METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF DECITABINE IN PHARMACEUTICAL DOSAGE FORM BY RP-HPLC METHOD

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IN

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Submitted by

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Certificate

This is to certify that the dissertation work entitled “Method Development and validation for the estimation of Decitabine in pharmaceutical dosage form by RP-HPLC” is a bonafide work of **Mr.PRABAHARAN. P** carried out in U Win Life sciences,malappuram,,kerala under my guidance and supervision of Dr.Hashim .K.M,Director,U Win life sciences for the partial fulfillment of the award of degree of Master of Pharmacy in Pharmaceutical Analysis, RVS college of Pharmaceutical Sciences, Sulur, Coimbatore, affiliated to The Tamilnadu Dr. M.G.R Medical University, Chennai.

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PRABAHARAN.P

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LIST OF ABBREVIATIONS USED

%	Percentage
^{0}C	Degree Celsius
AMA	Antimicrobial agent
ANDA	Abbreviated New Drug Applications
ARM	absorbance ratio method
BLA	Biologics License Applications
CAS No	Chemical Abstract service Number
CV	Coefficient of variation
D^0	Zero order
D^1	First Derivative
D^2	Second serivative
FDA	Food and Drug ADMINISTRATION
GFC	Gel Filtration chromatography
GPC	Gel permeation chromatography
HILIC	Hydrophilic interaction chromatography
HPTLC	High Performance Thin layer Chromatography
ICH	International Conference on Harmonisation
L	Lit
LIN	Linezolid
LOD	Limit of Detection
LOQ	Limit of Qualification
M	Molarity
mg	Milligram
mM	Millimole
NPC	Normal phase chromatography
ng/ul	Nano Gram per micro litre
NDA	New Drug Applications
No.	number
ODS	Octa Decyl Silence
OPA	Ortho Phosphoric Acid
PDA	Photo Array Detector
PEEK	Poly Ether Ketone

ppm	Parts per million
QC	Quality control
R^2	Correlation coefficient
Rf	Retention factor
RPC	Reverse phase chromatography
RP-HPLC	Reverse phase High performance Liquid chromatography
RS	Reference Standard
RSD	Relative standard Deviation
S	Slope
Sec	Second
SEC	Size exclusion chromatography
SFC	Super critical fluid chromatography
V/v	volume by volume
µl	Microlitre
µg/ml	Microgram per Millilitre
σ	Standard Deviation

Chapter 1: INTRODUCTION

A drug includes all medicines intended for internal or external use for or in the diagnosis, treatment, mitigation or prevention of disease or disorder in human beings or animals, and manufactured exclusively in accordance with the formulae mentioned in authoritative books.[1]

Pharmaceutical analysis is a branch of chemistry involving a process of identification, determination, quantification, purification and separation of components in a mixture or determination of chemical structure of compounds. There are two main types of analysis – Qualitative and Quantitative analysis.

Qualitative analysis is performed to establish composition of a substance. It is done to determine the presence of a compound or substance in a given sample or not. The various qualitative tests are detection of evolved gas, limit tests, color change reactions, determination of melting point and boiling point, mass spectroscopy, determination of nuclear half life etc.

Quantitative analysis techniques are mainly used to determine the amount or concentration of analyte in a sample and expressed as a numerical value in appropriate units. These techniques are based on suitable chemical reaction and either measuring the amount of reagent added to complete the reaction or measuring the amount of reaction product obtained the characteristic movement of a substance through a defined medium under controlled conditions, electrical measurement or measurement of spectroscopic properties of the compound. [2]

1.1 High Performance Liquid Chromatography

Chromatography is the method of separation that finds applications in all branches of science. It was first invented by Russian Botanist Mikhail Twsett. This technique was used separate various plant pigments like chlorophylls and xanthophylls by passing solutions of these compounds through a glass column packed with finely divided calcium carbonate. The

separated species appeared as colored bands on the column hence the name of the process (Greek *chroma* meaning “color” and *graphein* meaning “writing”). [3]

Chromatography is defined as a non- destructive procedure for resolving multi-component mixture of trace, minor, or major constituents into its individual fractions. In chromatography, the sample is dissolved in the mobile phase which may be a gas, liquid, or a supercritical fluid. The principle involved in HPLC is that when a mixture containing different compounds is introduced into the mobile phase and allowed to flow over a stationary phase, the individual compounds travel at different speeds and get separated based on the relative affinities to the stationary phase and the mobile phase. The compounds are separated based on the polarity of the stationary phase and the mobile phase.

Chromatography is of various types based on the physical state of the stationary and the mobile phase:

1. ***Solid - Liquid type*:** The stationary phase is a solid and the mobile phase is a liquid. Ex: Thin layer chromatography, High performance liquid chromatography (HPLC).
2. ***Liquid - Liquid type*:** The stationary phase is a liquid and the mobile phase is also a liquid. Ex: Paper Chromatography, HPLC.
3. ***Liquid - Gas Type*:** The stationary phase is a liquid and the mobile phase is a gas. Ex: Gas chromatography.

High Performance Liquid Chromatography is the most widely used of all the analytical separation techniques. The reasons for its popularity are its sensitivity, ready adaptability to quantitative determination, suitable for non-volatile and thermally fragile species, wide applicability to variety of substances such as amino acids, carbohydrates, nucleic acids, proteins, hydrocarbons, terpenoids, pesticides, steroids, metal-organic species and inorganic species. As high pressures (around 3000 psi) are used for the separation of the analytes down the column, it is often termed as High Pressure Liquid Chromatography. [4, 5, 6]

1.2 Types Of HPLC

HPLC is classified into various types

a) Based on polarity of stationary and mobile phase

- Normal Phase Chromatography
- Reverse Phase Chromatography

b) Based on the principle of separation

- Adsorption Chromatography
- Partition Chromatography
- Ion Pair Chromatography
- Size Exclusion Chromatography
- Chiral Phase Chromatography

c) Based on elution technique

- Isocratic Elution
- Gradient Elution

d) Based on scale of operation

- Analytical HPLC
- Preparative HPLC

1.2.1.a Based on the polarity of the stationary phase and the mobile phase, it is of two types:

Normal Phase (NP) HPLC

In this type, the stationary phase is polar and the mobile phase is non-polar, polar compounds are retained for a longer periods because of more affinity towards the stationary phase, hence non-polar compounds travel faster and are eluted first.

Reverse Phase (RP) HPLC

In this type, the stationary phase is non-polar and the mobile phase is polar, non-polar compounds are retained for longer periods as they have more affinity towards the stationary phase. Hence, polar compounds travel faster and are eluted first. [3, 4, 5, 6]

***Table 1.1*:** Types of HPLC based on polarity of stationary phase and mobile phase.

Types	Normal Phase	Reverse Phase
Stationary phase	Polar	Non polar
Mobile phase	Non polar	Polar
Compound eluted first	Non polar	Polar
Compound eluted last	Polar	Non polar

1.2.1.b Based on the principle of separation, chromatography is divided into various types,

Adsorption chromatography

It is one of the oldest types of chromatography. The principle involved is adsorption. Adsorption is a surface phenomenon in which the separation mechanism depends on the difference in the polarity of various drug molecules. The more polar a molecule, the more strongly it will be adsorbed by a polar stationary phase. Similarly the more non-polar a molecule, the more strongly it will be adsorbed by a non-polar stationary phase. [7, 8]

During a surface adsorption chromatography process, a competition for stationary phase adsorption sites exists between the materials to be separated and the mobile phase. In a mixture, molecules of low polarity spend proportionally more time in the mobile phase than those molecules that are highly polar, which are retained for a longer period of time. Therefore the components of a mixture are eluted in order of increasing polarity. [9]

Partition Chromatography

The principle involved is partition in which the solute molecules distribute themselves between the mobile and the stationary phases. In partition chromatography the stationary phase is a non-volatile liquid which is held as a thin layer (or film) on the surface of an inert solid. The mixture to be separated is carried by a gas or a liquid as the mobile phase. With the use of liquid as mobile phases, there is a tendency for the stationary liquid phase to

be removed or dissolved. Therefore, the stationary liquid phase has to be chemically bonded to the solid bonding support such as silica gel, cellulose powder, or kieselguhr (hydrated silica). The solute molecules in the mobile phase move through the system at rates determined by their relative solubilities in the stationary and mobile phases. [9, 10]

Ion Exchange Chromatography

Ion Exchange Chromatography is based on the reversible interaction between a charged protein and an oppositely charged chromatography medium. Biomolecules such as proteins, peptides, nucleic acids with even small differences in net surface charge can be separated, and very high resolution is obtained by choosing the optimal ion exchanger and separation conditions. The net surface charge of a protein varies according to the surrounding pH. Typically, when the pH is above its isoelectric point, a protein will bind to a positively charged anion exchanger. Below its isoelectric point, a protein will bind to a negatively charged cation exchanger.

Anion and cation exchangers are classified as strong or weak, depending on how much the ionization state of the functional groups vary with pH. A strong ion exchanger has the same charge density on its surface over a broad pH range, whereas the charge density of a weak ion exchanger changes with pH. The selectivity and the capacity of a weak ion exchanger are different at different pH values. [11, 12]

Size Exclusion Chromatography

This process is also known as Gel Permeation Chromatography. In this method the mixture of compounds with various molecular sizes are separated according to size or molecular weight. The stationary phase consists of a porous cross-linked polymeric gel. The pores of the gel normally small and exclude the larger solute molecules, but allows smaller molecules to enter the gel, causing them to flow through a larger volume. This causes the larger molecules to pass through the column at a faster rate than the smaller ones. The components of a mixture therefore elute in order of decreasing size or molecular weight. [9, 13]

Size Exclusion Chromatography is used extensively in the biochemical industry to remove small molecules and inorganic salts from valuable higher molecular weight products such as peptides, proteins and enzymes.

Chiral Phase Chromatography

In this type of chromatography, optical isomers are separated using chiral stationary phases. Two approaches for chiral separation are available, indirect, which utilizes derivatizing agents, and direct, which uses chiral stationary phases or chiral mobile phase additives.

In the indirect method, a racemic mixture is made to react with a chiral reagent to form a pair of diastereomers and then chromatographed using an achiral column. Because diastereomers possess different physiochemical properties, they can be separated in an achiral environment.

Direct separation of enantiomers on an achiral column using a chiral mobile phase additive is applied only in HPLC. In this method, enantiomeric separation is accomplished by the formation of a pair of transient diastereomeric complexes between racemic analyte and the chiral mobile phase additive. Chiral discrimination is due to differences in the stabilities of the diastereomeric complexes, solvation in the mobile phase, and/or binding of the complexes to the solid support.[14]

1.2.1.c Based on elution technique, chromatographic methods are of two types,

Isocratic Elution

In this technique, the composition of the mobile phase is maintained as constant throughout the process of separation. All the compounds in the sample are eluted in a reasonable amount of time, by changing the ratio of polar to non-polar compounds in the mobile phase during the sample run while maintaining peak resolution. [15]

Gradient Elution

A steady change of the mobile phase composition during the chromatographic run is called gradient elution. This is widely used technique when a sample contains components of a wide range of polarities. For a reverse phase gradient, the solvent starts out relatively polar and slowly becomes more non-polar. The gradient elution offers the most complete separation of the peaks, without taking much time. A sample containing compounds of a wide range of polarities can be separated by a gradient elution in a shorter time period without a loss of resolution in the earlier peaks or excessive broadening of later peaks. [15, 16]

Gradient elution also increases quasi-efficiency of the column. In the isocratic elution, the longer a component is retained, the wider its peak. In gradient elution, the tail of the peak is always under the influence of the stronger mobile phase when compared to the peak front. Thus, molecules on the tail of the chromatographic peak will move faster. This will tend to compress zone and narrow the resultant peak. [16]

1.2.1.d Based on the scale of operation, chromatography is of two types,

Analytical HPLC

In Analytical HPLC, quantitative and qualitative determination of a compound is done. It is the most widely used technique. In this method, the sample amount applied to the column is typically in the µg range or lower quantities. The mass ratio of compound to the stationary phase on the column is less than 1: 100000. Under these conditions good separations with sharp and symmetrical peaks are achieved. Recovery of samples is not possible as small quantities of the sample are used. [17]

Preparative HPLC

In Preparative HPLC, isolation and purification of a product is done. It is an expensive technique, when compared to the traditional purification techniques like distillation, crystallization or extraction.

Preparative HPLC is used for the isolation and purification of valuable products in the chemical and pharmaceutical industry as well as in biotechnology and biochemistry. It starts in the µg range for isolation of enzymes in biotechnology (micro purification). For identification and structure elucidation of unknown compounds in synthesis or natural product chemistry it is necessary to obtain pure compounds in amounts ranging from one to a few milligrams. Larger amounts, in gram quantity, are necessary for standards, reference compounds and compounds for toxicological and pharmacological testing. Industrial scale or production scale preparative HPLC, that is, kg quantities of compound, is often done nowadays for valuable pharmaceutical products. [17]

1.3 Instrumentation of HPLC

The main components of HPLC are as given below and are schematically represented in Fig 1.1

1. Solvent Reservoir
2. Solvent Delivery System (Pump)
3. Injection Port/ Autosampler
4. Column
5. Detector
6. Data Acquisition system

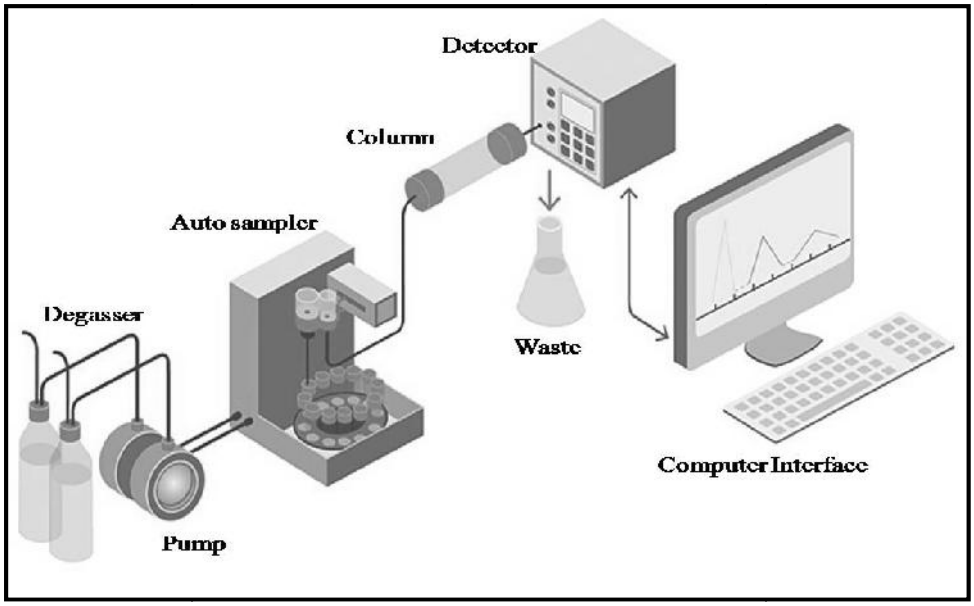

***Fig. 1.1*:** Schematic diagram of instrumentation of HPLC. [18]

1.3.1 Solvent Reservoir

Solvent Reservoirs are used to store mobile phase. Scott Duran bottles are commonly used as solvent reservoirs. The solvent reservoir must be made of inert material such as glass and must be smooth so as to avoid growth of microorganisms on its walls. It may be transparent or can be amber colored. A graduated bottle gives a rough estimate of mobile-phase volume in the bottle. Solvent reservoirs are placed above HPLC system (at higher level) in a tray. They should never be kept directly above the system as any spillage of solvent on the system may damage electronic parts of HPLC.

1.3.2 Solvent Delivery System (Pump)

Pumps are an important component of the HPLC system. It delivers a constant flow of mobile phase such that the separation of components of mixture takes place in a reasonable time. There are different types of pumps:

a) Reciprocating Pumps

b) Displacement Pumps

c) Pneumatic Pumps

In reciprocating pumps, a motor driven reciprocating piston controls the flow of mobile phase with the help of two ball check valves that opens and closes with the piston movement. The flow is thus not continuous and as damping of flow is necessary, it is accomplished using pulse dampers which are a long coiled capillary tube. Reciprocating pumps are most widely used in HPLC systems.

Displacement pumps, also known as syringe pump is composed of a one directional motor driven plunger that pushes the mobile phase present in a syringe like chamber. The volume of displacement pumps is limited. A constant flow rate is usually obtained with syringe like pumps.

Pneumatic pumps are the simplest where the mobile phase is pushed out of the mobile phase container by the pressure of a pressurized gas. The flow is dependent on the back pressure of the column and usually the flow is limited to pressures below 2000 psi. [20]

1.3.3 Injection Port or Auto sampler

The Sample Injector enables small liquid samples to be injected into the HPLC system without the sample passing through the pump. The function of the injector is to place an accurate volume of the sample into the high-pressure flow of the mobile phase so that the sample enters the column as a homogeneous, low-volume plug. It is of two types; manual injector or an auto injector. Injection is done through specially designed 6-port rotary injection valve or a Rheodyne valve as shown in Fig 1.5. The sample is introduced at atmospheric pressure by a syringe into a constant volume loop. In the LOAD position the loop is not in the path of the mobile phase. By rotating to the INJECT position the sample in the loop is moved by the mobile phase stream into the column. It is important to allow some sample to flow into waste from loop so as to ensure there are no air bubbles in the loop and previously used sample is completely washed out to prevent previous sample effects. The sample loading can be varied by part filling of the loop or by changing the loop volume. [21, 22]

Automatic injection improves laboratory productivity and eliminates personal errors. Present day advanced HPLC systems are equipped with an auto injector along with an auto sampler. The software programs help filling of the loop and delivery of the sample to the column. The computer controls the sequence of samples for injection from vials kept in numbered positions of the auto sampler. It is important to adopt precautions to ensure consistency of results. [22]

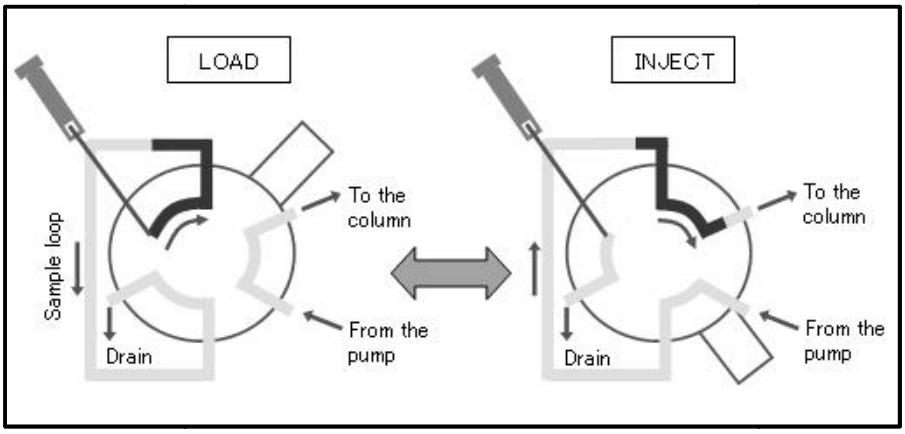

***Fig. 1.2*:** Flow Path of a Manual Injector. [23]

1.3.4 HPLC Column

The HPLC column is referred to as the heart of the process. The stationary phase of the column is used in separating the individual fractions of a sample mixture by using various physical and chemical parameters. Columns for analytical HPLC are of various sizes ranging from 10 - 25 mm in length and 2.6 – 4.6 mm internal diameter (i.d). Columns for preparative HPLC are in size range of 50 – 250 mm long and i.d greater than 4.6 mm. The columns are generally constructed of stainless steel SS-316 grade to cope with high back pressure (caused by the small particles of the stationary phase) and are glass lined to prevent metal catalysis of solvent- solute reactions at high column pressures (due to force of pumping of the mobile phase through the columns). Columns made of glass are used for the separation of Biomolecules and columns made of PEEK polymer are biocompatible and chemically inert to most solvents. [24]

Stationary phase

Separation of pharmaceuticals is usually achieved by partition of compounds in the test solution between the mobile and the stationary phases. HPLC systems consisting of polar stationary phases and non-polar mobile phases are described as normal-phase chromatography; those with non-polar stationary phases and polar mobile phases are called reversed-phase chromatography.

There are many types of stationary phases used in HPLC including:

- Unmodified silica, alumina, or porous graphite, used in normal-phase chromatography, where separation is based on differences in adsorption;

- A variety of chemically modified supports prepared from polymers, silica, or porous graphite, used in reverse-phase HPLC, where separation is based principally on partition of the molecules between the mobile phase and the stationary phase;

- Resins or polymers with acid or basic groups, used in ion-exchange chromatography, where separation is based on competition between the ions to be separated and those in the mobile phase;

- Porous silica or polymers, used in size-exclusion chromatography, where separation is based on the relative molecular mass of the molecules.

Most separations are based on partition mechanisms using chemically modified silica as the stationary phase and polar solvents as the mobile phase (reverse-phase HPLC). The surface of the support, e.g. the silanol groups of silica, is reacted with various silane reagents to produce covalently bonded silyl derivatives covering a varying number of active sites on the surface of the support. The nature of the bonded phase is an important parameter for determining the separation properties of the chromatographic system.

For the separation of enantiomers, special chemically modified stationary phases (chiral chromatography) are available, e.g. cyclodextrins, albumins, etc.

Generally, silica-based reverse-phase columns are generally considered to be stable in mobile phases with an apparent pH in the range 2.0 - 8.0, but the column manufacturer's instructions should be consulted before using the column. Columns containing particles of polymeric materials such as styrene divinyl benzene copolymer are stable over a wider pH range.

For analytical separations the particle size of the most commonly used stationary phases varies between 3 µm and 10 µm. The particles may be spherical or irregular, of different porosities and specific surface area. In the case of reversed-phase, the extent of bonding of the stationary phase is expressed as the carbon-loading. Furthermore, stationary phases may be "end-capped", i.e. the number of residual silanol groups is reduced by methylation. These parameters contribute to the chromatographic behavior of a particular stationary phase. Tailing of peaks, particularly for basic substances, can occur when residual silanol groups are present. [25]

1.3.5 Detector

A detector in the HPLC system measures the compounds after their separation from the column. The detector selected should be capable of responding to changes in the concentration of all the components in the sample with adequate sensitivity even to measure trace amounts. Basically there are two types of detectors: bulk property and solute property detectors. The bulk property detectors, function on some bulk property of the eluent such as refractive index (RI) and is not suitable for gradient elution and are usually less sensitive than solute property detectors. Solute property detectors perform by measuring a physical or chemical property that is specific to the solute. Detectors should have high linear dynamic range and should give faster response. The various detectors used and their applications are given in Table 1.2

***Table 1.2*:** Commonly used detectors and their application. [26, 27]

Detector	Analyte detected	Solvent requirements	Uses
UV-Visible	Any compounds with chromophore	Non UV absorbing solvents	Wavelength at which maximum absorption occurs can be selected. Has a high degree of selectivity and is useful for many applications.
Photo diode array (PDA) detector	Any compound with chromophore	Non UV absorbing solvents	Detects an entire spectrum simultaneously. Useful for analysis of related substances and impurities.
Fluores-cence	Fluorescent compounds	Non UV absorbing solvents	Highly selective and sensitive. Wavelength at which fluorescence occurs is selected. Often used to analyze derivatised samples after treatment with DANSYL chloride to form fluorescent compounds.
Refractive index (RI)	Compound with a different RI to that of the mobile phase	Cannot run mobile phase gradients	Virtually it is a universal detector but has limited sensitivity. Used for the detection of sugars.
Conduct-ivity	Charged or polar compounds	Mobile phase must be conducting	Excellent detector for ion exchange methods.
Electro-chemical	Readily oxidized or reduced compounds	Mobile phase must be conducting	Very sensitive and selective. Used for detection of Biological compounds.
Evaporative light scattering detector (ELSD)	All compounds are detected virtually	Volatile solvents and volatile buffers must be used.	Universal detector, Highly sensitive but not selective. But can be use for gradient analysis. Used for the detection of lipids, sugar and high molecular weight analytes.
Optical rotation detector	Optically active compounds	Mobile phase should be an asymmetric environment	Detects *R* and *L* type of isomers

Mass Spectro-meter (MS)	Broad range of compounds	Volatile solvents and volatile buffers must be used	Compounds are detected by difference in mass numbers. Highly sensitive and a two dimensional analytical tool. Used to quantify low detection limit of molecular and elemental compounds

1.3.6 Data Collection devices

Signals from the detector may be collected on chart recorders or electronic integrators that vary in complexity and in their ability to process, store and reprocess chromatographic data. The data storage capacity of these devices is usually limited

Modern data stations are computer based and have a large storage capacity to collect process and store data for possible subsequent reprocessing. Analytical reports can often be customized to the needs of the analyst.

Integration of peak areas and the setting of threshold levels are not normally problematic in an assay since the peak of the substance to be analyzed should be free of interference. However, in a test for impurities, the selection of the peak area integrator parameters becomes very important, particularly when baseline separations are not always attainable. If baseline separations cannot be obtained, valley-to-valley integration should be employed

METHOD DEVELOPMENT AND VALIDATION

1. 4 Introduction to Method Development

The number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing one. Often a time lag exists from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors. Under these conditions, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs. [28, 29]

Analytical methods should be used within good manufacturing practice (GMP) and good laboratory practice (GLP) environments, and must be developed using the protocols set out in the International Conference on Harmonization (ICH) guidelines (Q2A and Q2B). [30, 31]

Method development is a continuous process that progresses in parallel with the evolution of the drug product. The goal and purpose of the method should reflect the phase of drug development. During early drug development, the methods may focus on API behavior. They should be suitable to support preclinical safety evaluations, pre-formulation studies, and prototype product stability studies. As drug development progresses, the analytical methods are refined and expanded, based on increased API and drug product knowledge. The methods should be robust and uncomplicated, while still meeting the appropriate regulatory guidelines. Scouting experiments are frequently performed during method development to establish the performance limits of the method, prior to formal validation experiments. These may include forced degradation studies, which are an integral part of development of a stability-indicating method. API is typically subjected to degradation by acid, base, peroxide, heat, and light. This allows for a

determination of the capability of the method to separate and quantify degradation products, while providing insight into the main mechanisms of degradation. Once a stability-indicating method is in place, the formulated drug product can then be subjected to heat and light in order to evaluate potential degradation of the API in the presence of formulation excipients. [32]

1.4.1 Need for the Development of a New Method

Several reasons are available for the development of a new method of analysis.

- There may not be a suitable method for a particular analyte in the specific sample matrix.
- Existing methods may be too erroneous, artefact and/or contamination prone, or they may be unreliable (having poor accuracy or precision).
- Existing methods may be too expensive, time consuming, or energy intensive, or they may not be easily automated.
- Existing methods may not provide adequate sensitivity or analyte selectivity in samples of interest.
- Newer instrumentation and techniques may have evolved that provide opportunities for improved methods, including analyte identification or detection limits, greater accuracy or precision, or better return on investment.
- There may be a need for alternative method to confirm, for legal or scientific reasons, analytical data originally obtained by existing methods. [33]

1.5 Method Development Using HPLC

In method development, an attempt to select the best chromatographic conditions like the best column, the best mobile phase, the detection wavelength etc. to be used for routine analysis of any drug is done. For the method development by HPLC method some information about the sample is very essential i.e. number of components present in the sample, pKa values of different components, UV-Visible Spectra of each analyte, solubility in

different solvents, concentration range of each component, nature of sample etc. Prior to method development there must be some technical information i.e. chromatography method selection according to the sample properties, the sample when analyzed with HPLC, the condition where all compounds elute in a reasonable time, optimization of HPLC method with regard to analysis time, resolution, selectivity and sensitivity. [28]

1.5.1 Analyte Standard Characterization

All the information about the analyte is gathered regarding the structure, physical and chemical properties, toxicity, purity, hygroscopicity, solubility and stability. The availability of the 100% pure standard analyte is determined along with its storage and disposal information. If multiple components are to be analyzed in a sample matrix, the number of components to be analyzed is noted and the availability of the standard for each component is checked. [29, 33]

1.5.2 Literature Search and Prior Methodology

The available literature is searched for all types of information related to the analyte. Availability of information regarding the synthesis, physical and chemical properties, solubility or relevant analytical methods is determined. Books, periodicals, regulatory agency compendia, such as IP, USP/NF, BP etc. should be referred. Chemical Abstracts Service (CAS) automated/ computerized literature searches also should be used.

Information pertaining to prior analytical work on the analyte has to be determined within the company and compile the available data, results, reports, memos and publications. [33]

1.5.3 Choosing a Suitable Method

Using the available literature and previous methodology, the methods are adapted and modified. Sample preparation and instrument conditions are adopted to make use of the latest methods and instrumentation.

If no previous methods exist for the analyte in the literature, work from analogy to investigate compounds that are similar in structure and properties. Usually a compound with analytical method exists that is similar to the analyte of interest. [33]

1.5.4 Optimization

a) Choice of method

The most commonly used chromatographic methods are normal phase chromatography, reverse phase chromatography, reverse phase ion-pair chromatography and ion-exchange chromatography. In the selection of suitable chromatographic method for organic compounds, first reversed phase should be tried, if not successful, normal phase should be tried, then reverse phase ion-pair chromatography should be tried, ion-exchange chromatography at the end. [29]

b) Choice of Mobile Phase

In reversed phase chromatography the selection of mobile phase is very important for the analysis of the drug. We can use acetonitrile frequently as it is suitable for the entire UV range, methanol and Isopropanol are not suitable below wavelength of 210 nm, acetic acid is suitable above a wavelength of 240 nm, for the preparation of buffers, both K_2HPO_4 and KH_2PO_4 can be used in entire UV range, freshly distilled THF is suitable for HPLC above a wave length of 240 nm, TEA is suitable above 240 nm, ammonium acetate can be used above 215 nm, EDTA can be used in entire UV range, sodium phosphate is suitable above 210 nm. [29]

The organic phase concentration required for the mobile phase can be estimated by gradient elution method. For aqueous sample mixtures, the best way to start is with gradient reversed phase chromatography. Gradient can be started with 10% organic phase in the mobile phase and organic phase concentration (Methanol & Acetonitrile) can be increased up to 100% within 20 - 60 min. Separation can then be optimized by changing the initial mobile phase composition according to the chromatogram obtained from preliminary

run. The initial mobile phase composition can be estimated on the basis of where the compounds of interest were eluted, at what mobile composition.

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

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

When separating acid or bases, buffered mobile phase is required to maintain consistency in retention time and selectivity. Buffered salts reduce peak tailing for basic compounds by effectively masking silanol groups and also reduce potential ion-exchange interactions with a protonated silanol groups. As potassium is a stronger counter ion than sodium, it provides improved results compared to sodium (Na^+). Potassium phosphate is used for preparation of buffers of various pH. If band tailing is observed for basic amphoteric compounds few drops of diluted triethylamine or ammonium acetate is added, for acidic or amphoteric compounds, few drops of diluted triethylamine or ammonium acetate is tried. For neutral compounds, the aqueous eluent used in method development is water, for weak to medium acidic compounds, 100 mM H_3PO_4 buffer of pH 2.3, for weak to medium basic or acidic compounds in ionized form 100 mM H_3PO_4 buffer of pH 4.0, 50 mM H_3PO_4 buffer of pH 7.5 are used. Unknown sample should be analyzed first with water, then with an acidic and a neutral buffer.

During mixing of the solvents in the preparation of mobile phase, the difference in partial pressure of the individual solvent at a ratio they are to be combined should be considered. The solvent system must be miscible with

the previously used mobile phase, if not intermediate solvent may be used, the one that is miscible with previous mobile phase and new mobile phase.

c) Choice of Column

Columns being the heart of HPLC for optimum separation, Stable, high performance column with good selectivity, efficiency is essential requirement for rugged and reproducible method. These characteristics are dependent on the columns manufacturer's production of good quality columns and packing materials.

Column length

- Longer columns are chosen for increased resolution.
- Shorter columns are chosen for shorter analysis time, lower back pressure, fast equilibration and less solvent consumption.

Column internal diameter

- Wider diameter columns are chosen for greater sample loading.
- Narrow columns are chosen for more sensitivity and reduced mobile phase consumption.

Particle shape

- Columns with spherical particle shapes are preferred when lower back pressure column stability and greater efficiency is required.
- Columns with irregular particle shapes are preferred when large surface area and high capacity is required.

Particle size

- Columns with small particle size of 3 - 4 µm are preferred for complex mixtures with similar components. Combination of a short column (10 - 50 mm) with small particle size is used for fast, high resolution separations.

- Columns with larger particle size of 5 – 10 µm are preferred for structurally different compounds.
- Columns with large particle of 15 – 20 µm are used for preparative separations.

Surface area

- Columns with high surface area packing are selected for more capacity, greater resolution and longer retention.
- Columns with low surface area packing are selected for quicker equilibration time.

Carbon load

- Columns with high carbon load are chosen for greater column capacities and resolution.
- Columns with low carbon load for faster analysis time.

End capping

- Columns with end capped packing are selected to eliminate unpredictable secondary interactions with base material
- Columns with non-end capped packing are selected for selectivity differences for polar compounds by controlling secondary interactions. [29]

d) Choice of Detector

Detectors are eyes of the liquid chromatography system and measure the compounds after their separation on the column. Selected detector should be capable of responding to change in concentrations of all the components in the sample with adequate sensitivity even to measure trace substances. The detectors must have certain characteristics i.e. high sensitivity, higher linear dynamic range, application to most of the solutes, does not contribute to band broadening, non-destructive, faster response. [29]

1.5.5 Further Optimization

After the selection of a suitable method, mobile phase, column and detector, further optimization can be done to obtain a well developed method.

For shorter analysis time

- Change to isocratic method. The suitable mobile phase composition is estimated from the gradient run.
- Use of shorter column, if proper resolution is obtained.

For better resolution

- Use of longer column.
- Use of stationary phase with smaller particles (3 – 4 µm).

For better selectivity and sensitivity

- Other stationary phases e.g. phenyl, CN etc.
- pH control with ion-forming compounds
- Use of methanol or THF instead of acetonitrile.
- Detection at the absorption maximum of the substance
- All factors which leads to narrower and higher peaks as gradient elution, smaller particle, micro bore columns. [35]

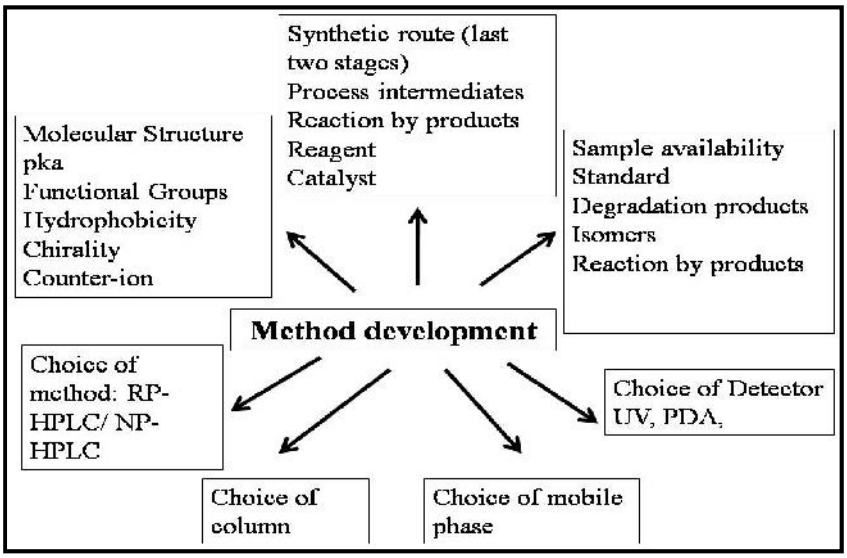

Fig 1.3: Outline of the process involved in method development

1.6 Parameters Affecting Changes in Chromatograph

The various parameters affecting the changes in chromatographic conditions are

1. Flow rate
2. Temperature
3. pH
4. Ion pair reagent
5. Column efficiency
6. Capacity factor
7. Resolution
8. Retention time
9. Peak asymmetry

1.6.1 Effect of Flow Rate

The efficiency of a HPLC column varies with flow rate. It is sometimes useful and readily utilized to increase the resolution. A faster flow rate of the eluent minimizes the time required to run a column and thereby minimizes diffusion, resulting in a better separation (less band broadening). However, the maximum flow rate is limited because a finite time is required for analyte to equilibrate between stationary phase and mobile phase. A slower flow rate will decrease the column back pressure and a corresponding increase in the run time is observed. [36, 37]

***Table 1.3*:** Flow rates for column with different internal diameter. [36]

Internal diameter of column (mm)	Standard flow rate (µL/ min)
4.6	1000
2.1	200
1.0	50
0.30	4

1.6.2 Effect of Temperature

Elevated temperatures decrease viscosity and increase solubility and diffusivity. Retention, peak shape, column efficiency, and total analysis time are affected by temperature because both the thermodynamics and kinetics of adsorption processes are functions of temperature. Additionally, temperature control results in improved reproducibility. In liquid chromatography temperature is used in the optimization of a separation. At higher temperature, peaks will be sharper and elute earlier. System pressure is affected by temperature. The viscosity of the mobile phase decreases with increasing temperature. For example if the HPLC system pressure is too high for a given solvent system temperature of the column may be raised to 40 ^{0}C or even 60 ^{0}C. Higher temperature will lead to a shorter column lifetime and some columns may not be able to tolerate 60 ^{0}C. The combination of smaller diameter packing with shorter column lengths at elevated temperatures facilitates efficient and fast separations, meeting the high throughput performance requirements of the pharmaceutical industry. [38, 39]

1.6.3 Role of pH

For some preparations the effect of changing pH is minimal. However for acids and bases, a small change in pH is significant. Changing the pH changes the degree of ionization of molecules in solution, affecting polarity of the solution thereby changing the retention times in an HPLC separation. In a sample mixture, the retention time of the components of the mixture are also changed to different extents. Hence it affects the degree of selectivity, where the peaks become further apart or at a particular pH they may co-elute and then the peak elution order will change. Selection of a proper buffer pH is necessary to reproducibly separate ionizable compounds by RP-HPLC. Selection of an improper pH for ionizable analytes leads to asymmetric peaks that are broad, tall or split.

During the selection of a buffer, pKa of the analyte should be considered. A buffer with 2 pH units above or below pKa of the analyte is

recommended for a good peak shape. From Henderson- Hasselback equation,

$$pH = pKa + \log ([A^-]/ [HA])$$

It can be determined that 99% of the analyte is in a single form, Good peak shape is possible only when an analyte is in a single form. [40, 41, 42]

1.6.4 Role of Ion-Pair Reagent

Most of these compounds are ionic or polar; hence the use of reversed phase-high performance liquid chromatography (RP-HPLC) is somewhat restricted. Initially when deciding to select RP-HPLC or RP-HPLC with ion-pairing, the nature of the analyte of interest is considered. If the sample is neutral, RP-HPLC is used first; and if the sample is ionic, RP-HPLC with ion pairing is used. Thus RP-HPLC and RP-HPLC with ion pairing are similar except that the latter consists of an ion-pair reagent in the mobile phase to improve the selectively of ionic samples. The ionic pair reagents are large ionic molecules having a charge opposite to the analyte of interest, as well as a hydrophobic region to interact with the stationary phase. The counter-ion combines with the ions of the eluent, becoming ion pairs in the stationary phase. This results in different retention, thus facilitating separation of analytes.

The use of an ion-pair reagent is suggested only when separation is not adequate with reversed-phase HPLC. This is because using an ion-pair reagent introduces additional experimental parameters that need to be controlled, such as selection of a suitable ion-pair reagent to use and its concentration. Because of this added variable, reversed-phase HPLC should be utilized on any ionic analyte first before trying ion-pair reversed–phase HPLC. The approach used in RP-HPLC to separate charged analytes is ionic suppression. This technique is based on the pH adjustment of the mobile phase to result in a non-ionized analyte. However, this requires extensive

method development and is only suitable for single compounds or simple mixtures where the pKa's of the analytes lie close together. [43]

1.6.5 Column Efficiency (N)

The efficiency of a chromatographic column is given terms of number of theoretical plates (plate number), *N*

$$N = 16 \left(\frac{t_r}{w} \right)^2$$

Where, t_r- retention time measured from the time of injection, *w- peak width* peak width obtained by drawing tangents to the sides of the Gaussian curve at the inflection points and extrapolating the tangents to intercept the baseline as in fig 2.3

Efficiency of the column is also expressed as height equivalent to theoretical plate, (or plate height) HETP (or *h*)

$$? = \frac{L}{N}$$

Where**,** *L*- length of the column,

N- number of theoretical plates. [4, 29, 44]

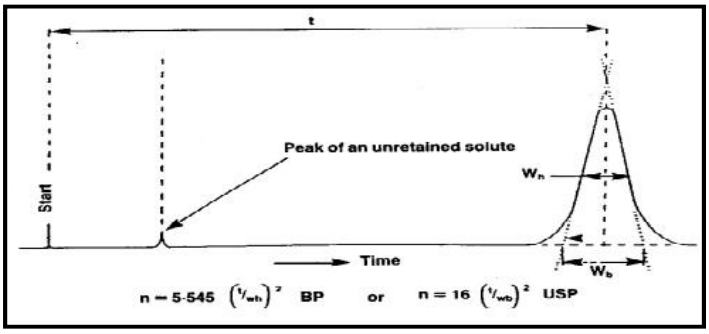

***Fig. 1.4*:** Pictorial Representation of number of theoretical plates. [44]

1.6.6 Capacity Factor (k')

It is measure of the position of a sample peak in the chromatogram, being specific for a given compound, a parameter which specifies the extent of delay of substance to be separated.

$$k' = \frac{t_r - t_m}{t_m}$$

Where, t_r– retention time of the solute, t_m – retention time of the unretained compound by the column packing. Fig 2.4 shows capacity factor of a solute.

k' depends at stationary phase, mobile phase, temperature and quality of column packing. For good chromatographic performance with isocratic separation, k' value should be in the range of 1-10. If k' < 1.0, the bands are inadequately separated from excessively unretained material, if k' > 10 separation takes too long and bands broadened, if k' > 30, satisfactory isocratic separation using present column and mobile phase is not obtained and gradient elution should be tried. [4, 29, 44]

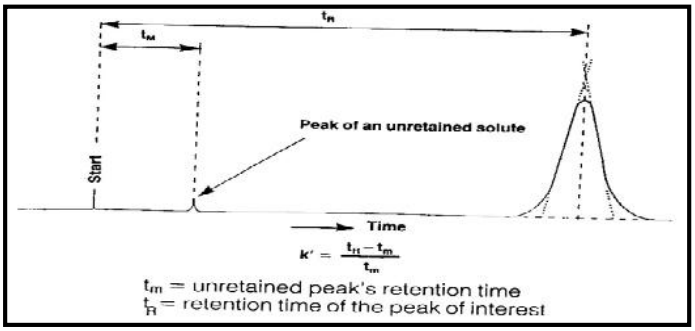

***Fig.1.5*:** Pictorial representation of capacity factor. [44]

1.6.7 Resolution (R_s)

The ability of the column to separate two solutes. In a chromatogram it is the distance of separation of two peaks.

$$R_s = \frac{t_{r2} - t_{r1}}{0.5(w_1 - w_2)}$$

Where,t_{r1} ,t_{r2} – retention time of two immediately adjacent peaks,

W_1, W_2 – peak widths of two immediately adjacent peaks as shown in fig 2.5. **4, 29, 44**

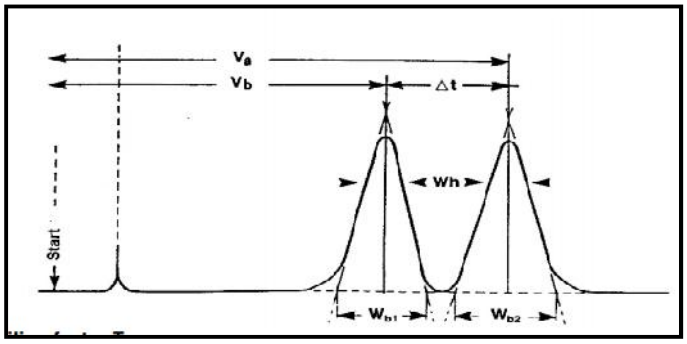

***Fig.1.6*:** Pictorial Representation of Resolution. **44**

1.6.8 Selectivity factor (α) / Relative Retention

This describes the relative position of two adjacent peaks. Ideally, it is calculated using the capacity factor because the peaks separation depends on the components interaction with the stationary phase.

$$\alpha = \frac{k'_b}{k'_a}$$

Where,

k'_a, k'_b– capacity factors of peak a and peak b respectively.

The value of the separation factor is always greater than unity. The separation factor is also identical to the ratio of the corresponding distribution constants. If the capacity factor is used, the separation factor should be consistent for a given column, mobile phase composition and specified temperature, regardless of the instrument used. **4, 29, 44** Fig 2.6 shows a pictorial representation of measurement of selectivity factor

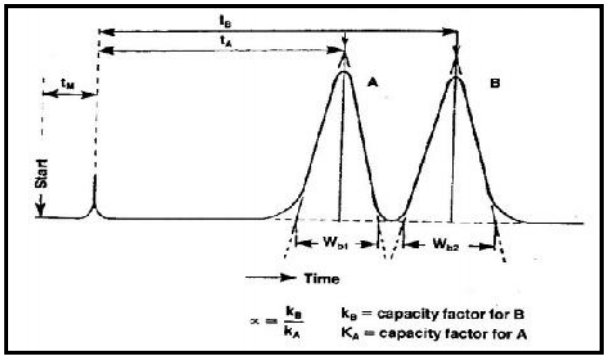

***Fig.1.7*: Pictorial representation of selectivity factor. [44]**

1.6.9 Peak Asymmetry (A_s)

It is also known as tailing factor. The asymmetry factor for a peak can be calculated using the following formula.

$$A_S = \frac{W_{0.05}}{2f}$$

Where, $W_{0.05}$– peak width at 5% height from the base line,

f – distance between maximum and leading edge of the peak as in Fig 2.7

It is also calculated from

$$A_S = \frac{b}{a}$$

Where, *b* - distance from the point at peak maxima to the trailing edge,

a – distance from the leading edge of the peak to the peak maxima (both measured at 10% height of the baseline) as shown in Fig 2.8. [29, 45]

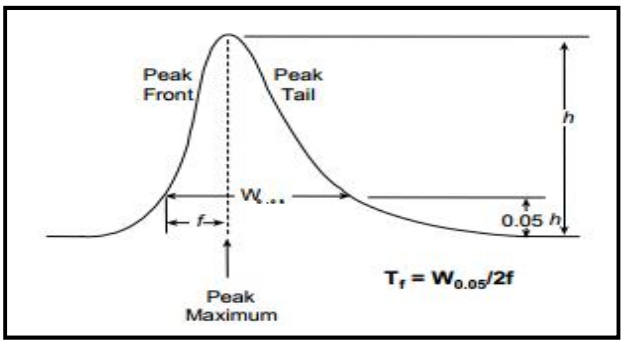

***Fig. 1.8*: Pictorial representation of peak asymmetry. [44]**

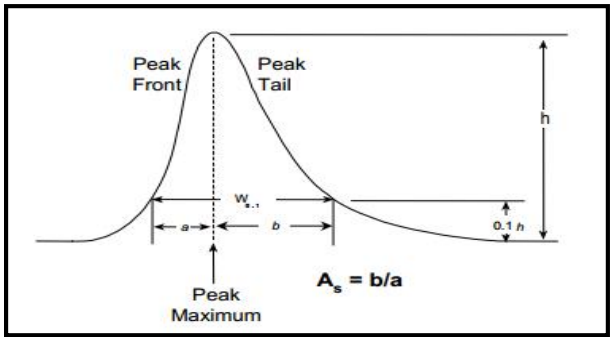

***Fig.1.9*:** Pictorial representation of peak asymmetry. [44]

1.7 Introduction to Method Validation

Validation is an integral part of quality assurance; it involves the systematic study of systems, facilities and processes aimed at determining whether they perform their intended functions adequately and consistently as specified. Validation in itself does not improve processes but confirms that the processes have been properly developed and are under control. [45]

Method validation is defined as the process of proving (through scientific studies) that an analytical method is acceptable for its intended use. To ensure compliance with quality and safety standards, the United States, Europe, Japan, and other countries have published compendia, or pharmacopeias, that describe official test methods for many marketed drug products. For example, analytical methods found in United States Pharmacopeia (USP) are legally recognized analytical procedures under section 501 (b) of the Federal Food, Drug, and Cosmetic Act. For these compendia methods, USP provides regulatory guidance for method validation. In addition, validation of analytical methods is covered by the United States Code of Federal Regulations (CFR). A great deal of effort has been devoted to the harmonization of pharmaceutical regulatory requirements in the United States, Europe, and Japan. As part of this initiative, the International Conference on Harmonization (ICH) has issued guidelines for analytical method validation. The recent FDA methods validation draft guidance documents as well as U.S. both refer to ICH guidelines. [46]

The required validation parameters, also termed analytical performance characteristics or analytical figs of merit. Methods should be validated or revalidated

- Before their introduction and routine use;
- Whenever the conditions change for which the method has been validated, e.g., instrument with different characteristics.
- Wherever the method is changed and the change is outside the original scope of the method. [45]

The validation of analytical procedures is directed to the four most common types of analytical procedures: Identification tests; Quantitative tests for impurities' content; Limit tests for the control of impurities; Quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product. [30]

1.8 Method Validation (ICH Guidelines)

1. Accuracy,
2. Precision,
    - Repeatability,
    - Intermediate precision.
3. Specificity / Selectivity,
4. Limit of Detection,
5. Limit of Quantitation,
6. Linearity,
7. Range,
8. Robustness,
9. System Suitability.

The validation terminology in ICH guidelines differ from the validation given in USP with two exceptions. Ruggedness is not included in ICH guideline and treats system suitability as a part of method validation, whereas the USP considers it in a separate chapter.

1.8.1 Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Accuracy should be established across the specified range of the analytical procedure. [32]

Accuracy is measured as the percentage of the analyte recovered by assay, spiking samples in a blind study.

Accuracy studies for drug substance and drug product are recommended to be performed at the 80, 100 and 120% levels of label claim as stated in the Guidelines for Submitting Samples and Analytical Data for Methods Validation

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. [33, 34, 35]

The acceptance criterion for accuracy is the Relative Standard Deviation (RSD) for all the recovery values should not be more than 2.0%.

1.8.2 Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. [32]

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.[30, 31, 32]

The relative standard deviation (RSD) for the assay of six sample preparations should not be more than 2.0%.

1.8.3 Specificity

Specificity is the ability to assess accurately the analyte in the presence of components which may be expected to be present in the sample matrix. Typically these might include impurities, degradants, matrix, etc. it is a measure of the degree of interference from such other things such as other active ingredients, excipients, impurities, and degradation products, ensuring that a peak response is due to a single component only. [32]

Specificity is divided into two separate categories: identification and assay/ impurity tests. For identification purpose, specificity is demonstrated by the ability to discriminate between compounds of closely related structures or comparison to a known reference standard. For assay/ impurity tests, specificity is demonstrated by the resolution of the two closely eluting compounds. These compounds are usually the major component or the active ingredient and an impurity. [30, 31, 32]

1.8.4 Limit of Detection (LOD)

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. It is a limit test that specifies whether or not an analyte is above or below a certain value.

It is expressed as a concentration at a specified signal to noise ratio usually a 2 or 3-to-1 ratio. Two other method can also be used to determine LOD: Visual non-instrumental methods and a means of calculation: Visual non-instrumental methods may include techniques such as thin-layer chromatography (TLC) or titrations. LODs may also be calculated based on the standard deviation (SD) of the response and the slope (S) of the calibration curve at levels approaching the LOD according to the formula:

$$LOD = 3.3 \frac{SD}{S}$$

Where,

SD- standard deviation

S- Slope

The standard deviation of the response can be determined based on the standard deviation of the blank, on the residual standard deviation of the regression line, or the standard deviation of y-intercepts of regression lines. The method used to determine LOD should be documented and supported, and an appropriate number of samples should be analyzed at the limit to validate the level. [30, 31, 32]

1.8.5 Limit of Quantitation (LOQ)

The limit of quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method.

The ICH has recognized the 10-to-1 signal –to-noise ratio as typical, and as for LOD, lists the same two additional options that can be used to determine LOQ: visual non-instrumental methods and a means of calculation. The calculation method is again based on the standard deviation (SD) of the response and the slope (S) of the calibration curve according to the formula,

$$LOD = 10 \frac{SD}{S}$$

Where,

SD- standard deviation

S- Slope

Again, the standard deviation of the response can be determined based on the standard deviation of the blank, on the residual standard deviation of the regression line, or the standard deviation of y-intercepts of regression lines. As with LOD, the method used to determine LOQ should be

documented and supported, and an appropriate number of samples should be analyzed at the limit to validate the level. [30, 31, 32]

1.8.6 Linearity and Range

Linearity is the ability of the method to elicit test results that are directly proportional to analyte concentration within a given range. Linearity is generally reported as the variance of the slope of the regression line. Range is the (inclusive) interval between the upper and lower levels of analyte that have been demonstrated to be determined with precision, accuracy, and linearity using the method. The range is normally expressed in the same units as the test results obtained by the method.

A minimum of five concentration levels, along with certain minimum specified ranges are to be determined. For assay tests, the minimum specified range is 80-120% of the target concentration. For impurity tests, the minimum range is from the reporting level of each impurity to 120% of the specification. [30, 31, 32]

The relationship between the concentration (in %) of drug in sample and area of should be linear in the specified range and the correlation should not be less than 0.9.

1.8.7 Robustness

Robustness is the capacity of a method to remain unaffected by small deliberate variations in method parameters. The robustness of a method is evaluated varying method parameters such as percent organic solvent, pH, ionic strength, or temperature and determining the effect (if any) on the results of the method. [30, 31, 32]

The RSD for the assay of drugs in a sample under deliberately modified chromatographic conditions should not be more than 2.0%.

1.8.8 System Suitability

System suitability tests are an integral part of gas and liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as a whole. [47]

System suitability is the checking of a system to ensure system performance before or drying the analysis unknowns. Parameters such as plate count, tailing factor, resolution and reproducibility (% RSD retention time and area for repetitive injections) are determined and compared against the specifications set for the method. These parameters are measured during the analysis of a system suitability sample that is a mixture of main components and expected by-products. [32]

***Table 1.4*:** Acceptance Limits for System Suitability Test. [32]

Parameter	Limit
Capacity Factor	$k' > 2$
Injection precision	RSD < 1% for n ≥ 5
Resolution	$R_s > 2$
Tailing factor	$A_s \leq 2$
Theoretical plates	$N > 2000$

1.9 Definitions and Formulas Used in Method Validation

1.9.1 Mean/ Average (x_i)

The average result (ā) is calculated by summing the individual results and dividing the sum by the number (n) of individual values.

$$x_i = \frac{x_1 + x_2 + x_3 \ldots}{n}$$

Where, x_1, x_2, x_3.. = Values of individual results

n = Number of individual results

1.9.2 Standard Deviation (SD)

It is the root mean square deviation of values from their average.

$$SD = \overline{\frac{\sum(x - x_i)}{n - 1}}$$

Where p = Sum of observations

x_i = Mean or arithmetic average ($\Sigma x / n$)

x = Individual observed value

$x - x_i$ = Deviation of a value from the mean

n = Number of observations

1.9.3 Relative Standard Deviation (RSD)

It is defined as standard deviation expressed as the percentage of mean.

$$RSD = \frac{SD}{x_i} \times 100$$

Where

SD = Standard deviation

x_i = Mean or arithmetic average (Σx / n)

1.9.4 Correlation Co-Efficient (R)

The correlation coefficient is used to indicate the relationship of two random variables. It provides a measure of the strength and direction of the correlation varying from -1 to +1. Positive values indicate that the two variables are positively correlated, meaning the two variables vary in the same direction. Negative values indicate that the two variables are negatively correlated, meaning the two variables vary in the contrary direction. Values close to +1 or -1 reveal the two variables are highly related.

$$R = \frac{n(\sum xy) - (\sum x)(\sum y)}{[\sum x^2 - (\sum x)^2]\,[[\sum y^2 - (\sum y)^2]}$$

Where *n* = number of observations

x = first value

y = second value

p*xy* = sum of products of first and second value

p*x* = sum of first values

p*y* = sum of second values

px^2 = sum of squares of first value

$p y^2$ = sum of squares of second value

1.9.5 Linear Regression

A regression is a statistical analysis assessing the association between two variables. It is used to find the relationship between two variables.

The equation of straight line is

$$y = a + bx$$

Where *b* = slope

a = intercept

$$Slope\ (b) = [n(\Sigma xy) - (\Sigma x)(\Sigma y)] / [n(\Sigma x^2) - (\Sigma x)^2]$$

$$Intercept\ (a) = [(\Sigma y) - b(\Sigma x)] / n$$

Where *n* = number of observations

x = first value

y = second value

p*xy* = sum of products of first and second value

p*x* = sum of first values

p*y* = sum of second values

$p x^2$ = sum of squares of first value

$p y^2$ = sum of squares of second value

Chapter 2: REVIEW OF LITERATURE

ZHANG et. al.,

HPLC-MS/MS method was developed and validated for the simultaneous determination of decitabine and valdecitabine in rat plasma. The analytes were separated on a C18 column (150mm×4.6mm,3.5μm) and a triple-quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source was applied for detection. A clean solid-phase extraction procedure with cation exchange cartridge was employed to extract the analytes from rat plasma with high recovery of decitabine (>82%). The calibration curves were linear over a concentration range of 10-10,000ng/mL for decitabine and 5-500ng/mL for valdecitabine. The lower limit of quantitation (LLOQ) of decitabine and valdecitabine was 10 and 5 ng/mL, respectively. The intra-day and inter-day precisions were less than 15% and the relative error (RE) was all within ±15%. [49]

PRAKASH CHANDAR et. al.,

Fast Analysis of Decitabine Using a Solid Core HILIC Column method was developed by prakash Chandra group by using Accucore HILIC 2.6 μm 150 mm x 3.0 mm as column, 20 mM ammonium acetate in water / acetonitrile (5:95 v/v) as mobile phase, 0.5 mL/min flowrate and detection was carried out at 244 nm. The decitabine was eluted at 5.30min. [50]

TONGQIN et. al.,

Determination of the Content of Decitabine for Injection and Its Related Substances by HPLC method was developed on a Shimpack VP ODS column(250 mm×4.6 mm, 5μm) with 0.01 mol/L solution of potassium phosphate buffer (adjust to pH 6.8 with phosphoric acid)-methanol (98:2) as mobile phase at the flow rate of 1.0 mL/min. The detection wavelength was set at 220 nm. The linear concentration range of decitabine was 5.028-201.12 μg/mLwith the correlation coefficient of 0.999 8. The recoveries(*n*=3) of low, medium and high concentration were 99.8%, 100.2%, 99.6%, and RSD were

0.27%, 0.51%, 0.60%, respectively. Determination of the content of three samples were 100.6%, 99.3%, 99.5%, the related substances were 0.56%, 0.49%, 0.44%.[51]

GLORY HEPSIBA et. al.,

Stability indicating Reverse phase high performance liquid chromatographic method has been developed for the quantitative analysis of Decitabine drug present in tablet formulation and bulk drug. The HPLC separation was achieved on Zorbax bonus C18 Column (250mm x 4.6 mm, i.d, 5 µm particle size) with the mobile phase and detection at 254nm.The proposed method provided linear responses within the concentration range 400-1200 µg/ml for Decitabine and its related compounds. LOD and LOQ values for the active substance were 0.26 and 0.8 µg/mL, respectively. Correlation coefficients (*r*) of the regression equations for the impurities were greater than 0.999 in all cases. The precision of the method was demonstrated using intra- day assay RSD% values which were less than 1% in all instances. [52]

YOUXI ZHANG et. al.,

HPLC–MS/MS method was developed and validated for the simultaneous determination of decitabine and valdecitabine in rat plasma. The analytes were separated on a C18 column (150 mm × 4.6 mm, 3.5 µm) and a triple-quadrupole mass spectrometer equipped with electro spray ionization (ESI) source was applied for detection. A clean solid-phase extraction procedure with cation exchange cartridge was employed to extract the analytes from rat plasma with high recovery of decitabine (>82%). The calibration curves were linear over a concentration range of 10–10,000 ng/mL for decitabine and 5–500 ng/mL for valdecitabine. The lower limit of quantitation (LLOQ) of decitabine and valdecitabine was 10 and 5 ng/mL, respectively. The intra-day and inter-day precisions were less than 15% and the relative error (RE) was all within ±15%.[53]

DRUG PROFILE

2.2 DECITABINE [48]

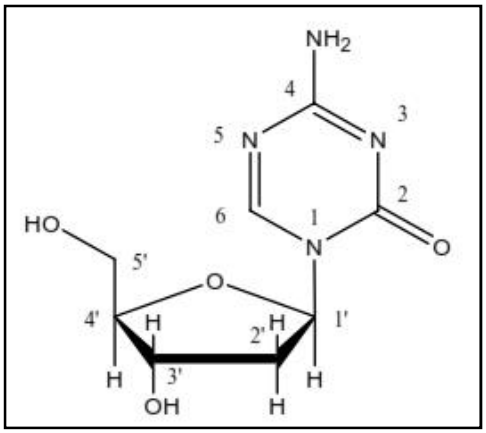

Fig. 3.1: Chemical structure of Decitabine.

IUPAC Name: 4-amino-1-(2-6 deoxy-β-D-erythro-pentofuranosyl)-1,3,5-triazin-2(1*H*)-one

Molecular Formula: $C_8H_{12}N_4O_4$

Molecular Weight: 228.21

Category: Antineoplastic.

Description: A white to off-white crystalline powder.

Solubility: Sparingly soluble in water and soluble in DMSO.

Storage: should be kept in a tightly closed container, protected from light.

Mechanism of action:

Decitabine is believed to exert its antineoplastic effects after phosphorylation and direct incorporation into DNA and inhibition of DNA methyltransferase, causing hypomethylation of DNA and cellular differentiation or apoptosis.

Chapter 3: AIM AND PLAN OF WORK

3.1 AIM

To develop New Rapid, Precise, Accurate RP HPLC method for the estimation of decitabine in pharmaceutical dosage form.

3.2 PLAN OF WORK

- Solubility determination of Decitabine in various solvents and buffers.
- Determine the absorption maxima of both the drugs in UV–Visible region in different solvents/buffers and selecting the solvents for HPLC method development.
- Optimize the mobile phase and flow rates for proper resolution and retention times.
- Validate the developed method as per ICH guidelines.

4. MATERIALS AND METHODS

4.1 MATERIALS

Instruments

***Table 4.1*:** Instruments used.

UV-Visible Spectrophotometer	Analytical Technologies Ltd
HPLC	Cyberlab (Salo Terrace, Millbury, USA)
Ultra Sonicator	Citizen, Digital Ultrasonic Cleaner
pH meter	Elico
Electronic balance	Shimadzu
Syringe	Hamilton
HPLC Column	Develosil Rp Aqueous-AR-5 (150 x 4.6 mm , 5µm)

Chemicals

***Table 4.2*:** Chemicals and Solvents used.

Ammonium Acetate	Rankem/ AR Grade
Acetonitrile	Merck/ HPLC Grade
Water	Merck/ HPLC Grade
Methanol	Merck/ HPLC Grade
Ortho phosphoric acid	Rankem/ AR Grade

Drug samples

***Table 4.3*:** Drugs used.

Decitabine bulk drugs	Gift samples obtained from Natco Pharma Ltd, HYD
Decitabine dosage form	Obtained from local pharmacy

4.2 METHODS

Preparation of Ammonium Acetate buffer pH 4.5:

3.85 gm of Ammonium Acetate was weighed and dissolved in 1000 mL of water. Adjust the pH to 4.5 ± 0.02 using ortho phosphoric acid. The buffer was filtered through 0.45µm filters to remove all fine particles and gases.

Preparation of mobile phase:

The mobile phase was prepared by mixing the buffer and acetonitrile in the ratio of 985:15, sonicate for 5 min. ofter the solution was filtered through 0.45µm to remove all fine particles and gases.

Chapter 5: RESULTS AND DISCUSSIONS

5.1 Solubility Studies

These studies are carried out at 25 ^{0}C

***Table 4.1*:** Solubility studies.

Solvent Name	Decitabine
Water	Soluble
DMSO	Freely Soluble
Ethanol	Sparingly Soluble

5.2 Determination of Working Wavelength (λ_{max})

In simultaneous estimation of two drugs isobestic wavelength is used. Isobestic point is the wavelength where the molar absorptivity is the same for two substances that are interconvertible. So this wavelength is used in simultaneous estimation to estimate both drugs accurately.

5.2.1 Preparation of Standard solution

About 100 mg of decitabine weighed into a 100 mL volumetric flask, to this 25 mL of mobile phase was added, sonicated and the volume was made up to mark with the mobile phase.

5.2.2 Dilutions

Necessary dilutions are made from standard stock solutions to get the concentration range of 100 µg/mL of decitabine.

The wavelength of maximum absorption (λ_{max}) of the solution of the drugs in mobile phase were scanned using UV-Visible spectrophotometer within the wavelength region of 200–400 nm against mobile phase as blank. The absorption

curve shows characteristic absorption maxima at 244 nm for decitabine (Fig.5.1).Thus 255 nm was selected as detector wavelength for the HPLC chromatographic method.

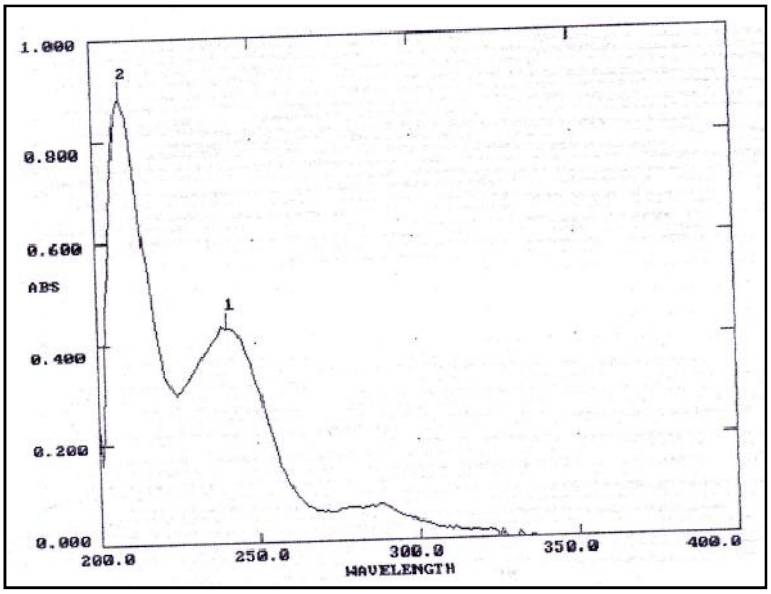

***Fig. 5.1*:** UV-VIS Spectrum of Decitabine

5.3 METHOD DEVELOPMENT FOR ASSAY

Trial -1

Chromatographic conditions

Column	:	Develosil Rp Aqueous-AR-5 (150 x 4.6 mm , 5 µm)
Elution mode	:	Isocratic
Mobile phase	:	phosphate buffer pH 6.5: ACN [40:60]
Flow rate	:	1.0 mL /min
Detection wavelength	:	226 nm
Injection volume	:	20 µL
Run time	:	7 min

Above conditions mixed standard solution is used for recording chromatogram (Fig. 5.2.).

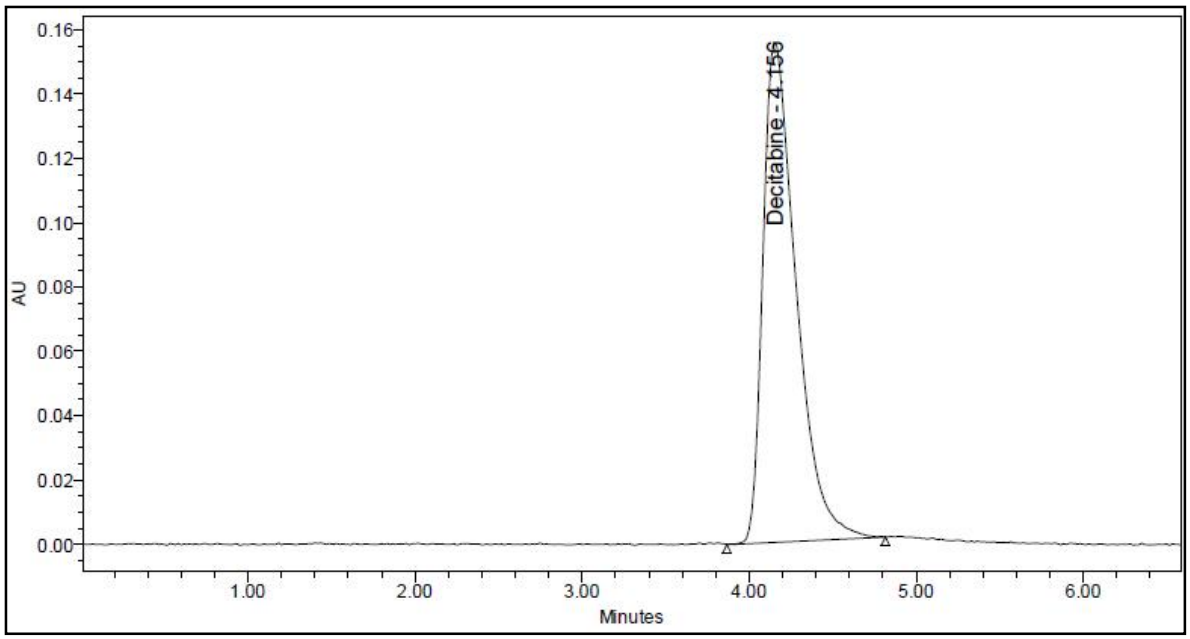

***Fig. 5.2*:** Chromatogram of Trail 1

***Table 5.2*:** Results for Trail 1

S.NO	Name	RT	Area	TP	TF
1	Decitabine	4.156	2145991	2067	1.62

Observation

The two peaks are not well resolved. Resolution was found to be less than 2 (Table 5.2). So this trial is not considered.

Trial -2

Chromatographic conditions

Column : Develosil Rp Aqueous-AR-5 (150 x 4.6 mm , 5 µm)

Elution mode : Isocratic

Mobile phase : ACN:Methanol: Buffer pH 5 (20:40:40)

Flow rate : 1.0 mL/min

Detection wavelength : 228 nm

Injection volume : 20 µL

Run time : 9 min

Mixed standard solution is used for recording chromatogram.

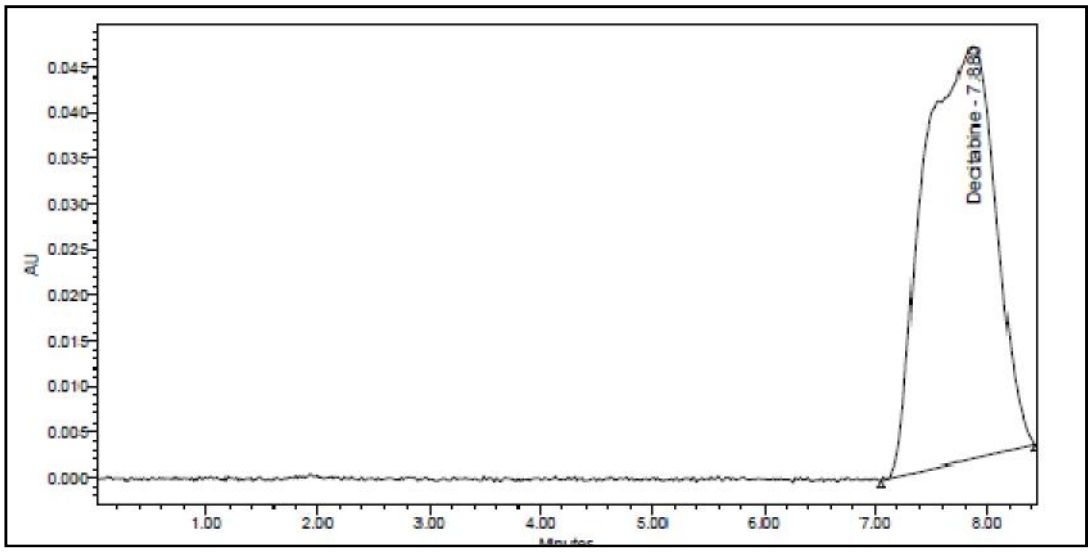

Fig.5.3: Chromatogram of Trail 2

Table 5.3**:** Results for Trail 2

S.NO	Name	RT	Area	TP	TF
1	Decitabine	7.880	2032510	805	0.84

Observation

It was observed that theoretical plates are very low. So this trial was not considered.

Trial -3

Chromatographic conditions

Column : Develosil Rp Aqueous-AR-5 (150 x 4.6 mm , 5 µm)

Elution mode : Isocratic

Mobile phase : Ammonium Acetate buffer pH4.5: Acetonitrile (985:15)

Flow rate : 1.5 mL/min

Detection wavelength : 244 nm

Injection volume : 20 µL

Run time : 10 min

Mixed standard solution is used for recording chromatogram.

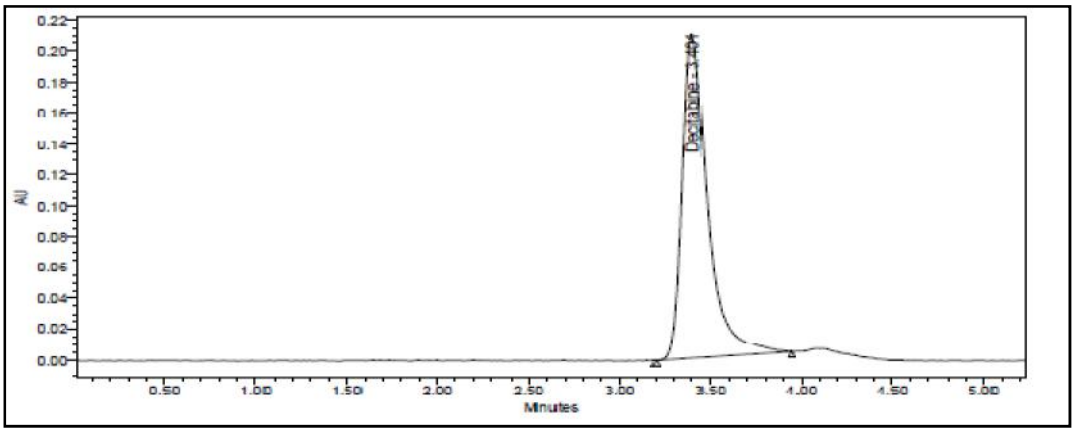

***Fig. 5.4*:** Chromatogram of Trail 3

***Table 5.4*:** Results for Trail 3

S.NO	Name	RT	Area	TP	TF
1	Decitabine	3.410	2001568	3396	1.51

Observation

The decitabine peak was observed at 3.410 min with peak area 2001568, theoretical plates 3396 and tailing factor 1.51. The Theoritical plates, tailing factor and resolution were found to be within limits. So this trail was considered and validated according to ICH guidelines.

Conclusion: Hence this method was finalized for the estimation of decitabine.

5.4 OPTIMIZED CHROMATOGRAPHIC CONDITIONS FOR ASSAY

***Table 5.5*:** Optimised condition.

Mobile phase	Ammonium Acetate buffer pH 4.5:ACN (985:15)
Column	Develosil Rp Aqueous-AR-5 (150 x 4.6 mm , 5 µm)
Flow rate	1.5 mL/min
Column temperature	Room temperature(20-25^{o}C)
Sample temperature	Room temperature(20-25^{o}C)
Wavelength	244 nm
Injection volume	20 µL
Run time	5 min
Retention time	3.401 min for decitabine

Preparation of samples for Assay

Preparation of Standard solution

About 100 mg of decitabine was weighed into a 100 mL volumetric flask, to this 25mL of mobile phase was added, sonicated and the volume was made up with the mobile phase.

Preparation of Sample solution

Sample name : Decogen

Manufacture name : Pfizer

Weigh a quantity of powder equivalent to 100 mg of decitabine in 100 mL volumetric flask and make up mark with mobile phase. From above solution Pipette 1 mL of the clear solution in to 10 mL volumetric flask and make up volume with mobile phase. The resulting solution is used to record the chromatogram (Fig. 7.9).

$$\% \text{ Assay} = \frac{AT}{AS} \times \frac{\text{WS}}{DS} \times \frac{\text{DT}}{WT} \times \frac{\text{P}}{100} \times \frac{\text{AW}}{LC} \times 100$$

Where,

AS: Average peak area due to standard preparation

AT: Peak area due to assay preparation

WS: Weight of decitabine in mg

WT: Weight of sample in assay preparation

DT: Dilution of assay preparation

DS: Dilution of standard preparation

P: Purity of decitabine

AV: Average weight of tablets in mg

LC: Labelled claim of decitabine

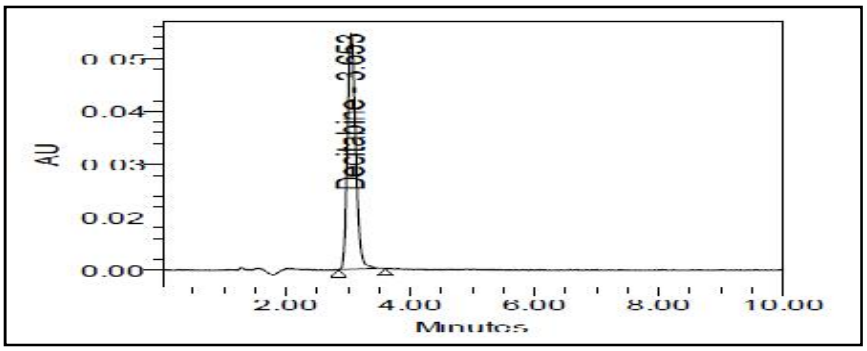

***Fig. 5.5*:** Chromatogram of Decitabine.

***Table 5.6*:** Results for Decitabine.

S.NO	Name	RT	Area	TP	TF
1	decitabine	3.653	345505	6599	1.18

***Table 5.7*:** Results of assay

Drug	**Label claim(mg)**	**Amount found(mg)**	**% Assay**
Decitabine	50	49.95	99.9

Observation

So the % assay found to be within the limits. The percentage purity of Decitabine was found to be within the limits that is 98-102 %.

5.5 HPLC METHOD VALIDATION

5.5.1 System Suitability

To verify that the analytical system is working properly and can give accurate and precise results were evaluated by 100 µg/mL of Decitabine was injected six times and the chromatograms were recorded for the same.

***Table 5.8*:** Results for system suitability of decitabine.

Injection	RT	Peak area	Theoretical plates (TP)	Tailing factor (TF)
1	3.793	345849	6679	1.22
2	3.799	345177	6441	1.23
3	3.72	344045	6552	1.22
4	3.726	345849	6551	1.24
5	3.797	347590	6283	1.21
6	3.799	345784	6329	1.23
Mean	3.772333	345715.7	-	-
SD	0.038323	1151.924	-	-
%RSD	1.015901	0.3332		--

Acceptance criteria

1. The % RSD for the retention times of decitabine Peaks from 6 replicate injections of each Standard solution should be not more than 2.0

2. The % RSD for the peak area responses of decitabine peaks from 6 replicate injections of each standard solution should be not more than 2.0%.

3. The number of theoretical plates (N) for the decitabine peaks is not less than 2000.

4. The Tailing factor (TP) for the decitabine peak is not more than 2.0.

Result

The plate count and tailing factor results were found to be satisfactory and are found to be within the limit. The % RSD was found to be 0.33.

5.5.2 Precision

5.5.2.1 System precision

The system precision was determined by analysing standard preparation of decitabine for six times. The chromatograms were recorded and the results were summarized in Table 5.6.

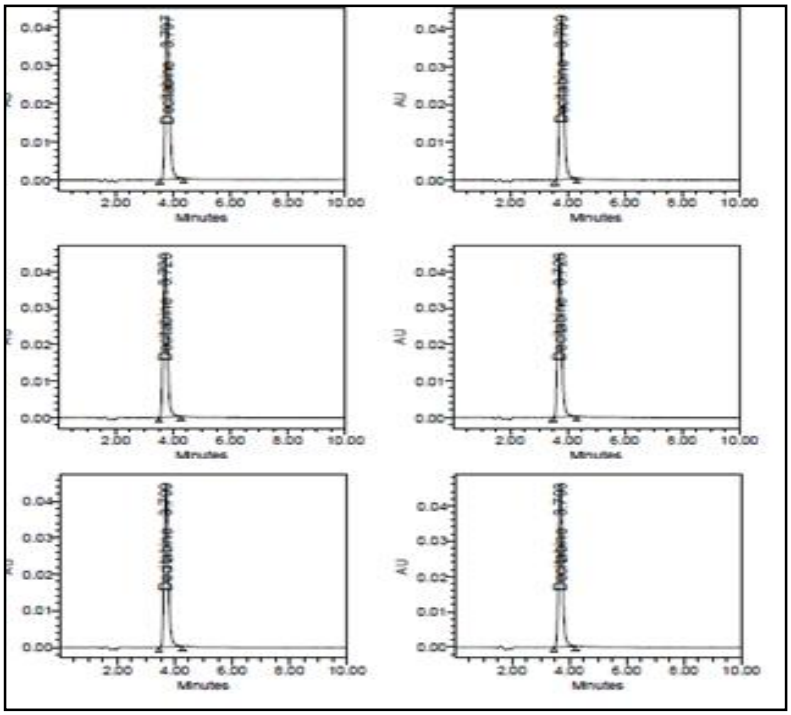

***Fig. 5.6*:** List of Chromatograms for system precision

***Table 5.9*:** Results for system precision

Injection	Decitabine			
	Retention times	**Area**	**TP**	**TF**
1	3.793	345849	6679	1.22
2	3.799	345177	6441	1.23
3	3.72	344045	6552	1.22
4	3.726	345849	6551	1.24
5	3.797	347590	6283	1.21
6	3.799	345784	6329	1.23
Average	3.772333	345715.7	6472.5	1.225
SD	0.038323	1151.924	-	-
%RSD	1.015901	0.3332	-	-

Result

% RSD of 6 determinations of decitabine for System precision found to be within the acceptance criteria of less than 2.0%.

5.5.2.2 Method precision

Method precision was determined by injecting sample solutions of concentration decitabine (100 µg/mL) for six times are prepared separately.

The chromatograms were recorded and the results were summarized in Table 5.10.

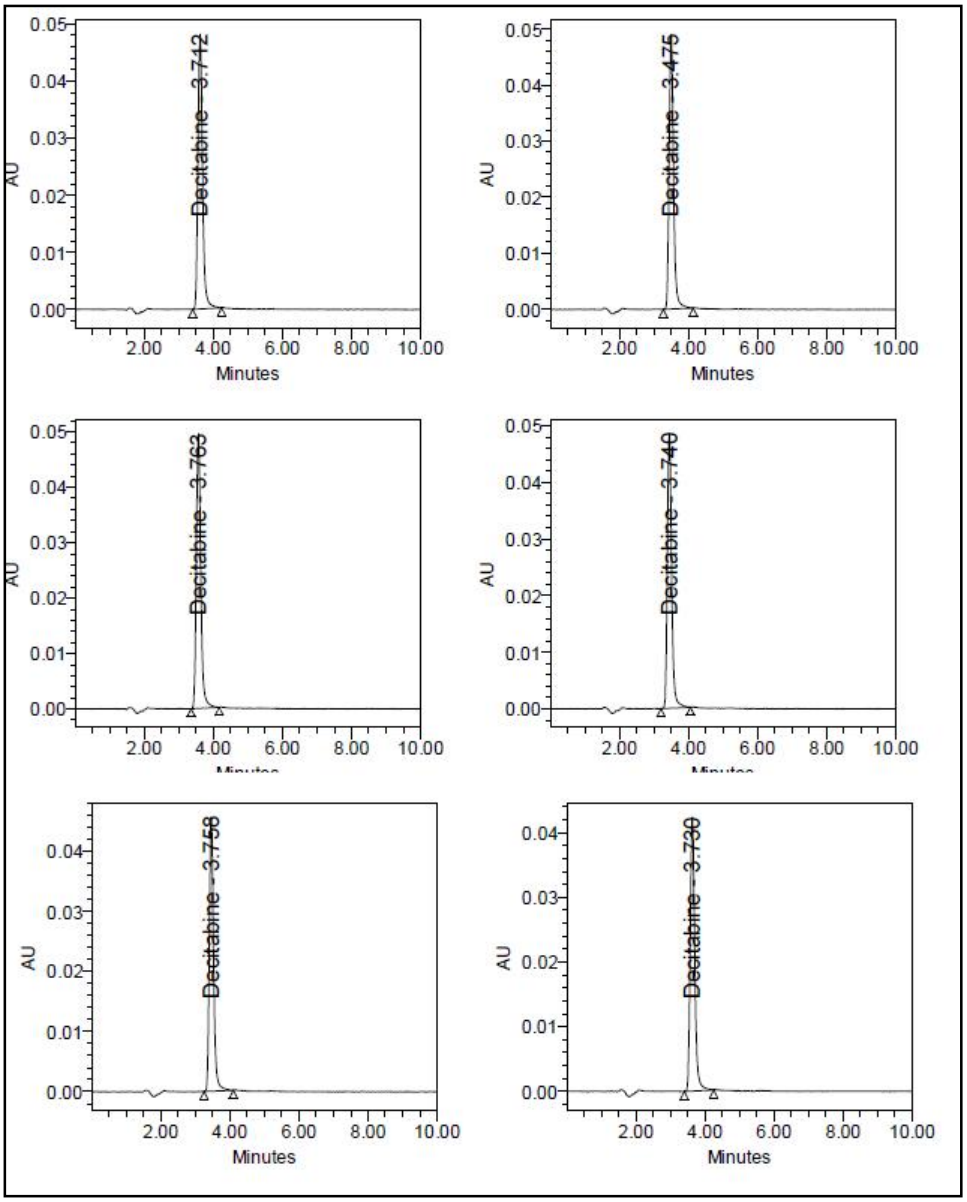

Fig. 5.7: List of Chromatograms for method precision

***Table 5.10*:** Results for method precision

Injection	Decitabine			
	Retention times	**Area**	**TP**	**TF**
1	3.74	345746	6883	1.25
2	3.758	344016	6140	1.23
3	3.795	347992	6829	1.22
4	3.763	346110	6874	1.22
5	3.712	345853	6822	1.21
6	3.73	345690	6932	1.22
Average	3.749667	345901.2	6746.667	1.225
SD	0.028987	1268.072	-	-
%RSD	0.773065	0.3666	-	-

Result

The %RSD of 6 determinations of decitabine for System precision found to be within the acceptance criteria of less than 2.0%.

5.5.3 Linearity and range

Preparation of standard stock solution

Standard stock solutions of decitabine (mg/mL) were prepared by dissolving 100 mg of decitabine in 100 mL of mobile phase. After that filtered the solution using 0.45-micron syringe filter and Sonicated for 5 min further dilutions were given in the Table 8.17.

***Table 5.11*:** Linearity Preparations.

Preparations	Volume from standard stock transferred in mL	Volume made up in mL (with mobile phase)	Conc. obtained (µg/mL)
			Decitabine
Preparation 1	0.5	10	50
Preparation 2	0.7	10	70
Preparation 3	1	10	100
Preparation 4	1.2	10	120
Preparation 5	1.5	10	150

The above prepared dilutions were in injected into the system and the chromatograms were recorded as given in Fig. 5.8, 5.9, 5.10, 5.11, 5.12 and the results of the chromatograms are given in Tables 5.12, 5.13, 5.15, 5.15, 5.16 respectively.

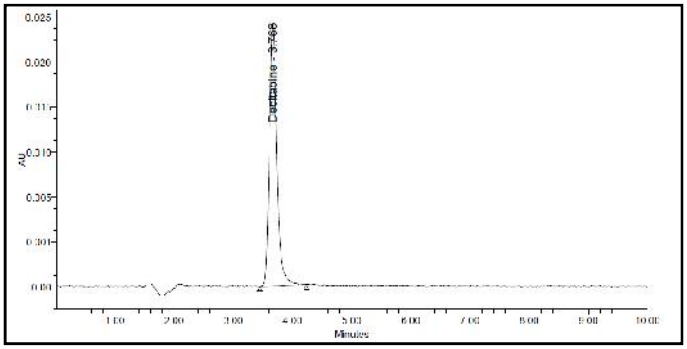

***Fig 5.8*:** Chromatogram of linearity for preparation 1.

***Table 5.12*:** Results for preparation 1.

S.NO	Name	RT	Area	TP	TF
1	Decitabine	3.768	174482	4317	1.24

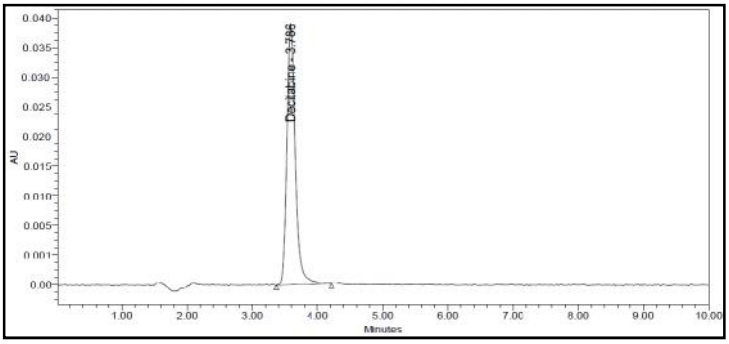

***Fig 5.9**:* Chromatogram of linearity for preparation 2.

***Table 5.13*:** Results for preparation 2.

S.NO	Name	RT	Area	TP	TF
1	Decitabine	3.786	232730	4107	1.24

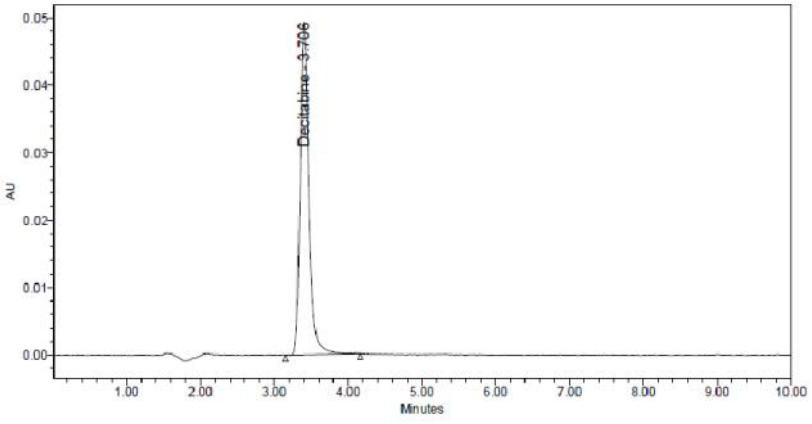

***Fig 5.10*:** Chromatogram of linearity for preparation 3.

***Table 5.14*:** Results for preparation 3.

S.NO	Name	RT	Area	TP	TF
1	Decitabine	3.706	345818	6334	1.24

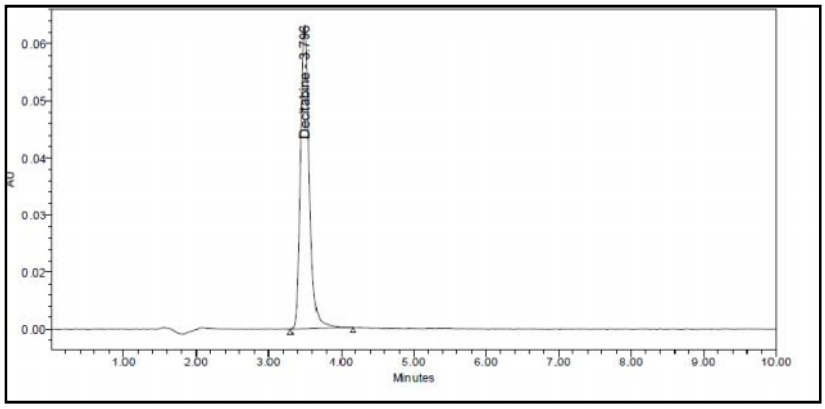

***Fig 5.11*:** Chromatogram of linearity for preparation 4.

***Table 5.15*:** Results for preparation 4.

S.NO	Name	RT	Area	TP	TF
1	Decitabine	3.796	423221	6019	1.20

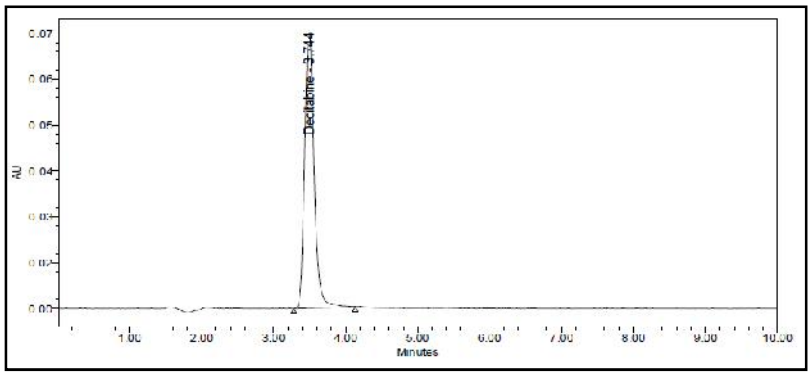

***Fig 5.12*:** Chromatogram of linearity for preparation 5.

***Table 5.16*:** Results for preparation 5.

S.NO	Name	RT	Area	TP	TF
1	Decitabine	3.794	518543	6623	1.20

A graph was plotted against the concentrations of the solutions and the peak areas (Table 8.17). The correlation coefficient R^2 was determined and was found to be 0.998 (Fig. 8.13).

***Table 5.17*:** Linearity data of Decitabine.

S.No	Concentration (µg/mL)	Area
1	50	174482
2	70	232730
3	100	345818
4	120	423221
5	150	518543

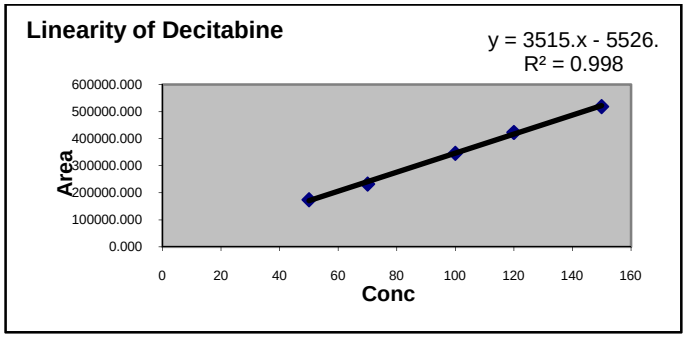

***Fig 5.13*:** Graph for Linearity data of Decitabine.

Table 5.18: Observation for linearity.

S.No	Parameter	Decitabine
1	Correlation coefficient	0.998
2	Slope	3515
3	Intercept	5526

Acceptance criteria

The relationship between the concentration (in %) and area of decitabine should be linear in the specified range and the correlation should not be less than 0.99.

Result

The correlation coefficient for linear curve obtained between concentration vs. Area for standard preparations of decitabine was found to be 0.998 respectively.

5.5.4 Limit of Detection and Limit of Quantitation

LOD and LOQ is calculated from standard deviation of response from precision and slope from linearity

$$LOQ = 10\ \sigma / S$$

$$LOD = 3.3\ \sigma / S$$

Where

σ is standard deviation from response

S is slope from calibration curve

The LOD for this method was found to be 0.0003 µg/mL. The LOQ for this method was found to be 0.0009 µg/mL.

5.5.5 Specificity

The standard solution 100 µg/mL of decitabine was injected and the chromatogram was recorded for the same as given in Fig. 5.14.

The sample solution 100 µg/mL of decitabine was injected and the chromatogram was recorded for the same as given in Fig. 5.15.

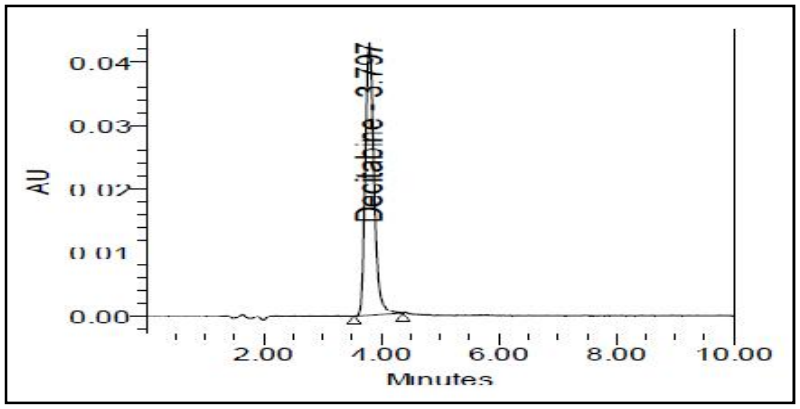

***Fig 5.14*:** Chromatogram of standard.

***Table 5.19*:** Results for standard.

S.NO	Name	RT	Area	TP	TF
1	Decitabine	3.793	345849	6679	1.22

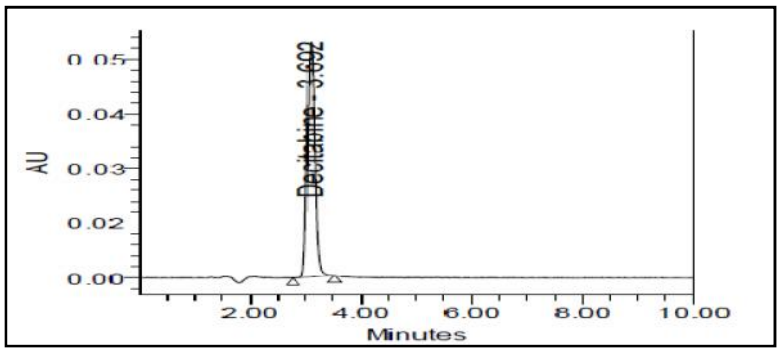

***Fig 5.15*:** Chromatogram of formulation.

***Table 5.20*: *R*esults for formulation.**

S.NO	Name	RT	Area	TP	TF
1	Decitabine	3.653	345505	6599	1.18

Result

It was observed that diluent or excipient peaks do not interfere with analyte peak.

5.5.6 Accuracy

Accuracy of the method was determined by Recovery studies. To the formulation (pre analysed sample), the reference standards of the drugs were added at the level of 50%, 100%, 150%. The recovery studies were carried out three times and the percentage recovery and percentage mean recovery were calculated for drug is shown in Table 8.24.

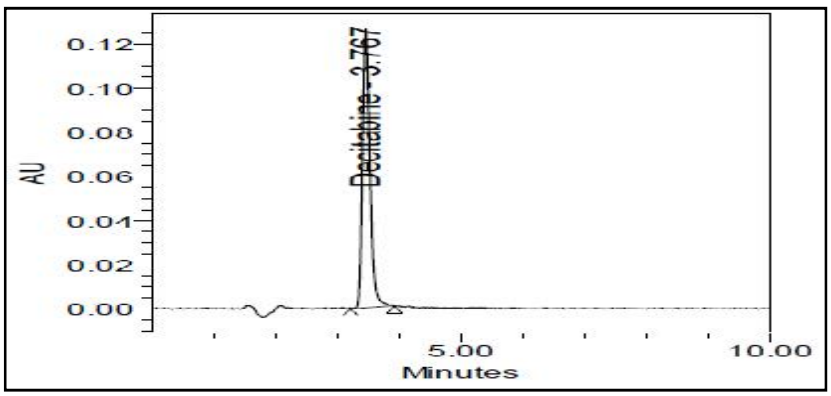

Fig 5.16: Chromatogram of 50% recovery

***Table 5.21*: Results for 50% Recovery.**

S.NO	Name	RT	Area	TP	TF
1	Decitabine	3.795	172873	6241	1.26

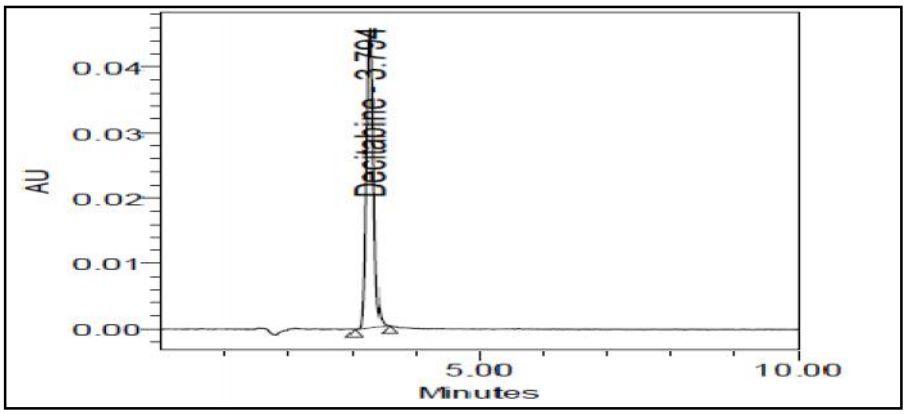

***Fig 5.17*:** Chromatogram of 100% recovery

***Table 5.22*:** Results for 100% Recovery.

S.NO	Name	RT	Area	TP	TF
1	Decitabine	3.770	344556	6395	1.11

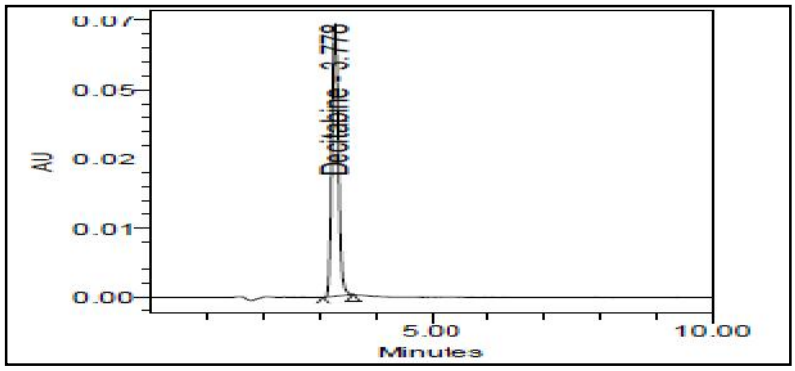

***Fig 5.18**:* Chromatogram of 150% Recovery.

***Table 5.23*:** Results for 150% Recovery.

S.NO	Name	RT	Area	TP	TF
1	Decitabine	3.751	518619	6732	1.06

***Table 5.24*:** Results for Recovery of Decitabine.

Conc	Amount present (µg/mL)	Amount added (µg/mL)	Amount found (µg/mL)*	Percent Recovery *	% Mean Recovery
50%	40	10	49.56	99.13	99.77
100%	90	10	100.02	100.02	
150%	140	10	150.27	100.18	

***** Mean of three observations

Acceptance criteria

The % recovery of Decitabine should lie between 98% and 102%.

Result

The percentage mean recovery of Decitabine was found to be 99.77% respectively.

5.5.7 Robustness

The Robustness of the method was determined. The results obtained by deliberate variation in method parameters are summarized below in Table 5.29.

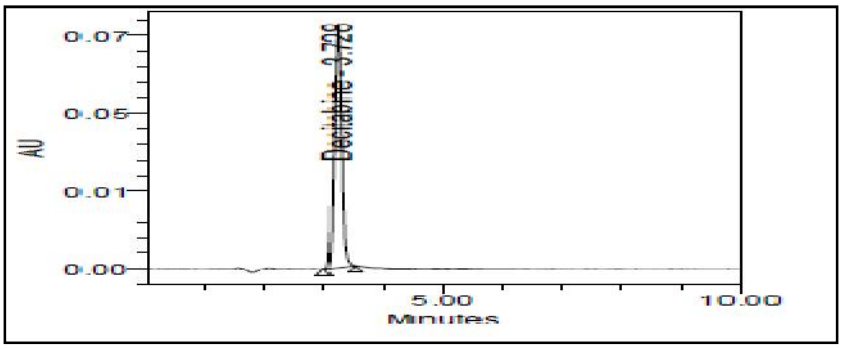

***Fig. 5.19**:* Chromatogram of mobile phase composition 987:13.

***Table 5.25*:** Results of mobile phase composition 987:13.

S.NO	Name	RT	Area	TP	TF
1	Decitabine	3.728	343650	6027	1.05

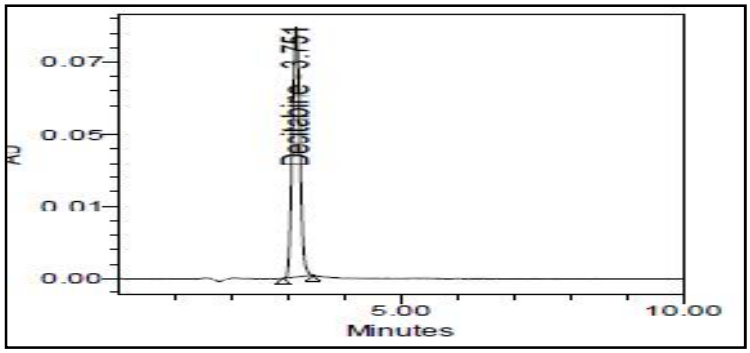

***Fig.5.20*:** Chromatogram of mobile phase composition 983:17.

***Table 5.26*:** Results of mobile phase composition 983:17.

S.NO	Name	RT	Area	TP	TF
1	Decitabine	3.751	344556	6732	1.06

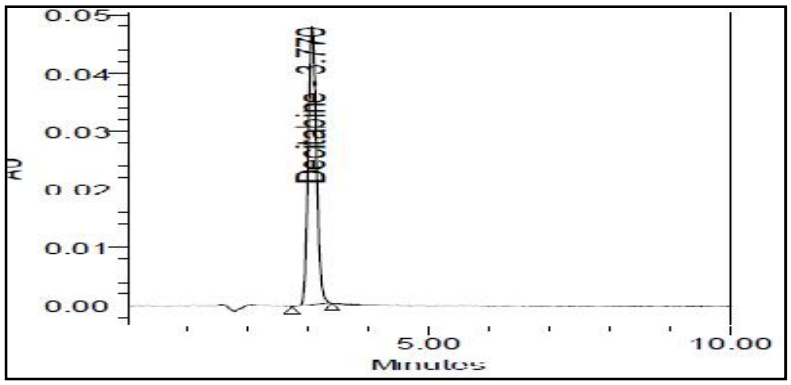

***Fig. 5.21*:** Chromatogram of wavelength from 244 to 246 nm.

***Table 5.27*:** Results of wavelength from 244 to 246 nm.

S.NO	Name	RT	Area	TP	TF
1	Decitabine	3.770	344556	6395	1.11

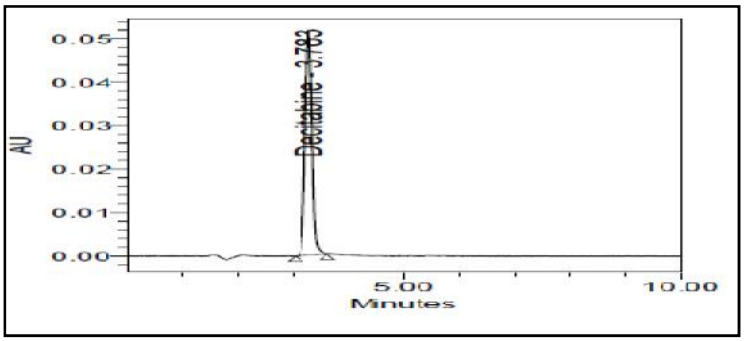

***Fig. 5.22*:** Chromatogram of wavelength from 244 to 242 nm.

***Table 5.28*:** Results of wavelength from 244 to 242 nm.

S.NO	Name	RT	Area	TP	TF
1	Decitabine	3.783	345432	6459	1.07

***Table 5.29*:** Results for Robustness of Decitabine.

Chromatographic changes		**Retention time(min)**	**Tailing factor**
wavelength (nm)	242	3.783	1.07
	244	3.768	1.24
	246	3.770	1.11
Mobile phase composition(v/v)	983:18	3.751	1.06
	985:15	3.768	1.24
	987:13	3.728	1.05

Result

The tailing factor was found to be within the limits on small variation of flow rate and wavelength.

PChapter 6: SUMMARY

Each and every day a number of diseases are being diagnosed. Several cancer diseases are on a rise not only for the rich but also for the poor. So, various pharmaceutical organizations are working to develop new drug molecules and new combinations of anticancer drugs for better treatment. This is the reason for a greater competition in the pharmaceutical sector, and the future scenario is likely to be the same.

The scope of developing and validating a method is to ensure a suitable strategy for evaluation of a particular analyte which is more specific, accurate and precise. The main focus is drawn to achieve improvement in the manufacturing and analytical conditions and making proper amendments in the standard operating procedures being followed.

The above review indicates that there are fewer methods for the estimation of Decitabine in pharmaceutical formulations. But the buffers used in this method was at acidic pH which may affect the column life and some method were with more a run time. So my aim was to develop a new method with minimum run time and less solvent consumption for the estimation of Decitabine in combination of drugs. Hence the present study aims to develop rapid, precise and accurate methods for the determination of Decitabine by RP-HPLC in pharmaceutical dosage forms.

CONCLUSION

A new precise, accurate, rapid method has been developed for the simultaneous estimation of Decitabine in pharmaceutical dosage form by RP-HPLC.

The optimum wavelength for the determination of Decitabine was selected at 244 nm. Various trials were performed with different mobile phases in different ratios, but Ammonium Acetate buffer pH 4.5: ACN (985:15)

was selected as good peak symmetry. The Retention time of decitabine was found to be 3.786 min.

The different analytical performance parameters such as linearity, precision, accuracy, and specificity, LOD, LOQ were determined according to International Conference on Harmonization ICH Q2B guidelines. The calibration curves were obtained by plotting peak area versus the concentration over the range of 50-150 µg/mL. From linearity the correlation coefficient R^2 value was found to be 0.998. The proposed HPLC method was also validated for system suitability, system precision and method precision. The % RSD in the peak area of drug was found to be less than 2%. The number of theoretical plates was found to be more than 2000, which indicates efficient performance of the column. The LOD for this method was found to be 0.0003µg/mL. The LOQ for this method was found to be 0.0009 µg/mL, indicates the sensitivity of the method. The percentage of recovery of was found to be 99.77 shows that the proposed method is highly accurate.

Hence the proposed method is highly sensitive, precise and accurate and it successfully applied for the quantification of API content in the commercial formulations of decitabine in Educational institutions and Quality control laboratories.

BIBLIOGRAPHY

1. The Drugs and Cosmetics Act and Rules, 1940.
2. Methods of Analysis-

   http://www.pharmatutor.org/pharma-analysis
3. Douglas, A.; Skoog, F.; James, H.; Stanley, R. C. Liquid Chromatography. In *Instrumental Analysis*, 9th ed.; Cengage Learning India Pvt. Ltd.: New Delhi, 2007; 893 - 934.
4. Skoog**;** Holler**;** Crouch**;** Liquid Chromatography**.** In *Instrumental Analysis***,** Cengage Learning India**.:**New Delhi**.** 2011**;** 893**.**
5. Chatwal**,** R**.** G**.;** Anand**,** K**.** S**.** High Performance Liquid Chromatography**.** In *Instrumental Methods Of Chemical Analysis***,** 5th ed**.;** Himalaya Publishers**.:** Mumbai, 2010**;** 2.570 - 2.629**.**
6. Sharma**,** B**.** K**.** High Performance Liquid Chromatography**.** In *Instrumental Methods Of Chemical Analysis***,** 24th ed**.;** Goel Publishers**.:** Meerut**,** 2005**;** 295 - 300**.**
7. Alfonso, R. G.; Ara, H. D. M.; Glen, R. H.; Thomas, M.; Nicholas, G. P.; Roger, L.S.; Steve, H. W. Chromatography. In *Remington: The Science and Practice of Pharmacy*, 20th ed.; Lippincott Williams & Wilkins: Philadelphia, 2000; 587
8. Adsorption Chromatography-
   http://www.separationprocesses.com/Adsorption/AD_Chp05a.htm
9. Adsorption Chromatography-
   http://cemca.org/andcollege/andcwebsite/subject01/CHEtext.pdf
10. Types of Chromatography-
    http://www.separationprocesses.com/Adsorption/AD_Chp05a.htm
11. Partition Chromatography -
    http://media.rsc.org/Modern%20chemical%20techniques/MCT5%20Chromatography.pdf

12. Ion Exchange Chromatography - http://www.gelifesciences.com/webapp/wcs/stores/servlet/catalog/en/GELifeSciences-IN/products/ion-exchange-chromatography-iex/
13. Ion Exchange Chromatography- http://wolfson.huji.ac.il/purification/PDF/IonExchange/AMERSHAM_iIEXandChromatofocManual.pdf
14. Size exclusion chromatography- http://www.rpi.edu/dept/chemeng/BiotechEnviron/CHROMO/be_types.htm
15. Chiral phase chromatography- http://scholar.lib.vt.edu/theses/available/etd32298223814/unrestricted/ch_02.pdf
16. Types of elution- http://chemwiki.ucdavis.edu/@api/deki/pages/402/pdf
17. Types of elution- http://hplc.chem.shu.edu/NEW/HPLC_Book/Rev.-Phase/rp_grad.htmL
18. Types of HPLC- http://www.chem.agilent.com/Library/primers/Public/59896639EN.pdf
19. Diagram of HPLC - http://hiq.lindegas.com/international/web/lg/spg/like35lgspg.nsf/docbyalias/image_hplc
20. Solvent Delivery System - http://www.monzirpal.net/Instrumental%20Analysis/Lectures/Lectures%2021-/L39.pdf
21. Injection valves - http://www.dolomitemicrofluidics.com/webshop/flowaccessoriesinjection-valves-c-17_18/sample-injection-valve-p-783
22. Injection valves - http://weather.nmsu.edu/Teaching_Material/SOIL698/Student_Material/HPLCHP1090/HPLCINJ.HTM

23. Flow path of a Manual Injector - http://polymer.ustc.edu.cn/xwxx_20/xw/201109/P020110906263097048536.pdf

24. Braithwaite, A.; Smith, F. J. Liquid Phase Chromatography on Columns. In Chromatographic methods, 5th ed.; Kluwer Academic Publishers: Netherlands, 1999; 129.

25. Columns - International pharmacopeia, 4th edition - http://apps.who.int/phint/en/p/docf/

26. Detectors –

http://lipidlibrary.aocs.org/topics/detect92/file.pdf

27. Detectors –

http://www.shodex.net/index.php?seitenid=1&applic=1485

28. Method development - http://www.pharmainfo.net/reviews/introductionanalyticalmethoddevelopmentpharmacutical-formulations

29. Manoj, K. S.; Pramod, K. S.; Sambhu, C. M.; Preet, K. K.; Nitin, K.;Rupesh, D. A perspective review on method development and validation by HPLC. *International Journal of Pharmaceutical Sciences.* **2011**, *4*, 1387-1413.

30. International Conference on Harmonization, "Q2A: Text on Validation of Analytical Procedures," *Federal Register.* **1995**, *60*, 11260–11262.

31. International Conference on Harmonization, "Q2B: Validation of Analytical Procedures: Methodology; Availability," *Federal Register*. **1997**, *62*, 27463–27467.

32. Michael Swartz, E.; Ira Krull, S, Analytical Method development. In Analytical Method Development and Validation, 1st ed.; Marcel Dekker, Inc: New York, 2009; 17-80.

33. Particle Sciences Drug Development Services. Analytic Method Development and Validation. *Technical Brief*. **2009**, *5*, 1-2.

34. Ghulam, A. S. PLC Method Development and Validation for Pharmaceutical Analysis. *Pharmaceutical Technology Europe*. **2004**, 7, 55 -63.

35. Radhika, R.; Alfred, D. G. Guidance for Industry- Analytical Procedures and Methods Validation. *Federal Register*, **2000**, *2396*, 1-32.

36. Effect of flow rate - http://www.ionsource.com/tutorial/chromatography/rphplc.htm

37. Effect of flow rate –

http://www.ionsource.com/Card/linvelocity/linvol.htm

38. Brian, L. H.; Thomas, E. B. The Influence of Column Temperature on HPLC Chiral Separation on Macrocyclic Glycopeptide CSPs. Advanced Separation Technologies Inc. (Astec). New Jersey, USA.

39. Effect of temperature –

http://www.pharmtech.com/pharmtech/Analytical/UsingHighTemperature HPLC--for-ImprovedAnalysis/ArticleStandard/Article/detail/97082

40. Effect of pH-

http://www.laserchrom.co.uk/LaserchromHPLC-pHBufferGuide.htm

41. Effect of pH - Technical tips-selecting buffer ph in reverse phase HPLC

42. Effect of pH- http://www.sigmaaldrich.com/content/dam/sigmaaldrich/docs/Fluka/General_InformatIon/analytixnotes_lpc_lowres.pdf

43. Effect of ion-pair reagent- http://www.standardbase.com/tech/HPLC%20validation%20PE.pdf

44. Peak shapes - http://www.chem.agilent.com/Library/eseminars/Public/secrets%20of%20good%20peak%20shape%20in%20hplc.pdf

45. Rajesh, K. P. Overview of Pharmaceutical Validation and Process Controls In Drug Development. *Der Pharmacia Sinica*. **2010**, *1*, 11 - 19.

46. Jay, B.; Kevin, J.; Pierre, B. Understanding and Implementing Efficient Analytical Methods Development and Validation. *Pharmaceutical Technology Analytical Chemistry & Testing*. **2003**, *5*, 6 - 13.

47. Ludwig, H. Validation of Analytical Methods. Agilent technologies. **2007**, 1- 65.

48. Decitabine – http://www.rxlist.com/dacogen-drug.htm

49. Zhang, Y.; Sun, J.; Gao, Y.; Kong, Y.; Xu, Y.; Jia, W.; Liao, C.; Zhang, P.; Lian, H.; Han, X.; Li, D.; Geng, Y.; He, Z. An HPLC-MS/MS method for simultaneous determination of decitabine and its valyl prodrug valdecitabine in rat plasma. Chromatogr B Analyt Technol Biomed Life Sci. 2013, 917-918.

50. Prakash Chander, Tushar N. Mehta. Fast Analysis of Decitabine Using a Solid Core HILIC Column themoscientific.2013, 1-3.

51. JI Tongqin, ZHAO Yanrong, HAN Jiyong and CHEN Xiangfeng. JMAP, 2012, 29, 352-357.

52. Glory hepsiba, B.B.Teja, K.Ashok kumar, Y.Ravindra reddy.Stability indicating RP-HPLC Method Devlopment and Validation of Decitabine Drug in Formulation International Journal of PharmTech Research. 2011, 3910, 237-243.

53. Youxi Zhanga, Jin Suna, Yikun Gaoa, Ying Konga, Youjun Xub, Weiru Jiac, Chuanrong Liaod, Peng Zhanga, He Liana, Xiaopeng Hana, Dongpo Lia, Yajie Genga, Zhonggui Hea. An HPLC–MS/MS method for simultaneous determination of decitabine and its valyl prodrug valdecitabine in rat plasma. Journal of Chromatography B. 2013, 918, 78-83.