DEVELOPMENT OF NEW RP- HPLC METHOD FOR THE ESTIMATION OF DOLUTEGRAVIR IN THE TABLET DOSAGE FORM AND VALIDATION OF THE METHOD

A Dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY
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In partial fulfillment of the requirements for the award of degree of
MASTER OF PHARMACY
IN
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Submitted by
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This is to certify that the dissertation entitled **"Development of new RP-HPLC method for the estimation of Dolutegravir in the tablet dosage form and validation of the method."** submitted to The Tamilnadu Dr. M.G.R Medical University, Chennai, is a bonafide project work of **Ms. K. SOWMYASREE (Reg. No: 261731107),** carried out in the Department of Pharmaceutical Analysis, Jaya College of Paramedical Sciences, College of Pharmacy, Thiruninravur, Chennai-602 024, in partial fulfillment for the degree of **MASTER OF PHARMACY** under the guidance of **Dr. C.ROOSEWELT, M.Pharm., Ph.D.,** Professor cum HOD, Department of Pharmaceutical Analysis, Jaya College of Paramedical Sciences, College of Pharmacy Thiruninravur Chennai-602 024.

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DECLARATION

I hereby declare that this dissertation entitled **"Development of new RP-HPLC method for the estimation of Dolutegravir in the tablet dosage form and validation of the method"** was a bonafide and original research work carried out in the Department of Pharmaceutical Analysis, Jaya College of Paramedical Sciences, College of Pharmacy, Thiruninravur, Chennai-602 024, under the guidance of **Dr. C. ROOSEWELT, M.Pharm., Ph.D.,** Professor cum HOD, Department of Pharmaceutical Analysis, Jaya College of Paramedical Sciences, College of Pharmacy, Thiruninravur, Chennai-602 024. This dissertation submitted to The Tamilnadu Dr. M.G.R. Medical University, Chennai, as a partial fulfillment for the award of DEGREE OF MASTER OF PHARMACY in **Pharmaceutical Analysis** during the academic year 2018-2019.

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EVALUATION CERTIFICATE

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LIST OF ABBREVIATIONS

AR	:	Analytical Reagent
API	:	Active Pharmaceutical Ingredients
CV	:	Coefficient of Variation
Conc	:	Concentration
EMR	:	Electro Magnetic Radiation
gm	:	Gram
HBr	:	Hydrobromide
GLC	:	Gas Liquid Chromatography
HPLC	:	High Performance Liquid Chromatography
HPTLC	:	High Performance Thin Layer Chromatography
HPLC-MS	:	High Performance Liquid Chromatography and Mass Spectroscopy
ICH	:	International Conferenceon Harmonization
IUPAC	:	International Union of Pure and Applied Chemistry
LC	:	Liquid Chromatography
LC-MS	:	Liquid Chromatography and Mass Spectroscopy
LOD	:	Limit of Detection
LOQ	:	Limit of Quantitation
mcg	:	Microgram
ml	:	Milliliter

mg	:	Milligram
nm	:	Nano meter
N	:	Theoritical plates
pg	:	Picogram
R_f	:	Retardation Factor
R_S	:	Resolution
RP-HPLC	:	Reverse Phase High Performance Liquid Chromatography
RP-TLC	:	Reverse Phase Thin Layer Chromatography
RSD	:	Relative Standard Deviation
R_t	:	Retention time
SD	:	Standard Deviation
TLC	:	Thin Layer Chromatography
T	:	Tailing factor
UV	:	Ultra-violet
USP	:	United States of Pharmacopoeia
v/v	:	Volume/Volume
WHO	:	World Health Organization
λ	:	Wavelength
λmin	:	Wavelength Minima
λmax	:	Wavelength Maxima

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INTRODUCTION

Analytical chemistry is the science that seeks ever improved means of measuring the chemical composition of natural and artificial materials. Chemical composition is the entire picture (composition) of the material at the chemical scale and includes geometric features such as molecular morphologies and distributions of species within a sample as well as single dimensional features such as percent composition and species identity.[1]

To be effective and efficient, analyzing samples requires expertise in

1. The chemistry that can occur in a sample.
2. Analysis and sample handling methods for a wide variety of problems (the tools-of-the-trade).
3. Accuracy and precision of the method.
4. Proper data analysis and record keeping**.**

The major stages of an analytical process are described as follows:

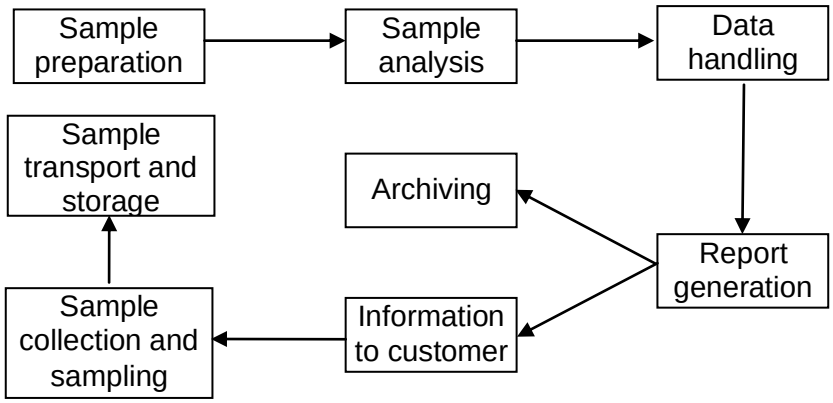


Fig : 1 Steps in analytical cycle

The pharmaceutical analysis comprises the procedures necessary to determine the "identity, strength, quality and purity" of such compounds. It also includes the analysis of raw material and intermediates during manufacturing process of drugs.

Types

Qualitative analysis

- Qualitative inorganic analysis seeks to establish the presence of a given element or inorganic compound in a sample.
- Qualitative organic analysis seeks to establish the presence of a given functional group or organic compound in a sample.

Quantitative analysis

- Quantitative analysis seeks to establish the amount of a given element or compound in a sample.

Methods of detecting analytes

1. Physical means
    - Mass
    - Color
    - Refractive index
    - Thermal conductivity
2. With electromagnetic radiation (Spectroscopy)
    - Absorption
    - Emission
    - Scattering

3. By an electric charge

   - Electrochemistry

Traditional analytical techniques

Titration

Titration involves the addition of a reactant to a solution being analyzed until some equivalence point is reached. Often the amount of material in the solution being analyzed may be determined.

Gravimetry

Gravimetric analysis involves determining the amount of material present by weighing the sample before and/or after some transformation. A common example used in the determination of the amount of water in a hydrate by heating the sample to remove the water such that the difference in weight is due to the water lost.

Instrumental Analysis

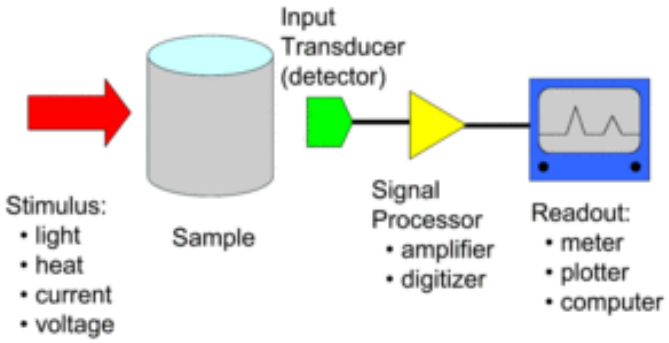


Fig : 2 Analytical instruments showing the stimulus and measurement of response

1. Separation Techniques

Separation processes are used to decrease the complexity of material mixtures. Chromatography and electrophoresis is representative of this field.

Chromatographic methods

Chromatography is a technique by which the components in a sample, carried by the liquid or gaseous phase, are resolved by sorption-desorption steps on the stationary phase.

There are various advanced chromatographic techniques, which are most reliable and widely used for the estimation of multicomponent drugs in their formulation namely,

a) Gas chromatography (GC)
b) High Performance Thin Layer Chromatography (HPTLC)
c) High Performance Liquid Chromatography (HPLC)

High performance liquid chromatographic separation is based on interaction and differential partition of the sample between the mobile phase and stationary phase. The commonly used chromatographic methods can be roughly divided into the following groups,

I. Chiral
II. Ion-exchange
III. Ion pair/affinity
IV. Normal phase
V. Reverse phase
VI. Size exclusion

When compared to classical column chromatography, this technique is preferred because of its improved performance in terms of rapidity, specificity, sensitivity, accuracy, convenience, ease of automation and the cost of analysis.

Advance in column technology, high pressure pumping system and sensitive detectors have transformed liquid column chromatography into a high speed, efficient, accurate and highly resolved method of separation.

2. Hybrid Techniques

Combination of the above techniques produces 'hybrid' or 'hyphenated' techniques. Several examples are in popular use today and new hybrid techniques are under development. For example

a) GC-MS
b) LC-MS
c) HPLC/ ESI-MS
d) LC-DAD
e) CE-MS
f) CE-UV

3. Microscopy

The visualization of single molecules, single cells, biological tissues and nano- micro materials is very important and attractive approach in analytical science. Also, hybridization with other traditional analytical tools is revolutionizing analytical science. Microscopy can be categorized into three different fields: optical microscopy, electron microscopy, and scanning probe microscopy. Recently, this field is rapidly progressing because of the rapid development of computer and camera industries.

4. Lab-on-a-chip

Miniaturized analytical instrumentation, which is also called as micro fluidics or micro total analysis system (μTAS). The beauty of lab-on-a-chip system is that a whole device can be visualized under a microscop

Method of data analysis

Standard Curve

A standard method for analysis of concentration involves the creation of a calibration curve. This allows for determination of the amount of a chemical in a material by comparing the results of unknown sample to those of a series known standards.

Internal Standard

Sometimes an internal standard is added at a known concentration directly to an analytical sample to aid in quantitation. The amount of analyte present is then determined relative to the internal standard as a calibrant.

Quality assurance plays a central role in determining the safety and efficacy of medicines. Highly specific and sensitive analytical technique holds the key to design, development, standardization and quality control of medicinal products.

The efficacy and safety of a medicinal product can be assured by analytical monitoring of its quality. It is important that analytical procedure proposed of a particular active ingredient or its dosage form should be systematically sound under the condition in which it is to be applied.

New Drug Discovery

New drugs have been discovered from two major sources

- Synthetic chemicals
- Natural products including plants, animal and microbes.

The number of drugs introduced into the market has been increasing at an alarming rate. Newer analytical methods are developed for these drugs or drug combinations because of the following reasons

i) The drug or combination may not be official in any pharmacopoeia.

ii) A literature search may not reveal an analytical procedure for the drug or its combination.

iii) Analytical methods may not be available for the drug combination due to the interference caused by excipients.

iv) Analytical methods for the quantification of drug or drug combination with other drugs may not be available.

On the other hand, the existing procedure may

- Require expensive instruments, reagents, solvents etc.
- Involve any tedious extraction or separation steps which may be quite time consuming.
- Not be rapid, reliable or sensitive.
- The newly developed analytical methods find their importance in various fields such as
- Research institutions
- Quality control department in industries

- Approved testing laboratories
- Bio-pharmaceutical and bio-equivalence studies
- Clinical pharmacokinetic studies

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High performance liquid chromatography is a very sensitive analytical technique most widely used for quantitative and qualitative analysis of pharmaceuticals. The principle advantage of HPLC compared to classical column chromatography is improved resolution of the separated substance, faster separation times and the increased accuracy, precision and sensitivity[8].

Principle of Separation and its type

There are four types of chromatography in which the mobile phase is a liquid. The mobile phase is pumped through the packed column, under high pressure.

a. Partition chromatography
    i. Normal phase chromatography
    ii. Reverse phase chromatography
b. Adsorption or liquid solid chromatography
c. Ion exchange chromatography
d. Size exclusion or gel permeation chromatography

Normal Phase Chromatography

In normal phase mode, the stationary phase (e.g. silica gel) is polar in nature and the mobile phase is non- polar in this technique, non-polar compounds travel faster and are eluted first. This is because less affinity between solute and stationary

phase. Polar compounds are retained for longer time in the column because more affinity towards stationary phase and takes more time to be eluted from the column. This is not advantageous in pharmaceutical applications since most of the drug molecules polar in nature and takes longer time to be eluted and detected. Hence this technique is not widely used in pharmacy.

Solute Polarity

Adsorption strengths increased with increasing solute polarity and this is favorable interaction between the polar solutes and the polar stationary phase increases the elution time (note:-the interaction strength not only depends on the functional groups in the enlight molecule, but also stearic factors).

Reverse phase chromatography

In RP-HPLC the stationary phase is non-polar often a hydrocarbon and the mobile phase is relatively polar such as water, methanol or Acetonitrile. In RPC the solutes are eluted in the order of their decreasing polarities. These are prepared by treating the surface of silanol group with an organochlorosilane reagent.

Reverse phase mode

Non-polar stationary phase and polar mobile phase is used here.

Mechanism

Retention by interaction of the stationary phase non-polar hydrocarbon chain with non-polar parts and sample molecules.

Adsorption or liquid solid chromatography

The principle of separation is adsorption, separation of components takes place because of difference in affinity of compounds towards stationary phase. This

principle is seen in normal phase as well as reverse phase mode, where adsorption will takes place

Ion – Exchange chromatography

In ion exchange chromatography, retention is based on the attraction between solute ions and charged sites bound to stationary phase. Ions of the same charge are excluded. Some types of ion Exchangers include :(1) polystyrene resins-allows cross linkage which increases the stability of the chain. Higher cross linkage reduces swerving, which increases the equilibration time and ultimately improves selectivity. (2)Cellulose and dextran ion-exchangers (gels) these possess larger pore sizes and low charge densities making them suitable for protein separation. (3) controlled-pore glass or porous silica

In general, ion-exchangers favor the binding of ions of higher charge and smaller radius. An increase in counter ion (with respect to the functional groups in resins) concentration reduces the retention time. An increase in ph reduces the retention time in cation exchange while a decrease in ph reduces the retention time in anion exchange

Size exclusion chromatography

Size exclusion chromatography (SEC) also known as gel permeation chromatography or gel filtration chromatography, separates particles on the basics of size. It is generally a low resolution chromatography and thus it is often reserved for the final, “polishing” step of purification. It is also useful for determining the tertiary structure and quaternary structure of purified proteins, and is the primary technique for determining the average molecular weight of natural and synthetic polymers.

SEC resins consist of a porous matrix of spherical particles that lack reactivity and adsorptive properties. After sample has been applied, molecules larger than the

pores are unable to diffuse into the beads, so they elute first. Molecules that range in size between the very big and very small can penetrate the pores to varying degrees based on their size. If a molecule is smaller than the smallest of the pores in the resin, it will be able to enter the total pore volume. Molecules that enter the total pore volume are eluted last. Samples are eluted isocratically so there is no need to use different buffers during the separation.

Methods of quantitative analysis:

The sample or solute is analyzed quantitatively in HPLC by either peak height or peak area measurements. Peak areas are proportional to the amount of the material eluting from the column as long as the solvent flows at constant rate. Peak heights are proportional to the amount of the material only when peak widths are constant and are strongly affected by the sample injection techniques. They are five principles evaluation methods for quantifying the solute[10]

(a) Calibaration by Standards:

Calibration curves for each component are prepared from pure standards, using identical injection volumes of operating conditions for standards and samples. The concentration of solute is read from its curve if the curve is linear

$$X=K \times Area$$

Where, x=concentration.

K=proportionality constant (slope of the curve).

In this evaluation method, only the area of the peaks of interest is measured. Relative response factors must be considered when converting areas to volume and when the response of the given detector differs for each molecular type of compounds.

(b) Internal standard method:

In this technique, a non quantity of internal standard is chromatographed and area Vs concentration is ascertained. Then a quantity of internal standard is added to the raw sample prior to any sample pretreatment or separation operations. The peak area of the standard in the sample run is compared with the peak are when the standard is run separately. This ratio serves as correction factor for variation in sample size, for losses in any preliminary pretreatment operations, or for incomplete elution of the sample. The material selected for the internal standard must be completely resolved from adjacent sample components and should not interfere with the sample components and never be present in samples

$$\text{Area ratio} = \frac{\text{Area of sample}}{\text{Area of internal standard}}$$

$$\text{Sample concentration} = \frac{\text{Area of sample}}{\text{Area of internal standard}} \times \text{concentration of standard}$$

(c) Area normalization

This technique is often used for the sample having identical components. It is used to evaluate the absolute purity of the sample. The procedure is to total up the areas under all peaks and then calculates the percentage of the total area that is contributed by the compound of interest. For this method the entire sample must be eluted, all components must be separated and each peak must be completely resolved

(d) Standard addition method

If only few samples are to be chromatographed, it is possible to employ the method of standard edition (s).the chromatogram of the unknown is recorded, then a

known amount of analyte (s) is added and the chromatogram is repeated using same reagents, instruments and other conditions. From the increase in the peak area (or peak height), the original concentration can be computed by interpolation.

If an instrumental reading(area/height) 'Rx' is obtained, from a sample of unknown 'x' and a reading 'Rt' is obtained from the sample to which a known concentration 'a' of analyte has been added, then 'x' can be calculated from

$$\frac{x}{x+a} = \frac{Rx}{Rt}$$

A correction for dilution must be made if the amount of standard added, changes the total sample volume significantly. It is always advisable to check the result by adding at least one other standard.

Principle of separation

HPLC is based on the mechanism of adsorption, partition, ion exchange or size exclusion, depending on the type of stationary phase used. HPLC involves a solid stationary phase, normally packed inside a stainless- steel column, and a liquid mobile phase. Separation of the components of a solution results from difference in the relative distribution ratios of the solutes between the two phases. The mobile phase flows through the stationary phase and carries the components of the mixture with it. Sample components that display stronger interactions with the stationary phase will move more slowly through the column than components with weaker interactions. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres. That makes it much faster.

Depending on the chemical structure of the analyte, the molecules are retarded while passing the stationary phase. The specific intermolecular interactions between the molecules of a sample and the packing material define their time "on-column".

Hence, different constituents of a sample are eluted at different times. Thereby, the separation of the sample ingredients is achieved.

A detection unit (e.g. UV detector) recognizes the analytes after leaving the column. The signals are converted and recorded by a data management system (computer software) and then shown in a chromatogram. After passing the detector unit, the mobile phase can be subjected to additional detector units, a fraction collection unit or to the waste. In general, a HPLC system contains the following modules: a solvent reservoir, a pump, an injection valve, a column, a detector unit and a data processing unit. The solvent (eluent) is delivered by the pump at high pressure and constant speed through the system. To keep the drift and noise of the detector signal as low as possible, a constant and pulseless flow from the pump is crucial. The analyte (sample) is provided to the eluent by the injection valve.

Depending on the composition of the mobile phase, two different modes are generally applicable. If the makeup of the mobile phase remains constant during the separation process, the HPLC system is defined as an isocratic elution system. When the composition of the mobile phase is changed during separation, the HPLC system is defined as a gradient elution system. Using a gradient system, two different techniques are available: a low-pressure gradient (LPG) and a high-pressure gradient (HPG). A low-pressure gradient means that the mixing of the solvents is carried out upstream of the pump (suction side). In a high-pressure gradient system, the different solvents are supplied by individual pumps and mixed after the pumps (discharge side)

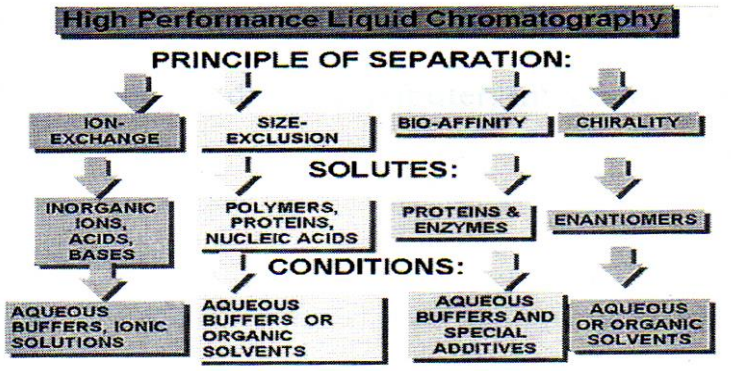


Fig : 3 High Performance Liquid chromatography

The majority of the HPLC separation are done with Reversed phase separation, probably over 90%.In reversed phase separation organic molecules are separated based on the degree of hydrophobicity. There is a correlation between the degree of lipophylicity and retention in the column. This is the list of mobile phase parameters effecting retention and separation in reversed phase .Elution order in normal phase HPLC shows that the polar solutes elute later then non polar lypophilic ones

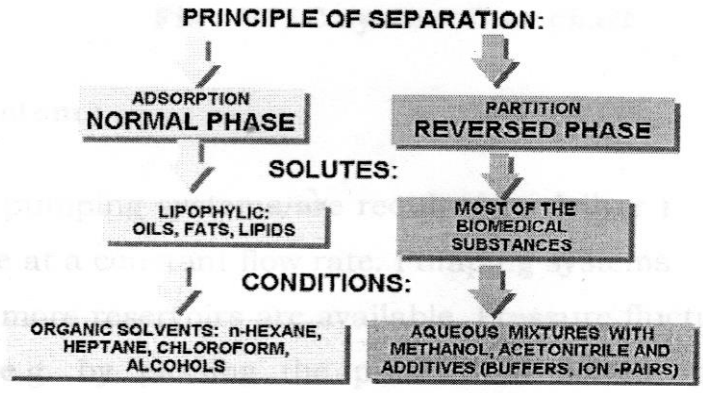


Fig : 4 Principle of Separation

Method development in RP-HPLC

Retention in RP-HPLC

The Reverse phase chromatography retention of a compound is determined by its polarity and experimental conditions, mobile phase, column and temperature.

1. Mobile phase effects

Retention (compound K value) can be preferably adjusted by changing mobile phase composition or solvent strength in RPC. Retention is less for stronger, less polar mobile phases. Solvent strength depends on

i) Choice of organic solvent or choice of % B.

ii) Concentration of the organic solvent in the mobile phase A: % B where A is water, B is the organic phase and % is volume % v/v.

iii) A retention range of $0.5<K<20$ are allowable for sample to be separated using isocratic condition but $1<K<10$ is generally preferred.

a) Choice of organic phase

A mobile phase of 100% Acetonitrile is a stronger polar solvent, which might result in ($K<0.2$), so weaker mobile phase is required to retain the compound. By decreasing the percentage of Acetonitrile, retention time will increase. If organic phase is decreased by 10%, the K value increases 3 times approximately. Systematic decrease of % B to investigate sample retention is a simple and convenient way to determine the best mobile phase composition for a given sample.

b) Mobile-Phase strength

Mobile phase strength in RPC depends upon both % B and the type of organic solvent. RPC solvent strength varies as water (weakest) < methanol <

Acetonitrile < ethanol < tetrahydrofuran < propanol < methylene chloride (strongest). Solvent strength increases as solvent polarity decreases. Any of the above solvents can be used with water for Reverse phase choromatography, except methylene chloride since it is not water miscible. Acetonitrile is the best initial choice of organic solvent for the mobile phase. The next best organic solvent is methanol followed by tetrahydrofuran.

2. Selectivity in RP-HPLC

Three main variables can be used in RPC to change selectivity (α) for neutral samples like mobile phase composition, column type and temperature. Overall sample retention acceptable is (0.5< K< 20).

a) Solvent-strength selectivity

The best sample resolution will occur for a % B value, where both pairs have the same resolution peak spacing can be explored while % B is varied for optimum sample retention (0.5< K< 20).

b) Solvent type selectivity

A change in organic solvent type is often used to change peak spacing and improve resolution. The selection of RPC solvents for this purpose is guided by solvent properties that are believed to affect selectivity, acidity, basicity and dipolarity. Only a slight increase (2 to 5 %) in the selectivity (α value) for a critical band pair may be necessary to achieve acceptable resolution. Changing solvent type in RPC is usually the most effective procedure to alter selectivity and achieve the separation of multicomponent neutral samples.

c) Column type selectivity

A change in column type can produce useful changes in selectivity and over

all sample retention. Retention is greater (run time longer) on the stronger (C_8 and phenyl column) vs. the weaker cyano column. A change of the column is usually less useful than a change in mobile phase type hence this should be tried only after the use of solvent strength or solvent type selectivity has failed. Usually a C_8 or C_{18} column should be tried first followed by a cyano, then by a phenyl column. Column padings bonded with cyclodextrin (CD) are useful in separation of enantiomeric isomers.

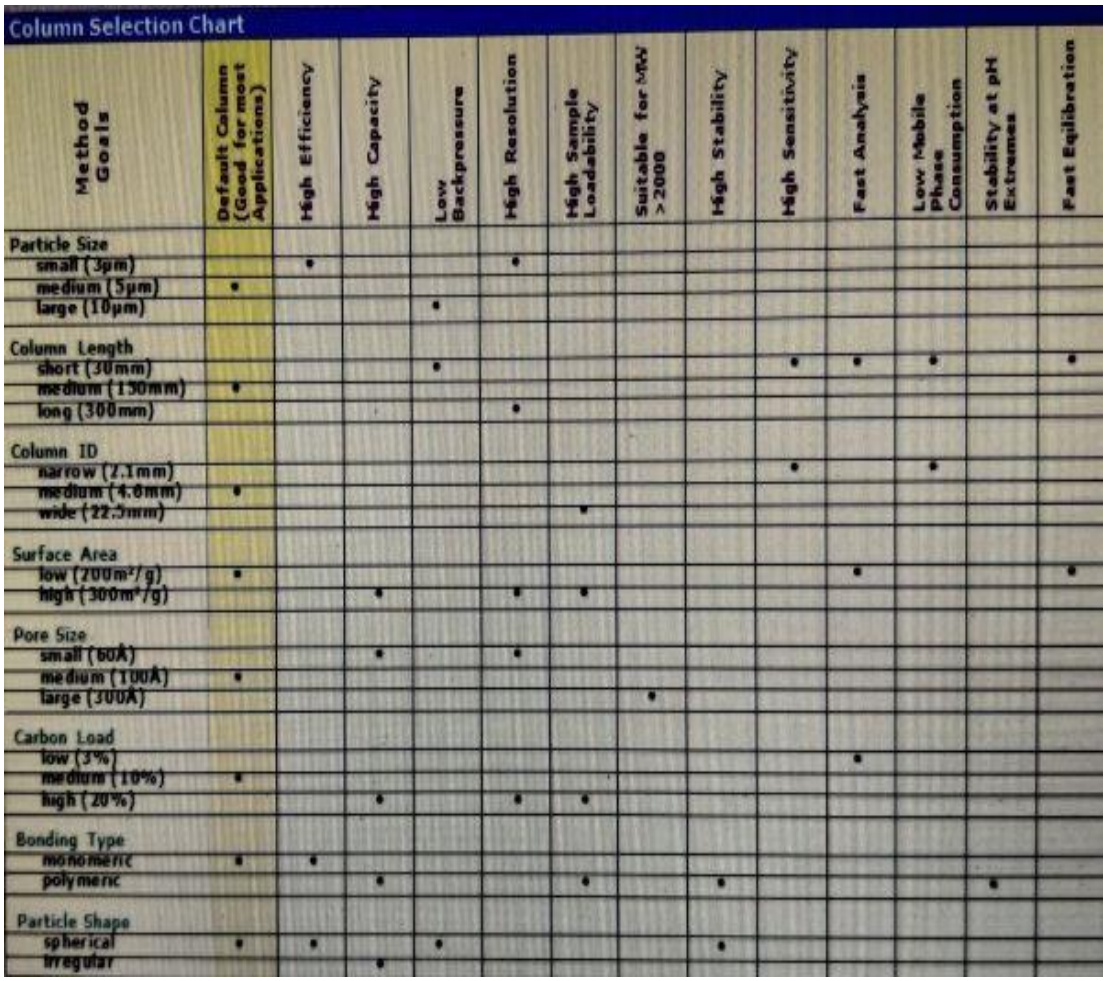


Fig : 5 Column Selection chart

d) Temperature selectivity

Values of K decreases at higher temperature for the RPC separation of neutral compounds. This is less effective for non-ionic compounds as a mean of altering selectivity for improved separation. As the temperature is increased, the relative retention of the polar compounds decreases more rapidly than for the non-polar compounds.

Fundamentals of Separation

Column efficiency (N)

Column efficiency is called as number of theoretical plates. It measures that the band spreading number of theoretical plate is higher. If it is higher it indicates good column and system performance[9].

Column performance can be defined on terms of values of N.

$$N = 16(t_R/w)^2 \text{ or } 3500 \text{ L (cm)}/ \text{ dp } (\mu m)$$

Plate height, H = N /L where L = length

Capacity factor (K')

It is the measure of how well the sample molecule is retained by the column during an isocratic separation. It is affected by solvent composition, separation, aging and temperature of separation.

$$K^1 = \frac{t_R - t_0}{t_0}$$

t_R = band retention time

t_0 = column dead volume

Resolution

The quality of separation is usually measured by resolution R, of adjacent bands.

$$R_s = \frac{2(t_2 - t_1)}{W_1 + W_2}$$

t_1 and t_2 are retention times of the first and second adjacent bands. w_1 and w_2 are baseline bandwidths.

Asymmetry

The asymmetry is a tool for quickly determining how much if any, of an eluting peak profile deviates in shape from a normal distribution. The subscript 'x' refers to the percentage of peak height at which the asymmetry is determined.

E.g.: A_{10} (determined at 10% peak height)

The equation for determining peak asymmetry is

$$A_x = b/a,$$

Where,

'b' = the distance between the perpendicular connecting the baseline to peak maximum and the latest eluting portion of the curve.

'a' = the distance between the perpendicular connecting the baseline to the peak maximum and the earliest eluting portion of the curve.

Selectivity

It measures relative retention of two components. Selectivity is the function

of chromatographic surface (column), melting point and temperature.

Equipment for HPLC:

The essential features of modern HPLC are illustrated in the block diagram and compromise of components

1. Pumping system
2. An injector
3. Chromatographic column
4. Detector
5. Data collection device (computer, integrator, or recorder)

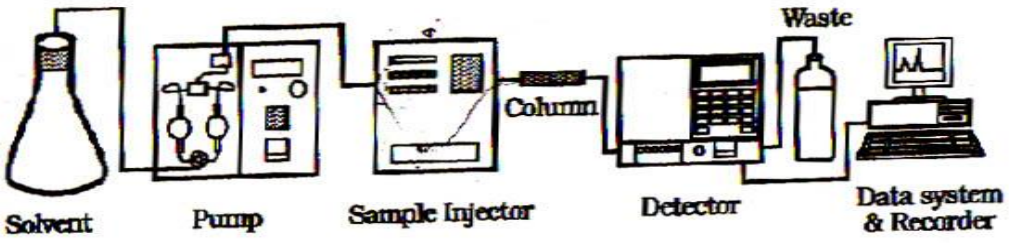


Fig : 6 A schematic diagram of HPLC equipment

Pumping system:

HPLC pumping systems are required to deliver metered amounts of mobile phase at a constant flow rate. Pumping systems that deliver solvent from one or more reservoirs are available. Pressure fluctuations should be minimized, e.g. by passing the pressurized solvent through a pulse dampning device.Tubing and connections should be capable of withstanding the pressures developed by the pumping systems. Many HPLC pumps are fitted with a facility for “bleeding” the system of entrapped air bubbles.

Modern computer or micro processor controlled pumping systems are capable of delivering a mobile phase of either constant (isocratic elution) or varying (gradient elution) composition, according to a defined programmed. In the case of gradient elution, solvent mixing can be achieved on either the low or high- pressure side of the pump(S) depending on the flow rate and composition of mobile phase, operating pressure of up to 42000 kPa (6000 psi) can be generated during routine analysis

An injector:

The sample solution is usually introduced into the flowing mobile phase at or near the head of the column using an injection system based on an injection valve design which can operate at high pressure. Such an injection system has a fixed – loop or a variable column device which can be operated manually or by an auto sampler. Partially filling of tuber may lead to poorer injection volume precision

Chromatographic column:

Columns are usually made of polished stainless steel, or between 50 and 300 mm long, and have an internal diameter of between 2 to 5 mm. They are commonly filled with a stationary phase with a particle size of 5 -1o µm. columns with internal diameters of less than 2 mm are often reoffered to as micro bore columns .ideally, the temperature of the mobile phase and the column should be kept to constant during an analysis. Most separations' are performed at an ambient temperature, but columns may be heated to give better efficiency normally, columns should not be heated above 6ooC because of the potential for stationary phase degradation or changes occurring to the composition of the mobile phase

Detector:

Ultraviolet/visible (UV/vis) absorption spectrometer are the most commonly used detectors for pharmaceutical analysis. In specific cases fluorescence

spectrophotometers, differential refracting meters, electro chemical detectors, light scattering detectors, mass spectrometers, or other special detectors may be used. Here an analyte possesses a chromophoric group that absorbs UV/vis radiation, the UV/vis detector is the most appropriate because of its sensitivity and stability. Such a detector is not suitable for detecting analyter with very weak chromophores.

A variant on the UV/vis type of detector, which is becoming increasingly popular because of its ability to furnish detailed spectral information, is the diode array detector. This type of detector acquires absorbance data over a certain UV/vis range and can provide chromatograms at multiple, selectable wave lengths, together with spectra for the eluted peaks. In addition, the detector and accompanying computer programs can be used to assess the spectral homogeneity of peaks, which may provide information on the chromatographic purity of the peaks. This can be especially useful in method development and validation

Enhanced sensitivity may be achieved in certain cases by using pre-column or post-column derivatization techniques,(These techniques are to be avoided for control of impurities.)

Data collection devices:-

Signals from 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 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 become very important, particularly when baseline separations

are not always attainable. If baseline separations cannot be obtained, valley-to-valley integration should be employed.

HPLC allows limits to be set for individual impurities and for the sum of impurities, but there is a level at which peaks should not be integrated. This "disregard level" is set in relation to the area of the peak in the chromatogram of the prescribed reference solution and is usually equivalent to 0.05% or the substance being examined.

ANALYTICAL PARAMETERS FOR VALIDATION

Validation may be defined as a process involving confirmation or establishing by laboratory studies that a method/ system/ analyst gives accurate and reproducible result for intended analytical application in a proven and established range. The analytical procedure refers to the way of performing the analysis. It should describe in detail the steps necessary to perform each analytical test. This may include but is not limited to the sample, the reference standard and the reagents preparations, use of the apparatus, generation of the calibration curve, use of the formulae for the calculation etc.

Validation parameters

The parameters for method validation as defined by the ICH guidelines are summarized below.

System Suitability

System suitability testing originally believed by the industry of pharmaceuticals to decide whether a chromatographic system is being utilized day today in a routine manner in pharmaceutical laboratories where quality of results is most important which is suitable for a definite analysis. The parameters used in the system suitability tests (SST) report are as follows:

- Number of theoretical plates or Efficiency (N).
- Capacity factor (K).
- Separation or Relative retention (α).
- Resolution (Rs).
- Tailing factor (T).
- Relative Standard Deviation (RSD).

Number of theoretical plates/Efficiency (N): In a specified column, efficiency is defined as the measurement of the degree of peak dispersion and it should have the column characteristics. The efficiency is conveyed in terms of number of theoretical plates.

Sigma/tangential method (USP method): The plate number depends on column length. Theoretical plate number is the measure of column efficiency. As stated by plate theory, the analyte will be in instant equilibrium with stationary phase and column has to be divided into number of hypothetical plates and each plate consists of a fixed height and analyte spends finite time in the plate. Height equivalent to theoretical plate (HETP) is given by following formula:

HETP = L/N, Where,

L = length of column, N = plate number.

Capacity ratio or Capacity factor (k): Capacity factor sometimes is called as a retention factor which has no dimension and independent from flow rate of mobile phase as well as column dimensions which is the measure of extent of retention

relating to an analyte relative to an un-retained peak. Where t_R implies retention time of the sample peak and retention time of an un-retained peak is t_M.

k' = 0 means no compound is left in the column. Generally the value of k' is > 2.

Relative retention or separation factor (α):

$$\alpha = t_2\text{-}t_a / t_1\text{-}t_a$$

α =Relative retention.

t_2= Retention time calculated from point of injection.

t_a = Unretained peak time (Retention time (t_R) of an inert component not retained by the column).

t_1 = the retention time from the point of injection of reference peak defined. (Suppose no reference peak is found, value would be zero).

Resolution (Rs): Resolution is the capability of the column to separate 2 drugs in 2 individual peaks or chromatographic zones and it is improved by enhancing column length, reduction of particle size and rising temperature, altering the eluent or stationary phase. It can be told in terms of ratio of separation of the apex of two peaks by the tangential width average of the peaks.

Resolution factor (R): Resolution is a function of capacity factor, function of selectivity and a function of efficiency (or) number of theoretical plates (N). In order to separate any two peaks you must have right capacity factor ideally between 2 and 10, but appropriate selectivity is required i.e., ideally 1.2 and enough efficiency i.e., number of theoretical plates (more than 2000 theoretical plates). Resolution should be ≥ 1.5. 1.5 defines baseline resolution.

Tailing factor or Asymmetry factor: Chromatographic peak assumed to have a Gaussian shape under ideal conditions. However in practical conditions, there is always a deviation from normal distribution which indicates non-uniform migration and non-uniform distribution process. Hence the regulatory organizations like USP and EP have recommended this as one of the system suitability parameter. The asymmetry factor and tailing factor are roughly same and rarely accurate and equal in most cases. Values should normally between 1.0-1.5 and values greater than 2 are unacceptable. The peak asymmetry is computed by utilizing the following formula.

$$A_s = B/A \quad \text{Where,}$$

A_s = peak asymmetry factor.

B = distance from the point at peak midpoint to the trailing edge. (measured at 10 % of peak height).

A = distance from the leading edge of peak to the midpoint. (measured at 10 % of peak height). Ideally, peaks should be Gaussian in shape or totally symmetrical.

Specificity

Specificity is the ability to assess unequivocally the analyte in presence of components which may be expected to be present. An investigation of specificity should be conducted during the validation of identification tests, the determination of impurities and the assay. The procedures used to demonstrate specificity will depend on the intended objective of the analytical procedure

Linearity

It is the ability of the method to elicit test result that is directly proportional to analytic concentration within a given range. It is generally reported as variance of

slope of regression line. It is determined by series of three to six injections of five of more standards.

Precision

It is a measure of degree of repeatability of an analytical method under normal operation and it is normally expressed as % of relative standard deviation (% RSD). This involves

a) Repeatability

b) Reproducibility

c) Intermediate precision

$$\% \text{ RSD} = 100 \, S/X$$

Where, S = Standard deviation

X = Mean

It is determined at three levels.

a) Repeatability

Precision of the method when repeated by the same analysts, same test method and under same set of laboratory conditions (reagent, equipments), within a short interval of time, the only difference being the sample.

b) Reproducibility

When the subject method is carried out by different analysts in different laboratories using different equipments, reagents and laboratory settings and on different days of variability of analytical results as function of analyst, day to day, laboratory to laboratory, equipment to equipment etc., using the samples from same homogenous

batch.

c) Intermediate precision

It is determined by comparing the results of a method within the same laboratory but different days, analysts, equipments and reagents. The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. The applicant should establish the effects of random events on the precision of the analytical procedure. Typical variations to be studied include days, analysts, equipment, etc. It is not considered necessary to study these effects individually. The use of an experimental design (matrix) is encouraged.

Accuracy

Defined as the closeness of agreement between the actual (true) value and mean analytical value obtained by applying the test method a number of times. Accuracy is acceptable if the difference between the true value and mean measured value does not exceed the RSD values obtained for repeatability of the method.

One can design experiments for recovery of known or spiked samples in presence of expected matrix, keeping the matrix constant. Accuracy can also be determined by comparing the results with those obtained using an alternative method which has already been validated.

Limit of Detection (LOD)

It is defined as the lowest concentration of an analyte in a sample that can be detected but not quantified. LOD is expressed as a concentration at a specified signal to noise ratio. The LOD will not only depend on the procedure of analysis but also on the type of instrument.

In chromatography, detection limit is the injected amount that results in a

peak with a height at least twice or thrice as high as baseline noise level.

S/N = 2/1 or 3/1

Limit of Quantification (LOQ)

It is defined as lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy and reliability by a given method under stated experimental conditions. The procedure usually followed is to analyze samples containing decreasing known quantity of the analyte and determine the Lowest level at which acceptable level of accuracy is attained.

LOQ is expressed as a concentration at a specified signal to noise ratio. In chromatography, limit of quantification is the injected amount that results in a peak with a height, ten times as high as base line noise level.

S/N = 10/1

Ruggedness

Degree of reproducibility of test results obtained by analyzing the same sample under variety of normal test conditions such as different analysts, instruments, days, reagents, column and TLC plates.

Robustness

It is the measure of the capacity of the analytical method to remain unaffected by small but deliberate variation in procedure. It provides an indication about variability of the method during normal laboratory conditions. The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters.

THE BASIC STEPS INVOLVED IN METHOD DEVELOPMENT LITERATURE COLLECTION

Search for literature (USP, EP, JP, IP, Chromatography journal, Patents, Internet etc.) for the same molecule or for similar molecules having similar structures.

Take the method from literature to check suitability to meet the requirements are modify the method to suite the requirements, such as resolution of possible impurities as per the synthetic process. Based on the synthetic process, select the components to be checked in the HPLC method. Consider all possible impurities, at least penultimate stage and key raw materials for any stage. Impurities to be considered are intermediates, process impurities and degradants. Collect samples, standards and all possible impurities in each stage.

Chemical structure

By observing the structure, based on the functional groups present in the molecule it can be determined whether the compound is basic, acidic and neutral.

Based on the nature of the compound pH of the mobile phase selected. If compound is acidic, acidic mobile phase is preferable. For a basic compound, low pH and basic mobile phase are preferable. For a neutral compound neutral mobile phase is suitable. Elution of the compounds will follow on 'like solves like' concept.

Molecular weight

If the molecular weight is less than 500 and based on solubility and nature (acid/base/ionic/nonionic) of the molecule we can select the techniques given below.

Reversed phase chromatography (for acids, bases and nonionic samples) ion-pair chromatography (for ionic samples), ion-exchange chromatography (for ionic samples), normal phase chromatography (for isomers, non-polar/nonionic samples,

chiral molecules), size exclusion chromatography (for protein related samples), capillary electrophoresis (for 'red-ox' samples).

pH- pKa Value of compounds

Based on pH- pKa values the nature of the compound (acidic, basic and neutral in nature) and polarity of the compound can be assumed.

Sample solubility

Check the solubility of all components in the solutions like mobile phase, mobile phase organic mixtures, water – organic mixtures and mixture of mineral acids like perchloric acid , phosphoric acid etc.

Column selection

Column specifications

Particle size	:	300 A^o
Pore size	:	5 µm
Bonded phase	:	C_{18}
Column dimensions	:	4.6 X 250 mm

- In the commercial market, there are so many types of vendors available. Before choosing the column, we need to consider the quality of the column with respect to batch to batch and lot to lot reproducibility.
- Column parameters like, internal diameter, particle surface area, pore volume and size also to be checked, how it is impacting the system suitability criteria.
- The C_8 columns are non polar when compare to C_{18} columns. So in the reverse phase analysis the retention times are less in C_8 when compared to C_{18} columns.

- Depending on the mobile phase nature, the type of the column, reverse phase and normal phase can be defined.
- In reverse phase analysis the mobile phase is highly polar and the column is non polar. With this conditions C_8, C_{18}, Cyano and Amino columns can be used against a more polar mobile phase. Similar way, in the normal phase analysis the column is more polar when compared to mobile phase. With this conditions Cyno, Phenyl and Silica columns are used against non polar mobile phases consisting of solvents like n-hexane, chloroform, dichloromethane etc.
- Generally increasing the particle size leads to decreasing of the retention time of components.
- If surface area increases, there is increase of retention time if columns internal diameter decrease the retention time will increase shortly
- In modern times, pH stable columns are available in the market [1-13 pH].

As well as understanding the hydrophobicity of a molecule, there are also a variety of different modes in which a molecule can interact with the HPLC stationary phase, including:

- **Dispersive** – These interactions will exist between all organic molecules to a certain extent and is the major retention mechanism for alkyl phases. In general the degree of retention on a C_{18} phase will be dependent on the hydrophobicity, log P, of the molecule.
- **Charge transfer** – Sometimes referred to as π-π or dipole-dipole interactions, this mode of interaction is primarily seen with aromatic compounds or with compounds that are unsaturated. These modes of interactions are enhance with methanol and are clearly dominant with aromatic phases (Phenyl, PFP, etc.).

- **Hydrogen bonding and dipole-dipole interactions** – The analyte acts as a proton donor with proton accepting groups on the stationary phase surface.
- **Ion exchange** – the analyte can lose or gain one or more protons resulting in a charged species which can interact with the oppositely charged species on the surface of the stationary phase.

DRUG PROFILE

Dolutegravir

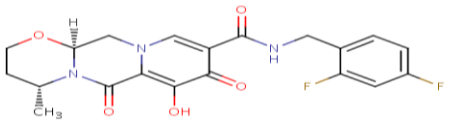


Fig: 7Dolutegravir Structure

Chemical Data

IUPAC Name: (3S, 7R)-N-[(2,4-difluorophenyl)methyl]-11-hydroxy-7-methyl-9,12-dioxo-4-oxa-1,8-diazatricyclo[8.4.0.0^{3,8}]tetradeca-10,13-diene-13-carboxamide

Chemical formula : $C_{20}H_{19}F_2N_3O_5$

Molecular weight : 419.3788

CAS No : 1051375-16-6

pKa : 8.7

Physical Data

Description : a white crystalline powder

Solubility :water solubilty.

Category : Dolutegravir is indicated for HIV-1 infection for adults and children and adolescents ≥12 years of age and weighing ≥40 kg. It is marketed as Tivicay as dolutegravir sodium. 52.6 mg of dolutegravir sodium is equivalent to 50 mg dolutegravir free acid.

Mechanism of action: Dolutegravir is an HIV-1 antiviral agent. It inhibits HIV integrase by binding to the active site and blocking the strand transfer step to retroviral DNA integration. This is an essential step of the HIV replication cycle and will result in an inhibition of viral activity. Dolutegravir has a mean EC50 value of 0.5 nM(0.21 ng/mL) to 2.1 nM (0.85 ng/mL) in peripheral blood mononuclear cells (PBMCs) and MT-4 cells.

Drug Effects during Lactation

Dolutegravir is detectable in maternal milk and infant plasma during breastfeeding. It appears that elimination by newborn infants is prolonged. In the United States and other developed countries, HIV-infected mothers should generally not breastfeed their infants. No published information is available on the use of dolutegravir during breastfeeding. In countries in which no acceptable, feasible, sustainable and safe replacement feeding is available, World Health Organization guidelines recommend that all women with an HIV infection who are pregnant or breastfeeding should be maintained on antiretroviral therapy for at least the duration of risk for mother-to-child transmission. Mothers should exclusively breastfeed their infants for the first 6 months of life; breastfeeding with complementary feeding should continue through at least 12 months of life up to 24 months of life.

The first choice regimen for nursing mothers is tenofovir, efavirenz and either lamivudine or emtricitabine.

If these drugs are unavailable, alternative regimens include: 1) zidovudine, lamivudine and efavirenz; 2) zidovudine, lamivudine and nevirapine; or 3) tenofovir, nevirapine and either lamivudine or emtricitabine. Exclusively breastfed infants should also receive 6 weeks of prophylaxis with nevirapine.

Therapeutic Uses

HIV Integrase Inhibitors

- The recommended dose of TIVICAY in pediatric patients aged 12 years and older and weighing at least 40 kg is 50 mg administered orally once daily. If efavirenz, fosamprenavir/ritonavir, tipranavir/ritonavir, or rifampin are coadministered, the recommended dose of TIVICAY is 50 mg twice daily. Safety and efficacy of TIVICAY have not been established in pediatric patients younger than 12 years or weighing less than 40 kg, or in pediatric patients who are INSTI-experienced with documented or clinically suspected resistance to other INSTIs (raltegravir, elvitegravir).
- TIVICAY (dolutegravir) is indicated in combination with other antiretroviral agents for the treatment of human immunodeficiency virus type 1 (HIV-1)

infection in adults and children aged 12 years and older and weighing at least 40 kg. The following should be considered prior to initiating treatment with TIVICAY: Poor virologic response was observed in subjects treated with TIVICAY 50 mg twice daily with an integrase strand transfer inhibitor (INSTI)-resistance Q148 substitution plus 2 or more additional INSTI-resistance substitutions, including L74I/M, E138A/D/K/T, G140A/S, Y143H/R, E157Q, G163E/K/Q/R/S, or G193E/R.

- Renal clearance of unchanged drug is a minor pathway of elimination for dolutegravir. In a trial comparing 8 subjects with severe renal impairment (CrCl<30 mL/min) with 8 matched healthy controls, AUC, Cmax, and C24 of dolutegravir were decreased by 40%, 23%, and 43%, respectively, compared with those in matched healthy subjects. The cause of this decrease is unknown. Population pharmacokinetic analysis using data from SAILING and VIKING-3 trials indicated that mild and moderate renal impairment had no clinically relevant effect on the exposure of dolutegravir. No dosage adjustment is necessary for treatment-naive or treatment-experienced and INSTI-naive patients with mild, moderate, or severe renal impairment or for INSTI-experienced patients (with certain INSTI-associated resistance substitutions or clinically suspected INSTI resistance) with mild or moderate renal impairment. Caution is warranted for INSTI-experienced patients (with certain INSTI-associated resistance substitutions or clinically suspected INSTI resistance with severe renal impairment, as the decrease in dolutegravir concentrations may result in loss of therapeutic effect and development of resistance to TIVICAY or other coadministered antiretroviral agents. Dolutegravir has not been studied in patients requiring dialysis.
- Dolutegravir is primarily metabolized and eliminated by the liver. In a trial comparing 8 subjects with moderate hepatic impairment (Child-Pugh Score B) with 8 matched healthy controls, exposure of dolutegravir from a single 50-mg dose was similar between the 2 groups. No dosage adjustment is necessary for patients with mild to moderate hepatic impairment (Child-Pugh Score A or B). The effect of severe hepaticimpairment (Child-Pugh Score C) on the pharmacokinetics ofdolutegravir has not been studied. Therefore, TIVICAY is not recommended for use in patients with severe hepatic impairment.

Drug Warnings

- The Centers for Disease Control and Prevention recommend that HIV-1-infected mothers in the United States not breastfeed their infants to avoid risking postnatal transmission of HIV-1 infection. Studies in lactating rats and their offspring indicate that dolutegravir was present in rat milk. It is not known whether dolutegravir is excreted in human milk. Because of both the potential for HIV transmission and the potential for adverse reactions in nursing infants, mothers should be instructed not to breastfeed if they are receiving TIVICAY.
- Pregnancy Category B. There are no adequate and well-controlled studies in pregnant women. Because animal reproduction studies are not always predictive of human response, and dolutegravir was shown to cross the placenta in animal studies, this drug should be used during pregnancy only if clearly needed.
- Dolutegravir (TIVICAY) should not be used with etravirine without coadministration of atazanavir/ ritonavir, darunavir/ ritonavir, or lopinavir / ritonavir.
- Immune reconstitution syndrome has been reported in patients treated with combination antiretroviral therapy, including TIVICAY. During the initial phase of combination antiretroviral treatment, patients whose immune systems respond may develop an inflammatory response to indolent or residual opportunistic infections (such as Mycobacterium avium infection, cytomegalovirus, Pneumocystis jirovecii pneumonia (PCP), or tuberculosis), which may necessitate further evaluation and treatment. Autoimmune disorders (such as Graves' disease, polymyositis, and Guillain-Barre syndrome) have also been reported to occur in the setting of immune reconstitution; however, the time to onset is more variable and can occur many months after initiation of treatment.
- Redistribution/accumulation of body fat, including central obesity, dorsocervical fat enlargement (buffalo hump), peripheral wasting, facial wasting, breast enlargement, and "cushingoid appearance" have been observed in patients receiving antiretroviral therapy. The mechanism and long-term

consequences of these events are currently unknown. A causal relationship has not been established.

- Patients with underlying hepatitis B or C may be at increased risk for worsening or development of transaminase elevations with use of TIVICAY. In some cases the elevations in transaminases were consistent with immune reconstitution syndrome or hepatitis B reactivation particularly in the setting where anti-hepatitis therapy was withdrawn. Appropriate laboratory testing prior to initiating therapy and monitoring for hepatotoxicity during therapy with TIVICAY are recommended in patients with underlying hepatic disease such as hepatitis B or C.
- Hypersensitivity reactions have been reported and were characterized by rash, constitutional findings, and sometimes organ dysfunction, including liver injury. The events were reported in 1% or fewer subjects receiving TIVICAY in Phase 3 clinical trials. Discontinue TIVICAY and other suspect agents immediately if signs or symptoms of hypersensitivity reactions develop (including, but not limited to, severe rash or rash accompanied by fever, general malaise, fatigue, muscle or joint aches, blisters or peeling of the skin, oral blisters or lesions, conjunctivitis, facial edema, hepatitis, eosinophilia, angioedema, difficulty breathing). Clinical status, including liver aminotransferases, should be monitored and appropriate therapy initiated. Delay in stopping treatment with TIVICAY or other suspect agents after the onset of hypersensitivity may result in a life-threatening reaction. TIVICAY should not be used in patients who have experienced a previous hypersensitivity reaction to TIVICAY.

Pharmacological Action

HIV-1 infected subjects on dolutegravirmonotherapy demonstrated rapid and dose-dependent reduction of antiviral activity with declines of HIV-1 RNA copies per ml. The antiviral response was maintained for 3 to 4 days after the last dose. The sustained response obtained in clinical trials indicates that dolutegravir has a tight binding and longer dissociative half-life providing it a high barrier to resistance. The combination therapy (ripivirine and dolutegravir) presented the same viral

suppression found in previous three-drug therapies without integrase strand transfer inhibitor mutations or rilpivirine resistance.

Dolutegravir is an orally bioavailable integrase strand-transfer inhibitor (INSTI), with activity against human immunodeficiency virus type 1 (HIV-1) infection. Upon oral administration, dolutegravir binds to the active site of integrase, an HIV enzyme that catalyzes the transfer of viral genetic material into human chromosomes. This prevents integrase from binding to retroviral deoxyribonucleic acid (DNA), and blocks the strand transfer step, which is essential for the HIV replication cycle. This prevents HIV-1 replication.

Absorption, Distribution and Excretion

Absorption

When 50 mg of dolutegravir once daily was orally administered to HIV-1 infected adults, the AUC, Cmax, and Cmin is 53.6 mcg h/mL, 3.67 mcg/mL, and 1.11 mcg/mL, respectively. The peak plasma concentration was observed 2 to 3 hours post-dose. Steady state is achieved within approximately 5 days with average accumulation ratios for AUC, Cmax, and C24h ranging from 1.2 to 1.5. When 50 mg once daily is given to pediatric patients (12 to < 18 years and weighing ≥40 kg) the Cmax, AUC, and C24 is 3.49 mcg/mL, 46 mcg.h/mL, and 0.90 mcg/mL respectively

Route of Elimination

When a single oral dose of dolutegravir is given, nearly all complete dose is recovered in a proportion of 53% excreted unchanged in the feces and 31% excreted in urine. The renal eliminated recovered dose consists of ether glucuronide of dolutegravir (18.9%), a metabolite formed by oxidation at the benzylic carbon (3.0%), a hydrolytic N-dealkylation product (3.6%) and unchanged drug (< 1%).

Volume of Distribution

The administration of a dose of 50 mg of dolutegravir presents an apparent volume of distribution of 17.4 L. The median dolutegravir concentration in CSF was 18 ng/mL after 2 weeks of treatment.

Clearance

- The apparent clearance rate of dultegravir is 1.0 L/h.
- After a single oral dose of [14C]dolutegravir, 53% of the total oral dose was excreted unchanged in feces. Thirty-one percent of the total oral dose was excreted in urine, represented by an ether glucuronide of dolutegravir (18.9% of total dose), a metabolite formed by oxidation at the benzylic carbon (3.0% of total dose), and its hydrolytic N-dealkylation product (3.6% of total dose). Renal elimination of unchanged drug was low (<1% of the dose).
- Dolutegravir is highly bound (=98.9%) to human plasma proteins based on in vivo data and binding is independent of plasma concentration of dolutegravir. The apparent volume of distribution (Vd/F) following 50-mg once-daily administration is estimated at 17.4 L based on a population pharmacokinetic analysis.
- Food increased the extent of absorption and slowed the rate of absorption of dolutegravir. Low-, moderate-, and high-fat meals increased dolutegravir AUC(0-8) by 33%, 41%, and 66%; increased Cmax by 46%, 52%, and 67%; and prolonged Tmax to 3, 4, and 5 hours from 2 hours under fasted conditions, respectively.
- Following oral administration of dolutegravir, peak plasma concentrations were observed 2 to 3 hours postdose. With once-daily dosing, pharmacokinetic steady state is achieved within approximately 5 days with average accumulation ratios for AUC, Cmax, and C24 h ranging from 1.2 to 1.5. Dolutegravir plasma concentrations increased in a less than dose-proportional manner above 50 mg. Dolutegravir is a P-glycoprotein substrate in vitro. The absolute bioavailability of dolutegravir has not been established.

Metabolism/Metabolites

Dolutegravir is highly metabolized through three main pathways and it forms no long-lived metabolites. The first pathway is defined by the glucuronidation by UGT1A1, the second pathway by carbon oxidation by CYP3A4 and the third pathway is what appears to be a sequential oxidative defluorination and glutathione conjugation. The

main metabolite found in blood plasma is the ether glucuronide form (M2) and its chemical properties disrupt its ability to bind metal ions, therefore, it is inactive.

Dolutegravir is primarily metabolized via UGT1A1 with some contribution from CYP3A. ... etherglucuronide of dolutegravir (18.9% of total dose), a metabolite formed by oxidation at the benzylic carbon (3.0% of total dose), and its hydrolytic N-dealkylation product (3.6% of total dose).

Biological Half-Life

- The half-life of dolutegravir is 14 hours.
- Dolutegravir has a terminal half-life of approximately 14 hours and an apparent clearance (CL/F) of 1.0 L/h based on population pharmacokinetic analyses.

Uses

- Medication
- HIV Integrase inhibitors

Storage Conditions

Store at 25 °C (77 °F); excursions permitted 15 deg to 30 °C (59 deg to 86 °F)

Toxicity

Dolutegravir is metabolized by UGT1A1 with some contribution from CYP3A. Dolutegravir is also a substrate of UGT1A3, UGT1A9, BCRP, and P-gp in vitro. Drugs that induce those enzymes and transporters may decrease dolutegravir plasma concentration and reduce the therapeutic effect of dolutegravir. Coadministration of dolutegravir and other drugs that inhibit these enzymes may increase dolutegravir plasma concentration. Etravirine significantly reduced plasma concentrations of dolutegravir, but the effect of etravirine was mitigated by coadministration of lopinavir/ritonavir or darunavir/ ritonavir, and is expected to be mitigated by atazanavir/ ritonavir. Darunavir/ ritonavir, lopinavir / ritonavir , rilpivirine , tenofovir , boceprevir, telaprevir, prednisone, rifabutin, and omeprazole had no clinically significant effect on the pharmacokinetics of dolutegravir.

Antidote and Emergency Treatment

Immediate first aid: Ensure that adequate decontamination has been carried out. If patient is not breathing, start artificial respiration, preferably with a demand valve resuscitator, bag-valve-mask device, or pocket mask, as trained. Perform CPR if necessary. Immediately flush contaminated eyes with gently flowing water. Do not induce vomiting. If vomiting occurs, lean patient forward or place on the left side (head-down position, if possible) to maintain an open airway and prevent aspiration. Keep patient quiet and maintain normal body temperature. Obtain medical attention. /Poisons A and B/

LITERATURE REVIEW

Susan L. Ford *et al,*

Dolutegravir (DTG) and GSK1265744 are HIV integrase inhibitors (INIs) in clinical development. The oral formulation of rilpivirine (RPV), a nonnucleoside reverse transcriptase inhibitor (NNRTI), has been approved for treatment-naive HIV infection. Long-acting depot injections of GSK1265744 and RPV are also being developed. This study evaluated the potential for drug interactions between RPV and these INIs. This phase 1, open-label, two-cohort, three-period, single-sequence crossover study evaluated oral coadministration of RPV with DTG or GSK1265744. Healthy subjects received DTG (50 mg every 24 h for 5 days) or GSK1265744 (30 mg every 24 h for 12 days) in period 1 followed by a washout, RPV (25 mg every 24 h for 11 or 12 days) in period 2, immediately followed by RPV (25 mg every 24 h) plus DTG (50 mg every 24 h) for 5 days or GSK1265744 (30 mg every 24 h) for 12 days in period 3. Steady-state pharmacokinetic (PK) parameters were estimated using noncompartmental analysis of data collected on the last day of each period. The combinations of RPV and DTG (n = 16) and of RPV and GSK1265744 (n = 11) were well tolerated; no grade 3 or 4 adverse events (AEs) or AE-related discontinuations were observed. The 90% confidence intervals for the area under the curve from time zero until the end of the dosage interval [$AUC_{0-\tau}$] and maximum concentration of drug in serum (C_{max}) geometric mean ratios were within 0.8 to 1.25. Following administration of DTG + RPV, DTG and RPV $C\tau$ increased by 22% and 21%, respectively. Following administration of GSK1265744 + RPV, RPV $C\tau$ decreased 8%. DTG and GSK1265744 can be administered with RPV without dosage adjustment for either agent. These results support coadministration of RPV with DTG or GSK1265744 as either oral or long-acting depot injection regimens.

M Grégoire *et al,*

A liquid chromatography-tandem mass spectrometry assay requiring a 100µL aliquot of human plasma for simultaneous determination of rilpivirine, a second generation non-nucleoside reverse transcriptase inhibitors of HIV and dolutegravir, a novel integrase stand transfer inhibitors of HIV concentrations has been developed. Sample pre-treatment is limited to protein precipitation with a mixture of methanol and zinc sulfate. After centrifugation the supernatant is injected in

the chromatographic system, which consists of on-line solid phase extraction followed by separation on a phenyl-hexyl column.

This 2.5min method, with its simple sample preparation provides sensitive (the limit of quantitation is 25ng/mL for each compound), accurate and precise (the intra-day and inter-day imprecision and inaccuracy are lower than 15%) quantification of the plasma concentration of these drugs and can be used for therapeutic drug monitoring in patients infected with HIV.

Burugula L *et al*,

An analytical method based on liquid chromatographic-tandem mass spectrometry (LC-MS/MS) was developed for the determination of the non-nucleoside reverse transcriptase inhibitor rilpivirine in human plasma using nevirapine as an internal standard. Analyte and the internal standard were extracted from human plasma by liquid-liquid extraction. The reconstituted samples were chromatographed on a C(18) column using a mixture of acetonitrile and 0.1% formic acid buffer (80:20, v/v) as the mobile phase at a flow rate of 0.5 mL/min. The linearity was confirmed in the concentration range 0.51-200 ng/mL in human plasma. Multiple reaction monitoring mode was used for quantification of ion transitions at m/z 367.2/195.1 and 267.1/226.1 for the drug and the internal standard, respectively. The results of the intra- and inter-day precision and accuracy studies were well within the acceptable limits. Extraction recoveries of drug from plasma were >69.5%. A run time of 2.50 min for each sample made it possible to analyze more than 300 plasma samples per day. The developed method is simple, rapid and sensitive for the determination of rilpivirine concentrations in real-time plasma samples obtained from pharmacokinetic studies.

AIM AND PLAN OF WORK

AIM

Development of new RP-HPLC method for the estimation of Doltugranavir in the tablet dosage form and then validation of the method.

PLAN OF WORK:

Literature reveals different methods for their analysis in their formulations. But our present plan is to develop a new, simple, precise& accurate method for its analysis in formulation after a detailed study a new RP-HPLC method was decided to be developed and validated.

1. Study of physicochemical properties of drug (pH, pKa, solubility and molecular weight.
2. Selection of chromatographic condition (mobile phase, column, flow rate, etc.).
3. Optimization of method.
4. Study of system suitability parameters.
5. Validation of proposed method.
6. Applying developed method to marketed formulation.

MATERIALS, INSTRUMENTS AND METHODS

Reagents Used:

Acetonitrile	HPLC Grade
Methanol	HPLC Grade
Water	HPLC water

Table 1

Instruments Used:

Waters HPLC with Alliance with Auto sampler with Empower 2.0 software

Column: Waters $C_{18,\ 150 * 4.6mm,\ 5\mu m}$

Labindia pH – Meter

Vaccum Pump

Afcoset Digital Balance

METHOD DEVELOPMENT AND OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS

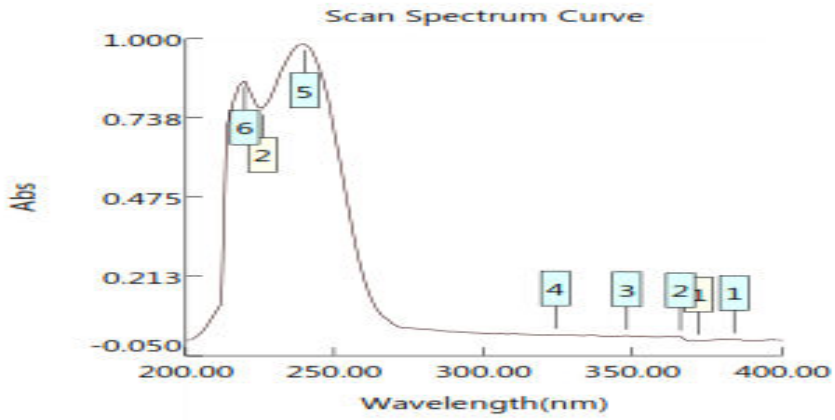
Solubility:

According to literature, Doltugranavir is freely soluble in acetonitrile. And it was checked for different dilutions of acetonitrile and methanol for solubility of Doltugranavir. Finally methanol was choosen as solvent for present work.

Selection of wavelength: (λ_{max})

From the UV Visible spectrophotometric results, the detection wavelength of 240 nm (for Doltugranavir) was selected because at this wavelength they show maximum absorbance. At 240 nm the chromatogram was observed in PDA detector. So the chromatographic condition was optimized at 240 nm.

UV Spectrum for Doltugranavir:



No.	P/V	Wavelength(nm)	Abs	Comment
1	Peak	384.00	0.005	
2	Peak	366.00	0.013	
3	Peak	348.00	0.017	
4	Peak	324.00	0.019	
5	Peak	240.00	0.982	
6	Peak	220.00	0.861	
1	Valley	372.00	-0.001	
2	Valley	226.00	0.768	

Fig : 7 UV Spectrum for Doltugranavir

Selection of initial chromatographic conditions:

Appropriate selection of chromatographic method depends upon the characteristic nature of the sample (ionic or ionisable or neutral), its molecular weight and solubility. The nature of Doltugranavir is polar. Hence reverse phase chromatography is used. The reverse phase HPLC was selected for initial chromatographic condition because of its simplicity and suitability.

METHOD DEVELOPMENT TRIALS

TRAIL-I

OBJECTIVE: To develop a method for the assay of Doltugranavir by HPLC.

Chromatographic conditions:

Column : C_{18} (4.6 X 150 mm; 5µm Waters)

Column temperature : room temperature

Flow rate : 1 ml/min.

Injection volume : 20 µl.

Wavelength : 240 nm.

Run time : 10 min.

Mobile phase preparation: buffer (pH 4.6): Acetonitril (60:40%v/v)

Diluents: mobile phase

Standard preparation: (stock preparation)

Accurately weighed quantity of 10 mg of Doltugranavir was transferred to 10 ml volumetric flask, dissolve and dilute to volume with mobile phase mixed.

Stock solution:

Doltugranavir : 1000 ppm

Diluted standard preparation:

From the standard stock preparation 0.3 ml of standard stock solution in 10ml volumetric flask and further diluted with mobile phase

Doltugranavir : 30 ppm

Result :

A peak eluted at 2.195 minute of Doltugranavir the peak shape was not good. Tailing and fronting observed with the analyte peak.

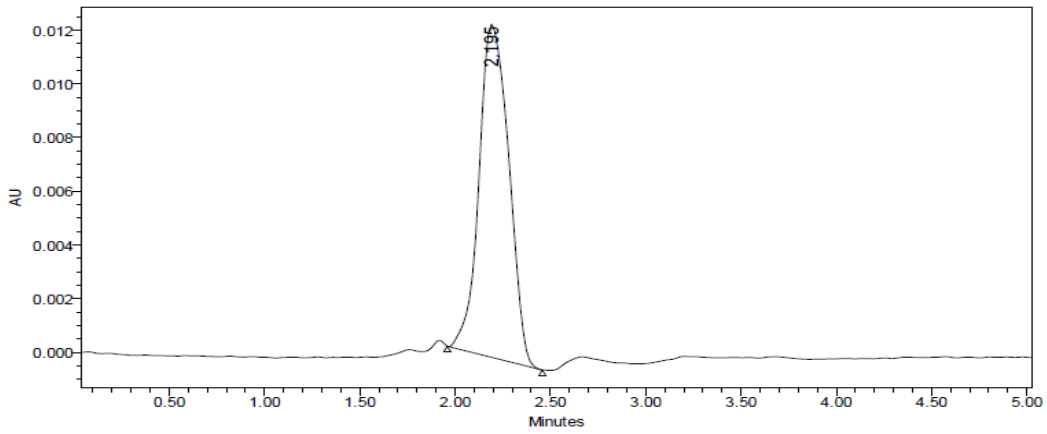


Fig: 8 Trial -1

TRAIL-II

OBJECTIVE: To develop a method for the assay Doltugranavir by HPLC.

Chromatographic conditions:

Column : C_{18} (4.6 X 150 mm; 5µm Waters).

Column temperature: room temperatures

Flow rate : 1 ml/min.

Injection volume : 20 µl.

Wavelength : 240 nm.

Run time : 10 min.

Mobile phase preparation: Water: Acetonitrile (40:60%v/v)

Diluents: mobile phase

Standard preparation: (stock preparation)

Accurately weighed quantity of 10 mg of Doltugranavir was transferred to 10 ml volumetric flask, dissolve and dilute to volume with mobile phase mixed.

Stock solution:

Doltugranavir: 1000ppm

Diluted standard preparation:

From the standard stock preparation 0.3ml of standard stock solution in 10 ml volumetric flask and further diluted with mobile phase

Doltugranavir: 30 ppm

Result:

A peak was eluted at 2.355 minute Doltugranavir which showed Tailing. The peak shape was not good.

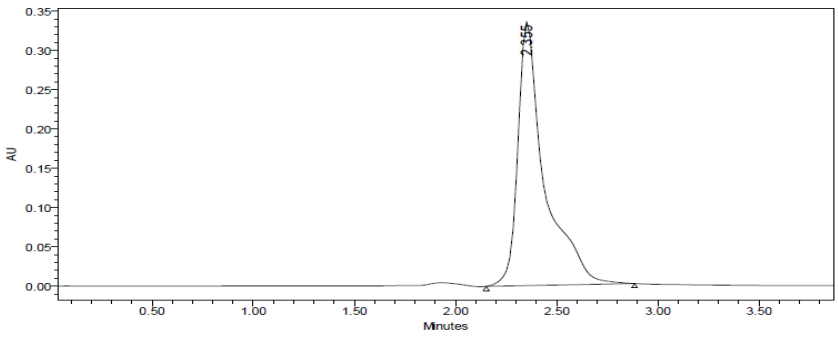


Fig: 9 Trial -2

TRAIL-3

OBJECTIVE: to develop a method for the assay of Doltugranavir by HPLC.

Chromatographic conditions:

Column : Agilent C_{18} (4.6 X 150 mm; 5µm Waters).

Column temperature: room temperatures

Flow rate : 1 ml/min.

Injection volume : 20 µl.

Wavelength : 240 nm.

Run time : 10 min.

Mobile phase preparation: methanol: Acetonitrile (60:40%V/v)

Diluents: mobile phase

Standard preparation: (stock preparation)

Accurately weighed quantity of 10 mg of Doltugranavir was transferred to 10 ml volumetric flask, dissolve and dilute to volume with mobile phase mixed.

Stock solution:

Doltugranavir: 1000ppm

Diluted standard preparation:

From the standard stock preparation 0.3ml of standard stock solution in 10 ml volumetric flask and further diluted with mobile phase

Doltugranavir: 30 ppm

Result:

A peak was eluted at 2.425 minute for Doltugranavir, which showed fronting.

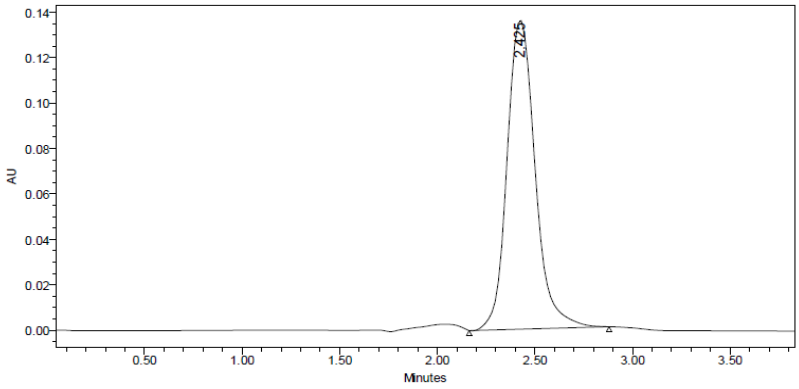


Fig: 10 Trial - 3

TRAIL - 4

Optimized Chromatographic conditions:

Column	: Symmetry C_{18} (4.6 X 150 mm; 5µm Waters).
Column temperature	: 250C
Flow rate	: 1 ml/min.
Injection volume	: 20 µl.
Wavelength	: 240nm.
Run time	: 10 min.
Diluent	: mobile phase.

Mobile phase composition : methanol: water (60:40%v/v).

Injector : Rheodyne.

Stationary phase : C_{18} (4.6 X 150 mm; 5µm Waters)

Operating temperature : Room temperature.

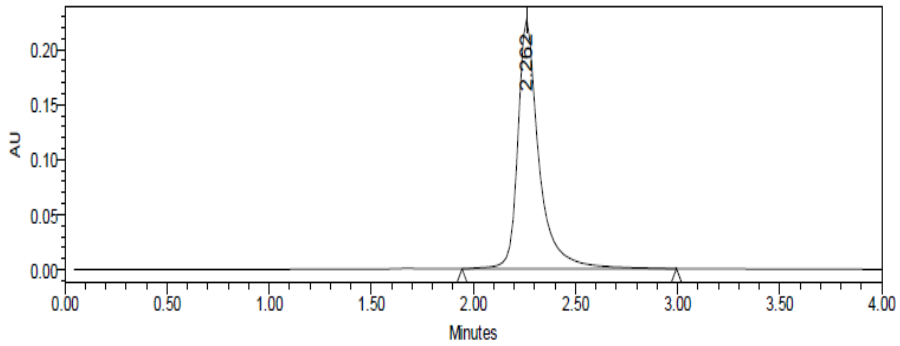


Fig:11 Trial - 4

Observation: Peak shape was good and retention time was good , so it is used as an optimized metho

ASSAY OF PROPOSED METHOD:

Preparation Mobile phase:

Mix a mixture of above methanol (60%), 400 mL of HPLC water (40%) and degas in ultrasonic water bath for 5 minutes. Filter through 0.45 µ filter under vacuum filtration.

Standard Solution Preparation

Accurately weigh and transfer 10 mg of Doltugranavir working standard into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Sample Solution Preparation:

Accurately weigh and transfer equivalent to 368.0 mg of Doltugranavir sample into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.3ml of Doltugranavir of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure:

Inject 20 µL of the standard, sample into the chromatographic system and measure the areas for Doltugranavir peaks and calculate the %Assay by using the formulae.

System Suitability:

Tailing factor for the peaks due to Doltugranavir in Standard solution

Should not be more than 2.0

Theoretical plates for the Doltugranavir peaks in Standard solution

Should not be less than 2000

Calculation: (For Doltugranavir)

Assay % =

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{\text{Avg. Wt}}{\text{Label Claim}} \times 100$$

Where:

AT = average area counts of sample preparation.

As= average area counts of standard preparation.

WS = Weight of working standard taken in mg.

P = Percentage purity of working standard

LC = LABEL CLAIM OF Doltugranavir in mg/ml.

RESULTS:

System Suitability Results:

1). Tailing factor Obtained from the standard injection is 1.5

2). Theoretical Plates Obtained from the standard injection is 2804.8

Assay Results:

1635162 10 0.3 10 10 99.8 368

---------- x ----------x-------- x --------- x-------- x ----------x---------- X 100 = 99.6

1637535 10 10 18.4 0.3 100 200

VALIDATION PARAMETERS

SYSTEM SUITABILITY PARAMETERS

Table 2

S.No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	2396.0	1.3
2	*Actual	2804.8	1.5
3	10% more	2218.0	1.4

Results for actual Mobile phase composition (60:40 methanol: water) have been considered from Accuracy standard.

ACCURACY

Sample preparation:

The accuracy shell be carried out using samples prepared for assay accuracy studies was conducted using triplicate determination as per the test method.

Accuracy for Doltugranavir:

Table 3

%Concentration (at specification Level)	**Area**	**Amount Added (mg)**	**Amount Found (mg)**	**% Recovery**	**Mean Recovery**
50%	823686.2	5.0	5.0	100.1%	99.5%
100%	1634793	10	9.93	99.3%	
150%	2451939	15.0	14.9	99.3%	

Linearity and Range:

Standard preparation:

Doltugranavir working standard solutions were prepared across the range of the analytical method with a minimum of 5 concentrations that are within the specified range (10-50 µg/ml) low level (10 µg/ml)and higher level (50µg/ml) for 5 replicating injections were taken and calculated the %RSD

The degree of linearity was estimated by calculating the correlation coefficient, Y-intercept, slope of the regression line and residue some of squers a plot of data for analyte response Vs its concentration was established**.**

Linearity Data for Doltugranavir:

Table 4

S.No	Linearity Level	Concentration	Area
1	I	10ppm	682741
2	II	20ppm	1201305
3	III	30ppm	1627183
4	IV	40ppm	2180552
5	V	50ppm	2716958
Correlation Coefficient			0.999

Parameters	Doltugranavir
Linearity Range	10-50 µg/ml
Correlation Coefficient	0.999
Slope (m)	

Linearity curve for Doltugranavir:

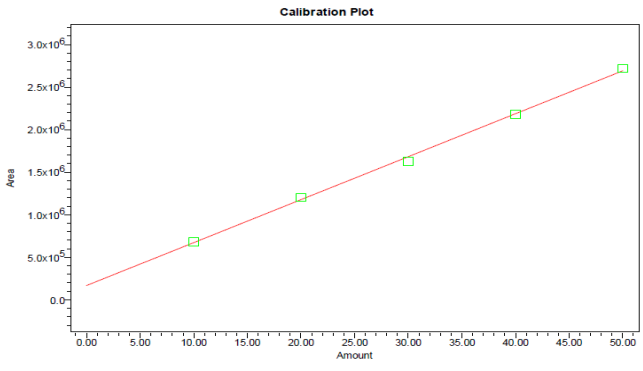


Fig 13 Linearity curve

PRECISION

Precision:

The system precision of the test method was performed by injecting 5 replicate determination of standard preparation injections were injected and the % RSD was calculated.

For Doltugranavir:

Table 5

Injection	Area
Injection-1	1631295
Injection-2	1630511
Injection-3	1636464
Injection-4	1628557
Injection-5	1635684
Average	1632502.2
Standard Deviation	3420.4
%RSD	0.2

Intermediate Precision/Ruggedness:

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day by using different make column of same dimensions.

Intermediate Precision:

The system precision of the test method was performed by injecting 5 replicate determination of standard preparation injections were injected and the % RSD was calculated

Table 6

Injection	Area
Injection-1	1639701
Injection-2	1645897
Injection-3	1640705
Injection-4	1637036
Injection-5	1638609
Average	1640389.4
Standard Deviation	3365.9
%RSD	0.2

ROBUSTNESS

Effect of flow rate

Robustness of assay method was carried out with variation of flow rate. Standard preparation was prepared and performed analysis as per test method and evaluated the system suitability parameters.

Table 7

S.No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	**USP Tailing**
1	0.8	3353.0	1.5
2	1	2804.8	1.5
3	1.2	2384.0	1.4

Effect of Organic Solvent:

Robustness of assay method was carried out with variation of Organic Solvent. Standard preparation was prepared and performed analysis as per test method and evaluated the system suitability parameters.

Table 8

S.No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	**USP Tailing**
1	10% less	2396.0	1.3
2	*Actual	2804.8	1.5
3	10% more	2218.0	1.4

LIMIT OF DETECTION(LOD) :

The lowest amount of analyte in sample that can be detected, but not necessary quantified was determined by comparison of measured signal with 0.02 µg/ml of Doltugranavir standard solutions with those of blank (mobile phase).

LIMIT OF QUANTITATION (LOQ):

The lowest amount of analyte in the sample that can be determined with acceptable precision and accuracy was determined by the comparison of measured signal with 0.05 µg/ml of Doltugranavir.

Chromatographs

1. System Suitability

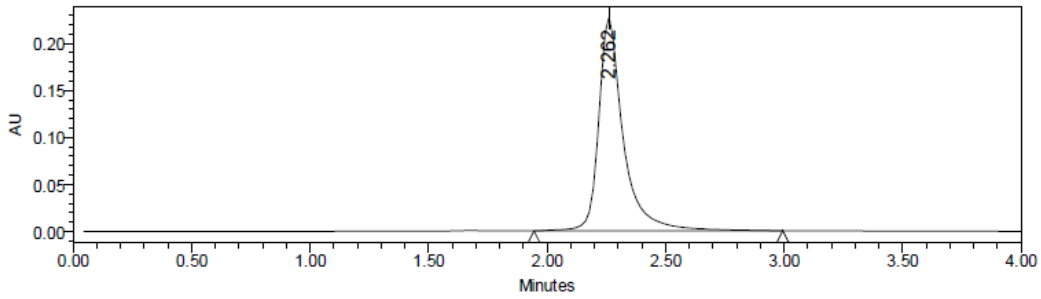


Fig: 13 System Suitability

2. Specificity

Mobile phase blank:

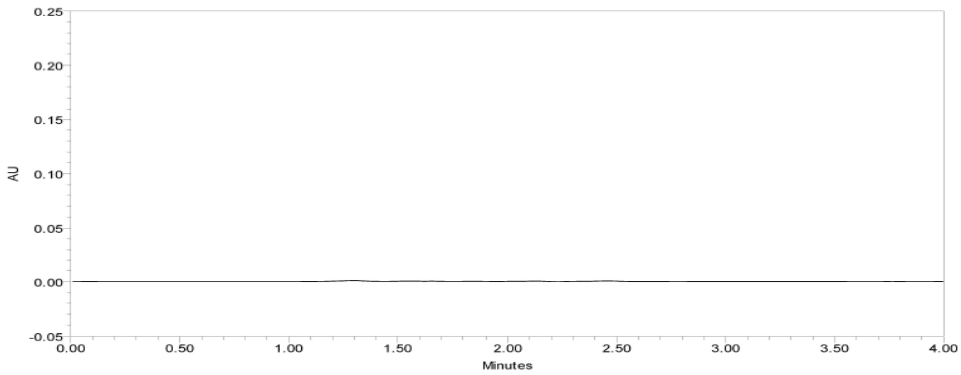


Fig: 14 Blank

1. **Assay standard:**

Standard: Injection 1

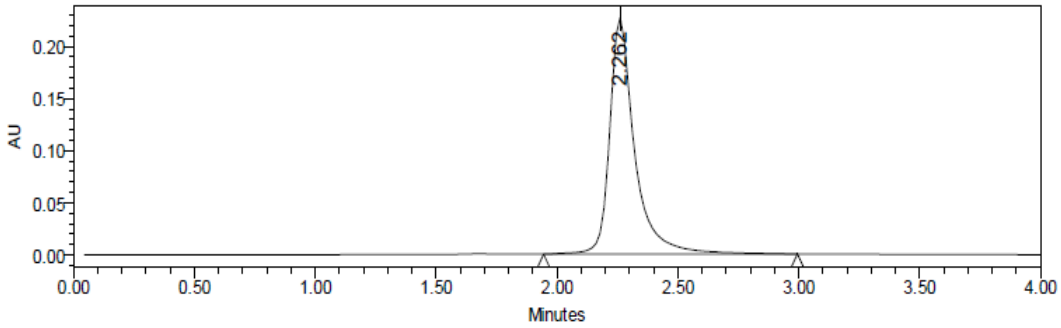


Fig: 15 Inj 1

Standard: Injection 2

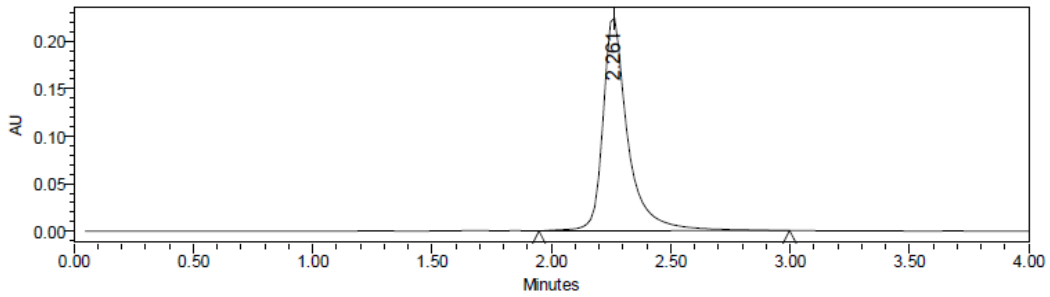


Fig: 16 Inj 2

2. Assay sample:

Sample injection 1:

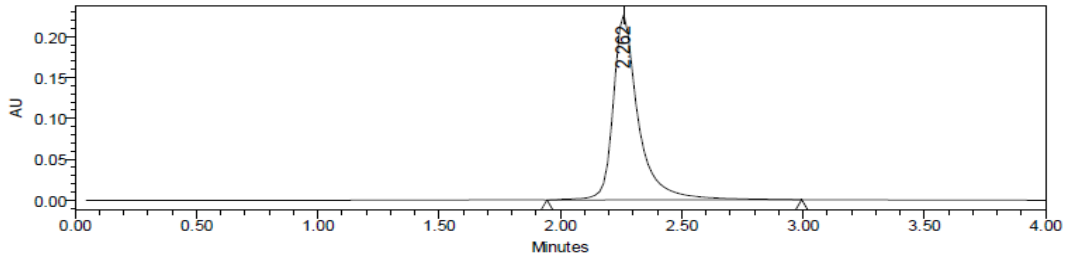


Fig: 17 Spl Inj 1

Sample injection 2:

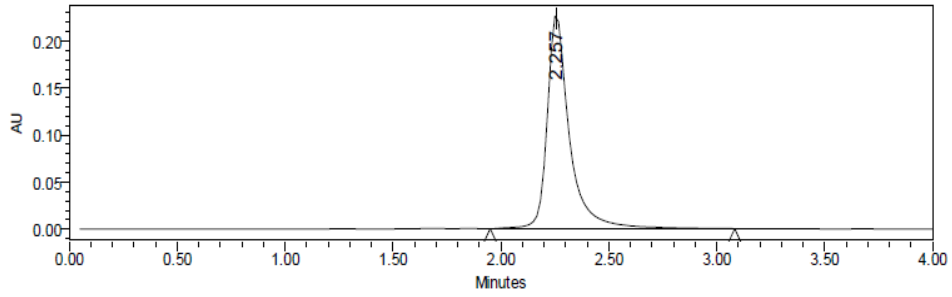


Fig: 18 Spl Inj 2

3. Accuracy:

Standard:

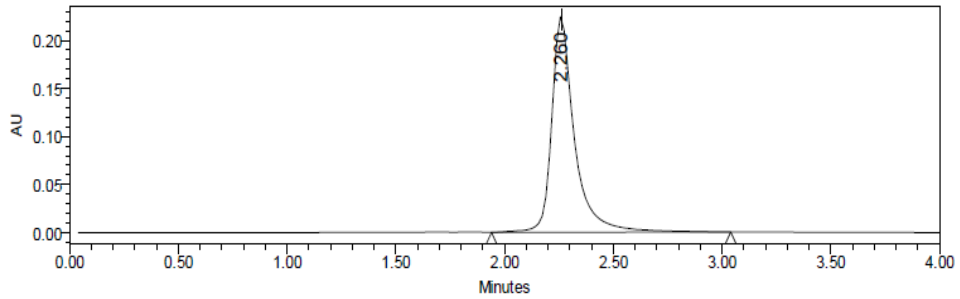


Fig: 19 inj-01_Accuracy-01

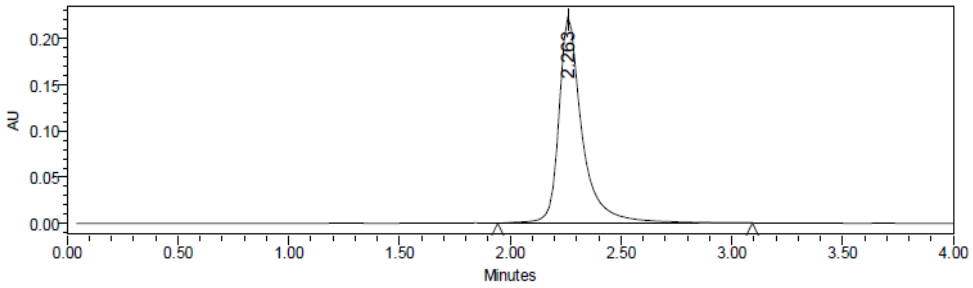


Fig: 20 inj-02_Accuracy-02

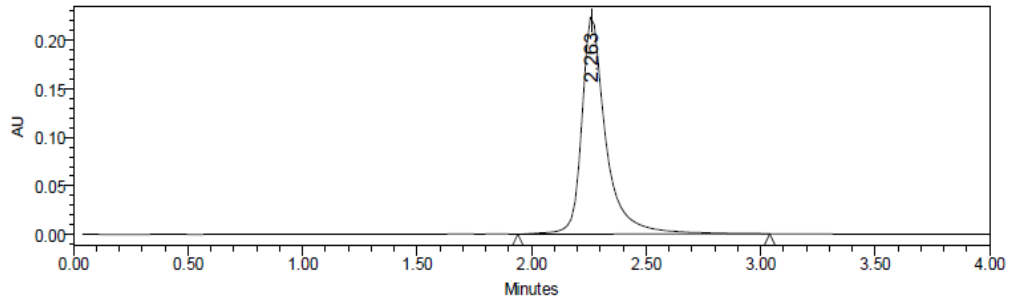


Fig : 21 inj-03_Accuracy-03

Accuracy 50%:

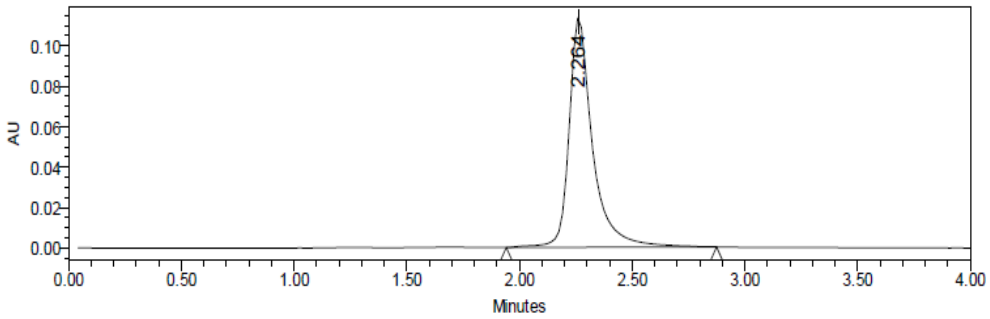


Fig: 22 inj-01_Accuracy-01

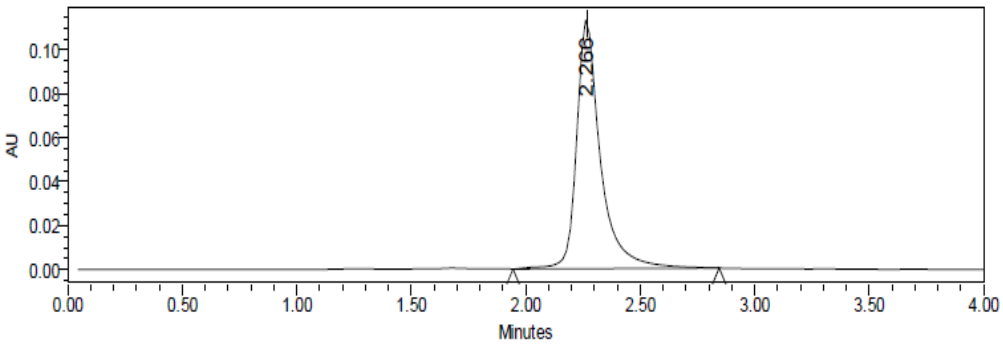


Fig: 23 inj-02_Accuracy-02

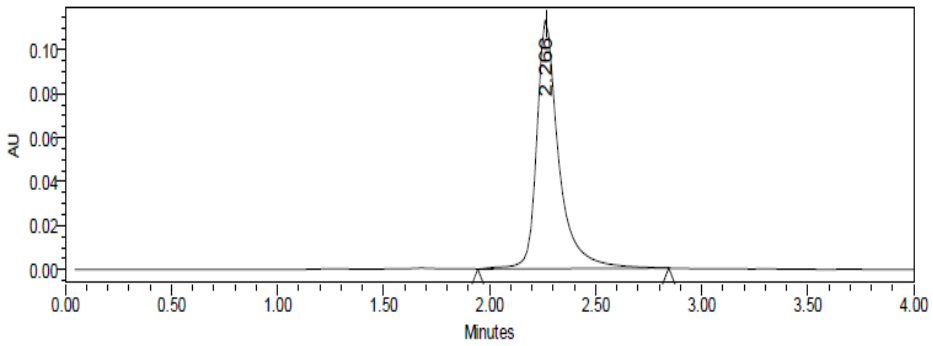


Fig: 24 inj-03_Accuracy-03

Accuracy 100%:

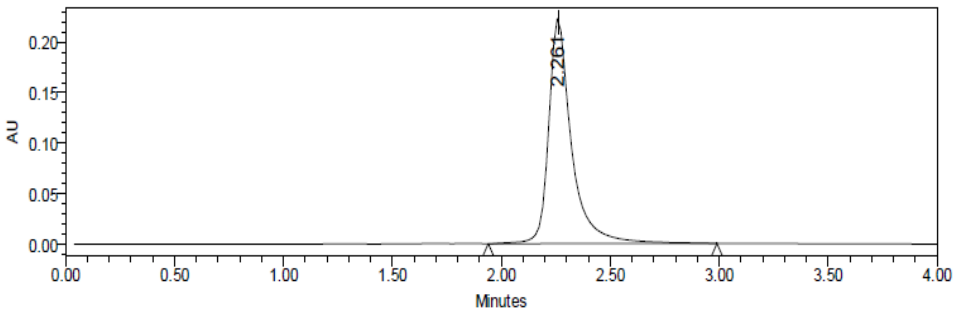


Fig: 25 inj-01_Accuracy-01

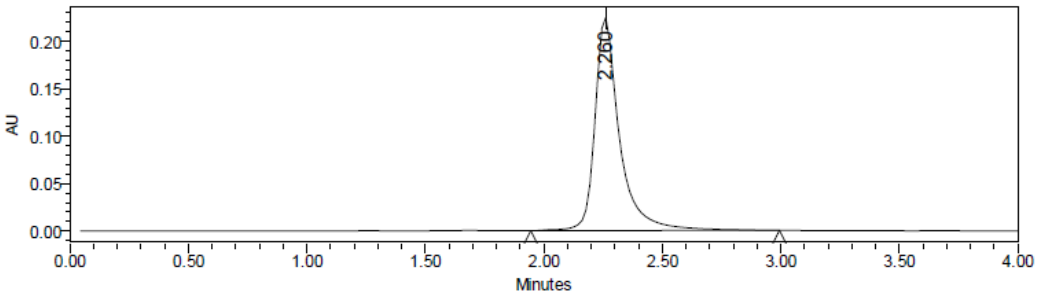


Fig: 26 inj-02_Accuracy-02

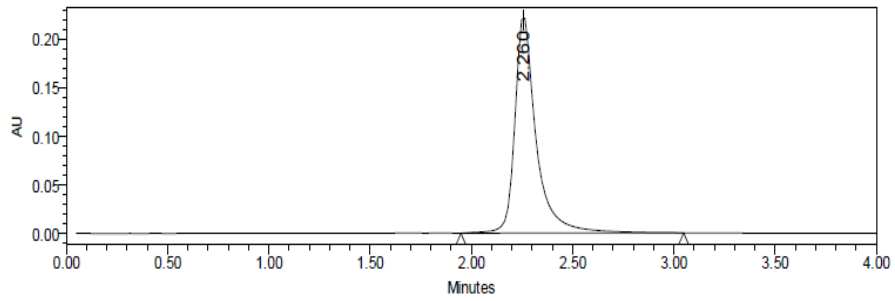


Fig: 27 inj-03_Accuracy-03

Accuracy 150%:

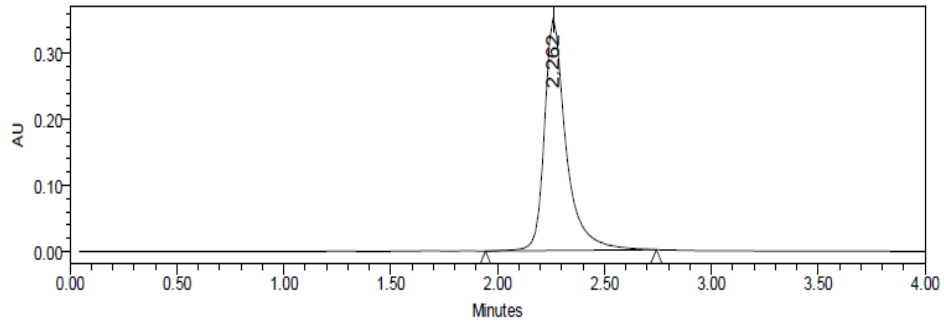


Fig : 28 inj-01_Accuracy-01

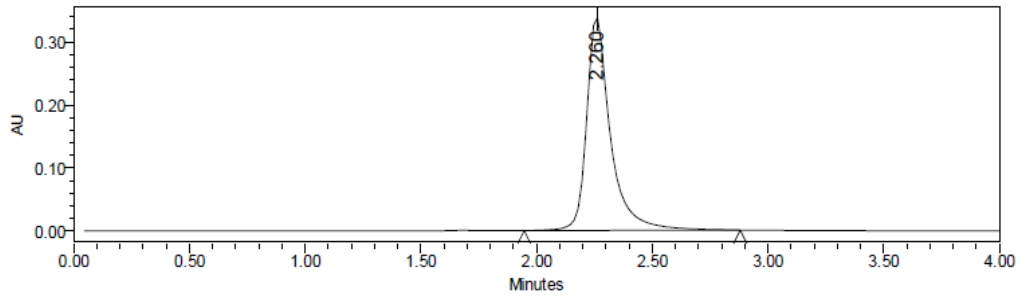


Fig : 29 inj-02_Accuracy-02

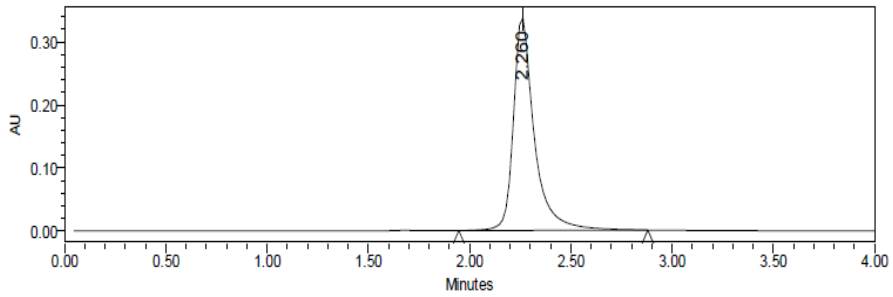


Fig : 30 inj-03_Accuracy-03

4. Linearity:

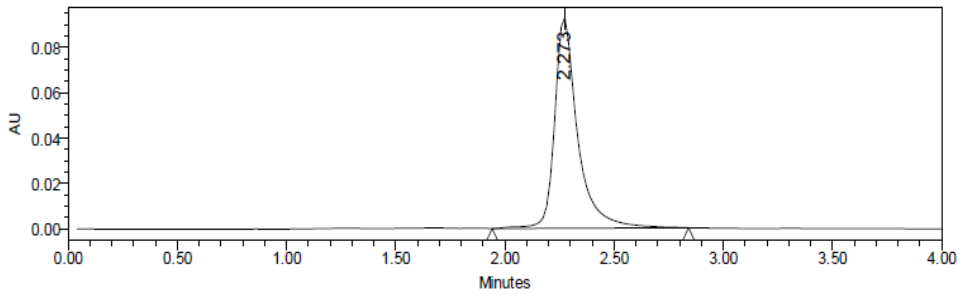


Fig : 31 inj-01_linearity-01

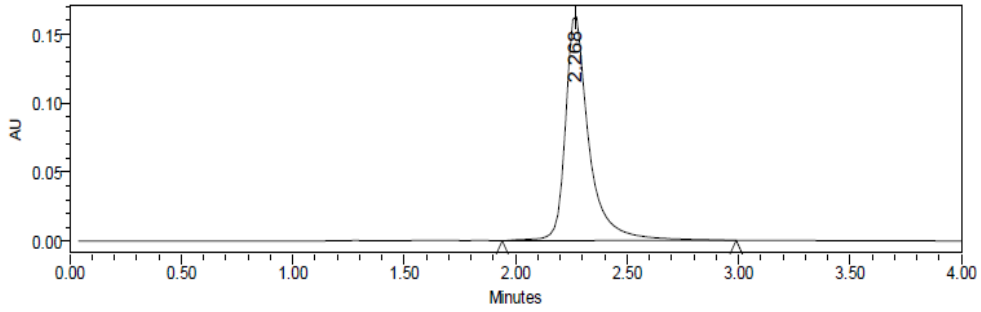


Fig : 32 inj-02_linearity-02

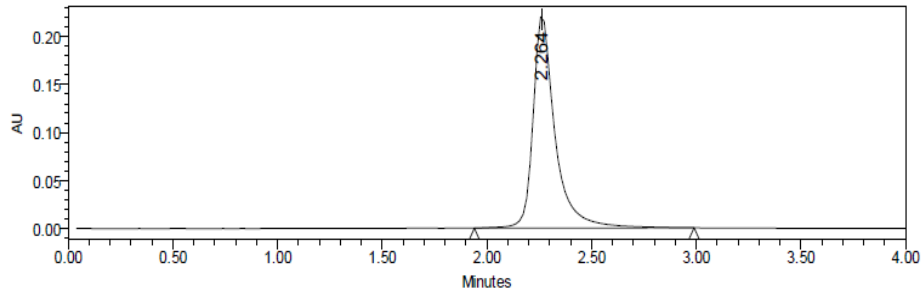


Fig : 33 inj-03_linearity-03

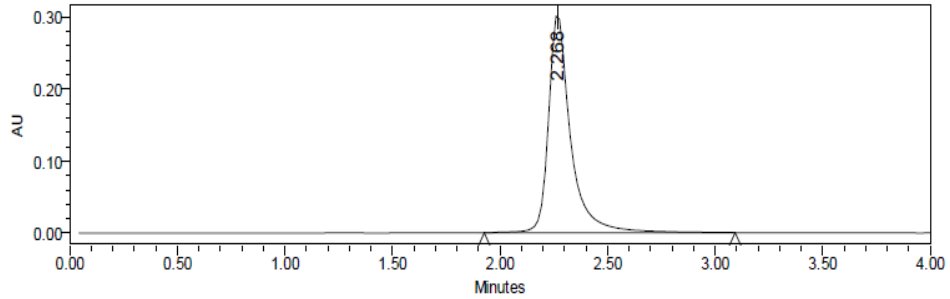


Fig : 34 inj-04_linearity-04

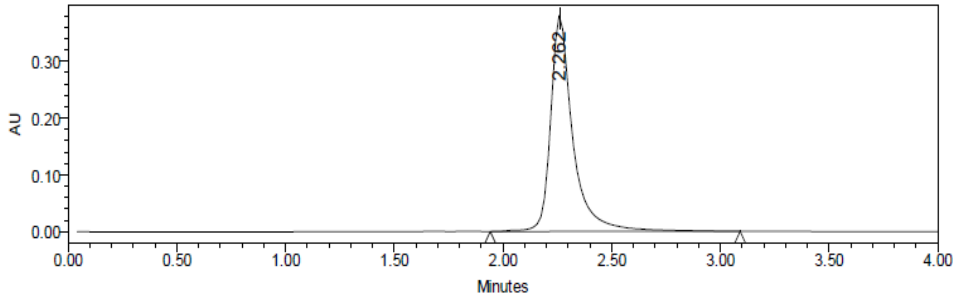


Fig : 35 inj-05 _linearity-05

5. Precision:

Repeatability

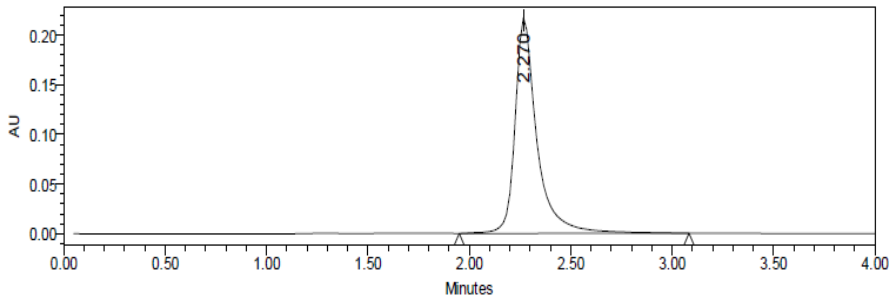


Fig : 36 inj-01 _Precision-01

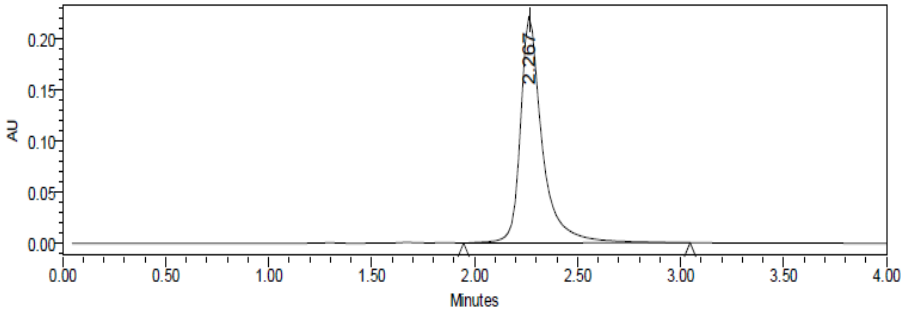


Fig : 37 inj-02 _Precision-02

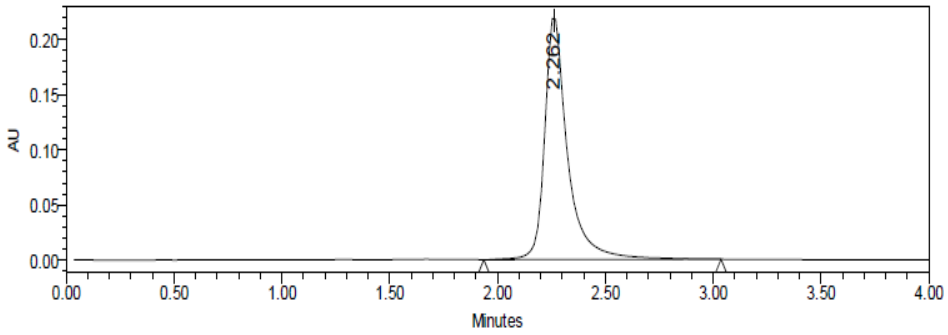


Fig : 38 inj-03 _Precision-03

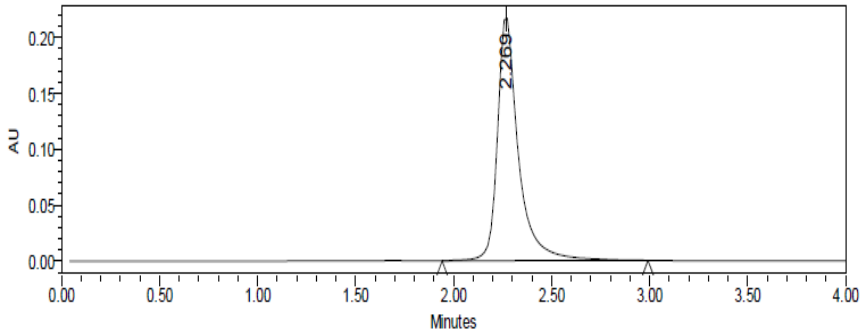


Fig : 39 inj-04 _Precision-04

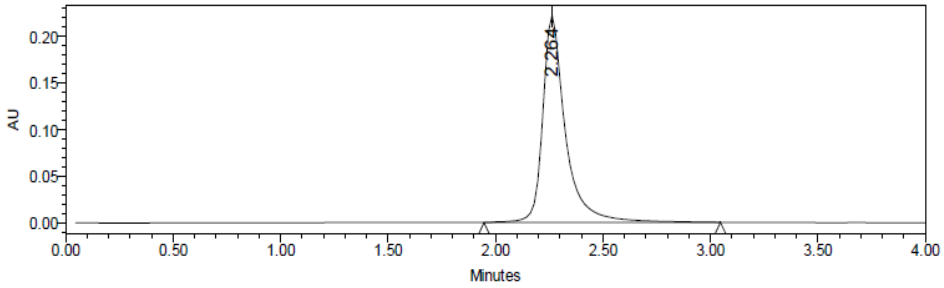


Fig : 40 inj-05 _Precision-05

Intermediate precision:

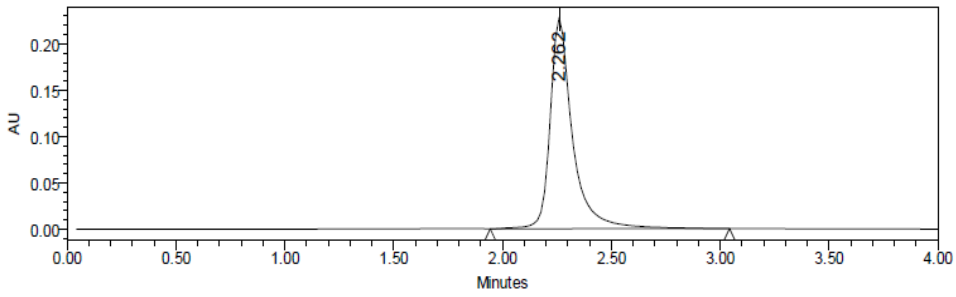


Fig : 41 inj-01 _Intermediate Precision-01

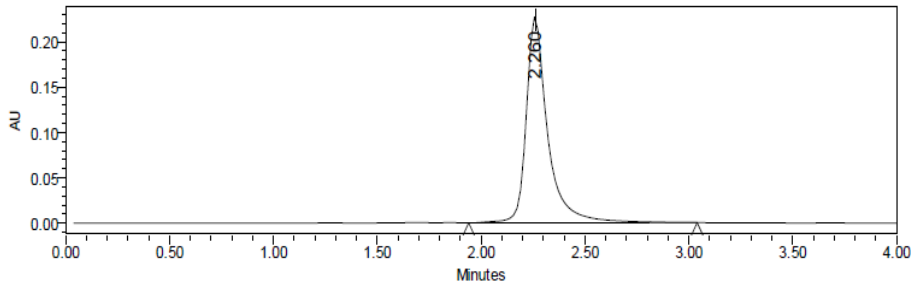


Fig : 42 inj-02 _Intermediate Precision-02

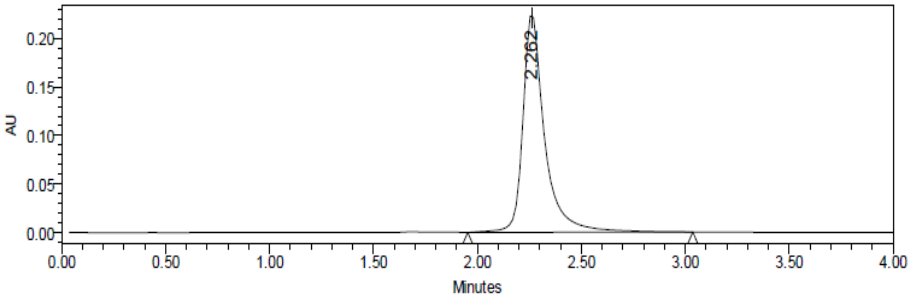


Fig : 43 inj-03 _Intermediate Precision-03

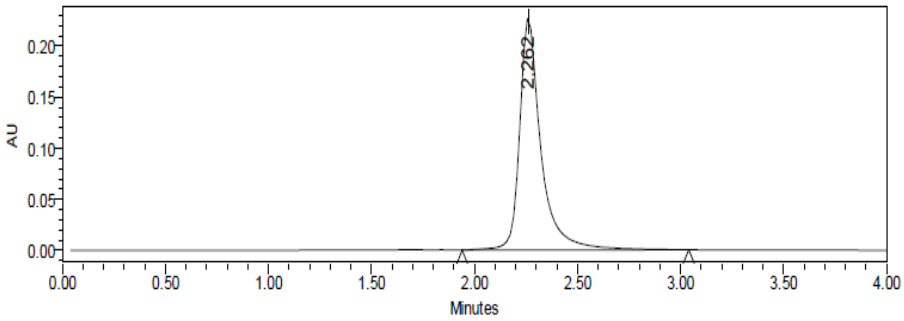


Fig : 44 inj-04 _Intermediate Precision-04

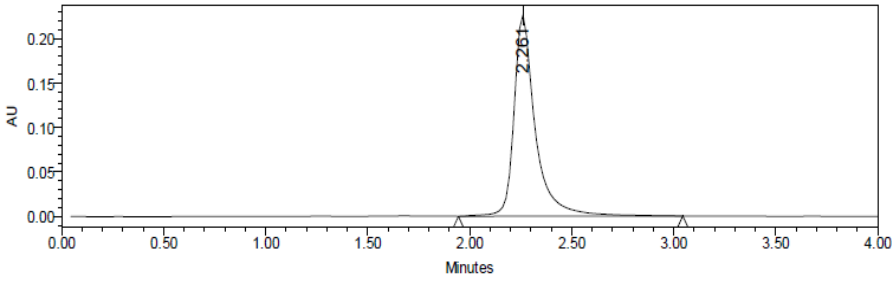


Fig : 45 inj-05 _Intermediate Precision-05

6. Robustness:

Effect of flow rate(Less flow)

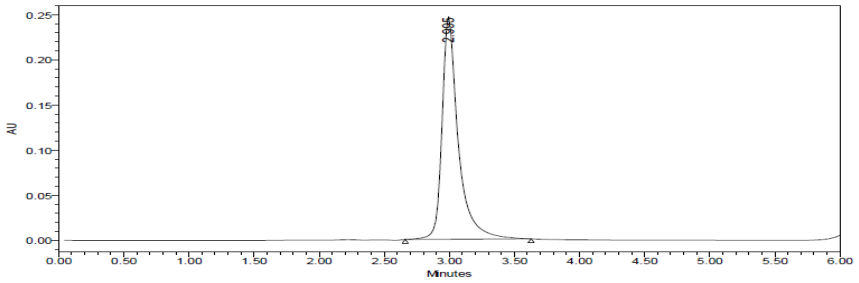


Fig : 46 inj-01_flowrate_+0.2

Effect of flow rate(More flow)

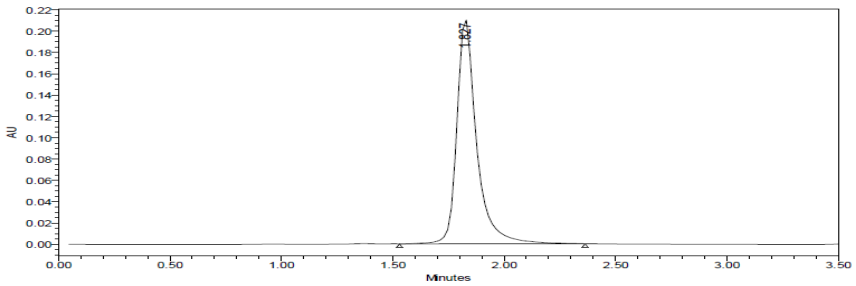


Fig : 47 inj-02_flowrate_--0.2

Effect of Organic Solvent(Less org)

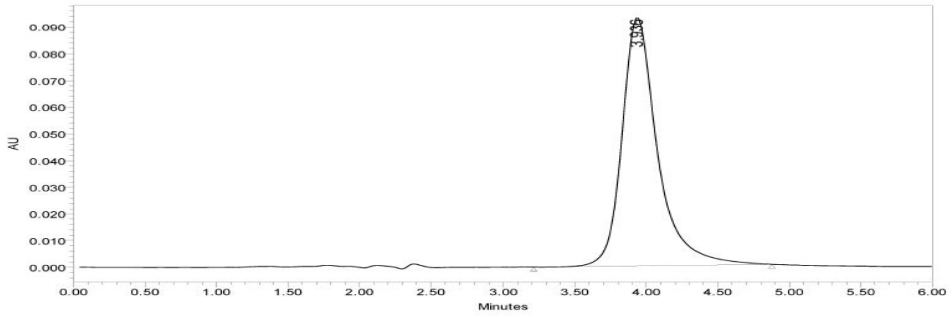


Fig : 48 inj-01_ Organic Solvent_+10

Effect of Organic Solvent:(More org)

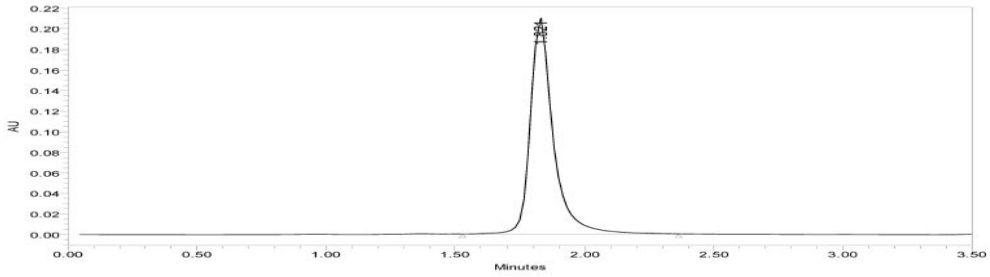


Fig : 49 inj-01_ Organic Solvent_--10

7. LOD:

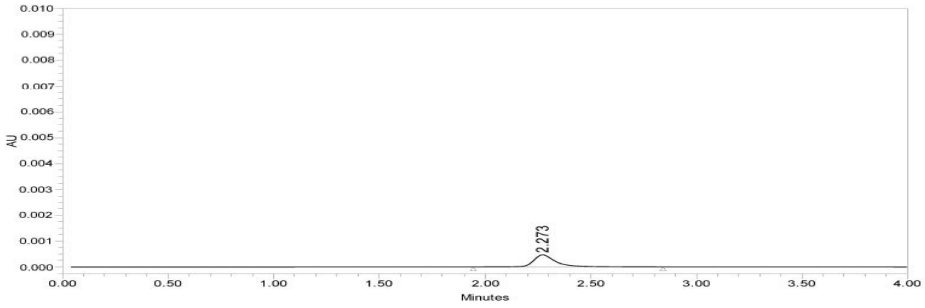


Fig : 50 inj_LOD

8. LOQ:

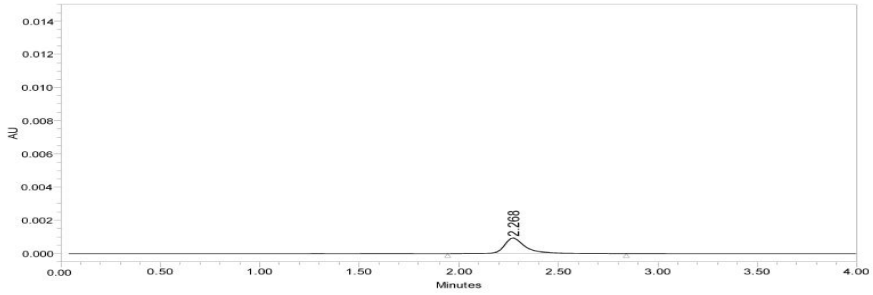


Fig : 51 Inj_loq

Standards

Doltugranavir:

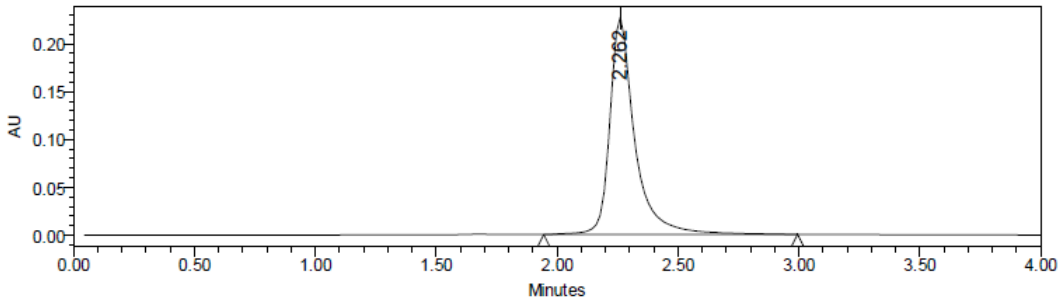


Fig : 52 Inj_standard

RESULT AND DISCUSSION

A simple, precision and accuracy HPLC method was developed the estimation of Doltugranavir analysis of uncoated formulation, consisting of an Methanol: water (60: 40 % v/v). The chromatographic condition was set at a Flow rate of 1 ml/min with the UV detector at 240 nm. The above method was optimized with a view to develop an assay method for Doltugranavir.

Several mobile phase compositions were tried to resolve the peaks of Doltugranavir. The optimum mobile phase containing methanol: water (60: 40 % v/v) was selected because it was found ideal to resolve the analyte peaks of the drug. Quantification was achieved with UV detections at 240 nm based on peak area and absorbence. As per USP requirements system suitability studies were carried out and freshly prepared standard solutions of Doltugranavir. Various parameters obtained with 20 µl of injection volume are summarized in the table given below.

Validation and system suitability parameters

S.NO	PARAMETERS	LIMIT	OBSERVATION
1	System suitability (%RSD of tailing factor)	suitable	1.0
2	Precision: A) Precision B).Intermediate Precision	RSD NMT 2.0%	0.2 0.2
3	Linearity	Correlation coefficient NLT 0.999	0.998
4	Accuracy	%Recovery range98-102 %	99.5%
5	Robustness	RSD NMT 2%	Robustted
6	LOD	S:N Ratio should be more than 3:1	2.92
7	LOQ	S:N ratio should be more than 10:1	9.95

The system is suitable for tailing factor, theoretical plate, and resolution.

The method was specific for the drug.

The data obtained from the precision experiments. The R.S.D. value for precision was indication that the method was efficiently precise.

It is evident that the response for Doltugranavir was strictly linear in the studied concentration range, which is evident from the R.S.D values, slope, intercept and correlation. The method worked well in the range from 10µg/ml to 50µg/ml which

suggests full capacity for the quantification of Doltugranavir. The regression coefficient was found to be 0.998.

Percentage recovery was calculated from 50% to 150% by injecting to HPLC. The excellent recovery was made at each added concentration.

There is allowable variation in flow rate, wave length which indicates that method is robust enough.

The LOD for Doltugranavir was found to be 0.02µg/ml.

The LOQ for Doltugranavir was found to be 0.05 µg/ml.

The chromatogram of sample showed a single peak at the retention time (2.273) of Doltugranavir indicating that there is no interference of the changing the persons for injecting the sample to the instrument.

SUMMARY AND CONCLUSION

- The reliability and suitability of the method could be seen from recovery studies. Further there is no interference due to excipients.
- System suitability parameters were calculated which includes efficiency, resolution and tailing factor.
- Precision of the methods were studied by making repeated injections of the samples and system precision values were determined.
- The method was validated for linearity, accuracy, precision, robustness.
- The method is simple, specific & easy to perform and requires short to analyse the samples.
- Low limit of Quantification and limit of detection makes this method suitable for Quality control.
- The method was found to be accurate, precise and robusted.
- Hence it was concluded that the RP-HPLC method developed was very much suit for routine analysis. Emtricitabine in tablet formulations and future planings use this method for estimation Emtricitabine in clinical trials.

FUTURE SCOPE OF WORK:

In the above mentioned HPLC method for estimation of Emtricitabine in single tablet dosage form, as the drug Emtricitabine are estimated with hence the present method is faster, can be used for routine analysis of these drugs from tablet formulation.

BIBLIOGRAPHY

1. Ravindra, Development and validation of uv spectrophotometric method for estimation of dolutegravir sodium in bulk and pharmaceutical formulations, world journal of pharmaceutical research, Vol 19 No 6 (2015): 1156 – 1163.

2. T.Sudha, V.R.Ravikumar 2 P.V. Hemalatha, 2 RP-HPLC Method for the Simultaneous Estimation of Lamivudine and Abacavir Sulphate in Tablet Dosage Form, International Journal on Pharmaceutical and Biomedical Research) (IJPBR), Vol. I(4), 2010, 108-113

3. Sarat, M & Krishna,P & Rambabu, Cheedalla, Development and Validation of RP-UPLC Method for Simultaneous Estimation of Abacavir Sulphate and Lamivudine in Combined Tablet Dosage Form, International Journal of Chem Tech Research CODEN (USA) Vol 10, Issue 1 (Jan –Feb), 2018

4. Mohideen, Shafiullah & Vinaykumar, G. Surendranath, Y. Sureshkumar, P. Krishnan, Validated RP-HPLC for simultaneous estimation of Abacavir and Lamivudine in Tablet Dosage Form, International Journal of Pharmacy and Pharmaceutical Sciences, January 2012, 349-356.

5. Brij Bhushan, Uttam Singh Baghel, Ramandeep Singh, RP-HPLC method development for the estimation of levocitrizine and phenylephrine hydrochloride in combined dosage form, International Journal of Pharmaceutical and Medical Research, 2013, I(2):85-90.

6. Susan L. Ford, Int. Med. 35(9): 749–751 (1996).

7. M Grégoire-American journal research committe1 (1): 9–11 (2008).

8. Grégoire M, IJPhS 63(5): 433–436 (2001).

9. ICH, Q1A (R2) Stability Testing of New Drug Substances and Products, International Conference on Harmonization, Geneva, Q1A (R2), current step 4 ,1-24, Feb-2003.

10. Burugula L, 2nd ed. pp.135-148. John Wiley and Sons, Inc. New York, U.S.A.1986.

11. B.H.M. Mruthyunjayaswamy, S. M. M. Patil, and S. A. Raju. Spectrophotometric methods for the estimation of Doltugranavir in pharmaceutical formulations, Indian J. Pharmaceuit. Sci. 63: 433-436 (2001).

12. S. Appala Raju, M. Shobha and S. Manjunath. Spectrophotometric determination of Doltugranavir in bulk drug and formulations, Asian J. Chem. 14: 520-522 (2002).

13. J. Lalla, P. Hamrapukar and T. Wadhwa. High performance thin layer chromatographic determination of Doltugranavir in its dosage form, J. Planar Chromatography. 16: 447-450 (2003).

14. M. B. Patel, K. M. Patel, G. S. Patel and B. N. Suhaghia, A. M. Prajapati. Development and validation of a stability indicating HPTLC densitometric method for Doltugranavir, J. Lid. Chromatography related Technol. 30: 2459-2471 (2007).

15. Balasaheb, B.G. Balasaheb, A.K. Subhash, T.R. Jijabapu, K. Sudhakar. Development and validation of UV spectrophotometric method for estimation of dolutegravir sodium in tablet dosage form. Malaysian Journal of Analytical Sciences 19(6),1156-1163, 2015.