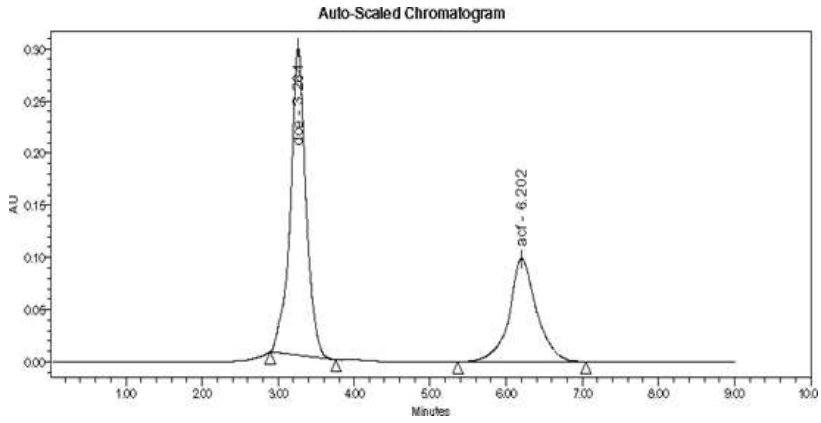


Figure 3.2 In the optimized conditions Diacerein has a Rt=3.26 and Aceclofenac has a Rt=6.20. The major peaks are well separated. There are no other potentially interfering peaks at the retention time of the two drugs, thus showing the specificity of the method

DEGRADATION STUDIES:

Forced degradation or stress studies are undertaken to deliberately degrade the sample. These studies are used to evaluate an analytical method's ability to measure an active ingredient and its degradation products, without interference, by generating potential degradation products. During validation samples of the drug product and drug substances are exposed to heat, light, acid, base and oxidizing agent to produce approximately 10% to 30% degradation of the active substance.

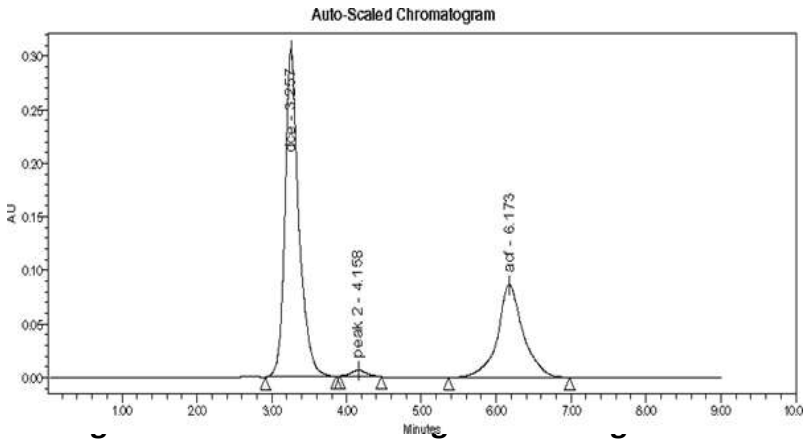
In the present research work the samples of Diacerein and Aceclofenac were subjected under forced degradation.

Diacerein + Aceclofenac tablets were subjected to acid, base, oxidative and thermal degradation to obtain degradation of about 10% - 30% in at least one of the forced degradation conditions. The samples required for forced degradation studies were prepared as per the following procedure.

1) Acid degradation

Five tablets were weighed and their average weight was calculated. These tablets were powdered and 0.2349 gm of powdered sample was transferred to a 100mL volumetric flask, 10mL of dimethyl sulfoxide was added and sonicated for 10minutes and 20mL of diluent was added in it. Then 10mL of 0.1N HCl was added and sonicated for 5 minutes. The contents were allowed to stand for 15 minutes in water bath at 70^0C temperature. Then the solution was cooled at room temperature and it was neutralized with 0.1N NaOH solution with continuous shaking. Then the solution was diluted with diluent and filtered through syringe filter. 5mL of this filtered solution was diluted to 50mL in a dilution flask with diluent. This sample solution was injected into a chromatogram three times and average peak area was calculated. From the mean peak area the % of degradation was calculated. Following chromatogram represents the degradation of Diacerein and Aceclofenac.

HPLC Chromatogram of (Acid degradation) sample solution



(0.1NHCl)

2) Alkali degradation

0.2349 gm of powdered sample was transferred to a 100mL volumetric flask, 10mL of dimethyl sulfoxide was added and sonicated for 10minutes, 20mL of diluent was added to it. 10mL of 0.1N NaOH was then added and sonicated for 5 minutes. The contents were allowed to stand for 15 minutes in water bath at a temperature of 70^0C. The solution was cooled at room temperature and it was neutralized with 0.1N HCl solution with continuous shaking. The solution was then diluted with diluent and filtered through syringe filter. 5mL of this filtered solution was diluted to 50mL in a dilution flask with a diluent. This sample solution was injected three times into a HPLC system and the average peak area was calculated. From the mean peak area the % of degradation was calculated. Following chromatogram represents the degradation of Diacerein and Aceclofenac

HPLC Chromatogram of (Alkali degradation) sample solution

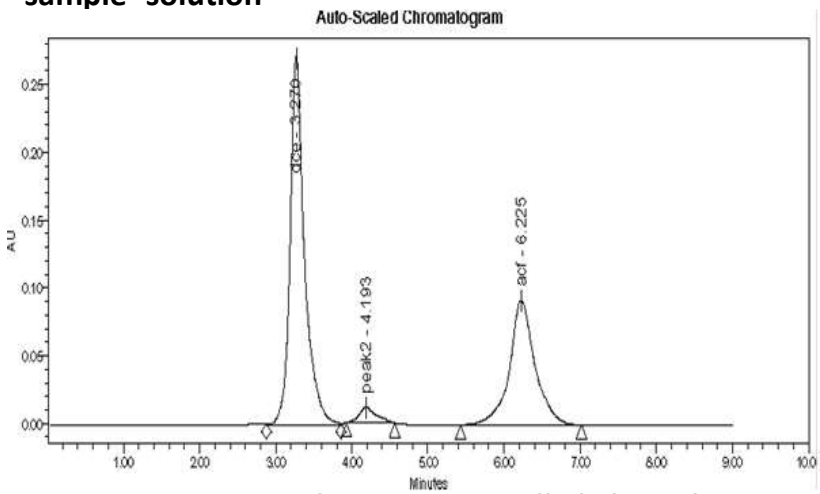


Figure 3.4 HPLC Chromatogram alkali degradation (0.1N NaOH)

3) Oxidative (H_2O_2) degradation

0.2349 gm of powdered sample was transferred to a 100mL volumetric flask, 10mL of dimethyl sulfoxide was added and sonicated for 10minutes, 20mL of diluent was added to it. 10mL of 10% H_2O_2 was then added and sonicated for 5 minutes. The contents were allowed to stand for 15 minutes in water bath at a temperature of 70^0C. The solution was then cooled at room temperature. Then the solution was diluted with diluent and filtered through syringe filter. 5mL of this filtered solution was diluted to 50mL in a dilution flask with a diluent. This sample solution was injected three times into a chromatogram and the average peak area was calculated. From the mean peak area the % of degradation was calculated. Following chromatogram represents the degradation of Diacerein and Aceclofenac.

HPLC Chromatogram of (oxidative degradation) sample solution

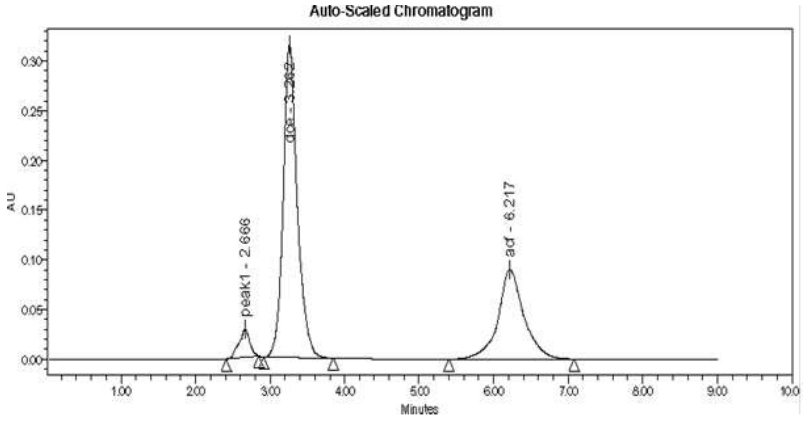
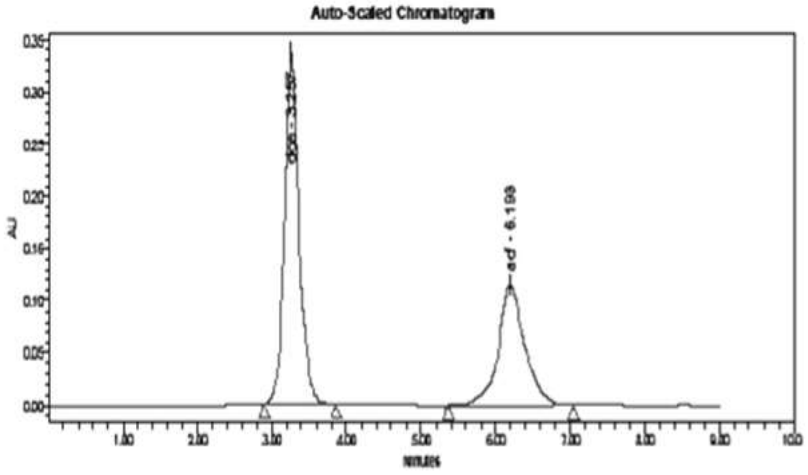


Figure 3.5 HPLC Chromatogram oxidative degradation (10% H_2O_2)

4) Thermal degradation:

Diacerein – A tablet Containing diacerein and Aceclofenac was exposed to 105^0C for 24 hrs and transferred to a 100mL volumetric flask then 10mL of dimethyl sulfoxide was added and sonicated for 10minutes then diluted to 100mL with a diluent. The solution was mixed and allowed to settle for 5 minutes. The solution was then filtered through a syringe filter. 5mL of this filtrate was diluted to 50mL with a diluent and this solution was used for further analysis. The chromatogram shows no degradation takes place in the presence of heat.



showed degradation of the analytes with acid, alkali and oxidation, while there was no thermal degradation of the analytes. It indicates that the proposed developed HPLC method is stability indicating method. Following tables shows degradation study of diacerein and Aceclofenac.

For Diacerein

Table 3.2 Degradation study of Diacerein

Obs No	Conc. in µg/ml	Peak Area				
		100% SPL	Acid degradation	Alkali degradation	10% H_2O_2 degradation	Thermal degradation
1	50	4625154	3940729	3568012	4220549	4611215
2	50	4610125	3939851	3570014	4218985	4608845
3	50	4584451	3937877	3569546	4147882	4598878

Mean		4606577	3939486	3569191	4195805	4606313
S.D.		20582	1461	1047	41510	6547
%RSD		0.45	0.04	0.03	0.99	0.14

For Aceclofenac

Table 3.3 Degradation study of Aceclofenac

Obs NO	Conc. in µg/ml	Peak Area				
		100% SPL	Acid degradation	Alkali degradation	10% H_2O_2 degradation	Thermal degradation
1	100	2443014	2030486	2118256	2148855	2443359
2	100	2442798	2032028	2117546	2144451	2441611
3	100	2441061	2031881	2109830	2145671	2441016
Mean		2442291	2031465	2115211	2146326	2441995
S.D.		1071	851	4673	2274	1218
%RSD		0.04	0.04	0.22	0.11	0.05

Table 3.4 % Degradation of Diacerein and Aceclofenac

Conditions	Temp	Time	% degradation	
			Diacerein	Aceclofenac
0.1N NaOH	70^0C	15min	22.58%	13.49%
0.1N HCl	70^0C	15min	15.34%	16.91%
10% H_2O_2	70^0C	15min	10.47%	12.15%
Thermal	105^0C	24 hrs	No degradation	No degradation

SYSTEM SUITABILITY TEST:

A system suitability test should be carried out to see if the HPLC system is performing properly. System suitability tests were performed as per the ICH guidelines to confirm the suitability and the reproducibility of the system. The test was carried out by injecting standard solutions of Diacerein and Aceclofenac of the strength 100%

level. The standard deviation (S.D) and Relative standard deviation (%RSD) values of the five determinations are presented in table 3.5. The %RSD values were found to be satisfactory and meeting the requirements.

Table 3.5 System suitability test for Diacerein and Aceclofenac

Obs No	100% Level			
	Diacerein		Aceclofenac	
	Peak area	Retention time	Peak area	Retention time
1	4517486	3.27	2597754	6.21
2	4484345	3.26	2583826	6.22
3	4497438	3.25	2592180	6.20
4	4509368	3.26	2597554	6.22
5	4490984	3.27	2585250	6.20
Mean	4499924	3.26	2585250	6.21
S.D	13476	0.0065	19199	0.01
% RSD	0.30	0.20	0.40	0.16

LINEARITY RANGE FOR DIACEREIN AND ACECLOFENAC:

This experiment was carried out to demonstrate the range over which the response of the detector is linear with respect to concentration of Diacerein and Aceclofenac. In a series of volumetric flasks varying volumes of a solution containing Diacerein (500µg/mL) and Aceclofenac (1000µg/mL) was taken and then diluted upto the mark with diluent. Each level was injected three times and average area was calculated. Preparation of linearity levels were as per the following table.

Table 3.6 Preparation of mixed standard solution for Linearity levels

Linearity Levels	Volume of std stock solution added in mL	Volume made upto in mL	Concentration of Diacerein in ppm	Concentration of Aceclofenac in ppm
40%	2	50	20	40
60%	3	50	30	60
80%	4	50	40	80
100%	5	50	50	100
120%	6	50	60	120
160%	8	50	80	160
200%	10	50	100	200

Table 3.7 Linearity of Diacerein at different concentration levels

Obs No.	Conc. level %	Conc. in µg/ml	Peak Area			Mean peak area	S.D.	% RSD
			1	2	3			
1	40	20	1835145	1833892	1834978	1834672	680.4	0.037
2	60	30	2646201	2644749	2644983	2645311	779.6	0.029
3	80	40	3592534	3590201	3590870	3591202	1201.4	0.033
4	100	50	4480172	4485016	4465995	4477061	9884.8	0.221
5	120	60	5483225	5482947	5485171	5483781	1211.8	0.022
6	160	80	7278554	7270036	7240652	7263081	19885	0.274
7	200	100	8873693	8876883	8878046	8876207	2253.8	0.025

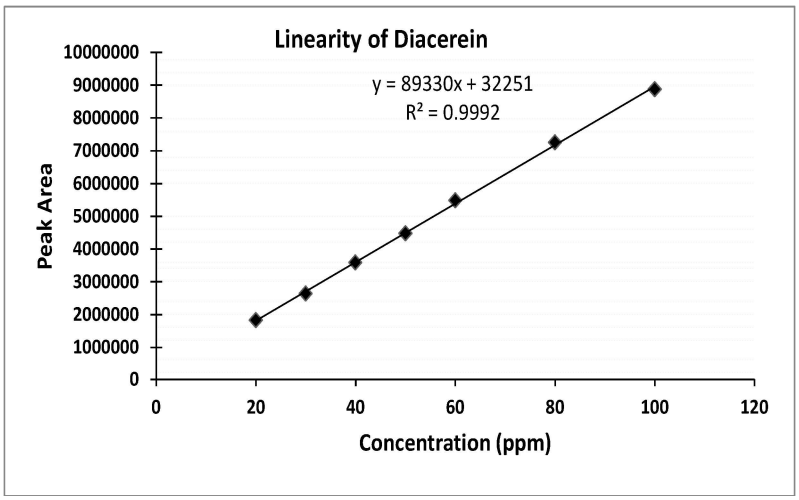


Fig 3.7 Linearity plot for Diacerein

Table 3.8 Linearity of Aceclofenac at different concentration levels

Obs No.	Conc. level %	Conc. in µg/ml	Peak Area			Mean peak area	S.D.	% RSD
			1	2	3			
1	40	40	1046986	1047571	1048874	1047810	966.49	0.092
2	60	60	1525815	1525042	1523052	1524636	1425.47	0.093
3	80	80	2065126	2063019	2060692	2062946	2217.91	0.108
4	100	100	2579981	2578180	2575801	2577987	2096.65	0.081
5	120	120	3160716	3161103	3159061	3160293	1084.63	0.034
6	160	160	4204097	4202963	4201167	4202742	1477.41	0.035
7	200	200	5153131	5152144	5149287	5151521	1996.37	0.039

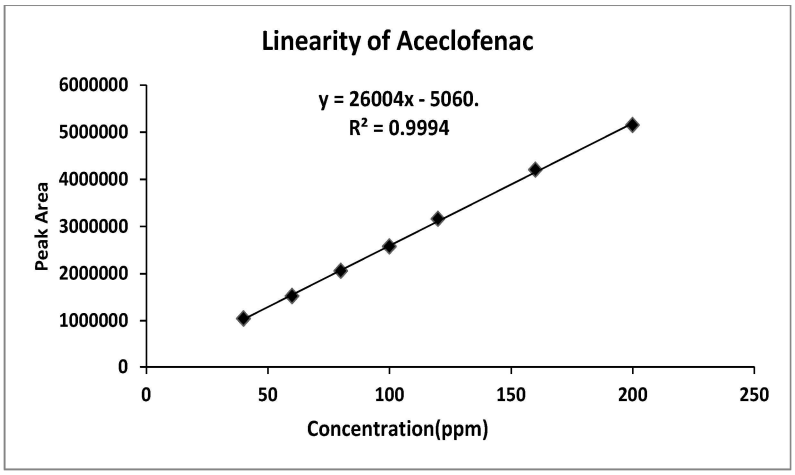


Fig 3.8 Linearity Level plot for Aceclofenac

REGRESSION ANALYSIS:

The calibration data range 20µg/mL to 100µg/mL for Diacerein and 40µg/mL to 200µg/mL for Aceclofenac was further considered for regression analysis. The regression analysis of the calibration data was carried out to determine the relationship between the dependent variable (Peak area) and the independent variable (Drug) concentration.

Applying the linearity plot from fig. 3.7 For Diacerein and fig 3.8 for Aceclofenac the values for the regression equation ***y = mx + C*** are found to be,

For Diacerein:

y = 8.93e+004 X + 3.25e+004

m = 89330 (Slope)

C = 32251 (Intercept)

x = Found concentration of the drug

y = Calculated peak area

For Aceclofenac:

y = 2.60e+004 X -5.060e+003

m = 26004 (Slope)

C = -5060 (Intercept)

x = Found concentration of the drug

y = Calculated peak area

The regression equation indicates that one unit increase in the concentration of the Diacerein and Aceclofenac will increase the detector response by 89330 and 26004 units respectively. Also by using the regression equation the actual values of the drug injected can be back calculated by substituting the value of y i.e. area of the drug. The correlation (r^2) for Diacerein and Aceclofenac was found to be 0.9992 and 0.9994 respectively.

SYSTEM PRECISION:

As a part of method validation, system precision was performed to determine the repeatability of the method. Six samples of standard were prepared at 100% level and assayed according to the procedure.

Table 3.9 Precision study for Diacerein

Obs No	Peak area	True value Conc. in µg/ml	Experimental values Conc. in µg/ml
1	4514360	50	50.17
2	4520807	50	50.25
3	4527757	50	50.32
4	4536582	50	50.42
5	4545052	50	50.52
6	4498154	50	49.99
Mean	4523785		50.28
S.D			0.17
%RSD			0.34
%Accuracy			100.56

Table 3.10 Precision study for Aceclofenac

Obs NO	Peak area	True value Conc. in µg/ml	Experimental values Conc. in µg/ml
1	2597754	100	100.09
2	2583826	100	99.56
3	2592180	100	99.88
4	2597554	100	100.09
5	2585250	100	99.61
6	2591313	100	99.85
Mean	2591313		99.85
S.D			0.21
%RSD			0.21
%Accuracy			99.85

METHOD PRECISION (REPRODUCIBILITY)

The method precision study was performed to determine the reproducibility of the method. Six samples of tablets were prepared at 100% level and assayed according to the procedure. The amount of the drug in mg per tablet was calculated by the following way.

Table 3.11 Reproducibility study for Diacerein

Obs No	Peak area	True value Conc. in µg/ml	Experimental values Conc. in µg/ml
1	4536934	50	50.15
2	4645042	50	51.34
3	4585307	50	50.68
4	4580737	50	50.63
5	4577334	50	50.59
6	4576124	50	50.58
Mean	4583579		50.66
S.D			0.35
%RSD			0.69
%Accuracy			101.32

Table 3.12 Reproducibility study for Aceclofenac

Obs NO	Peak area	True value Conc. in µg/ml	Experimental values Conc. in µg/ml
1	2498142	100	99.28
2	2505907	100	99.57
3	2515156	100	99.94
4	2525432	100	100.35
5	2529621	100	100.51
6	2537251	100	100.82
Mean	2518585		100.08
S.D			0.54
%RSD			0.54
%Accuracy			100.08

ACCURACY: (% RECOVERY)

Recovery experiments were carried out to check the efficiency of the method to extract and determine drugs in the presence of excipients present in the formulation. A standard addition method was employed for this experiment. A known quantity of each drug substance corresponding to 100%, 110%, 120% and 130% of the label claim of each drug was added. Each set of addition was repeated three times. The accuracy was expressed as a percentage of analytes recovered by the assay. In the present research work 100ppm sample solution was considered as 100% (0 level) and 10%, 20% and 30% standard drugs were added into it. Table 3.13 lists the recoveries of the drugs from a series of spiked concentrations.

Table 3.13 Accuracy study for Diacerein

Levels in %	Area of sample	Amt added in ppm	Amt found in ppm	% recovery
0%	4636934	50	51.25	102.50
	4612541	50	50.98	101.96
	4598120	50	50.82	101.64
10%	5015381	55	55.43	100.79
	5016312	55	55.44	100.81
	5015850	55	55.44	100.80
20%	5407672	60	59.77	99.62
	5406550	60	59.76	99.59
	5399102	60	59.67	99.46
30%	5905535	65	65.27	100.42
	5898887	65	65.20	100.31
	5894445	65	65.15	100.23
			Mean	100.68
			S.D.	0.53
			%RSD	0.53
			Range of % recovery	99.46-102.50

Table 3.14 Accuracy study for Aceclofenac

Levels in %	Area of sample	Amt added in ppm	Amt found in ppm	% recovery
0%	2545142	100	98.22	98.22
	2555768	100	98.63	98.63
	2556861	100	98.67	98.67
10%	2799320	110	108.03	98.21
	2805535	110	108.27	98.42
	2825342	110	109.03	99.12
20%	3044326	120	117.61	98.00
	3055101	120	117.90	98.25
	3050212	120	117.71	98.09
30%	3380629	130	130.46	100.35
	3355250	130	129.48	99.60
	3312180	130	127.82	98.32
			Mean	98.65
			S.D.	0.77
			%RSD	0.79
			Range of % recovery	98.00-100.35

LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTITATION (LOQ):

The limit of detection (LOD) is the amount of analyte which can be reliably detected under the stated experimental conditions. The limit of quantitation (LOQ) is the amount of analyte which can be reliably quantified under the stated experimental conditions. The limit of quantification (LOQ) and limit of detection (LOD) was established using signal-to-noise ratio. The LOQ and LOD of Diacerein and Aceclofenac were experimentally determined. The LOD of Diacerein and Aceclofenac was found to be 0.0025 µg/mL and 0.020 µg/mL respectively. The LOQ of Diacerein and Aceclofenac was found to be 0.005 and 0.045 µg/mL respectively.

SOLUTION STABILITY STUDIES:

During validation the stability of standards and samples is established under normal bench top conditions, normal storage conditions and sometimes in the instrument to determine if special storage conditions are necessary, for instance refrigeration or protection from light. Stability is determined by comparing the response of aged standards or samples to the response of freshly prepared standards. For checking the solution stability, standard and sample solutions having assay concentration of individual analytes was prepared as per the method given above. Injections from these solutions were analysed initially at different time intervals of 0hrs, 6 hrs, 12 hrs and 24 hrs at ambient temperature.

Table 3.15 Solution stability of Diacerein standard solution

Conditions	Level in %	Peak area	% Relative Deviation
Initial - 0 hrs	100	4527874	-
Initial -6hrs	100	4516356	-0.254
Initial -12hrs	100	4506158	-0.226
Initial -24hrs	100	4501147	-0.111
	Mean	4512884	
	S.D.	10243	
	%RSD	0.23	

Table 3.16 Solution stability of Diacerein sample solution

Conditions	Level in %	Peak area	% Assay in mg/tab	% Label claim	% Relative Deviation
Initial - 0 hrs	100	4635089	51.23	102.46	-
Initial -6hrs	100	4612140	50.98	101.95	-0.50
Initial -12hrs	100	4598023	50.82	101.64	-0.31
Initial -24hrs	100	4578018	50.60	101.20	-0.44
	Mean	4605817	50.91	101.81	
	S.D.	20798	0.23	0.5	
	%RSD	0.45	0.45	0.45	

Table 3.17 Solution stability of Aceclofenac standard solution

Conditions	Level in %	Peak area	% Relative Deviation
Initial - 0 hrs	100	2589226	-
Initial -6hrs	100	2583826	-0.21
Initial -12hrs	100	2546186	-1.46
Initial -24hrs	100	2533692	-0.49
	Mean	2563232	
	S.D.	23785	
	%RSD	0.93	

Table 3.18 Solution stability of Aceclofenac sample solution

Conditions	Level in %	Peak area	% Assay in mg/tab	% Label claim	% Relative Deviation
Initial - 0 hrs	100	2498142	99.26	99.26	-
Initial -6hrs	100	2508916	99.69	99.69	0.43
Initial -12hrs	100	2516843	100.01	100.01	0.32
Initial -24hrs	100	2527360	100.42	100.42	0.42
	Mean	2512815	99.85	99.85	
	S.D.	10703	0.43	0.4	
	%RSD	0.43	0.43	0.43	

INTERMEDIATE PRECISION (RUGGEDNESS):

Precision consist of two components: repeatability and intermediate precision. Intermediate precision refers to variation within a laboratory such as different days, within different instruments and by different analyst. This was formerly known as ruggedness. During the validation a second analyst repeats the repeatability on a different day using different conditions and different instruments.

Table 3.19 Ruggedness study, mixed standard solution

Obs NO	100% level			
	Diacerein		Aceclofenac	
	Peak Area	Retention time	Peak Area	Retention time
1	4517486	3.27	2540616	6.23
2	4484345	3.26	2520144	6.22
3	4497438	3.25	2512044	6.22
4	4509368	3.27	2506152	6.22
5	4490984	3.26	2508412	6.22
6	4483367	3.26	2510506	6.22
Mean	4497165	3.26	2516312	6.22
S.D	12615	0.0068	12829	0.0046
%RSD	0.28	0.21	0.510	0.07

Amount of Diacerein:

Average area of Diacerein standard: 4497165

Table 3.20 % RSD of Diacerein in Intermediate Precision (Ruggedness)

Obs NO	Area of Diacerein in sample	Content in mg/tab	% LC
1	4508956	50.13	100.26
2	4519340	50.25	100.49
3	4501577	50.05	100.10
4	4513286	50.18	100.36
5	4511231	50.16	100.31
6	4517258	50.22	100.45
Mean	4511941	50.16	100.33
S.D	5797	0.06	0.13
%RSD	0.13	0.13	0.13
Limits for %RSD	NMT 2.00%		

Table 3.21 Cumulative % RSD of Diacerein in method precision (M.P.) and Intermediate precision(I.P)

Obs NO	Content in mg/tab	% LC
M.P 1	50.15	100.29
M.P 2	51.34	102.68
M.P 3	50.68	101.36
M.P 4	50.63	101.26
M.P 5	50.59	101.18
M.P 6	50.58	101.16
I.P 1	50.13	100.26
I.P 2	50.25	100.49
I.P 3	50.05	100.10
I.P 4	50.18	100.36
I.P 5	50.16	100.31
I.P 6	50.22	100.45
Mean	50.41	100.83
S.D.	0.37	0.74
%Cumulative RSD	0.73	0.73
Limits	NMT 2.00%	

Amount of Aceclofenac:

Average area of Aceclofenac standard 2516312

Table 3.22 % RSD of Aceclofenac in Intermediate Precision (Ruggedness)

Obs No	Area of Aceclofenac in sample	Content in mg/tab	% LC
1	2539261	100.91	100.91
2	2538547	100.88	100.88
3	2549216	101.31	101.31
4	2566980	102.01	102.01
5	2541231	100.99	100.99
6	2547258	101.23	101.23
Mean	2547082	101.22	101.22
S.D	9736	0.39	0.39
%RSD	0.38	0.38	0.38
Limits for %RSD	NMT 2.00%		

Table 3.23 Cumulative % RSD of Aceclofenac in method precision (M.P.) and Intermediate Precision (I.P.)

Obs N0	Content in mg/tab	% LC
M.P 1	99.28	99.28
M.P 2	99.59	99.59
M.P 3	99.95	99.95
M.P 4	100.36	100.36
M.P 5	100.53	100.53
M.P 6	100.83	100.83
I.P 1	100.91	100.91
I.P 2	100.88	100.88
I.P 3	101.31	101.31
I.P 4	102.01	102.01
I.P 5	100.99	100.99
I.P 6	101.23	101.23
Mean	100.66	100.66
S.D.	0.736	0.736
%Cumulative RSD	0.73	0.73
Limits	NMT 2.00%	

ROBUSTNESS:

A robust or rugged method is one which is not adversely affected by minor changes in experimental variables of the order that might reasonably be expected to take place during the course of the operation of the method. This is therefore tested by carrying out the chromatography on columns of the same type but with different histories and/or using different batches of mobile phase, perhaps even intentionally making some minor changes in the composition. In the above ruggedness study the cumulative % relative standard deviations with respect to precision values were found to be below 2% for Diacerein and Aceclofenac respectively. The robustness study was also carried out by changing the pH of the mobile phase by ±0.2 units, the wavelength by ±2nm and mobile phase composition by ±5%. The results obtained were within the limits as per the validation criteria.

Mean % assay of Diacerein

Table 3.24 Robustness study for Diacerein

Name of the Sample	Change in wavelength 263nm(-2)	Change in wavelength 267nm(+2)	Change in pH of the mobile phase 5.7 (-0.2)	Change in pH of the mobile phase 6.1 (+0.2)	Change in organic phase composition - 5%	Change in organic phase composition +5%
Sample -1	98.92	97.69	99.21	100.02	99.52	99.16
Sample-2	99.65	98.98	98.74	99.58	98.39	99.46
Sample -3	99.20	98.84	99.28	98.47	99.63	98.01
Mean	99.26	98.50	99.08	99.36	99.18	98.88
S.D	0.37	0.71	0.29	0.80	0.69	0.77
RSD	0.37	0.72	0.30	0.80	0.69	0.77

Mean % assay of Aceclofenac

Table 3.25 Robustness study for Aceclofenac

Name of the Sample	Change in wavelength 263nm(-2)	Change in wavelength 267nm(+2)	Change in pH of the mobile phase 3.5 (-0.2)	Change in pH of the mobile phase 3.9 (+0.2)	Change in organic phase composition -5%	Change in organic phase composition +5%
Sample -1	98.21	97.94	99.03	98.15	99.24	99.53
Sample-2	99.65	99.21	98.10	99.71	98.47	97.56
Sample -3	99.57	98.84	99.68	98.49	99.36	98.50
Mean	99.14	98.66	98.94	98.78	99.02	98.53
S.D	0.81	0.65	0.79	0.82	0.48	0.99
RSD	0.82	0.66	0.80	0.83	0.49	1.00

SUMMARY OF VALIDATION AND RESULTS:

In order to develop an isocratic reverse phase stability indicating HPLC method for the determination of Diacerein & Aceclofenac in the combined dosage form the chromatographic conditions were optimized. For better separation and resolution different buffers were tried. It has been found that potassium dihydrogen phosphate buffer, pH 5.9 adjusted with 0.1% triethylamine gave better peak shape than other buffers. The different composition of mobile phase was tried for getting better separation of analytes. Thus the mobile phase composed of the mixture of methanol, acetonitrile and buffer (0.02MKH_2PO_4, pH 5.9 adjusted with 0.1% triethylamine) in the ratio of (50:20:30v/v/v) was finalized. The better separation, peak symmetry and reproducibility were obtained with Inertsil C_{18}, 250mm x 4.6mm, 5ìm column compared to Thermo BDS Hypersil

C8, 150mm x 4.6mm, 5ìm column. Both the analytes gave better response at 265nm wavelength using UV detector. The flow rate was maintained at 1.0mL/min. There was no peak tailing observed under these optimized chromatographic conditions. The retention times of Diacerein and Aceclofenac were found to be 3.2 mins and 6.2 mins respectively. The analytes were subjected to forced degradation under different conditions. It has been found that there was no interference from the excipients and degraded products of the analytes at the retention times of the analytes. Thus the developed method was stability indicating method.

The proposed stability indicating method showed short elution time and good separation between Diacerein & Aceclofenac. The system suitability test was performed as per the USP and international conference of harmonization (ICH) guidelines to confirm the suitability and the reproducibility of the method. The standard solution was injected Six consecutive times and evaluated for repeatability, tailing factor, theoretical plates and resolution. % RSD values were found to be 0.30 & 0.40 for Diacerein and Aceclofenac respectively. The tailing factor and theoretical plates were found to be perfectly within the limits.

The method was linear over the range 20–100µg/mL for diacerein and 40–200 µg/mL for aceclofenac. The calibration curve was constructed by plotting response factor against concentration of drugs. The slope and intercept value for calibration curve was

$Y = 8.93e+004 X + 3.25e+004$ ($r^2 = 0.9992$) for Diacerein and

$Y = 2.60e+004 X - 5.060e+003$ ($r^2 = 0.9994$) for Aceclofenac. The results show an excellent correlation between response factor and concentration of drugs.

The limit of quantification (LOQ) and limit of detection (LOD) was established using signal-to-noise ratio. The LOQ and LOD of Diacerein and Aceclofenac were experimentally determined. The LOD of Diacerein and Aceclofenac was found to be 0.0025 µg/mL and 0.020 µg/mL respectively. The LOQ of Diacerein and Aceclofenac was found to be 0.005 and 0.045 µg/mL respectively.

The developed method was validated for system precision (repeatability) and method precision. Six injections of mixed standards of 50 µg/mL of Diacerein and 100 µg/mL of Aceclofenac were injected and %RSD calculated for injection repeatability. Six samples were prepared at 100% levels and assayed according to the procedure. The average assay of three replicate analysis was found to be 101.32 % for Diacerein and 100.08% for Aceclofenac with a relative standard deviation of 0.69 and 0.54 respectively.

The accuracy of the method was determined by the standard addition method at three different levels. The sample solution of 100% level was considered as a zero level and 10%, 20% and 30% of the standard drug of analytes were added respectively. Each determination was performed in triplicates. The accuracy was then calculated as the percentage of the standard drug recovered by the recovery study. The results are well within the acceptance limit and hence the method is accurate.

The stability of both the standard and the sample was determined by monitoring the peak area responses of the standard solution and the sample solution of Diacerein and Aceclofenac at 6, 12 and 24 hours at room temperature. The results showed that there were no significant differences.

The specificity of the method was determined by exposing 100% sample solutions of Diacerein and Aceclofenac to stress conditions i.e. 0.1M NaOH, 0.1M HCl, 10% H_2O_2 and thermal degradation. Typical chromatograms obtained from the assay of pure sample and stressed samples are shown in the figures. The degradation products were separated from their parent compounds. The results of the forced degradation studies indicated a high degree of selectivity & specificity of this method for Diacerein and Aceclofenac. Diacerein and Aceclofenac were found to be stable under dry heat conditions, but in acid, alkali and 10 % H_2O_2 the drugs undergo degradation. Under acid and alkali degradation the degradation product of diacerein shows the peak at 4.15mins while in oxidative degradation, the peak appears at 2.60mins.

CONCLUSION:

The isocratic RP- HPLC method has proved to be simple, specific, precise and accurate and is suitable for simultaneous quantification of Diacerein and Aceclofenac. The proposed method gives a good resolution among the analytes. High percentage of recovery shows that the method is accurate. The forced degradation study shows that there is no interference of the excipients and the degraded products at the retention times of Diacerein and Aceclofenac. The stability data of the drugs carried out by this method shows that the stock solutions are stable for longer time than that taken for analysis. The linearity, precision, accuracy, ruggedness of the method proves that the method is easily reproducible in any quality control set up provided all the parameters are accurately followed. The proposed HPLC method summarizes the overall

method for the intended purpose of successfully applying for routine quality control analysis and checking the purity of Diacerein and Aceclofenac from their fixed dosage form as well as the active pharmaceutical ingredient.

EFERENCES:

1) P. Nicolas, M. Tod, C. Padoin, O. Petitjean, Clin. *Pharmecokinet,* 35 (5) (1998) 347.

2) T.S.A. Fidelix, B.G.D.O. Soares, V.F.M. Trevisani, *Cochrane Database Syst. Rev., 1* (2006) CD005117.

3) A.C. Moffat, M.D. Osselton, E.G.C. Clarke, B. Widdop, L.Y. Galichet, Clarke's analysis of drugs and poisons, third ed., *Pharmaceutical Press,* vol. 2, (2004) 570–571.

4) S. Narade; S. Patil; S. Surve; D. Shete; Y. Pore, Simultaneous UV Spectrophotometric method for the determination of Diacerein and Aceclofenac in tablets. *Journal of Pharmaceutical Sciences and Research,* (2010), 2(2), 137-142.

5) K.S. Topagi; P.K. Sinha; R.M. Jeswani; M.C. Damlela, Spectrophotometric methods for simultaneous estimation of Diacerhein and Aceclofenac. *International Journal of ChemTech Research,* (2009), 1(4), 991-995.

6) S. Bhalerao; S. Tambe; V. Pareek; R. Shinde; L. Gupta, A solid-liquid extraction and high performance thin layer chromato- graphic determination of Diacerein and Aceclofenac in pharmaceutical tablet dosage form. *Asian Journal of Pharmaceutical and Clinical Research,* (2010), 3(1), 25-30.

7) J.R. Bhinge; R.V. Kumar; V.R. Sinha, A simple and sensitive stability-indicating RP- HPLC assay method for the determination of Aceclofenac. *Journal of Chromatographic Science,* (2008), 46(5), 440-444.

8) R. Siva; N. Srisutherson; W.D. Sam Solomon; N.P Kumar; R. Venkatnarayanan, Validated RP-HPLC method for the simultaneous estimation of Aceclofenac and Diacerein in bulk and formulation. *International Journal of PharmTech Research,* (2010), 2(1), 940-944.

9) V. Giannellini; F. Salvatore; G. Bartolucci;S.A. Coran; M. Bambagiotti-Alberti, A validated HPLC stability-indicating method for the determination of Diacerhein in bulk drug substance. *Journal of Pharmaceutical and Biomedical Analysis,* (2005), 39(3-4), 776-780.

10) A. Goyal; S. Jain.; A validated HPLC stability-indicating method for the determination of Diacerhein in bulk drug substance. *Rajendra Acta Pharmaceutica Sciencia,* (2007), 49(2), 147-151.

11) A. Ojha; R. Rathod; H. Padh, Simultaneous HPLC-UV determination of Rhein and Aceclofenac in human plasma. Analytical technologies in the biomedical and life sciences. *Journal of chromatography B,* (2009), 877(11-12), 1145-1148.

12) V. Sekar; S. Jayaseelan; E. Udhaya Kumar; N. Subash; M. Prakash; P. Perumal, Development and validation of RP-HPLC method for the simultaneous estimation of Diacerein and Aceclofenac in dosage form. *International Journal of ChemTech Research,* (2010), 2(1), 168-171.

13) Harikrishnan, N.; Gunasekaran, V.; Sathishbabu, A.; Srinivasarao, G.; Roosewelt, C. Validated high performance liquid chromato- graphy method for simultaneous estimation of Paracetamol and Aceclofenac in tablet dosage forms. *Asian Journal of Chemistry* (2007), 19(7), 5596-5600.

CHAPTER - IV

Simultaneous determination of Paracetamol, Chlorzoxazone and Diclofenac Sodium in tablet dosage form by high performance liquid chromatography (HPLC)

METHODOLOGY

SELECTION OF CHROMATOGRAPHIC MODE:

Proper selection of the method depends upon the nature of the sample (ionic/ ionizable/ neutral molecule), its molecular weight and solubility. All the drugs selected in the present study are polar in nature.

Paracetamol is an odorless, slightly bitter taste white crystalline powder. It is soluble in organic solvent such as methanol and etha nol but slightly soluble in water and ether. Its pH range is 5.5 - 6.5 based on saturated aqueous solution. Molecular weight of Paracetamol is 151.16. Chlorzoxazone is polar in nature, its molecular weight is 169.56 and its experimental hydrophobicity is 1.6. Diclofenac sodium is less polar in character. Its experimental hydrophobicity is 3.9 and molecular weight is 296.149. From the nature of the analyte it has been clear that reverse phase high performance liquid chromatography (RP-HPLC) was the suitable mode of separation.

Selection of stationary phase:

The availability of a stable high performance column is essential in developing rugged, reproducible method. When selecting an HPLC column it is necessary to consider

column to column reproducibility. The selection of the column depends upon the nature of the analyte.

Based on reversed phase HPLC mode C18 columns from various manufactures were tried, Kromasil 100-5C18 250x4.6mm, i.d particle size 5µm, Inertsil ODS-3V C18, 250x4.6mm, 5µm, Hypersil Phenyl C18 250x4.6, 5µm, Agilent Zorbax C18 150x4.6, 5µm, Thermo C8 BDS Hypersil 150x4.6mm, 5µm. From the trials it was found that Inertsil, ODS-3V C18 (250mm × 4.6mm, i.d.) with particle size 5 µm is the most suitable column with good separation and peak shape.

Hence the column with 250mm × 4.6mm i.d with a 5 µm particle size Inertsil C18 **column was employed as the stationary phase in the separation of analytes.**

Selection of mobile phase:

Selection of the mobile phase depends upon the properties of the analytes. The hydrophobicity of Paracetamol, Chlorzoxazone and Diclofenac Sodium is 0.4, 1.6 and 3.9 respectively. Paracetamol has low hydrophobicity indicates that it will be retained for a lesser time in the column. While diclofenac has higher hydrophobicity indicates that it will be retained for a longer time in the column. For a given stationary phase the retention of the given solute depends upon the mobile phase composition. In the present research work the composition of the organic solvent is kept higher as compared to the polar solvent. Solvent polarity is the key factor in chromatographic separations, since the polar mobile phase gives rise to high solute retention in reverse phase liquid chromatography.

Acetonitrile and methanol are most popularly used organic solvents in HPLC. Paracetamol, Chlorzoxazone and

Diclofenac are soluble in methanol and acetonitrile. For getting intermediate selectivity in the separation of critical composition of analytes the use of organic solvent mixtures is necessary. Hence Buffer: Acetonitrile: Methanol combination was used for trials. Finally Buffer: ACN: Methanol combination was selected for the method development.

Selection of buffer for mobile phase:

Selection of buffer for mobile phase was made according to the nature of the drugs. Different buffers were used in the study of separation of the drug components. Initially 0.01M and 0.10M NaH_2PO_4 was used with Methanol. But proper separation of analytes was not achieved. In some cases it also showed merging of the two peaks. KH_2PO_4 was the second choice of buffer and it was used at the concentration level of 0.02M. In presence of KH_2PO_4 good separation of the three components was observed. Multiple trials were carried out using Buffer, acetonitrile and methanol at different ratios. Finally 0.02M KH_2PO_4 : ACN : MeOH solvent combination was selected in the ratio of 25 : 25 : 50v/v/v.

Selection of pH for mobile phase:

pH of the mobile phase plays a vital role in the separation of analytes. It depends upon the properties and nature of analytes. The pKa/isoelectric point values of Paracetamol, Chlorzoxazone and Diclofenac sodium are 9.38, 8.0 and 4.15 respectively. For proper separation and resolution the pH of the mobile phase was adjusted with orthophosphoric acid. At pH 3.7 all the analytes were properly separated and resolved. Hence pH 3.7 was finalized as the drugs were eluted within 10 minutes.

Thus mobile phase was finalized as Buffer (0.02M KH_2PO_4 pH 3.7, adjusted with dilute orthophosphoric acid), acetonitrile and methanol in the ratio

(25: 25: 50v/v/v)

SELECTION OF DETECTOR AND DETECTION WAVELENGTH:

A large number of LC detectors have been developed over the past thirty years based on variety of the different sensing principles. The four dominant detectors used in HPLC are the UV detector, electrical conductivity detector, the fluorescence detector and refractive index detector. The drugs selected for the analysis have absorbance in the UV region of the electromagnetic spectrum. Hence, a UV detector was chosen for the detection of the eluent in the chromatographic process.Multi component pharmaceutical preparations contain more than one active ingredient with variable concentrations. Detecting each component at their maximum absorbance, the purpose of using HPLC for their simultaneous determination is lost. Hence in such cases one has to explore the possibility of selecting the wavelength at which all the components are detected. This single wavelength is referred to as the "most suitable wavelength". In this particular method, 220nm wavelength is selected as the detection wavelength because at this wavelength all the analytes gives best response.

Table 4.1 FINALISED CHROMATOGRAPHIC CONDITIONS FOR HPLC METHOD

Chromatographic mode Chromatographic condition

Chromatographic mode	Chromatographic condition
Standard solution	100 µg/mL of Paracetamol , 100 µg/mL of Chlorzoxazone and 10 µg/mL of diclofenac Na
Equipment system	
Pump	Waters HPLC Waters 501 isocratic pump
Detector	Waters 486 tunable absorbance UV-detector
Data processor	Millenium^{32} chromatographic software
Injector	Waters ^{TM} 717plus autosampler
Stationary phase/Column	Inertsil C18 column (250 X 4.6 mm , i.d 5µm)
Buffer solution	Aqueous buffer solution of 0.02M KH_2PO_4
Mobile phase	Buffer(0.02M KH_2PO_4, pH 3.7 adjusted with orthophosphoric acid) : Acetonitrile : Methanol (25 : 25 : 50v/v/v)
Detection wavelength	220 nm
Flow rate	1.0mL/min
Injection Volume	20µl
Column temperature	Ambient
Pump Mode	Isocratic

PREPARATION OF STOCK SOLUTIONS

Preparation of Buffer

Buffer solution was prepared by dissolving accurately weighed 2.72 g of Potassium dihydrogen phosphate (KH_2PO_4) in 1 liter distilled water (HPLC Grade). In order to have quick dissolution of the salt in distilled water, the solution was sonicated for 2min. The concentration of the solution obtained was 0.02M KH_2PO_4 solution.

Preparation of Mobile phase

Mobile phase was prepared by using the above prepared buffer, acetonitrile, methanol and mixing them in the volume ratio of (25:25:50,v/v/v). The pH of this mixture was then adjusted to 3.7 with orthophosphoric acid. The mobile phase was then sonicated for 10min to remove the dissolved gases which may cause interference in the HPLC system.

Preparation of Standard stock solution

50mg of Paracetamol and 50 mg of Chlorzoxazone and 5mg of Diclofenac sodium were accurately weighed and transferred to a 100mL volumetric flask. It was dissolved in a minimum quantity of methanol and then diluted up to the mark with methanol. The concentration of the solution obtained was 500µg/mL for Paracetamol, 500 µg/mL for Chlorzoxazone and 50µg/mL for Diclofenac sodium **(Solution A).** 2 mL of this **solution A** was diluted to 10 mL in a volumetric flask with the mobile phase. The concentration of the solution obtained was 100 µg/mL for Paracetamol, 100 µg/mL for Chlorzoxazone and 10 µg/mL for Diclofenac sodium.

Preparation of Sample solution

Twenty tablets (Powergesic MR) were weighed and their average weight was calculated. These tablets were powdered and weight equivalent to one tablet containing 500 mg of Paracetamol and 500 mg of Chlorzoxazone and 50 mg of Diclofenac sodium was taken in a 100mL dilution flask. Then about 50 mL of diluent (Mobile phase) was added to it, sonicated for 20-25mins at an ambient temperature with intermittent swirling, cooled and diluted upto the mark with diluent and mixed well. Then the solution from the flask was filtered through a syringe filter (0.45µ).

METHOD IN BRIEF:

A simple, precise and rapid isocratic reverse phase high performance liquid chromatographic method has been developed for the simultaneous determination of Paracetamol, Chlorzoxazone and Diclofenac Sodium from tablet dosage form. The chromatographic separation was performed on a Inertsil C18 column (250mm × 4.6mm i.d 5µm particle size). Mobile phase consisted of a mixture of phosphate buffer (0.02M KH_2PO_4, pH adjusted to 3.7 using orthophosphoric acid), Acetonitrile and Methanol in the ratio of

(25 : 25 : 50v/v/v) at a flow rate of 1.0 mL/min. The wavelength was set at 220nm. The proposed method was validated for linearity, accuracy, precision, ruggedness, solution stability LOD and LOQ. The calibration was linear over the range of 50 – 150 µg/mL for Paracetamol, 50 - 150 µg/mL for Chlorzoxazone and 5 – 15 µg/mL for Diclofenac Sodium. The method can be easily adopted for quality control analysis.

METHOD VALIDATION OF HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF PARACETAMOL, CHLORZOXAZONE AND DICLOFENAC SODIUM

The following experiment was carried out to determine the working concentration range for Paracetamol, Chlorzoxazone and Diclofenac sodium. The proposed developed method was subjected to method validation process to determine its suitability for intended use. With reference to the following parameters the method was validated.

1) Selectivity and Specificity

2) System suitability

3) Linearity

4) Precision (Repeatability)

5) Accuracy (% Recovery)

6) Solution stability

7) Intermediate precision (Ruggedness)

8) Robustness

SELECTIVITY AND SPECIFICITY:

Specificity is the measure of the degree of the interference in the analysis of the complex sample mixtures such as analyte present in the matrix containing endogenous substances and related chemical compounds, etc. To validate specificity of the method following samples were prepared and injected to the HPLC system and chromatograms were recorded.

Blank solution i.e. diluent

Blank + individual drug having assay concentration of individual drug

Blank + all drugs having assay concentration of individual drug

By performing this exercise it has been found that there were no interferences of excipients or placebo at the retention time of the individual drugs.

Representative chromatogram of sample solution is shown in the following figure.

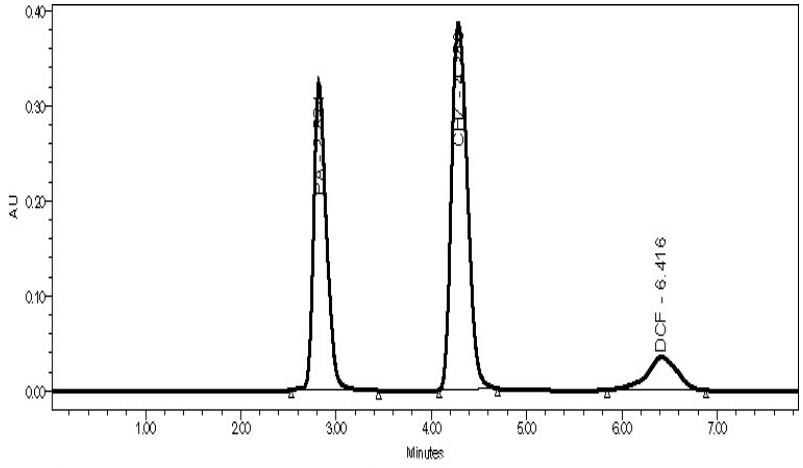


Figure 4.1 In the optimized conditions Paracetamol has a Rt=2.80, Chlorzoxazone has a Rt=4.23 and Diclofenac sodium has 6.41. The major peaks are well separated. There are no other potentially interfering peaks at the retention time of the three drugs, thus showing the specificity of the method

SYSTEM SUITABILITY TEST:

A system suitability test should be carried out to check if the HPLC system is performing properly. System suitability tests were performed as per the ICH guidelines to confirm the suitability and the reproducibility of the

system. The test was carried out by injecting standard solutions of Paracetamol, Chlorzoxazone and Diclofenac Sodium of the strength 100% level. The standard deviation (S.D) and Relative standard deviation (%RSD) values of the five determinations are presented in table 4.2.

The %RSD values were found to be satisfactory and meeting the requirements.

Table 4.2 System suitability tests for Paracetamol, Chlorzoxazone and Diclofenac Na

Obs No	100% Level					
	Paracetamol		Chlorzoxazone		Diclofenac Na	
	Peak area	Retention time	Peak area	Retention time	Peak area	Retention time
1	2855236	2.81	4359708	4.26	700169	6.41
2	2862403	2.81	4378725	4.27	705528	6.40
3	2874550	2.82	4397350	4.27	706536	6.39
4	2882082	2.81	4396982	4.26	708275	6.39
5	2892565	2.81	4408500	4.26	702214	6.41
Mean	2873367	2.81	4388253	4.26	704544	6.4
S.D	14957	0.0030	19199	0.005	3295	0.0089
% RSD	0.52	0.11	0.4	0.2	0.5	0.14

LINEARITY RANGE FOR PARACETAMOL, CHLORZOXAZONE AND DICLOFENAC SODIUM:

This experiment was carried out to demonstrate the range over which the response of the detector is linear with respect to concentration of Paracetamol Chlorzoxazone and Diclofenac sodium. In a series of volumetric flasks varying volumes of solution A containing Paracetamol, Chlorzoxazone and Diclofenac sodium was taken and then diluted upto the mark with diluent. Preparation of linearity levels were as per the following table 4.3

Table 4.3 Preparation of mixed standard solution for Linearity level

Linearity Levels	Volume of std stock solution added in mL	Volume made upto in mL	Concentration of Paracetamol in ppm	Concentration of Chlorzoxazone in ppm	Conc. of Diclofenac Na in ppm
50%	5	50	50	50	5
75%	7.5	50	75	75	7.5
90%	9	50	90	90	9.0
100%	10	50	100	100	10
125%	12.5	50	125	125	12.5
140%	14	50	140	140	14.0
150%	15	50	150	150	15.0

Table 4.4 Linearity of Paracetamol at different concentration level

Obs No.	Conc. level %	Conc. in µg/ml	Peak Area			Mean peak area	S.D	%RSD
			1	2	3			
1	50	50	1444507	1446980	1446535	1446007	1318.24	0.091
2	75	75	2181428	2184332	2182450	2182737	1473.07	0.067
3	90	90	2599146	2598356	2608121	2601874	5424.17	0.208
4	100	100	2913604	2914680	2915995	2914760	1197.49	0.041
5	125	125	3636923	3636985	3637665	3637191	411.665	0.011
6	140	140	4038820	4038103	4034541	4037155	2291.72	0.057
7	150	150	4291450	4292993	4291244	4291896	955.88	0.022

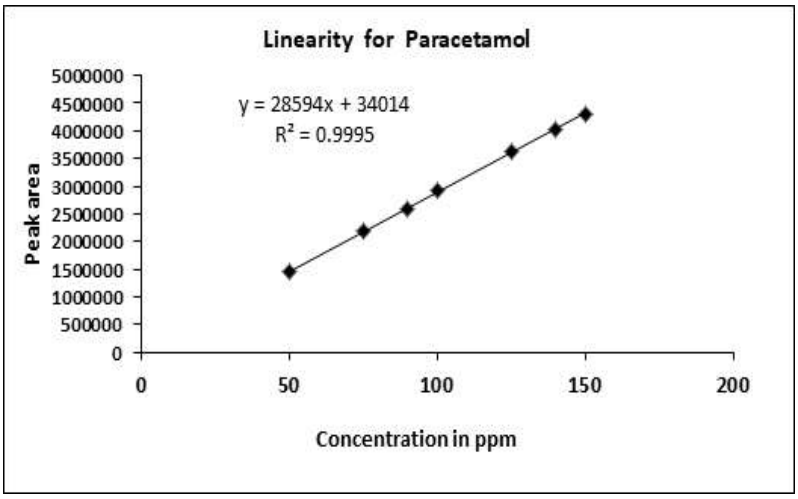


Fig 4.2 Linearity Level plot for Paracetamol

Table 4.5 Linearity of Chlorzoxazone at different concentration levels

Obs No.	Conc. level %	Conc. in µg/ml	Peak Area			Mean peak area	S.D	%RSD
			1	2	3			
1	50	50	2157685	2157845	2159874	2158468	1220.26	0.057
2	75	75	3291483	3291050	3289554	3290696	1012.14	0.031
3	90	90	3961090	3960682	3962994	3961589	1234.03	0.031
4	100	100	4450880	4452836	4451054	4451590	1082.57	0.024
5	125	125	5532037	5534515	5534154	5533569	1338.69	0.024
6	140	140	6147350	6150452	6151459	6149754	2141.66	0.035
7	150	150	6527137	6525113	6527250	6526500	1202.51	0.018

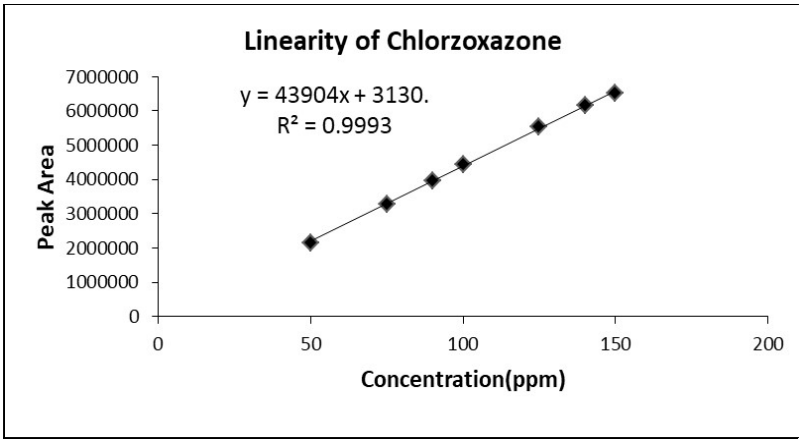


Fig 4.3 Linearity Levels plot for Chlorzoxazone

Table 4.6 Linearity of Diclofenac Sodium at different concentration Levels

Obs No.	Conc. in µg/ml	Peak Area			Mean peak area	S.D	%RSD
		1	2	3			
1	5	372930	374022	374652	373868	871.26	0.233
2	7.5	563252	561522	562878	562551	910.26	0.162
3	9	647056	649722	649878	648885	1586.16	0.244
4	10	726775	729984	732212	729657	2733.21	0.375
5	12.5	895254	897809	894495	895853	1736.22	0.194
6	14	1001884	1002398	1006912	1003731	2766.50	0.276
7	15	1073125	1076301	1073617	1074348	1709.43	0.159

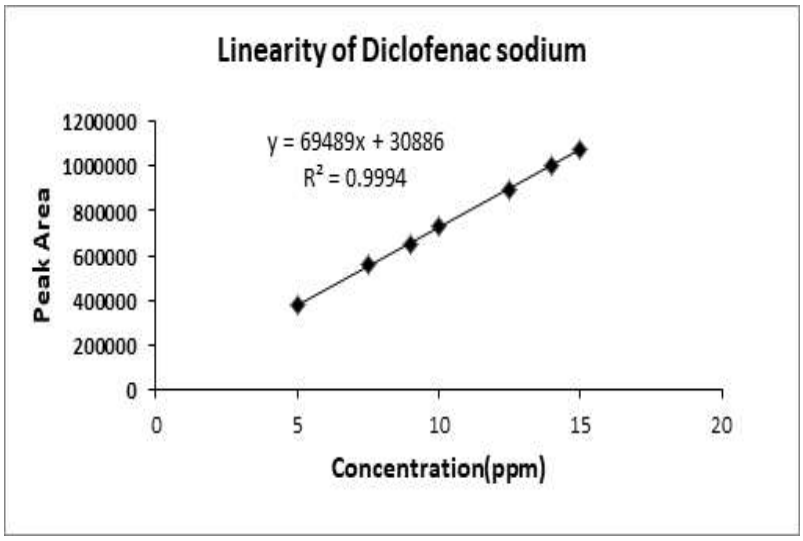


Fig 4.4 Linearity level plot for Diclofenac sodiu

Regression analysis

The calibration data range 50µg/mL to 150µg/mL for Paracetamol, 50µg/mL to 150 µg/mL for Chlorzoxazone and 5µg/mL to 15µg/mL for Diclofenac sodium was further considered for regression analysis. The regression analysis of the calibration data was carried out to determine the relationship between the dependent variable (Peak area) and the independent variable (Drug) concentration.

Applying the linearity plot from fig. 4.2 for Paracetamol and fig 4.3 for Chlorzoxazone and fig 4.4 for Diclofenac sodium the values for the regression equation y= mx + C are found to be,

For Paracetamol	**For Chlorzoxazone**
y = 28594x + 34014	y = 43904x + 3130
m = 28594 (Slope)	m = 43904 (Slope)
C = 34014 (Intercept)	C = 3130 (Intercept)

For Diclofenac sodium

x = Found concentration of the drug

y = 69489x + 30886 y = Calculated peak area

m = 69489 c = 30886

The regression equation indicates that one unit increase in the concentration of the Paracetamol, Chlorzoxazone and Diclofenac sodium will increase the detector response by 28594, 43904 and 69489 units respectively. Also by using the regression equation the actual values of the drug injected can be back calculated by substituting the value of y i.e. area of the drug. The correlation (r^2) for Paracetamol, Chlorzoxazone and Diclofenac sodium was found to be 0.9995, 0.9993 and 0.9994 respectively.

SYSTEM PRECISION (REPEATABILITY)

The system precision study was performed to determine the repeatability of the method. Six samples of the standard were prepared at 100% level and assayed according to the procedure.

Table 4.7 Precision study for Paracetamol

Obs NO	Peak area	True value Conc. in µg/ml	Experimental values Conc. in µg/ml
1	2855236	100	98.65
2	2862403	100	98.90
3	2874550	100	99.32
4	2882082	100	99.59
5	2892565	100	99.95
6	2885123	100	99.69
Mean	2875326	-	99.35
S.D	-	-	0.45
%RSD	-	-	0.46
%Accuracy	-	-	99.35

Table 4.8 Precision study for Chlorzoxazone

Obs NO	Peak area	True value Conc. in µg/ml	Experimental values Conc. in µg/ml
1	4359708	100	99.23
2	4378725	100	99.66
3	4397350	100	100.09
4	4396982	100	100.08
5	4408500	100	100.34
6	4412024	100	100.42
Mean	4392214	-	99.97
S.D	-	-	0.41
%RSD	-	-	0.41
%Accuracy	-	-	99.97

Table 4.9 Precision study for Diclofenac sodium

Obs NO	Peak area	True value Conc. in µg/ml	Experimental values Conc. in µg/ml
1	710169	10	9.78
2	715528	10	9.85
3	716536	10	9.87
4	718275	10	9.89
5	718514	10	9.90
6	718815	10	9.90
Mean	716306	-	9.86
S.D	-	-	0.04
%RSD	-	-	0.44
%Accuracy	-	-	98.64

Method Precision (Reproducibility)

The method precision study was performed to determine the reproducibility of the method. Six samples of tablets were prepared at 100% level and assayed according to the procedure. The results are shown in following tables.

Table 4.10 Reproducibility study for Paracetamol

Obs No	Peak area	True value Conc. in µg/ml	Experimental values Conc. in µg/ml
1	2894493	100	100.67
2	2900817	100	100.89
3	2909183	100	101.18
4	2917275	100	101.46
5	2924780	100	101.72
6	2885231	100	100.34
Mean	2905296	-	101.04
S.D	-	-	0.47
%RSD	-	-	0.46
%Accuracy	-	-	101.04

Table 4.11 Reproducibility study for Chlorzoxazone

Obs No	Peak area	True value Conc. in µg/ml	Experimental values Conc. in µg/ml
1	4360282	100	99.27
2	4370706	100	99.51
3	4391360	100	99.98
4	4410899	100	100.43
5	4410479	100	100.42
6	4401212	100	100.20
Mean	4390823		99.97
S.D			0.44
%RSD			0.44
%Accuracy			99.97

Table 4.12 Reproducibility study for Diclofenac sodium

Obs NO	Peak area	True value Conc. in µg/ml	Experimental values Conc. in µg/ml
1	700169	10	9.77
2	705528	10	9.85
3	706536	10	9.86
4	708275	10	9.89
5	702214	10	9.80
6	710152	10	9.91
Mean	705479	-	9.85
S.D	-	-	0.05
%RSD	-	-	0.48
%Accuracy	-	-	98.49

ACCURACY: (% RECOVERY)

Recovery experiments were carried out to check the efficiency of the method to extract and determine drugs in the presence of excipients present in the formulation. A standard addition method was employed for this experiment. A known quantity of each drug substance corresponding to 100%, 110%, 120% and 130% of the label claim of each drug was added. Each set of addition was repeated three times. The accuracy was expressed as a percentage of analytes recovered by the assay. In the present research work 100ppm sample solution considered as 100% (0 level) and 10%, 20% and 30% standard drug was added to it. Table lists the recoveries of the drugs from a series of spiked concentrations.

Table 4.13 Accuracy study for Paracetamol

Obs No	Levels in %	Area of sample	Initial amount in ppm	Amt added in ppm	Amt found in ppm	% recovery
1	0%	2892493	100	0	100.60	100.60
2		2887122	100	0	100.41	100.41
3		2867864	100	0	99.74	99.74
1	10%	3168275	100	10	110.19	100.17
2		3167110	100	10.5	110.15	99.68
3		3165021	100	10.5	110.08	99.62
1	20%	3447529	100	20	119.90	99.92
2		3446215	100	20.5	119.85	99.46
3		3445034	100	20.5	119.81	99.43
1	30%	3761034	100	30	130.80	100.62
2		3760214	100	30.5	130.78	100.21
3		3758189	100	30.8	130.70	99.93
					Mean	**99.89**
					S.D.	**0.37**
					%RSD	**0.37**
					Range of % recovery	**99.43-100.62**

Table 4.14 Accuracy study for Chlorzoxazone

Obs No	Levels in %	Area of sample	Initial amount in ppm	Amt added in ppm	Amt found in ppm	% recovery
1	0%	4359789	100	0	99.26	99.26
2		4370706	100	0	99.51	99.51
3		4392360	100	0	100.00	100.00
1	10%	4874190	100	10	110.97	100.88
2		4872545	100	10.6	110.94	100.30
3		4871014	100	10.8	110.90	100.09
1	20%	5276479	100	20	120.13	100.11
2		5274124	100	20.5	120.08	99.65
3		5275147	100	20.5	120.10	99.67
1	30%	5757550	100	30	131.09	100.83
2		5755542	100	30.5	131.04	100.41
3		5754889	100	30.8	131.02	100.17
					Mean	**100.08**
					S.D.	**0.46**
					%RSD	**0.46**
					Range of % recovery	**99.26-100.88**

Table 4.15 Accuracy study for Diclofenac sodium

Levels in %	Area of sample	Initial amount in ppm	Amt added in ppm	Amt found in ppm	% recovery
0%	710141	10	0	9.91	99.14
	711914	10	0	9.94	99.39
	717458	10	0	10.02	100.16
10%	781228	10	1	10.91	99.15
	785415	10	1.1	10.96	98.78
	779858	10	1.1	10.89	98.08
20%	852142	10	2	11.90	99.14
	850156	10	2.1	11.87	98.09
	849778	10	2.1	11.86	98.04
30%	923156	10	3	12.89	99.14
	930255	10	3.2	12.99	98.39
	927831	10	3.2	12.95	98.13
				Mean	98.80
				S.D.	0.61
				%RSD	0.62
				Range of % recovery	98.04-100.16

LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTITATION (LOQ):

The limit of detection (LOD) is the amount of analyte which can be reliably detected under the stated experimental conditions. The limit of quantitation (LOQ) is the amount of analyte which can be reliably quantified under the stated experimental conditions. The limit of quantification (LOQ) and limit of detection (LOD) was established using signal-to-noise ratio. The LOQ and LOD Paracetamol, Chlorzoxazone and Diclofenac sodium were experimentally determined. The LOD of Paracetamol,

Chlorzoxazone and Diclofenac sodium was found to be 0.0020 µg/mL, 0.040 µg/mL and 0.025 µg/mL respectively. The LOQ of Paracetamol, Chlorzoxazone and Diclofenac sodium was found to be 0.0065 µg/mL, 0.085 µg/mL and 0.070 µg/mL respectively.

SOLUTION STABILITY STUDY:

The purpose of this study is to check the stability of analytes in solvents or mixtures of solvents used in preparation of samples. Sometimes the drugs may undergo degradation after going into the solution form. Hence it becomes necessary to check whether the samples have ample stability. For checking the solution stability, standard and sample solutions having assay concentration of individual analytes were prepared as per the method given above. Injections from these solutions were analysed initially at different time intervals of 0hrs, 6hrs, 12hrs, and 24 hrs at ambient temperature.

For Paracetamol:

Table 4.16 Solution stability of Paracetamol standard solution

Conditions	Level in %	Peak area	% Relative Deviation
Initial - 0 hrs	100	2854564	-
Initial -6hrs	100	2853456	-0.038
Initial -12hrs	100	2812549	-1.433
Initial -24hrs	100	2806574	-0.212
	Mean	2831785	
	S.D.	22327	
	%RSD	0.79	

Table 4.17 Solution stability of Paracetamol sample solution

Conditions	Level in %	Peak area	% Assay in mg/tab	% Label claim	% Relative Deviation
Initial - 0 hrs	100	2894498	503.79	100.76	-
Initial -6hrs	100	2898223	504.43	100.89	0.13
Initial -12hrs	100	2890124	503.02	100.60	-0.28
Initial -24hrs	100	2841879	494.63	98.93	-1.67
	Mean	2881181	501.47	100.29	
	S.D.	22871	3.98	0.8	
	%RSD	0.79	0.79	0.79	

For Chlorzoxazone:

Table 4.18 Solution stability of Chlorzoxazone standard solution

Conditions	Level in %	Peak area	% Relative Deviation
Initial - 0 hrs	100	4358521	-
Initial -6hrs	100	4345123	-0.31
Initial -12hrs	100	4342584	-0.06
Initial -24hrs	100	4312876	-0.68
	Mean	4339776	
	S.D.	16669	
	%RSD	0.38	

Table 4.19 Solution stability of Chlorzoxazone sample solution

Conditions	Level in %	Peak area	% Assay in mg/tab	% Label claim	% Relative Deviation
Initial - 0 hrs	100	4405874	502.00	100.40	-
Initial -6hrs	100	4382241	499.31	99.86	-0.54
Initial -12hrs	100	4387587	499.92	99.98	0.12
Initial -24hrs	100	4356781	496.41	99.28	-0.70
	Mean	4383120	499.41	99.88	
	S.D.	17551	2.00	0.4	
	%RSD	0.40	0.40	0.40	

For Diclofenac sodium:

Table 4.20 Solution stability of Diclofenac sodium standard solution

Conditions	Level in %	Peak area	% Relative Deviation
Initial - 0 hrs	100	715008	
Initial -6hrs	100	714668	-0.048
Initial -12hrs	100	713551	-0.156
Initial -24hrs	100	712159	-0.195
	Mean	713846	
	S.D.	1113.4	
	%RSD	0.16	

Table 4.21 Solution stability of Diclofenac sodium sample solution

Conditions	Level in %	Peak area	% Assay in mg/tab	% Label claim	% Relative Deviation
Initial - 0 hrs	100	710152	49.61	99.23	-
Initial -6hrs	100	712564	49.78	99.57	0.34
Initial -12hrs	100	709863	49.59	99.19	-0.38
Initial -24hrs	100	709214	49.55	99.10	-0.09
	Mean	710448	49.64	99.27	
	S.D.	1267	0.09	0.18	
	%RSD	0.18	0.18	0.18	

INTERMEDIATE PRECISION (RUGGEDNESS):

The intermediate precision or ruggedness of an analytical method is the degree of reproducibility of the test results obtained by the analysis of the same samples under a variety of conditions, such as different laboratories, analysts, instruments, lots of reagents, elapsed assay times or days. The ruggedness of an analytical method is determined by aliquots from homogeneous lots in different laboratories, by different analysts. In ruggedness the analysis was performed by other analysts. By changing the analyst the ruggedness of the method was tested. As a part of the method validation, Intermediate precision was also examined by carrying out the same assay procedure on a different instrument on different days. The experimental conditions were kept same but the HPLC system was changed.

Sample preparation: (Same as precision study)

On different days six sample preparation of Paracetamol, Chlorzoxazone and Diclofenac sodium tablets (Powergesic MR) were analyzed as per the methodology. This analysis was carried out on different day and on different make of HPLC system. With reference to the below tables the cumulative % relative standard deviation was found to be 0.68, 0.46 & 0.58 for Paracetamol, Chlorzoxazone and Diclofenac sodium respectively. The percentage value for the assay lies close to the theoretical value (100%) which indicates that the method is accurate. Low value of cumulative % RSD of assay of precision study and Intermediate precision study showed that the method is rugged. The results are well below 2.00%. Following tables represents the ruggedness of the method.

Table 4.22 Ruggedness study, standard solution

Obs NO	100% level					
	Paracetamol		Chlorzoxazone		Diclofenac sodium	
	Peak Area	Retention time	Peak Area	Retention time	Peak Area	Retention time
1	2855236	2.81	4359708	4.26	700169	6.41
2	2862403	2.81	4378725	4.27	705528	6.4
3	2874550	2.82	4397350	4.27	706536	6.39
4	2882082	2.81	4396982	4.26	708275	6.39
5	2892565	2.81	4408500	4.26	702214	6.41
6	2885123	2.81	4388253	4.26	704544	6.4
Mean	2875326	2.81	4388253	4.3	704544	6.4
S.D	12974	0.0027	15676	0.0042	2690	0.0082
%RSD	0.45	0.10	0.36	0.10	0.38	0.13

Amount of Paracetamol

Average area of Paracetamol standard: 2875326

Table 4.23 % RSD of Paracetamol in Intermediate Precision (Ruggedness)

Obs NO	Area of Paracetamol in sample	Content in mg/tab	% LC
1	2894493	503.78	100.76
2	2870598	499.63	99.93
3	2875233	500.43	100.09
4	2859512	497.70	99.54
5	2865577	498.75	99.75
6	2884214	502.00	100.40
Mean	2874938	500.38	100.08
S.D	11640	2.03	0.41
%RSD	0.40	0.40	0.40
Limits for %RSD		NMT 2.00%	

Table 4.24 Cumulative % RSD of Paracetamol in precision and Intermediate precision

Obs NO	Content in mg/tab	% LC
M.P 1	503.78	100.76
M.P 2	504.89	100.98
M.P 3	506.34	101.27
M.P 4	507.75	101.55
M.P 5	509.06	101.81
M.P 6	502.17	100.43
I.P 1	503.78	100.76
I.P 2	499.63	99.93
I.P 3	500.43	100.09
I.P 4	497.70	99.54
I.P 5	498.75	99.75
I.P 6	502.00	100.40
Mean	503.02	100.60
S.D.	3.428	0.686
%Cumulative RSD	0.68	0.68
Limits for % of RSD	NMT 2.00%	

M.P – Method precision, I.P- Intermediate precision

Amount of Chlorzoxazone

Average area of Chlorzoxazone standard: 4388253

Table 4.25 % RSD of Chlorzoxazone in Intermediate Precision (Ruggedness)

Obs NO	Area of Chlorzoxazone in sample	Content in mg/tab	% LC
1	4360282	497.26	99.45
2	4370706	498.45	99.69
3	4391360	500.80	100.16
4	4410899	503.03	100.61
5	4410479	502.98	100.60
6	4401212	501.93	100.39
Mean	4390823	500.74	100.15
S.D	19301	2.20	0.44
%RSD	0.44	0.44	0.44
Limits for %RSD	**NMT 2.00%**		

Table 4.26 Cumulative % RSD of Chlorzoxazone in precision and Intermediate precision

Obs NO	Content in mg/tab	% LC
M.P 1	497.26	99.45
M.P 2	498.45	99.69
M.P 3	500.80	100.16
M.P 4	503.03	100.61
M.P 5	502.98	100.60
M.P 6	501.93	100.39
I.P 1	499.00	99.80
I.P 2	498.34	99.67
I.P 3	494.97	98.99
I.P 4	501.58	100.32
I.P 5	500.44	100.09
I.P 6	499.35	99.87
Mean	499.84	99.97
S.D.	2.313	0.4627
%Cumulative RSD	0.46	0.46
Limits	**NMT 2.00%**	

M.P – Method precision, I.P- Intermediate precision

Amount of Diclofenac Sodium:

Average area of Diclofenac sodium standard: 704544

Table 4.27 % RSD of Diclofenac Na in Intermediate Precision (Ruggedness)

Obs NO	Area of Chlorzoxazone in sample	Content in mg/tab	% LC
1	710124	50.44	100.88
2	708814	50.35	100.70
3	702010	49.86	99.73
4	701023	49.79	99.59
5	699916	49.72	99.43
6	698301	49.60	99.20
Mean	703365	49.96	99.92
S.D	4477	0.32	0.64
%RSD	0.64	0.64	0.64
Limits for %RSD	**NMT 2.00%**		

Table 4.28 Cumulative % RSD of Diclofenac sodium in precision and Intermediate precision

Obs No	Content in mg/tab	% LC
1	49.73	99.47
2	50.11	100.23
3	50.19	100.37
4	50.31	100.62
5	49.88	99.76
6	50.44	100.89
7	50.44	100.88
8	50.35	100.70
9	49.86	99.73
10	49.79	99.59
11	49.72	99.43
12	49.60	99.20
Mean	50.04	100.07
S.D.	0.292	0.585
% Cumulative RSD	0.58	0.58
Limits	**NMT 2.00%**	

M.P – Method precision, I.P- Intermediate precision

ROBUSTNESS:

A robust or rugged method is one which is not adversely affected by minor changes in experimental variables of the order that might reasonably be expected to take place during the course of the operation of the method. This is therefore tested by carrying out the

chromatography on columns of the same type but with different histories and/or using different batches of mobile phase, perhaps even intentionally making some minor changes in the composition. In the above ruggedness study the cumulative % relative standard deviations with respect to precision values were found to be below 2% for Paracetamol, Chlorzoxazone and Diclofenac sodium respectively. The robustness study was also carried out by changing the pH of the mobile phase by ± 0.2 units, the wavelength by ±2nm and mobile phase composition by ± 5%. The results obtained were within the limits as per the validation criteria.

Mean % assay of Paracetamol

Table 4.29 Robustness study for Paracetamol

Name of the Sample	Change in wavelength 218nm (-2)	Change in wavelength 222nm(+2)	Change in pH of the mobile phase 3.5 (-0.2)	Change in pH of the mobile phase 3.9 (+0.2)	Change in organic phase composition -5%	Change in organic phase composition +5%
Sample -1	100.67	99.95	99.65	100.48	98.96	99.06
Sample-2	100.1	100.12	98.56	100.26	98.12	99.17
Sample -3	99.39	99.28	99.82	98.81	99.87	98.47
Mean	100.05	99.78	99.34	99.85	98.98	98.90
S.D	0.641	0.444	0.684	0.907	0.875	0.376
RSD	0.641	0.445	0.688	0.909	0.884	0.381

% RSD is not more than 2%

Mean % assay of Chlorzoxazone

Table 4.30 Robustness study for Chlorzoxazone

Name of the Sample	Change in wavelength 218nm(-2)	Change in wavelength 222nm(+2)	Change in pH of the mobile phase 3.5 (-0.2)	Change in pH of the mobile phase 3.9 (+0.2)	Change in organic phase composition -5%	Change in organic phase composition +5%
Sample -1	99.97	97.23	97.95	99.62	97.19	98.15
Sample-2	98.36	97.86	99.13	99.56	97.9	98.52
Sample -3	98.21	98.97	98.78	99.01	99.06	99.29
Mean	98.85	98.02	98.62	99.40	98.05	98.65
S.D	0.976	0.881	0.606	0.336	0.944	0.582
RSD	0.987	0.899	0.615	0.338	0.963	0.590

Mean % assay of Diclofenac sodium

Table 4.31 Robustness study for Diclofenac sodium

Name of the Sample	Change in wavelength 218nm(-2)	Change in wavelength 222nm(+2)	Change in pH of the mobile phase 3.5 (-0.2)	Change in pH of the mobile phase 3.9 (+0.2)	Change in organic phase composition -5%	Change in organic phase composition +5%
Sample -1	99.47	100.05	100.51	101.36	99.33	99.49
Sample-2	100.23	99.61	99.16	100.21	98.26	98.62
Sample -3	100.47	99.27	99.57	99.57	99.75	97.54
Mean	100.06	99.64	99.75	100.38	99.11	98.55
S.D	0.522	0.391	0.692	0.907	0.768	0.977
RSD	0.522	0.392	0.694	0.904	0.775	0.991

SUMMARY OF VALIDATION AND RESULTS:

To develop an isocratic reverse phase HPLC method for the determination of Paracetamol, Chlorzoxazone and Diclofenac sodium in combined dosage form the chromatographic conditions were optimized. Depending upon the nature and properties of the analytes solvent selection and composition of the mobile phase was finalized by number of trials. For better separation and resolution the different buffers were tried. It has been found that potassium dihydrogen phosphate buffer, pH 3.7 adjusted with orthophosphoric acid gave better peak shape than other buffers. Thus the mobile phase composed of the mixture of buffer (0.02M$KH_2PO_{4,}$ pH 3.7 adjusted with orthophosphoric acid) acetonitrile, methanol in the ratio of (25: 25: 50, v/v/v) was finalized. The better separation, peak symmetry and reproducibility were obtained with Inertsil C18, 250mm x 4.6mm, 5ìm column. All the three analytes gave better response at 220nm wavelength using UV detector. The flow rate was maintained at 1.0mL/min. There was no peak tailing observed under these optimized chromatographic conditions. Under these conditions the retention times of Paracetamol, Chlorzoxazone and Diclofenac sodium were found to be 2.8 mins, 4.2 and 6.4 mins respectively.

The proposed method showed short elution time and good separation between Paracetamol, Chlorzoxazone and Diclofenac sodium. The system suitability test was performed as per the USP and ICH guidelines to confirm the suitability and the reproducibility of the method. Six consecutive injections of the standard solution were analysed and evaluated for repeatability, tailing factor, theoretical plates and resolution. % RSD values were found

to be 0.52, 0.40 and 0.50 for Paracetamol, Chlorzoxazone and Diclofenac sodium respectively. The tailing factor and theoretical plates were found to be perfectly within the limits.

The method was linear over the range 50–150µg/mL for Paracetamol, 50–150 µg/mL for Chlorzoxazone and 5-15 µg/mL for Diclofenac sodium. The calibration curve was constructed by plotting response factor against concentration of drugs. The slope and intercept value for calibration curve was

$Y = 28594x + 34014$ ($r^2 = 0.9995$) for Paracetamol, $Y = 43904x + 3130$ ($r^2 = 0.9993$) for Chlorzoxazone and $Y = 69489x + 30886$ ($r^2 = 0.9994$) for Diclofenac sodium.

The results show an excellent correlation between response factor and concentration of drugs.

The limit of Detection (LOD) and limit of Quantification (LOQ) of the developed method was determined by injecting progressively low concentrations of the standard solutions using the developed RP-HPLC method. The limit of quantification (LOQ) and limit of detection (LOD) was established using signal-to-noise ratio. The LOQ and LOD of Paracetamol, Chlorzoxazone and Diclofenac sodium were experimentally determined. The LOD of Paracetamol, Chlorzoxazone and Diclofenac sodium was found to be 0.0020 µg/mL, 0.040 µg/mL and 0.025 µg/mL respectively. The LOQ of Paracetamol, Chlorzoxazone and Diclofenac sodium was found to be 0.0065 µg/mL, 0.085 µg/mL and 0.070 µg/mL respectively.

The system precision study was performed to determine the repeatability of the method. Six samples of standard were prepared at 100% level and assayed

according to the procedure. The method precision study was performed to determine the reproducibility of the method. Six samples of tablets were prepared at 100% level and assayed according to the procedure.

The accuracy of the method was determined by the standard addition method at three different levels. Each determination was performed in triplicates. The accuracy was then calculated as the percentage of the standard drug recovered by the recovery study. The results are well within the acceptance limit and hence the method is accurate. The solution stability study revealed that the stock solutions were stable for longer time. The ruggedness of the method was determined by carrying out the experiment on a different instrument like Jasco HPLC by different operators using different columns of similar type like Hypersil C18. Robustness of the method was determined by making slight changes in the chromatographic conditions. It was observed that there were no marked changes in the chromatograms, which demonstrated that the RP-HPLC method developed is rugged and robust.

CONCLUSION:

The short elution time makes the method very valuable for quality control and stability testing of drugs and their pharmaceutical preparation. In the present work all the analytes were eluted and separated within 7 minutes.

The isocratic RP- HPLC method has proved to be simple, specific, precise and accurate and is suitable for simultaneous quantification of Paracetamol, Chlorzoxazone and Diclofenac sodium. The proposed method gives a good resolution among the analytes. The method is very simple,

rapid and no complicated sample preparation is needed. High percentage of recovery shows that the method is accurate.

The solution stability data on the drugs carried out by this method shows that the stock solutions are stable for a longer time than that taken for analysis. The linearity, precision, accuracy, ruggedness study of the method proves that the method is easily reproducible in any quality control set up. Thus this method can be used for routine quality control analysis.

REFERENCES:

1) Goodman and Gilman's (1992), The Pharmacological Basis of Therapeutics, Eighth edn. Volume I, *Macmillan Publishing Company,* Singapore 656-657.

2) Bailey L.C, Remington (1995), The Science and Practice of Pharmacy, Nineteenth edition Volume II, *Mack Publishing Company, Pennsylvania* 1208.

3) *British Pharmacopoeia* (2003), HMSO, Volume II, London 582-583.

4) Bailey L.C., Remington (1995), The Science and Practice of Pharmacy, Nineteenth edition, Volume II, *Mack Publishing Company, Pennsylvania* 1211.

5) *The Merck Index* (2001), Thirteenth edition, Merck and Co. Inc.USA

379- 380.

6) Bailey L.C., Remington (1995), The Science and Practice of Pharmacy, Nineteenth edition, Volume II, *Mack Publishing Company*, Pennsylvania 1033.

7) Bhatia, Manish S.; Dhaneshwar, S.R. Simultaneous spectrophotometric determination of Diclofenac sodium

Chlorzoxazone and Paracetamol from combined dosage forms. *Indian Drugs* (1995), 32(9), 446-50.

8) Bhatia, M. S.; Kaskhedikar, S. G.; Chaturvedi, S. C. Comparative evaluation of different spectrophotometric methods developed for simultaneous estimation of Diclofenac sodium Chlorzoxazone and Paracetamol from combined dosage forms. *Indian Drugs* (1997), 34(3), 149-153

9) Garg, G.; Saraf, Swarnlata; Saraf, S. Simultaneous estimation of Aceclofenac, Paracetamol and Chlorzoxazone in tablets. *Indian Journal of Pharmaceutical Sciences* (2007), 69(5), 692-694.

10) Ravisankar, S.; Vasudevan, M.; Nanjan, M. J.; Bijukurian; Suresh, B. Reversed phase HPLC method for the estimation of Paracetamol, Chlorzoxazone and Diclofenac sodium in formulations. *Indian Drugs* (1997), 34(11), 663-665.

11) Goyal, Anju; Jain, Sandeep. Rajendra, Simultaneous estimation of Paracetamol, Chlorzoxazone and Diclofenac sodium in pharmaceutical formulation by a novel HPLC method. *Acta Pharmaceutica Sciencia* (2007), 49(2), 147-151.

12) Chawla, Jyoti L.; Sodhi, Renu A.; Sane, R.T. Simultaneous determination of Chlorzoxazone, Paracetamol and Diclofenac sodium by different chromato- graphic techniques. *Indian Drugs* (1996), 33(4), 171-178.

13) Shinde, V. M.; Desai, B. S.; Tendolkar, N. M. Simultaneous determination of Paracetamol, Diclofenac sodium and Chlorzoxazone by HPLC from tablet. *Indian Journal of Pharmaceutical Sciences* (1995), 57(1), 35-7.

CHAPTER - V

Simultaneous estimation of Ampicillin Sodium and Sulbactam Sodium in injectable dosage form by high performance liquid chromatography (HPLC)

METHOD DEVELOPMENT

Method development in chromatography is the setting up of an analytical procedure that will be approximate for the analysis of a particular sample. It starts with the choice of the techniques. In the present research work high performance liquid chromatography (HPLC) was the technique used for the separation and simultaneous estimation of the Ampicillin sodium and Sulbactam sodium. In HPLC method development the selection of chromatographic mode, selection of the stationary phase, optimization of chromatographic conditions played a vital role. Efficient method development requires expert knowledge of chromatographic science and extensive practical experience.

Selection of chromatographic mode:

All the drugs selected in the present study are polar in nature. Ampicillin sodium (AMP) and Sulbactam sodium (SUB) are antibacterial drugs. They are freely soluble in water, sparingly soluble in acetone, practically insoluble in fatty oils and in liquid paraffin. Molecular weights of Ampicillin sodium and Sulbactam sodium are 371.39 and 255.22 respectively. As both analytes are water soluble the reverse phase mode of separation was selected.

Selection of stationary phase/Chromatographic column:

Traditionally the stationary phase used in HPLC has been silica gel which separates solutes largely on the basis of polarity. The bonded phase being introduced to provide a material that would separate solutes by dispersive interaction. The bonded phase was also based on silica gel.

The availability of a stable high performance column is essential in developing rugged, reproducible method. When selecting an HPLC column it is necessary to consider column to column reproducibility. The selection of the column depends upon the nature of the analyte.

From the trials it was found that Inertsil, ODS-3V C18 (250mm × 4.6mm,i.d.) with particle size 5 µm is the most suitable column with good separation and peak shape.

Selection of mobile phase:

As both the analytes are strongly polar in nature, a polarizable stationary phase would be appropriate to separate the solutes by polar and induced polar interactions. If the solutes are weakly polar then a strong polar stationary phase would be require to separate the solute by polar interactions. Many solvents have been tried to prepare the mobile phase. Both the analytes are highly soluble in water, hence water was the major component of the mobile phase and the organic solvent has the minimum composition in the mobile phase. Initially water and methanol were taken in the ratio 50:50, but proper separation of analytes was not found. Then by increasing the composition of water and by changing the organic solvent such as acetonitrile the separation of the analytes was achieved. Hence water: ACN

mixture was selected as a mobile phase for the separation.

Selection of buffer for mobile phase:

Buffer solution plays a vital role in the separation of analytes. Selection of buffer for mobile phase was done according to the nature of the drugs. Different buffers were used in the study of separation of the drug components. Initially 0.01M and 0.10M NaH_2PO_4 was used with acetonitrile. But proper separation of analytes was not observed. In some cases it also showed merging of the two peaks. Ammonium acetate was the second choice of buffer and it was used in the concentration level of 0.01M. The composition of buffer and ACN in the ratio of 83:17 was finalized to separates the analytes. The pH of the buffer solution was 6.0.

Thus the mobile phase was finalized as Buffer (0.01M CH_3COONH_4 pH 6.0) ,acetonitrile in the ratio (83 : 17 v/v)

Selection of detector and detection wavelength:

The function of the detector in HPLC is to monitor the mobile phase as it emerges from the column. The UV absorption detector is the most widely used detector in HPLC, being based on the principle of absorption of UV visible light as the effluent of the column is passed through a small flow cell and held in the radiation beam. In the present research work UV detector was selected. The detection wavelength was set at 230nm because both the analytes shows maximum absorbance at this wavelength.

Table 5.1 FINALISED CHROMATOGRAPHIC CONDITIONS FOR HPLC METHOD

Chromatographic mode	Chromatographic condition
Standard solution	200 µg/mL of Ampicillin sodium , 100 µg/mL of Sulbactam sodium
Equipment system	Waters HPLC
Pump	Waters 501 isocratic pump
Detector	Waters 486 tunable absorbance UV-detector
Data processor	Millenium^{32} chromatographic software
Injector	Waters ^{TM} 717plus autosampler
Stationary phase/Column	Inertsil C18 column (250 X 4.6 mm , i.d 5µm)
Buffer solution Mobile phase	Aqueous buffer solution of 0.01M CH_3COONH_4 Buffer(0.01M CH_3COONH_4, pH= 6.0) : Acetonitrile (83 : 17v/v)
Detection wavelength	230 nm
Flow rate	1.0 mL/min
Injection Volume	20µl
Column temperature	Ambient
Pump Mode	Isocratic

PREPARATION OF STOCK SOLUTIONS

Preparation of Buffer

Buffer solution was prepared by dissolving accurately weighed 0.081g of ammonium acetate (CH_3COONH_4) in 1 liter of distilled water (HPLC Grade). The concentration of the buffer solution was 0.01M.

Preparation of Mobile phase

Mobile phase was prepared by using the above prepared buffer and acetonitrile, mixing them in the volume ratio of (83:17, v/v). The pH of this mixture was 6.0. The mobile phase was then sonicated for 10min to remove the dissolved gases which may cause interference to the HPLC system.

Preparation of Standard stock solution

50mg of Sulbactam (SUB) sodium and 100 mg of Ampicillin sodium (AMP) was accurately weighed and transferred to a 100mL volumetric flask. It was dissolved in a minimum quantity of double distilled water and then diluted up to the mark with water. The concentration of the solution obtained was 500 µg/mL for Sulbactam and 1000 µg/mL for Ampicillin sodium (Solution A).

5mL of this solution A was taken in a 25 mL volumetric flask and diluted upto the mark with distilled water. The concentration of the solution obtained was 100 µg/mL for SUB and 200 µg/mL for AMP.

Preparation of Sample solution

The sample solution of Sulbactam sodium and Ampicillin sodium was prepared from injectable dosage form. The content of the Injectable dosage form was 0.5gm of anhydrous Sulbactam sodium and 1.0 gm anhydrous Ampicillin sodium. 150mg of the sample was taken in 100 mL volumetric flask. It was dissolved and diluted by sterile water upto the mark. Then 5mL of this solution was taken in a 25 mL volumetric flask and diluted up to the mark with sterile water.

METHOD IN BRIEF:

A reverse phase high performance liquid chromatographic method was developed for the simultaneous estimation of Ampicillin sodium and Sulbactam sodium in a injectable formulation. The separation was achieved by Inertsil C18 column (250mm × 4.6mm i.d 5µm particle size) and Ammonium acetate buffer (pH = 6.0) : Acetonitrile (83 : 17) as mobile phase at a flow rate of 1.0mL/min. The retention times of Ampicillin sodium and Sulbactam sodium were found to be 5.8 mins & 4.3mins respectively. The proposed method was validated for linearity, accuracy and precision. The calibration curve was linear over the range of 50 – 150 µg/mL for Sulbactam sodium and 100 - 300 µg/mL for Ampicillin sodium. The proposed method can be applied successfully in routine analysis.

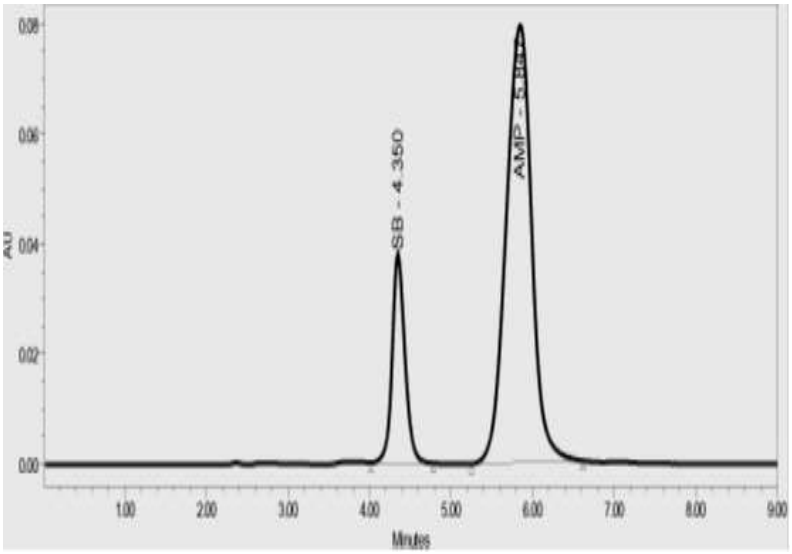
METHOD VALIDATION:

A validation of the method for simultaneous determination of Ampicillin and Sulbactam from injectable dosage form using HPLC was done according to the ICH guidelines. The proposed method which was developed subjected to method validation process to determine its suitability for the intended use. The method was validated for specificity, linearity, accuracy, precision and recovery and solution stability.

SPECIFICITY:

The specificity of the method was checked by injecting the sample solution and standard solution. The chromatogram shows that there was no interference at the retention time of individual drugs due to other components.

Figure 5.1 In the optimized conditions Ampicillin sodium has a Rt=5.84 and Sulbactam sodium has 4.35. The major peaks are well separated. There are no other potentially interfering peaks at the retention time of the two drugs, thus showing the specificity of the method



SYSTEM SUITABILITY TEST:

System suitability test is an integral part of many analytical procedures. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated. It should be performed prior to initiation of the analysis.

Standard solution having assay concentration of individual analyte (100% level) was prepared as described earlier and injected into the HPLC system. The %RSD values were found to be satisfactory and meeting the requirements.

Table 5.2 System suitability tests for Ampicillin sodium and Sulbactam Sodium

Obs No	100% Level			
	Ampicillin Sodium		Sulbactam Sodium	
	Peak area	Retention time	Peak area	Retention time
1	1710642	5.83	429650	4.34
2	1717879	5.84	421922	4.35
3	1719028	5.84	425219	4.34
4	1721134	5.83	425079	4.36
5	1723316	5.85	429852	4.35
Mean	1718400	5.84	426344	4.35
S.D	4809	0.0084	3378	0.0083
% RSD	0.280	0.143	0.792	0.192

LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION

A calibration curve was prepared using concentrations in the range of 100-300 µg/mL for Ampicillin and 50-150 µg/mL Sulbactam sodium. The standard deviations of *y*-intercepts of regression lines were determined and substituted in the following equation for the determination of detection limit and quantitation limit. Detection limit = 3.3ó /s; quantitation limit = 10ó /s; where ó is standard deviation of *y*-intercepts of regression lines and s is the slope of the calibration curve. Detection limit and quantitation limit can also be estimated using signal to noise ratio and relative standard deviation method.

The LOQ and LOD of Ampicillin and Sulbactam were experimentally determined. The LOD of Ampicillin and Sulbactam was found to be 0.8 µg/mL and 0.4 µg/mL respectively. The LOQ of Ampicillin and Sulbactam was found to be 2.0 µg/mL and 1.0 µg/mL respectively.

LINEARITY RANGE FOR AMPICILLIN SODIUM AND SULBACTAM SODIUM:

This experiment was carried out to demonstrate the range over which the response of the detector is linear with respect to concentration of Ampicillin and Sulbactam. In a series of volumetric flasks varying volumes of solution A containing Ampicillin and Sulbactam was taken and then diluted upto the mark with a diluent. Preparation of linearity levels were as per the following table 5.3

Table 5.3 Preparation of mixed standard solution for Linearity levels

Linearity Levels	Volume of std stock solution added in mL	Volume made upto in mL	Concentration of Ampicillin in ppm	Concentration of Sulbactam in ppm
50%	2.5	25	100	50
60%	3	25	120	60
80%	4	25	160	80
100%	5	25	200	100
120%	6	25	240	120
150%	7.5	25	300	150

Table 5.4 Linearity levels for Ampicillin sodium

Obs No.	Conc. level %	Conc. in µg/ml	Peak Area			Mean peak area	S.D.	% RSD
			1	2	3			
1	50	100	851698	851956	851722	851792	142.53	0.017
2	60	120	1058401	1059914	1058630	1058982	815.50	0.077
3	80	160	1444364	1442056	1442334	1442918	1259.96	0.087
4	100	200	1765680	1764258	1766010	1765316	930.99	0.053
5	120	240	2195824	2198019	2197080	2196974	1101.31	0.050
6	150	300	2753101	2750983	2752931	2752338	1176.83	0.043

Fig 5.2 Linearity Level plot for Ampicillin sodium

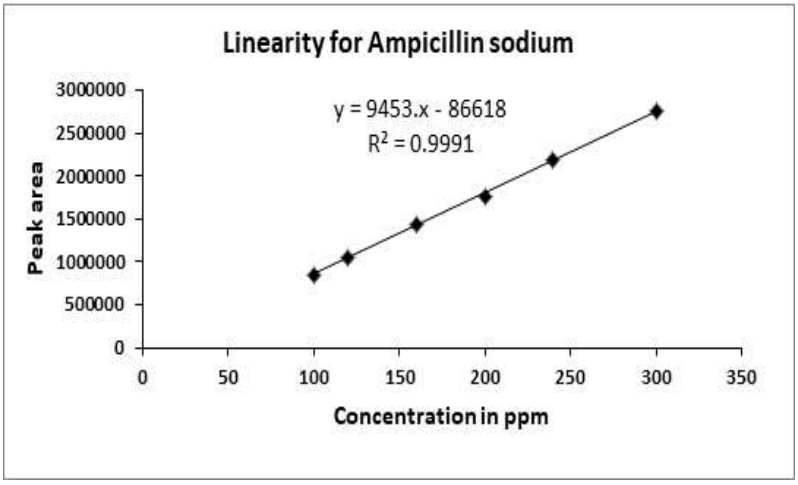


Table 5.5 Linearity levels for Sulbactam sodium

Obs No.	Conc. level %	Conc. in µg/ml	Peak Area			Mean peak area	S.D.	% RSD
			1	2	3			
1	50	50	211541	211884	211932	211786	213.24	0.101
2	60	60	253550	254111	253957	253873	289.85	0.114
3	80	80	344056	343563	343665	343761	260.24	0.076
4	100	100	437647	438551	437897	438032	466.80	0.107
5	120	120	522510	522178	522403	522364	169.46	0.032
6	150	150	653444	654411	653621	653825	514.87	0.079

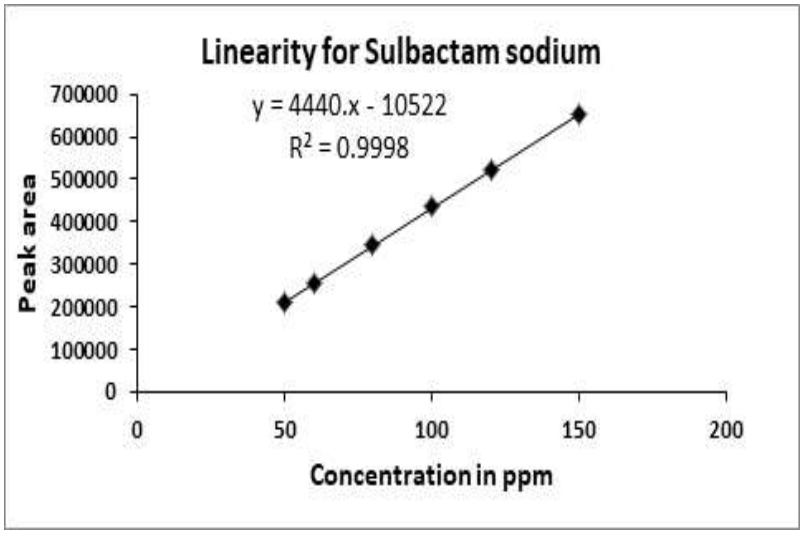


Fig 5.3 Linearity Level plot for Sulbactam sodium

Regression analysis

The calibration data range 100µg/mL to 300µg/mL for Ampicillin sodium and 50µg/mL to 150 µg/mL for Sulbactam sodium was further considered for regression analysis. The regression analysis of the calibration data was carried out to determine the relationship between the dependent variable (Peak area) and the independent variable (Drug) concentration.

Applying the linearity curve from fig. 5.2 for Ampicillin and fig 5.3 for Sulbactam the values for the regression equation y= mx + C are found to be,

For Ampicillin	**For Sulbactam**
y = 9453x + (-86618)	y = 4440x + (-10522)
m = 9453 (Slope)	m = 4440 (Slope)
C = -86618 (Intercept)	C = -10522 (Intercept)
x = Found concentration of the drug	y = Calculated peak area

The regression equation indicates that one unit increase in the concentration of the Ampicillin and Sulbactam will increase the detector response by 9453 and 4440 units respectively. Also by using the regression equation the actual values of the drug injected can be back calculated by substituting the value of y i.e. area of the drug. The correlation (r^2) for Ampicillin sodium and Sulbactam sodium was found to be 0.9991 and 0.9998 respectively.

PRECISION:

The precision of an analytical method is the closeness of a series of individual measurements of an analyte when the analytical procedure is applied to multiple aliquots of

a sample solution. The precision is calculated as a coefficient of variation i.e relative standard deviation (RSD).

System Precision:

System precision study was performed by injecting six samples of the mixed standard solutions of Ampicillin sodium and Sulbactam sodium (100%level) into the system and assayed according to the procedure. The results of the precision study are given as follows.

Table 5.6 Precision study for Ampicillin sodium

obs No	Peak Area	True value Conc. in µg/ml	Experimental values Conc. in µg/ml
1	1761251	200	195.47
2	1765841	200	195.95
3	1762326	200	195.58
4	1766628	200	196.04
5	1771254	200	196.53
6	1772542	200	196.66
Mean	-	-	196.04
S.D	-	-	0.48
%RSD	-	-	0.247
%Accuracy	-	-	98.02

Table 5.7 Precision study for Sulbactam sodium

Obs No	Peak Area	True value Conc. in µg/ml	Experimental values Conc. in µg/ml
1	429650	100	99.14
2	421922	100	97.40
3	425219	100	98.14
4	425079	100	98.11
5	429852	100	99.18
6	426344	100	98.39
Mean	-	-	98.39
S.D	-	-	0.68
%RSD	-	-	0.69
%Accuracy	-	-	98.39

Method Precision (Reproducibility)

The method precision study was performed to determine the reproducibility of the method. Six samples were prepared at 100% level and assayed according to the procedure.

For Ampicillin Sodium

Table5.8 Reproducibility study of Ampicillin sodium

Obs NO	Peak area	True value Conc. in µg/ml	Experimental values Conc. in µg/ml
1	1760487	200	195.39
2	1762135	200	195.56
3	1764229	200	195.78
4	1772548	200	196.66
5	1775847	200	197.01
6	1769642	200	196.35
Mean	-	-	196.13
S.D	-	-	0.59
%RSD	-	-	0.30
%Accuracy	-	-	98.06

For Sulbactam Sodium

Table5.9 Reproducibility study of Sulbactam sodium

Obs NO	Peak area	True value Conc. in µg/ml	Experimental values Conc. in µg/ml
1	428624	100	98.91
2	428011	100	98.77
3	428862	100	98.96
4	430123	100	99.24
5	429214	100	99.04
6	427958	100	98.76
Mean	-	-	98.95
S.D	-	-	0.17
%RSD	-	-	0.17
%Accuracy	-	-	98.95

ACCURACY: (% RECOVERY)

The accuracy of the method was studied by recovery experiments. The recovery experiments were performed by adding known amounts of the drugs into a 100% level sample solution. The recovery was performed at three levels (110%, 120%, and 130%) of the label claim per injectable dosage (0.5gm of SUB and 1.0 gm of AMP). Three samples were prepared for each recovery level. The sample solution contains 100mg of Ampicillin and 50mg of Sulbactam. The 0% level can be considered as 100% level. In this sample solution 10%, 20% and 30% of the standard drugs were added and injected into the system.

For Ampicillin sodium:

Table 5.10 Accuracy study of Ampicillin sodium

Obs No	Levels in %	Area of sample	Amt added in mg	Amt found in mg	% recovery
1	0%	1760542	0	99.65	99.65
2		1762154	0	99.75	99.75
3		1766982	0	100.02	100.02
1	10%	1985661	10.2	112.40	101.99
2		1968220	10.6	111.41	100.73
3		1970157	10.5	111.52	101.38
1	20%	2126025	20	120.34	100.29
2		2125124	20.2	120.29	100.08
3		2126845	20.2	120.39	100.16
1	30%	2308783	30	130.69	100.53
2		2305036	30.2	130.48	100.21
3		2312159	30.4	130.88	100.37
				Mean	100.43
				S.D.	0.67
				%RSD	0.67
				Range of % recovery	**99.65-101.99**

For Sulbactam sodium:

Table 5.11 Accuracy study of Sulbactam sodium

Obs No	Levels in %	Area of sample	Amt added in mg	Amt found in mg	% recovery
1	0%	428624	0	50.27	100.53
2		428412	0	50.24	100.49
3		427874	0	50.18	100.36
1	10%	511135	10.2	59.94	99.91
2		508515	10.6	59.64	99.39
3		506518	10.5	59.40	99.00
1	20%	585211	20	68.63	98.04
2		585487	20.2	68.66	98.09
3		586298	20.2	68.76	98.23
1	30%	669459	30	78.51	98.14
2		670589	30.2	78.64	98.31
3		671550	30.4	78.76	98.45
				Mean	99.08
				S.D.	1.01
				%RSD	1.02
				Range of % recovery	98.04-100.53

SOLUTION STABILITY STUDY:

The stability of the analytical solutions was determined in terms of the assay of the drugs in the standard preparation and the assay preparation at room temperature. These solutions were analyzed at 0, 6, 12, and 24 hrs against a freshly prepared standard at each time interval. The relative standard deviations for the assay values, determined up to 24 hrs for Ampicillin and Sulbactam in assay preparation and standard preparation, were 0.21% and 0.09%, respectively. The assay values were within ±2% after 24 hrs. The results showed that the solutions were stable for 24 hrs at room temperature.

For Ampicillin sodium:

Table 5.12 Solution stability of Ampicillin standard solution

Conditions	Level in %	Peak area	% Relative Deviation
Initial - 0 hrs	100	1745156	
Initial -6hrs	100	1755415	0.59
Initial -12hrs	100	1750265	-0.29
Initial -24hrs	100	1752564	0.13
	Mean	1750850	
	S.D.	3759	
	%RSD	0.21	

Table 5.13 Solution stability of Ampicillin sodium sample solution

Conditions	Level in %	Peak area	% Assay in Injectable sample	% Label claim
Initial - 0 hrs	100	1760546	99.99	99.99
Initial -6hrs	100	1765849	100.30	100.30
Initial -12hrs	100	1763624	100.17	100.17
Initial -24hrs	100	1755129	99.69	99.69
	Mean	1761287	100.04	100.04
	S.D.	4023	0.23	0.2
	%RSD	0.23	0.23	0.23

For Sulbactam sodium:

Table 5.14 Solution stability of Sulbactam sodium standard solution

Conditions	Level in %	Peak area	% Relative Deviation
Initial - 0 hrs	100	428415	
Initial -6hrs	100	427879	-0.13
Initial -12hrs	100	427456	-0.099
Initial -24hrs	100	428259	0.188
	Mean	428002.25	
	S.D.	370.8	
	%RSD	0.09	

Table 5.15 Solution stability of Sulbactam sodium sample solution

Conditions	Level in %	Peak area	% Assay in Injectable sample	% Label claim
Initial - 0 hrs	100	427102	50.09	100.18
Initial -6hrs	100	427628	50.15	100.30
Initial -12hrs	100	427893	50.18	100.36
Initial -24hrs	100	419927	49.25	98.49
	Mean	425637	49.92	99.83
	S.D.	3309	0.39	0.8
	%RSD	0.78	0.78	0.78

INTERMEDIATE PRECISION (RUGGEDNESS):

Intermediate precision of the method was assessed by analyzing the samples six times on different days, by different chemists, by using different analytical columns of the same manufacture and different HPLC systems. The percentage assay was calculated using the area of the mixed standard preparation. The assay results are shown as follows.

Ruggedness study, mixed standard solution of Ampicillin sodium and Sulbactam sodium

Table 5.16 Ruggedness study, standard solution

Obs No	Ampicillin		Sulbactam	
	Peak Area	Retention time	Peak Area	Retention time
1	1742514	5.81	428271	4.31
2	1739831	5.82	427652	4.32
3	1749069	5.80	425119	4.31
4	1743150	5.81	429718	4.32
5	1747201	5.81	427356	4.33
6	1753998	5.82	427567	4.31
Mean	1745961	5.81	427613	4.32
S.D	5160	0.01	1494	0.01
%RSD	0.296	0.130	0.349	0.189

Amount of Ampicillin sodium:

Average area of Ampicillin standard: 1745961

Table 5.17 % RSD of Ampicillin in Intermediate Precision (Ruggedness)

Obs No	Area of Ampicillin in sample	% Assay in mg/sample	% LC
1	1759443	100.77	100.77
2	1752456	100.37	100.37
3	1748789	100.16	100.16
4	1746658	100.04	100.04
5	1744267	99.90	99.90
6	1739834	99.65	99.65
Mean	1748574	100.15	100.15
S.D	6809	0.390	0.390
% RSD	0.389	0.389	0.389

Table 5.18 Cumulative % RSD of Ampicillin in method precision (M.P.) and Intermediate precision(I.P.)

Obs No	Content in mg/Sample	% LC
M.P 1	99.65	99.65
M.P 2	99.74	99.74
M.P 3	99.86	99.86
M.P 4	100.33	100.33
M.P 5	100.52	100.52
M.P 6	100.17	100.17
I.P 1	100.77	100.77
I.P 2	100.37	100.37
I.P 3	100.16	100.16
I.P 4	100.04	100.04
I.P 5	99.90	99.90
I.P 6	99.65	99.65
Mean	100.10	100.10
S.D.	0.36	0.36
%Cumulative RSD	0.36	0.36
Limits for % of RSD	NMT 2.00%	

Amount of Sulbactam Sodium

Average area of Sulbactam sodium standard: 427613

Table 5.19 % RSD of Sulbactam in Intermediate Precision (Ruggedness)

Obs No	Area of Sulbactam in sample	Content in mg/Sample	% LC
1	425916	49.80	99.60
2	425130	49.71	99.42
3	419923	49.10	98.20
4	422569	49.41	98.82
5	424382	49.62	99.24
6	421891	49.33	98.66
Mean	423301.83	49.50	98.99
S.D	2248.859	0.263	0.526
%RSD	0.531	0.531	0.531
Limits for %RSD	**NMT 2.00%**		

Table 5.20 Cumulative % RSD of Sulbactam in method precision (M.P.) and Intermediate precision(I.P.)

Obs No	Content in mg/Sample	% LC
M.P 1	50.27	100.53
M.P 2	50.20	100.39
M.P 3	50.30	100.59
M.P 4	50.44	100.89
M.P 5	50.34	100.67
M.P 6	50.19	100.38
I.P 1	49.80	99.60
I.P 2	49.71	99.42
I.P 3	49.10	98.20
I.P 4	49.41	98.82
I.P 5	49.62	99.24
I.P 6	49.33	98.66
Mean	49.89	99.78
S.D.	0.456	0.910
% Cumulative RSD	0.914	0.912
Limits	**NMT 2.00%**	

ROBUSTNESS:

A robust or rugged method is one which is not adversely affected by minor changes in experimental variables of the order that might reasonably be expected to take place during the course of the operation of the method. This is therefore tested by carrying out the chromatography on columns of the same type but with different histories and/or using different batches of mobile phase, perhaps even intentionally making some minor changes in the composition. In the above ruggedness study the cumulative % relative standard deviations with respect to precision values were found to be below 2% for Ampicillin sodium and Sulbactam sodium respectively. The robustness study was also carried out by changing the pH of the mobile phase by ± 0.2 units, the wavelength by ± 2nm and mobile phase composition by ±5%. The results obtained were within the limits as per the validation criteria.

Mean % assay of Ampicillin sodium

Table 5.21 Robustness study for Ampicillin sodium

Name of the Sample	Change in wavelength 228nm(-2)	Change in wavelength 232nm(+2)	Change in pH of the mobile phase 5.8 (-0.2)	Change in pH of the mobile phase 6.2 (+0.2)	Change in organic phase composition -5%	Change in organic phase composition +5%
Sample -1	100.77	99.25	98.94	99.36	99.37	98.57
Sample-2	100.37	98.23	98.62	99.76	99.82	97.53
Sample -3	100.16	98.61	97.95	100.05	98.27	98.85
Mean	100.43	98.70	98.50	99.72	99.15	98.32
S.D	0.310	0.515	0.505	0.346	0.797	0.696
RSD	0.309	0.522	0.513	0.347	0.804	0.707

Mean % assay of Sulbactam sodium

Table 5.22 Robustness study for Sulbactam sodium

Name of the Sample	Change in wavelength 228nm(-2)	Change in wavelength 232nm(+2)	Change in pH of the mobile phase 5.8 (-0.2)	Change in pH of the mobile phase 6.2 (+0.2)	Change in organic phase composition -5%	Change in organic phase composition +5%
Sample -1	98.75	99.06	100.25	97.8	98.99	99.15
Sample-2	97.35	99.87	99.62	98.25	97.32	98.19
Sample -3	99.02	98.26	99.27	98.93	98.08	99.6
Mean	98.37	99.06	99.71	98.33	98.13	98.98
S.D	0.896	0.805	0.497	0.569	0.836	0.720
RSD	0.911	0.813	0.498	0.579	0.852	0.728

SUMMARY OF VALIDATION AND RESULTS:

A reverse-phase HPLC procedure was proposed as a suitable method for the simultaneous determination of Ampicillin sodium and Sulbactam sodium in a combined dosage form. Method development was started with 80% methanol in water, but no peaks were observed. The mobile phase was then adjusted by mixing potassium dihydrogen phosphate (0.02 M) with methanol in the ratio 20:80. This resulted in distorted signals that were not well defined. As the analytes are water soluble, the composition of buffer has been increased as compared to the organic solvent. The chromatographic conditions were adjusted to provide adequate retention and resolution of Ampicillin sodium and Sulbactam sodium. A mixture of Ammonium acetate buffer and acetonitrile, in the ratio of 83:17, v/v with pH 6.0. at a flow rate of 1 mL/min, was found to be an appropriate mobile phase, allowing adequate separation of active substances of the combined dosage form. The wavelength was set at 230nm. Typical chromatogram obtained by using

the aforementioned mobile phase, for sample solution of Ampicillin sodium and Sulbactam sodium illustrated in Fig. 5.1. The retention times of Ampicillin and Sulbactam were 5.81 and 3.31 mins, respectively.

The specificity of the method was checked by injecting the mixed standard solution and sample solution of Ampicillin and Sulbactam. The chromatogram shows that there was no interference found at the retention time of the analytes.

The LOD of Ampicillin sodium and Sulbactam sodium was found to be 0.8 µg/mL and 0.4 µg/mL respectively. The LOQ of Ampicillin and Sulbactam was found to be 2.0 µg/mL and 1.0 µg/mL respectively.

The method was linear over the range 100–300µg/mL for Ampicillin and 50–150 µg/mL for Sulbactam. The calibration curve was constructed by plotting the response factor against concentration of drugs. The slope and intercept value for calibration curve was

$y = 9453x + (-86618)$ ($r^2 = 0.9991$) for Ampicillin,

$y = 4440x + (-10522)$ ($r^2 = 0.9998$) for Sulbactam

The high values of the correlation coefficients were indicative of linear relationships between analyte concentration and peak area.

The accuracy of the method was determined by the standard addition method at three different levels. Each determination was performed in triplicates. The accuracy was then calculated as the percentage of the standard drug recovered by the recovery study. The results are well within the acceptance limit and hence the method is accurate. The recovery studies of Ampicillin sodium and Sulbactam sodium were found in the range of 99.65 to 101.99 and 98.04 to 100.53 respectively.

CONCLUSION:

The proposed developed RP-HPLC method for the estimation of Ampicillin sodium and Sulbactam sodium was found to be simple, economical and useful with high accuracy, precision, low detection limit and quantitation limit. The proposed methods did not utilize any extraction step for recovering the drug from the formulation excipients matrixes and thereby decreased the degree of error, time in the estimation of drugs and the overall cost of the analysis. The solvent system used was a simple mobile phase compared to the reported method. The method gives good resolution between Ampicillin sodium and Sulbactam sodium. The method was validated and found to be simple, sensitive, accurate, precise and economical. Therefore, the proposed method can be used for routine analysis of Ampicillin sodium and Sulbactam sodium in their pharmaceutical dosage form.

REFERENCES:

1) Dhandapani, B.; Thirumoorthy, N.; Rasheed, Shaik Harun; Kotaiah, M. Rama; Chandrasekhar, K. B. RP-HPLC method development and validation for the simultaneous estimation of Cefoperazone and Sulbactam in parenteral preparation. *International Journal of ChemTech Research* (2010), 2(1), 752-755.

2) Jiang, Hong; Hu, Changqin; Jin, Shaohong. Determination of Amoxicillin and Sulbactam pivoxil and related substances in tablets. Hubei Institute for Drug Control, Wuhan, Peop. Rep. China, *Yaowu Fenxi Zazhi* (2002), 22(2), 91-94.

3) Chen, Yingying; Chen, Yunlei; Wang, Hongyan. HPLC determination of Amoxicillin and Sulbactam sodium in

compound injection. *Zhongguo Yaoxue Zazhi (Beijing, China)* (2001), 36(3), 191-192

4) Wenzel M; Wildfeuer A; Gutsche F Pharmacokinetics of Ampicillin/Sulbactam in patients undergoing colorectal surgery: measurements in serum, the colonic wall and in tissue at the incision site. *International journal of antimicrobial agents* (1996), 6 Suppl S35-9.

5) Siddiqui, Masoom Raza; Tariq, Abu; Chaudhary, Manu; Reddy, K. Dinesh; Negi, Prithvi Singh; Yadav, Jitendra; Srivastava, Nitya; Shrivastava, Sanjay Mohan; Singh, Rajkumar. Development and validation of high performance liquid chromatographic method for the simultaneous determination of Ceftazidime and Sulbactam in spiked plasma and combined dosage form-Zydotam. *American Journal of Applied Sciences* (2009), 6(10), 1781-1787.

6) Tsou, Tai-Li; Huang, Yu-Chuan; Lee, Chiu-Wey; Lee, An-Rong; Wang, Hsian-Jenn; Chen, Su-Hwei. Simultaneous determination of Ampicillin, Cefoperazone, and Sulbactam in pharmaceutical formulations by HPLC with â -cyclodextrin stationary phase. *Journal of Separation Science* (2007), 30(15), 2407-2413.

7) Aparicio, Irene; Bello, Miguel Angel; Callejon, Manuel; Guiraum, Alfonso. Simultaneous determination of Rifampicin and Sulbactam in mouse plasma by high-performance liquid chromatography. *Biomedical Chromatography* (2006), 20(8), 748-752.

8) Qi, Meiling; Wang, Peng; Sun, Yujing; Wang, Jun. An LC method for simultaneous determination of Amoxicillin and Sulbactam pivoxil in a combination formulation. *Journal of Liquid Chromatography & Related Technologies* (2003), 26(12), 1927-1936.

9) Qi, Mei-Ling; Chen, Rong-Liang; Cong, Rui-Hua; Wang, Peng. A validated method for simultaneous determination of Piperacillin sodium and Sulbactam sodium in sterile powder for injection by HPLC. *Journal of Liquid Chromatography & Related Technologies* (2003), 26(4), 665-676.

10) Pajchel, Genowefa; Pawlowski, Krzysztof; Tyski, Stefan. CE versus LC for simultaneous determination of Amoxicillin/ Clavulanic acid and Ampicillin/Sulbactam in pharmaceutical formulations for injections. *Journal of Pharmaceutical and Biomedical Analysis* (2002), 29(1-2), 75-81.

11) Argekar, A. P.; Kunjir, S. S. Simultaneous determination of Sulbactam and Ampicillin sodium in pharmaceutical preparations by high performance liquid chromatography - reverse phase (HPLC - RP). *Indian Drugs* (1996), 33(7), 352-354

12) Sulochana, K. N.; Bhooma, V.; Madhavan, H. N.; Ramakrishnan, S.; Biswas, A. High performance liquid chromatographic method for simultaneous determination of Ampicillin and Sulbactam in biological samples. *Indian Journal of Pharmacology* (1995), 27(3), 189-92.

13) Haginaka, Jun; Nishimura, Yuki. Simultaneous determination of Ampicillin and Sulbactam by liquid chromatography: post-column reaction with sodium hydroxide and sodium hypochlorite using an active hollow-fiber membrane reactor. *Journal of Chromatography, Biomedical Applications* (1990), 532(1), 87-94.

CHAPTER - VI

Simultaneous determination of Paracetamol and Aceclofenac by high performance thin layer chromatography (HPTLC) in tablet dosage form

INTRODUCTION

Thin-layer chromatography (TLC) is without doubt one of the most versatile and widely used separation method in chromatography. Commercially, many sorbents on a variety of backings are now available. Most stages of the technique are now automated (can now be operated instrumentally) and modern HPTLC (High performance thin-layer chromatography) allows the handling of a large number of samples in one chromatographic run. Speed of separation (development time), high sensitivity and good reproducibility all result from the higher quality of chromatographic layers and the continual improvement in instrumentation. In addition TLC has remained relatively inexpensive and one can easily see why it is still popular today. It has found a use in a wide range of application areas as the concept of TLC is so simple and samples usually require only minimal pretreatment.

Thin layer chromatography (TLC), a branch of liquid chromatography is one of the most popular and extensively used separation technique. This is because it requires less analysis time, low solvent use, high degree of precision, ecofriendly and the results obtained are accurate. Thus TLC is a powerful and reliable method for qualitative and quantitative analysis.

In High Performance Thin Layer Chromatography the constituents of a mixture are separated by principle based on the interaction with the stationary phase and then quantified using a suitable detector. The method is one of the best methods for the simultaneous determination of Paracetamol and Aceclofenac.

In High Performance Thin Layer Chromatography the components of the mixture are separated based on their interaction with the stationary phase and then quantified using a suitable detector. In TLC the mobile phase is liquid and the stationary phase is solid or liquid, which is in the form of a thin layer on the flat surface made of glass, synthetic material or metal. The liquid stationary phase employed could be in the form of immobilized liquid or modified chemically bonded liquids by chemical reactions. For example

1) Immobilized liquid can be water or triethylene glycol, physically adsorbed silica gel or alumina.

2) Chemically bonded phases like alkyl chlorosilanes, which react with silica gel. The most frequent alkyl groups in practice are C18 or C8 in reverse phase chromatography. Functional groups like diol, cyano, amino dimethylamino etc. are examples.

3) Chemically modified phases used in normal phase.

The mobile phase is moved up by capillary forces across the layer of the stationary phase. The mobile phase serves as a transport medium for the solutes to be separated. The separation in TLC is the result of two opposite acting forces viz, the driving force of the mobile phase and retarding action of the sorbent. The driving force of the mobile phase

moves the solute from the origin in the direction of the mobile phase. The retarding action of the sorbent hampers the movements of the solute with the mobile phase by dragging it back into the sorbent. Depending on the difference in the speeds of the various solutes, they travel through different distances on the stationary phase. At the end of the chromatographic development, each sample component elutes at a certain distance from the point of application of the sample. The differential migration is the result of varying degrees of affinity of the components sample, for the stationary phase and mobile phase. Different separation mechanisms are involved during the chromatographic separations.

The degree of attraction of solute for the stationary phase is described in the following equation.

$$K = \frac{Cs}{Cm}$$

Where

K = Partition coefficient

Cs = equilibrium concentration of the solute in the stationary phase

Cm = equilibrium concentration of the solute in the mobile phase

The solute with a large K value will have grater affinity for the stationary phase and will travel slowly through the stationary phase.

METHOD IN BRIEF:

The present section of the chapter describes a normal phase high performance thin layer chromatographic

method for simultaneous determination of Paracetamol and Aceclofenac from its combined dosage form. The proposed HPTLC method involves the use of HPTLC plates precoated with silica gel $60F_{254}$ on aluminum sheets. A mobile phase composed of Acetonitrile : Toluene : Acetic acid in the volume ratio of (6 : 4 : 0.1) was employed for the elution. After development of the chrome plate, the detection was carried out using UV scanning densitometer set a wavelength 270nm. The HPTLC method was subjected to statistical validation and was applied for the simultaneous determination of Paracetamol & Aceclofenac from its combined dosage form.

The various steps involved in TLC/HPTLC are shown in fig 6.1 Thin layer chromatography is an off-line technique as compared to HPLC/GC but the properties that govern the selection of a method and its components are essentially the same. Fig 6.2 gives schematic representation of the steps in the method development in HPTLC.

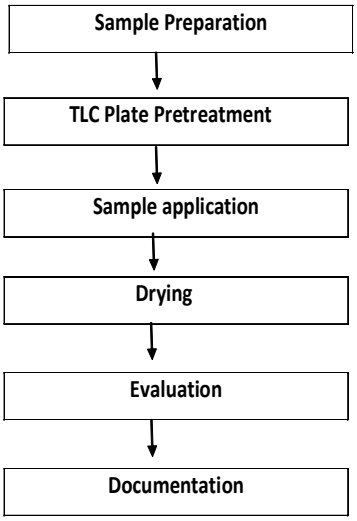


Fig 6.1 Various Steps in TLC/HPTLC

Traditional TLC is inexpensive, simple to use and require minimal instrumentation, laboratory space and maintenance. However to achieve good precision accuracy and reproducibility, a certain degree of instrumentation is required and densitometric evaluation is necessary for quantification.

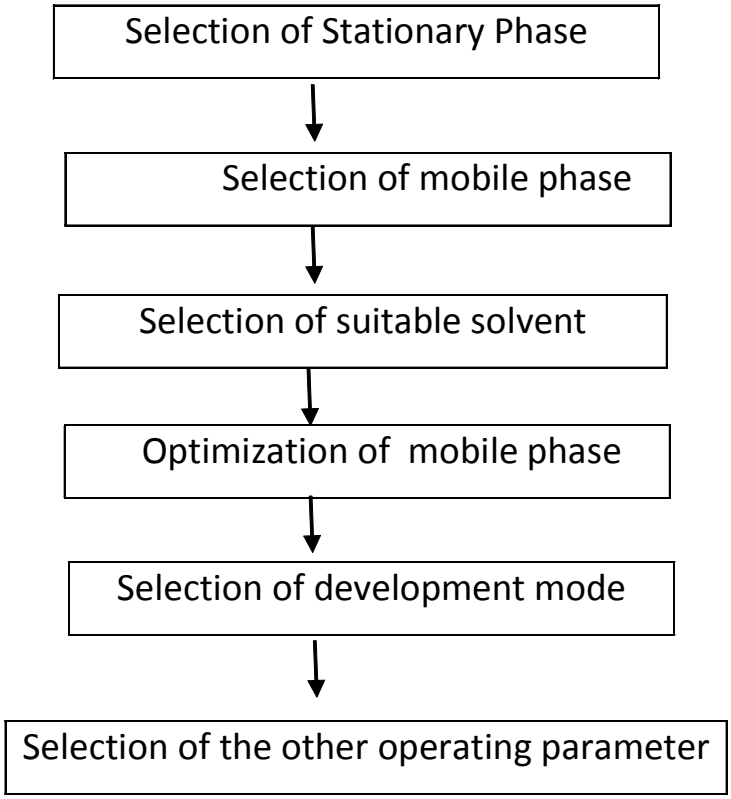


Fig 6.2 Various Steps in Method development

STANDARDIZATION OF EXPERIMENTAL CONDITIONS:

The extent of separation of various components of a mixture by a given TLC method depends on the separation efficiency and selectivity of the separating system. The

separation of the analytes depends upon the following factors.

- Type of stationary phase
- Type of Pre-coated plate(TLC/HPTLC)
- Layer thickness
- Binder in the layer
- Mobile phase
- Solvent purity
- Size of the developing chamber
- Saturation of the chamber(Pre- equilibrium)
- Relative humidity
- Temperature
- Separation distance

Instrument:

- DESAGA SARSTEDT GRUPPE AS 30 - SAMPLE APPLICATOR
- CD 60 - DENSITOMETER / SCANNER EQUIPPED WITH PRO.QUANT SOFTWARE
- TWIN TROUGH CHAMBER

Reagents:

All solvents of HPLC grade were used throughout the experiment

- Toluene
- Methanol
- Acetonitrile

PREPARATION OF STANDARD SOLUTIONS:

75mg of Paracetamol and 15 mg of Aceclofenac was accurately weighed and transferred to a 50mL volumetric

flask. It was dissolved in a minimum quantity of methanol and then diluted up to the mark with methanol. The concentration of the solution obtained was 1500µg/mL for Paracetamol and 300 µg/mL for Aceclofenac (Solution A). 5 mL of this solution A was diluted to 10 mL in a volumetric flask with methanol. The concentration of the solution obtained was 750µg/mL for Paracetamol and 150µg/mL for Aceclofenac.

SELECTION OF VARIOUS CHROMATOGRAPHIC CONDITIONS :

Mode of Separation:

There are a number of modes or mechanisms into which chromatography is divided. In HPTLC method of separation a normal phase mode of separation is mostly used. But in case of HPLC a normal phase mode of separation is frequently used. In HPTLC process in reverse phase mode of separation water will be one of the components of the mobile phase. While in normal phase mode of separation, water cannot be used because stationary phase is more polar and mobile phase is less polar therefore instead of water, organic solvents are used in the separation process.

Also organic solvents are less viscous and more volatile and the plate develops very quickly and also dries faster.

For TLC/HPTLC the stationary phase may be modified or unmodified but silica is the most commonly used.

Hence normal phase mode of separation was chosen for HPTLC determination

SELECTION OF STATIONARY PHASE:

The drugs selected for the study were Paracetamol and Aceclofenac. As compared to Paracetamol, Aceclofenac

is less polar as it has larger hydrophobicity. In this experiment silica gel which is polar was used as a stationary phase. The surface of the silica gel contains active sites that interact with sample molecules. These sites consist of silica groups (Si –O – Si), silanol groups and water hydrogen bonded to silanol groups.

HPTLC plates Pre-coated with silica gel $60F_{254}$ on aluminum sheets 0.25mm thickness were employed in the research work. The plates are heat resistant and compatible with most organic solvents.

Hence HPTLC plate precoated with silica gel $60F_{254}$ on aluminum sheets were used as a stationary phase for the research work.

PREWASHING OF PLATES:

Sorbents with large surface area adsorb not only water vapors and other impurities from the atmosphere but other volatile substances often condense after the plate is exposed to laboratory atmosphere for a long time. Such impurities along with the eluted components from the binder usually give dirty zone and fail to give reproducible results. To avoid any possible interference from the impurities in the chromatographic separation, it is always recommended to clear the plates before the experiment. This technique is called prewashing of the plates. The washing liquid generally used is methanol. After washing the plates are dried to remove the liquid. As a result of prewashing the signal to noise ratios are significantly low and base line is straighter. Thus this operation is essential for quantitative determination.

PLATE ACTIVATION:

Separation process on silica is a function of the surface hydroxyl content, which is responsible for selective adsorption onto the particle. Hence the hydroxyl content must be controlled. The higher the water uptake the more active is the sorbent layer. Activated plate produce sharper and better separation than inactivated plate. In the process of activation the silica gel plate was dried for one hour at 110^0C to remove the physically adsorbed water from the moisture present in the silica gel surface.

In the present research work the plates were prewashed with methanol and dried in an oven at 110^0C for one hour before use.

SAMPLE APPLICATION:-

Sample application is the most critical step in the thin layer chromatography for obtaining good resolution and quantification. In the present research work, the samples were applied as sharp bands of 5mm width on the HPTLC plate. These bands were applied with the help of Desaga AS 30 - sample applicator at a distance of 10mm from X axis and 15mm from Y axis at the edge of the HPTLC plate with the speed of 150nl/sec for methanol.

MOBILE PHASE OPTIMIZATION:

The mobile phase in TLC is generally selected by controlled trial and error method. In normal phase TLC separation was carried out on a polar stationary phase (Silica gel) using a non-aqueous mobile phase. Developing solvent usually, is a mixture of non-polar organic solvent (Toluene) with a polar modifier such as methanol, ethyl acetate, acetone and acetonitrile to control the solvent strength and selectivity. Sometimes a small amount of a

third component such as acetic acid, ammonia, triethylamine or formic acid is added to the mobile phase because they partially deactivate the surface of the silica gel, keeping the acidic and basic centers in a molecule non ionized thereby leading to a decrease in the tailing of polar sample components. The general strategy for optimizing the mobile phase in TLC is to adjust the solvent strength by replacing the pure solvent by another or by varying the properties of the weak solvent and a strong solvent in a mixture so that the R_f values are in the range of 0.2 – 0.7.

In the present research work, initially the mobile phase used was toluene: methanol and toluene: ethyl acetate. It was observed that analytes moved from the origin with toluene and ethyl acetate but not convincingly. Hence ethyl acetate was replaced by acetonitrile, which gave better separation and resolution. Along with that glacial acetic acid was also incorporated in the mobile phase as the modifier to reduce tailing and achieve sharper spots. Hence the mobile phase was changed to acetonitrile: toluene: acetic acid in the ratio of 6 : 4 : 0.1 v/v/v. With this mobile phase, good resolution between all the three components was seen with suitable R_f values.

Thus optimized mobile phase used for separation was Acetonitrile : Toluene : Glacial acetic acid in the volume ratio 6 : 4 : 0.1, v/v/v.

CHAMBER SATURATION:

It is important to prevent the solvent evaporating from the plate surface during development, as this would change the composition of the solvent. If the solvents used are volatile and the evaporation rate becomes equal to the migration rate then this will also result in a static “solvent

front" and arrest the chromatographic development. The saturated solvent vapours in the chamber not only prevents solvent evaporation from the plate surface but also plays a role in controlling the retention mechanism by deactivating the surface of the stationary phase. The components of the mobile phase are selectively adsorbed on the surface of the stationary phase causing the solutes to interact, not with the silica surface, but with the silica surface covered with the most strongly interacting solvent.

When a TLC plate development is carried out in an unsaturated chamber, it is observed that the spots near the edge of the TLC plate travel faster than those near the center. This is because the developing solvent evaporates more rapidly at the edge than those near the center of the plate. Thus resulting into a curved solvent front. This effect is referred to as the "edge effect". The chamber selected in the present work was Degasa twin trough chamber which allows the plate equilibrium to be carried out before the development of the plate.

The best results were obtained when chamber saturation was carried out with solvent vapours for 15 minutes prior to the chromatographic development.

DEVELOPMENT AND DRYING:

Ascending mode of chromatographic development was chosen for the present work. After the development of the plate upto a distance of 8cm from the point of application, it was removed from the chamber and kept at a room temperature for the solvent to evaporate before the densitometric scanning.

VISUALIZATION, DETECTION AND QUANTIFICATION:

There are many methods for the detection and visualization of the separated components on a TLC plate. Densitometry is the insitu instrumental measurement of visible, UV absorbance and fluorescence directly on the adsorbent layer without resorting to scrapping and elution. Since, chromatographic zones re-emit lower light intensity than the environment around it. The purpose of the scanner is to convert the spot /band on the layer into a chromatogram. Paracetamol and Aceclofenac exhibit absorbance in the UV region of the electromagnetic spectrum. Hence detection and quantification was performed in the absorbance mode using Degasa TLC scanner with Pro-Quant software. During the method development the spots on the TLC plate were visualized in a UV chamber equipped with a UV lamp

(ë =254nm). The developed TLC plate was scanned between 200 and 400nm wavelength using CD-60 Densitometer/scanner. The wavelength chosen for further quantification was 270nm.

SELECTION OF THE MOST SUITABLE WAVELENGTH FOR DENSITOMETRIC SCANNING:

In case of multicomponent pharmaceutical preparations containing more than one active ingredient with variable concentrations, if the TLC plate is scanned at maximum absorbance of each component the purpose of using TLC is lost. Hence in such cases one has to explore the possibility of selecting a wavelength at which the entire chromatogram can be scanned without losing any vital information. The single wavelength is referred to as the “most suitable wavelength” In the present research work

the most suitable wavelength was found to be 270nm as could be seen from the UV spectrum Fig 6.3

Fig 6.3 UV Spectrum for Paracetamol and Aceclofenac mixture in methanol

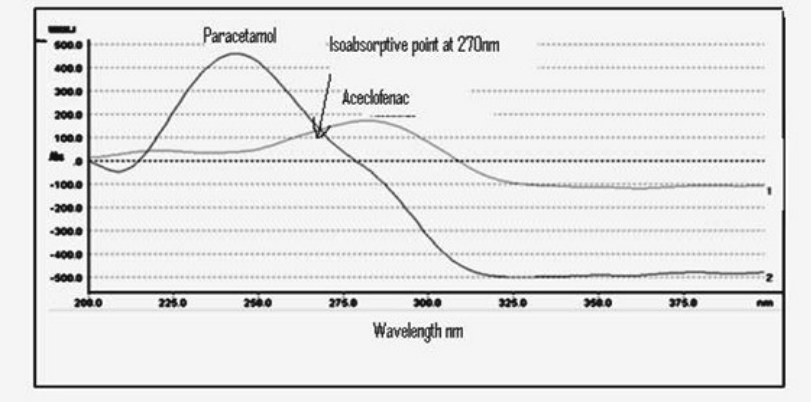


Table 6.1 Optimized chromatographic conditions for HPTLC method

Parameters	Chromatographic Conditions
Development chamber	Twin trough chamber
Stationary phase	Silica gel
Mobile Phase	Acetonitrile : Toluene : Acetic acid (6 : 4 : 0.1 v/v/v)
Chamber saturation	15 min
Sample applicator	AS 30 - SAMPLE APPLICATOR
Band	8mm
Space	12mm
Scanning Speed	20mm/sec
Development distance	8cm
Drying of plate	Room temperature
Densitometric scanner	CD 60 - DENSITOMETER / SCANNER
Lamp	Deuterium
Wavelength	270nm
Volume	10µl

METHOD VALIDATION:

The following experiment was carried out to determine the working concentration range for Paracetamol and Aceclofenac. The proposed developed method was subjected to method validation process to determine its suitability for intended use. The method was validated with reference to the following parameters.

1) Selectivity and Specificity

2) System suitability

3) Linearity

4) Precision (Repeatability)

5) Accuracy (% Recovery)

6) Solution stability

7) Intermediate precision (Ruggedness)

SPECIFICITY:

An investigation of specificity was conducted during the validation of identification tests, the determination of impurities and the assay. Demonstration of specificity requires that there should not be any interference of impurities and excipients. In practice this was done by taking the chromatogram of the sample solution and the assay result was unaffected by the presence of the extraneous material. It has been found that there was no interference of the diluent/placebo at the peak of the drug substance.

SYSTEM SUITABILITY TEST:

A system suitability test should be carried out to see if the HPTLC system is performing properly. System suitability tests were performed as per the USP to confirm

the suitability and the reproducibility of the system. The experiment was carried out using 100% level mixed standard solution of Paracetamol and Aceclofenac. This solution was spotted five times on the chromatographic plate under the optimized chromatographic conditions. Parameters that were studied to evaluate the suitability of the system was % RSD of peak area and % RSD of retention factor of the drug the peak.

Table 6.2 System suitability for Paracetamol and Aceclofenac

Obs No	100% Level			
	Paracetamol		Aceclofenac	
	Peak area	Rf value	Peak area	Rf value
1	2588	0.64	1139	0.49
2	2499	0.62	1102	0.46
3	2451	0.63	1086	0.48
4	2459	0.62	1070	0.49
5	2412	0.61	1020	0.45
Mean	2481	0.62	1083	0.47
S.D	66.92	0.0102	39.098	0.016
% RSD	2.70	1.63	3.61	3.43

From the above table it has been found that the value of % Relative standard deviation for 5 replicates, the peak area of the bands should be less than 5.0%. Whereas % Relative standard deviation of Rf value should be less than 10.0%. The results show that the % relative standard deviation for all parameters is well below the required limit.

LINEARITY AND RANGE:

The linearity of a method is a measure of how well a calibration plot of detector response against concentration approximates a straight line. The following experiment was

carried out to determine the working concentration range for Paracetamol and Aceclofenac. Preparation of linearity levels were as per the following table 6.3

Table 6.3 Preparation of mixed standard solution for Linearity levels

Linearity Levels	Volume of std stock solution added in mL	Volume made upto in mL	Concentration of Paracetamol in ppm	Concentration of Aceclofenac in ppm
50%	2.5	10	375	75
60%	3	10	450	90
90%	4.5	10	675	135
100%	5	10	750	150
120%	6	10	900	180
140%	7	10	1050	210
150%	7.5	10	1125	225

To a prewashed and activated thin layer chromatographic plate 10µl of the above solutions of different concentrations were applied at a distance of 10mm from the base of the plate with the help of Degasa sample applicator in the form of 8mm bands with a distance of 12mm between the adjacent bands. The optimized mobile phase was prepared and poured on one side of the twin trough chamber. The chamber was allowed to saturate with the mobile phase for 15 minutes. The plate was then kept in the mobile phase and allowed to develop until the solvent front reached 8cm above the position of the sample application. After development, the plate was removed

from the chamber.The plate was dried at room temperature. The developed plate was then scanned using a scanning CD-60 densitometer at a wavelength of 270nm. The chromatogram was recorded and the peak areas were computed. A typical HPTLC chromatogram is shown in fig 6.4. The peak area for paracetamol and Aceclofenac are presented in Tables 6.4 and 6.5 respectively.

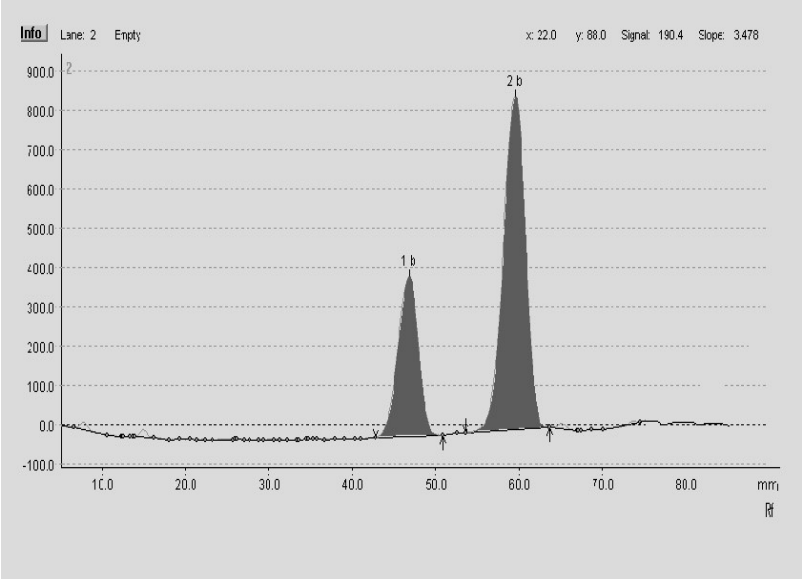


Fig 6.4 Typical HPTLC chromatogram 1) Aceclofenac 2) Paracetamol

In the optimized chromatographic conditions the major peaks of Aceclofenac and Paracetamol are separated from all its excipients. There are no other potentially interfering peaks at the Rf values of the drugs, as noted after injecting the sample solution of formulation thus showing the specificity of the method.

Results of the linear working range for Paracetamol:

Table 6.4 Linearity for Paracetamol

Sr.No	Level %	Conc. in ppm	Peak Area			Mean	S.D.	% RSD
			1	2	3			
1	50%	375	1249	1256	1244	1250	6.028	0.482
2	60%	450	1553	1563	1549	1555	7.211	0.463
3	90%	675	2324	2341	2329	2331	8.737	0.374
4	100%	750	2572	2583	2569	2575	7.371	0.286
5	120%	900	3070	3079	3086	3078	8.021	0.260
6	140%	1050	3579	3567	3586	3577	9.609	0.268
7	150%	1125	3866	3856	3849	3857	8.544	0.221

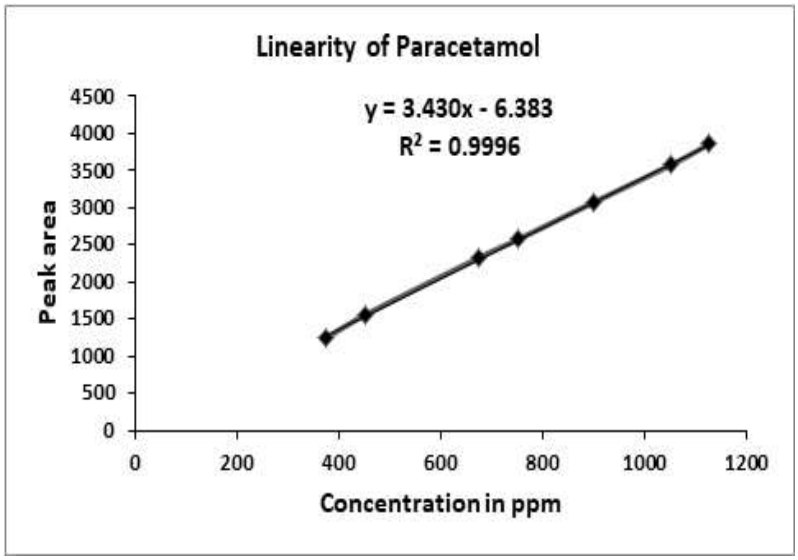


Fig 6.5 Linearity Plot for Paracetamol

(HPTLC Method)

Results of the linear working range for Aceclofenac:

Table 6.5 Linearity for Aceclofenac

Sr. No	Level %	Conc. in ppm	Peak Area			Mean	S.D.	% RSD
			1	2	3			
1	50%	75	541	564	589	565	24.007	4.25
2	60%	90	719	742	698	720	22.008	3.06
3	90%	135	1012	1045	1090	1049	39.154	3.73
4	100%	150	1183	1225	1165	1191	30.790	2.59
5	120%	180	1397	1426	1448	1424	25.580	1.80
6	140%	210	1598	1640	1683	1640	42.501	2.60
7	150%	225	1780	1762	1792	1778	15.100	0.85

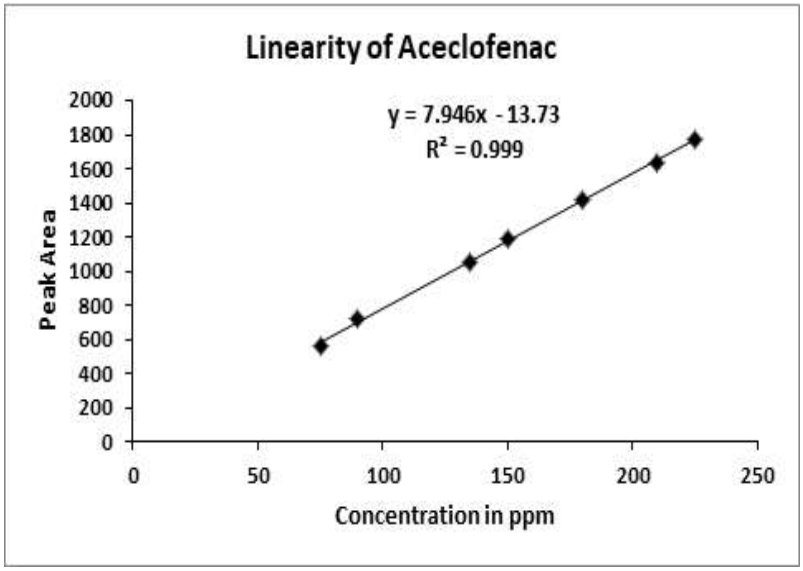


Fig 6.5 Linearity Plot for Aceclofenac (HPTLC Method)

REGRESSION ANALYSIS:

The calibration data range 50% to 150% for Paracetamol and Aceclofenac was further considered for regression analysis. The regression analysis of the calibration data was carried out to determine the relationship between the dependent variable (Peak area) and independent variable(drug concentration).

The regression equation

$y = mx + C$ was found to $y = 3.430X + -6.383$ for Paracetamol

$y = 7.976X + -13.73$ for Aceclofenac

Where y = dependent variable m = Slope of the regression line

x = independent variable C = intercept on y axis

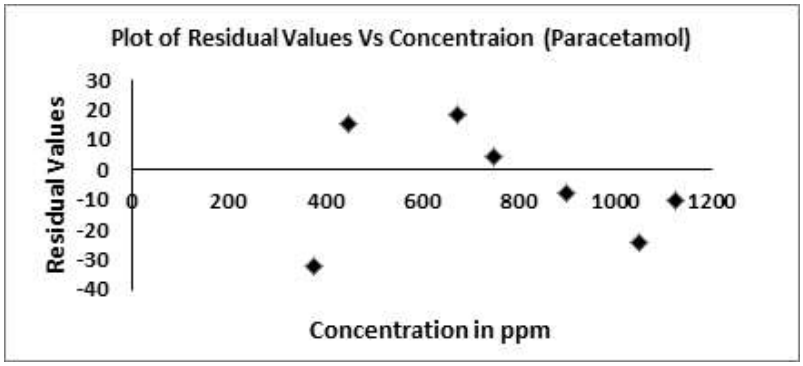
For the method to have a good correlation between analyte concentration and response, the residuals should be equally distributed above and below the zero residual line indicating the random precision of the method.

Regression analysis data (Paracetamol):

Table 6.6 Regression analysis data of Paracetamol

Obs No	X value Conc in ppm	Y Value observed	Y Value Calculated	Residual value
1	375	1250	1282	-32
2	450	1555	1540	15
3	675	2331	2313	18
4	750	2575	2571	4
5	900	3078	3086	-8
6	1050	3577	3601	-24
7	1125	3849	3859	-10

Slope	3.430
Correlation coefficient	0.9996
Std error of Y estimate	20.52



Regression analysis data (Aceclofenac):

Table 6.7 Regression analysis data of Aceclofenac

Obs No	X value Conc in ppm	Y Value observed	Y Value Calculated	Residual value
1	75	565	582	-17
2	90	720	701	19
3	135	1049	1059	-10
4	150	1191	1178	13
5	180	1424	1417	7
6	210	1640	1655	-15
7	225	1778	1774	4

Slope	7.946
Correlation coefficient	0.9990
Std error of Y estimate	15.48

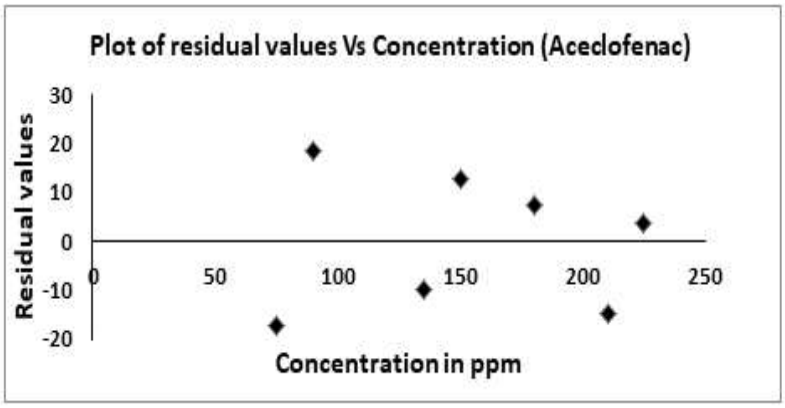


Fig 6.7 Plot of residual value Vs Concentration (Aceclofenac)

APPLICATION OF THE PROPOSED METHOD FOR SIMULTANEOUS DETERMINATION OF PARACETAMOL AND ACECLOFENAC FROM THEIR PHARMACEUTICAL FORMULATION

Dosage form : Tablet

Strength : 100mg Aceclofenac and 500mg of Paracetamol

Procedure for sample preparation:

Twenty tablets were weighed and the average weight was calculated. These tablets were powdered and a weight equivalent to one tablet was taken in a 100mL volumetric flask. This was dissolved in a minimum amount of methanol and was sonicated for about 15 minutes then diluted upto the mark with methanol. This solution was filtered through syringe filter. Then 15mL of the sample solution was taken in a 50mL dilution flask and was diluted upto the mark with methanol (Solution A). From this stock solution 100%

level sample solution was prepared by diluting 5mL of solution A to 10mL with methanol.

The procedure was repeated six times, by individually weighing the tablets powder each time. 10µl of each of these solutions were spotted on the plate under the optimized chromatographic conditions. The peak area of Paracetamol and Aceclofenac were seen. The amount of Paracetamol and Aceclofenac present in this solution was determined by using peak area obtained from the system suitability experiment that was carried out along with the assay.

Amount of drug in mg/tablet was calculated by the following formula.

Amount of active in mg per tablet

$$\text{Mg content} = \frac{\text{Area of sample}}{\text{Area of STD}} \times \frac{\text{Dil factor for STD}}{\text{Dil factor for sample}} \times \frac{\text{\% Purity}}{100}$$

Amount of active in mg/tab =

$$\frac{A1 \times W \times 5 \times 100 \times 50 \times 10 \times M \times P}{A2 \times 50 \times 10 \times S \times 15 \times 50}$$

W = Weight of the standard drug

S = Weight of the sample

A_1 = Average peak area of standard

A_2 = Observed peak area of sample

M = Average weight of the sample

P = Purity of standard

Results of replicate analysis for Paracetamol (Precision study)

Table 6.8 Precision study results for Paracetamol

Obs NO	Peak Area	Amount of Paracetamol in mg/tab	% Assay
1	2521	508.06	101.61
2	2512	506.25	101.25
3	2507	505.24	101.05
4	2499	503.63	100.73
5	2442	492.14	98.43
6	2474	498.59	99.72
	Mean	502.32	100.46
	S.D.	5.419	1.187
	% RSD	1.079	1.182

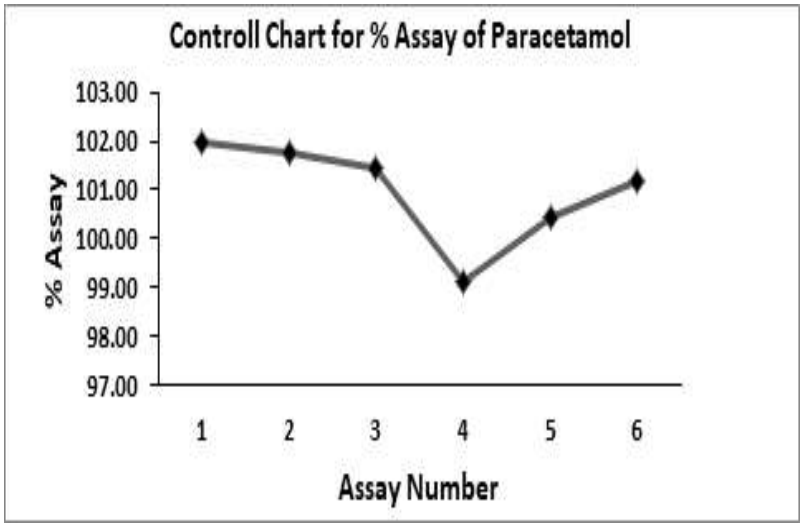


Fig 6.8 Control Chart for % Assay

Results of replicate analysis for Aceclofenac

(Precision study)

Table 6.9 Precision study results for Aceclofenac

Obs No	Peak Area	Amount of Aceclofenac in mg/tab	% Assay
1	1101	101.66	101.66
2	1081	99.82	99.82
3	1072	98.98	98.98
4	1067	98.52	98.52
5	1055	97.41	97.41
6	1076	99.35	99.35
	Mean	100.00	99.29
	S.D.	1.307	1.298
	% RSD	1.307	1.307

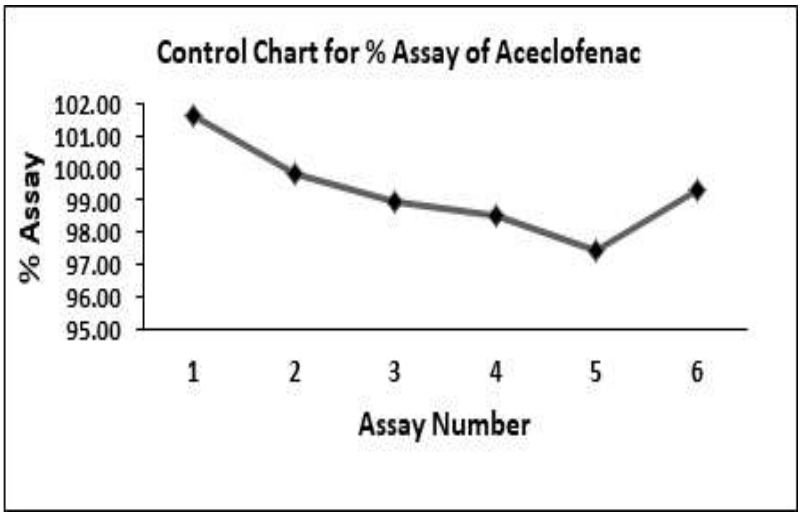


Fig 6.9 Control Chart for % Assay

Accuracy :(Recovery Experiment)

% recovery experiments were carried out to check for the presence of positive and negative interferences from excipients present in the formulation. The standard addition method was employed for this experiment. The recovery of the added standard was studied at three different levels namely 110%, 120% and 130% of the estimated amount of the drug. The experiment was carried out as follows.

Set I

A Preanalyzed sample equivalent to one tablet weight was weighed and transferred to a 100mL standard volumetric flask. The content was dissolved in minimum amount of methanol and sonicated for 10-15minutes. It was then diluted with methanol upto the mark. This solution was filtered through a syringe filter. 15mL of the sample solution was then taken in a 50mL dilution flask and diluted upto the mark with methanol. From this solution 100% level sample solution was prepared by diluting 5mL of solution A to 10mL by methanol. This served as the zero level.

Set II, III, IV

A fixed amount of Preanalyzed sample was taken in three different flasks to which of them sufficient amount of methanol was added and the content dissolved. The solutions were filtered through a syringe filter. To this filtered solution 75mg of Paracetamol and 15mg of Aceclofenac were added in the form of solution and the mixture was diluted upto the mark.

This gave the first level i.e. of 110%. The same procedure was followed for the 120% and 130% of the drug. These solutions were then chromatographed into the

system and the amount of drug present at each level was determined. The recovery was calculated using the formula.

$$\text{\% Recovery of drug} = \frac{\text{Amount found}}{\text{Amount added}} \text{ X 100}$$

Results of Recovery Experiment (Paracetamol):

Table 6.10 Amount of % recovery for Paracetamol

Obs No	Levels in %	Area of sample	Amt added in mg	Amt found in mg	% recovery
1	0%	2482	0	500.20	100.04
2		2499	0	503.63	100.73
3		2459	0	495.57	99.11
1	10%	2713	50	546.76	99.41
2		2725	50.5	549.17	99.85
3		2739	50.8	552.00	100.36
1	20%	2926	100	589.68	98.28
2		2919	100.25	588.27	98.05
3		2953	100.75	595.12	99.19
1	30%	3170	150	638.86	98.29
2		3198	151	644.50	99.15
3		3221	151	649.13	99.87
				Mean	99.28
				S.D.	0.85
				%RSD	0.86
				Range of % recovery	**98.05-100.73**

Result of Recovery Experiment (Aceclofenac):

Table 6.11 Amount of % recovery for Aceclofenac

Obs No	Levels in %	Area of sample	Amt added in mg	Amt found in mg	% recovery
1	0%	1110	0	102.49	102.49
2		1098	0	101.39	101.39
3		1073	0	99.08	99.08
1	10%	1181	10	109.05	99.14
2		1191	10.6	109.97	99.97
3		1206	10.5	111.36	101.23
1	20%	1301	20	120.13	100.11
2		1287	20.5	118.84	99.03
3		1289	20.5	119.02	99.18
1	30%	1410	30	130.19	100.15
2		1394	30.2	128.72	99.01
3		1383	30.3	127.70	98.23
				Mean	99.82
				S.D.	1.24
				%RSD	1.24
				Range of % recovery	**98.23-102.49**

INTERMEDIATE PRECISION (RUGGEDNESS):

Precision consist of two components: repeatability and intermediate precision. Intermediate precision refers to variation within a laboratory such as different days, with different instruments and by a different analyst. This was formerly known as ruggedness. During the validation a second analyst repeats the repeatability on a different day using different conditions and different instruments.

Procedure and Calculation for sample preparation:

It is same as described in precision study. Analysis was carried out on a different day, done by different analyst and on a different instrument. With reference to the below

tables the cumulative % relative standard deviations were found to be low. Values of standard deviation and coefficient of variation indicate high precision and ruggedness of the method. The results were found to be satisfactory and meeting the requirements.

Intermediate Precision Results:

For Paracetamol Content:

Table 6.12 Ruggedness study results for Paracetamol

Obs No	Peak Area	Amount of Paracetamol in mg/tab	% Assay
1	2514	506.65	101.33
2	2501	504.03	100.81
3	2507	505.24	101.05
4	2474	498.59	99.72
5	2443	492.34	98.47
6	2449	493.55	98.71
	Mean	500.08	100.01
	S.D.	5.629	1.233
	% RSD	1.126	1.233

Table 6.13 Cumulative % RSD of Paracetamol in method precision (M.P.) and Intermediate precision (I.P)

Obs NO	Content in mg/tab	% LC
M.P 1	508.06	101.61
M.P 2	506.25	101.25
M.P 3	505.24	101.05
M.P 4	503.63	100.73
M.P 5	492.14	98.43
M.P 6	498.59	99.72
I.P 1	506.65	101.33
I.P 2	504.03	100.81
I.P 3	505.24	101.05
I.P 4	498.59	99.72
I.P 5	492.34	98.47
I.P 6	493.55	98.71
Mean	501.20	100.24
S.D.	5.639	1.127
%Cumulative RSD	1.125	1.125
Limits	NMT 2.00%	

For Aceclofenac Content:

Table 6.14 Ruggedness study results for Aceclofenac

Obs No	Peak Area	Amount of Aceclofenac in mg/tab	% Assay
1	1098	101.39	101.39
2	1084	100.09	100.09
3	1070	98.80	98.80
4	1076	99.35	99.35
5	1058	97.69	97.69
6	1067	98.52	98.52
	Mean	99.31	99.31
	S.D.	1.185	1.185
	% RSD	1.193	1.193

Table 6.15 Cumulative % RSD of Aceclofenac in method precision and Intermediate precision

Obs NO	Content in mg/tab	% LC
M.P 1	101.66	101.66
M.P 2	99.82	99.82
M.P 3	98.98	98.98
M.P 4	98.52	98.52
M.P 5	97.41	97.41
M.P 6	99.35	99.35
I.P 1	101.39	101.39
I.P 2	100.09	100.09
I.P 3	98.80	98.80
I.P 4	99.35	99.35
I.P 5	97.69	97.69
I.P 6	98.52	98.52
Mean	99.30	99.30
S.D.	1.244	1.244
%Cumulative RSD	1.253	1.253
Limits	NMT 2.00%	

SOLUTION STABILITY:

Stability of sample solution was checked by using sample preparation from precision study stored at room temperature for 24 hours, withdrawn at the intervals of 2hrs, 4 hrs, 12 hrs and 24 hrs and then applied on the chromatoplate. After development, the chromatogram was evaluated for additional spots if any. There was no indication of compound instability in the sample solution.

VALIDATION SUMMARY AND RESULTS:

A simple, precise and rapid normal phase high performance thin layer chromatographic method was developed for the simultaneous determination of Paracetamol and Aceclofenac from a tablet dosage form. The chromatographic separation was performed on a stationary phase silica gel $60F_{254}$ on aluminum sheets. Mobile phase consisted of a mixture of Acetonitrile : Toluene : Acetic acid (6 : 4 : 0.1v/v/v). The wavelength was set at 270nm. The proposed method was validated for Specificity, Linearity, Accuracy, Precision, System suitability, Solution stability and Ruggedness.

The calibration was linear over the range of 375 – 1125 µg/mL for Paracetamol and 75-225 µg/mL for Aceclofenac. The regression analysis reveals that the proposed method was linear.

The precision study of the proposed method gave the results in the prescribed limits of relative standard deviation. This is less than 2% for both the analytes.

The developed method was applied for simultaneous estimation of Paracetamol and Aceclofenac in tablet dosage form. The % recovery values for Paracetamol and Aceclofenac are from 98.05% - 100.73% and 98.23% - 102.49% respectively. The % recovery data shows that the method is accurate.

The intermediate precision (Ruggedness) study showed that the results are within the limits as per the ICH guidelines. Low value of cumulative % RSD of assay of precision study and Intermediate precision study showed that the method is rugged.

The solution stability study showed that the sample solution and standard solution were stable for longer time and it has been found that both the analytes did not degrade.

The results obtained from robustness study were within the specified limits indicating that the developed method is robust. The HPTLC method for the determination of Paracetamol and Aceclofenac from their pharmaceutical dosage form was found to be accurate, precise, sensitive and less time consuming. Thus the developed method can be applicable for the simultaneous determination of Paracetamol and Aceclofenac in tablet dosage form.

CONCLUSION:

The HPTLC method for the determination of Paracetamol and Aceclofenac from their pharmaceutical dosage form was found to be accurate, precise, specific and rapid. The results of the recovery studies performed show the high degree of accuracy of the proposed method. The advantage of this method is that it is less time consuming and a cost effective method. Less amount of solvents were consumed during the analysis. Therefore the proposed method can be applied successfully in routine analysis.

REFERENCES:

1) Argekar, A. P.; Sawant, J. G. Simultaneous determination of Paracetamol and Mefenamic acid in tablets by HPTLC. *Journal of Planar Chromatography—Modern TLC* (1999), 12(5), 361-364.

2) Bhalerao, Santosh; Tambe, Santosh; Pareek, Vikas; Shinde, Rupali; Gupta, Lalit Kumar. A solid-liquid extraction and high performance thin layer chromatographic

determination of Diacerein and Aceclofenac in pharmaceutical tablet dosage form. *Asian Journal of Pharmaceutical and Clinical Research* (2010), 3(1), 25-30.

3) Yadav, Alok; Singh, Raman M.; Mathur, Satish C.; Saini, Pawan K.; Singh, Gyanendra N. A simple and sensitive HPTLC method for simultaneous analysis of Domperidone and Paracetamol in tablet dosage forms. *Journal of Planar Chromatography—Modern TLC* (2009), 22(6), 421-424.

4) Vaidya, V. V.; Singh, G. R.; Choukekar, M. P.; Kekare, M. B. Simultaneous RP HPLC determination of Aceclofenac, Paracetamol and Tizanidine in pharmaceutical preparations. *E-Journal of Chemistry* (2010), 7(1), 260-264.

5) Godse, V. P.; Deodhar, M. N.; Bhosale, A. V.; Sonawane, R. A.; Sakpal, P. S.; Borkar, D. D.; Bafana, Y. S. Reverse phase HPLC method for determination of Aceclofenac and Paracetamol in tablet dosage form. *Asian Journal of Research in Chemistry (*2009), 2(1), 37-40.

6) Dongre, Vaijanath G.; Shah, Sweta B.; Bayes, Gunaji S.; Phadke, Manisha; Jadhav, Vivek K. Simultaneous Determination of Etodolac and Acetaminophen in Tablet Dosage Form by RP-LC. *Chromatographia* (2009), 69(9-10), 1019-1023.

7) Vidya V. Dighe, Ramesh T. Sane, Shashikumar N. Menon, Harsha N. Tambe, Sreedevi Pillai[1], Vijay N. Gokarn. Simultaneous determination of Diclofenac sodium and Paracetamol in a pharmaceutical preparation and in bulk drug powder by high-performance thin-layer chromatography. *JPC - Journal of Planar Chromatography - Modern TLC.* 2006; 19(112), 443-448

8) L. G. Lala , P. M. D'Mello and S. R. Naik HPTLC determination of Diclofenac sodium from serum. *Journal of Pharmaceutical and Biomedical analysis ,* 2002, 29 (3), 539-544

9) Vidya V. Dighe, Ramesh T. Sane, Shashikumar N. Menon, Harsha N. Tambe, Sreedevi Pillai, Vijay N. Gokarn TLC analysis of non-steroidal anti-inflammatory drugs and videodensitometric determination of Fenbufen in tablets. *JPC - Journal of Planar Chromatography - Modern TLC.* 2004; 17(5) 383-387

10) Sethi P D, High Performance Thin Layer Chromatography, Quantitative Analysis of Pharmaceutical Formulations, *2nd Ed., CBS Publishers and distributors, New Delhi, India,* 1996.

11) Harikrishnan, N.; Gunasekaran, V.; Sathishbabu, A.; Rao, G. Srinivasa; Roosewelt, C. Simultaneous estimation of Aceclofenac and Paracetamol by HPTLC in pure and pharmaceutical dosage form. *Asian Journal of Chemistry* (2007), 19(5), 3918-3922.

12) Gandhi S.V. Barhate N.S., Patel B.R., Panchal D.D., and Bothara K.G., A validated densitometric method for analysis of Aceclofenac and Paracetamol as the bulk drugs and in combined tablet dosage forms. *Acta Chromatogr.,* 2008, 20,175, 182.

CHAPTER - VII

Simultaneous estimation Of Paracetamol and aceclofenac in tablet dosage form by ultraviolet (Uv) spectrophotometry

INTRODUCTION:

Spectroscopy is the study of interaction between the electromagnetic radiation and matter. Electromagnetic radiations results from the transfer and propagation of energy by alternating associated electric and magnetic fields. It is considered to be transmitted by wave motion. Spectroscopy is essentially a technical procedure by which the energy differences between the allowed states of the system are measured by determining the frequencies of the corresponding light absorbed. In short the absorbed radiation brings about different kinds of excitations in a molecule and each requires its own distinctive energy. That is each type of excitations corresponds to the absorption of light in the different region of the electromagnetic spectrum. Ultraviolet and visible light brings about movement of valence shell electrons, typically from a filled bonding molecular orbital to an unfilled molecular orbital.

Molecular spectra arise both due to emission and absorption of energy by molecules. Absorption spectra are obtained when a molecule absorbs energy from a beam of electromagnetic radiation and jumps to a higher energy state. If the radiation after its interaction with the molecule is observed in a spectrophotometer, then it will be found

that certain frequencies, which have been absorbed, are missing. This leaves dark lines or bands in their places. The observed spectrum is called as the absorption spectrum of the molecule.

In spectrophotometric analysis a source of radiation is used that extends into the ultraviolet region of the spectrum.

Absorption spectrometry is the measurement of selected absorption by atoms, molecules or ions of electromagnetic radiation having a definite and narrow wavelength range approximating monochromatic energy. The amount of absorption depends on the wavelength of radiation and the structure of the compound. The absorption of radiation is due to the subtraction of energy from the radiation beam when electrons in orbital of lower energy are excited into orbital of higher energy. Since this is in electron transition phenomenon, UV is sometimes called electronic spectroscopy. The technique of UV visible spectrophotometry is one of the most frequently employed in pharmaceutical analysis. It involves the measurement of the amount of ultraviolet (190 380nm) or visible (380 800nm) radiation absorbed by a substance in a solution.

The basis of all spectrophotometric methods for multicomponent sample analysis is the property that all wavelengths:

· the absorbance of a solution is the sum of absorbances of individual components or

· the measured absorbance is the difference between total absorbance of the solution in the sample cell and that of the solution in the reference (blank) cell.

The various spectrophotometric methods which are used for estimation of the drug in the combined dosage form include simultaneous equation method, absorbance ratio method, geometric correction method, orthogonal polynomial method, difference spectrophotometry, derivative spectrophotometry, and absorption correction method, multicomponent method of analysis and two wavelength quantitation method.

SIMULTANEOUS EQUATION METHOD OR (VIERODT'S METHOD)

If a sample contains two absorbing drugs (X and Y) each of which absorbs at the ëmax of the other. It may be possible to determine both drugs by the technique of simultaneous equations (Vierodt's method) provided that certain criteria apply.

The information required is

- the absorptivities of X at $\ddot{e}_1$ and $\ddot{e}_2$ ax_1 and ax_2 respectively
- the absorptivities of Y at $\ddot{e}_1$ and $\ddot{e}_2$ ay_1 and ay_2 respectively
- the absorbances of the diluted sample at $\ddot{e}_1$ and $\ddot{e}_2$, A_1 and A_2 respectively. Let Cx and Cy be the concentrations of X and Y respectively in the diluted sample. Two equations are constructed

At $\ddot{e}_1$ $A_1 = a X_1 b Cx + aY_1 b Cy$ (1)

At $\ddot{e}_2$ $A_2 = a X_2 b Cx + aY_2 b Cy$ (2)

For measurements in 1 cm cells b=1

Rearrange eq. (2)

$Cy = A_2 \; a X_2 b Cx / aY_2$

Substituting for Cy in eq. (1) and rearranging

$Cx = A_2 aY_1 \; A_1 aY_2 / a X_2 aY_1 \; a X_1 aY_2$ (3)

$Cy = A_1\ a\ X_2 - A_2\ a\ X_1 / a\ X_2\ aY_1\ \ a\ X_1 aY_2$ (4)

As an exercise you should derive modified equation containing a symbol (b) for path length for application in situations where A1 and A2 are measured in cells other than 1 cm path length. Criteria for obtaining maximum precision based upon absorbance ratios have been suggested that place limits on the relative concentration of the components of the mixture. The criteria are that the ratios.

$A_2D\ \ A_1 / aX_2D\ \ a\ X_1$ and $aY_2D\ \ aY_1 / A_2D\ \ A_1$ should lie out side the range 0.1 2.0 for the precise determination of Y and X respectively. These criteria are satisfied only when the ëmax of two components are reasonably dissimilar, an additional criterion is that the two components don't interact chemically thereby negating the initial assumption that the total absorbance is the sum of individual absorbances. The additive of the absorbance should always be confirmed in the development of a new application of this techniques based upon the fact that at $ë_1$ and $ë_2$ the absorbance of the mixture is the sum of the individual absorbance of X and Y.

METHOD IN BRIEF:

The present work describes an ultraviolet spectrophotometric method for the quantitative simultaneous determination of Paracetamol and Aceclofenac from its bulk drug and pharmaceutical preparation. Paracetamol and Aceclofenac absorb the radiation in the ultraviolet region. The proposed ultraviolet spectrophotometric method is based on the measurement of absorbed ultraviolet radiations by both the analytes. The absorbance measurement was carried out at $\mathbf{ë_{max}}$ of Paracetamol and

Aceclofenac. Paracetamol shows maximum absorbance at 247nm wavelength while Aceclofenac shows at 276nm. The molar absorptivities of both the analytes were found at their respective ëmax value. Using the molar absorptivities of both the analytes the simultaneous equation was constructed and the concentration of analytes was determined. The proposed ultraviolet spectrophotometric method was subjected to statistical validation to determine its accuracy and precision.

EXPERIMETNAL DETAILS:

Instrument:

LAMBDA 25 UV/Visible Spectrophotometer, Range: 190 nm - 1100 nm, Bandwidth: 1 nm

Reagents:

All the solvents employed for the determination were of spectroscopic grade.

Preparation of standard drug solution:

50mg of Paracetamol and 10 mg of Aceclofenac was separately weighed and transferred to a 50mL volumetric flask. It was dissolved in a minimum quantity of methanol and then diluted up to the mark with methanol. The concentration of the solution obtained was 1000µg/mL for Paracetamol and 200 µg/mL for Aceclofenac. 10mL of each of this solution was diluted to 100 mL in a volumetric flask with methanol. The concentration of the solution obtained was 100µg/mL for Paracetamol and 20µg/mL for Aceclofenac.

Preparation of Sample solution:

Twenty tablets (AROFF PLUS, UNICHEM) were weighed and the average weight was calculated. These tablets were

powdered and 0.0840 gm of powdered tablet was taken in a 100 mL volumetric flask, 10 mL of methanol was added and sonicated for 20minutes and shaken by mechanical means for 20minutes at 250rpm. Further the solution was diluted with methanol. The solution was mixed and allowed to settle for 5 minutes. The solution was filtered through Whatman filter paper No 41. Then 2.5 mL of the filtrate was diluted to 100mL with diluents and mixed. The concentrations obtained were 12.5 µg/mL of Paracetamol and 2.5 µg/mL of Aceclofenac. This sample solution was used for further determination.

OPTIMIZATION OF EXPERIMENTAL CONDITIONS:

The parameters that need to be standardized while developing an ultraviolet spectrophotometric method are as follows.

- Selection of the solvent
- Spectral Characteristics

The choice of the solvent in spectrophotometric methods is depend on the following factors.

- Solubility of the absorbing species.
- Absorbance value of the analyte.
- Interference from the solvent at the wavelength of maximum absorbance.

The solvents that are commonly used in spectrophotometric analysis are water, dilute bases and organic solvents. Most of the drugs are soluble in organic solvents like methanol, acetonitrile etc. In the present study the drugs used are Paracetamol and Aceclofenac. Both the drugs are highly soluble in methanol. Other solvents were also tried but methanol gives higher $E_{1\%}$ Value.

Table 7.1 $E_{1\%}$ Value for Paracetamol and Aceclofenac

Solvent	Paracetamol		Aceclofenac	
	Conc. in µg/mL	$E_{1\%}$	Conc. in µg/mL	$E_{1\%}$
Methanol	12.5	**919.2**	2.5	**484**

From the above $E_{1\%}$ value data it has been found that methanol was chosen as a solvent for the preparation of solution as it gives higher $E_{1\%}$ value.

SPECTRAL CHARACTERISTICS:

To find out the wavelength for maximum absorbance, standard solutions of Paracetamol and Aceclofenac in methanol was prepared in the given range of concentration. Paracetamol and Aceclofenac range from 5 µg/mL to 20 µg/mL and 1 µg/mL to 4.5 µg/mL respectively. The standard solutions of these analytes were scanned on a spectrophotometer from 200nm to 400nm against methanol as the reagent blank. Paracetamol shows the maximum absorbance at 247nm wavelength and Aceclofenac shows at 276nm wavelength. The spectra of Paracetamol, Aceclofenac and overlain spectra for both analytes are as shown below.

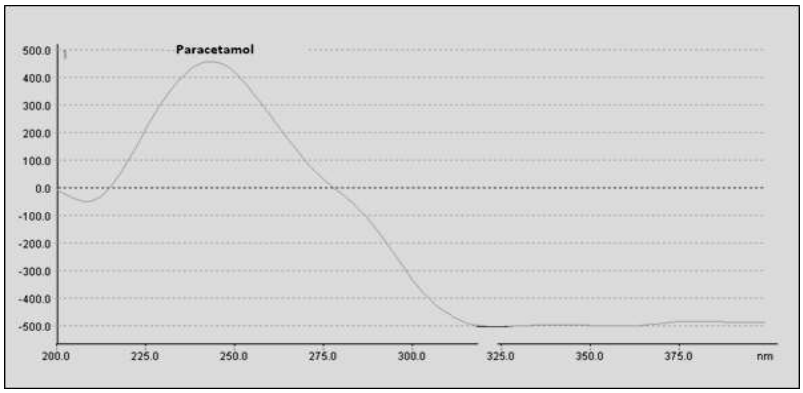


Fig 7.1 Absorption spectrum of Paracetamol showing ëmax at 247nm

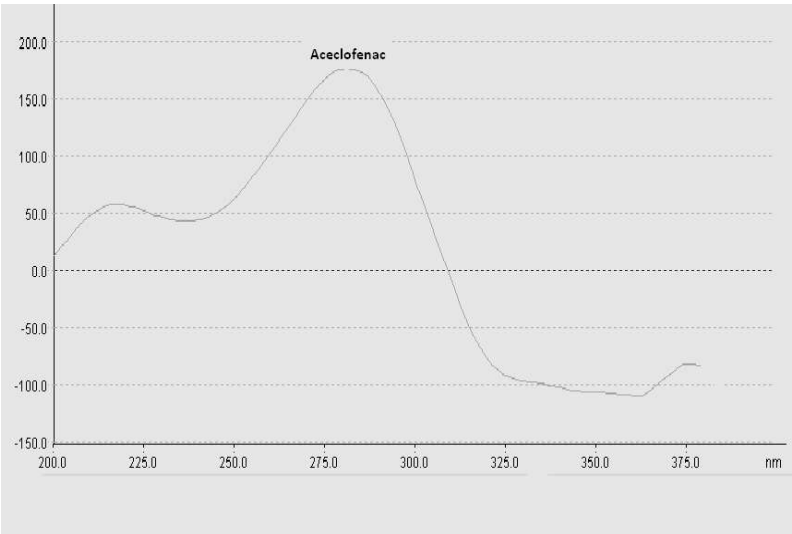


Fig 7.2 Absorption spectrum of Aceclofenac showing ëmax at 276nm

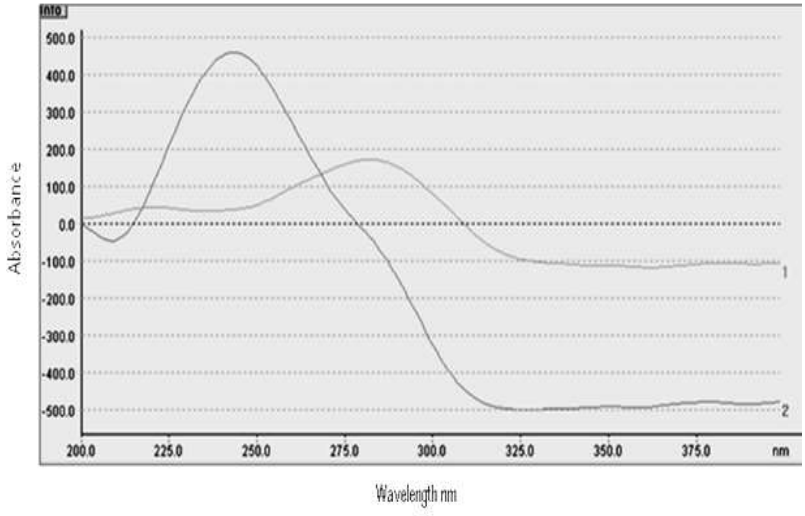


Fig 7.3 Overlain spectra of Paracetamol and Aceclofenac

Development of Method:

Simultaneous equation method:

Analytical wavelength selection was done by diluting the stock solution of Paracetamol and Aceclofenac at a concentration of 100µg/mL and 20µg/mL respectively. They were scanned in the wavelength range of 400nm-200nm. The overlain spectra was taken and the ë max value of Paracetamol and Aceclofenac were determined. Two series of different concentration in the range of 5 - 20 µg/mL for Paracetamol and 1 - 5.5 µg/mL for Aceclofenac were prepared from the working standard solutions. The calibration curves were plotted at 247nm and 276nm. The absorptivities of both the drugs at both the wavelengths were determined. The absorptivity values at a particular wavelength were calculated and simultaneous equation was constructed. Absorbance and corresponding absorptivity of different concentration for Paracetamol and Aceclofenac are shown in the table

Absorbance and Molar absorptivities of Paracetamol:

Table 7.2 Absorbance and corresponding absorptivity of Paracetamol at ëmax 247nm and ëmax 276 nm

Obs. No	Conc. in µg/mL	Absorbance at 247nm	ax_1	Absorbance at 276nm	ax_2
1	5	0.566	0.1132	0.11	0.0220
2	6	0.59	0.0983	0.125	0.0208
3	7.5	0.706	0.0941	0.156	0.0208
4	9	0.845	0.0939	0.18	0.0200
5	10	0.993	0.0993	0.218	0.0218
6	12.5	1.155	0.0924	0.258	0.0206
7	15	1.398	0.0932	0.313	0.0209
8	16	1.491	0.0932	0.322	0.0201
9	17.5	1.593	0.0910	0.339	0.0194
10	20	1.82	0.0910	0.366	0.0183
		Mean	**0.0960**	**Mean**	**0.0205**

Absorbance and Molar absorptivities of Aceclofenac:

Table 7.3 Absorbance and corresponding absorptivity of Aceclofenac at ëmax 247nm and ëmax 276 nm

Obs. No	Conc. in µg/mL	Absorbance at 247nm	ay_1	Absorbance at 276nm	ay_2
1	1	0.021	0.021	0.055	0.0550
2	1.5	0.029	0.0193	0.080	0.0533
3	2	0.037	0.0185	0.102	0.0510
4	2.5	0.045	0.0180	0.125	0.0500
5	3	0.054	0.0180	0.139	0.0463
6	3.5	0.062	0.0177	0.158	0.0451
7	4	0.072	0.0180	0.178	0.0445
8	4.5	0.080	0.0178	0.199	0.0442
9	5	0.091	0.0182	0.225	0.0450
10	5.5	0.101	0.0184	0.248	0.0451
		Mean	**0.0185**	**Mean**	**0.0480**

To determine simultaneously the concentration of Paracetamol and Aceclofenac the following equation was constructed using the molar absorptivities.

$$Cx = \frac{A_2\, ay_1 \; - \; A_1\, ay_2}{ax2\; ay1 \; - \; ax1\; ay2}$$

$$Cy = \frac{A_2 0.0185 \; - \; A_1\, 0.0480}{0.0205 \times \; 0.0185 - \; 0.0960 \times \; 0.0480}$$

Therefore $$Cx = \frac{A_2 0.0185 \; - \; A_1\, 0.0480}{0.0205 \times \; 0.0185 - \; 0.0960 \times \; 0.0480}$$

and $$Cy = \frac{A_1 \times 0.0205 \; - \; A_2 \times 0.0960}{0.0205 \times \; 0.0185 - \; 0.0960 \times \; 0.0480}$$

Where A_1 and A_2 are absorbance of sample solution at 247nm and 276nm respectively

Cx and Cy are concentrations of Paracetamol and Aceclofenac respectively in the sample solution. The validity of the formed equation was checked by preparing five mixed standard solutions of Paracetamol and Aceclofenac.

METHOD VALIDATION OF ULTRAVIOLET SPECTROPHOTOMETRY METHOD FOR SIMULTANEOUS DETERMINATION OF PARACETAMOL AND ACECLOFENAC

The following experiment was carried out to determine the working concentration range for Paracetamol and Aceclofenac. The proposed method was subjected to method validation process to determine its suitability for the intended use. With reference to the following parameters the method was validated.

1) Specificity

2) Linearity

3) System suitability

4) Precision

5) Accuracy (% Recovery)

6) Intermediate precision (Ruggedness)

7) Robustness

SPECIFICITY:

Specificity is the measure of the degree of the interference in the analysis of the complex sample mixtures such as analyte present in the matrix containing endogenous substances and related chemical compounds, etc. To validate specificity of the method the sample solution of the analytes were prepared (100% level) and their

corresponding absorbance being measured at wavelength 247nm and 276nm. By comparing the absorbance values of mixed standard solution of Paracetamol and Aceclofenac, it has been found that there was no variation which indicates that the proposed method was specific. The results shows that the absorbance of mixed standard and sample solution were not differ from each other. Thus there was no interference of the excipients at the absorbance of the pure drugs.

Absorbance of mixed standard and Sample solution of Paracetamol and Aceclofenac:

Table 7.4 Absorbance of Mixed standard and Sample solution

Obs.No	Level	Absorbance			
		Mixed standard solution		Sample solution	
		247nm	276nm	247nm	276nm
1	100%	1.241	0.349	1.245	0.352
2	100%	1.244	0.343	1.232	0.350
3	100%	1.231	0.346	1.237	0.355
4	100%	1.236	0.339	1.243	0.349
5	100%	1.225	0.341	1.248	0.351
	Mean	1.235	0.344	1.241	0.351
	S.D.	0.0076	0.0040	0.0064	0.0023
	% RSD	0.0062	0.0116	0.0052	0.0066

Preparation of Calibration curve:

Method validation is a process by which it is established by laboratory studies that the performance characteristics of a method meet the requirements for its intended application. To demonstrate that the proposed method is adequate for its intended purpose, the proposed

method was subjected to statistical validation. An essential step after developing a new analytical method is to determine the concentration range over which the detector exhibits linear response for the analyte. The following experiment was performed in order to determine the linear working range for Paracetamol and Aceclofenac.

Preparation of standard solution for linear working range:

To determine linear working range of Paracetamol and Aceclofenac, separately the standard solution of each analyte was taken and diluted with methanol. 100 µg/mL and 20 µg/mL stock solutions of Paracetamol and Aceclofenac respectively were used in the following linearity levels.

Table 7.5 Preparation of standard solution for linearity levels

Linearity Levels	Volume of std stock solution added in mL	Volume made upto in mL	Concentration of Paracetamol in ppm	Concentration of Aceclofenac in ppm
40%	0.5	10	5.0	1
60%	1.5	20	7.5	1.5
80%	1.0	10	10.0	2.0
100%	2.5	20	12.5	2.5
120%	1.5	10	15.0	3.0
140%	3.5	20	17.5	3.5
160%	2	10	20.0	4.0

Absorbance measurements were performed at 247nm for Paracetamol and 276nm for Aceclofenac against methanol as the reagent blank. The absorbance readings for Paracetamol and Aceclofenac are given in a table 7.3 and 7.4 respectively. A graph of absorbance values against the concen-tration was plotted. The linearity plots for Paracetamol and Aceclofenac are shown in a fig 7.2 and 7.3 respectively.

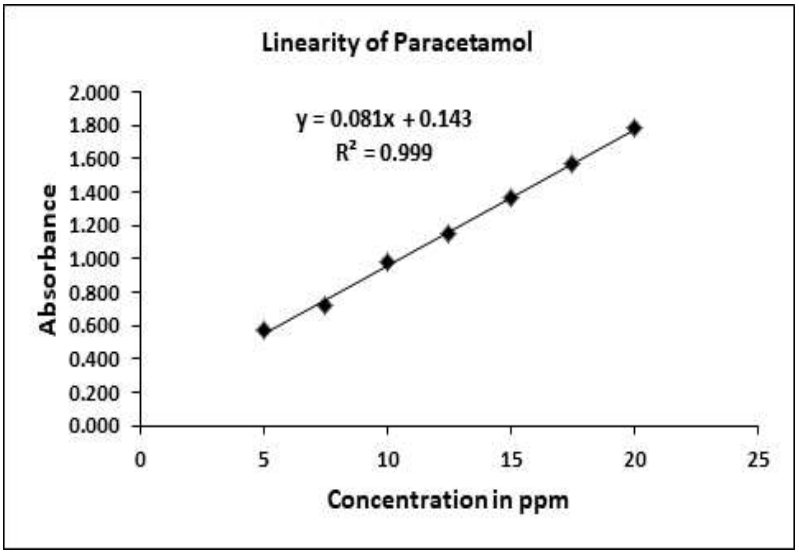
Linear response was observed in the concentration range of 5 µg/mL to 20 µg/mL for Paracetamol and 1 µg/mL to 4.5 µg/mL for Aceclofenac. The experiment was repeated thrice in this linear working range in order to check the reproducibility of the method. The mean, standard deviation and RSD of the three observations at each concentration level of the calibration curve were calculated.

Linearity of Paracetamol at different concentration level:

Table 7.6 Linearity level for Paracetamol

Obs No.	Conc. level %	Conc. in µg/ml	Absorbance			Mean	S.D	%RSD
			1	2	3			
1	40	5	0.571	0.577	0.575	0.574	0.0031	0.532
2	60	7.5	0.715	0.719	0.725	0.720	0.0050	0.699
3	80	10	0.982	0.985	0.974	0.980	0.0057	0.580
4	100	12.5	1.148	1.147	1.157	1.151	0.0055	0.479
5	120	15	1.363	1.368	1.375	1.369	0.0060	0.440
6	140	17.5	1.56	1.571	1.58	1.570	0.0100	0.638
7	160	20	1.76	1.788	1.797	1.782	0.0193	1.083

Fig 7.4 Linearity plot for Paracetamol



Linearity of Aceclofenac at different concentration level:

Table 7.7 Linearity level for Aceclofenac

Obs No.	Conc. level %	Conc. in µg/ml	Absorbance			Mean	S.D	%RSD
			1	2	3			
1	40	1	0.056	0.057	0.056	0.056	0.0006	1.025
2	60	1.5	0.081	0.080	0.080	0.080	0.0006	0.719
3	80	2	0.099	0.098	0.099	0.099	0.0006	0.585
4	100	2.5	0.123	0.122	0.122	0.122	0.0006	0.472
5	120	3	0.136	0.137	0.138	0.137	0.0010	0.730
6	140	3.5	0.154	0.156	0.157	0.156	0.0015	0.981
7	160	4	0.176	0.178	0.175	0.176	0.0015	0.866

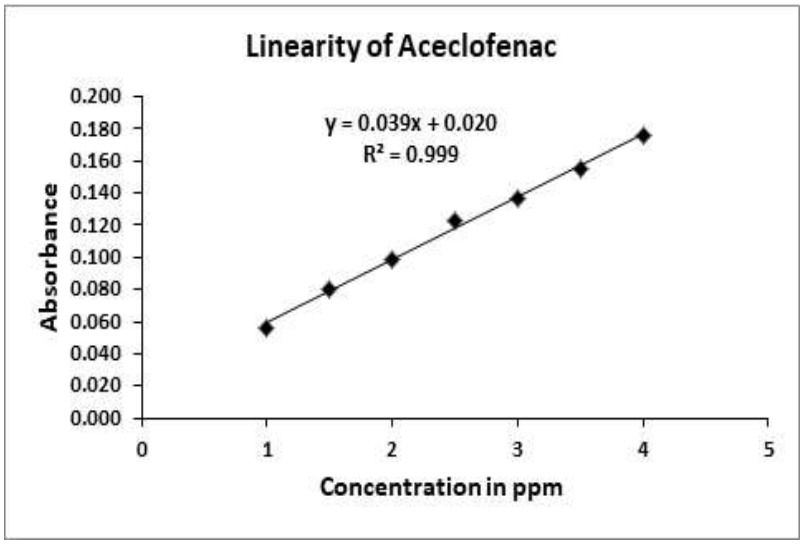


Fig 7.5 Linearity plot for Aceclofenac

REGRESSION ANALYSIS:

The calibration data range 40% to 160% for Paracetamol and Aceclofenac was further considered for regression analysis. The regression analysis of the calibration data was carried out to determine the relationship between the dependent variable (Peak area) and independent variable (drug concentration).

The regression equation

y = mx + C was found to

y = 0.081X + 0.143 for Paracetamol

y = 0.039X + 0.020 for Aceclofenac

Where y = dependent variable

m = Slope of the regression line

x = independent variable C = intercept on y axis

For the method to have good correlation between analyte concentration and response, the residuals should be

distributed above and below the zero residual line indicating the random precision of the method.

Regression analysis data (Paracetamol):

Table 7.8 Regression analysis data of Paracetamol

Obs No	X value Conc in ppm	Y Value observed	Y Value Calculated	Residual value
1	5	0.574	0.548	0.026
2	7.5	0.720	0.751	-0.031
3	10	0.980	0.953	0.027
4	12.5	1.151	1.156	-0.004
5	15	1.369	1.358	0.011
6	17.5	1.570	1.561	0.010
7	20	1.782	1.763	0.019

Slope	0.081
Correlation coefficient	0.9990
Std error of Y estimate	0.022

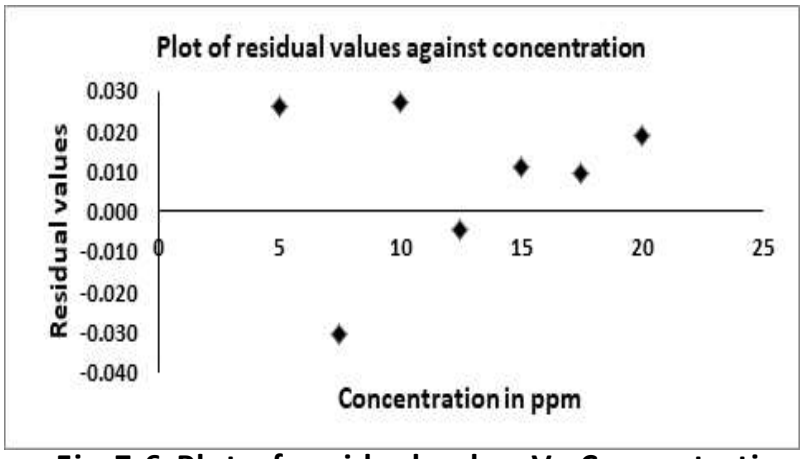


Fig 7.6 Plot of residual value Vs Concentration (Paracetamol)

Regression analysis data (Aceclofenac)

Table 7.9 Regression analysis data of Aceclofenac

Obs No	X value Conc in ppm	Y Value observed	Y Value Calculated	Residual value
1	1	0.056	0.059	-0.003
2	1.5	0.08	0.079	0.002
3	2	0.099	0.098	0.001
4	2.5	0.122	0.118	0.004
5	3	0.137	0.137	0.000
6	3.5	0.156	0.157	-0.001
7	4	0.176	0.176	0.000

Slope	0.039
Correlation coefficient	0.9990
Std error of Y estimate	0.0020

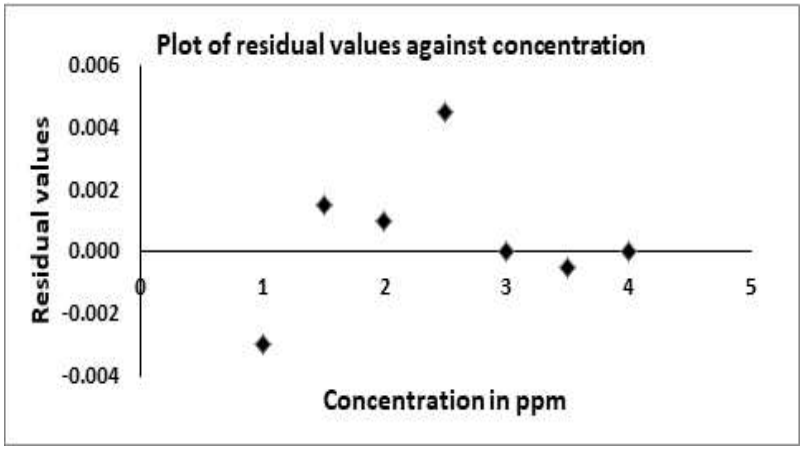


Fig 7.7 Plot of residual value Vs Concentration (Aceclofenac)

SENSITIVITY PARAMETERS:

The molar absorptivity and Sandell sensitivity values of Paracetamol and Aceclofenac were calculated and given in table 7.10

MOLAR ABSORPTIVITY:

The Molar absorption coefficient, molar extinction coefficient, or molar absorptivity, is a measurement of how strongly a chemical species absorbs light at a given wavelength. It is an intrinsic property of the species; the actual absorbance, *A*, of a sample is dependent on the path length l and the concentration *c* of the species via the Beer-Lambert law, A = åcl.

The molar absorptivity value for Paracetamol and Aceclofenac were calculated from the slope of the calibration curve using the following formula.

$$A = å\,c\,l$$

Therefore

$$å = \frac{A}{c\,l}$$

Molar absorptivity å = slope x Molecular weight x 10^3 lit.mol^{-1} cm^{-1}

Molar absorptivity of Paracetamol = 0.081 x 151.16 x 10^3 lit.mol^{-1} cm^{-1}

= 12.244 x 10^3 lit.mol^{-1} cm^{-1}

= 1.244 x 10^4 lit.mol^{-1} cm^{-1}

For Aceclofenac:

Molar absorptivity of Aceclofenac = 0.039 x 354.2 x 10^3 lit.mol^{-1} cm^{-1}

= 13.814 x 10^3 lit.mol^{-1} cm^{-1}

= 1.3814 x 10^4 lit.mol^{-1} cm^{-1}

SANDELL SENSITIVITY:

Sensitivity of the methods for drugs individually was determined by calculating Sandell's sensitivity (ìg/ cm^2 / 0.001/ABS unit) which can be defined as the smallest weight of the substance that can be detected in the column of the solution of unit cross section. The weight of the sample can be conveniently expressed in ìg and area in cm^2, if the molar extinction coefficient of the substance is å and molecular weight is M then Sandell's sensitivity is given by

$$\text{Sandell Sensitivity} = \frac{\text{Molecular Wt.}}{\text{Molar Absorptivity}} \quad \mu g/cm^3/cm^2$$

For Paracetamol

$$\text{Sandell Sensitivity} = \frac{151.16}{1.244 \times 10^4} \quad \mu g/cm^3/cm^2$$

$$= 0.01215\ \mu g/cm^3/cm^2$$

For Aceclofenac

$$\text{Sandell Sensitivity} = \frac{354.2}{1.384 \times 10^4} \quad \mu g/cm^3/cm^2$$

$$= 0.02559\ \mu g/cm^3/cm^2$$

High value of Molar absorptivity and low value of Sandell sensitivity indicate high sensitivity of the proposed method. Sensitivity Parameters for Paracetamol and Aceclofenac are given in table 7.10

Table 7.10 Sensitivity Parameters for Paracetamol and Aceclofenac

Analyte	λ max (nm)	Molar absorptivity	Sandell's Sensitivity
Paracetamol	247	1.244×10^4 lit.mol^{-1} cm^{-1}	0.01215 µg/cm^3/cm^2
Aceclofenac	276	1.3814×10^4 lit.mol^{-1} cm^{-1}	0.02559 µg/cm^3/cm^2

SYSTEM SUITABILITY TEST:

After a method has been validated, system suitability test should be performed to confirm if the operating system is performing properly. System suitability test is used to verify that the reproducibility of the equipment is adequate for the analysis to be done. System suitability test was performed as per the USP 25 to confirm the suitability and reproducibility of the system. The test was performed with 12.5 µg/mL (100% level) for Paracetamol and 2.5 µg/mL (100% level) for Aceclofenac. Absorbance measurements were performed at $ë_{max}$ value of both the analytes. The entire procedure was repeated for five times. The S.D and R.S.D values of these five determinations were calculated and are given in following tables as follows.

Table 7.11 Results of system suitability test for Paracetamol

Sr.No	Concentration in µg/mL	Absorbance at 247nm
1	12.5	1.155
2	12.5	1.157
3	12.5	1.152
4	12.5	1.559
5	12.5	1.154
	Mean	1.1552
	S.D	0.0024
	% R.S.D	0.21

Table 7.12 Results of system suitability test for Aceclofenac

Sr.No	Concentration in µg/mL	Absorbance at 247nm
1	2.5	0.123
2	2.5	0.124
3	2.5	0.125
4	2.5	0.123
5	2.5	0.122
	Mean	0.1234
	S.D	0.0011
	% R.S.D	0.92

PRECISION:

The method precision study was performed to determine the reproducibility of the method. Six samples of tablets were prepared at 100% level and assayed according to the simultaneous equation method. The results are given in table 7.13.

Table 7.13 Method precision study for Paracetamol and Aceclofenac

Obs.No	Absorbance		True value conc. in µg/ml		Experimental values Conc. in µg/ml	
	A_1 247nm	A_2 276nm	Paracetamol	Aceclofenac	Paracetamol Cx	Aceclofenac Cy
1	1.250	0.349	12.5	2.5	12.497	2.454
2	1.244	0.343	12.5	2.5	12.438	2.435
3	1.252	0.346	12.5	2.5	12.534	2.378
4	1.252	0.339	12.5	2.5	12.515	2.468
5	1.241	0.341	12.5	2.5	12.399	2.471
6	1.24	0.345	12.5	2.5	12.403	2.407
Mean	-	-	-	-	12.464	2.436
S.D	-	-	-	-	0.059	0.037
%RSD	-	-	-	-	0.470	1.520
% Accuracy	-	-	-	-	99.71	97.43

Method precision study shows that the results are within the limits. The concentration of Paracetamol and Aceclofenac were calculated by solving simultaneous equation method. The results obtained are within the range.

ACCURACY (% RECOVERY):

The accuracy of the method was assessed by taking the known amounts of Paracetamol and Aceclofenac in a sample solution of 100% level and absorption was determined at ëmax of both the drugs. Concentration of both the drugs in the mixture solution was determined by simultaneous equation method. According to this method if the sample contain two absorbing drugs X and Y and each of which has absorption maxima at (ë1 ‘“ ë2), it may be possible to determine both drugs by simultaneous equation. The total absorbance of a solution at a given wavelength is the sum of absorbance of individual component at the given wavelength.

Table 7.14 Accuracy study of Paracetamol

Obs No	Levels in %	Absorbance		Initial amount in mg	Amt added in mg	Amt found in mg	% recovery
		at 247nm	at 276nm				
1	0%	1.250	0.349	12.5	0	12.497	99.98
2		1.253	0.351	12.5	0	12.521	100.17
3		1.254	0.350	12.5	0	12.537	100.30
1	10%	1.375	0.384	12.5	1.25	13.746	99.97
2		1.38	0.386	12.5	1.25	13.793	100.31
3		1.381	0.387	12.5	1.30	13.799	100.36
1	20%	1.505	0.420	12.5	2.50	15.047	100.31
2		1.498	0.421	12.5	2.50	14.963	99.75
3		1.507	0.422	12.5	2.60	15.06	100.40
1	30%	1.621	0.451	12.5	3.75	16.189	99.62
2		1.619	0.449	12.5	3.80	16.171	99.21
3		1.617	0.453	12.5	3.80	16.158	99.13
						Mean	99.96
						S.D	0.44
						%RSD	0.44
						Range of Recovery	99.13-100.40

INTERMEDIATE PRECISION (RUGGDENESS):

Obs No	Levels in %	Absorbance		Initial amount in mg	Amt added in mg	Amt found in mg	% recovery
		at 247nm	at 276nm				
1	0%	1.250	0.349	2.5	0	2.454	98.16
2		1.253	0.351	2.5	0	2.468	98.73
3		1.254	0.35	2.5	0	2.471	98.84
1	10%	1.375	0.384	2.5	0.25	2.702	98.26
2		1.38	0.386	2.5	0.25	2.726	99.11
3		1.381	0.387	2.5	0.26	2.744	99.78
1	20%	1.505	0.42	2.5	0.50	2.951	98.35
2		1.498	0.421	2.5	0.50	3.004	100.13
3		1.507	0.422	2.5	0.52	2.987	99.58
1	30%	1.621	0.456	2.5	0.75	3.260	100.31
2		1.619	0.455	2.5	0.76	3.246	99.88
3		1.617	0.453	2.5	0.76	3.210	98.47
						Mean	99.13
						S.D	0.77
						%RSD	0.78
						Range of Recovery	98.16-100.31%

As a part of method validation, Intermediate precision was performed by carrying out the same assay procedure on a different instrument on a different day under similar experimental conditions.

Sample preparation:

Sample solution of 100% level concentration was prepared as mentioned in system precision. On a different day six samples of Paracetamol and Aceclofenac tablets, were prepared and analysed as per the methodology. This analysis was carried out on a instrument TSUV 75 UV-Visible spectrophotometer.

Ruggedness study, mixed standard solution of Paracetamol and Aceclofenac:

Table 7.16 Ruggedness study, standard solution

Obs No	100% level	
	Absorbance(A_1)	**Absorbance(A_2)**
	At 247nm	**276nm**
1	1.246	0.348
2	1.248	0.352
3	1.243	0.353
4	1.241	0.347
5	1.239	0.356
6	1.251	0.347
Mean	1.245	0.351
S.D	0.0045	0.0037
%RSD	0.362	1.064

Amount of Paracetamol and Aceclofenac:

Table 7.17 Ruggedness study results for Paracetamol and Aceclofenac

Obs No	Absorbance A_1	Absorbance A_2	PCM mg/tab	ACF mg/tab	% LC PCM	% LC ACF
1	1.238	0.347	499.03	98.71	99.81	98.71
2	1.240	0.349	498.12	99.05	99.62	99.05
3	1.241	0.35	498.38	99.78	99.68	99.78
4	1.245	0.345	503.08	101.30	100.62	101.30
5	1.249	0.352	501.64	100.19	100.33	100.19
6	1.250	0.355	502.29	99.12	100.46	99.12
Mean	-	-	500.42	99.69	100.08	99.69
S.D	-	-	2.166	0.954	0.433	0.954
%RSD	-	-	0.433	0.957	0.433	0.957

Table 7.18 Cumulative % RSD of Paracetamol & Aceclofenac in Precision and Intermediate precision

Obs No	Paracetamol mg/tab	% LC Paracetamol	Aceclofenac mg/tab	% LC Aceclofenac
M.P 1	501.48	100.30	100.74	100.74
M.P 2	499.12	99.82	99.96	99.96
M.P 3	502.97	100.59	97.62	97.62
M.P 4	502.21	100.44	101.31	101.31
M.P 5	497.55	99.51	101.44	101.44
M.P 6	497.71	99.542	98.81	98.81
I.P 1	499.03	99.806	98.71	98.71
I.P 2	498.12	99.624	99.05	99.05
I.P 3	498.38	99.676	99.78	99.78
I.P 4	503.08	100.62	101.3	101.3
I.P 5	501.64	100.33	100.19	100.19
I.P 6	502.29	100.46	99.12	99.12
Mean	500.30	100.06	99.84	99.84
S.D.	2.161	0.432	1.214	1.214
Cumulative % RSD	0.432	0.432	1.216	1.216
Limits	NMT 2.00%		NMT 2.00%	

ROBUSTNESS:

Robustness of the proposed method was determined by minor changes in the ëmax of both the analytes. Since the absorbance was not significantly affected, the proposed method could be considered as robust.

Mean % assay of Paracetamol and Aceclofenac

Table 7.19 Robustness study for Paracetamol and Aceclofenac

Name of the sample	Change in wavelength +0.5nm		Change in wavelength -0.5nm	
	Paracetamol	Aceclofenac	Paracetamol	Aceclofenac
Sample -1	99.81	98.71	99.26	98.05
Sample -2	99.62	99.05	98.62	98.23
Sample -3	99.68	99.78	98.16	99.17
Mean	99.70	99.180	98.680	98.483
S.D.	0.097	0.547	0.552	0.601
%RSD	0.097	0.551	0.560	0.611

RESULTS AND DISCUSSION:

A study of overlain spectra of Paracetamol and Aceclofenac in methanol shows that at 247nm Paracetamol shows maximum absorbance whereas Aceclofenac shows at 276nm. The overlain spectrum also shows that both the analytes shows considerable absorbances at their ëmax values. Hence it was possible to construct simultaneous equation.

The study of the system suitability test showed that the operating system has given good results and verified the reproducibility of the method.

Linearity

Linearity of the method was tested from 40% to 160% of the targeted level of the assay concentration (12.5 µg/mL Paracetamol and 2.5 µg/mL Aceclofenac) for the two analytes. The standard solutions containing 5 – 12.5 µg/mL Paracetamol and 1.0- 4.0µg/mL Aceclofenac were prepared from the standard stock solutions of Paracetamol

and Aceclofenac. Linearity test solutions were injected and analyzed in triplicate. The calibration graphs were plotted by using absorbance of the analytes against the concentration of the drug (in micrograms per milliliter). In the simultaneous determination, the calibration graphs were found to be linear for both the analytes in the mentioned concentration ranges. The regression equations for Paracetamol and Aceclofenac were found to be

$y = 0.081X + 0.143$ and $y = 0.039X + 0.020$, and the correlation coefficients for the regression lines were 0.9990 and 0.9990, respectively.

Sensitivity

Sandell's sensitivity of Paracetamol and Aceclofenac was found to be sufficiently low. Table 7.10 which shows that very less amount of both the drugs can be effectively detected by this method.

Recovery

The accuracy of the method was determined by the standard addition method at three different levels. The sample solution of 100% level was considered as a zero level and 10%, 20% and 30% of the standard drug of analytes were added respectively. Each determination was performed in triplicates. The accuracy was then calculated as the percentage of the standard drug recovered by the recovery study. The recovery of Paracetamol and Aceclofenac from the standard mixture solution was found to be 99.13% -100.40% and 98.16-100.31% respectively. The recovery results shows that Paracetamol and Aceclofenac could be quantified by this procedure simultaneously. The results are well within the acceptance limit and hence the method is accurate.

Intermediate precision

Precision and Intermediate precision study shows that the proposed method was precise. Table 7.18 shows the cumulative % RSD of Paracetamol & Aceclofenac in precision and Intermediate precision. The cumulative % of RSD for Paracetamol and Aceclofenac were within the limits.

Robustness

Robustness study shows that the proposed method was found to be robust where minor variation in wavelength could not alter the assay of the analytes.

COMPARISON OF HPTLC AND UV METHOD:

Table 7.20 Comparison of HPTLC method and UV method

Validation Parameters		By HPTLC		By UV	
		Paracetamol	Aceclofenac	Paracetamol	Aceclofenac
Correlation coefficient		0.9996	0.9990	0.9990	0.9990
Precision	% LA	100.01	99.31	100.08	99.69
	%RSD	1.233	1.193	0.433	0.957
Intermediate precision	% Cumulative RSD	1.125	1.253	0.432	1.216

Comparison of HPTLC and UV method for simultaneous determination of Paracetamol and Aceclofenac from tablet formulation is shown in above table. There is no statistically significance difference between the mean values for samples. Therefore both analytical methods were found to be equally good and could be used for routine determination of assay of Paracetamol and Aceclofenac tablets.

CONCLUSION:

The proposed simultaneous equation method is found to be very simple and can be performed by using any spectrophotometer and it does not requires any costly instrument equipped with special package. It also shows good linearity values and sensitivity. The proposed method was found to be simple, accurate, precise, rugged and robust. The proposed method was successfully applied to the simultaneous determination of Paracetamol and Aceclofenac from pharmaceutical tablet formulation. Therefore it can be concluded that the proposed method provides an alternative procedure for quality control of Paracetamol & Aceclofenac in pharmaceutical formulation.

REFERENCES:

1) An, Duong Thi Thuy; Vu, Dang Hoang. Simultaneous determination of Paracetamol and Codeine phosphate in combined tablets by first-order derivative and ratio spectra first-order derivative UV spectrophotometry. *Asian Journal of Research in Chemistry* (2009), 2(2), 143-147.

2) Chitlange, Sohan S.; Soni, Ranjana; Wankhede, Sagar B.; Kulkarni, Amol A. Spectrophotometric methods for simultaneous estimation of Dexibuprofen and Paracetamol. *Asian Journal of Research in Chemistry* (2009), 2(1), 30-33.

3) Srinivasan, K. K.; Alex, J.; Shirwaikar, A. A.; Jacob, S.; Kumar, M. R. Sunil; Prabu, S. L. Simultaneous derivative spectrophotometric estimation of Aceclofenac and Tramadol with Paracetamol in combination solid dosage forms. *Indian Journal of Pharmaceutical Sciences* (2007), 69(4), 540-545.

4) Selvan, P. Senthamil; Gopinath, R.; Saravanan, V. S.; Gopal, N.; Kumar, A. Sarvana; Periyasamy, K. Simultaneous estimation of Paracetamol and Aceclofenac in combined dosage forms by RP-HPLC method. *Asian Journal of Chemistry* (2007), 19(2), 1004-1010.

5) Sena, Marcelo M.; Freitas, Camilla B.; Silva, Lucas C.; Perez, Caridad Noda; de Paula, Ydilla O. Simultaneous spectrophotometric determination of Paracetamol and Ibuprofen in pharmaceutical formulations using multivariate calibration. *Quimica Nova* (2007), 30(1), 75-79.

6) Momin, M. Y.; Yeole, P. G.; Puranik, M. P.; Wadher, S. J. Reverse phase HPLC method for determination of Aceclofenac and Paracetamol in tablet dosage form. *Indian Journal of Pharmaceutical Sciences* (2006), 68(3), 387-389.

7) Dinc, E.; Kokdil, G.; Onur, F. Derivative ratio spectra-zero crossing spectrophotometry and LC method applied to the quantitative determination of Paracetamol, Propyphenazone and Caffeine in ternary mixtures. *Journal of Pharmaceutical and Biomedical Analysis* (2001), 26(5-6), 769-778.

8) Srinivasan KK, Shirwaikar A., Joseph A., Jacob S., Prabu SL. Simultaneous estimation of Aceclofenac & Paracetamol in solid dosage form by ultraviolet spectro-photometry. *Indian Drugs*. 2006; 43 (2): 141 – 145.

9) Shirkhedkar, A. A.; Shaikh, Afsar; Surana, S. J. Spectrophotometric method for simultaneous determination of Paracetamol and Piroxicam in tablets. *Asian Journal of Chemistry* (2008), 20(3), 2470-2472.

10) Wadher, S. J.; Momin, M. Y.; Puranik, M. P.; Yeole, P. G. Simultaneous estimation of Aceclofenac and Paracetamol in combined dosage form by two wavelength spectrophotometry. *Pharma Review* (2007), 5(30), 164-166.

11) Harikrishnan, N.; Kalaivani, K.; Meher, Vijay Kumar; Gunasekaran, V.; Babu, A. Sathish; Roosewelt, C. Simultaneous estimation of Paracetamol, Aceclofenac and Chlorzoxazone in oral dosage form using spectrophotometric method. *Analytical Chemistry* (Rajkot, India) (2007), 5(1-6), 46-50.

12) Jain, Anurekha; Jain, Avijeet; Vyas, Vivek; Subedar, Niharika; Gupta, Arun. B.R. Simultaneous estimation of Aceclofenac and Paracetamol in tablet dosage form by UV spectroscopy. *Asian Journal of Chemistry* (2007), 19(6), 4920-4922.

13) P.R.Mahaparale, J.N. Sangshetti and B.S. Kuchekar Simultaneous spectrophotometric estimation of Aceclofenac and Paracetamol in tablet dosage form. *Indian journal*

of Pharmaceutical scinces (2007), 69(2); 289- 14) Maheshwari RK, Chaturvedi SC., Jain NK. Analysis of Aceclofenac in tablets using hydrotropic solublisation technique. *Indian Drugs.* 2006; 43(6): 516 – 518.

15) Shanmugam S., Cednil Kumar A., Vetrichelvan T., Manavalan R., Venkappyya D., Pandey V.P Spectrophotometric method for estimation of Aceclofenac in tablets. *Indian Drugs.* 2005; 42(2): 106 – 107.

16) Ali SM. EI Moghazy. Determination of Aceclofenac in bulk and pharmaceutical formulation. J. Pharm. Biomed. *Anal.* 2002; 27(1): 243-251.

17) El.Kousy N.M., Spectrophotometric and spectrofluorimetric determination of Etodolac and Aceclofenac, *J. Pharm. Biomed. Anal.,* 1999, 20,185- 94

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