METHOD DEVELOPMENT AND VALIDATION OF SIMULTANEOUS ESTIMATION OF OLMESARTAN MEDOXIMIL AND CILNIDIPINE BY RP-HPLC IN PHARMACEUTICAL DOSAGE FORM

DISSERTATION

Submitted to

THE TAMIL NADU DR.M.G.R.MEDICAL UNIVERSITY,

CHENNAI.

In partial fulfillment for the award of the degree of

MASTER OF PHARMACY
In
(Pharmaceutical Analysis)

By

261230011

Under the Guidance of

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This is to certify that the project entitled **"METHOD DEVELOPMENT AND VALIDATION OF SIMULTANEOUS ESTIMATION OF OLMESARTAN MEDOXIMIL AND CILNIDIPINE BY RP-HPLC IN PHARMACEUTICAL DOSAGE FORM"** submitted **by 261230011** in partial fulfillment for the award of degree of **Master of Pharmacy.** It was carried out at Indian Institute Of Chemical Technology, Hyderabad and at C.L.Baid Metha college of Pharmacy, Chennai-96 under the guidance of **Dr. Shantha Arcot, M.Sc. (Pharm).,Ph.D.,** HOD, Department of Pharmaceutical Analysis **during the year 2013-2014.**

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DECLARATION

The thesis entitled **"METHOD DEVELOPMENT AND VALIDATION OF SIMULTANEOUS ESTIMATION OF OLMESARTAN MEDOXIMIL AND CILNIDIPINE BY RP-HPLC IN PHARMACEUTICAL DOSAGE FORM"** was carried out by me in Department of Pharmaceutical Analysis, C.L.Baid Metha College of Pharmacy, Chennai – 97 during the academic year 2013-2014. The work embodied in this thesis is original, and is not submitted in part or full for any other degree of this or any other University.

Date: **[Reg.No: 261230011]**

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ACKNOWLEDGEMENTS

I would like to express my appreciation and sincere thanks for the efforts of everyone who have directly or indirectly contributed their ideas and energy in the successful completion of my project.

It gives me an immense pleasure and pride to express my deep sense of gratitude and respect for my teacher and guide ***Dr. SHANTHA ARCOT, M.Sc. (Pharm)., Ph.D.,*** *HOD, Department of Pharmaceutical Analysis, C.L.Baid Metha College of Pharmacy, Chennai – 97, for her evergreen expertise and inspiring guidance for the timely completion of this work.*

I would like to express my sincere thanks with deep sense of gratitude, to my respected guide, ***Dr. K. Suresh Babu****, Scientist, NPL, Organic Division-I, Indian institute of Chemical Technology, Hyderabad for his immense guidance, help, undeterred support, dedication, intellectual supervision, professional expertise, freedom of thought, speech and trust he has bestowed upon me throughout the period of my work.*

It's my privilege to express my grateful and sincere gratitude to ***Dr. GRACE RATHNAM, M.Pharm., Ph.D, Principal****, and HOD of pharmaceutics, C.L.Baid Metha College of Pharmacy.*

I acknowledge my sincere thanks ***to Mrs. VIJAYA NAGARAJAN, M.Pharm., (Ph.D).,*** *Assistant professor,* ***Mrs. VIJAYA GEETHA, M.Pharm., (Ph.D).,*** *Assistant professor,* ***Mrs. G. UMA, M.Pharm., (Ph.D).,*** *Assistant professor,* ***Mrs. S. YAMUNA M.Pharm., (Ph.D.,)****Lecture Department of pharmaceutical Analysis, for their valuable suggestions throughout my thesis work.*

I thank ***Dr.M LAKSHMI KANTHAM*** *Director, IICT, Hyderabad and Dr. S. Chandrashekhar, Head of Department, Organic Division I, IICT for giving me opportunity to do project in IICT.*

I would like to thank ***Dr. V U M SHARMA****, IICT, Hyderabad for providing the facilities for performing method development and validation of my work and for constant support and Mrs.B Manjula for helping me in the process.*

I would like to show my greatest appreciation to my ***seniors****, Ch.Pavan ,B.Poornima,and B.Siva for their tremendous guidance, support and suggestions during my project and I take this opportunity to thank all my senior colleagues V Rajesh,K Hari Babu, Ramesh, K kumar, Ashok, A.Venkanna and my labmate Saheethi for their unreserved help and encouragement during my project.*

*I specially thank Ms.****G.Surya, M.Sc, Incharge of Ceeal lab*** *for her guidance and source of inspiration that I have received from her throughout my project work.*

I extend my sincere thanks to Chief Librarian ***M. Rajalakshmi,*** *C.L.Baid Metha College of Pharmacy in helping me to utilize the library facilities for references.*

I thank all non teaching staff members of our college including ***Mrs.R.Usha, Mrs.Valli*** *and* ***Mrs.A.P.Kalpakam*** *for their help extended during my project work.*

I cannot ignore the support and help which I received from my friend and M. Pharmacy classmates SONJA JOSE AND FRANCIS XAVIER during the course of my training period

My gratitude, affection and lots of love saying special thanks to my ***Parents*** *Mr.N V R Prasad and Mrs. N Sarada who mean the world to me and to whom I owe everything for what I'm today, having unwavering confidence in me and showing me a way to my will. Thanks for being a friend, guide, my positive energy, driving force, spirit and ever ending support and encouragement in every path of my life and thus completing me.*

I might have missed a few names and I am sorry for that, but they are always in my memories.

Place: CHENNAI

Date: *NIHARIKA NANDI*

CONTENT

LIST OF TABLES

CHROMATOGRAMS LIST

ABBREVIATIONS

Conc.	Concentration
^{0}C	Degree centigrade
gm	Grams
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
ICH	International Conference on Harmonization
λ	Lambda
LOD	Limit of Detection
LOQ	Limit of Quantification
mg	Milligrams
Min	Minutes
ml	Milliliter
mM	Millimolar
mm	Millimeter
Mol.Wt.	Molecular weight
µg	micro gram
µl	micro liter
µm	micro meter
nm	Nanometer
NLT	Not Less Than
NMT	Not More Than
PDA	Photo Diode Array
IP	Indian Pharmacopoeia
BP	British Pharmacopoeia
EP	European Pharmacopoeia
USP	United States Pharmacopoeia

pH	Negative Logarithm Of Hydrogen ion
pKa	Dissociation Constant
%	Percentage
RI	Refractive Index
LC-MS	Liquid Chromatography-Mass Chromatography
RSD	Relative Standard Deviation
Rt	Retention Time
SD	Standard Deviation
Std	Standard
µg	Micro gram
UV	Ultra-violet
Vs	Versus
v/v	Volume By Volume

1. INTRODUCTION

PHARMACEUTICAL ANALYSIS[1]

Pharmaceutical analysis[1] plays a vital role in the pharmaceutical product development. Pharmaceutical analysis is a specialized branch of analytical chemistry. Analytical chemistry involves separating, identifying, and determining the relative amounts of components in a sample matrix.

Pharmaceutical analysis derives its principles from various branches of sciences like physics, microbiology, nuclear science, and electronics etc. Qualitative analysis reveals the chemical identity of the sample. Quantitative analysis establishes the relative amount of one or more of these species or analytes in numerical terms. Qualitative analysis is required before a quantitative analysis can be undertaken.

A separation step is usually a necessary part of both a qualitative and quantitative analysis. The results of typical quantitative analysis can be computed from two measurements. One is the mass or volume of sample to be analyzed and second is the measurement of some quantity that is proportional to the amount of analyte in that sample and normally completes the analysis.

Instruments play a key role in the quantitative analysis of pharmaceutical chemistry.

INSTRUMENTAL METHODS OF ANALYSIS

Instrumental methods are exciting and fascinating part of chemical analysis that interacts with all areas of chemistry and with many other areas of pure and applied sciences. Analytical instrumentation plays an important role in the production and evaluation of new products and in the protection of consumers and environment.

This instrumentation provides lower detection limits required to assure safe foods, drugs, water and air. Instrumental methods are widely used by Analytical chemists to save time, to avoid chemical separation and to obtain increased accuracy. Most instrumental techniques fit into one of the four-principle areas mentioned below.

a) Spectrophotometric techniques

- UV and Visible Spectrophotometry
- Fluorescence and Phosphorescence Spectrophotometry

- Atomic Spectrophotometry (Emission &Absorption)
- Infrared Spectrophotometry
- Raman Spectrophotometry
- X-Ray Spectrophotometry
- Nuclear Magnetic Resonance Spectroscopy
- Mass Spectroscopy
- Electron spin Resonance Spectroscopy

b) Electrochemical Techniques

- Potentiometry
- Voltametry
- Electrogravimetry
- Conductometry
- Amperometry

c) Chromatographic Techniques

- High Performance Liquid Chromatography
- Gas chromatography
- High Performance Thin Layer Chromatography
- Thin Layer Chromatography
- GC- MS (Gas chromatography - Mass Spectroscopy
- LC-MS (Liquid Chromatography - Mass Spectroscopy

d) Miscellaneous techniques

- Thermal analysis
- Kinetic techniques

Chromatographic techniques are predominantly used in the pharmaceutical industry for a large variety of samples. HPLC is one of the chromatographic techniques is widely used for checking the purity of new drug candidates, monitoring changes or scale ups of synthetic procedures, evaluating new formulations, and scrutinizing quality control/assurance of final drug products.

1.1 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY[2]

High performance liquid chromatography[2] is basically a highly improved form of column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres, that makes it much faster. It also allows using a very much smaller particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it. This allows a much better separation of the components of the mixture. The other major improvement over column chromatography concerns the detection methods which can be used. These methods are highly automated and extremely sensitive.

HPLC employs a liquid mobile phase and a very finely divided stationary phase. In order to obtain satisfactory flow rate, liquid must be pressurized to a few thousands of pounds per square inch. The rate of distribution of drugs between stationary and mobile phase is controlled by diffusion process. If diffusion is minimized, a faster and effective separation can be achieved. The technique of high performance liquid chromatography is so called because of its improved performance when compared to classical column chromatography. Advances in column technology, high-pressure pumping system and sensitive detectors have transformed liquid column chromatography into high speed, efficient, accurate and highly resolved method of separation.

The HPLC is the method of choice in the field of analytical chemistry, since this method is specific, robust, linear, precise and accurate and the limit of detection is low and also it offers the following advantages.

- Speed (many analysis can be accomplished in 20 min or less)
- Greater sensitivity (various detectors can be employed)
- Improved resolution (wide variety of stationary phases)
- Reusable columns (expensive columns but can be used for many analysis)
- Ideal for the substances of low viscosity
- Easy sample recovery, handling and maintenance.
- Instrumentation leads itself to automation and quantification (less time and less labor)
- Precise and reproducible
- Integrator itself does calculations.

SEPARATION PRINCIPLES OF HPLC

Adsorption chromatography employs high-surface area particles that adsorb the solute molecules. Usually a polar solid such as a silica gel, alumina or porous glass beads and a non-polar mobile phase such as heptane, octane or chloroform are used in adsorption chromatography.

In adsorption chromatography, adsorption process is described by competition model and solvent interaction model. Competition model assumes that entire surface of the stationary phase is covered by mobile phase molecules as result of competition for adsorption site. In solvent interaction model the retention results from the interaction of solute molecule with the second layer of adsorbed mobile phase molecules. The differences in affinity of solutes for the surface of the stationary phase account for the separation achieved.

In partition chromatography, the solid support is coated with a liquid stationary phase. The relative distribution of solutes between the two liquid phases determines the separation. The stationary phase can either be polar or non polar. If the stationary phase is polar and the mobile phase is non polar, it is called normal phase partition chromatography. If the opposite case holds, it is called reverse-phase partition chromatography. In normal phase mode, the polar molecule partition preferentially in to the stationary phase and are retained longer than non-polar compounds. In reverse phase partition chromatography, the opposite behaviour is observed.

TYPES OF HPLC TECHNIQUES

Based on modes of chromatography:

- Normal phase chromatography
- Reverse phase chromatography

Based on principle of separation:

- Adsorption chromatography
- Ion exchange chromatography
- Size exclusion chromatography
- Affinity chromatography

- Chiral phase chromatography

Base on elution technique:

- Isocratic separation
- Gradient separation

Based on the scale of operation:

- Analytical HPLC
- Preparative HPLC

REVERSED PHASE CHROMATOGRAPHY[3]

In 1960s, chromatographers started modifying the polar nature of the silanol group by chemically reacting silicon with organic silanes. The object was to make silica less polar or non-polar so that polar solvents can be used to separate water-soluble polar compounds. Since the ionic nature of the chemically modified silica in now reversed i.e., it is non-polar or the nature of the phase is reverted, the chromatographic separation carried out with such silica is referred to as reverse-phase chromatography.

Reverse phase liquid chromatography (RPLC) is considered as the method of choice for the analysis of pharmaceutical compounds for several reasons, such as its compatibility with aqueous and organic solutions as well as with different detection systems and its high consistency and repeatability. Sensitive and accurate RPLC analysis, whether in the pharmaceutical or bioanalytical field, necessitates the use of stationary phases which give symmetrical and efficient peaks.

Therefore, manufacturers of stationary phases are continuously improving and introducing new RPLC products, and the selection of various types of reverse phase stationary phases is high. The needs for consistency as well as the globalization of the pharmaceutical companies require that the methods will be transferred from site to site, using either the same column brands or their equivalents. Therefore, an extensive categorization or characterization of the rich selection of stationary phases has been done in recent years.

The stationary phase in the Reverse Phase chromatographic columns is a hydrophobic support that is consisted mainly of porous particles of silica gel in various shapes (spherical or irregular) at various diameters (1.8, 3, 5, 7, 10 μm etc.) at various pore sizes (such as 60, 100, 120, 300).

The surface of these particles is covered with various chemical entities, such as various hydrocarbons (C1, C6, C4, C8, C18, etc). In most methods C18 columns are used currently to separate medicinal materials, which sometimes are called ODS (octedecylsilane) or RP-18. A polar solvent is used as mobile phase.

The parameters that govern the retention in Reversed Phase systems are the following:

A. The chemical nature of the stationary phase surface.

B. The type of solvents that compose the mobile phase

C. pH and ionic strength of the mobile phase

A. The chemical nature of the stationary phase

The chemical nature is determined by the size and chemistry of hydrocarbon bonded on the silica gel surface, its bonding density (units of μmole/m^2), and the purity and quality of the silica gel support. As a rule, the more carbons in a bonded hydrocarbon the more it retains organic solutes (as long as similar % coverage is compared). The higher the bonding density the longer the organic solutes are retained. A column is considered relatively hydrophobic if its bonding density exceeds 3 μmole/m^2.

Very important modifiers of the stationary phase's surface are surface-active substances used as mobile phase's additives, acting as ion-pair reagents. These are substances such as tri-ethylamine or tetrabutylamine or hexyl, heptyl, octyl sulfonate. They are distributed between the mobile phase and the hydrophobic surface and cover it with either positive (alkylamines) or negative (alkyl sulfonates) charges. This change of the surface into charged surface affects the retention significantly, especially on charged species in the sample.

B. Composition of the mobile phase

As a rule, the weakest solvent in Reverse Phase is the most polar one, water. The other polar organic solvents are considered stronger solvents, where the order of solvent strength follows more

or less their dielectric properties, or polarity. The less polar the solvent added to the mobile phase, the stronger it gets, shortening the retention times.

C. PH and ionic strength of the mobile phase

When the samples contain solutes of ionizable functional groups, such as amines, carboxyls, phosphates, phosphonates, sulfates and sulfonates, it is possible to control their ionization degree with the help of buffers in the mobile phase. As a rule, the change of an ionizable molecule to an ion makes it more polar and less available to the stationary phase.

NORMAL PHASE CHROMATOGRAPHY

In normal phase chromatography, the stationary phase is polar adsorbent. The mobile phase is generally a mixture of non-aqueous solvents. The silica structure is saturated with silanol group at the end in normal phase separations. These OH groups are statistically distributed over the whole of the surface. The silanol groups represent the active sites (very polar) in the stationary phase.

This forms a weak bond with many molecules in the vicinity when any of the following interactions are present. Dipole-induced dipole, dipole-dipole, hydrogen bonding, π-complex bonding. These situations arise when the molecule has one or several atoms with lone pair electrons or a double bond. The adsorption strengths and hence 'K' value (elution series) increase in the following order. Saturated hydrocarbon < olefins < aromatic < organic < halogen compounds < sulphides < ethers < esters < aldehydes and ketones < amines < sulphones < amides < carboxylic acids. The strength of interactions depends not only on the functional groups in the sample molecule but also on stearic factors. If a molecule has several functional groups, then the most polar one determines the reaction properties.

Chemically modified silica, such as aminopropyl, cyanopropyl and diol phases are the stationary phases alternative to silica gel in normal phase chromatography.

The aminopropyl and cyanopropyl phases provide opportunities for specific interactions between the analyte and the stationary phase and thus offer additional options for the optimization of separations. Other advantages of bonded phases lie in the increased homogeneity of the stationary phase surface. Polar modifiers such as acetic acid or triethylamine (TEA) are added to the mobile phase, to deactivate the more polar adsorption sites on the surface of stationary phase, which in turn will improve peak shape as well as the reproducibility of the retention times.

ADSORPTION CHROMATOGRAPHY

The stationary phase is an adsorbent (like silica gel or any other silica based packing) and the separation is based on repeated adsorption-desorption steps.

ION EXCHANGE CHROMATOGRAPHY

Separation is based on the charge-bearing functional groups, anion exchange for sample negative ion, or cation exchange - for sample positive ion. Gradient elution by pH is common.

AFFINITY CHROMATOGRAPHY

Separation is based on a chemical interaction specific to the target species. The more popular reverse phase mode uses a buffer and an added counter-ion of opposite charge to the sample with separation being influenced by pH, ionic strength, temperature, concentration of and type of organic co-solvent(s). Affinity chromatography, common for macromolecules, employs a ligand (biologically active molecule bonded covalently to the solid matrix) which interacts with its homologous antigen (analyte) as a reversible complex that can be eluted by changing buffer conditions.

CHIRAL CHROMATOGRAPHY:

Separation of the enantiomers can be achieved on chiral stationary phases by formation of diastereomers via derivatizing agents or mobile phase additives on a chiral stationary phase. When used as an impurity test method, the sensitivity is enhanced if the enantiomeric impurity elutes before the enantiomeric drug.

ISOCRATIC SEPARATION

In this technique, the same mobile phase combination is used throughout the process of separation. The same polarity or elution strength is maintained the process.

GRADIENT SEPARATION In this technique, a mobile phase combination of lower polarity or elution strength is used followed by gradually increasing the polarity or elution strength.

ANALYTICAL HPLC

In this only analysis of the samples are done. Recovery of the samples for reusing is normally not done, since the samples used are very low.

PREPARATIVE HPLC

Where analysis of the individual fractions of pure compounds can be collected using fraction collector. The collected samples are reused.

ION-PAIR CHROMATOGRAPHY[11]

Ion Pair Chromatography (IPC) is used to separate ionic analytes on a Column. An Ion Pair reagent is added to modulate retention of the ionic analytes. Ion-pair chromatography is commonly used in combination with UV detection, in which case it is referred as reverse phase ion-pair chromatography (RPIPC).

Principle of ion-pairing:

With the aid of ion pair chromatography it is possible to separate the same analytes as in ion exclusion chromatography, but the separation mechanism is completely different. The stationary phases used are completely polar reverse phase materials such as are used in distribution chromatography. A so-called ion pair regent is added to the eluents; this consists of anionic or cationic surfactants such as tetra alkyl ammonium salts or n-alkylsulfonic acids. Together with the oppositely charged analyte ions the ion pair reagents form an uncharged ion pair, which can be retarded at the stationary phase by hydrophobic interactions. Separation is possible because of the formation constants of the ion pairs and their different degrees of adsorption. Figure 1 shows a simplified static ion exchange model in which it is assumed that interactions with the analytes only occur after adsorption of the ion pair reagent at the stationary phase.

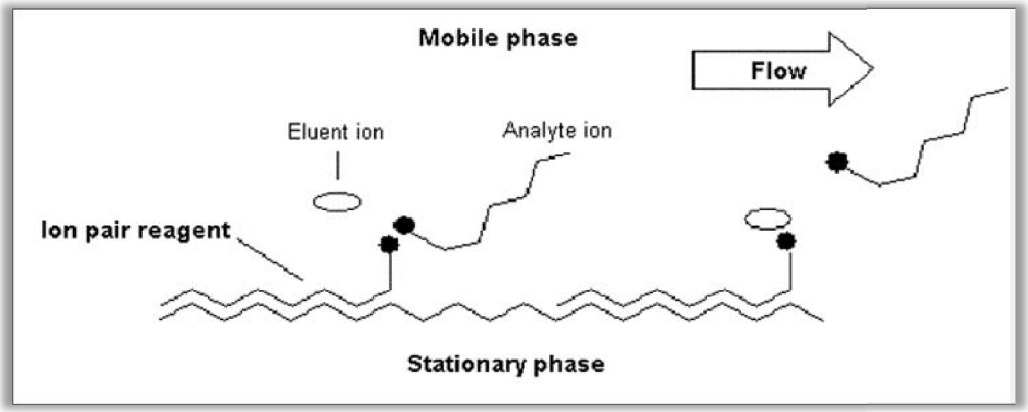


Fig No.1: Ion Pairing

Separation mechanism:

Ion-exchange selectivity is mediated by both the mobile phase and stationary phase. In contrast the selectivity of an ion-pair separation is determined primarily by the mobile phase. The two major components of aqueous mobile phase are the ion-pair reagent and the organic solvent; the type and concentration of each component can be varied to achieve desired separation. The ion-pair reagent is a large ionic molecule that carries a charge opposite to analyte of interest. It usually has both hydrophobic region to interact with the analyte. Stationary phases used for ion-pair are neutral, hydrophobic resins such as polystyrene, divinyl benzene or bonded silica. A single stationary phase can be used for either anion or cation analysis.

Although the retention mechanism of ion-pair chromatography is not fully understood, three major theories have been proposed:

- Ion pair formation
- Dynamic ion exchange
- Ion interaction

In first model, the analyte and ion-pair reagent form a neutral pair, which is then partitions between the mobile phase and stationary phase. Retention can be controlled by varying the concentration of organic solvent in the mobile phase as in reverse phase chromatography.

The dynamic ion-exchange model maintains that the hydrophobic portion of ion-pair reagent adsorbs to the hydrophobic stationary phase to form a dynamic ion-exchange surface. The analyte

is retained on this surface, as it would in classic Ion Chromatography. Using this scenario, solvents used in the mobile phase can be used to impede interaction of ion-pair reagent with the stationary phase, there by altering the capacity of the column.

A third model describes an electrical double layer that is formed when ion-pair reagent permeates the stationary phase, carrying with it an associated counter-ion. Retention of analyte ion in this model is dependent upon a combination of factors including those described in first two models.

Typical IP reagents are divided into two categories

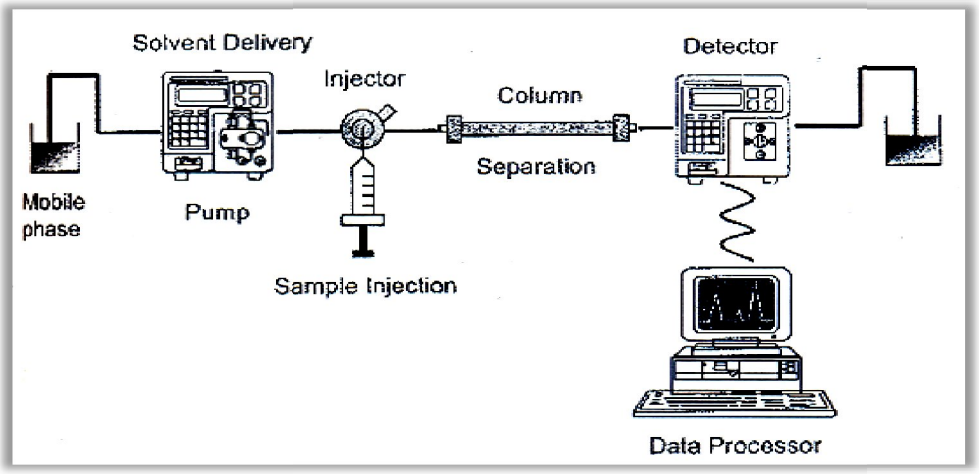
- **Cationic:** These are used for anion analysis. Cationic ion-pair reagents include ammonium and tetra methyl-, tetraethyl-, tetra propyl-, and tetra butyl ammonium.
- **Anionic:** These reagents used for cation analysis. Anionic ion-pair reagents include hydrochloric, perchloric, and perfluorocarboxylic acids and pentane-, hexane-, heptane-, and octane sulfonic acids.

INSTRUMENTATION OF HPLC[4]

The individual components HPLC and their working functions are described below.

- Mobile phase and reservoir
- Solvent degassing system
- Pump
- Injector
- Column
- Detector
- Data system

Fig No.2: Block diagram of HPLC



MOBILE PHASE AND RESERVOIR

The most common type of solvent reservoir is a glass bottle. Most of the manufacturers supply these bottles with special caps, Teflon tubing and filters to connect to the pump inlet and to the purge gas (helium) used to remove dissolved air.

The mobile phase is pumped under pressure from one or several reservoirs and flows through the column at a constant rate. With micro particulate packing, there is a high-pressure drop across a chromatography column. Eluting power of the mobile phase is determined by its overall polarity, the polarity of the stationary phase and the nature of the sample components. For normal phase separations, eluting power increases with increasing polarity of the solvent but for reversed phase separations, eluting power decreases with increasing solvent polarity. Optimum separating conditions can be achieved by making use of mixture of two solvents. Some other properties of the solvents, which need to be considered for a successful separation, are boiling point, viscosity, detector compatibility, flammability and toxicity.

Mobile phases used for HPLC typically are mixtures of organic solvents and water or aqueous buffers. Isocratic methods are preferable to gradient methods. Gradient methods will sometimes be required when the molecules being separated have vastly different partitioning properties. When a gradient elution method is used, care must be taken to ensure that all solvents are miscible.

The following points should also be considered when choosing a mobile phase

It is essential to establish that the drug is stable in the mobile phase for at least the duration of the analysis.

Excessive salt concentrations should be avoided. High salt concentrations can result in precipitation, which can damage HPLC equipment.

The mobile phase should have a pH 2.5 and pH 7.0 to maximize the lifetime of the column.

Reduce cost and toxicity of the mobile phase by using methanol instead of acetonitrile when possible minimize the absorbance of buffer. Since trifluoroacetic acid, acetic acid or formic acid absorb at shorter wavelengths, they may prevent detection of products without chromophores above 220 nm. Carboxylic acid modifiers can be frequently replaced by phosphoric acid, which does not absorb above 200 nm.

Use volatile mobile phases when possible, to facilitate collection of products and LC-MS analysis. Volatile mobile phases include ammonium acetate, ammonium phosphate, formic acid, acetic acid, and trifluoroacetic acid. Some caution is needed as these buffers absorb below 220 nm.

Ionizable compounds in some cases can present some problems when analyzed by reverse phase chromatography. Two modifications of the mobile phase can be useful in reverse phase HPLC for ionizable compounds. One is called ion suppression and other ion pairing chromatography. In both techniques, a buffer is used to ensure that the pH of the solution is constant and usually at least 1.5 pH units from a pKa of the drug to ensure that one form predominates.

If pH is approximately equal to pKa, peak broadening can occur. In ion suppression chromatography, the pH of the aqueous portion of the mobile phase is adjusted to allow the neutral form of the drug to predominate. This ensures that the drug is persistent in only one form and results in improvement of the peak shape and consistency of retention times.

In ion pairing chromatography, the pH of the mobile phase is adjusted so that the drug is completely ionized. If necessary to improve peak shape or lengthen retention time, an alkyl sulfonic acid salt or bulky anion such as trifluoroacetic acid is added to the ion pair to cationic drugs or a quaternary alkyl ammonium salt is added to ion-pair to anionic drugs. Ion pairing chromatography also allows the simultaneous analysis of both neutral and charged compounds.

SOLVENT DEGASSING SYSTEM[12]

The constituents of the mobile phase should be degassed and filtered before use. Several methods are employed to remove the dissolved gases in the mobile phase.

They include heating and stirring, vacuum degassing with an aspirator, filtration through 0.45 filter, vacuum degassing with an air-soluble membrane, helium purging ultra signification or purging or combination of these methods. Helium purging and storage of the solvent under helium is not sufficient for degassing aqueous solvents. It is useful to apply a vacuum for 5-10 min and then keep the solvent under a helium atmosphere.

HPLC systems are also provided an online degassing system, which continuously removes the dissolved gases from the mobile phase.

PUMP:

High pressure pumps are needed to force solvents through packed stationary phase beds. Smaller bed particles require higher pressures. There are many advantages to using smaller particles, but they may not be essential for all separations.

The most important advantages are: higher resolution, faster analyses, and increased sample load capacity. However, only the most demanding separations require these advances in significant amounts.

Many separation problems can be resolved with larger particle pickings that require less pressure. Flow rate stability is another important pump feature that distinguishes pumps. Very stable flow rates are usually not essential for analytical chromatography.

However, if the user plans to use a system in size exclusion mode, then there must be a pump which provides an extremely stable flow rate. An additional feature found on the more elaborate pumps is external electronic control. Although it adds to the expense of the pump, external electronic control is a very desirable feature when automation or electronically controlled gradients are to be run.

Alternatively, this becomes an undesirable feature (since it is an unnecessary expense) when using isocratic methods. The degree of flow control also varies with pump expense. More expensive pumps include such state of-the-art technology as electronic feedback and multi headed configurations.

It is desirable to have an integrated degassing system, either helium purging, or membrane filtering.

INJECTOR

Sample introduction can be accomplished in various ways. The simplest method is to use an injection valve.

In more sophisticated LC systems, automatic sampling devices are incorporated where the sample is introduced with the help of auto samplers and microprocessors. In liquid chromatography, liquid samples may be injected directly and solid samples need only be dissolved in an appropriate solvent.

The solvent need not be the mobile phase, but frequently it is judiciously chosen to avoid detector interference, column/component interference, and loss in efficiency or all of these.

It is always best to remove particles from the sample by filtering over a 5 μm filter, or centrifuging, since continuous injections of particulate material will eventually cause blockages in injection devices or columns. Sample sizes may vary widely.

The availability of highly sensitive detectors frequently allows use of the small samples which yield the highest column performance. Sample introduction techniques can be used with a syringe or an injection valve.

COLUMN

The heart of the system is the column. The choice of common packing material and mobile phases depends on the physical properties of the drug.

Many different reverse phase columns will provide excellent specificity for any particular separation. It is therefore best to routinely attempt separations with a standard C_8 or C_{18} column and determine if it provides good separations. If this column does not provide good separation or the mobile phase is unsatisfactory, alternate methods or columns should be explored.

Reverse phase columns differ by the carbon chain length, degree of end capping and percent carbon loading. Diol, cyano and amino groups can also be used for reverse phase chromatography.

Typical HPLC columns are 5, 10, 15 and 25 cm in length and are filled with small diameter (3, 5 or 10 μm) particles. The internal diameter of the columns is usually 4.6 mm; this is considered the best compromise for sample capacity, mobile phase consumption, speed and resolution. However, if pure substances are to be collected (preparative scale), then larger diameter columns may be needed. Packing the column tubing with small diameter particles requires high skill and specialized equipment.

For this reason, it is generally recommended that all but the most experienced chromatographers purchase prepacked columns, since it is difficult to match the high performance of professionally packed LC columns without a large investment in time and equipment.

In general, LC columns are fairly durable and one can expect a long service life unless they are used in some manner which is intrinsically destructive, as for example, with highly acidic or basic eluents, or with continual injections of 'dirty' biological or crude samples.

It is wise to inject some test mixture (under fixed conditions) into a column when new, and to retain the chromatogram. If questionable results are obtained later, the test mixture can be injected again under specified conditions.

The two chromatograms may be compared to establish whether or not the column is still useful.

DETECTOR

Today, optical detectors are used most frequently in liquid chromatographic systems. These detectors pass a beam of light through the flowing column effluent as it passes through a low Volume (~ 10µl) flow cell.

The variations in light intensity caused by UV absorption, fluorescence emission or change in refractive index, from the sample components passing through the cell, are monitored as changes in the output voltage.

These voltage changes are recorded on a strip chart recorder and frequently are fed into a computer to provide retention time and peak area data. The most commonly used detector in LC is the ultraviolet absorption detector.

A variable wavelength detector of this type, capable of monitoring from 190 to 400 nm, will be found suitable for the detection of the majority samples.

Other detectors in common use include: Photo Diode Array UV detector (PDA), refractive index (RI), fluorescence (FLU), electrochemical (EC).
The RI detector is universal but also the less sensitive one. FLU and EC detectors are quite sensitive (up to 10-15 pmole) but also quite selective.

DATA SYSTEM

Since the detector signal is electronic, using modern data collection techniques can aid the signal analysis. In addition, some systems can store data in a retrievable form for highly sophisticated computer analysis at a later time.

The main goal in using electronic data systems is to increase analysis accuracy and precision, while reducing operator attention.

There are several types of data systems, each differing in terms of available features. In routine analysis, where no automation (in terms of data management or process control) is needed, a pre-programmed computing integrator may be sufficient.

If higher control levels are desired, a more intelligent device is necessary, such as a data station or minicomputer. The advantages of intelligent processors in chromatographs are found in several areas.

First, additional automation options become easier to implement. Secondly, complex data analysis becomes more feasible. These analysis options include such features as run parameter optimization and deconvolution (i.e. resolution) of overlapping peaks.

Finally, software safeguards can be designed to reduce accidental misuse of the system.

HPLC THEORY[5,6]:

System Suitability Parameters

High performance liquid chromatography is defined as a separation of mixtures of compounds due to differences in their distribution equilibrium between two phases, the stationary phase packed inside columns and the mobile phase, delivered through the columns by high pressure pumps.

Components whose distribution into the stationary phase is higher, are retained longer, and get separated from those with lower distribution into the stationary phase. The theoretical and practical foundations of this method were laid down at the end of 1960s and at the beginning of 1970s.

The theory of chromatography has been used as the basis for System- Suitability tests, which are set of quantitative criteria that test the suitability of the chromatographic system to identify and quantify drug related samples by HPLC at any step of the pharmaceutical analysis.

Retention Time (t_R), Capacity Factor k' & Relative Retention Time (RRT)

The time elapsed between the injection of the sample components into the column and their detection is known as the Retention Time (t_R).

The retention time is longer when the solute has higher affinity to the stationary phase due to its chemical nature. For example, in reverse phase chromatography, the more lypophilic compounds are retained longer.

Therefore, the retention time is a property of the analyte that can be used for its identification. A non retained substance passes through the column at a time t_0, called the Void Time.

Retention factor is calculated as follows:

$$k' = \frac{t_R - t_0}{t_o}$$

The Capacity Factor describes the thermodynamic basis of the separation and its definition is the ratio of the amounts of the solute at the stationary and mobile phases within the analyte band inside the chromatographic column:

$$k' = \frac{C_s}{C_m} \Phi$$

Where C_s is the concentration of the solute at the stationary phase and C_m is its concentration at the mobile phase and phi is the ratio of the stationary and mobile phase volumes all within the chromatographic band.

The Retention Factor is used to compare the retention of a solute between two chromatographic systems, normalizing it to the column's geometry and system flow rate.

The retention factor value should be in between 1-20. The need to determine the void time can be tricky sometimes, due to the instability of the elution time of the void time marker, t_0, therefore, when the chromatogram is complex in nature, and one known component is always present at a certain retention time, it can be used as a retention marker for other peaks. In such cases the ratio

between the retention time of any peak in the chromatogram and the retention time of the marker is used ($t_{R\ (Peak)} / t_{R\ (Marker)}$) and referred to as the Relative Retention Time (RRT).

RRT is also used instead of the capacity ratio for the identification of the analyte as well as to compare its extent of retention in two different chromatographic systems.

The sharpness of a peak relative to its retention time is a measure of the system's efficiency, calculated as N, plate count. Band-broadening phenomena in the column such as eddy diffusion, molecular diffusion, and mass-transfer kinetics and extra-column effects reduce the efficiency of the separation.

The sharpness of a peak is relevant to the limit of detection and limit of quantification of the chromatographic system. The sharper the peak for a specific area, the better is its signal-to-noise; hence the system is capable of detecting lower concentrations.

Therefore, the efficiency of the chromatographic system must be established by the system suitability test before the analysis of low concentrations that requires high sensitivity of the system, such as the analysis of drug impurities and degradation products.

Efficiency: Plate Count N and Peak Capacity P_c

The efficiency of the separation is determined by the Plate Count N when working at isocratic conditions, whereas it is usually measured by Peak Capacity P_c when working at gradient conditions.

The following equation for the plate count is used by the United States Pharmacopoeia (USP) to calculate N:

$$N = 16 \times \left(\frac{t_R}{w_{(Baseline)}}\right)^2$$

Where w is measured from the baseline peak width calculated using lines tangent to the peak width at 50 % height. European and Japanese Pharmacopoeias use the peak width at 50% of the peak height, hence the equation becomes:

$$N = 5.54 \times \left(\frac{t_R}{w_{(50\%)}}\right)^2$$

Peak Capacity P_c is defined as number of peaks that can be separated within a retention window for a specific pre-determined resolution. In other words, it is the runtime measured in peak width units (34). It is assumed that peaks occur over the gradient chromatogram.

Therefore, Peak Capacity can be calculated from the peak widths $\underline{w}$ in the chromatogram as follows:

$$1+\frac{t_g}{(1/n)\sum_1^n w}$$

Where n is the number of peaks at the segment of the gradient selected for the calculation, t_g. Thus peak capacity can be simply the gradient run time divided by the average peak width. The sharper the peaks the higher is the peak capacity, hence the system should be able to resolve more peaks at the selected run time as well as detect lower concentrations.

Another measure of the column's chromatographic efficiency is the Height Equivalent to Theoretical Plate (HETP) which is calculated from the following equation:

HETP = (L/N)

Where L is column length and N is the plate count. HETP is measured in micrometer.

The behavior of HETP as function of linear velocity has been described by various equations. It is frequently called "The Van-Deemter curve", and it is frequently used to describe and characterize various chromatographic stationary phases' performance and compare them to each other.

The lower are the values of HETP, the more efficient is the chromatographic system, enabling the detection of lower concentrations due to the enhanced signal-to-noise ratio of all the peaks in the chromatogram.

Peak Asymmetry Factor A_f and Tailing Factor T

The chromatographic peak is assumed to have a Gaussian shape under ideal conditions, describing normal distribution of the velocity of the molecules populating the peak zone migrating through the stationary phase inside the column.

Any deviation from the normal distribution indicates non-ideality of the distribution and the migration process therefore might jeopardize the integrity of the peak's integration, reducing the accuracy of the quantification. This is the reason why USP Tailing is a peak's parameter almost always measured in the system suitability step of the analysis.

The deviation from symmetry is measured by the Asymmetry Factor, A_f or Tailing Factor T. The calculation of Asymmetry Factor, A_f is described by the following equation:

$$A_f = \frac{A_{(10\%h)}}{B_{(10\%h)}}$$

Where A and B are sections in the horizontal line parallel to the baseline, drawn at 10% of the peak height

The calculation of Tailing Factor, T, which is more widely used in the pharmaceutical industry, as suggested by the pharmacopeias, is described by the following equation:

$$T = \frac{A_{(5\%h)} + B_{(5\%h)}}{2A_{(5\%h)}}$$

Where A and B are sections in the horizontal line parallel to the baseline, drawn at 5% of the peak height. The USP suggests that Tailing Factor should be in the range of 0.5 up to 2 to assure a precise and accurate quantitative measurement.

Selectivity Factor: Alfa and Resolution Factor Rs

The separation is a function of the thermodynamics of the system. Substances are separated in a chromatographic column when their rate of migration differs, due to their different distribution between the stationary and mobile phases.

The Selectivity Factor, α, and Resolution Factor, Rs, measure the extent of separation between two adjacent peaks. The Selectivity Factor accounts only for the ratio of the Retention Factors, k', of the two peaks (k'_2/k'_1), whereas the Resolution Factor, Rs, accounts for the difference between the retention times of the two peaks relative to their width

The equation that describes the experimental measurement of the Resolution Factor, Rs, is as follows:

$$Rs = \frac{t_{R(2)} - t_{R(1)}}{0.5(w_2 + w_1)}$$

Where t_R is the retention time of peaks 1 and 2 respectively and w is their respective peak width at the tangents' baseline. According to the pharmacopeias Rs should be above 1.5 for an accurate quantitative measurement.

The resolution is a critical value when working with complex samples such as drug impurities and degradation products, or when the formulation is complex and excipients might interfere with the quantitative measurements. Therefore, it is an essential part of the system suitability measurement stage before the quantitative work of these types of samples.

The sample used for the measurements of Rs during the system suitability runs is sometimes called Resolution Solution, It usually contains the components that are the most difficult to resolve.

The theoretical description of the Resolution Factor Rs equation is shown in Equation. It includes some of the above parameters, the plate count N, the selectivity α and the average of the two peaks' capacity factors k':

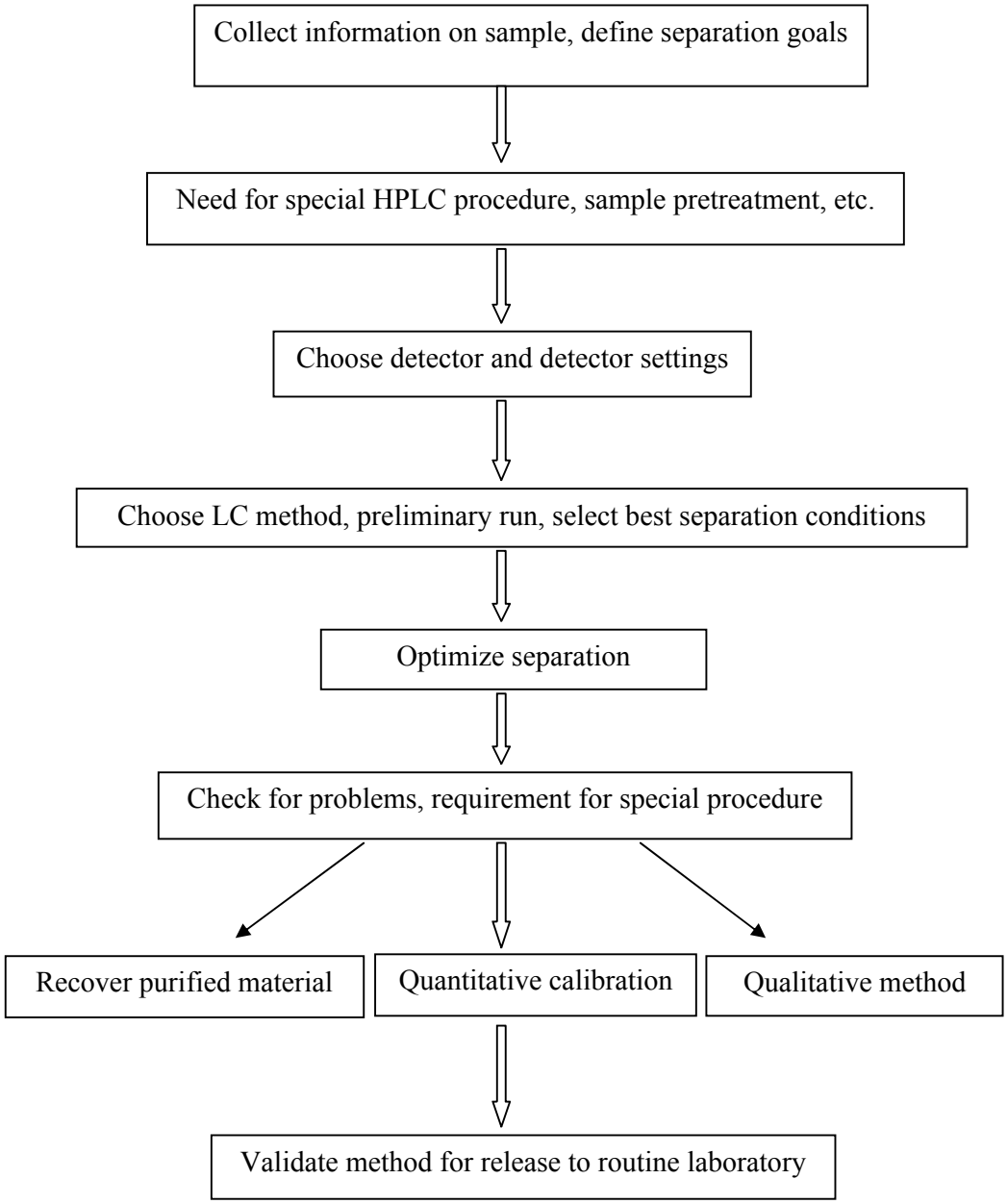
$$Rs = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_{ave}'}{k_{ave}'+1}\right)$$

It can be clearly seen from this equation that the plate count is the most effecting parameter in the increase of the chromatographic resolution. Since the plate count increases with the reduction in particle diameter, it explains the reduction in particle diameter of the stationary phase material during the last 3 decades of HPLC.

This is also the rationale behind the recent trend in HPLC, the use of sub 2 micron particle columns and the development of a specially design of ultra performance HPLC systems to accommodate such columns.

1.2 ANALYTICAL METHOD DEVELOPMENT[7]

Method development has following steps:



A good method development strategy should require only as many experimental runs as are necessary to achieve the desired final result. Finally method development should be as simple as possible, and it should allow the use of sophisticated tools such as computer modeling.

The important factors, which are to be taken into account to obtain reliable quantitative analysis, are:

1. Careful sampling and sample preparation.
2. Appropriate choice of the column.
3. Choice of the operating conditions to obtain the adequate resolution of the mixture.
4. Reliable performance of the recording and data handling systems.
5. Suitable integration/peak height measurement technique.
6. The mode of calculation best suited for the purpose
7. Validation of the developed method.

Sample preparation

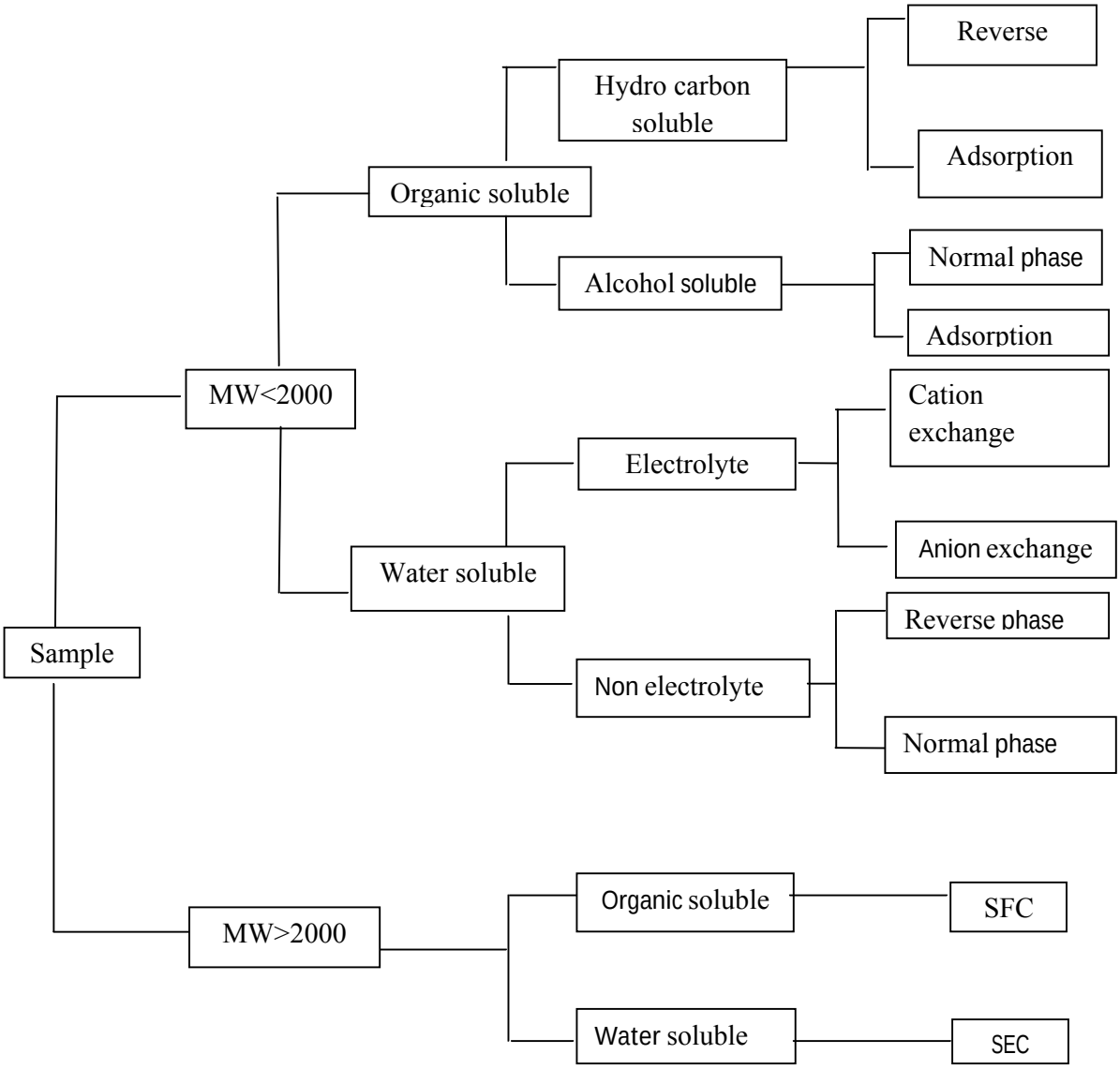
Samples come in various forms:

- Solutions ready for injection
- Solutions that require dilution, buffering, addition of an internal standard or other volumetric manipulation
- Solids must be dissolved or extracted
- Samples that require pretreatment to remove interferences and/or protect the column or equipment from damage.

Most samples for HPLC analysis require weighing and /or volumetric dilution before injection. Best results are often obtained when the composition of the sample solvent is close to that of the mobile phase since this minimizes baseline upset and other problems. Some samples require a partial separation (pretreatment) prior to HPLC, because of need to remove interferences, concentrate sample analytes or eliminate “column killers”.

SELECTING HPLC METHOD AND INITIAL CONDITIONS:

The mode of HPLC can be selected based on the following chart:



In many cases the development of an adequate sample pretreatment can be challenging for achieving a good HPLC separation. The samples may be of two types, regular or special. The regular samples are typical mixtures of small molecules (<2000Da) that can be separated by normal starting conditions.

Table No.1: Goals in method development[8]

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

OPTIMIZATION OF HPLC METHOD

During the optimization stage, the initial sets of conditions that have evolved from the first stages of development are improved or maximized in terms of resolution and peak shape, plate counts, asymmetry, capacity factor, elution time, detection limits, limit of quantification and overall ability to quantify the specific analyte of interest.

Optimization of a method can follow either of two general approaches:

- Manual
- Computer driven

The manual approach involves varying one experimental variable at a time, while holding all other constant and recording changes in response. The variables might include flow rate, mobile or stationary phase composition, temperature, detection wavelength and pH.

This univariate approach to system is slow, time consuming and potentially expensive. However, it may provide a much better understanding of the principles and theory involved and of interactions of the variables. In the second approach, computer driven automated method development, efficiency is optimized while experimental input is minimized. Computer driven automated approaches can be applied to many applications. In addition, they are capable of significantly reducing the time, energy and cost of all instrumental method development.

The various parameters that include to be optimized during method development are

- Selection of mode of separation.
- Selection of stationary phase.
- Selection of mobile phase.
- Selection of detector.

Selection of Mode of Separation

In reverse phase mode, the mobile phase is comparatively more polar than the stationary phase. For the separation of polar or moderately polar compounds, the most preferred mode is reverse phase.

The nature of the analyte is the primary factor in the selection of the mode of separation. A second factor is the nature of the matrix.

A useful and practical measurement of peak shape is peak asymmetry factor and peak tailing factor. Peak asymmetry is measured at 10% of full peak height and peak tailing factor at 5%. Reproducibility of retention times and capacity factor is important for developing a rugged and repeatable method.

Buffers if any and its Strength

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

- Phosphate buffers prepared using salts like KH_2PO_4, K_2HPO_4, NaH_2PO_4, Na_2HPO_4 etc.
- Phosphoric acid buffers prepared using H_3PO_4.
- Acetate buffers-Ammonium acetate, Sodium acetate etc.
- Acetic acid buffers prepared using CH_3COOH.

The retention also depends on the molar strengths of the buffer-Molar strength is increasingly proportional to retention times. The strength of the buffer can be increased, if necessary to achieve the required separations. The solvent strength is a measure of its ability to pull analyte from the column. It is generally controlled by the concentration of the solvent with the highest strength.

It is important to maintain the pH of the mobile phase in the range of 2.0 to 8.0 as most columns does not withstand to the pH which are outside this range. This is due to the fact that the siloxane linkages are cleaved below pH 2.0, while pH values above 8.0, silica may dissolve.

Mobile Phase Composition

Most chromatographic separations can be achieved by choosing the optimum mobile phase composition. This is due to the fact that fairly large amount of selectivity can be achieved by choosing the qualitative and quantitative composition of aqueous and organic portions.

Most widely used solvents in reverse phase chromatography are Methanol and Acetonitrile. Experiments should be conducted with mobile phases having buffers with different pH and different organic phases to check for the best separations of analyte peak.
A mobile phase which gives separation of analyte peak and which is rugged for variation of both aqueous and organic phase by at least ± 0.2% of the selected mobile phase composition should be used.

Selection of Detector

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

- High sensitivity facilitating trace analysis
- Negligible baseline noise to facilitate lower detection
- Large linear dynamic range.
- Low dead volume
- Inexpensive to purchase and operate

Pharmaceutical ingredients do not absorb all UV light equally, so that selection of detection wavelength is important. An understanding of the UV light absorptive properties of the organic impurities and the active pharmaceutical ingredient is very helpful.

For the greatest sensitivity λmax should be used. Ultra violet wavelengths below 200nm should be avoided because detector noise increases in this region. Higher wavelengths give greater selectivity.

Performance Calculations

Carrying out system suitability experiment does the performance calculations. System suitability experiments can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision.

The requirements for system suitability are usually developed after method development and validations have been completed. The criteria selected will be based on the actual performance of the method as determined during its validation. For example, if sample retention times form part of the system suitability criteria, their variation SD can be determined during validation.

System suitability might then require that retention times fall within a ±3 SD range during routine performance of the method.

The USP (2000) defines parameters that can be used to determine system suitability prior to analysis. These parameters include plate number (n), tailing factor (T), resolution (R_S) and relative standard deviation (RSD) of peak height or peak area for respective injections.

The RSD of peak height or area of five injections of a standard solution is normally accepted as one of the standard criteria. For assay method of a major component, the RSD should typically be less than 1% for these five respective injections.

The plate number and/ or tailing factor are used if the run contains only one peak. For chromatographic separations with more than one peak, such as an internal standard assay or an impurity method expected to contain many peaks, some measure of separations such as R_S is recommended. Reproducibility of t_R or k value for a specific compound also defines system performance.

The column performance can be defined in terms of column plate number. As the plate count is more the column is more efficient.

1.3 ANALYTICAL METHOD VALIDATION[9,10]

Method validation can be defined as (ICH*)* "Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics".

Method validation is an integral part of the method development; it is the process by which a method is tested by the developer or user for reliability, accuracy and preciseness of its intended purpose and demonstrating that analytical procedures are suitable for their intended use that they support the identity, quality, purity, and potency of the drug substances and drug products.
Data thus generated become part of the methods validation package submitted to Center for Drug Evaluation and Research (CDER). Simply, method validation is the process of proving that an analytical method is acceptable for its intended purpose.

All the variables of the method should be considered, including sampling procedure, sample preparation, chromatographic separation, and detection and data evaluation. For chromatographic methods used in analytical applications there is more consistency in validation practice with key analytical parameters includes namely

- System suitability
- Specificity
- Accuracy
- Precision
- Linearity
- Limit of Detection
- Limit of Quantification
- Ruggedness
- Robustness

SYSTEM SUITABILITY

According to the USP, system suitability tests are an integral part of chromatographic methods. These tests are used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. System suitability tests are based on the concept that the equipment,

electronics, analytical operations, and samples constitute an integral system that can be evaluated as a whole.

The purpose of the system suitability test is to ensure that the complete testing system (including instrument, reagents, columns, analysts) is suitable for the intended application.

Similar to the analytical method development, the system suitability test Strategy should be revised as the analysts develop more experience with the assay. In general, consistency of system performance. (E.g.: Replicate injections of the standard) and chromatographic suitability.(Eg: Tailing factor, column efficiency and resolution of the critical pair) are the main components of system suitability.

During the early stage of the method development process some of the more sophisticated system suitability tests may not be practical due to the lack of experience with the method. In this stage, usually a more "generic" approach is used. For example, evaluation of the tailing factor to check chromatographic suitability, and replicate injections of the system suitability solution to check injection precision may be sufficient for an HPLC impurities assay. As the method matures more experience is acquired for this method, a more sophisticated system suitability test may be necessary.

System suitability is the checking of a system to ensure system performance before or during the analysis of unknowns.

Parameters such as plate count, tailing factors; resolution and reproducibility (%RSD retention time and area for six repetitions) are determined and compared against the specifications set for the method. These parameters are measured during the analysis of system suitability "sample" that is a mixture of main components and expected by-products

Table No.2: System suitability parameters and recommendations

Parameter	Recommendation
Capacity Factor (k')	The peak should be well-resolved from other peaks and the void volume, generally k' 1 to 20
Repeatability	RSD $\leq$ 1% for N $\geq$ 5 is desirable.
Relative retention	Not essential as long as the resolution is stated.
Resolution (R_s)	R_s of > 2 between the peak of interest and the closest eluting potential interfering (impurity, excipient, degradation product, internal standard, etc.
Tailing Factor (T)	T of >0.5 and $\leq$ 2
Theoretical Plates (N)	N > 3000

SPECIFICITY/SELECTIVITY

The terms selectivity and specificity are often used interchangeably. According to ICH, the term specific generally refers to a method that produces a response for a single analyte only while the term selectivity refers to a method that provides responses for a number of chemical entities that may or may not be distinguished from each other. If the response is distinguished from all other responses, the method is said to be selective. Since there are very few methods that respond to only one analyte, the term selectivity is usually more appropriate.

Specificity is the ability of a method to discriminate between the analyte(s) of interest and other components that are present in the sample. Studies are designed to evaluate the degree of interference, if any, which can be attributed to other analytes, impurities, degradation products, reagent "blanks" and excipients. This provides the analyst with a degree of certainty that the

response observed is due to the single analyte of interest. The degree of specificity testing varies depending on the method type and the stage of validation. Specificity should be evaluated continually through the drug development process. Specificity is sometimes used interchangeably with the term "selectivity". The argument over which term is more correct is one of semantics. Although there is some dissention, the term "specificity" has been adopted by the regulatory guidance documents and should be used to prevent further confusion.

Non-Interference of Placebo

This portion of specificity evaluation applies to the finished drug product only. Excipients present in the formulation should be evaluated and must not interfere with the detection of the analyte. Individual solutions of each excipient prepared at several times the normal concentration of the component in the drug product ensure that any detector response from the excipient will be readily visible. Injecting individual solutions of each excipient into the HPLC system in comparison with a standard solution of the analyte is one means of performing this experiment. The absence of a peak eluting at the retention time of the active ingredient is sufficient to demonstrate specificity for excipients.

Challenge Study

Injecting solutions of known process impurities, degradation products, intermediates, homologues, dimers, etc. further challenges the specificity of a method. Identification of these compounds may require an extensive search in order to identify all possible species that may be present in the sample. For new chemical entities (NCE), this information may not be readily available. Probable suspects should be identified by careful review of the synthetic route and manufacturing process to identify any likely species that may be present in the sample.

DEGRADATION STUDIES

Degradation studies involve exposing the sample to a variety of stressed conditions to further evaluate the specificity of degradation products. In this study, the drug substance, drug product, and the combined excipients (or placebos) are each exposed to the stressed conditions.

These may include, but are not limited to, heat, light, acidic media, alkaline media, and oxidative environments. Other conditions may be used depending on the nature and chemistry of the test subject.

Forced degradation is usually evaluated with not more than 20% degradation of the drug substance, although more may be acceptable depending on the particular properties of the drug. A reasonable effort should be made to degrade samples in order to identify possible degradation products. If the planned experiments do not show any appreciable degradation, the strength and/or exposure time of the stress condition may be increased, but degradation is not required for every condition studied. There is a point beyond which the stress condition becomes extreme and unrealistic. Sound scientific judgment should be used to determine the extent and degree of degradation studies.

ACCURACY

Accuracy is the measure of how close the experimental value is to the true value. Accuracy should be established across the specified range of the analytical procedure.

Assay:

Drug Substance

Several methods of determining accuracy are available:

a) Application of an analytical procedure to an analyte of known purity (e.g. reference material);
b) Comparison of the results of the proposed analytical procedure with those of a second well-characterized procedure, the accuracy of which is stated and/or defined.
c) Accuracy may be inferred once precision, linearity and specificity have been established.

Drug Product

Several methods for determining accuracy are available:

a. Application of the analytical procedure to synthetic mixtures of the drug product components to which known quantities of the drug substance to be analyzed have been added.

b. In cases where it is impossible to obtain samples of all drug product components, it may be acceptable either to add known quantities of the analyte to the drug product or to compare the results obtained from a second, well Characterized procedure, the accuracy of which is stated and/or defined.

c. Accuracy may be inferred once precision, linearity and specificity have been established.

Impurities (Quantification)

Accuracy should be assessed on samples (drug substance/drug product) spiked with known amounts of impurities. In cases where it is impossible to obtain samples of certain impurities and/or degradation products, it is considered acceptable to compare results obtained by an independent procedure. The response factor of the drug substance can be used. It should be clear how the individual or total impurities are to be determined e.g., weight/weight or area percent, in all cases with respect to the major analyte.

Recommendations

Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g. 3 concentrations / 3 replicates each of the total analytical procedure). Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals.

PRECISION

Precision is the measure of how close the data values are to each other for a number of measurements under the same analytical conditions. ICH has defined precision to contain three components: repeatability, intermediate precision and reproducibility. Ruggedness as defined in USP XXII <1225>, 1990 incorporates the concepts described under the terms "intermediate precision", "reproducibility" and "robustness" of this guide.

Repeatability

(i) Injection Repeatability

Sensitivity is the ability to detect small changes in the concentration of the analyte in the sample. Sensitivity can be partially controlled by monitoring the specification for injection reproducibility (system suitability testing).

The sensitivity or precision as measured by multiple injections of a homogeneous sample (prepared solution) indicates the performance of the HPLC instrument under the chromatographic conditions and day tested.

The information is provided as part of the validation data and as a system suitability test. The specification, as the coefficient of variation in % or relative standard deviation (RSD), set here will determine the variation limit of the analysis. The tighter the value, the more precise or sensitive to variation one can expect the results. This assumes that the chromatograph does not malfunction after the system suitability testing has been performed. Keep in mind, however, that it does not consider variations due to the drug product manufacturing and laboratory sample preparation procedures. The set of four duplicate samples were injected sequentially. Variations in peak area and drift of retention times are noted.

Precision refers to the reproducibility of measurement within a set, that is, to the scatter of dispersion of a set about its central value. The term 'set' is defined as referring to a number (N) of independent replicate measurements of some property. One of the most common statistical terms employed is the standard deviation of a population of observation. Standard deviation is the square root of the sum of squares of deviations of individual results for the mean, divided by one less than the number of results in the set. The standard deviation S, is given by

$$s = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - \overline{x})^2}\,,$$

Standard deviation has the same units as the property being measured.

The square of standard deviation is called variance (S^2). Relative standard deviation is the standard deviation expressed as a fraction of the mean, i.e., S/x. It is sometimes multiplied by 100 and expressed as a percent relative standard deviation. It becomes a more reliable expression of precision.

% Relative standard deviation = S x 100 / x

Recommendations

As part of method validation, a minimum of 10 injections with an RSD of 2% is recommended. With the methods for release and stability studies, an RSD of 2% for precision of the system suitability tests for at least five injections (n=5) for the active drug either in drug substance or drug product is desirable. For low-level impurities, higher variations may be acceptable.

(ii) Analysis Repeatability

Determination, expressed as the RSD, consists of multiple measurements of a sample by the same analyst under the same analytical conditions.

For practical purpose, it is often combined with accuracy and carried out as a single study.

(iii) Intermediate Precision

Intermediate precision was previously known as part of ruggedness. The attribute evaluates the reliability of the method in a different environment other than that used during development of the method. The objective is to ensure that the method will provide the same results when similar samples are analyzed once the method development phase is over. Depending on time and resources, the method can be tested on multiple days, analysts, instruments, etc.

Intermediate precision in the test method can be partly assured by good system suitability specifications. Thus, it is important to set tight, but realistic, system suitability specifications.

LINEARITY

The linearity of a method is its ability to obtain test results that are directly proportional to the analyte concentration over a given range. For HPLC methods, the relationship between analyte concentration and detector response (peak area or height) is used to make this determination.

Concentration Ranges

The concentration range used for linearity should be large enough to encompass the desired range of the method. A minimum of five concentration ranges should be investigated and a plot of the detector response vs. the sample concentration should be generated. It is important that the concentration ranges selected for the linearity study are relatively equally spaced throughout the range of the method (e.g., 50%, 75%, 100%, 125% and 150%), and not clustered, as this will provide a skewed estimation of linearity.

Acceptance Criteria

Acceptance criteria should be evaluated to ensure that they are meaningful when compared with the performance of the method. Table 6 gives a list of suggested acceptance criteria for use in evaluating method linearity. The ranges in Table 6 are suggestions only and should be adjusted to ensure that all specification limits are within the validated linear range for any given method. Under most circumstances, regression coefficient (r) is 0.999. Intercept and slope should be indicated.

Statistical Analysis

Linearity data should be evaluated using appropriate statistical methods. A simple regression line of the detector response vs. the analyte concentration is the most common means of evaluation. Regulatory agencies require the submission of the correlation coefficient, y-intercept, slope of the regression line, and the residual sum of squares for linearity evaluation.

A graphical representation of the linearity data should also be generated. Additional analysis of the deviation of the actual values from the regression line is suggested, especially when the method uses a single-point calibration standard. The percent y-intercept is calculated by dividing the y-intercept by the detector response at the nominal concentration expressed as a percentage. For single-point calibration, this value should be less than 1-2% to ensure accurate results.

Table No.3: Statistical analysis

Test	Level	Range	Acceptance criteria
Assay	5	50% to 150%	R> 0.999,
Dissolution	5-8	10% to 150%	R > 0.99,
Impurity	5	LOQ to 2%	R > 0.98

LIMIT OF DETECTION

These limits are normally applied to related substances in the drug substance or drug product. Specifications on these limits are submitted with the regulatory impurities method relating to release and stability of both drug substance and drug product.Limit of detection is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantified, under the stated experimental conditions. With UV detectors, it is difficult to assure the detection precision of low-level compounds due to potential gradual loss of sensitivity of detector lamps with age, or noise level variation by detector manufacturer.

At low levels, assurance is needed that the detection and quantification limits are achievable with the test method each time. With no reference standard for a given impurity or means to assure detectability, extraneous peak(s) could "disappear/appear." A crude method to evaluate the feasibility of the extraneous peak detection is to use the percentage claimed for detection limit from the area counts of the analyte. Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental.

Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

Based on Signal-to-Noise

This approach can only be applied to analytical procedures which exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

Based on the Standard Deviation of the Response and the Slope

The detection limit (DL) may be expressed as:

$$DL = \frac{3.3\ \sigma}{S}$$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of s may be carried out in a variety of ways, for example

Based on the Standard Deviation of the Blank

Analyzing an appropriate number of blank samples and calculating the standard deviation of these responses perform measurement of the magnitude of analytical background response.

Based on the Calibration Curve

A specific calibration curve should be studied using samples containing an analyte in the range of DL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

Recommendations

The detection limit and the method used for determining the detection limit should be presented. If DL is determined based on visual evaluation or based on signal to noise ratio, the presentation of the relevant chromatograms is considered acceptable for justification.

In cases where an estimated value for the detection limit is obtained by calculation or extrapolation, this estimate may subsequently be validated by the independent analysis of a suitable number of samples known to be near or prepared at the detection limit.

LIMIT OF QUANTIFICATION

Limit of quantification is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. Several

approaches for determining the quantification limit are possible, depending on whether the procedure is a non-instrumental or instrumental.

Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. The quantification limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

Based on Signal-to-Noise Approach

This approach can only be applied to analytical procedures that exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

Based on the Standard Deviation of the Response and the Slope

The quantification limit (QL) may be expressed as:

$$QL = \frac{10\,\sigma}{S}$$

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of s may be carried out in a variety of ways.

Based on Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

Based on the Calibration Curve

A specific calibration curve should be studied using samples, containing an analyte in the range of QL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

Recommendations

The quantification limit and the method used for determining the quantification limit should be presented. The limit should be subsequently validated by the analysis of a suitable number of samples known to be near or prepared at the quantification limit. Otherwise the information that is expressed as % area or height of the drug substance peak from the same HPLC chromatogram will be biased. It should also be noted that the extraneous peak using area count does not consider the detection response that depends on the UV extinction coefficient or absorptivity of the compound.

RUGGEDNESS

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as different laboratories, analysts, instruments, reagents, elapsed assay times, assay temperatures, or days. It is normally expressed as the lack of influence on test results of operational and environmental variables of the analytical method.

Method Ruggedness is defined as the reproducibility of results when the method is performed under actual use conditions. Method ruggedness may not be known when a method is first developed, but insight is obtained during subsequent use of that method.

Recommendations

The ruggedness of an analytical method is determined by analysis of aliquots from homogeneous lots in different laboratories, by different analysts, using operational and environmental conditions that may differ but are still within the specified parameters of the assay.

The degree of reproducibility of test results is then determined as a function of the assay variables. This reproducibility may be compared to the precision of the assay under normal conditions to obtain a measure of the ruggedness of the method.

ROBUSTNESS

ICH defines robustness as a measure of the method's capability to remain unaffected by small, but deliberate variations in method parameters. Robustness can be partly assured by good system suitability specifications. The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters.

If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness should be that a series of system suitability parameters

(e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used.

Examples of typical variations are:

- Stability of analytical solutions
- Extraction time

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

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

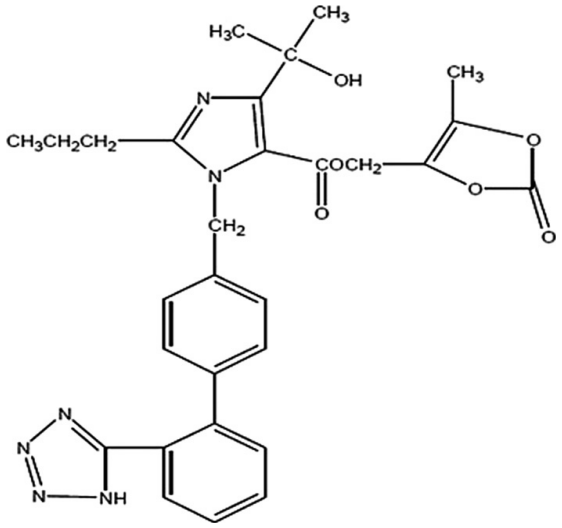
In the case of gas chromatography, examples of typical variations are

- Different columns (different lots and/or suppliers)
- Temperature.

2. DRUG PROFILE

OLMESARTAN MEDOXIMIL[13]:-

Chemical structure :



IUPAC name : 5-Methyl-2-oxo-1,3-dioxol-4-ylmethyl-4-(1-hydroxy-1-methylethyl)-2-propyl-1-[2'-(1H-tetrazol-5-yl)biphenyl-4-ylmethyl]imidazol-5-carboxylate.

Molecular Formula : C29-H30-N6-O6.

Molecular Weight : 558.59

CAS number : 0144689-63-4

General properties:-

Colour : White to off-white powder

State : Crystalline powder

Solubility : Insoluble in water, slightly soluble in ethanol, methanol.

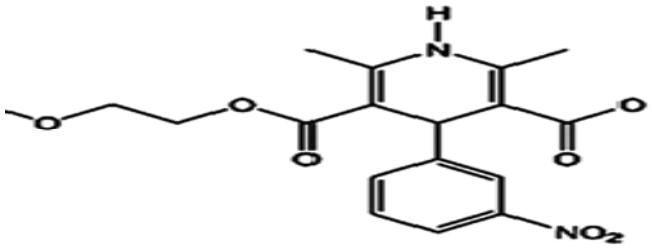
Melting point : 175-180^{0}C

Dosage form : Tablets

Category **:** Anti hypertensive agent

CILNIDIPINE[14]

Chemical structure :



IUPAC name : *o*3-(2-methoxyethyl) *O*5-[(*E*)-3-phenylprop-2-enyl] 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine- 3,5-dicarboxylate

Molecular Formula : C27-H28-N2-O7

Molecular Weight : 492.52

CAS number : 132203-70-4

General properties:-

Colour : light yellowish

State : Crystalline powder

Solubility : Insoluble in water, slightly soluble in ethanol, methanol.

Melting point : 107-112^{0}C

Dosage form : Tablets

Category : calcium channel blocker

Dosage form : Tablets

Marketed formulations: Nexovas-O

3. REVIEW OF LITERATURE

- **V GANDHI , *et al.*, (2013)**[22] developed a simple, accurate reverse phase liquid chromatographic method for estimation of simultaneous quantification of Telmisartan and Cilnidipine as the bulk drug and in tablet dosage forms. Separation was carried out on Jasco HPLC system equipped with HiQ sil C18 HS column (250 x 4.6mm i.d) and PDA detector using Methanol: 40mM Potassium dihydrogen ortho phosphate buffer (pH3) (90:10, v/v)as the mobile phase and detection was carried out at 245nm .Results were linear in the range of 1-10µg/ml for Cilnidipine and 5-30µg/ml for Telmisartan. The method was successfully applied for the analysis of drug in pharmaceutical formulation. Results of the analysis were validated statistically and by the recovery studies.
- **Manohara yagina *et al.*, (2013)**[19] A rapid simple and specific reversed phase HPLC method has been developed for estimation of Cilnidipine in Pharmaceutical formulation. HPLC analysis was performed on a C18 column using a 80:20 (v/v) mixture of Methanol and 0.05M Phosphate buffer at pH 3.0 as isocratic mobile phase at a flow rate of 1ml/min UV detection was carried out at 254 nm. The method was validated for accuracy, Precision, linearity, specificity. Validation revealed that the method is specific accurate precise relative and reproductive. Good linear correlation coefficient were obtained for calibration plots in the ranges tested. Limit od detection was 0.179µg/ml and limit if quantification was 0.544µg/ml. Intraday and interday RSD of retention time and peak areas are less than 1.729%. and recovey was obtained between 99.95% and 101.09%. The estimated HPLC method is appropriate enabling efficient quantitative analysis of Cilnidipine in pharmaceutical formulations.
- **Hamrapukar *et al.*,** (2013)[16] stability testing of an active substance or finished product provide evidence as to that quality it remains acceptable upto the stated period under storage condition as on label . With this objective a stability indicating HPLC has been established for the analysis of Olmesartan Medoximil in the presence of degradation products . The drug was subjected to sterss conditions of hydrolysis, photolysis , oxidation, thermal degradation. Extensive degradation was found in acid medium and alkaline medium. Minimum degradation in thermal degradation. While no degradation in photolytic condition .successful separation of drug from degradation product formed under stress condition was achieved on C18 column using Methanol and water (60:40% v/v) pH 3.75 was adjusted with 10mM ortho phosphoric acid mobile phase. Flow rate was 1ml/min and detector was set to at 270nm. The method was

validated for linearity , range , precision, and accuracy, limit of detection and limit of quantification .

- **Reema H, *et al.,*** (**2013**)[21]A simple, precise, and accurate RP-HPLC method has been developed and validated for the simultaneous assay of Telmisartan and Cilnidipine in tablets. Isocratic RP-HPLC method was developed on Waters C18 mm, 5 µm column using mobile phase as acetonitrile (ACN): buffer pH 3.0 with orthophosphoric acid (68 : 32) at a flow rate of 1.0 mL/min and the detection was carried out at 245 nm using photodiode array detector. Forced degradation study was carried out by oxidation, hydrolysis, photolysis, and heating the drug. The method was validated for specificity, linearity, precision, accuracy, robustness, and solution stability. The method was found to be linear in the concentration range of 40–160 µg/mL with correlation coefficient of 0.9990 for Telmisartan and 10–40 µg/mL with correlation coefficient of 0.9989 for Cilnidipine. Degradation products produced as a result of stress studies did not interfere with the detection of agomelatine; therefore, the assay can be considered to be stability indicating.

- **Padmanabh B *et al.,* (2012)** [20] A simple HPTLC method for the determination of Cilnidipine and Telmisartan in combined dosage form has been developed and validated. The separation was carried out on Merck aluminium plates precoated with silica gel 60 F_{254} using Toulene : Methanol : Ethyl acetate (8:2:1 v/v/v) as the mobile phase and detection was carried out in 260nm. Results were in the linear range of 200-1200 ng/gm for Cilnidipine and 400-4800ng/gm for Telmisartan. The method was successfully applied for the analysis of drugs in pharmaceutical formulation.The results of analysis were statistically and by recovery studies.. Present method can be applied for the determination of Olmesartan medoxomil in quality control of formulation without interference of the excipients

- **Kameswrao , *et al.,*** (2012)[17] The present method provides the detailed description of development and validation of a simple stability indicating re- verse phase column liquid chromatographic method for Olmesartan in the presence of its impurities namely Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F and Imp-G and degradation products generated from forced degradation studies. The drug substance was subjected to stress conditions of aqueous hydrolysis, Oxidative, photolytic and thermal stress degradation. The degradation of Olmesartan was observed under acid hydrolysis, base hydrolysis and peroxide. The drug

was found to be stable to other stress conditions attempted. Successful separation of the drug from synthetic impu- rities and degradation products formed under stress conditions was achieved on symmetry C18, 150 mm × 4.6 mm, 5 µ column using a phosphate buffer, Acetonitrile and Milli Q water. The developed LC method was validated with respect to specificity, linearity, accuracy, precision, raggedness and robustness. The assay method was found to be linear in the range of 250 µg·mL–1 to with 1000 µg·mL–1 correlation coefficient of 0.9999 and the linearity of the impurities was es- tablished from LOQ to 0.4%. Recoveries of assay and impurities were found between 98.5% and 101.2%. The devel- oped LC method to determine the related substances and assay determinations of Olmesartan can be used to evaluate the quality of regular production samples and stability samples.

- **Kumanan, *et al.,*** (2011)[18] stability testing of an active substance or finished product provide evidence as to that quality it remains acceptable upto the stated period under storage condition as on label . With this objective a stability indicating HPLC has been established for the analysis of Olmesartan Medoximil in the presence of degradation products . The drug was subjected to stress conditions of hydrolysis, photolysis , oxidation, thermal degradation.successful separation of drug from degradation product formed under stress condition was achieved on Luna C18 column using Acetonitrile and water (50:50% v/v) pH 4.5 was adjusted with 10mM Potassium hydrogen phosphate mobile phase. Flow rate was 1ml/min and detector was set to at 258nm. The method was validated for linearity , range , precision, and accuracy, limit of detection and limit of quantification .
- **Chaitanya Prasad, *et al.,*** (2011)[15] Reversed phase high performance liquid Chromatographic method was developed and validated for the estimation of Olmesartan medoxomil in bulk and formulation. Selected mobile phase was a combination of phosphate buffer with pH adjusted at 2.8 and acetonitrile (35:65% v/v) and wavelength selected was 250 nm. Retention time of Olmesartan medoxomil was 2.591 min. Linearity of the method was found to be 50-150 µg/ml, with the regression coefficient of 0.9993. Quantification was done by calculating area of the peak and the detection limit and quantification limit was 0.02µg/ml and 0.09µg/ml, respectively. There was no significant difference in the intraday and inter day analysis of Olmesartan medoxomil determined for three different concentrations using this method.
- **Chaitanya Prasad, *et al.,*** (2011)[15] Reversed phase high performance liquid Chromatographic method was developed and validated for the estimation of Olmesartan medoxomil in bulk and

formulation. Selected mobile phase was a combination of phosphate buffer with pH adjusted at 2.8 and acetonitrile (35:65% v/v) and wavelength selected was 250 nm. Retention time of Olmesartan medoxomil was 2.591 min. Linearity of the method was found to be 50-150 µg/ml, with the regression coefficient of 0.9993. Quantification was done by calculating area of the peak and the detection limit and quantification limit was 0.02µg/ml and 0.09µg/ml, respectively. There was no significant difference in the intraday and inter day analysis of Olmesartan medoxomil determined for three different concentrations using this method.

4. AIM AND OBJECTIVE

On the literature survey most of literatures related to HPTLC and HPLC methods have been reported determination of Cilnidipine and Olmesartan Medoximil separately . Few methods reported related to HPLC in tablet dosage form . Hence an attempt has made to develop a HPLC method for the determination of Cilnidipine and Olmesartan Medoximil in API and pharmaceutical dosage forms.

Present work is aimed at to develop a new, simple, fast, rapid, accurate, efficient and reproducible RP-HPLC method for the analysis of Olmesaratn medoximil and Cilnidipine in tablet dosage form and developed method will be validated according to ICH (Q2b) [14] guidelines.

OBJECTIVE

- To develop rapid, accurate, method with good reproducibility.
- To develop economic assay methods.
- To develop sensitive methods for analysis.
- To develop a precise method of analysis.
- To develop a selective method of estimation.

5. PLAN OF WORK

The experimental work has been planned as follows

STEP 1-

- Study of physico-chemical properties of drugs (pH, pka, solubility, and molecular weight)

- Preparation of drug standard and sample
- Develop simple, rapid and specific HPLC method for the quantitative simultaneous estimation of Olmesartan medoximil and Cilnidipine in tablet dosage form.
- Selection of stationary phase
- Selection of mobile phase
- Preparation of solutions
- Optimizing the chromatographic conditions

STEP 2-

- To validate the newly developed methods in accordance with the analytical validation parameters mentioned as ICH guidelines(Q2B)

6. MATERIALS AND METHODS

MATERIALS

Drug sample:

Olmesartan Medoximil and Cilnidipine reference standards are provided from Dr.reddys Hyderabad, India

REAGENTS AND CHEMICALS:

- Ammonium acetate
- Formic acid (0.1%)
- Methanol
- Olmesartan Medoximil (API)
- Cilnidipine (API)
- HPLC water (AR grade)

INSTRUMENTS USED:

Table No.4: Instruments used

INSTRUMENT	MAKE
HPLC System	Dionex
UV-Spectrometer	Perkin elmer
Analytical Balance	Shimadzu
Sonicator	Bandelin sonorex

METHOD DEVELOPMENT AND OPTIMISATION

Table No.5: Chromatographic conditions

Instrument	DIONEX
Column	SYNERGIFUSION RP,150×4.6mm,5µ
Detector	PDA
Flow rate	1ml /min
Wavelength	254nm
Injection volume	5µl
Column temperature	37^0C
Mobile phase	0.1% FA +Ammonium acetate : Methanol(20:80)
Elution technique	Isocratic

METHOD DEVELOPMENT AND OPTIMISATION

The objective of this experiment was to optimize the assay method for the estimation of Olmesartan Medoximil and Cilnidipine based on the literature survey. So here the trials mentioned describes how the optimization was done.

TRIAL-1

Preparation of mobile phase:

The mobile phase was prepared by mixing ammonium bicarbonate and methanol in the ratio of 50:50. The mobile phase is then sonicated using ultra sonicator for removing impurities and dissolved gases.

CHROMATOGRAPHIC CONDITIONS

- Technique: HPLC
- Column: SYNERGI RP FUSION,150×4.6mm,5µ
- Mobile phase:- ammonium bicarbonate:methanol 50:50
- Column temperature: 40^0C
- Wavelength: 254nm
- Flow rate: 1ml/min
- Run time: 8min
- Detector: PDA
- pressure: 1200-1300 psi
- **NOTE:** Merging problems faced

TRIAL-2

Preparation of mobile phase: The mobile phase was prepared by mixing ammonium bicarbonate and methanol in the ratio of 40:60. The mobile phase is then sonicated using ultra sonicator for removing impurities and dissolved gases.

CHROMATOGRAPHIC CONDITIONS

- Technique: HPLC
- Column: SYNERGI FUSION RP COLUMN ,150×4.6mm,5µ
- Mobile phase:- ammonium bicarbonate:acetonitrile 40:60
- Column temperature: 40^0C
- Wavelength: 254nm
- Flow rate: 1ml/min
- Run time: 8min
- Detector: PDA
- pressure: 1200-1400 psi

NOTE: got an uncleared Rt's

OPTIMIZEDMETHOD

PREPARATION OF MOBILE PHASE:

The mobile phase was prepared by mixing ammonium acetate and methanol in the ratio of 20:80. The mobile phase is then sonicated using ultra sonicator to removing impurities and dissolved gases.

CHROMATOGRAPHIC CONDITIONS

- Technique: HPLC
- Column: SYNERGI FUSION RP 150×4.6mm,5µ
- Mobile phase:- ammonium acetate:methanol:20:80
- Column temperature: 37^0C
- Wavelength: 254nm
- Flow rate: 1ml/min
- Run time: 8min

- Detector: PDA
- Pressure: 1200-1400 psi

METHODOLOGY

3.2.1Assay method development:

3.3 METHOD DEVELOPMENT OF LMESARTAN MEDOXIMIL AND CILNIDIPINE BYRP-HPLC

The method should be developed mainly based on pk_a concept of drug and also different mobile phase composition, flow rate, λ_{max}, different columns and column temperature. Olmesartan medoximil has two pk_a values that are 4.3, and 13 and Cilnidipine has pka of 2.3 .Generally pH of buffer solution should be select $\pm$ 1 of pk_a value of drug. In this method pKa value of 4.3 for olmesartan was selected because in HPLC, column may damage solutions with pH more than 10. So pk_a 13 of Olmesartan Medoximil was eliminated. Finally good peak was obtained at pH 3.5 of buffer and retention time also less than compared to other trials. So the method was optimized at these conditions.

Further validation study should be performed as per ICH guidelines.

Preparation of solutions:

Buffer Preparation:

Add 0.768gms of ammonium acetate in 1000ml of Millipore water and sonicate it in sonicator for 30mins and adjust the pH to 3.5 ± 0.05 with formic acid..

Mobile phase

Prepare a filtered (0.45µ*)* and degassed mixture of buffer preparation and methanol in ratio of 20: 80 v/v respectively.

Preparative Steps for Assay method development:

Standard Preparation:

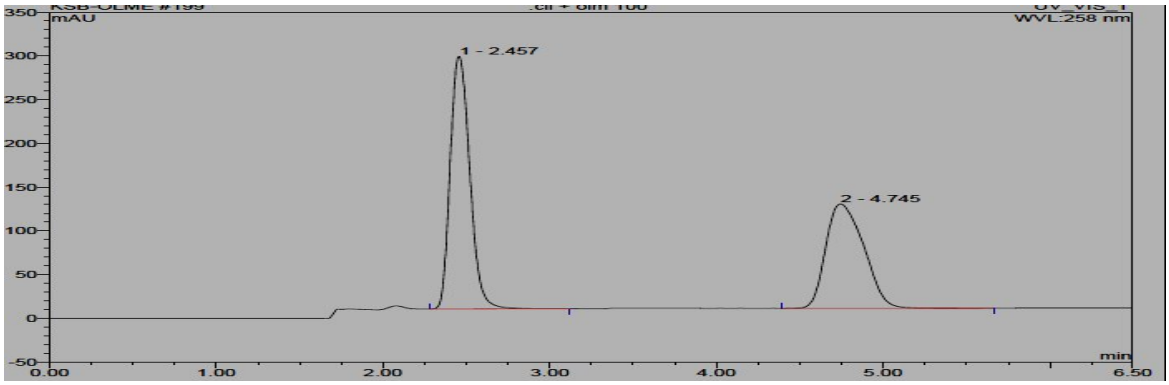
Weigh and transfer accurately 200 and 100 mg of Olmesartan Medoximil and Cilnidipine repectively working Standard into a 100 ml clean dry volumetric flask, sonicate to dissolve.

Cool the solution to room temperature . Then transfer 5.0 ml above solution into 50 ml volumetric flask and dilute with mobile phase.

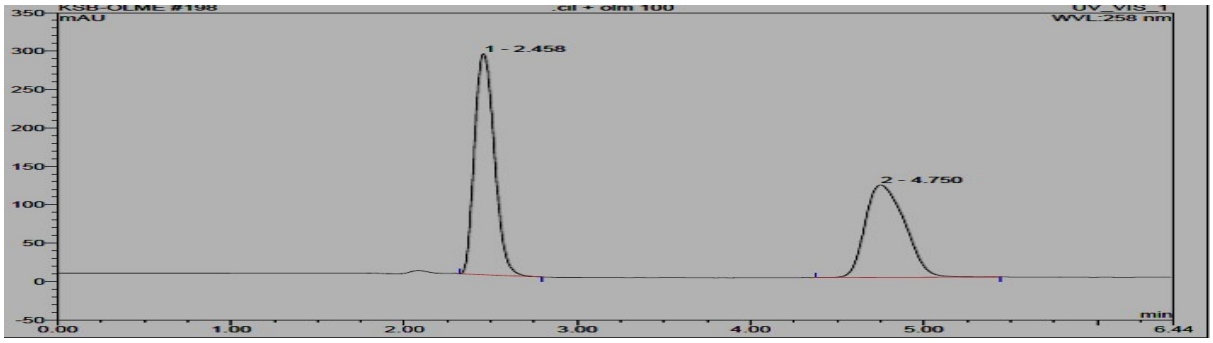
Sample preparation:

Weigh and finely powder 20 tablets and Transfer the powder equivalent to 30mg of Nexovas-o into 10ml of clean, dry, volumetric flask. Add about 10ml of mobile phase, shake on orbital shaker for 15 min and sonicated for 30 min with occasionally shaking. Cool the solution to room temperature and dilute volume with solvent mixture. Centrifuge the solution at 3000 RPM for 15 min. Then transfer 1.0 ml above solution into 10 ml volumetric flask and dilute with mobile phase

Standard chromatogram-1



Sample chromatogram-2



METHOD VALIDATION

The validation protocol is applicable to quantitative analytical method- Assay, which is used for the estimation of active content in support of regulatory requirements. This procedure covers the following parameters.

1) System Suitability

2) Specificity

3) Method precision

4) Accuracy

5) Linearity

6) Robustness

SYSTEM SUITABILITY

System suitability is defined by ICH as "the checking of a system performance, before or during analysis of unknowns, to ensure system performance."

System suitability criteria may include such factors as plate count, tailing, retention, and/or resolution, tailing factor. System suitability criteria should also include a determination of reproducibility (%RSD).

RETENTION TIME:

Retention time the difference in time between the point of injection and appearance of peak maximum.

RESOLUTION:

Resolution is the measure of the extent of separation of two components and the base line separation achieved. It can determine by following formula

$R_s = 2(Rt_2-Rt_1)/W_1+W_2$

Where n= no of theoretical plates

R_t = Retention time
W= peak width at base

NO OF THEORETICAL PLATES:

$n= 16* Rt^2/ W^2$
Where n= no of theoretical plates
R_t = Retention time

W= peak width at a base

THEORETICAL PLATE (PLATE THEORY):

A theoretical plate is an imaginary or hypothetical unit of a column where distribution of solute between stationary phase and mobile phase has attained equilibrium.

HETP (HEIGHT EQUIVALENT TO A THEORETICAL PLATE)

A theoretical plate count decides the efficiency of separation. And if HETP is less, The column is more efficient and vice versa.

HETP= length of the column /No of theoretical plates.

VANDEEMETER PRINCIPLE:

HETP is given by the VANDEEMETER Equation

HETP = A+B/u+Cu

Where A= Eddy's Diffusion term

B= Longitudinal Diffusion Term

C= Effect of Mass Transfer which depends on flow rate

u=flow rate

Preparation of standard stock solution:

Weigh accurately 20mg of Olmesartan medoximil and 10mg of Cilnidipine iin a 10 ml standard flask add diluent and sonicate for half an hour. Now make up to the mark by adding mobile phase. Then transfer the 0.1ml of above solution into 10ml volumetric flask and dilute with mobile phase

Procedure: Injected 5µl of standard stock solution in replicate (5 times) and recorded the peak area response of Olmesartan Medoximil and cilnidipine. Calculate the % RSD for the response of the standard Solution (for 5 replicates).

Acceptance criteria: The % RSD should not be more than 2.0%, USP tailing should not be more than 2.5, and Theoretical plates should not be less than 1500

SYSTEM SUITABILITY (chromotogram-3)

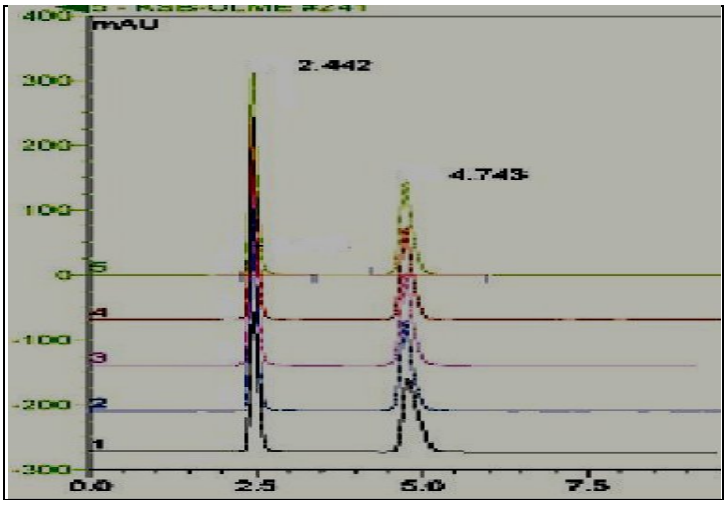


Table No.6: System suitability parameters for Olmesartan Medoximil

Injection no	Drug	Area(mAU*)	%Area	Retention time	Theoretical plate	Tailing factor
1	Olmesartan medoximil	40.78	53.71	2.44	2633	1.1
2	Olmesartan medoximil	40.79	53.70	2.47	2641	1.3
3	Olmesartan medoximil	40.76	53.22	2.49	2621	1.4
4	Olmesartan medoximil	40.80	53.45	2.46	2606	1.3
5	Olmesartan medoximil	40.78	53.47	2.51	2656	1.1
Mean		40.782				
STD.DEV		0.014832				
%RSD		0.03				

Table No.7: System suitability parameters for Cilnidipine

Injection no	Drug	Area(mAU*)	%Area	Retention time	Theoretical plate	Tailing factor
1	Cilnidipine	31.613	47.63	4.75	3539	1.1
2	Cilnidipine	31.655	47.67	4.76	3553	1.3
3	Cilnidipine	31.689	47.68	4.78	3509	1.4
4	Cilnidipine	31.699	47.69	4.72	3452	1.3
5	Cilnidipine	31.683	47.61	4.76	3469	1.1
Mean		31.6678				
STD.DEV		0.034716				
%RSD		0.107				

SPECIFICITY

Ability of an analytical method to measure the analyte free from interference due to other components.

PROCEDURE

To demonstrate the specificity blank, standard Olmesartan medoximil in the concentration of 100mcg/ml, Cilnidipine 100mcg/ml and blend solution 100mcg/ml were prepared and chromatogram was recorded

Preparation of standard solution:

Weigh accurately 20mg of Olmesartan medoximil and 10mg of Cilnidipine iin a 10 ml standard flask add diluent and sonicate for half an hour. Now make up to the mark by adding mobile phase.Then transfer the 0.1ml of above solution into 10ml volumetric flask and dilute with mobile phase

Preparation of sample solution:

Weigh and finely powder 20 tablets and Transfer the powder equivalent to 30mg of Nexovas-o into 10ml of clean, dry, volumetric flask. Add about 10ml of mobile phase, shake on orbital shaker for 15 min and sonicated for 30 min with occasionally shaking. Cool the solution to room temperature and dilute volume with solvent mixture. Centrifuge the solution at 3000 RPM for 15 min. Then transfer 0.1 ml above solution into 10 ml volumetric flask and dilute with mobile phase

Acceptance criteria: There should be no interference peak due to other components

SPECIFICITY(chromotogram-4)

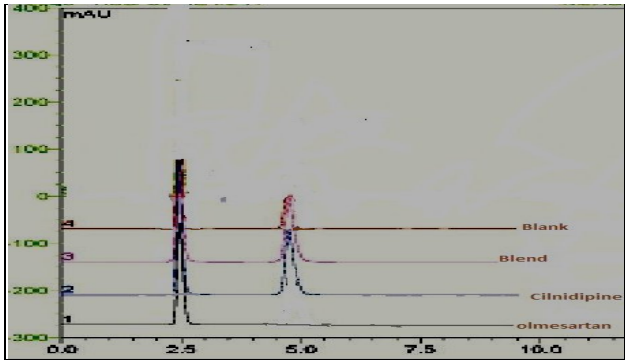


Table No.8: Specificity

Injection	Drug	Area	% Area	RT
1	Blank	Nil	Nil	Nil
1	Olmesartan Medoximil	39.428	100.00	2.46
1	Cilnidipine	32.897	100.00	4.73
Blend soln	Olmesartan Medoximil	41.865	52.867	2.44
	Cilnidipine	31.986	47.592	4.74

Report: From the results method was said to be specific for test results

LINEARITY

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

PREPARATION OF STANDARD STOCK SOLUTION: (for Olmesartan Mediximil)

Accurately weighed 20mg of Olmesartan Medoximil and 10mg of CIlnidipine in a 10 ml standard flask respectively and dilute with diluent , sonicate 30 minutes . Finally make upto the volume by using mobile phase. From above solution the following dilutions are made.

Preparation of 10% standard solution:

From the above solutions take take 0.02ml of Olmesartan Medoximil and Cilnidipine in a 10 ml standard flask and further dilute upto the mark with mobile phase in order to get the concentration 20mcg/ml.

Preparation of 25% standard solution:

From the above solutions take 0.05ml of Olmesartan Medoximil and Cilnidipine in a 10 ml standard flask and further dilute up to the mark with mobile phase in order to get the concentration 50mcg/ml.

Preparation of 50% standard solution:

From the above solutions take 0.1ml of Olmesartan Medoximil and Cilnidipine in a 10 ml standard flask and further dilute up to the mark with mobile phase in order to get the concentration 100mcg/ml.

Preparation of 75% standard solution:

From the above solutions take 0.15ml of Olmesartan Medoximil and Cilnidipine in a 10 ml standard flask and further dilute up to the mark with mobile phase in order to get the concentration 150mcg/ml.

Preparation of 100% standard solution:

From the above solutions take 0.2ml of Olmesartan Medoximil and Cilnidipine in a 10 ml standard flask and further dilute upto the mark with mobile phase in order to get the concentration 200mcg/ml.

PROCEDURE

Different standard concentrations of 20, 50, 100, 150, 200mcg/ml were prepared and one injection from each concentration was used to get chromatogram.

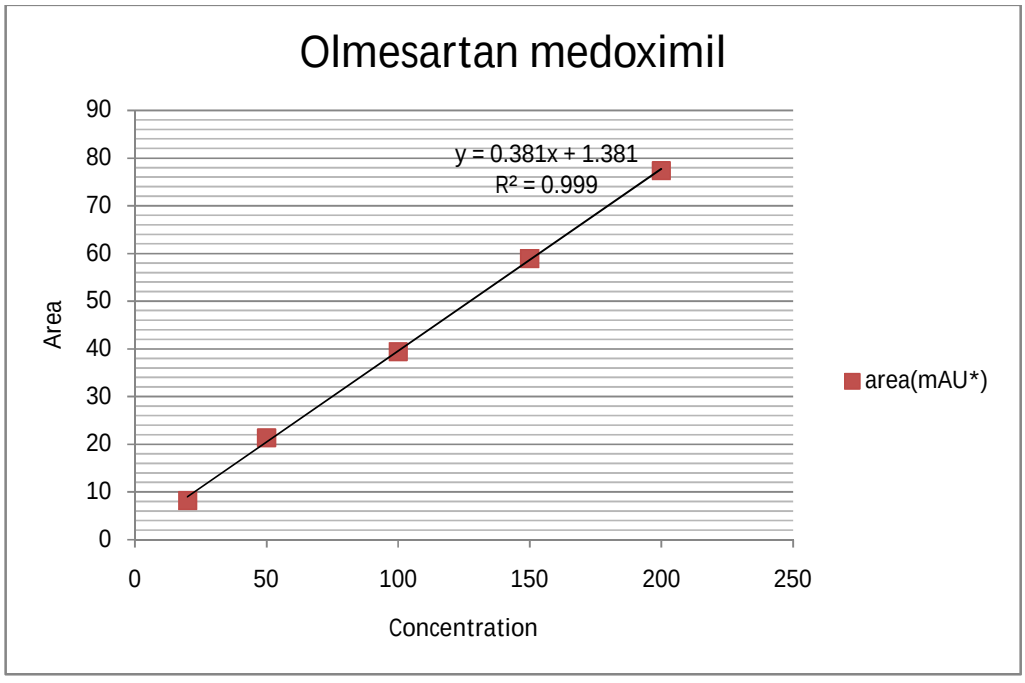
Acceptance criteria

The linearity regression should not be less than 0.995.

Linearity for Olmesartan Medoximil

Table No.9:

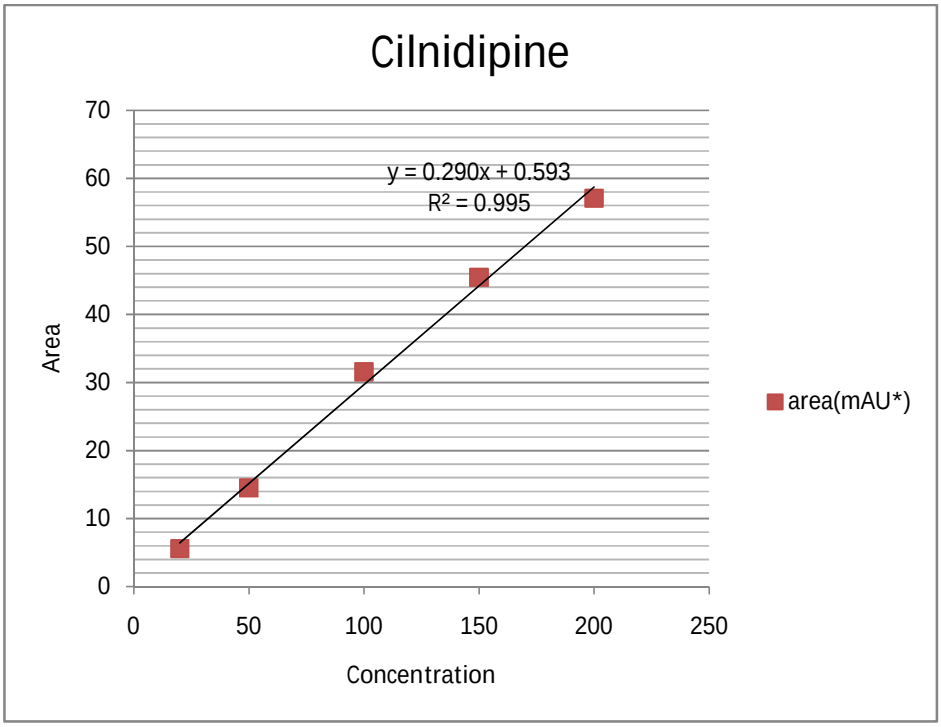
Serial no	Concentration(mcg/ml)	Area (mAU*)
1	20	8.216
2	50	21.389
3	100	39.430
4	150	58.956
5	200	77.371



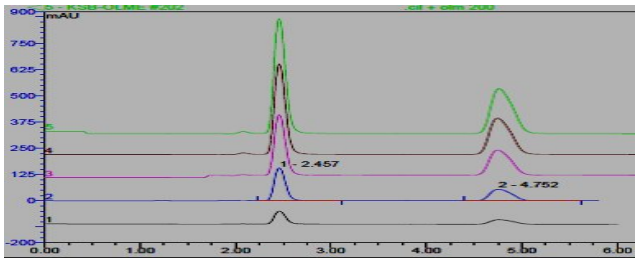
Linearity for Cilnidipine

Table No.10:

Serial no	Concentration(mcg/ml)	Area (mAU*)
1	20	5.552
2	50	14.502
3	100	31.541
4	150	45.443
5	200	57.072



LINEARITY (chromotogram-5)



ACCURACY:

The accuracy of an analytical procedure is the closeness of test results obtained by that procedure to the true value. The accuracy of analytical should be established across its range.

Determination: Accuracy is determined by three ways:

i) Comparison to a reference standard

ii) Recovery of the analyte spiked into blank matrix

iii) Standard addition of the analyte

Procedure: The accuracy of the method was determined by analyzing Atorvastatin and amlodipine solutions approximately 80%, 100%, and 160% of standard level and the results were summarized in the following table.

Preparation of standard stock solution:

Weigh accurately 20mg of Olmesartan medoximil and 10mg of Cilnidipine iin a 10 ml standard flask add diluent and sonicate for half an hour. Now make up to the mark by adding mobile phase.Then transfer the 0.1ml of above solution into 10ml volumetric flask and dilute with mobile phase

Preparation of sample solution:

Weigh and finely powder 20 tablets and Transfer the powder equivalent to 30mg of Nexovas-o into 10ml of clean, dry, volumetric flask. Add about 10ml of mobile phase, shake on orbital shaker for 15 min and sonicated for 30 min with occasionally shaking. Cool the solution to room temperature and dilute volume with solvent mixture. Centrifuge the solution at 3000 RPM for 15 min. Then transfer 0.1 ml above solution into 10 ml volumetric flask and dilute with mobile phase.

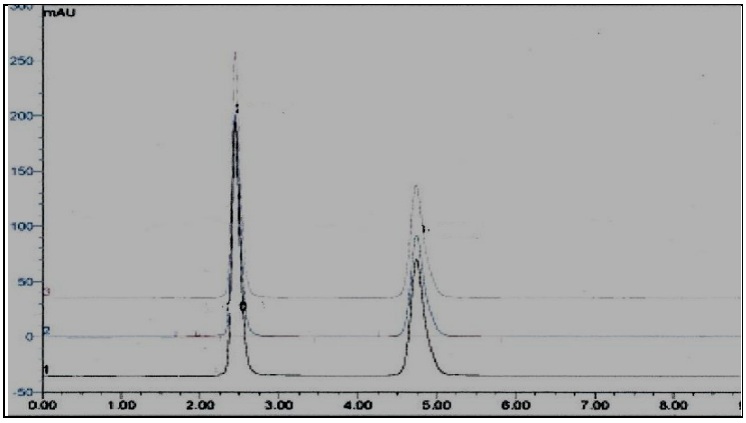
Acceptance criteria:

% recovery should be in the limits of 98% to 102%

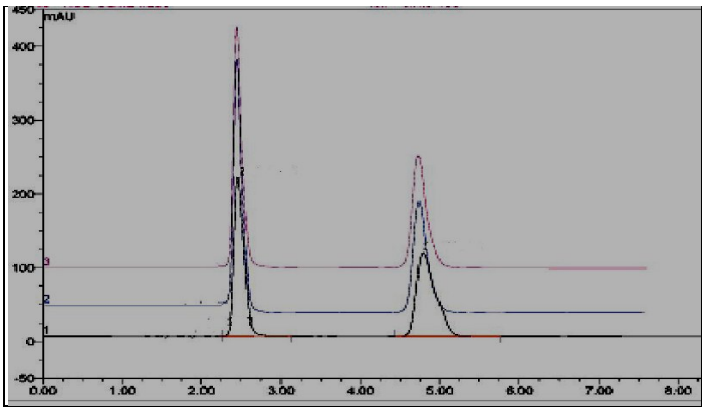
Table No: 11 Accuracy

Olme	**Level**	**Area(%mAU*)**	**%recovery**	**Cil**	**Level**	**Area (mAU*)**	**% recovery**
80%	A	31.345	98.75%	80%	A	23.889	100.425
	B	31.623			B	23.787	
	C	31.473			C	24.008	
100%	A	39.910	101.7%	100%	A	29.321	100.05
	B	41.120			B	30.081	
	C	39.421			C	29.456	
160	A	63.125	101.65%	160%	A	47.413	101.39%
	B	63.797			B	47.615	
	C	63.129			C	47.893	
		Average	100.7%			Average	100.62

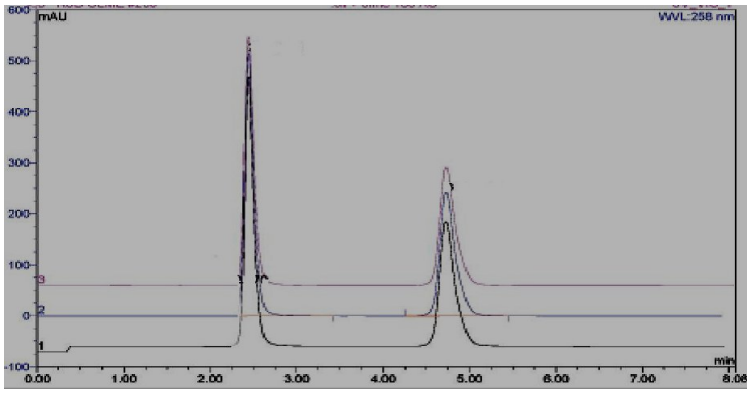
Accuracy 80(Chromatogram 6)



Accuracy 100(chromatogram-7)



Accuracy 160(chromatogram-8)



Report: The method was found to be accurate as the % recovery ranged from Olmesartan Medoximil 98.75% - 101.65%, Cilnidipine 100.425% - 101.39%, which is well within the acceptance range of 98-102

METHOD PRECISION OR REPEATABILITY:

Preparation of sample stock solution:

Weigh and finely powder 20 tablets and Transfer the powder equivalent to 30mg of Nexovas-o into 10ml of clean, dry, volumetric flask. Add about 10ml of mobile phase, shake on orbital shaker for 15 min and sonicated for 30 min with occasionally shaking. Cool the solution to room temperature and dilute volume with solvent mixture. Centrifuge the solution at 3000 RPM for 15 min. Then transfer 0.1 ml above solution into 10 ml volumetric flask and dilute with mobile phase

Procedure: Injected 5µl of standard stock solution in replicate (5 times) and recorded the peak area response of Olmesartan Medoximil and cilnidipine. Calculate the % RSD for the response of the standard Solution (for 5 replicates).

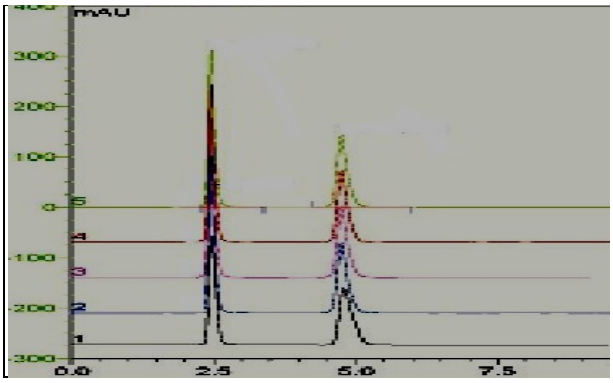
Acceptance criteria: The result obtained from the sample preparation shall be calculated and it should be within the limit of 98% to 102% from the assay value and RSD should not more than 2.0%

Table No.12: method precision

Injection	Olmesartan Medoximil			Cilnidipine		
	Area(mAU*)	%Area	Assay	Area(mAU*)	%Area	Assay
1	39.910	53.17	99.36	29.321	48.89	102.79
2	41.120	52.98	99.00	30.081	48.98	102.98
3	39.421	53.22	99.45	29.456	48.95	102.92
4	40.807	52.99	99.02	31.700	48.11	101.14
5	40.785	52.82	98.71	31.689	48.15	101.24
Avg			99.108			102.214
Std dev			0.299			0.937
%RSD			0.30			0.917

Report: As the results were within the acceptable limits, all the methods provide good precision

IMMEDIATE PRECISION (chromotogram-9)



Intermediate Precision/Ruggedness

To evaluate the intermediate precision (also known as Ruggedness) of the method,

Precision was performed on different day by using different make column of same dimensions.

Preparation of stock solution

Weigh and finely powder 20 tablets and Transfer the powder equivalent to 30mg of Nexovas-o into 10ml of clean, dry, volumetric flask. Add about 10ml of mobile phase, shake on orbital shaker for 15 min and sonicated for 30 min with occasionally shaking. Cool the solution to room temperature

and dilute volume with solvent mixture. Centrifuge the solution at 3000 RPM for 15 min. Then transfer 0.1 ml above solution into 10 ml volumetric flask and dilute with mobile phase

Procedure

The standard solution was injected for three times and measured the area for all three injections in HPLC. The %RSD for the area of three replicate injections was found to be within the specified limits.

Acceptance Criteria

- The % RSD for the area of three sample injections results should not be more than 2%

Intermediate precision (chromotogram-10,11)

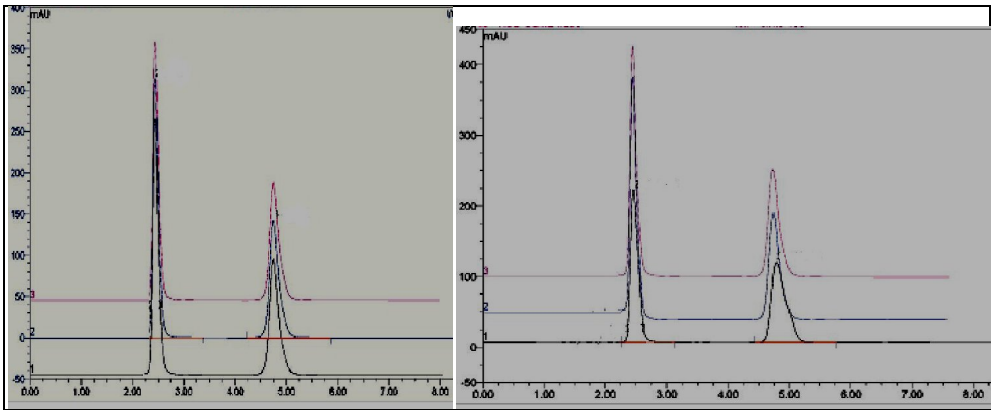


Table No.13: Immediate precision

Injection	Olmesartan Medoximil			Cilnidipine		
	Area (mAU*)	%Area	Assay	Area (mAU*)	%Area	Assay
1	39.910	53.17	99.36	29.321	48.89	102.64
2	41.120	52.98	99.00	30.081	48.98	102.84
3	39.421	53.22	99.45	29.456	48.95	102.77
Avg			99.27			102.75
Std dev			0.238			0.1014
%RSD			0.2			0.098

Table No.14: intermediate precision

Injection	Olmesartan Medoximil			Cilnidipine		
	Area (mAU*)	%Area	Assay	Area (mAU*)	%Area	Assay
1	41.832	52.80	98.67	31.889	48.29	101.53
2	40.889	52.71	98.5	31.203	48.36	101.68
3	40.145	52.81	98.69	30.749	48.21	101.36
Avg			98.62			101.52
Std dev			0.1044			0.160
%RSD			0.10			0.15

ROBUSTNESS

- ICH defines the robustness of an analytical procedure as a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters. It provides an indication of the procedure's reliability during normal usage.
- Robustness tests examine the effect that operational parameters have on the analysis results.
- For the determination of a method's robustness, a number of method parameters, such as pH, flow rate, column temperature, injection volume, detection wavelength or mobile phase composition, are varied within a realistic range, and the quantitative influence of the variables is determined. If the influence of the parameter is within a previouslyspecified tolerance, the parameter is said to be within the method's robustness range

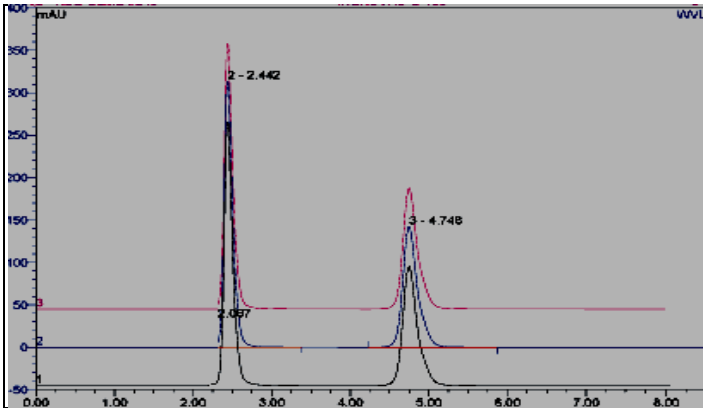
PROCEDURE:

To demonstrate the robustness of the method standard solution concentration of 100mcg/ml was prepared, from this solution three injections were used to get chromatogram.

Mobile phase composition:

Actual mobile phase composition 20:80(water(10mM ammonium acetate + 0.1%F .A) (changed mobile phase composition 27:.73 and 33:77)

ROBUSTNESS(chromatogram-12)



ROBUSTNESS(chromatogram-13)

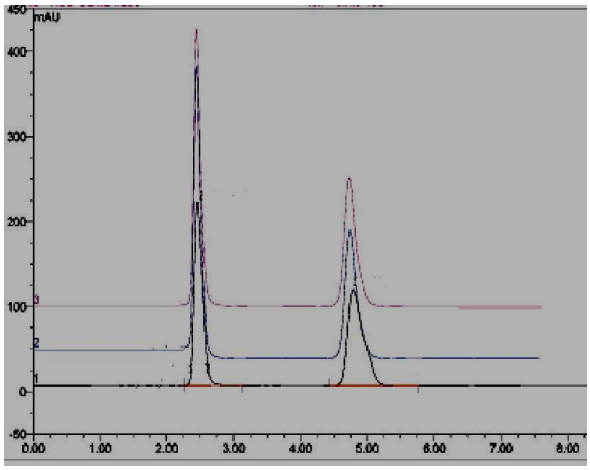


Table No.15: Robustness

Injection	Olmesartan Medoximil			Cilnidipine		
	Area(mAU*)	%Area	Assay	Area(mAU*)	%Area	Assay
1	42.832	52.56	98.22	32.879	47.45	99.76
2	39.879	52.89	98.88	31.427	47.75	100.39
3	41.156	52.49	98.80	30.749	48.21	101.36
Avg			98.63			100.50
Std dev			0.3601			0.8059
%RSD			0.36			0.80

Table no:16 Robustness

Injection	Olmesartan Medoximil			Cilnidipine		
	Area(mAU*)	%Area	Assay	Area(mAU*)	%Area	Assay
1	40.867	53.01	99.06	31.889	47.82	100.35
2	41.778	53.59	100.01	30.478	47.79	100.29
3	41.169	52.94	98.93	31.684	47.59	99.87
Avg			99.33			100.17
Std dev			0.589			0.261
%RSD			0.59			0.26

RESULTS AND DICCUSSION

An exertion has been made for a simple, accurate and precise method for the estimation of Olmesartan Medoximil and Cilnidipine in pharmaceutical dosage form by an isocratic RP-HPLC method.

Table No.17:

S.No	Drug	RT
1.	Olmesartan Medoximil	2.467
2.	Cilnidipine	4.731

Table No.18:

Validation parameters	Observed value		Acceptance criteria
	Olmesartan Medoximil	Cilnidipine	
System suitability			As per USP
Tailing factor	1.2	1.06	NMT 2.5
Theoretical plates (N)	2631	3504	NLT 1500
%RSD	0.03	0.10	NMT 2.0

Table No.19: Statistical analysis

S.No	Validation parameters	Acceptance criteria	Observed values
1	Precision	%RSD≤2	0.30-0.91
2	Accuracy	98%-102%	100.7-100.62%
3	Linearity	NLT 0.995	0.995-0.999
4	Robustness	%RSD≤2	0.26-0.80
5	Ruggedness	%RSD≤2	0.10-0.15
6	Specificity	No interferances	nil

- At 258nm Olmesartan Medoximil and Cilnidipine were eluted at 2.467min, 4.731min, respectively using ammonium acetate in 0.1% Formic acid and Methanol in the ratio (20:80) as mobile phase and a flow rate of 1mL/min.
- Specificity: Olmesartan Medoximil and Cilnidipine were shows no interferences due to othe components
- Precision: The % RSD values obtained for peak areas of Olmesartan Medoximil and Cilnidipine were in range of 0.30-0.91, which were within the acceptance criteria.
- Ruggedness: The % RSD values obtained for peak areas of Olmesartan Medoximil and Cilnidipine were in range of 0.10-0.15, which were within the acceptance criteria.
- Accuracy: The percentage recovery obtained values were in the range of 100.7-100.62% which were within acceptance criteria.
- Linearity: Correlation coefficient was found to be greater than 0.995.
- Robustness: The % RSD values obtained for peak areas of Olmesartan Medoximil and Cilnidipine were in range of 0.91-1.77%, which were within the acceptance criteria.

SUMMARY AND CONCLUSION

In order to develop a Liquid Chromatographic method effectively, most of the effort should be spent in method development and optimization as this will improve the final method performance.The method validations documents for its intended purpose.

A fast isocratic HPLC-PDA method was developed for the estimation of Olmesartan Medoximil and Olmesartan in tablet dosage form. The total run time was 8 minutes. Simple buffer was used. The mobile phases were easy to prepare and economical.

The baseline with low signal to noise ratio was obtained. Each peak eluted with good resolution and no carryover was observed. Since the solutions were stable only for 24 hrs at room temperature. The purity of each peak was observed in PDA, from which an inference can be drawn that there is no interference of other peaks in the particular peak.

The method allows high samples through put due to short run time. Thus, selective, simple and rapid method was developed.

The method was validated with different analytical performance characteristics and data was compiled. The results satisfied the acceptance criteria. This demonstrated that the HPLC method developed was specific, linear, accurate, precise and robust.

This work provided me a great opportunity to have an exposure with the instruments used in Pharmaceutical Industry.

BIBLIOGRAPHY

1. Skoog, Fundamentals of Analytical Chemistry, 8th Edn, Thomson Asia Pvt.Ltd., Singapore, P 2-11, 785-802.

2. Connors KA. A Textbook of Pharmaceutical Analysis, 3rd Edn, Wiley intersciencesInc, Delhi, 1994, P 1-3.

3. Vogel's text book of Quantitative Chemical Analysis by Denny R.C, Bassett j, Mendham J, 5th Edn, Pearson Education 2003, P11, 229.

4. Gurdeep.R.Chatwal, Sham.K.Anand, Instrumental method of Chemical Analysis, Himalaya Publishing house, 5th Edn Revised, P 2.568, 2.673, 2.617, 2.647, 2.624, 2.588, 2.656, 2.630.

5. Kasture AV, Wododkar S G, Mohadik K R, Morl H N, Instrumental Methods Of Pharmaceutical Analysis, Vol 2, 19th Edn, NiraliPrakashan, New Delhi, 2010, P 6-7.

6. Lindsay S, High Performance Liquid Chromatography, 1st Edn, London:John Wiley and sons, 1991, P 30-45.

7. SatinderAhuja and Michael W.Dong, Handbook of Pharmaceutical Analysis for HPLC, Elsevier, 2006 Edn, P 6.

8. Khopkar S.M, Basic concepts of Analytical Chemistry, New age International Publishers, P 1-5, 178-181, 215-33.

9. Beckett, J.B.Stenlak, Practical Pharmaceutical Chemistry, 4thEdn, CBS publishers and distributors, 2001, P 157-67.

10. Willard H, Instrumental methods of analysis, 7th Edn, CBS publishers and distributors, P 580-600.

11. Marvin C.McMaster, HPLC-A Practical Users Guide, 2nd Edn, John Wiley & Sons.Inc.Hoboken, New Jersey, 2007, P 1-14.

12. Skoog, Principles of Instrumental Analysis, Barkhanath publishers, 8th Edn, P 973-95.

13 .Olmesartan Medoximil description from www.chemicalland 21.com

14. Cilnidipine description from www.chemicalland 21.com

15. Chaitanya Prasad *et al.,*2011, development of rp-hplc method for estimation of olmesartan medoxomil in tablet dosage forms, **der pharma chemica**, 2011,vol. 3 (6),P-208-212.

16. Hamrapukar *et al.,* 2013, optimization and validation of rp - hplc stability indicating method for determination of olmesartan medoxomil and its degraded product, **international journal of applied science and engineering,** 2013, vol.119(2),P- 137-147.

17. kameswrao , *et al.,* 2012, development and validation of stability indicating lc method for olmesartan medoxomil ,**american journal of analytical chemistry**, 2012, vol. 3, P-153-160.

18. Kumanan, *et al.,* 2011, stability indicating rp-hplcmethod development and validation of olmesartan medoxomil ,**asian j pharm biol res.**

19. Manohara yagina *et al.,* 2013development and validation of rapid stability indicating chromatographic determination of cilnidipine in bulk and dosage form ,research **journal of pharmacy and technology**,vol.6,iss:3 ,P- 296-299

20. Padmanabh b *et al.,* 2012 hptlc determination of cilnidipine and telmisartan in a combined dosage form**. internation resesrch journal of pharmacy**. issn:2230-8407.

21. Reema h, *et al.,* 2013 method development and validation of simultaneous assay of cilnidipine and telmisartan in a tablet dosage form vol. 2013, article id 461461.

22. V gandhi , *et al.,* (2013) simultaneous rp-hplc estimation of cilnidipine and telmisartan combined tablet dosage form. iss no:0976-8505.