DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR ESTIMATION OF NELARABINE IN BULK AND PHAMACEUTICAL DOSAGE FORM

A dissertation submitted to

THE TAMILNADU Dr.M.G.R MEDICAL UNIVERSITY

CHENNAI- 600 032.

In partial fulfillment of the requirements for the award of Degree of

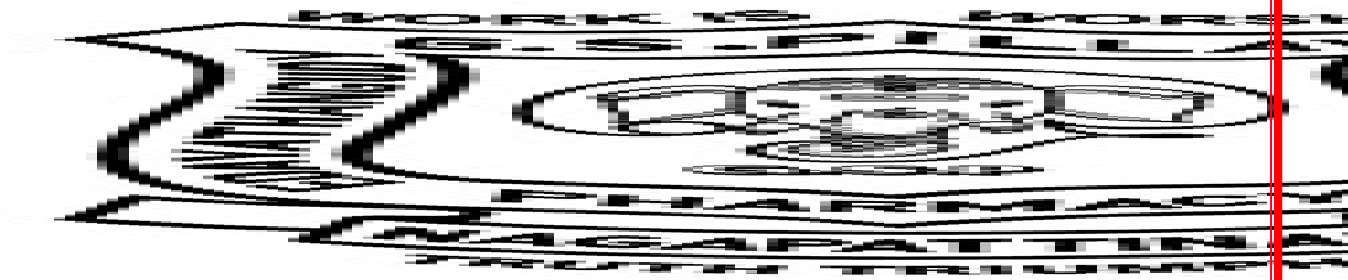
MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

Submitted
By

Reg No: 261230958



DEPARTMENT OF PHARMACEUTICAL ANALYSIS

EDAYATHANGUDY.G.S PILLAY COLLEGE OF PHARMACY

NAGAPATTINAM-611002

APRIL 2014

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Srinivas Ganta

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Under the guidance of

Prof.Dr. P. Dheen Kumar, M.Pharm., Ph.D.,

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CERTIFICATE

This is to certify that the dissertation entitled **"Development and validation of stability indicating RP- HPLC method for estimation of Nelarabine in bulk and pharmaceutical dosage form"** submitted by **Srinivas ganta** (Reg No: 261230958) in partial fulfillment for the award of degree of Master of Pharmacy to the Tamilnadu Dr. M.G.R Medical University, Chennai is an independent bonafide work of the candidate carried out under my guidance in the Department of Pharmaceutical Analysis, Edayathangudy.G.S Pillay College of Pharmacy during the academic year 2013-2014.

Place: Nagapattinam **(Prof. Dr. Dheen Kumar, M.Pharm., Ph.D.,)**

Date:

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Place: Nagapattinam (**Prof.Dr.D.Babu Ananth, M.Pharm., Ph.D.,**)

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INDEX

1. INTRODUCTION

The quality of a drug plays an important role in ensuring the safety and efficacy of the drugs. Quality assurance and control of pharmaceutical and chemical formulations is essential for ensuring the availability of safe and effective drug formulations to consumers. Hence Analysis of pure drug substances and their pharmaceutical dosage forms occupies a pivotal role in assessing the suitability to use in patients. The quality of the analytical data depends on the quality of the methods employed in generation of the data. Hence, development of rugged and robust analytical methods is very important for statutory certification of drugs and their formulations with the regulatory authorities.[1]

The wide variety of challenges are encountered while developing the methods for different drugs depending on its nature and properties. This along with the importance of achieving the selectivity, speed, cost, simplicity, sensitivity, reproducibility and accuracy of results gives an opportunity for researchers to come out with solution to address the challenges in getting the new methods of analysis to be adopted by the pharmaceutical industry and chemical laboratories.

1.1.ANALYTICAL METHODS

Analytical methods are defined as the set of techniques that allow us to determine qualitatively and / or quantitatively the composition of any material and chemical state in which it is located.[1]

Different physico-chemical methods are used to study the physical phenomenon that occurs as a result of chemical reactions.

Chemical methods:

The chemical methods include the gravimetric and volumetric procedures which are based on complex formation; acid-base, precipitation and redox reactions. Titrations in non-aqueous media and complexometry have also been used in pharmaceutical analysis.[2]

Instrumental (Physical methods) :

Table. No. 1.1. Classification of Instrumental methods

S.NO	Method	Examples
1.	Electrochemical methods [2]	Potentiometry
		Conductometry
		Electrogravimetry
		Polarography
		Coulometry
2.	Optical methods	Atomic absorption spectroscopy
		Raman spectroscopy
		Emission spectroscopy
		Refractometry
		Absorption spectrophotometry
		Turbidimetry
		Nephelometry
		Luminescence analysis
		X-Ray spectroscopy
3.	Radiometric methods	Isotopic dilution
4.	Mass spectroscopy	
5.	Nuclear magnetic resonance	
6.	Chromatography (separation and analytical method)	Thin layer Chromatography
		Paper Chromatography
		Column Chromatography
		High Performance Liquid Chromatography

		Ion Exchange Chromatography
		Gas Chromatography

1.2 CHROMATOGRAPHY

Chromatography (Chroma means 'color' and graphein means to 'write') is the collective term for a set of laboratory techniques for the separation of mixtures. It involves passing a mixture dissolved in a "mobile phase" through a stationary phase, which separates the analyte to be measured from other molecules in the mixture based on differential partitioning between the mobile and stationary phases.[3]

Table. No.1. 2.Different types of chromatographic techniques

S. No	Basic principle involved	Type of Chromatography
1.	Techniques by chromatographic bed shape	Column chromatography
		Paper chromatography
		Thin layer chromatography
2	Techniques by physical state of mobile phase	Gas chromatography
		Liquid chromatography
3	Affinity chromatography	Supercritical fluid chromatography
4	Techniques by separation mechanism	Ion exchange chromatography
		Size exclusion chromatography
5	Special techniques	Reversed phase chromatography
		Simulated moving-bed chromatography
		Pyrolysis gas chromatography
		Fast protein liquid chromatography

		Counter current chromatography
		Chiral chromatography

1.3.HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The modern form of column chromatography has been called high performance, high pressure, high-resolution and high-speed liquid chromatography.

High-Performance Liquid Chromatography (HPLC) is a special branch of column chromatography in which the mobile phase is forced through the column at high speed. As a result the analysis time is reduced by 1-2 orders of magnitude relative to classical column chromatography and the use of much smaller particles of the adsorbent or support becomes possible which increase the column efficiency substantially.[4]

CLASSIFICATION OF HPLC

Table. No. 1.3.Classification of Chromatography [4]

Sl. No		Type of Chromatography
1	Modes of Chromatography	Normal phase Chromatography
		Reverse phase Chromatography
2	Principle of separation	Adsorption Chromatography
		Partition Chromatography
		Ion exchange Chromatography
		Size exclusion Chromatography
		Affinity Chromatography
		Chiral phase Chromatography
3	Elution Technique	Isocratic Separation
		Gradient Separation
4	Scale of Operation	Analytical HPLC
		Preparative HPLC
5	Type of Analysis	Qualitative Analysis
		Quantitative Analysis

Partition chromatography

This method results from a thermodynamic distribution of analytes between two liquid phases. On the basis of relative polarities of stationary and mobile phase, partition chromatography can be divided into normal phase and reverse phase chromatography.

Normal Phase - High Performance Liquid Chromatography (NP-HPLC)

In Normal phase HPLC the stationary phase is polar and mobile phase is non-polar. Common solvents (such as hexane, heptanes, etc.) with the small addition of polar modifier (i.e., methanol, ethanol) are generally used. Packing materials traditionally used in NP-HPLC are usually porous oxides such as silica (SiO_2) or alumina (Al_2O_3). Surface of these stationary phases is covered with the dense population of OH groups, which makes these surfaces highly polar. Chemically modified stationary phases can also be used in NP-HPLC. Silica modified with trimethoxy glycidoxypropyl silanes (common name: diol-phase) is typical packing material with decreased surface polarity. Since NP-HPLC uses mainly non-polar solvents, it is the method of choice for highly hydrophobic compounds (which may show very stronger interaction with non polar mobile phases), which are insoluble in polar or aqueous solvents.[5]

Reversed Phase - High Performance Liquid Chromatography (RP-HPLC)

As opposed to NP-HPLC, RP-HPLC employs mainly dispersive forces (hydrophobic or vanderwal's interactions). The polarities of mobile and stationary phases are reversed, such that the surface of the stationary phase in RP-HPLC is hydrophobic and mobile phase is polar, where mainly water-based solutions are employed. RP-HPLC is by far the most popular mode of chromatography. Almost 90% of all analyses of low-molecular-weight samples are carried out using RP-HPLC. Dispersive forces employed in this separation mode are the weakest intermolecular forces, thereby making the overall

background interaction energy in the chromatographic system very low compared to other separation techniques. This low background energy allows for distinguishing very small differences in molecular interactions of closely related analytes. Adsorbents employed in this mode of chromatography are porous rigid materials with hydrophobic surfaces. The majority of packing materials used in RP-HPLC are chemically modified porous silica.[5]

Adsorption chromatography

The analyte interact with solid stationary surface and are displaced with eluent for active sites on surface.

Ion exchange chromatography (IEC)

IEC is based on the differences in affinities of the analyte ions for the oppositely charged ionic center in the resin or adsorbed counter ions in the hydrophobic stationary phase. Consider the exchange of two ions $\mathbf{A^+}$ and $\mathbf{B^+}$ between the solution and exchange resin $\mathbf{E^-}$:

$$A{\cdot}E + B^+ \leftrightarrow B{\cdot}E + A^+$$

The equilibrium constant for this process is shown in Eq. below:

$$\mathbf{K} = \frac{[A+][BE]}{[AE][B+]}$$

This essentially determines the relative affinity of both cations to the exchange centers on the surface. If the constant is equal to 1, no discriminating ability is expected for this system.Four major types of ion-exchange centers are usually employed [6]:

- SO_3^-—strong cation-exchanger
- CO_2^-—weak cation-exchanger
- Quaternary amine—strong anion-exchanger
- Tertiary amine—weak anion-exchanger

Analyte retention and selectivity in ion exchange chromatography are strongly dependent on the pH and ionic strength of the mobile phase.

Size exclusion chromatography (SEC):

SEC is the method for dynamic separation of molecules according to their size. The separation is based on the exclusion of the molecules from the porous

space of packing material due to their steric hindrance. Hydrodynamic radius of the analyte molecule is the main factor in determining its retention. This is the only chromatographic separation method where any positive interaction of the analyte with the stationary phase should be avoided.

In SEC, the higher the molecular weight of the molecule, the greater its hydrodynamic radius results in faster elution. [6]

INSTRUMENTATION OF HPLC

HPLC is a special branch of Column Chromatography in which the mobile phase is forced through the column at high speed. As a result, the analysis time is reduced by 1-2 orders of magnitude relative to classical Column chromatography and the use of much smaller particles of the absorbent or support becomes possible increasing the column efficiency substantially[7]. The basic HPLC Instrumentation is shown in the Fig. No. 1.1

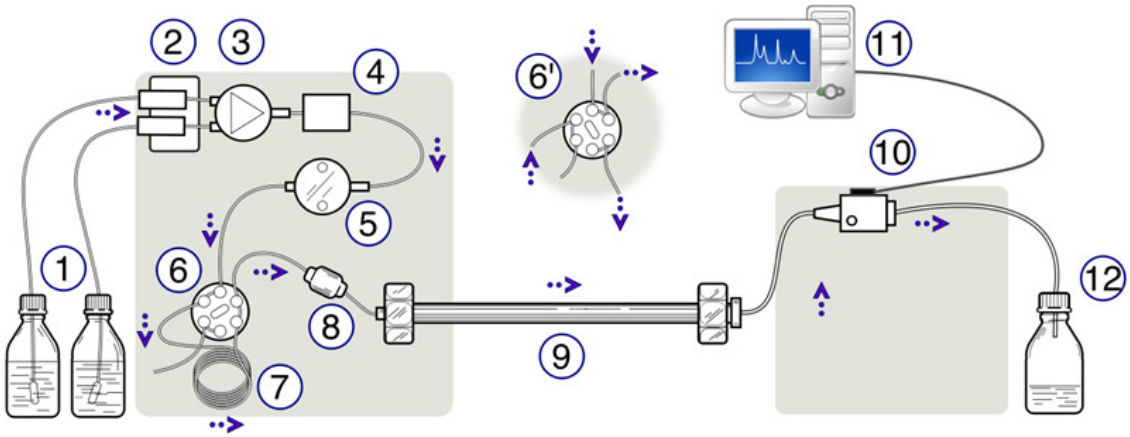


Figure .1.1. Instrumentation of a High Performance Liquid Chromatography.

(1) Solvent reservoirs,

(2) Solvent degasser,

(3) Gradient valve,

(4) Mixing vessel for delivery of the mobile phase,

(5) High-pressure pump ,

(6') Switching valve in "load position",

(6) Switching valve in "inject position",

(7) Sample injection loop,

(8) Pre-column or guard column

(9) Analytical column,

(10) Detector (i.e. IR, UV),

(11) Data acquisition,

(12) Waste or fraction collector.

i.Solvent delivery system:

The most important component of HPLC in solvent delivery system is the pump, because its performance directly effects the retention time, reproducibility and detector sensitivity. Among the several solvent delivery systems, (direct gas pressure, pneumatic intensifier, reciprocating etc.) reciprocating pump with twin or triple pistons is widely used, as this system gives less baseline noise, good flow rate reproducibility etc.

The pumping systems used in HPLC can be categorized in three different ways.

- The first classification is according to the eluent flow rate that the pump is capable of delivering: Standard bore systems, Micro bore systems
- The second classification is according to the construction materials : Metallic, non-metallic
- The final classification is according to the mechanism by which the pump delivers the eluent : syringe pumps and reciprocating-piston pump.

ii.Solvent degassing system

The constituents of the mobile phase should be degassed and filtered before use. Several methods can be applied to remove the dissolved gases in the mobile phase. They include

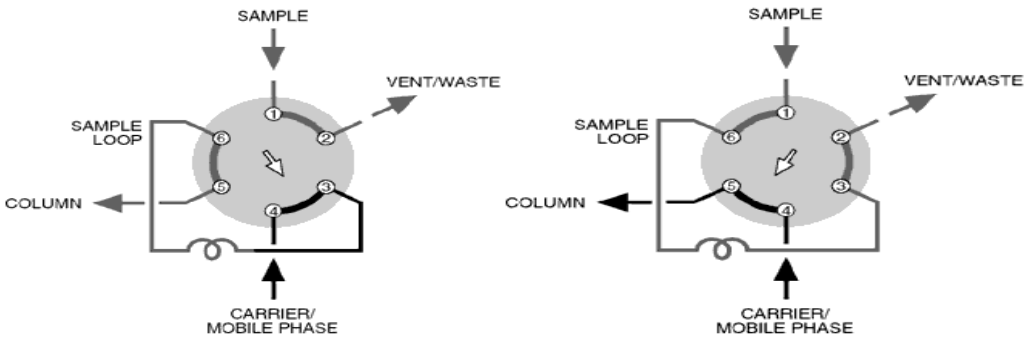
- heating and stirring,
- filtration through 0.45μm filters,
- vacuum degassing with an air-soluble membrane,
- Helium purging ultra signification or purging or combination of these methods.
- HPLC systems are also provided an online degassing system which continuously removes the dissolved gases from the mobile phase.[8]

iii.Sample introduction system:

Two means for analyte introduction on the column are injection into a flowing stream and a stop flow injection. These techniques can be used with a syringe or an injection valve. Automatic injector is a microprocessor-controlled version of the manual universal injector.

Injector

Injectors should provide the possibility of injecting the liquid sample within the range of 0.1 to 100 ml of volume with high reproducibility and under high pressure (up to the 4000 psi). They should also produce minimum band broadening and minimize possible flow disturbances. The most useful and widely used sampling device for modern LC is the micro sampling injector valve. With these sampling valves, samples can be introduced reproducibly into pressurized columns without significant interruption of flow even at elevated temperatures.[9]



LOAD (the sample loop) INJECT (move the sample loop

In the mobile phase

Fig. No.1.2 .Injection system

iv.Columns

The heart of the system is the column. Analytical column is the most important part of the HPLC which decides the efficiency of separation. The choice of

common packing material and mobile phases depends on the physical properties of the drug.[9]

The following properties of the column stationary phases play an important role in giving different selectivity for separations.

i) Particle size, ii) Particle shape, iii) Pore size / Pore volume, iv) Specific surface area, v) End capping, vi) % carbon loading.

The following are the most widely used columns with stationary phases for separation and quantification of wide variety of drugs.

i). Silica based columns with different bonding phases like C4, C6, C8, C18, C20 and bonding phases having functional groups like cyano, phenyl, naphthyl and amino.
ii). Silica based columns with polar embedded phases within chains of C8, C18, NH2.
iii). Strong cation exchange (SCX) and strong anion exchange (SAX) columns.
iv). Size Exclusion chromtography (SEC) or gel permeation chromatography (GPC) columns.
v). Silica based monolith columns.
vi). Fused core silica columns with bonding phases like C8, C18, CN, phenyl.
vii). Metal oxide columns like zirconia based and alumina based.
viii). Chiral columns.

Column-packing materials

Silica ($SiO_2.X\ H_2O$) is the most widely used substance for the manufacture of packing materials it consist of a network of siloxane linkages(Si-O-Si) in a rigid three dimensional structure containing inter connected pores.[10] Thus a wide range of commercial products are available with surface areas ranging

from 100 to 800 m^2/g and particle sizes from 3 to 50 µm. The silonol groups on the surface of silica give it a polar character, which is exploited in adsorption chromatography using non-polar organic elutents. Silica can be drastically altered by reaction with organochlorosilanes or organoalkoxysilanes giving Si-O-Si-R linkages with the surface. The attachment of hydrocarbon chain to silica produces a non polar surface suitable for reversed phase chromatography where mixtures of water and organic solvents are used as eluents. The most popular material is octa decyl silica (ODS) which contains C_{18}chains, but material with C_2, C_6, C_8 and C_{22} chains are also available.

The most popular brands of LC columns:

Inertsil, Hypersil, X-terra, X-bridge, Sun-fire, Atlantis, Aquity-BEH, Zorbax, Lichrosphere, Purosphere, Sperisorb, Luna, Kromasil, ACE, YMC, Symmetry, Chiralcel and Chiralpak.

The LC columns are supplied in different dimensions:

Column lengths - 10 mm, 50 mm, 100mm, 150mm, 250mm, 300mm, 500mm and Internal diameters -2.1mm, 3.0mm, 4.0mm, 4.6mm.

LC columns with stationary phases having different particle sizes like 5.0 µm, 4.0 µm 3.5 µm, 3.0 µm, 2.5 µm, 2.0 µm, 1.9 µm, 1.8 µm, 1.7 µm and 1.3 µm are available.

V.Mobile phase

Mobile phases used for HPLC are typically mixtures of organic solvents and water or aqueous buffers.

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

- ✓ It is essential to establish that the drug is stable in the mobile phase for at least the duration of the analysis.
- ✓ Excessive salt concentrations should be avoided. High salt concentrations can result in precipitation which can damage HPLC equipment.

Reduce cost and toxicity of the mobile phase by using methanol instead of acetonitrile whenever possible.

✓ Minimize the absorbance of buffer. Since trifluroacetic acid or formic acid absorb at shorter wavelengths. They may prevent detection of products without chromophores above 220 nm. Carboxylic acid modifiers can be frequently replaced by phosphoric acid which does not absorb above 200 nm.[11]

Physical properties of some HPLC solvents were summarized in Table: 1.4

Table No. 1.4. Physical properties of common HPLC solvents

Solvent	MW	BP	RI (25 °C)	UV Cut-off (nm)	Density g / ml (25 °C)	Viscosity cP (25 °C)	Dielectric Constant
Acetonitrile	41.0	82	1.342	190	0.787	0.358	38.8
Dioxane	88.1	101	1.420	215	1.034	1.26	2.21
Ethanol	46.1	78	1.359	205	0.789	1.19	24.5
Ethyl acetate	88.1	77	1.372	256	0.901	0.450	6.02
Methanol	32.0	65	1.326	205	0.792	0.584	32.7
CH_2Cl_2	84.9	40	1.424	233	1.326	0.44	8.93
Isopropanol	60.1	82	1.375	205	0.785	2.39	19.9
n-propanol	60.1	97	1.383	205	0.804	2.20	20.3
THF	72.1	66	1.404	210	0.889	0.51	7.58
Water	18.0	100	1.333	170	0.998	1.00	78.5

Vi. Detectors

The detection of UV light absorbance offers both convenience and sensitivity for molecules. When a chromophore is present, the wavelength of detection for a drug should be based on its UV Spectrum in the mobile phase and not in pure

solvents. The most selective wavelength for detecting a drug is frequently the longest λ_{max} to avoid interference from solvents, buffers and excipients. Other methods of detection can be useful are required in some instances.

- Solute specific detectors (UV-Vis, fluorescence, infra-red, radio activity)
- Bulk property detectors (refractive index, viscometer, conductivity)
- Desolvation detectors (flame ionization etc.)
- LC-MS detectors
- Reaction detectors

Applications of HPLC in pharmaceutical research :

- **Separation:** This can be accomplished using HPLC by utilizing the fact that, certain compounds have different migration rates given a particular column and mobile phase. The extent or degree of separation is determined by the choice of stationary phase and mobile phase along with parameters like flow, temperature and gradient programme.

- **Identification**: For this purpose a clean peak of known sample has to be observed from the chromatogram. Selection of column mobile phase and flow rate matter to certain level in this process. Identification is generally by comparing with reference compound based on retention time and also based on UV-Vis spectra in some cases. Identification can be assured by combining two or more detection methods, where necessary.

- **Quantification:** Analyte concentrations are estimated by measuring the responses (peak areas) known reference standards followed by unknown samples. Quantification of known and unknown components are done by various methods like - area normalization method, internal standard method, external standard method and diluted standard method along with relative response factors.

- **Isolation** : It refers to the process of isolation and purification of compounds using analytical scale or preparative scale HPLC. Volatile

buffers and solvents are preferred choice as mobile phases as it reduces the effort on purification. Solute purity and throughput is the key challenge in isolation and purification processes.

HPLC theory:

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

Retention time (t_R), capacity factor k' and relative retention time (RRT)

The time elapsed between the injection of the sample components into the column and their detection is known as the retention time (t_R). The retention time is longer when the solute has higher affinity to the stationary phase due to its chemical nature. For example, in reverse phase chromatography, the more lyophilised compounds are retained longer. Therefore, the retention time is a property of the analyte that can be used for its identification. A non retained substance passes through the column at a time t_0, called the void time. Retention factor is calculated as follows:

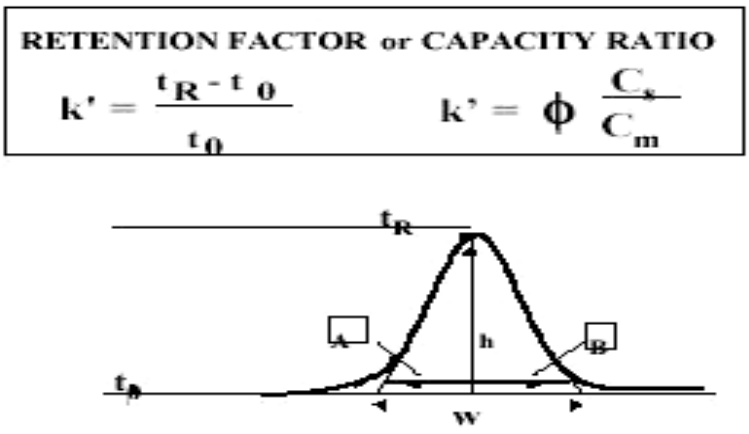


Fig. No.1.3. Figure showing retention factor

The capacity factor describes the thermodynamic basis of the separation and its definition is the ratio of the amounts of the solute at the stationary and mobile phases within the analyte band inside the chromatographic column: Where C_s is the concentration of the solute at the stationary phase and C_m is its

concentration at the mobile phase and phi is the ratio of the stationary and mobile phase volumes all within the chromatographic band. The Retention Factor is used to compare the retention of a solute between two chromatographic systems, normalizing it to the column's geometry and system flow rate. The retention factor value should be in between 1-20.[11]

Efficiency: Plate count N and peak capacity P_c:

The efficiency of the separation is determined by the plate count N when working at isocratic conditions, whereas it is usually measured by Peak Capacity P_c when working at gradient conditions. The following equation for the plate count is used by the United States Pharmacopoeia (USP) to calculate N:

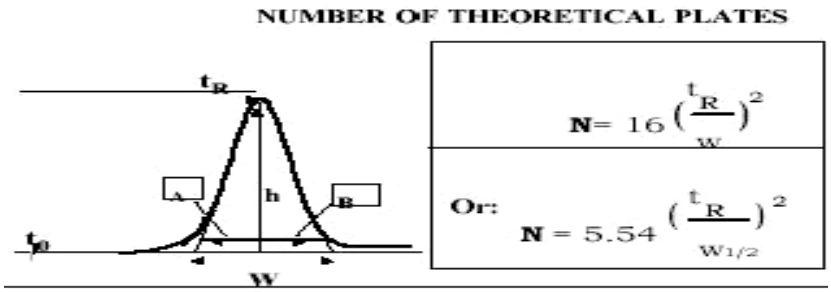


Fig. No.1.4. Figure showing Number of Theoretical Plates

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

Peak capacity P_c is defined as number of peaks that can be separated within a retention window for a specific pre-determined resolution. In other words, it is the runtime measured in peak width units . It is assumed that peaks occur over the gradient chromatogram. Therefore, peak capacity can be calculated from the peak widths in the chromatogram as follows:

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

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

Another measure of the column's chromatographic efficiency is the height equivalent to theoretical plate (HETP) which is calculated from the following equation:

$$HETP = (L/N)$$

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

The behaviour of HETP as function of linear velocity has been described by various equations. It is frequently called "The Van-Deemter curve", and it is frequently used to describe and characterize various chromatographic stationary phases' performance and compare them to each other. The lower are the values of HETP, the more efficient is the chromatographic system, enabling the detection of lower concentrations due to the enhanced signal-to-noise ratio of all the peaks in the chromatogram.

Peak asymmetry factor A_f and tailing factor T:

- The chromatographic peak is assumed to have a gaussian shape under ideal conditions, describing normal distribution of the velocity of the molecules populating the peak zone migrating through the stationary phase inside the column. Any deviation from the normal distribution indicates non-ideality of the distribution and the migration process therefore might jeopardize the

integrity of the peak's integration, reducing the accuracy of the quantitation. This is the reason why USP Tailing is a peak's parameter almost always measured in the system suitability step of the analysis.[12]

- The deviation from symmetry is measured by the asymmetry factor, A_f or tailing factor T. The calculation of asymmetry factor, A_f is described by the following equation:

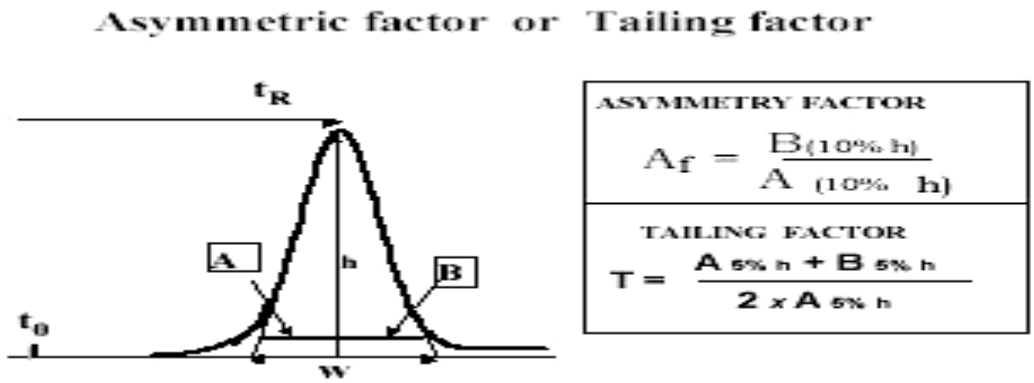


Fig. No.1.5. Figure showing Asymmetric Factor

Where A and B are sections in the horizontal line parallel to the baseline, drawn at 10% of the peak height. The calculation of tailing Factor, T, which is more widely used in the pharmaceutical industry, as suggested by the pharmacopeia's, where A and B are sections in the horizontal line parallel to the baseline, drawn at 5% of the peak height. The USP suggests that tailing factor should be in the range of 0.5 up to 2 to assure a precise and accurate quantitative measurement.

Selectivity Factor Alfa and Resolution Factor Rs:

The separation is a function of the thermodynamics of the system. Substances are separated in a chromatographic column when their rate of migration differs, due to their different distribution between the stationary and mobile phases. The selectivity factor, α, and resolution factor, Rs, measure the extent of separation between two adjacent peaks. The selectivity factor accounts only for the ratio of the retention factors, k', of the two peaks (k'_2/k'_1), whereas the

resolution factor, Rs, accounts for the difference between the retention times of the two peaks relative to their width

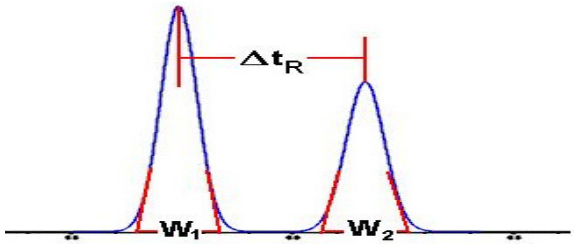


Fig. No.1.6. Figure showing Resolution Factor

The equation that describes the experimental measurement of the resolution factor, Rs, is as follows: $\mathbf{Rs = \Delta t_R / 0.5\ (W_1 + W_2)}$

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

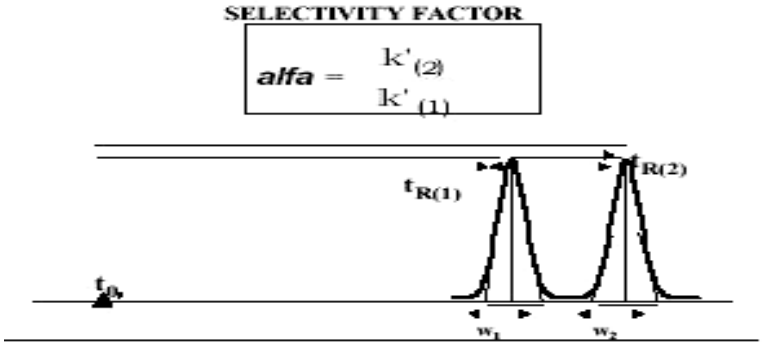


Fig. No.1.7. Figure showing selectivity

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

decades of HPLC. This is also the rational behind the recent trend in HPLC, the use of sub 2 micron particle columns and the development of a specially design of ultra performance HPLC systems to accommodate such columns.[12]

1.4..ANALYTICAL METHOD DEVELOPMENT

Methods are developed for new products when no official methods are available. Alternate methods for existing (Non-Pharmacopoeias) products are developed to reduce the cost and time for better precision and ruggedness. Trial runs are conducted, method is optimized and validated. When alternate method proposed is intended to replace the existing procedure, comparative laboratory data including merits / demerits should be made available

Steps involved in method development

Documentation starts at the very beginning of the development process.

1. Analyte standard characterization

a) All known information about the analyte and its structure is collected i.e., physical and chemical properties.
b) The standard analyte (100 % purity) is obtained. Necessary arrangement is made for the proper storage (refrigerator, desiccators and freezer).
c) When multiple components are to be analyzed in the sample matrix, the number of components is noted, data is assembled and the availability of standards for each one is determined.
d) Only those methods (spectroscopic, MS, GC, HPLC etc.,) that are compatible with sample stability are considered.

2. Method requirements

The goals or requirements of the analytical method that need to be developed are considered and the analytical figures of merit are defined. The required detection limits, selectivity, linearity, range, accuracy and precision are defined.[13]

3. Literature search and prior methodology

The literature for all types of information related to the analyte is surveyed. solubility profile (solubility of Drug in different solvents and at different pH conditions), analytical profile (Physico-chemical properties, Eg: pKa, melting point, degradation pathways, etc) and stability profile (sensitivity of the drug towards light, heat, moisture etc) and relevant analytical methods, books, periodicals, chemical manufacturers and regulatory agency compendia such as USP / NF, are reviewed.

4. Choosing a method

a) Using the information in the literatures and prints, methodology is adopted. The methods are modified wherever necessary. Sometimes it is necessary to acquire additional instrumentation to reproduce, modify, improve or validate existing methods for in-house analytes and samples.

b) If there are no prior methods for the analyte in the literature, from analogy, the compounds that are similar in structure and chemical properties are investigated and are worked out. There is usually one compound for which analytical method already exist that is similar to the analyte of interest.

c) 5. Instrumental setup and initial studies

The required instrumentation is setup. Installation, operational and performance qualification of instrumentation using laboratory standard operating procedures (SOP's) are verified. Always new consumables (e.g. solvents, filters and gases) are used. For example, method development is never started on a HPLC column that has been used earlier. The analyte standard in a suitable injection / introduction solution and in known concentrations and solvents are prepared. It is important to start with an authentic, known standard rather than with a complex sample matrix. If the sample is extremely close to the standard (e.g., bulk drug), then it is possible to start work with the actual sample.

6. Optimization

During optimization one parameter is changed at a time and set of conditions are isolated, rather than using a trial and error approach. Work has been done from an organized methodical plan, and every step is documented (in a lab notebook) in case of dead ends. **Table No.1.5-variables for improving separation**

Variable	Comment
Choice of organic solvent	A change from Methanol to Acetonitrile or THF often results in large changes in separation .
Mobile phase pH	A Change in pH may result in a major effect on band spacing for samples that contain ionic or ionisable compounds.
Solvent strength	A change in percent organic often provides significant changes in retention and separation.
Column type	This refers to the choice of bonded-phases for reversed-phase LC (C 18, C 8,Phenyl,cyano etc)
Concentration of mobile phase additives	The most common additives for varying band spacing include amine modifiers, acid modifiers, buffers and salts.
Temperature	The temperature can be varied between 0 to 70ºC for the purpose of controlling band spacing; however, temperatures of 25-60 ºC are more common.

7. Documentation of analytical figures of merit

The originally determined analytical figures of merit are limit of quantitation (LOQ), limit of detection (LOD), linearity, time per analysis, cost, sample preparation etc., are documented.

8. Evaluation of method development with actual samples

The sample solution should lead to unequivocal, absolute identification of the analyte peak of interest apart from all other matrix components.

9.Determination of percent recovery of actual sample and demonstration of quantitative sample analysis

Percent recovery of spiked, authentic standard analyte into a sample matrix that is shown to contain no analyte is determined. Reproducibility of recovery (average + / - standard deviation) from sample to sample and whether recovery has been optimized or not has been shown. It is not necessary to obtain 100 % recovery as long as the results are reproducible and known with a high degree of certainty. The validity of analytical method can be verified only by laboratory studies. [13]

Table No.1.6.Separation goals in HPLC method development

Goal	Comment
Resolution	Precise and rugged quantitative analysis requires that R_s be greater than 1.5.
Separation time	<5-10 min is desirable for routine procedures.
Quantitation	£2% for assays; £5% for less-demanding analyses ; 15% for trace analyses.
Pressure	<150 bar is desirable, <200 bar is usually essential (new column assumed).
Peak height	Narrow peaks are desirable for large signal/noise ratios.
Solvent consumption	Minimum mobile-phase use per run is desirable.

METHOD DEVELOPMENT PROCEDURE

The wide variety of equipment's, columns, eluent and operation preparations involved high performance liquid chromatography (HPLC) method development seems complex. The processes influenced by the nature of analytes and generally follow the following steps. [14]

Steps:

- Step 1 - Selection of the HPLC method and initial system
- Step 2 - Selection of initial conditions
- Step 3 - Selectivity optimization
- Step 4 - System optimization
- Step 5 - Method validation.

HPLC method development

Step 1 - selection of the HPLC method and initial system.

When developing an HPLC method, the first step is always to consult the literature to ascertain whether the separation has been previously performed and if so, under what conditions - this will save time doing unnecessary experimental work. When selecting an HPLC system, it must have a high probability of actually being able to analyse the sample; for example, if the sample includes polar analytes then reverse phase HPLC would offer both adequate retention and resolution, whereas normal phase HPLC would be much less feasible. Consideration must be given to the following:

Sample preparation:

- ✓ Does the sample require dissolution, filtration, extraction, preconcentration.

- ✓ Is chemical derivatization required to assist detection sensitivity or selectivity

Column dimensions:

For most samples (unless they are very complex), long columns (25 cm) are recommended to enhance the column efficiency. A flow rate of 1-1.5 ml/min should be used initially. packing particle size should be 3 or 5 μm.

Detectors:

Consideration must be given to the following:

- Do the analytes have chromophores to enable UV detection
- Is more selective/sensitive detection required
- What detection limits are necessary
- Will the sample require chemical derivatization to enhance detectability and/or improve the chromatography.

Fluorescence or electrochemical detectors should be used for trace analysis. For preparative HPLC, refractive index is preferred because it can handle high concentrations without over loading the detector. UV wavelength for the greatest sensitivity λ_{max} should be used, which detects all sample components that contain chromophores. UV wavelengths below 200 nm should be avoided because detector noise increases in this region. Higher wavelengths give greater selectivity. The excitation wavelength locates the excitation maximum; that is, the wavelength that gives the maximum emission intensity. The excitation is set to the maximum value then the emission is scanned to locate the emission intensity. Selection of the initial system could, therefore, be based on assessment of the nature of sample and analytes together with literature data, experience, expert system software and empirical approaches.

Step 2 - selection of initial conditions.

This step determines the optimum conditions to adequately retain all analytes; that is, ensures no analyte has a capacity factor of less than 0.5 (poor retention could result in peak overlapping) and no analyte has a capacity factor greater than 10–15 (excessive retention leads to long analysis time and broad peaks with poor detectability). Selection of the following is then required.

Mobile phase solvent strength:

The solvent strength is a measure of its ability to pull analytes from the column. It is generally controlled by the concentration of the solvent with the highest strength; for example, in reverse phase HPLC with aqueous mobile phases, the strong solvent would be the organic modifier; in normal phase HPLC, it would be the most polar one. The aim is to find the correct concentration of the strong solvent. With many samples, there will be a range of solvent strengths that can be used within the a fore mentioned capacity limits. Other factors (such as pH and the presence of ion pairing reagents) may also affect the overall retention of analytes.

Step 3 - selectivity optimization:

The aim of this step is to achieve adequate selectivity (peak spacing). The mobile phase and stationary phase compositions need to be taken into account. To minimize the number of trial chromatograms involved, only the parameters that are likely to have a significant effect on selectivity in the optimization must be examined. To select these, the nature of the analytes must be considered. Once the analyte types are identified, the relevant optimization parameters may be selected. Note that the optimization of mobile phase parameters is always considered first as this is much easier and convenient than stationary phase optimization.

Step 4 - system parameter optimization:

This is used to find the desired balance between resolution and analysis time after satisfactory selectivity has been achieved. The parameters involved

include column dimensions, column-packing particle size and flow rate. These parameters may be changed without affecting capacity factors or selectivity.

Step 5 - Method validation:

Proper validation of analytical methods is important for pharmaceutical analysis when ensure of the continuing efficacy and safety of each batch manufactured relies solely on the determination of quality. The ability to control this quality is dependent upon the ability of the analytical methods, as applied under well-defined conditions and at an established level of sensitivity, to give a reliable demonstration of all deviation from target criteria.

Analytical methods should be used within good manufacturing practice (GMP) and good laboratory practice (GLP) environments, and must be developed using the protocols set out in the international conference on harmonization (ICH) guidelines (Q2A and Q2B). The US food and drug administration (FDA) and US Pharmacopoeia (USP) both refer to ICH guidelines. The most widely applied validation characteristics are accuracy, precision (repeatability and intermediate precision), specificity, detection limit, quantitation limit, linearity, range, robustness and stability of analytical solutions. Method validation must have a written and approved protocol prior to use.[14]

1.5.ANALYTICAL METHOD VALIDATION

Method validation can be defined as (ICH*)*

"Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics".

Method validation study include system suitability, linearity, precision, accuracy, specificity, robustness, limit of detection, limit of quantification and stability of samples, reagents, instruments.

1. System Suitability

Prior to the analysis of samples of each day, the operator must establish that the HPLC system and procedure are capable of providing data of acceptable quality. This is accomplished with system suitability experiments, which can be defined as tests to ensure that the method can generate results of acceptable accuracy and Precision. The requirements for system suitability are usually developed after method development and validation have been completed.[15]

Table.No.1.7.System Suitability Parameters and Recommendations

PARAMETER	RECOMMENDATION
Capacity Factor (k')	The peak should be well-resolved from other peaks and the void volume, generally k'>2.0
Repeatability	RSD </= 1% for N >/= 5 is desirable.
Resolution (R_s)	R_s of > 2 between the peak of interest and the closest eluting Potential interferent (impurity, excipient, degradation product, internal standard)
Theoretical Plates (N)	In general should be > 2000
Tailing Factor (T)	T of </= 2

2. Linearity

The linearity of a method is a measure of how well a calibration plot of response vs. concentration approximates a straight line. Linearity can be assessed by performing single measurements at several analyte concentrations. The data is then processed using a linear least-squares regression. The resulting plot slope, intercept and correlation coefficient provide the desired information on linearity.

3. Range

The range is the interval between the upper and lower levels of the analytical method that have been demonstrated to obtain acceptable level of precision, accuracy and linearity.

Table.No.1.8. Range for the Analytical procedures

Analytical Procedure	Range
Assay of a drug substance or a finished product	80 to 120 % of the test concentration
Impurity(quantification)	Reporting threshold to 120% of Acceptance criteria
Assay and Impurity	One test with 100% standard Linearity: Reporting threshold to 120% assay Acceptance criteria
content uniformity	70 to 130 %of the test concentration
dissolution testing	+/-20 % over the specified range
Drug release testing	20% after 1 hour upto 90% after 24 hours 0-110% of label claim

4. Precision

Precision can be defined as "The degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogenous sample". A more comprehensive definition proposed by the International Conference on Harmonization (ICH) divides precision into three types:

1. Repeatability
2. Intermediate precision and
3. Reproducibility

Repeatability is the precision of a method under the same operating conditions over a short period of time.

Intermediate precision is the agreement of complete measurements (including standards) when the same method is applied many times within the same laboratory.

Reproducibility examines the precision between laboratories and is often determined in collaborative studies or method transfer experiments.

5. Accuracy

The accuracy of a measurement is defined as the closeness of the measured value to the true value. In a method with high accuracy, a sample (whose "true value" is known) is analyzed and the measured value is identical to the true value. Typically, accuracy is represented and determined by recovery studies. There are three ways to determine accuracy:

1. Comparison to a reference standard
2. Recovery of the analyte spiked into blank matrix or
3. Standard addition of the analyte.[15]

It should be clear how the individual or total impurities are to be determined. e.g.,Weight / weight or area percent in all cases with respect to the major analyte.

6. Specificity / selectivity

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

7. Robustness

The concept of robustness of an analytical procedure has been defined by the ICH as "a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters". A good practice is to vary important parameters in the method systematically and measure their effect on separation. The variable method parameters in HPLC technique may involves flow rate, column temperature, sample temperature, pH and mobile phase composition.

8. Limit of detection

Limit of detection (LOD) is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. Several approaches for determining the LOD are possible, depending on whether the procedure is a non-instrumental or instrumental.

- Based on visual evaluation
- Based on signal-to-noise
- Based on the standard deviation of the response and the slope

The LOD may be expressed as:

$$\mathbf{LOD = 3.3\ \sigma / S}$$

Where,

σ = Standard deviation of Intercepts of calibration curves

S = Mean of slopes of the calibration curves

The slope S may be estimated from the calibration curve of the analyte.

9. Limit of quantification

Limit of quantitation (LOQ) is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. Several approaches for determining the LOQ are possible depending on whether the procedure is a non-instrumental or instrumental.

- Based on visual evaluation
- Based on signal-to-noise Approach
- Based on the standard deviation of the response and the slope

The LOQ may be expressed as:

$$\mathbf{LOQ = 10\ \sigma / S}$$

Where,

σ = Standard deviation of Intercepts of calibration curves

S = Mean of slopes of the calibration curves

The slope S may be estimated from the calibration curve of the analyte.[16]

Table.No.1.9. Acceptance criteria of validation for HPLC

S.No	Characteristics	Acceptance criteria
1	Specificity	No interference
2	Accuracy	98-102%
3	Precision	RSD<2
4	Detection limit	S/N > 2or 3
5	Quantitation limit	S/N > 10
6	Linearity	$R^2 > 0.999$
7	Range	80-120%

10. Stability

To generate reproducible and reliable results, the samples, standards, and reagents used for the HPLC method must be stable for a reasonable time (e.g., one day, one week, one month, depending on need). Therefore, a few hours of

standard and sample solution stability can be required even for short (10 min) separation. When more than one sample is analyzed (multiple lots of one sample or samples from different storage conditions from a single lot), automated, overnight runs often are performed for better lab efficiency. Such practices add requirements for greater solution stability.

Degradation studies:

Degradation studies or stress testing is conducted in order to investigate the likely degradation products, which in turn helps to establish the degradation pathways and the intrinsic stability of the drug molecule and also to provide foundation for developing a suitable stability indicating method. Stress testing the drug molecule under particular stress condition generate samples containing degradation products. Use these samples to develop suitable analytical methods. The degradation products generated in the stressed samples are termed as "potential" degradation products that may or may not be formed under relevant storage conditions. Stress drug product, and placebo separately to understand the peaks due to placebo components, if any. Four major forced degradation studies are [17]

(i) Thermolytic Degradation,

(ii). Hydrolytic degradation,

(iii). Oxidative degradation,

(iv). Photolytic degradation.

2. LITERATURE REVIEW

2.1. Drug Profile

NELARABINE:-

Chemical structure :

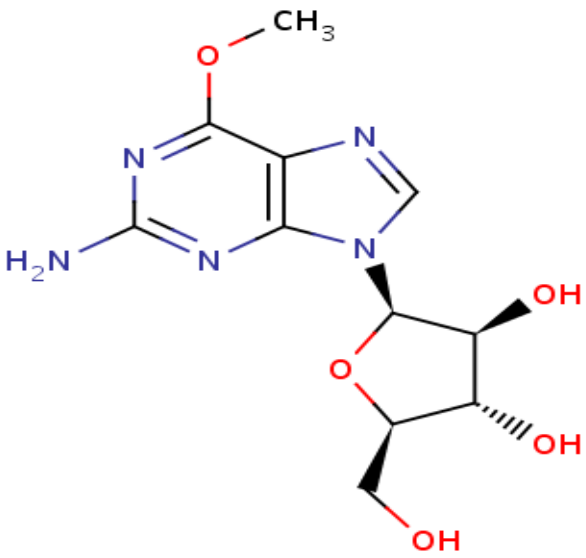


Fig no. 2.1. Structure of Nelarabine

- **IUPAC name :** (2R,3S,4S,5R)-2-(-2-amino-6-methoxy-9H-purin-9yl)-5-(hydroxymethyl)oxolane-3,4-diol

- **Molecular Formula** : $C_{11}H_{15}N_5O_5$
- **Molecular Weight** : 297.267
- **CAS number** : 121032-29-9
- **General properties:-**

  Colour : White powder

  State : Crystalline powder

  Solubility : Slightly soluble to soluble in water, soluble in methanol.

  Melting point : 209-217^{0}C

- **Dosage form** : Injection
- **Stability** : Nelarabine Injection is stable in polyvinyl- chloride (PVC) infusion bags and glass containers for 8 hours up to 30° C
- **Category** : Antineoplastic agent .Used in treatment of T-cell acute lymphoblastic leukemia.
- **Brand names** : Arranon (Glaxosmithkline), Atriance
- **Official** : Not official in any Pharmacopoeias
- **Mechanism of action** : Nelarabine is a pro-drug of the deoxyguanosine analogue 9-β-D-arabinofuranosylguanine (ara-G). Nelarabine is demethylated by adenosine deaminase (ADA) to ara-G, mono-phosphorylated by deoxyguanosine kinase and deoxycytidine kinase, and subsequently converted to the active 5'-triphosphate, ara-

GTP. Accumulation of ara-GTP in leukemic blasts allows for incorporation into deoxyribonucleic acid (DNA), leading to inhibition of DNA synthesis and cell death. Other mechanisms may contribute to the cytotoxic and systemic toxicity of nelarabine.

- **Side effects :** Nelarabine may cause serious side effects of the central nervous system.[18]

Formulation :

Table no. 2.1.Various brands of the formulation

Formulation	Nelarabine (mg/mL)	Manufacturer
Arranon [19]	5	GlaxoSmithKline
Atriance	5	GlaxoSmithKline

2.2 REVIEW OF LITERATURE

Huang Qiaoqiao *et al.,*(2012) established a nonaqueous titration method for the determination of Nelarabine using 0.1 mol/L perchloric acid as titrant. Effect of different solvents and indicators on the titration endpoint was compared in this paper. Using acetic acid as solvent and determining the endpoint potentiometrically 5 times, the average content of Nelarabine was found to be 99.8%(RSD=0.22%). The method is simple and precise,and can be used to determine the content of Nelarabine in APIs.[20]

Yoshiyuki Minamide1, Minamide, *et al.*,(2012) developed a highly sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) method for quantitation of arabinofuranosyl guanosine 5'-triphosphate (ara-GTP) in human peripheral blood mononuclear cells (PBMC) and validated using a standard addition method with the human Tlymphoblastoid cell line as an alternative blank matrix. Ara-GTP was extracted with methanol/250 mmol/L ammonium carbonate solution (7/3, v/v) from the cells at a density of 106 cells per 0.5 mL. Extracts were subjected to LC-MS/ MS using a TurboIon spray interface and selected reaction monitoring with the transitions of *m/z* 524 to *m/z* 152 for quantitation. Endogenous guanosine triphosphate in the extract was used as an internal standard. Separation of the analytes was achieved on a porous graphitic carbon column (100 mm length × 2.1 mm i.d., 5 µm particle size) by isocratic elution with 250 mmol/L ammonium carbonate buffer (pH 9.5)/water/acetonitrile (40/51.5/8.5, v/v/v) at a flow rate of 0.2 mL/min. The method was validated in the range of 2–250 pg/mL. The pharmacokinetic profile of ara-GTP in PBMC in a Phase I clinical study of nelarabine in relapsed or refractory T-ALL/T-LBL patients was successfully determined using this method.[21]

Jeanette Kaiser and Krämer *et al.,*(2011) developed a stability-indicating reversed-phase high performance liquid chromatography with ultraviolet detection for determining Physico-chemical stability of nelarabine infusion solution in ethylene vinyl acetate infusion bags. The column used was the Spherisorb ODS-2 C18, end-capped, 80 Å x 4.6 mm, particle size 3 µm, mobile

phase consisted of 80% 0.01 M potassium dihydrogen phosphate (pH 6.8) solution and 20% methanol.The flow rate was set at 1.0 mL/minute, with an injection volume of 10 µL. The detection wavelength was set at 265 nm. The stability tests revealed that nelarabine infusion solutions are physico-chemically stable for a minimum of four weeks. Nelarabine concentrations remained at a level of > 95% of the initial concentration independent of the storage conditions.[22]

N.Y.Sreedhar, C.Nageswara Reddy (2011) developed a robust, highly reliable and reproducible adsorptive stripping voltammetric procedure for the determination of nelarabine in pharmaceutical formulations and urine samples. The analytical procedure was based on the reduction of the >C=N- of the pyrozole ring of the drug molecule at the hanging mercury drop electrode(HMDE) surface in Universal buffer of pH 6.0. The optimal experimental parameters for the drug assay were, accumulation potential -0.78V (vs. Ag/AgCl), accumulation time 60sec,pulse amplitude 25mV and scan rate 40mV s^{-1} in universal buffer (pH.6.0).The linear concentration range of application was 1.0×10^{-2} to 1.0×10^{-7} M of nelarabine, with a relative standard deviation of 1.3% and a detection limit of 1.0×10^{-7} M. The method was successfully applied to the determination of nelarabine in human urine and pharmaceutical formulations.[23]

Takahero Yamauchi, Rie Nish *et al.,*(2009) developed a new, sensitive isocratic elution HPLC method for determining low production of 9-ß-D-arabinosylguanine triphosphate, an active metabolite of nelarabine in adult T-cell leukemia cells. The determination of ara-GTP production in cancer cells is informative for optimizing nelarabine administration. The developed method showed λ_{max} at 254nm, and samples were eluted isocratically by using phosphate buffer (80% 0.06M Disodium hydrogen phosphate pH and 20% ACN) at a constant flow rate 0.7ml/min and injection volume 10 µ L . Ara-

GTP was clearly separated from other nucleotides by using an anion-exchange column DEAE 2 SW(250×4.6mm) and it was quantitated by its peak area. The standard curve was linear with % CV less than 10 and a sensitive detection limit (10 pmol). This study was the first to evaluate the potential of ara-G against ATL cells.[24]

Berg SL, Brueckner C, Nuchtern JG, Dauser R, McGuffey L, Blaney SM (2007) studied Plasma and cerebrospinal fluid pharmacokinetics of nelarabine in nonhuman primates using LC-MS method. Nelarabine (35 mg/kg, approximately 700 mg/m2) was administered over 1 h through a surgically implanted central venous catheter to four nonhuman primates. Blood (four animals) and ventricular CSF (three animals) samples were obtained at intervals for 24 h for determination of nelarabine concentrations, which were measured by HPLC-mass spectrometry. The nelarabine plasma AUC (median+/-s.d.) was 2,820+/-1,140 microM min and the ara-G plasma AUC was 20,000+/-8,100 microM min. The terminal half-life of nelarabine in plasma was 25+/-5.2 min and clearance was 42+/-61 ml/min/kg. The excellent CSF penetration of nelarabine and ara-G supports further study of the contribution of nelarabine to the prevention and treatment of CNS leukemia.[25]

Carlos O. Rodriguez Jr., and William Plunkett *et al.*,(2000) developed a gradient anion-exchange high-performance liquid chromatographic assay for the simultaneous determination and quantitation of the cytotoxic triphosphates of arabinosylguanine (ara-GTP) and fludarabine (F-ara-ATP). To assess the clinical utility, perchloric acid extracts of circulating human leukemia cells isolated from patients treated with fludarabine and nelarabine were analyzed. Samples were eluted gradiently using 60% 0.005 M NH4 H2 PO4 (pH 2.8) and 40% 0.75 M NH4 H2 PO 4 (pH 3.6) at a constant flow-rate of 1.5 ml/min, in 10-SAX Partisil anion-exchange column (4.6×250 mm, Whatman, Clifton, NJ, USA) and UV absorption at 256 nm . The range of quantitation was 0.0125–10 nmol for the ara- and native NTPs in cellular extracts. This assay should be helpful in establishing the mechanistic rationales for drug scheduling

and combinations of nelarabine and fludarabine, and for correlating the therapeutic efficacy and levels of the cytotoxic triphosphates in target cells.[26]

2.3. RESEARCH ENVISAGED

From the literature survey it is clear that only one RP-HPLC method have been reported so far for Nelarabine in commercially available formulation. Very few methods have been reported in determination of arabinosyl guanine triphosphate, active form of nelarabine present in biological fluids. Hence an attempt has made to develop a RP-HPLC method for the estimation of Nelarabine in bulk and pharmaceutical dosage form.

Present work is aimed at to develop a new, simple, fast, rapid, accurate, efficient and reproducible RP-HPLC method for the analysis of Nelarabine and to validate the developed method according to ICH (Q2b) guidelines.

3.1. PLAN OF WORK

The experimental work has been planned as follows

STEP 1-

- Study of physico-chemical properties of drug (pH, pka, solubility, and molecular weight)
- Preparation of drug standard and sample solution
- Selection of stationary phase
- Selection of mobile phase
- Preparation of solutions
- Developing simple, rapid and specific RP-HPLC method for the quantitative estimation of Nelarabine in the dosage form.
- Optimizing the chromatographic conditions

STEP 2-To validate the newly developed method in accordance with the analytical validation parameters mentioned as ICH guidelines(Q2B)

- Selectivity/specificity
- Linearity and range
- Accuracy
- Precision (repeatability and reproducibility)
- Limit of detection (LOD)
- Limit of quantification (LOQ)
- Robustness
- Forced degradation studies

3.2 Materials and Methods

3.2.1 Chemicals and standards used

Table.No.3.1. List of Chemicals and standards used

S.NO	Chemicals/reagents	Grade	Manufacturer
1	Methanol	GR	Merck
2	Acetonitrile	GR	Merck
3	Purified water	Milli-Q	NA
4	Trifluoroacetic acid	GR	Merck

3.2.2 Equipment used during assay development

Table.No.3.2. List of equipment used

S. No.	Name	Manufacturer
1.	HPLC(empower-2software)	Aliance waters(2489)
2.	HPLC detector	UV/Visible detector
3.	pH Meter	Lab india
4.	UV Spectrophotometer	SHIMADZU
5.	Micro Balance	Mettle Toledo
6.	Water Purifier	Millipore

3.3. METHODOLOGY

3.3.1Assay method development:

The project work entitled as 'Development and validation of stability indicating RP-HPLC for estimation of Nelarabine in bulk and pharmaceutical dosage form' was carried out at Natco Pharma Limited, Kothur, Hyderabad.

A new RP-HPLC method was developed for the determination of nelarabine in i.v infusion . The HPLC method was then validated to indicate that the analytical procedure used is suitable for intended use by using various parameters like specificity, linearity, precision, accuracy, system suitability.

3.3.1.1 Selection of initial conditions for Method Development:

Determination of absorption maxima by UV/Visible Spectroscopy:

Accurately weighed and transferred about 100mg of nelarabine working standard into a 100ml volumetric flask, then added to it about 60 ml of methanol and sonicated for

10 minutes to dissolve and diluted up to mark with methanol and mix well. Further dilute 10ml ofthe above solution to 100ml with methanol and mix well. Finally dilute 10ml of above solution to 100ml with methanol. Final concentration of Nelarabine is about 10 ppm.

The solution was scanned over a range of 200-400nm and a UV spectrum was recorded. The best possible wavelength was chosen as 266nm.

3.4.METHOD DEVELOPMENT OF NELARABINE INFUSION BYRP-HPLC

The method was developed mainly basing on pk_a concept of drug and mobile phase composition, flow rate,λ_{max}, different columns and column temperature.Nelarabine has two pk_a values that are 12.45 and 3.45. Generally pH of buffer solution should be selected$^{\pm}$ 1 of pk_a value of drug. In this method pKavalue 3.45was selected because in HPLC, solution may damage column with pH more than10. So pk_a12.45 of Nelarabinewas eliminated. Then trials were performed by adjusting pH of buffer$^{\pm}$1 ofpKa value 3.45 and also by changing mobile phase composition. Finally good peak was obtained at pH 2.44 of buffer and retention time was also less compared to other trials. So the method was optimized at these conditions.

Further validation study was performed as per ICH guidelines.

Trial- 1

Chromatographic conditions:

Mobile phase **:** 0.1% Trifluoroacetic acid and acetonitrile in the ratio 60:40v/v

Column **:** Cosmicsil Adze C18 column (150×4.6mm,5µm)

Flow rate **:** 1.0 ml/min

Detector wavelength **:** 266 nm

Column temperature **:** 30^0C

Injection volume **:** 10 µL

Run time **:** 8 min

Retention time **:** 5.97 min

Inference: An extra peak and peak tailing appeared

Trial-2

Chromatographic conditions:

Mobile phase **:** 0.1% Trifluoroacetic acid and acetonitrile in the ratio 60:40v/v

Column **:** Cosmicsil Adze C18 column (150×4.6mm,5µm)

Flow rate **:** 1.3 ml/min

Detector wavelength **:** 266 nm

Column temperature **:** 30^0C

Injection volume **:** 10 µL

Run time **:** 8 min

Retention time **:** 5.77 min

Inference: Peak tailing appeared with improper baseline.

Trial-3

Chromatographic conditions:

Mobile phase **:**0.1% Trifluoroacetic acid and acetonitrile in the ratio 80:20v/v

Column **:** Cosmicsil Adze C18 column (150×4.6mm,5µm)

Flow rate **:** 1.3 ml/min

Detector wavelength **:** 266 nm

Column temperature **:** 30^0C

Injection volume **:** 10 µL

Run time **:** 8 min

Retention time **:** 5.42 min

Inference:peak tailing is obtained

Trial-4

Chromatographic conditions:

Mobile phase **:**0.01% Trifluoroacetic acid and acetonitrile in the ratio 60:40v/v

Column **:** Cosmicsil Adze C18 column (150×4.6mm,5µm)

Flow rate **:** 1.0 ml/min

Detector wavelength **:** 266 nm

Column temperature **:** 30^0C

Injection volume **:** 10 µL

Run time **:** 8 min

Retention time **:** 5.775 min

Inference: Tailing factor is more than 2 and baseline disturbance appeared.

Trial-5

Chromatographic conditions:

Mobile phase **:**0.01% Trifluoroacetic acid and acetonitrile in the ratio 80:20v/v

Column **:** Cosmicsil Adze C18 column (150×4.6mm,5µm)

Flow rate **:** 1.0 ml/min

Detector wavelength **:** 266 nm

Column temperature **:** 30^0C

Injection volume **:** 10 µL

Run time **:** 8 min

Retention time **:** 4.51min

Inference: Baseline is not proper

Trial-6

Optimized Chromatographic conditions:

Mobile phase **:** 0.01% Trifluoroacetic acid and acetonitrile in the ratio 85:15v/v

Column **:** Cosmicsil Adze C18 column (150×4.6mm,5µm)

Flow rate **:** 1.0 ml/min

Detector wavelength **:** 266 nm

Column temperature **:** 30^0

Injection volume **:** 10 µL

Run time **:** 8 min

Retention time **:** 3.83 min

Inference: Sharp peak was obtained at 3.83minutes.

3.4.1.Preparation of solutions:

Buffer Preparation:

100µL of Trifluoroacetic acid was transferred in to 1000mL of purified water and mixed well.Finally the solution was filtered through 0.45µm membrane filter and degassed.

Mobile phase

The buffer and acetonitrile were mixed in the ratio of 85:15 v/v respectively and degassed.

Diluent

Purified water was used as diluent.

3.4.2.Preparative Steps for Assay method development:

Standard Preparation:

Accurately 20.0mg of Nelarabine working Standard was weighed and transferred into a 100 ml clean ,dry volumetric flask, and 60 ml of diluent was added and sonicated to dissolve. The solution was cooled to room temperature and diluted to volume with diluent. Then 1.0 ml of the above solution was transferred into 10 ml volumetric flask and diluted with mobile phase.

Sample preparation:

2mL of sample was transferred into 50 ml of clean, dry volumetric flask. About 20 ml of diluents was added and sonicated for 15 min with occasional shaking. The solution was cooled to room temperature and diluted to 20ml with diluent.Then 1.0 ml of above solution was transferred into 10 ml volumetric flask and diluted with mobile phase.

Assay calculation-:

$$\%\text{Assay} = \frac{TA}{SA} \times \frac{SW}{100} \times \frac{1}{10} \times \frac{50}{TW} \times \frac{10}{1} \times \frac{P}{100} \times 100$$

Where,

TA = peak area response due to Nelarabine from sample

SA = peak area response due to Nelarabine from standard

SW = Weight of Nelarabine working standard takenin mg

TW = weight of sample taken in mg

P = purity of Nelarabine working standard taken on as is basis

3.4.3.SYSTEM SUITABILITY:

Chromatograph the standard preparation (Six replicate injections), measure the peak area responses for the analyte peak and evaluate the system suitability parameters as directed.

Standard Preparation:

Weigh and transfer accurately 100 mg of Nelarabine working standard into a 100 ml clean, dry volumetric flask and add about 60 ml of diluent and sonicate to dissolve. Cool the solution to room temperature and dilute to volume with solvent mixture. Then transfer 10.0 ml above solution into 100 ml volumetric flask and dilute with mobile phase.A Standard solution was prepared by using Nelarabine working standard as per test method and was injected six timesinto the HPLC system.

Acceptance criteria:

- %RSD for replicate injections of peak area response for Nelarabine peak from the standard preparation should be not more than 2.0.
- The Tailing factor for Nelarabine peak should be not more than 2.0.
- The number of Theoretical plates for Nelarabine peak should be not less than 2000

3.5. ANALYTICAL METHODVALIDATION

Validation was done for the developed method as per ICH Guidelines (Q2B).The method validation parameters for assay of Nelarabine include

- Specificity
- Accuracy
- Linearity and Range
- Precision

  Repeatability

  Intermediate precision (ruggedness)
- Detection Limit
- Quantitation Limit
- Robustness

3.5.1. SPECIFICITY:

Specificity of the developed method was determined by injecting blank,3 replicates of working standard solution and 3 replicates of working sample solution containing 20µg/ml of Nelarabine.

3.5.2. LINEARITY AND RANGE:

Preparation of stock solution

Weigh and transfer accurately 100mg Nelarabine working standard into a 100 ml clean,dry volumetric flask and add about 60 ml of diluentand sonicated to dissolve. Cool the solution to room temperature and dilute to volume with diluent. Then transfer 10.0 ml of theabove solution into 100 ml volumetric flask and dilute with mobile phase.

Preparation of Level – I (20 µg/mL)

2.0ml of stock solution was taken in to 10ml of volumetric flask and dilute up to the mark with mobile phase.

Preparation of Level – II (40 µg/mL)

4.0ml of stock solution was taken in to 10ml of volumetric flask and dilute up to the mark with mobile phase.

Preparation of Level – III (60 µg/mL)

6.0ml of stock solution was taken in to 10ml of volumetric flask and dilute up to the mark with mobile phase.

Preparation of Level – IV (80 µg/mL)

8.0ml of stock solution was taken in to 10ml of volumetric flask and dilute up to the mark with mobile phase.

Preparation of Level – V (100 µg/mL)

10ml of stock solution was taken in to 10ml of volumetric flask

Procedure

Each level was injected into the chromatographic system and peak area was measured.Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and the correlation coefficient was calculated.

Acceptance Criteria

Correlation coefficient should not be less than 0.999

RANGE

Based on precision, linearity and accuracy data it can be concluded that the assay method is precise, linear and accurate in the range of 50-150% of Nelarabine.

3.5.3 ACCURACY

Preparation of stock solution:

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

Preparation of 60 µg/ml solution:

Further pipette 0.6 ml of the above stock solution into a 10ml volumetric flask and dilute upto the mark with diluent. Mix well and filter through 0.45µm filter.

Preparation of Nelarabine sample solution:

For preparation of 50% solution (With respect to target Assay concentration):

1 mL of Nelarabine sample was taken into a 10 mL volumetric flask and about 7 mL of diluent was added.The resulted solution was sonicated and the volume was made to the mark with the same solvent. (Stock solution)

Pipette 0.6 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45µm filter.

For preparation of 100% solution (With respect to target Assay concentration):

1 mL of Nelarabine sample was taken into a 10 mL volumetric flask and about 7 mL of diluent was added.The resulted solution was sonicated and the volume was made to the mark with the same solvent. (Stock solution)

Pipette 1.2 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45µm filter.

For preparation of 150% solution (With respect to target Assay concentration):

1 mL of Nelarabine sample was taken into a 10 mL volumetric flask and about 7 mL of diluent was added.The resulted solution was sonicated and the volume was made to the mark with the same solvent. (Stock solution)

Pipette 1.8 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Mix well and filter through 0.45µm filter.

Procedure

The standard solution was spiked with sample of accuracy level 50% ,100%,150% and injected into chromatographic system. Calculate the amount found and amount added for Nelarabine and the individual % recovery and mean % recovery values were calculated.

Acceptance Criteria

The % Recovery for each level should be between 98.0 to 102.0%.

3.5.4 .PRECISION

Repeatability

Preparation of stock solution

Weigh and transfer accurately 100 mg of Nelarabine working Standard into a 100 ml clean, dry volumetric flask and add about 60 ml of diluent and sonicated to dissolve. Cool the solution to room temperature and dilute to volume with solvent mixture.

Sample Preparation:

Then transfer 2.0 ml above solution into 100 ml volumetric flask and dilute with mobile phase.

Procedure:

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

Acceptance Criteria

The % RSD for the area of six standard injections results should not be more than 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.

Preparation of stock solution

Weigh and transfer accurately 100 mg of Nelarabine working Standard into a 100 ml clean. dry volumetric flask and add about 60 ml of diluent sonicated to dissolve. Cool the solution to room temperature and dilute to volume with solvent mixture.

Sample Preparation:

Then transfer 2.0 ml above solution into 100 ml volumetric flask and dilute with mobile phase

Procedure

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

Acceptance Criteria

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

3.5.5 LIMIT OF DETECTION AND QUANTIFICATION

Detection Limit

The Detection Limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily qualtitated as an exact value.

Preparation of 60µg/mL solution:

Accurately weighed and transferred 10mg of Nelarabine working standard into 10mL volumetric flask.To that 7mL of diluent was added and sonicated. The volume was made up to the mark with the same diluent. (Stock solution).

Further 0.6mL of the above stock solution was pipetted into 10mL volumetric flask and diluted to the mark with diluent. The solution was mixed and filtered through 0.45µm filter.

Preparation of 1.0% solution At Specification level (0.029µg/ml solution):

1 mL of 10µg/mL solution was pipetted into 10mL volumetric flask and diluted up to the mark with diluent. Further 0.9 mL of above solution was pipette into 10mL volumetric flask and diluted up to the mark with diluent.

Further 0.3mL of above diluted solution was pipetted into 10mL volumetric flask and diluted up to the mark with diluent.

Quantitation Limit

The Quantitation limit of an analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

Preparation of 60µg/mL solution:

Accurately weighed and transferred 10mg of Nelarabine working standard into 10mL Volumetric flask.To that 7mL of diluent was added and sonicated. The volume was made up to the mark with the same diluent. (Stock solution)

Further 0.6mL of the above stock solution was pipetted into 10mL volumetric flask and diluted to the mark with diluent. The solution was mixed and filtered through 0.45µm filter.

Preparation of 0.9% solution At Specification level (0.095µg/ml solution):

1.0mL of above solution was pipetted into 10mL volumetric flask and diluted up to the mark with diluent.1.0mL of the solution was pipetted into 10mL volumetric flask and diluted up to the mark with diluent.

Further 0.9 mL of above diluted solution was pipetted into 10mL volumetric flask and diluted up to the mark with diluent.

3.5.6.ROBUSTNESS:

The Robustness of analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in procedural parameters listed in theprocedure documentation and indication of its suitability during normal usage.Examples of typical variations in assay method validation by HPLC are:

- ✓ Mobile phase flow rate

- ✓ Influence of variation in mobile phase composition etc.

± 0.1 ml/min flow rate:

Standard solution of 20µg/mL of Nelarabine was prepared and analysed at 0.9 mL/min and 1.1mL/min i.e. at ± 0.1 unit of optimised flow rate (1.0 mL/min).

± 5% in mobile phase composition:

Standard solution of 20µg/mL of Nelarabine was prepared by changing the mobile phase ratio by ± 5% and analysed

Acceptance Criteria

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

3.6. Forced degradation studies

Forced degradation studies are typically performed to assess physical and chemical stability of the drug product. These studies are also used to determine the degradation pathways of drug substances and drug products, the intrinsic stability of the drug substance molecule in solution and solid state.

Prepation of sample stock solution (100 µg/mL):

2 mL of sample was taken from the Nelarabine injection solution(250mg/50mL) in to a 100 mL volumetric flask and diluted to the volume using the diluent.

3.6.1 Acid degradation:

Acid degradation studies were carried out by adding 10 mL 1.5 N HCl to 5 mL of above solution and by refluxing the solution at 60^0C for 2 hours. Then 10mL of 1N NaOH was added and volume was made up to 50 mL with diluent.Then the samples were injected through proposed HPLC method.

3.6.2 Alkali degradation:

Base degradation studies were carried out by adding 10 mL of 1.5 N NaOH to 5 mL Nelarabine sample solution and by refluxing the solution at 60^0C for 2

hours. Then 10mL of 1N HCl was added and volume was made up to 50 mL with diluent.Then the samples were injected through proposed HPLC method.

3.6.3 Oxidative degradation:

Peroxide degradation studies were carried out by adding 1 mL of 30% H_2O_2 to 5mL of Nelarabine sample solution and by refluxing the solution at 40^0C for 2 hours.Then volume was made up to 50mL with diluent. Then the samples were injected through proposed HPLC method.

3.6.4 Thermal degradation:

Thermal degradation studies were carried out for 5mL Nelarabine sample solution in 50 mL volumetric flask which was diluted to mark using diluent. Resulting solution was subjected to 80^0C for 2 hours. Then the samples were injected through proposed HPLC method.

3.6.5 Photolytic degradation

Photolytic degradation studies were carried out by exposing 5mL Nelarabine sample solution to UV lamp in UV cabinate at 254nm for 2 hours. Finally the volume was made upto mark in 50mL volumetric flask using diluent. Then the samples were injected through proposed HPLC method.

4. RESULTS AND DISCUSSION

The present study was aimed to develop a new method for the estimation of Nelarabine by stability indicating RP-HPLC and to validate the developed method. The literature survey revealed that only one HPLC method was reported for Nelarabine in pharmaceutical dosage form. Hence an attempt has been made to develop a RP-HPLC method for the determination of Nelarabine in bulk and pharmaceutical dosage form.

4.1. ANALYTICAL METHOD DEVELOPMENT

4.1.1 Selection of wavelength:

The detection wavelength was determined by dissolving Nelarabine in Methanol to get a concentration of 10 µg/ml. The solution was scanned in U.V region from 200-400nm. The absorbance maximum was found to be 266nm. The spectrum was shown in fig.No.4.1.

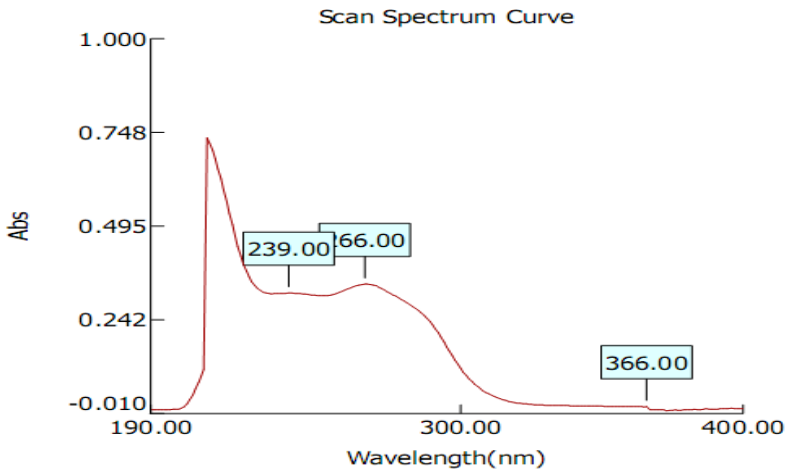


Fig.No.4.1. UV spectra of Nelarabine

4.1.2 Method Development and Optimization of Chromatographic Parameters:

The chromatographic method was developed for the estimation of Nelarabine by optimizing several parameters like flow rate, mobile phase, and run time and elution technique.

4.1.3. Method development trials:

Trial 1:

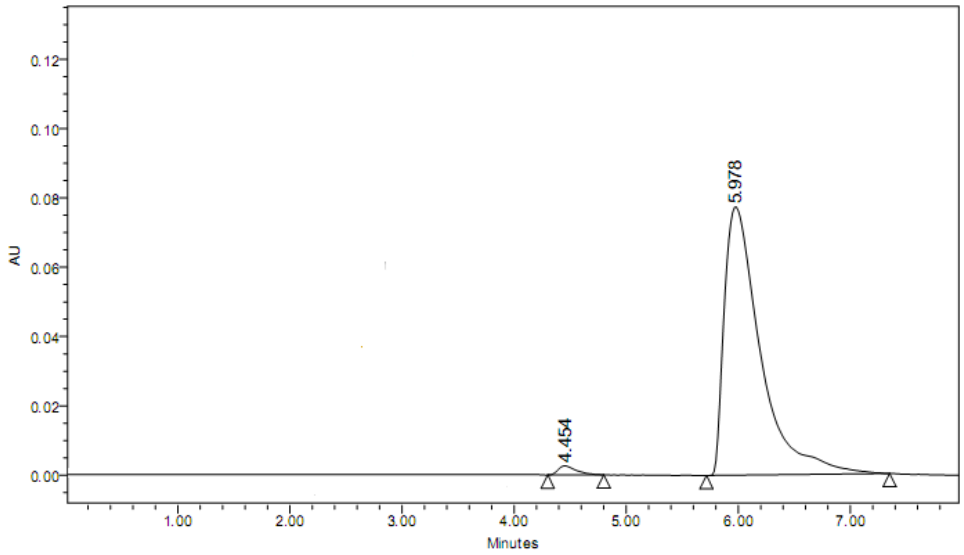


Fig.No.4.2. Chromatogram of Trial 1

Inference: An extra peak and peak tailing appeared.

Trail 2:

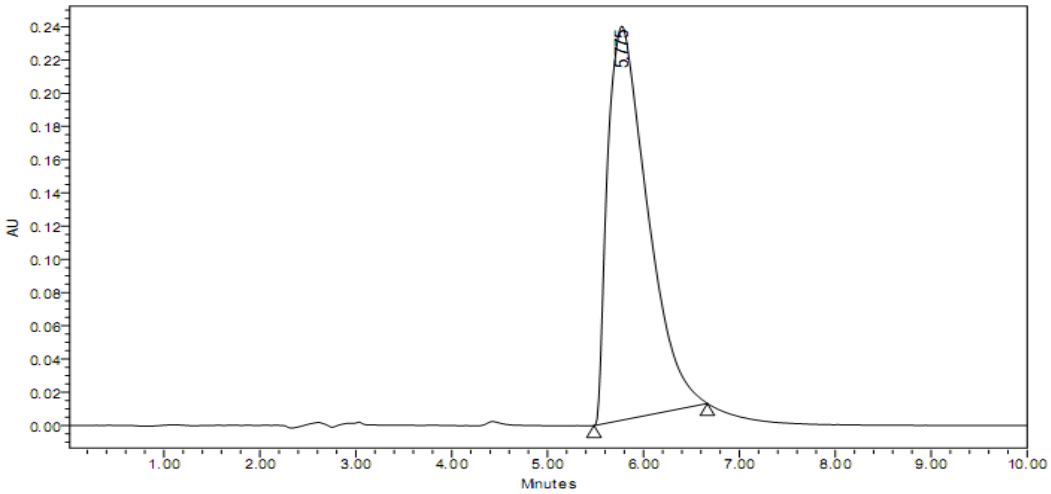


Fig.No.4.3. Chromatogram of Trial 2

Inference: Peak tailing appeared with improper baseline.

Trail 3:

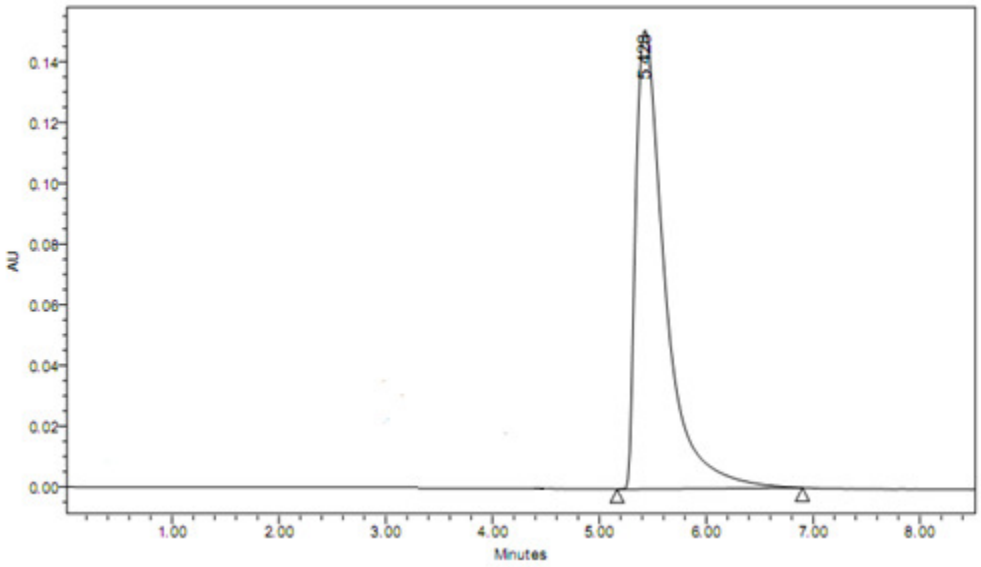


Fig.No.4.4. Chromatogram of Trial 3

Inference: peak tailing is obtained.

Trail 4:

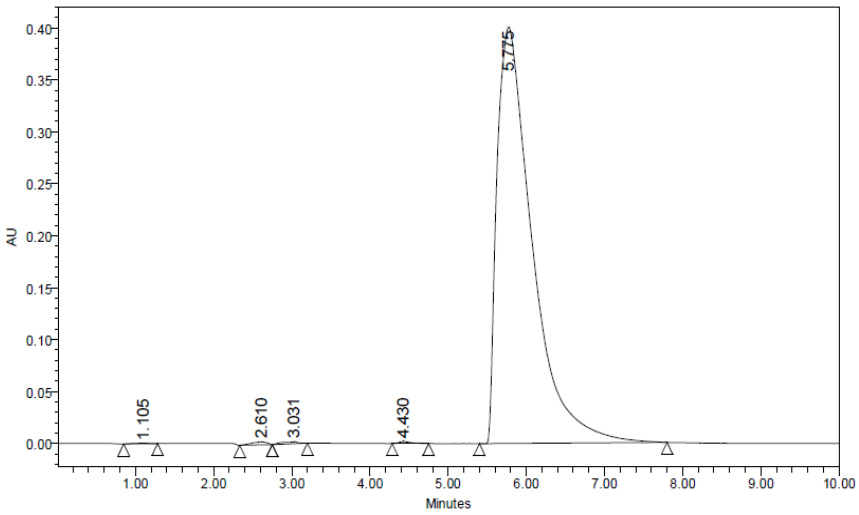


Fig.No.4.5. Chromatogram of Trial 4

Inference: Tailing factor is more than 2 and baseline disturbance appeared.

Trail 5:

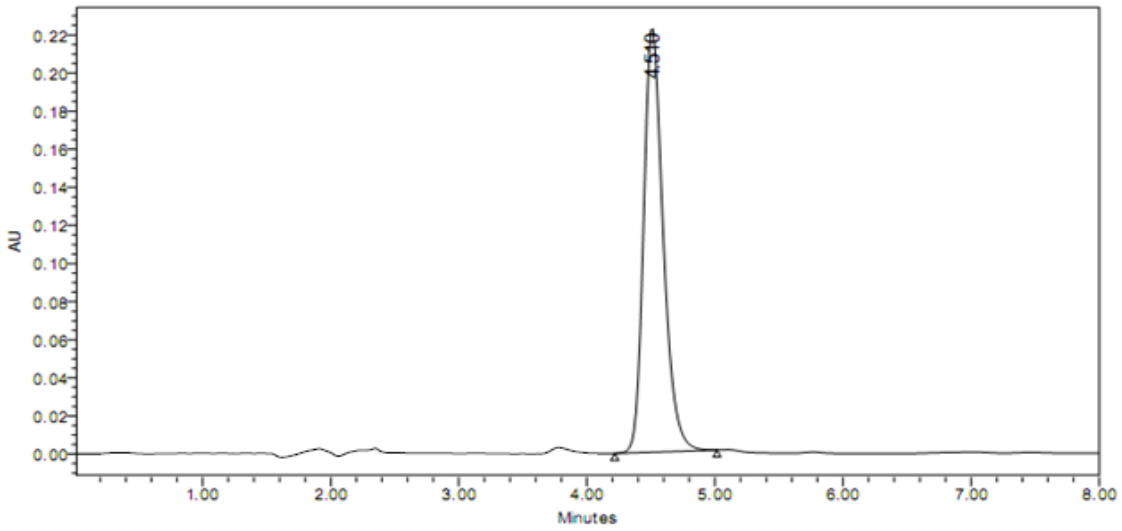


Fig.No.4.6. Chromatogram of Trial 5

Inference: Baseline is not proper**.**

Trail 6: Optimized Method

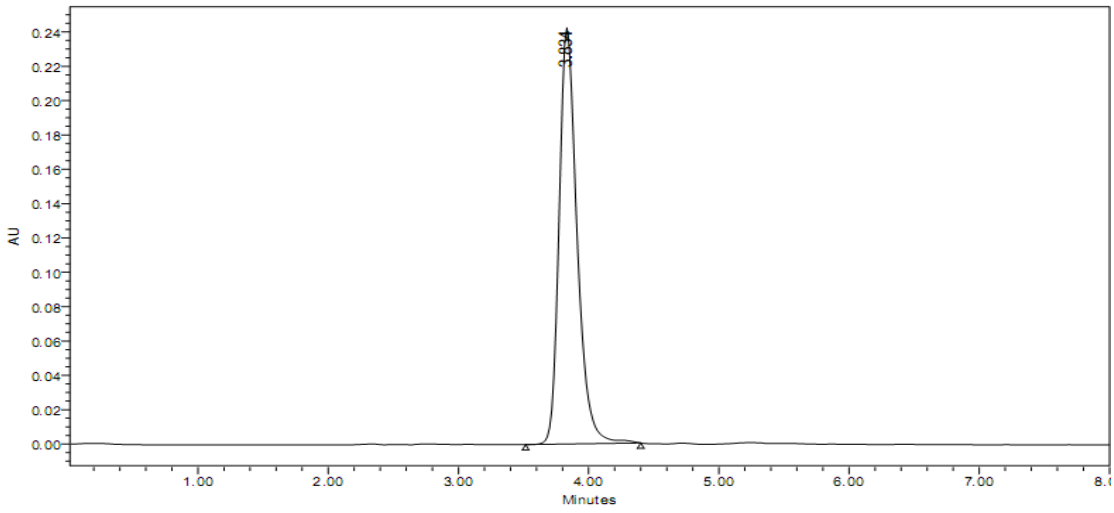


Fig.No.4.7. Chromatogram of Trial 6

Discussion: sharp peak was eluted at 3.83 min. Plate counts, Tailing factor and Resolution were within acceptable limits.

4.1.4. Optimized chromatographic conditions

The optimized chromatographic conditions for the method development and validation of Nelarabine are as follows

Table no.4.1. Optimized Method conditions

S.No	Parameter	Condition
1	Column	Cosmicsil Adze C18 column(150×4.6mm,5µm)
2	Mobile Phase	85:15 v/v 0.01% Trifloroacetic acid (pH 3.0) and Acetonitrile
3	Column Temperature	30^0C
4	Wavelength	266nm
5	Flow rate	1.0 ml/min
6	Auto sampler temperature	Ambient
7	Injection volume	10µL
8	Run time	8 minutes

4.2. Estimation of Nelarabine in formulation by developed RP-HPLC method:

A.Assay:

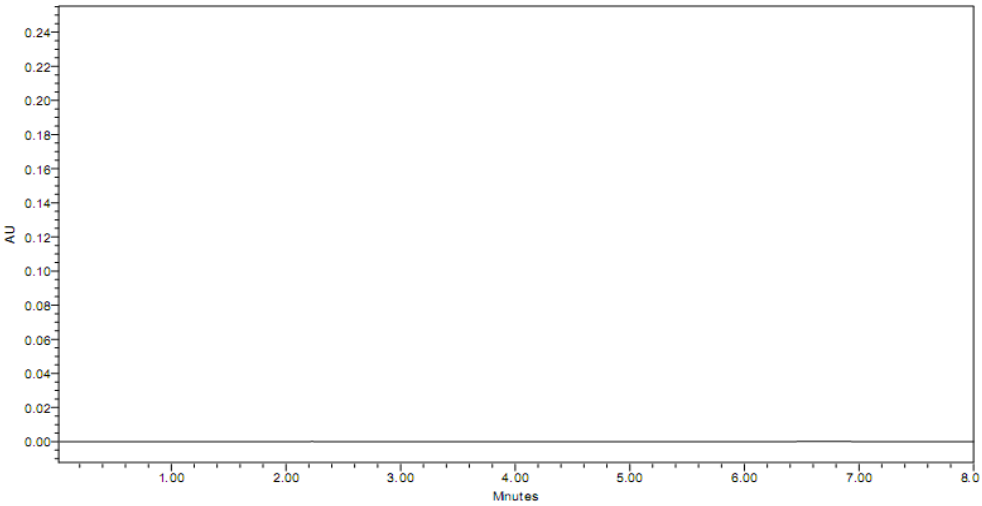


Fig.No.4.8. Chromatogram of Blank

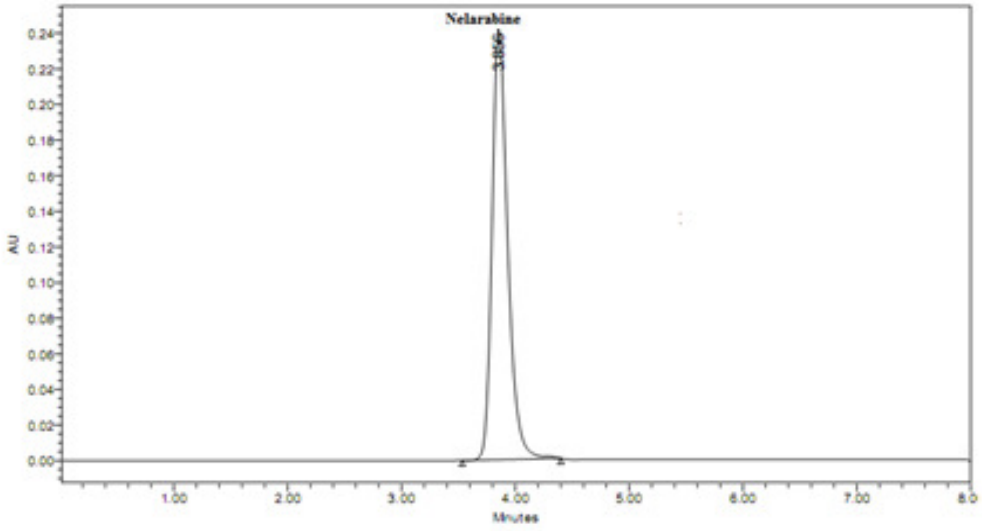


Fig.No.4.9. Chromatogram of Standard

	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Tailing	USP Plate Count
1		3.856	2309481	243220	1.3	3905.1

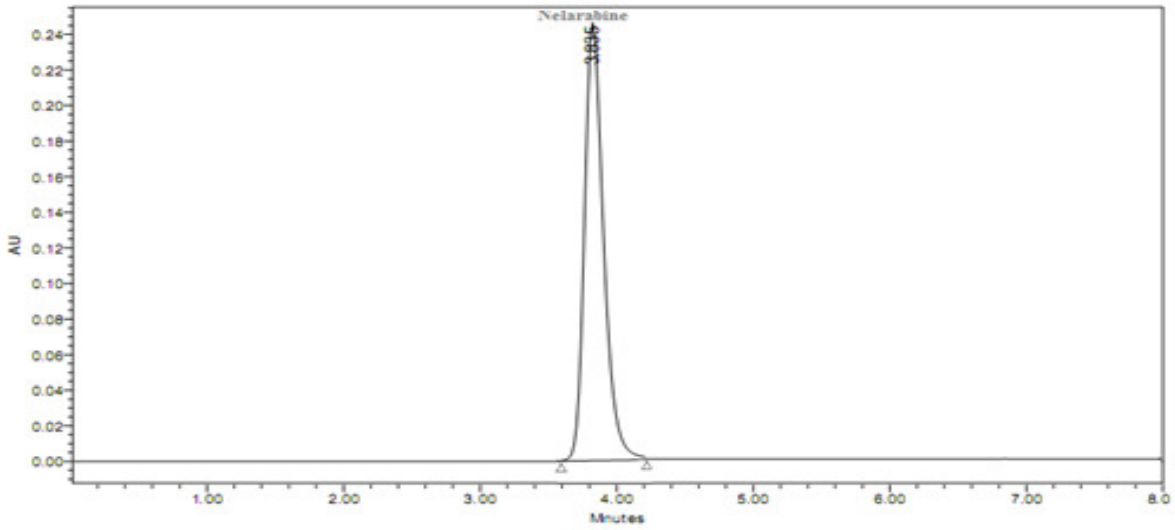


Fig.no.4.10. Chromatogram of Sample

	Name	Retention Time (min)	Area (µV*sec)	Height (µV)	USP Tailing	USP Plate Count
1		3.835	2308368	240742	1.3	3850.7

Peak results for test preparation (Assay)

Table 4.2. Product Label

Product name	Arranon (Nelarabine) injection
Active ingredient	Nelarabine
Label claim mg/mL	250mg/50mL in each vial
% purity	99.9

Table no.4.3. Peak responses

Name	Nelarabine
Peak Area of sample	2308368
Peak Area of standard	2309481
Weight of working standard in mg	20.0
Weight of sample in mg	10.0
% assay	99.4

Assay calculation-:

$$\%\text{Assay} = \frac{TA}{SA} \times \frac{SW}{100} \times \frac{1}{10} \times \frac{50}{TW} \times \frac{10}{1} \times \frac{P}{100} \times 100$$

Where,

TA = peak area response due to Nelarabine from sample

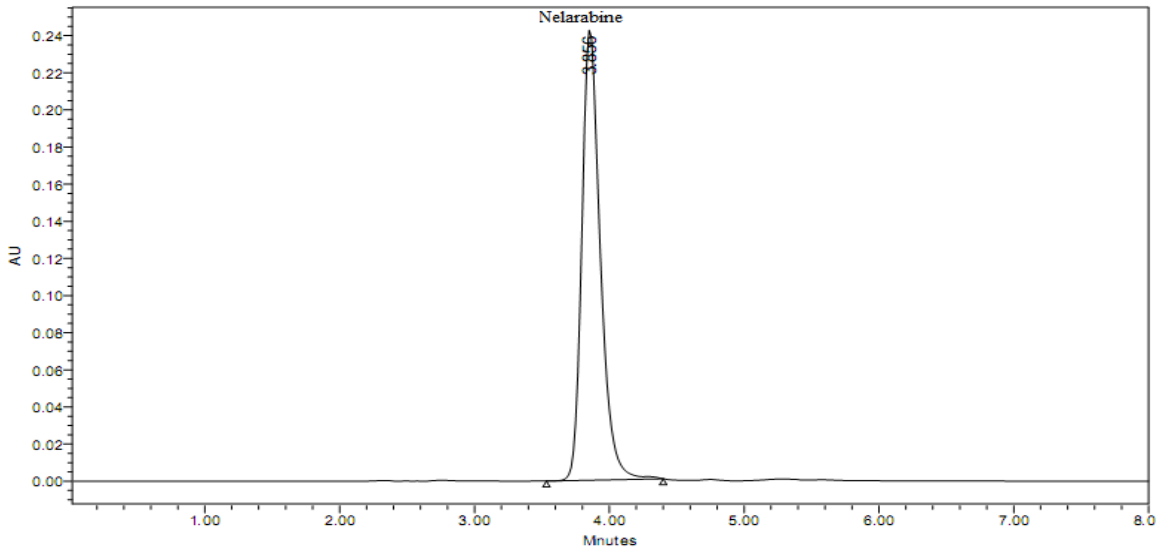
SA = peak area response due to Nelarabine from standard

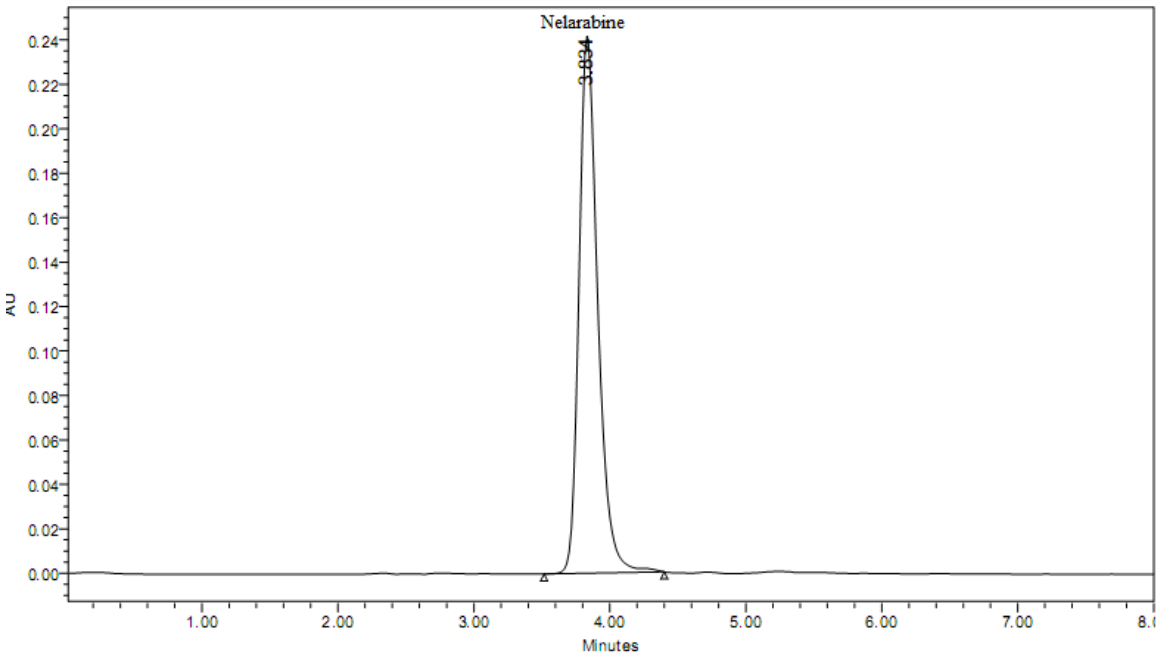
SW = Weight of Nelarabine working standard taken in mg

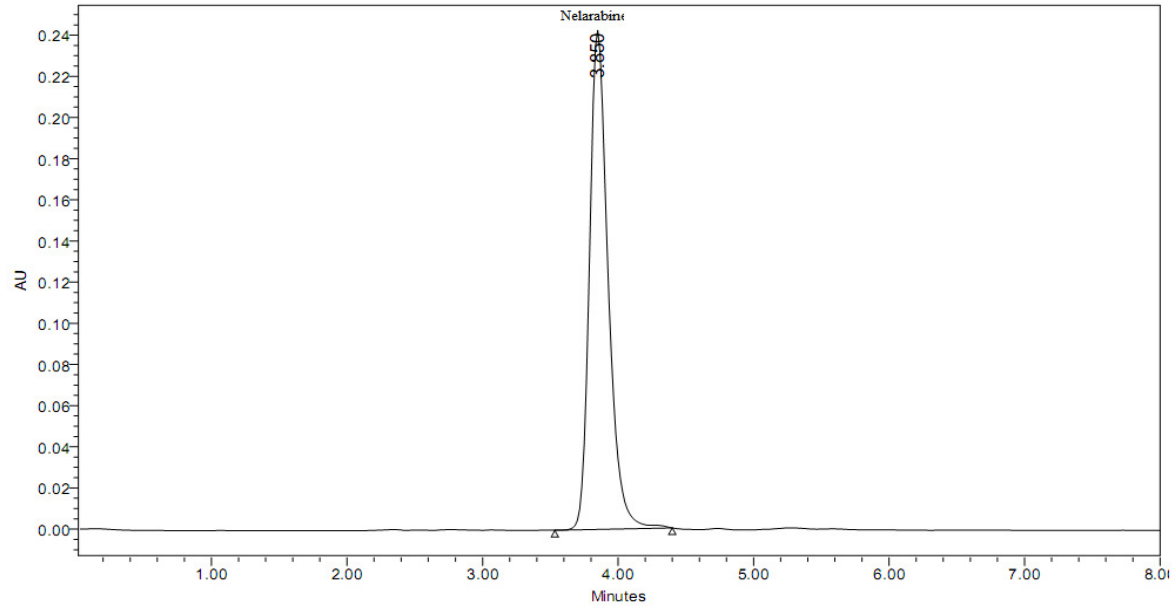
TW = weight of sample taken in mg

P = purity of Nelarabine working standard taken on as is basis

B. System Suitability Results:




Nelarabine
3.834
AU
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00
Minutes


Nelarabine
3.850
AU
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00
Minutes

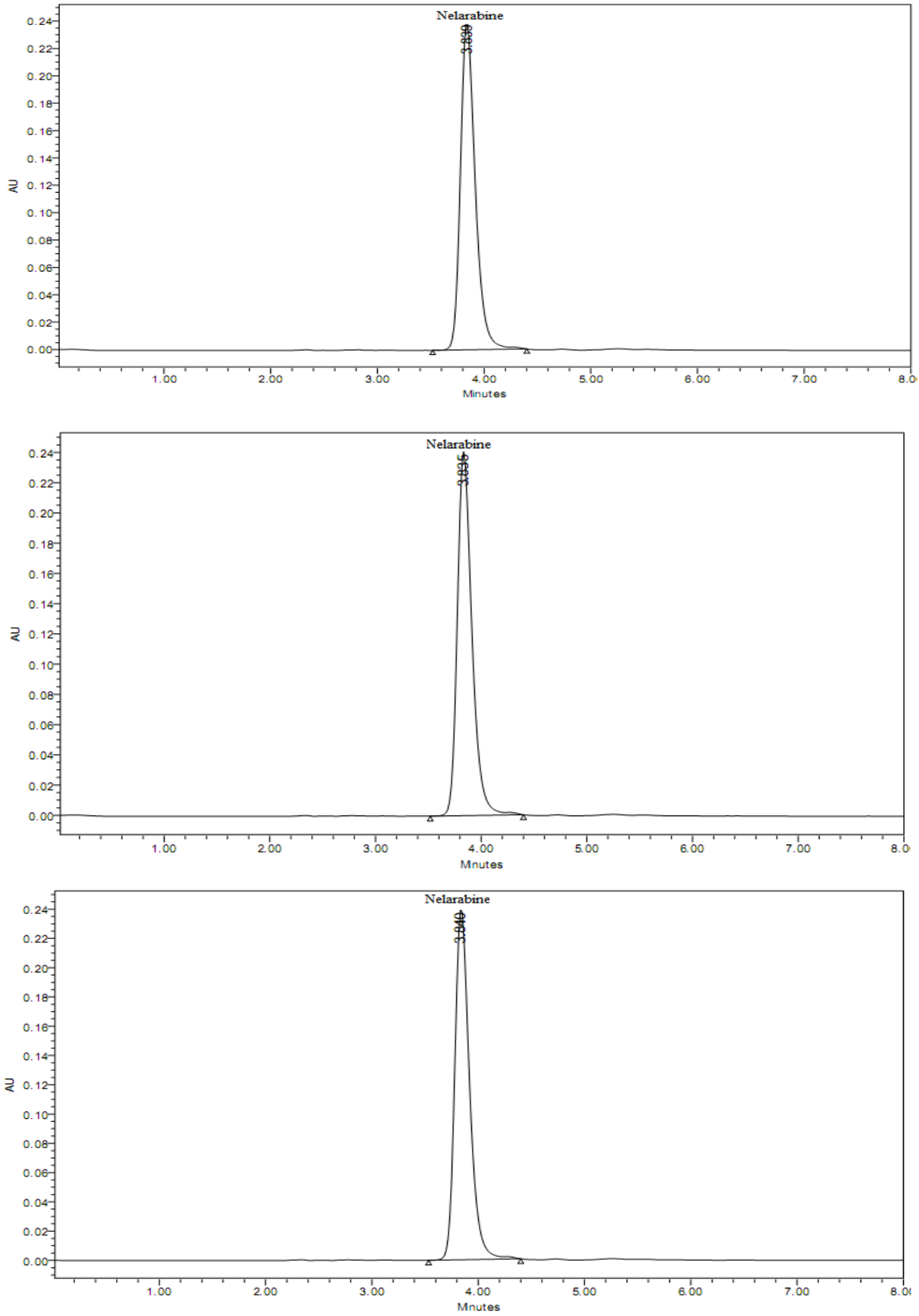


Fig.No.4.11. Chromatograms for system suitability

Table.No.4.4. system suitability data of Nelarabine

S.No	Rt (min)	Area	USP Tailing	Plate count	Height
1	3.856	2309481	1.3	3905.1	243220
2	3.834	2324212	1.3	3862.4	242034
3	3.850	2321743	1.3	3903.0	242111
4	3.839	2309743	1.3	3804.6	240877
5	3.835	2308368	1.3	3850.7	240742
6	3.840	2306722	1.3	3825.0	240983
Mean		2313375	1.3		
SD		7553.667			
%RSD		0.326			

Discussion: All the system suitability parameters were within limits.

4.3. ANALYTICAL METHOD VALIDATION OF RP-HPLC METHOD

Validation of an analytical method is the process to establish by laboratory studies that the performance characteristic of the method meets the requirements for the intended analytical application. Performance characteristics were expressed in terms of analytical parameters.

4.3.1. SPECIFICITY

The system suitability for specificity was carried out to determine whether there is any interference at the retention time of analytical peak.

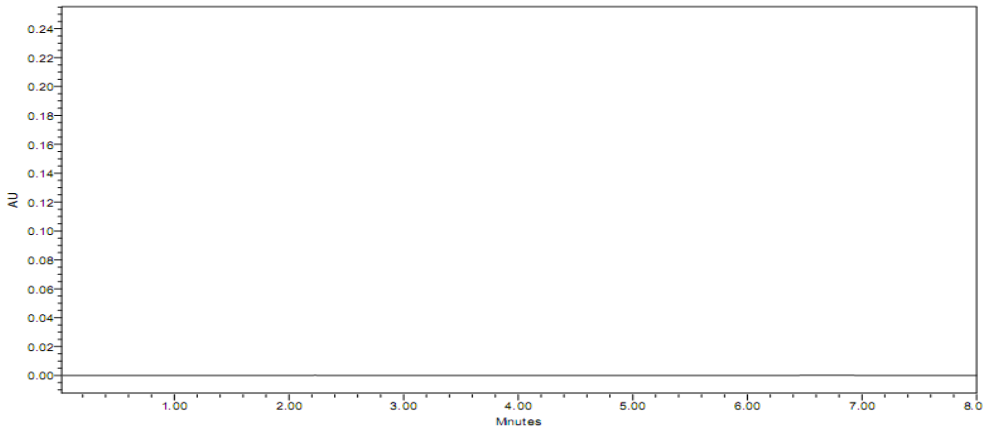
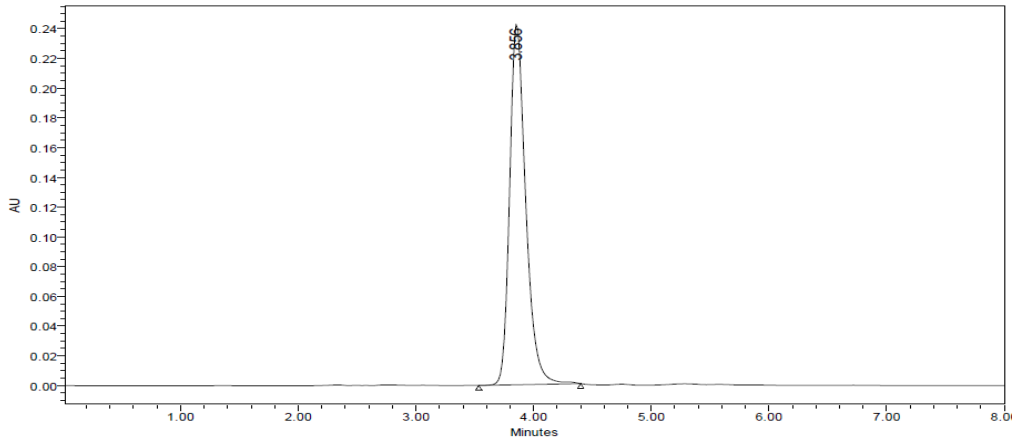
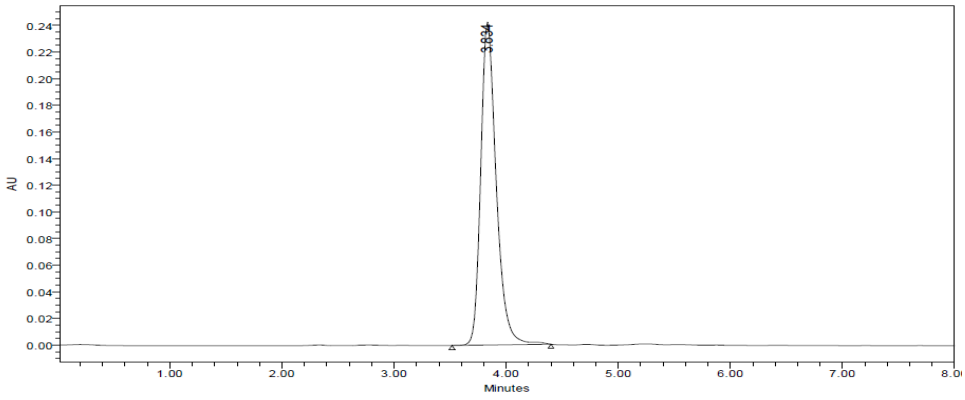


Fig.No.4.12. Chromatogram of Blank





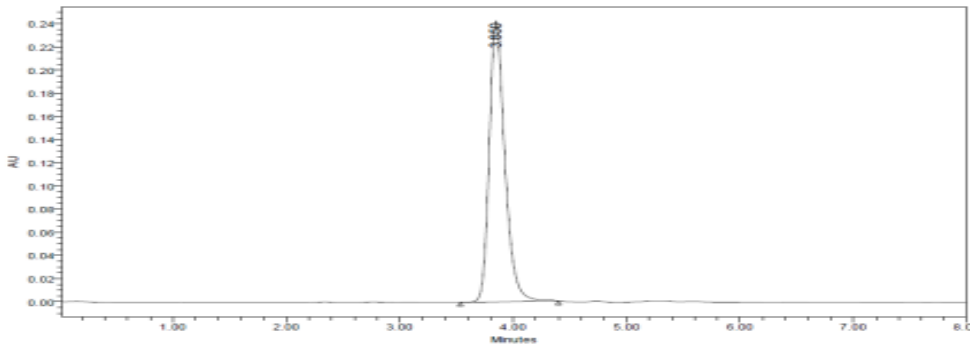


Fig.No.4.13. Chromatogram of Standard

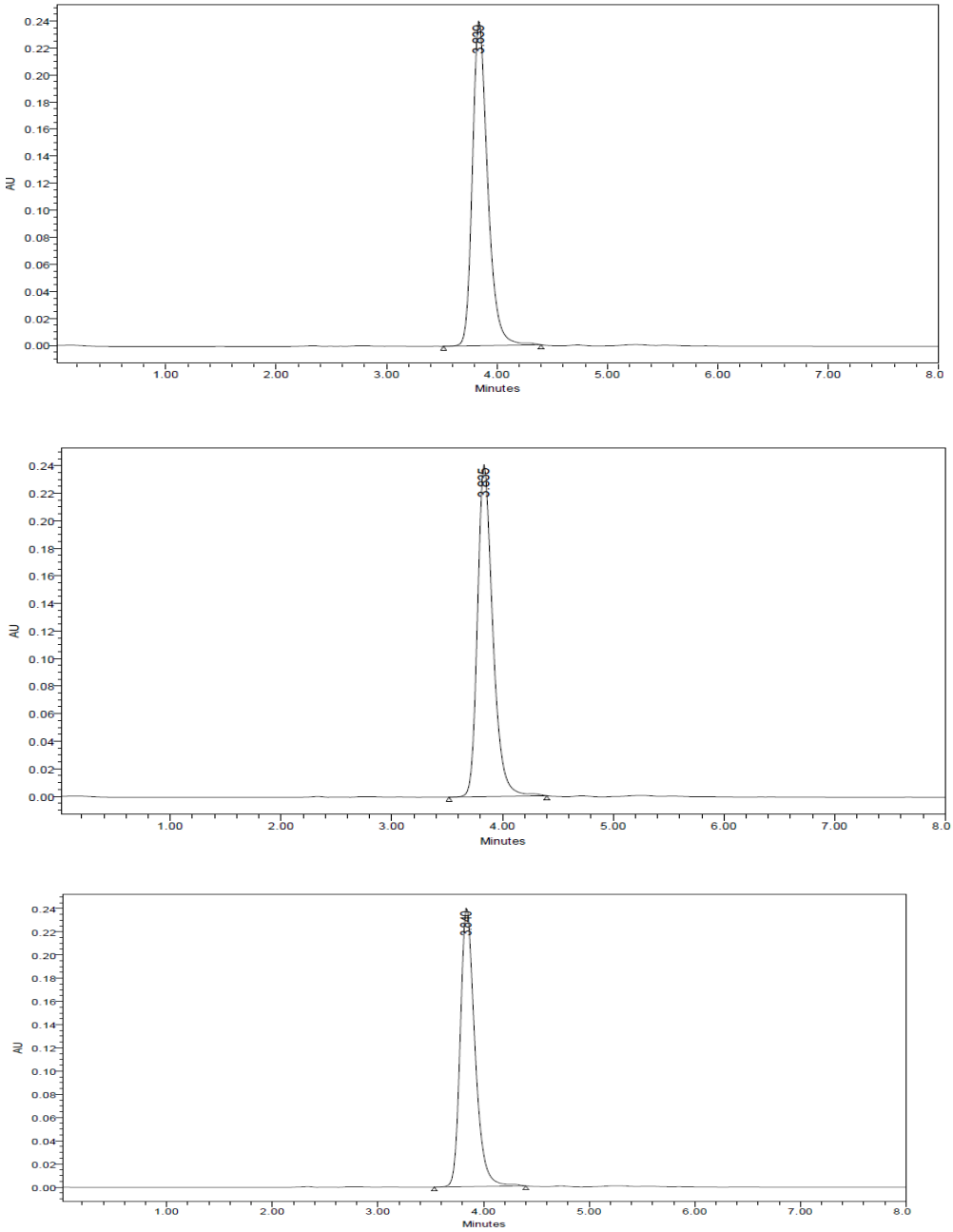


Fig.No.4.14. Chromatogram of sample

Discussion: No interference was observed at the retention time of the analyte.

4.3.2. LINEARITY AND RANGE

The linearity of a method is its ability to obtain test results that are directly proportional to the sample concentration over a given range. Linearity was established by least squares regression analysis of the calibration curve. The linearity study was performed for the concentration of 20µg/mL to 100µg/mL level. Each level was injected into chromatographic system. The calibration curve was plotted for Peak areas against respective concentrations and linear regression analysis was performed on the resultant curves. The chromatograms were shown in fig.No.4.15-4.19 and table no.4.5.

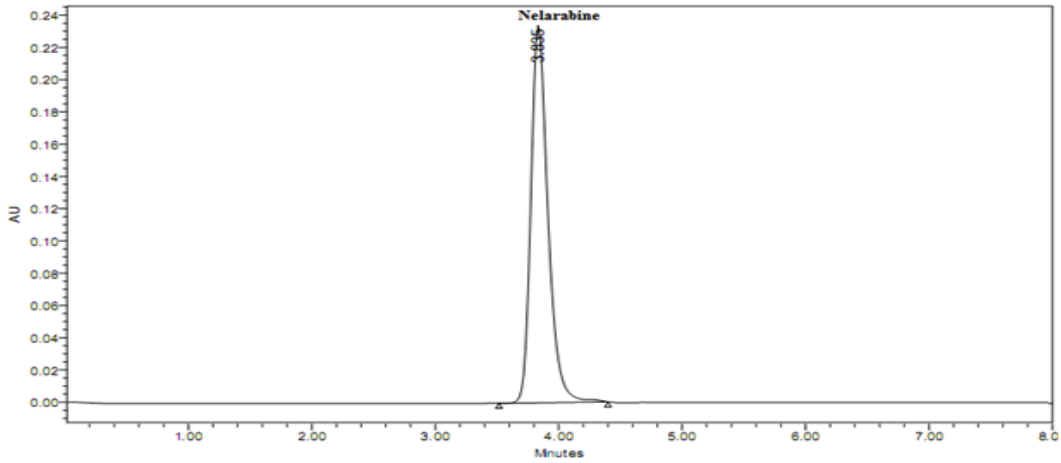


Fig.No.4.15. HPLC chromatogram of linearity 20µg/mL

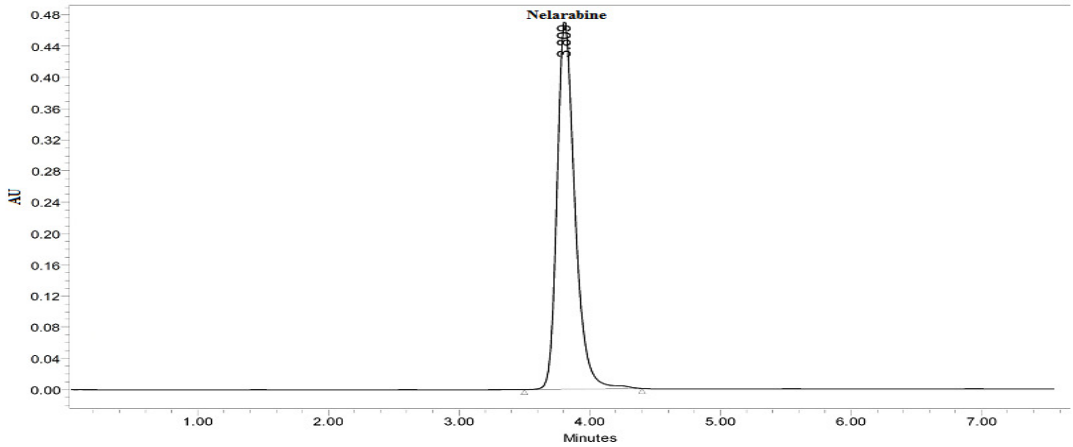


Fig.No.4.16. HPLC chromatogram of linearity 40µg/mL

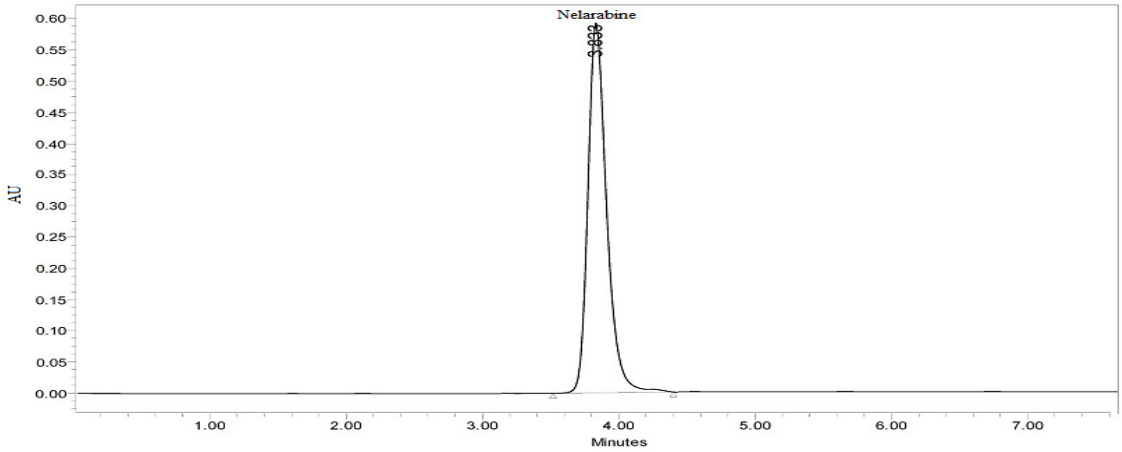


Fig.No.4.17. HPLC chromatogram of linearity 60µg/mL

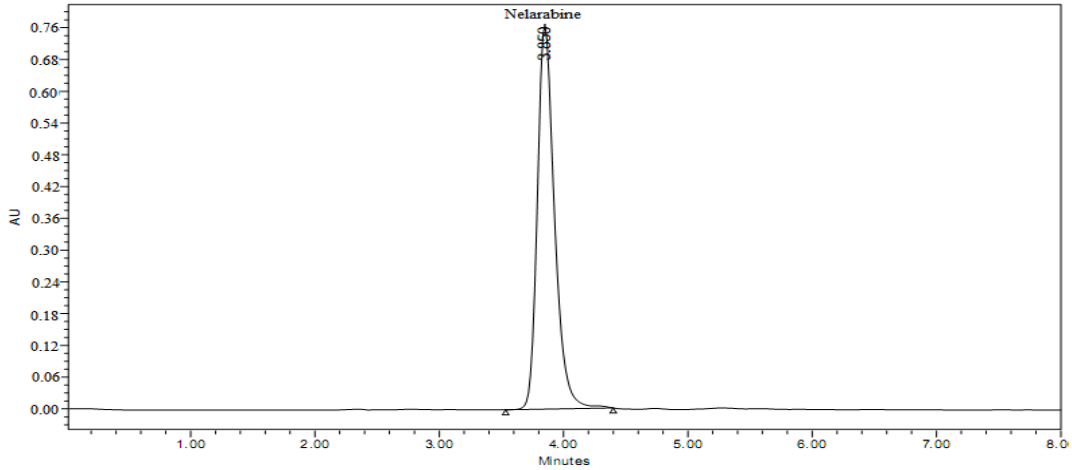


Fig.No.4.18. HPLC chromatogram of linearity 80µg/mL

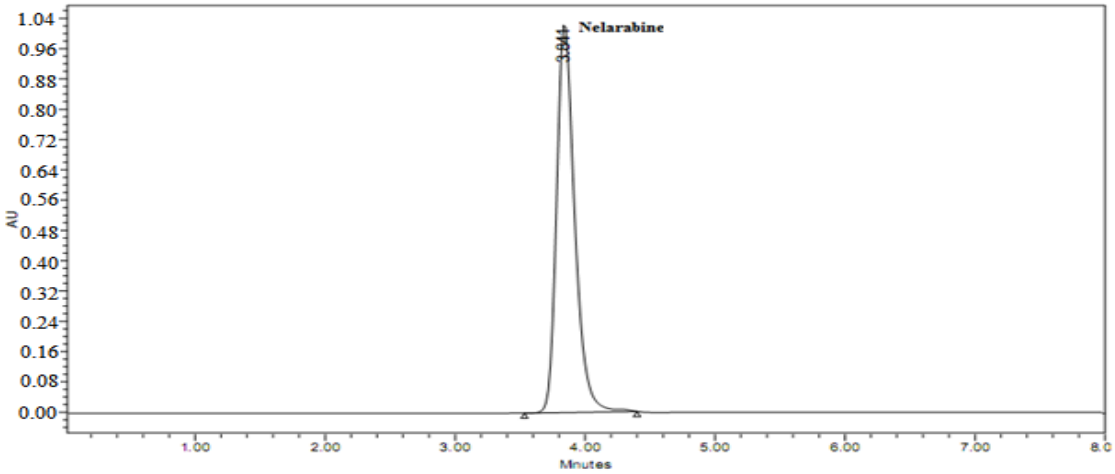


Fig.No.4.19. HPLC chromatogram of linearity 100µg/mL

Table.No.4.5. Linearity data of Nelarabine

S.No	Linearity Level	Concentration	Area
1	I	20 µg/mL	2242651
2	II	40 µg/mL	4236227
3	III	60 µg/mL	6251237
4	IV	80 µg/mL	8360119
5	V	100 µg/mL	10351126
Correlation Coefficient			**0.9995**

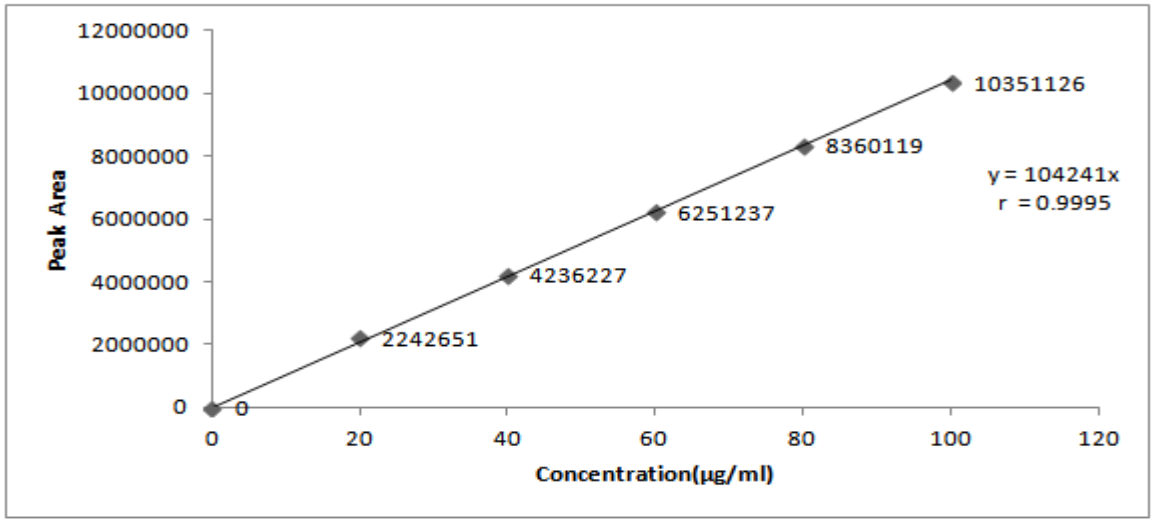


Fig.No.4.20. Linearity plot of Nelarabine

Discussion

Nelarabine was found to be linear over the range of 20-100µg/mL. Correlation coefficient value for calibration plot of Nelarabine was found to be 0.999.

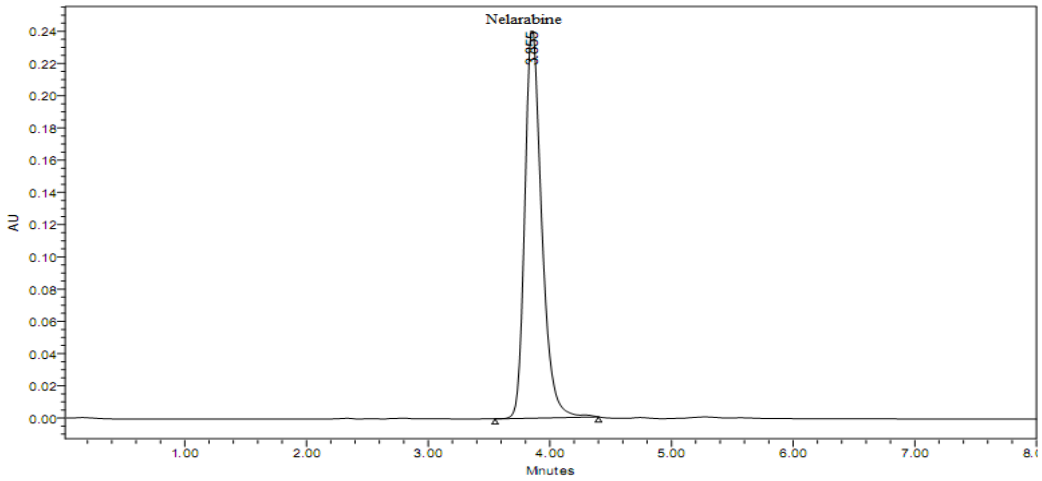
4.3.3. PRECISION

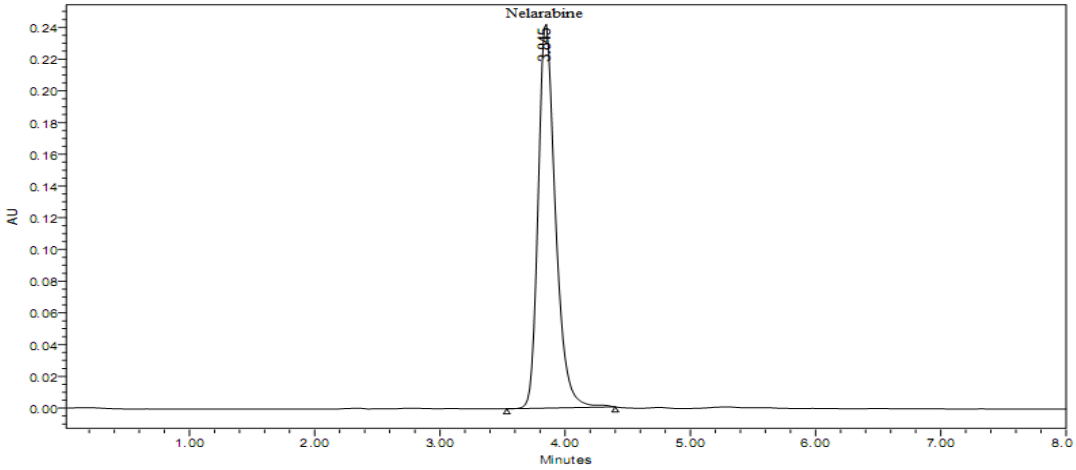
Precision of method was demonstrated by

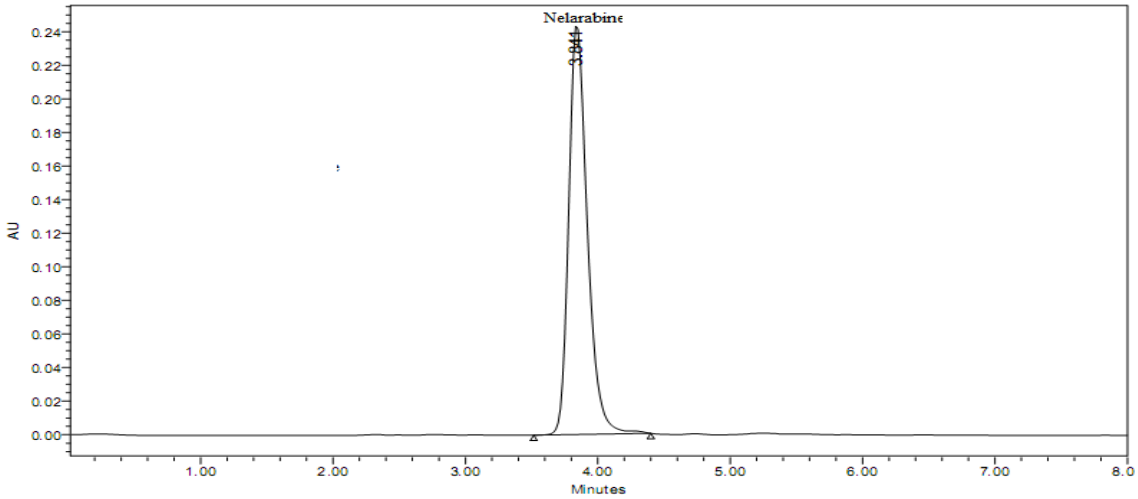
a) Repeatability
b) Intermediate precision
c) Reproducibility

a) Repeatability

The Repeatability studies were studied by six replicate measurements and the % RSD of peak area was calculated.


Nelarabine
3.855
AU
Minutes


Nelarabine
3.845
AU
Minutes


Nelarabine
3.841
AU
Minutes

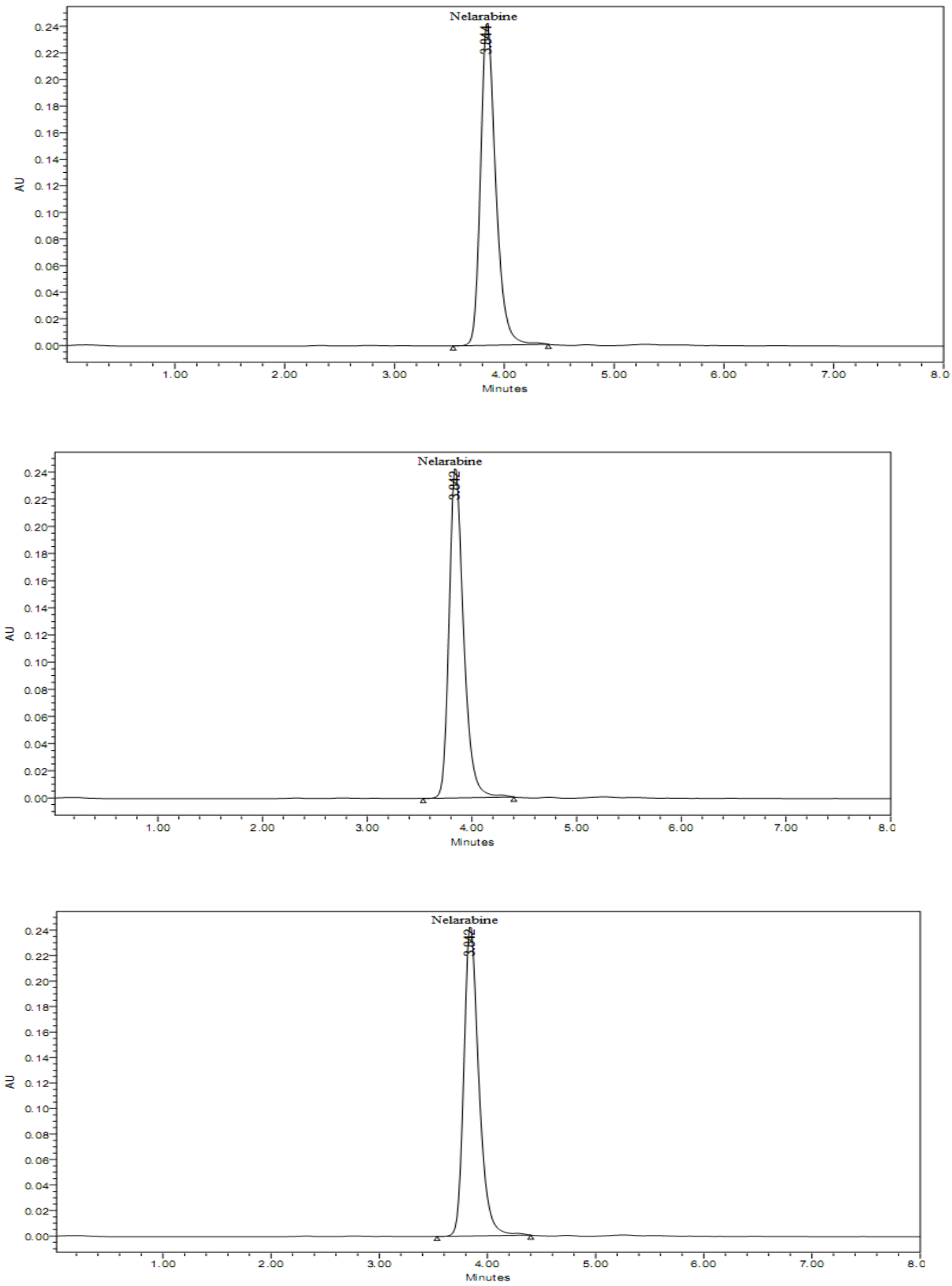


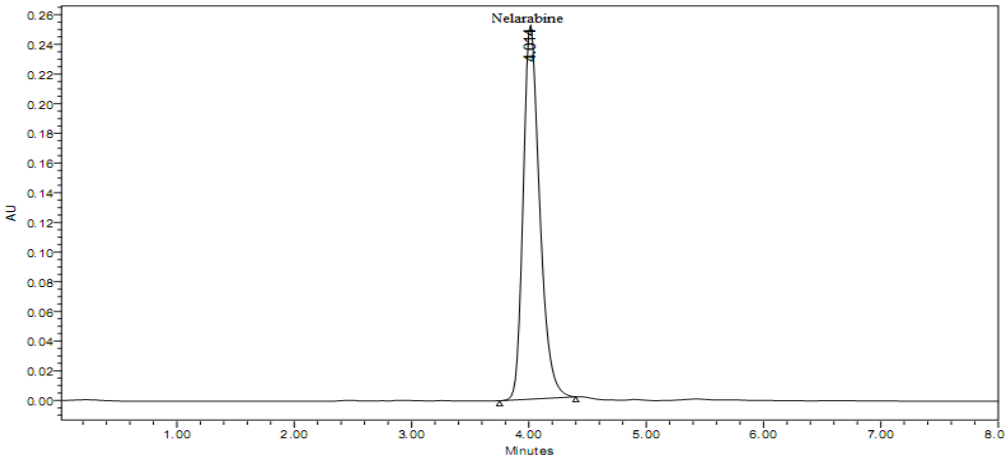
Fig.No.4.21. HPLC chromatograms for Repeatability

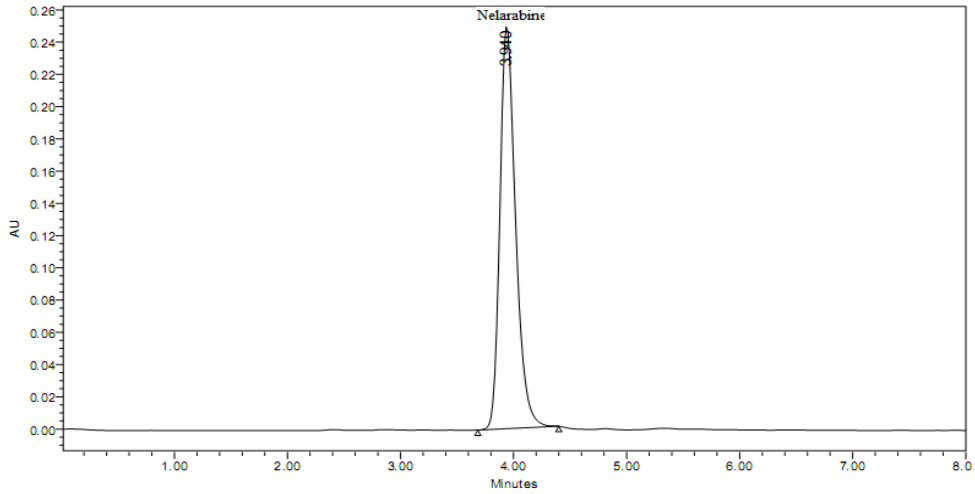
Table.No.4.6. Repeatability data of Nelarabine

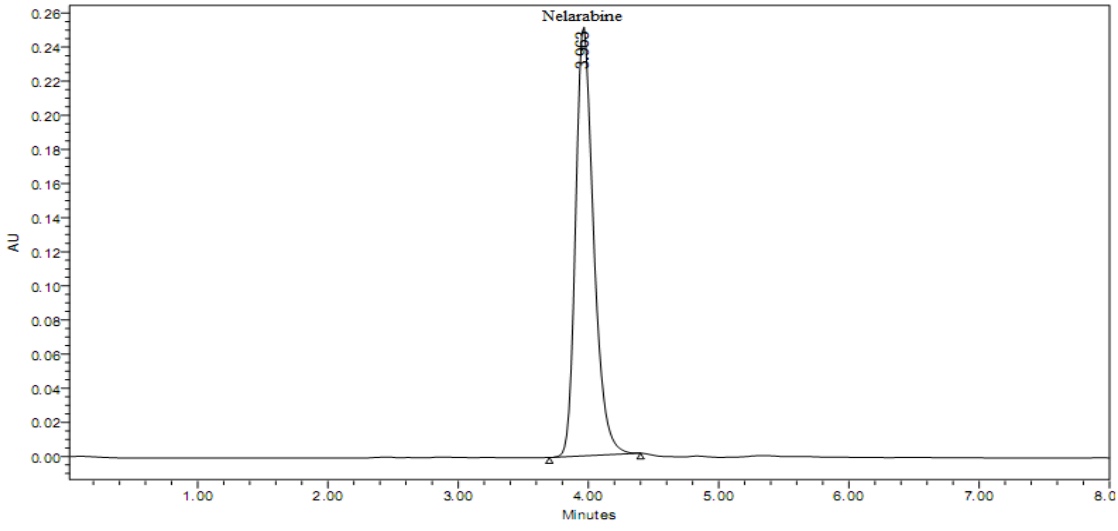
S.No	Area
1	2332801
2	2328717
3	2385913
4	2335516
5	2344025
6	2345394
Mean	2345394.3
SD	20870.7661
%RSD	0.889

b) Intermediate precision/Ruggedness

The intermediate precision was carried out on same HPLC system, using same column on another day. The Intermediate precision studies were studied by six replicate measurements and the % RSD of peak area was calculated. The chromatograms were shown in Fig.No.4.22 and results are tabulated in Table.No.4.7.


Nelarabine
4.044
AU
Minutes


Nelarabine
3.940
AU
Minutes


Nelarabine
3.963
AU
Minutes

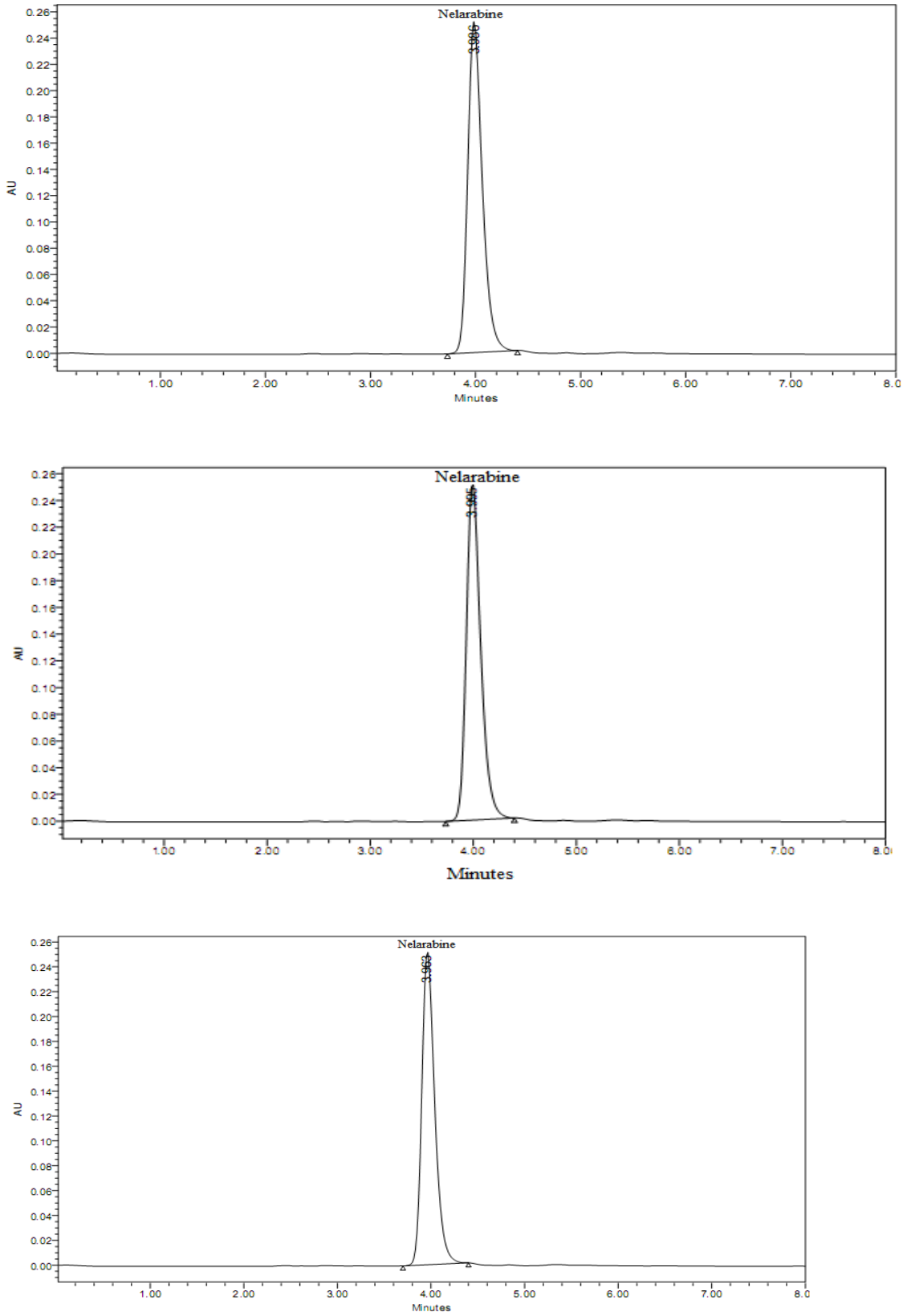


Fig.No.4.22. HPLC chromatograms for Intermediate precision

Table.No.4.7. Intermediate precision data of Nelarabine

S.No	Area
1	2471937
2	2413336
3	2423902
4	2437684
5	2445010
6	2438374
Mean	2438373.8
SD	20036.605
%RSD	0.821

Discussion: The %RSD values calculated for repeatability and intermediate precision were found to be within limits (%RSD < 2).

4.3.4. ACCURACY

The accuracy of the method shall be demonstrated through determination on samples in three concentrations from 50%, 100% and 150% three replicates each of the theoretical concentrations employed as per the usual procedure and the chromatograms were recorded and % recovery was calculated.

Table.No.4.8. Accuracy results of Nelarabine

%concentration (at specification level)	Area	Average area	Amount found(µg/mL)	Amount added(µg/mL)	%Recovery	Mean recovery
	2127297					
50	2121167	2126881	30.10	29.94	100.54	
	2132178					
	4236227					
100	4239885	4236598	59.97	59.88	100.15	99.81
	4233682					
	6251237					
150	6265225	6264031	88.67	89.82	98.72	
	6275631					

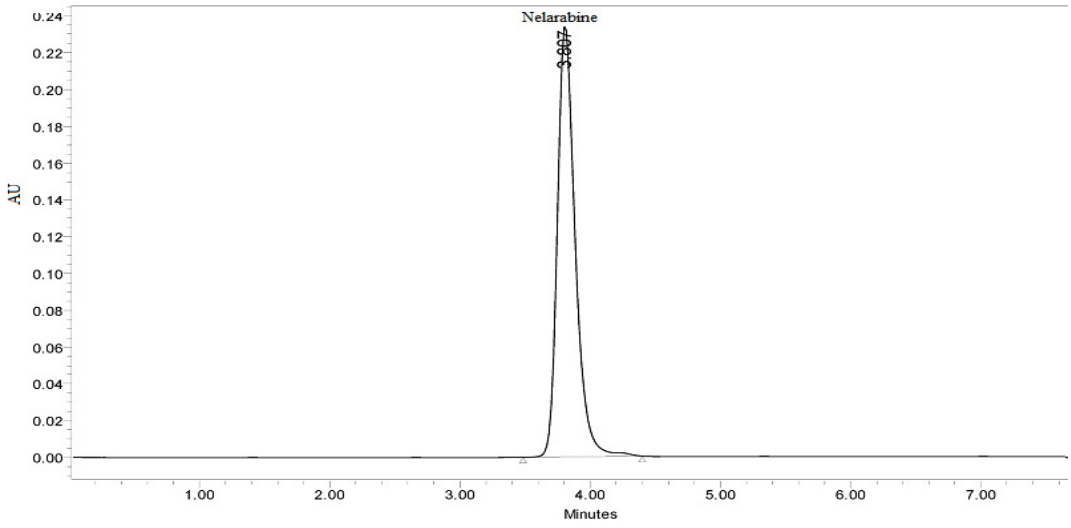


Fig.No.4.23. HPLC chromatogram for accuracy 50% (prep-1)

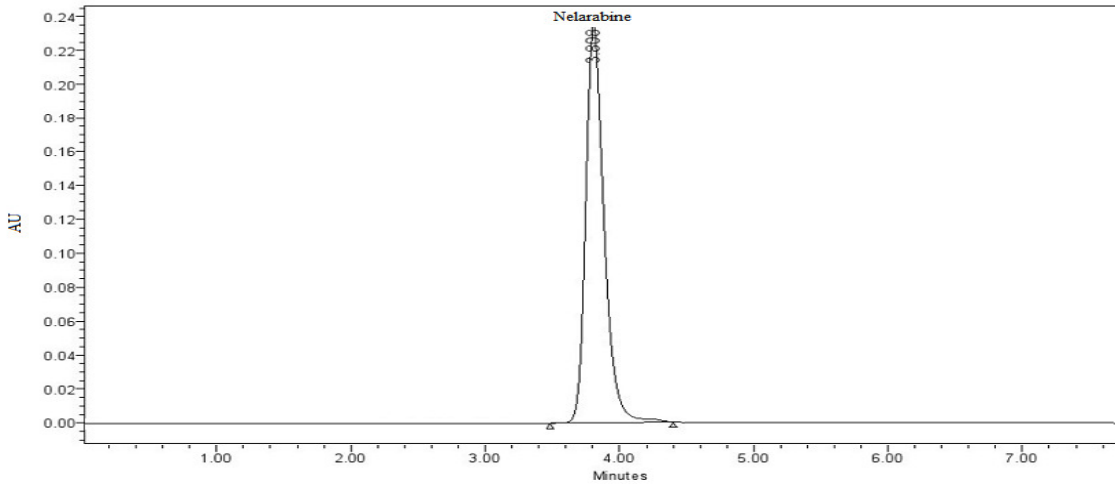


Fig.No.4.24. HPLC chromatogram for accuracy 50% (prep-2)

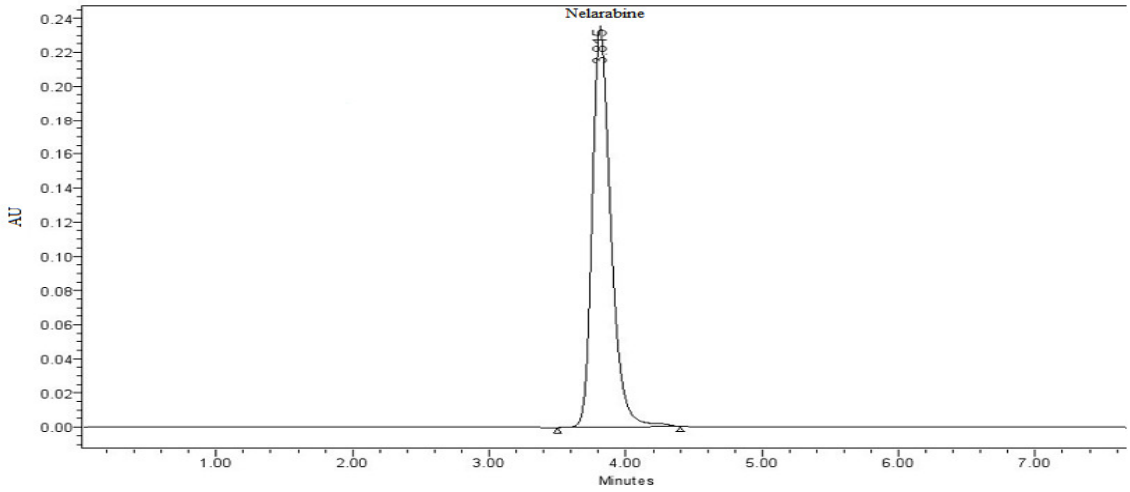


Fig.No.4.25. HPLC chromatogram for accuracy 50% (prep-3)

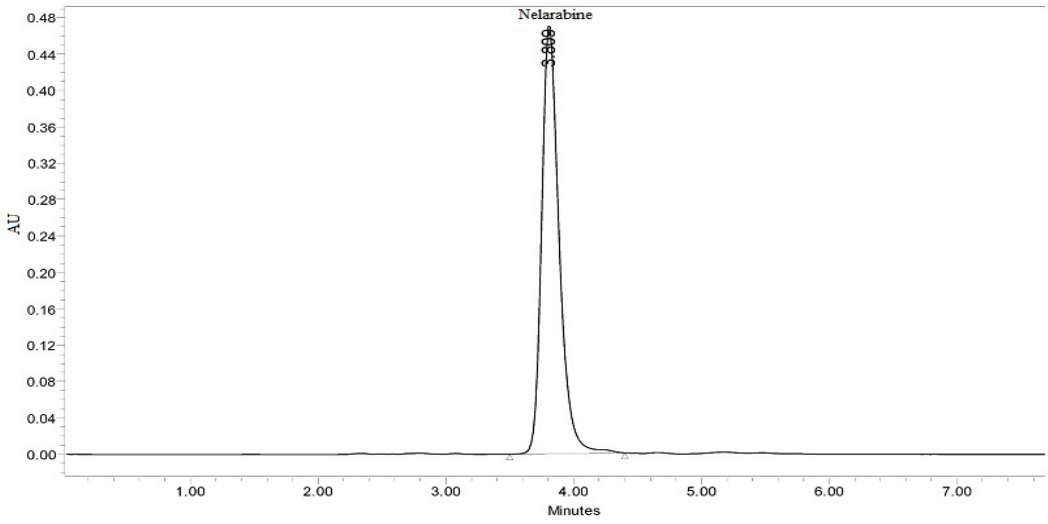


Fig.No.4.26. HPLC chromatogram for accuracy 100% (prep-1)

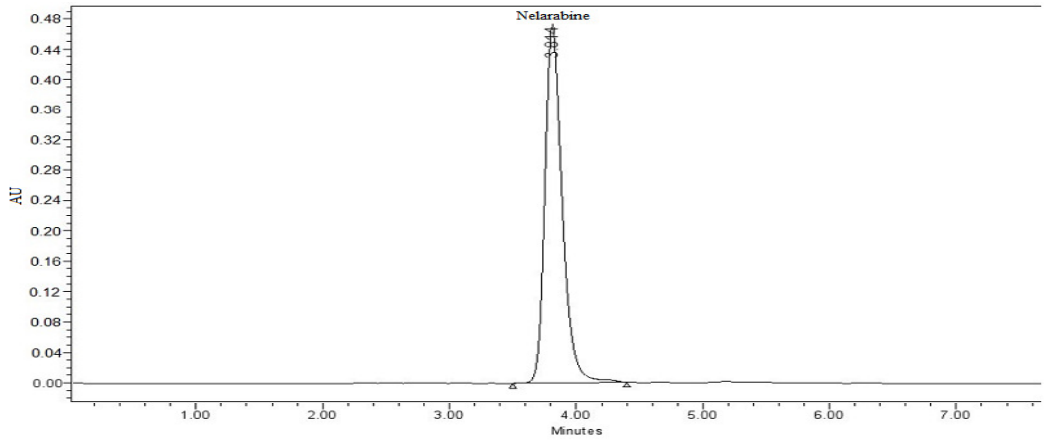


Fig.No.4.27. HPLC chromatogram for accuracy 100% (prep2)

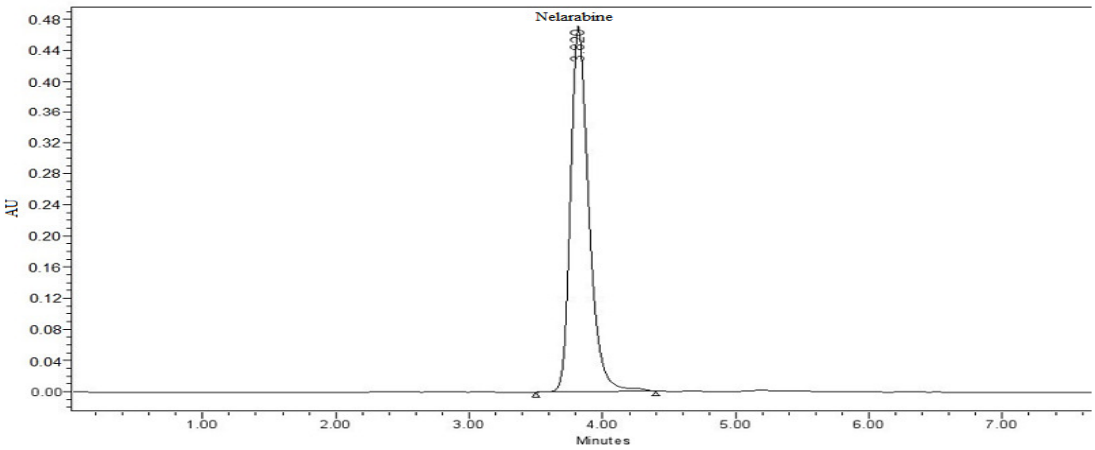


Fig.No.4.28. HPLC chromatogram for accuracy 100% (prep3)

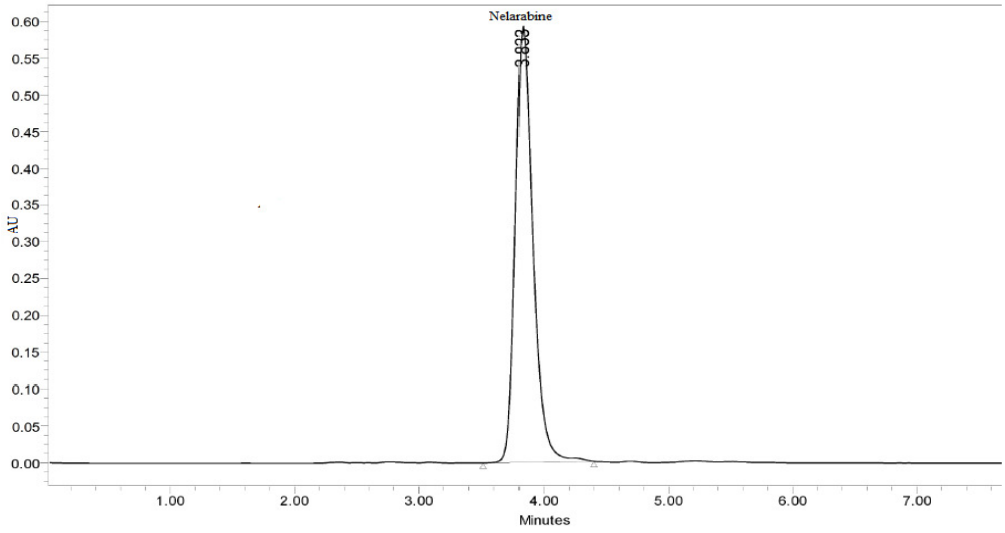


Fig.No.4.29. HPLC chromatogram for accuracy 150% (prep-1)

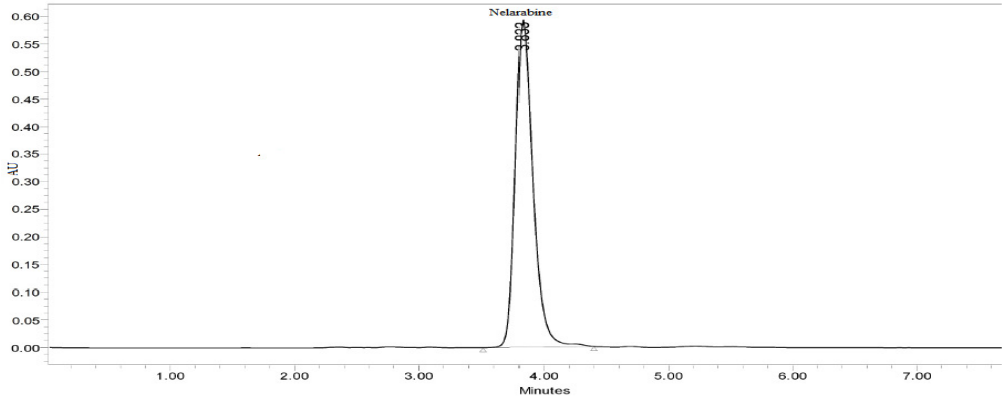


Fig.No.4.30. HPLC chromatogram for accuracy 150% (prep-2)

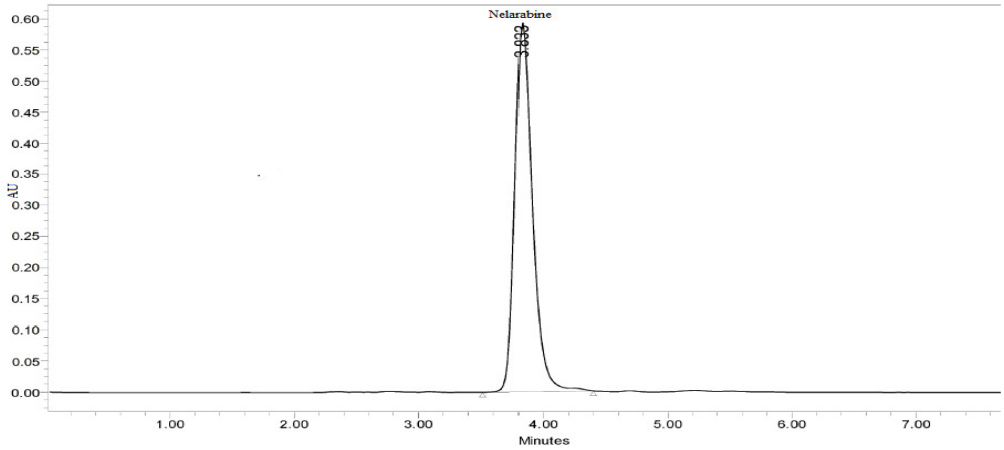


Fig.No.4.31. HPLC chromatogram for accuracy 150% (prep-3)

Discussion: The percentage recovery of Nelarabine was found to be 100.4%, 100.12% and 98.72% for accuracy 50%, 100% and 150% samples respectively. The percentage recovery was within 98% -102%. The %RSD of the samples was found to be less than 2.

4.3.5. Limit of detection (LOD)

LOD is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated under the stated experimental conditions. LOD was calculated by using standard deviation and slope values obtained from calibration curve. The chromatogram was shown in fig.4.32.

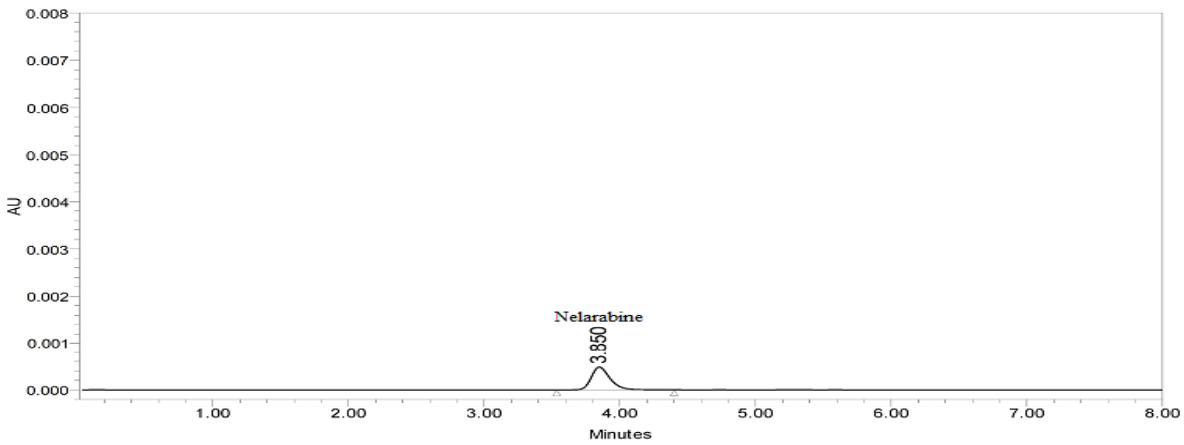


Fig.No.4.32. LOD chromatogram

Table.No.4.6.LOD results of Nelarabine

	RT	Name	Area	Height (µV)
1	3.850		1388	148

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank = 48 µV
Signal Obtained from LOD solution = 148 µV
S/N = 148/48 = 3.08

Discussion: The S/N ratio was found to be within limits and it was calculated as 3.08**:**1.

4.3.6 Limit of quantification (LOQ)

LOQ is the lowest amount of analyte in a sample, which can be quantitatively determined with acceptable accuracy and precision. LOQ was calculated by using standard deviation and slope values obtained from calibration curve. The chromatogram was shown in fig.No.4.33.

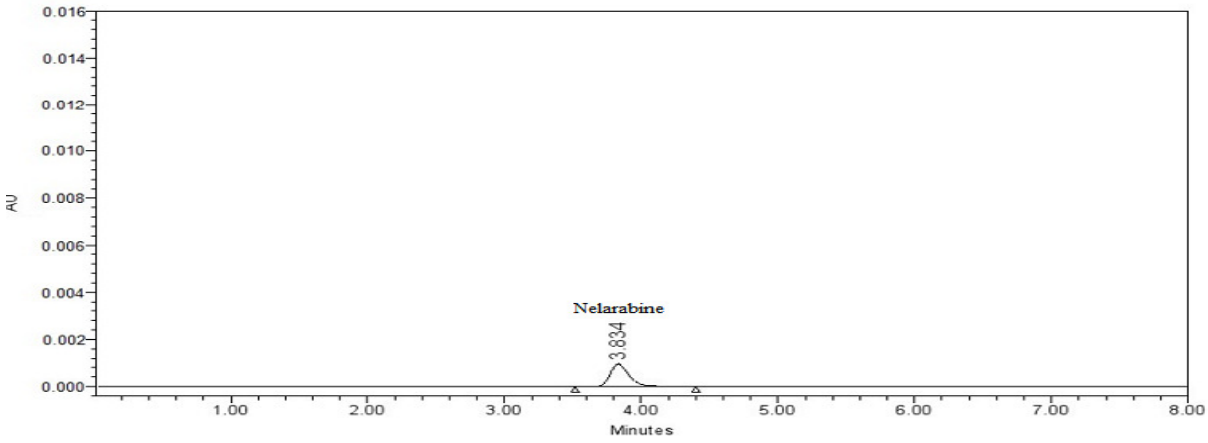


Fig.No.4.33.LOQ chromatogram

Table.No.4.7.LOQ results of Nelarabine

	RT	Name	Area	Height (µV)
1	3.834		4491	479

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank = 48 µV
Signal Obtained from LOQ solution = 479µV
S/N = 479/48 = 9.41

Discussion: The S/N ratio was found to be within limits and it was calculated as 9.41**:**1.

4.3.5. Robustness

Robustness of the method was investigated under a variety of conditions including changes in the composition of the mobile phase, column temperature and flow rate.% RSD of assay was calculated for each condition.

In order to demonstrate the robustness of the method, the following optimized conditions were slightly varied.

- 5% in mobile phase composition,
- 0.1 ml/min flow rate,

The degree of reproducibility of the results obtained as a result of the above small deliberate variations in the method parameters has proven that the method is robust.

a) Influence of Flow Variation

The robustness of the method was demonstrated by changing the flow rate to +/- 0.1mL/min of specified flow rate (1mL/min). By injecting the replicate injections of standard solution at a flow rate of 0.9mL/min and 1.1mL/min, it was found that the system suitability parameters were passed. The %RSD of peak area, tailing factor and theoretical plates were found to be within the limits. The chromatograms were shown in fig.No.4.34-4.35 and results were tabulated in table.No.4.9.

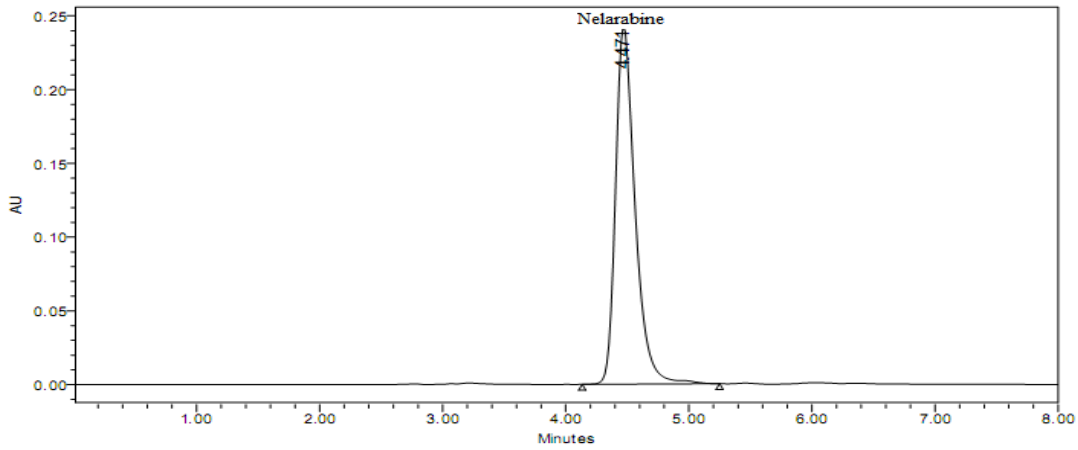


Fig.No.4.34. HPLC chromatogram with 0.9ml/min flowrate

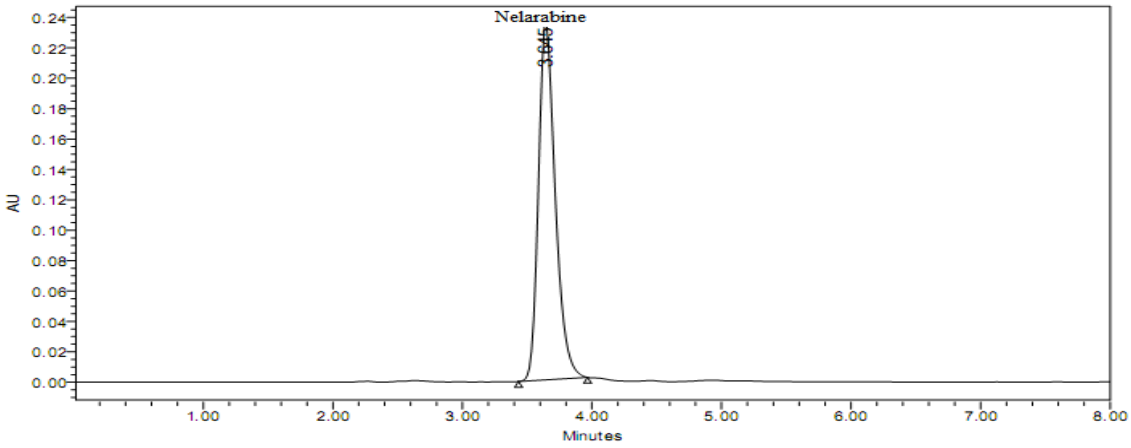


Fig.No.4.35. HPLC chromatogram with 1.1ml/min flowrate

Table.No.4.9. Influence of Flow Variation

Parameter	0.9mL/min		1.1mL/min	
Retention time	4.47		3.645	
Area	2680365	%RSD	2122468	%RSD
	2665296	0.98	2098529	0.86
Theoretical plates	4046.14		3818.45	

b) Influence on variation in Mobile phase composition:

The robustness of the method was demonstrated by changing the organic solvent ratio by +/-5%. By injecting the replicate injections of standard solution with 5% change in organic phase ratio, it was found that the system suitability parameters were passed. The %RSD of peak area, tailing factor and theoretical plates were found to be within the limits. The chromatograms were shown in fig.No.4.36-4.37 and results were tabulated in table.No.4.10.

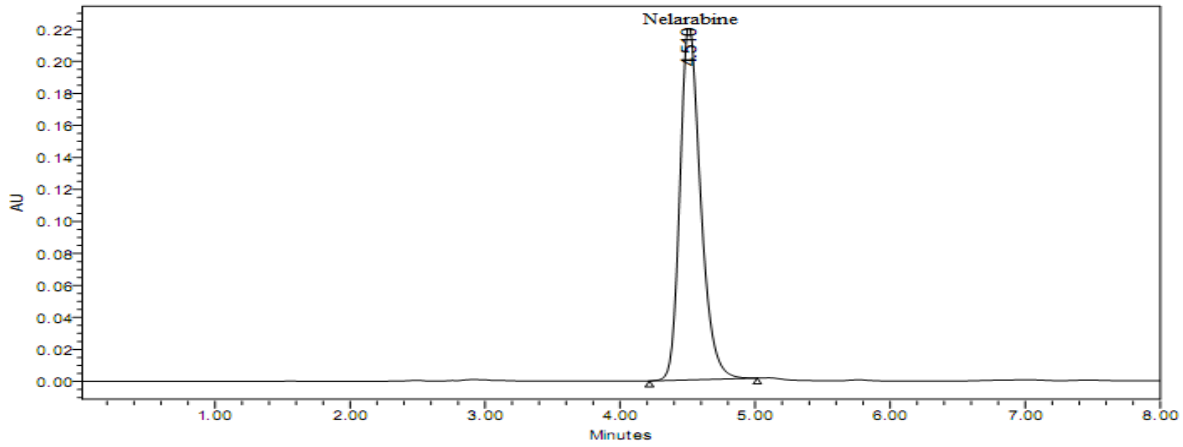


Fig.No.4.36. HPLC chromatogram with 5% more organic solvent

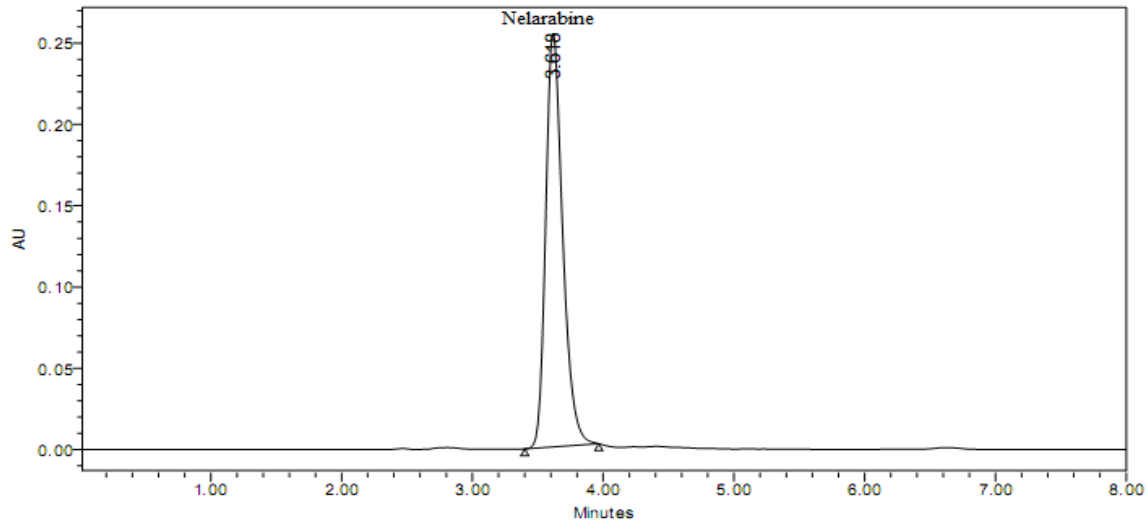


Fig.No.4.37. HPLC chromatogram with 5% less organic solvent

Table.No.4.10. Influence on variation in Mobile phase composition

Parameter	5% more organic solvent		5% less organic solvent	
Retention time	4.510		3.618	
Area	2360411	%RSD	2300176	%RSD
	2019654	1.02	2271134	0.99
Theoretical plates	4270.41		3809.67	

Discussion:

The robustness was tested by changing the flow rate, mobile phase composition It was found that the system suitability parameters were within the acceptance criteria. And the %RSD was found to be within limits (i.e., less than 2).

4.4. Forced degradation studies

Intentional degradation was attempted to stress conditions of acidic, basic/alkali, oxidative degradation and thermal treatment to evaluate the ability of the proposed method to separate Nelarabine from its degradation products.

4.4.1. Acid degradation

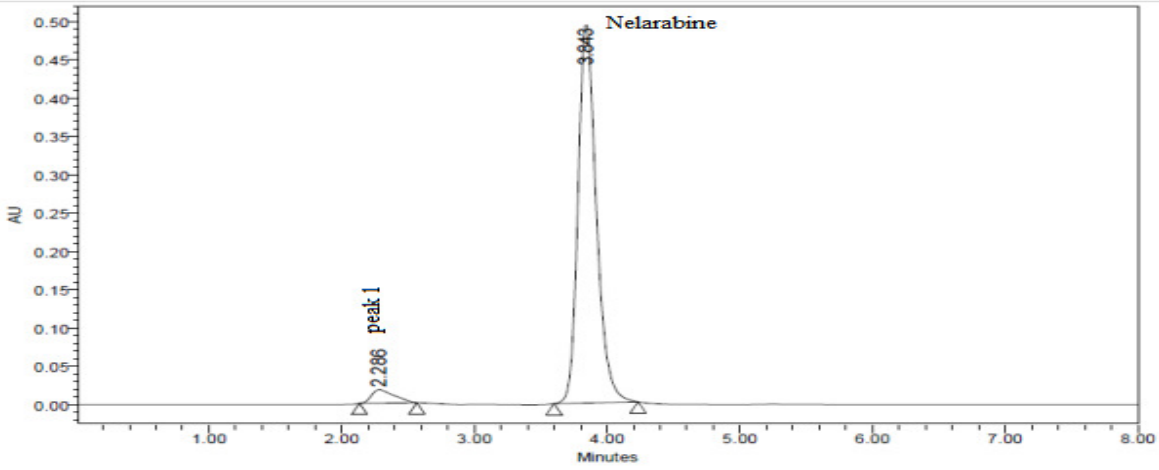


Fig.No.4.38.Chromatogram of acid degradation

S.no.	Peak name	RT	Area	%Area	USP plate count	Tailing factor
1	Peak 1	2.286	207808	4.21	653.39	1.49
2	Nelarabine	3.843	4736871	95.79	3839.22	1.24

4.4.2. Alkali degradation

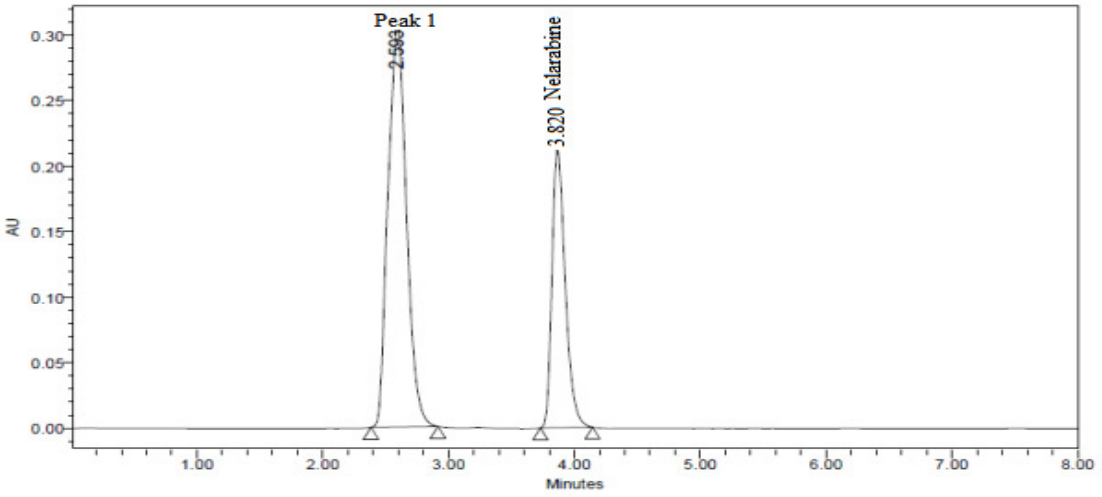


Fig.No.4.39.Chromatogram of alkali degradation

S.No	Peak name	RT	Area	%Area	USP plate count	Tailing facor
1	Peak_1	2.593	1162327	69.17	590	1.75
2	Nelarabine	3.820	517916	30.38	1237.71	1.15

4.4.3. Oxidative degradation

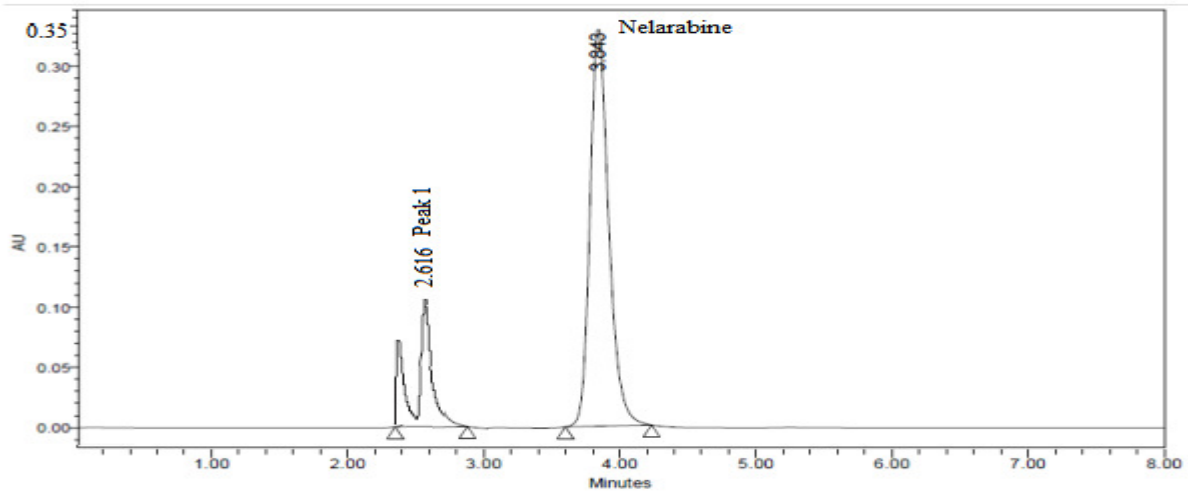


Fig.No.4.40.Chromatogram of oxidative degradation

S.no.	Peak name	RT	Area	%Area	USP plate Count	Tailing factor
1	Peak 1	2.616	2996989	33.41	588.41	1.09
2	Nelarabine	3.841	7972363	66.59	4272.27	1.21

4.4.4 Thermal degradation

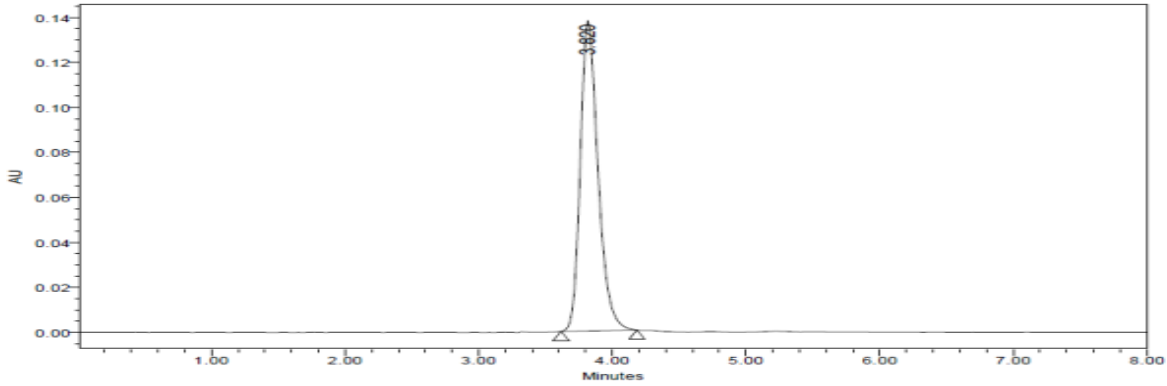


Fig.No.4.41.Chromatogram of Thermal degradation

S.no.	Peak name	RT	Area	%Area	USP plate count	Tailing factor
1	Nelarabine	3.820	1271327	100	3990.08	1.27

4.4.5. Photolytic degradation

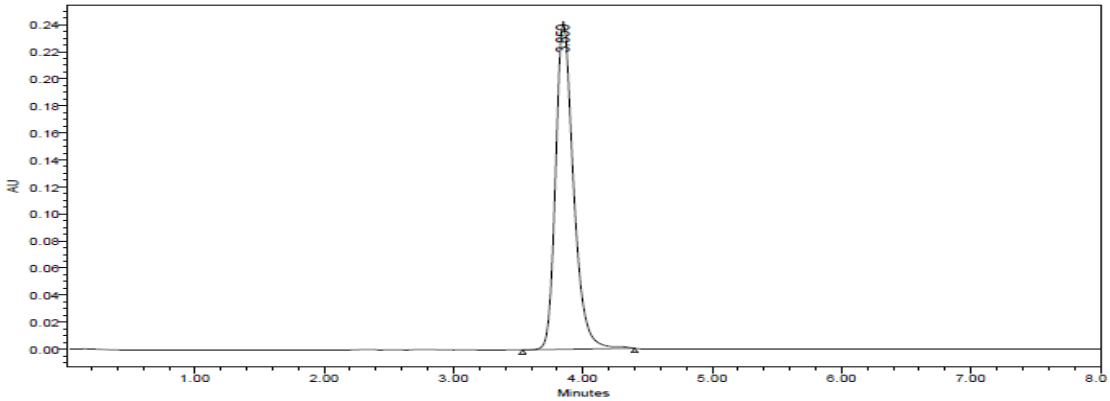


Fig.No.4.42.Chromatogram of Photolytic degradation

S.no.	Peak name	RT	Area	%Area	USP plate count	Tailing factor
1	Nelarabine	3.850	2371327	100	39985.1	1.29

Table.No.4.11. Forced degradation summary of Nelarabine

Degradation type	**Experimental condition**	**Storage condition**	**Interpretation**
Hydrolysis	Acid degradation (1.5 N HCL)	Refluxed at 60°c for 2hrs	No peak interference
	Base degradation (1.5 N NaOH)		No peak interference
Oxidative	30% peroxide	Refluxed at 40°c for 2hrs	No peak Interference
Thermal	Heat chamber	80°c for 2hrs	No peak Interference
Photolytic	UV lamp	254nm for 2hrs	No peak Interference

Conclusion: Forced degradation studies of Nelarabine were performed at different stress conditions. The HPLC chromatograms of degraded products showed no interference at the analyte peaks, hence the method was stability indicating. It was also concluded that Nelarabine is more unstable at alkaline pH.

5. SUMMARY

A new method has been developed for the estimation of Nelarabine by RP-HPLC method since it is a versatile tool for the qualitative and quantitative analysis of drugs and pharmaceuticals.

The chromatographic conditions were successfully developed for the separation of Nelarabine using Cosmicsil Adze C-18 (150×4.6mm), 5µm column, at a flow rate of 1.0mL/min, detection wave length of 266nm. The mobile Phase optimized was 0.01%Trifluoro acetic acid and Acetonitrile in the ratio of 85:15% v/v. The instrument used was WATERS HPLC auto sampler. The retention times was found to be 3.83 mins.

The developed method was validated in accordance with the ICH guidelines. The results obtained were within the limits.

- Correlation coefficient value for calibration plot of Nelarabine was 0.999 and the regression equation was found to be Y = 104241x.
- The %RSD for precision on replicate injection and intermediate precision was 0.88 and 0.82 respectively which indicates that the method was precise, robust and repeatable.
- The % Recovery was within limits (98%-102%) indicating that the proposed method was highly accurate.
- LOD value was 3.08 and LOQ value was 9.41, which indicates the sensitivity of the method.

Forced degradation studies of Nelarabine were performed at different stress conditions. The HPLC chromatograms of degraded products showed no interference at the analyte peaks, hence the method was stability indicating. It was also concluded that Nelarabine is more unstable at alkaline pH.

6. CONCLUSION

A simple, precise, robust, economical and accurate stability indicating RP-HPLC method was developed for the estimation of Nelarabine which can be used for the routine analysis of Nelarabine in API and Pharmaceutical dosage form.

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