Section A-Research paper
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"METHOD DEVELOPMENT AND VALIDATION OF DAUNORUBICIN BY RP- HPLC"

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ABSTRACT

Daunorubicin is an anthracycline antibiotic it used to treat cancer. A simple, sensitive and specific reverse phase high performance liquid chromatographic (RP HPLC) method for the determination of Daunorubicin was developed and validated by using Cosmosil C18 column

(250 mm x 4.6 mm, 5pm) using a mixture of Water(0.1% OPA) and Acetonitrile in the ratio 05:95 (v/v) as a mobile phase This was found to give sharp peak of Daunorubicin at a retention time of 4.3167 min. RP HPLC analysis of Daunorubicin was carried out Detection of wavelength by UV detector at 230 nm with a flow rate of 1ml/min. The applied RP HPLC method allowed Daunorubicin with good linearity (r2 = 0.999) in the studied concentration range. Limit of detection and limit of quantification were found to be 0.21µg/ml and 0.66µg/ml respectively. The method was validated as per the International Conference on Harmonization (ICH) guidelines. The developed method was employed with a high degree of precision and accuracy limit of quantification, limit of detection. The method was validated for accuracy, precision, robustness. The precision, accuracy, repeatability, short retention time and composition of the mobile phase indicated that this method is better for the /11 15:13 quantification of Daunorubicin,

Keywords: Daunorubicin, RP-HPLC Method Development, Validation

INTRODUCTION

Analytical chemistry is a branch of chemistry that deals with the separation, identification and determination of components in a sample. It is the science of making quantitative measurements, which requires background knowledge of chemical and physical concepts of chemistry.[1] Analytical chemistry may be defined as the "Science and art of determining the composition of materials in terms of the elements or compounds contained". Pharmaceutical analysis [2] plays a major role today, and it can be considered as an interdisciplinary subject. Pharmaceutical analysis derives its principles from various branches of science like Chemistry, Physics, Microbiology, Nuclear Science, Electronics, etc. Analytical method is a specific application of a technique to solve an analytical problem. Analytical instrumentation plays an important role in the production and evaluation of new products and in the protection of consumers and the environment. This instrumentation provides the lower detection limits required to assure safe foods, drugs, water and air, generally used for drug analysis are spectral methods, chromatographic methods, electro analytical techniques, and miscellaneous techniques like conventional titrimetric, gravimetric and polarimetric methods.

1. PHARMACEUTICAL ANALYSIS TECHNIQUES ARE APPLIED MAINLY IN TWO AREA[3]

Traditionally, analytical chemistry has been split into two main types, qualitative and Quantitative:

1.1. Qualitative

Qualitative analysis seeks to establish the presence of a given element or compound in a sample.

1.1.2 Quantitative

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

1.2 SPECIFIC TECHNOLOGIES AND INSTRUMENTATION[4]

1.2.1 Spectrometric techniques

- Ultraviolet and visible Spectrophotometry
- Fluorescence and phosphorescence Spectrophotometry
- Atomic Spectrometry (emission and absorption)
- Infrared Spectrophotometry
- Raman Spectroscopy
- X-Ray Spectroscopy
- Radiochemical Techniques including activation analysis
- Nuclear Magnetic Resonance Spectroscopy
- Electron Spin Resonance Spectroscopy

1.2.2 Electrochemical Techniques

- Potentiometry
- Voltametry
- Voltametric Techniques
- Amperometric Techniques
- Colorimetry
- Electrogravimetry
- Conductance Techniques

1.2.3 Chromatographic Techniques

- Gas Chromatography
- High performance Liquid Chromatography

- Thin Layer Chromatography
- Ultra performance Liquid Chromatography

1.2.4 Miscellaneous Techniques

- Thermal Analysis
- Mass Spectrometry
- Kinetic Techniques

1.3 ANALYTICAL METHOD DEVELOPMENT[5]

Methods are developed for new products when no official methods are available. Alternate methods for existing 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 merit / demerits are made available. Chromatography includes those methods having goal for the separation of different types of components from a mixture based upon their distribution capacity between a stationary and mobile phase. There are several valid reasons for developing new methods of analysis for determination of a compound or drug.

There may not be a suitable method available for a particular analyte in the specific sample matrix. Existing methods may be too error, artifact, and /or contamination prone, or they may be unreliable (having poor accuracy or precision). Existing methods may not provide adequate sensitivity or analyte selectivity in samples of interest. Newer instrumentation and techniques may have evolved that provide opportunities for improved analysis identification or detection limits, greater accuracy or precision, or better return on investment.

1.4 INTRODUCTION OF VALIDATION

1.4.1 Validation [9]

Validation of an analytical method is the process by which it is established, by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical applications. Validation is defined as follows by different agencies

1.4.1.1 Food and Drug administration (FDA)

Establishing documentation evidence, which provides a high degree of assurance that specific process, will consistently produce a product meeting its predetermined specification and quality

attributes.

1.4.1.2 World Health Organization (WHO)

Action of providing that any procedure, process, equipment, material, activity, or system actually leads to the expected results

1.4.1.3 European Committee (EC)

Action of providing in accordance with the principles of good manufacturing practice that any procedure, process, equipment, material, activity or system actually leads to the expected results. In brief validation is a key process for effective Quality Assurance.

1.4.2 Reasons for Validation [10]

There are two important reasons for validating assays in the pharmaceutical industry. The first, and by for the most important, is that assay validation is an integral part of the quality-control system. The second is that current good manufacturing practice regulation requires assay validation.

1.4.3 Steps followed for validation procedures

Proposed protocols or parameters for validations are established.

1. Experimental studies are conducted.
2. Analytical results are evaluated.
3. Statistical evaluation is carried out .
4. Report is prepared documenting all the results.

1.4.4 Objective and Parameters of Analytical Method Validation [11]

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. According to ICH guidelines, typical analytical performance characteristics that should be considered in the validation of the types of methods are

1.4.4.1 Accuracy

1.4.4.2 Precision

1.4.4.3 Specificity

1.4.4.4 Detection Limit

1.4.4.5 Quantization Limit

1.4.4.6 Linearity

1.4.4.7 Range

1.4.4.8 Ruggedness

1.4.4.9 Robustness

1.4.4.1 Accuracy

The accuracy is the closeness of the measured value to the true value for the sample. The ICH documents recommended that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentrations levels the specified range (i.e., three concentrations and three replicates of each concentration) Accuracy was tested (% Recovery and % RSD of individual measurements) by analyzing samples at least in triplicate, at each level (80,100 and 120 % of label claim) is recommended. For each determination fresh samples were prepared and assay value is calculated. Recovery was calculated from regression equation obtained in linearity study. Accuracy was determined from the mean relative error for a set of replicate analysis (i.e. the difference between measured and nominal concentration) for spiked samples.

1.4.4.2 Precision

The precision of an analytical procedure expresses the closeness of agreement between series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision of an analytical method is usually expressed as the standard deviation, relative standard deviation or coefficient of variations of a series of measurements. The ICH documents recommend the repeatability should be assessed using a minimum of nine determinations covering specified range of procedure. Precision may be measure of either the degree of reproducibility or of repeatability of the analytical method under normal operating conditions.

1.4.4.3 Repeatability:

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra –assay precision

1.4.4.3.1 Intermediate Precision: Intermediate precision expresses with in laboratories variations: different days, different analyst and different equipment.

1.4.4.3.2 Reproducibility: When the procedure is carried out by different analyst in different laboratories using different equipment, regents and laboratories setting. Reproducibility was determined by measuring repeatability and intermediate precision. Reproducibility is assessed by means of an inter-laboratory trial. An investigation of specificity should be conducted during the validation of identification tests, the determination of impurities. An ICH document defines

specificity as the ability to assess unequivocally the analytes in the presence compounds that may be expected to products and matrix components. The definition has the following implications.

1.4.4.3.3 Identification test: Suitable identification tests should be able to discriminate compounds of closely related structure which are likely to be present Ensure identity of an analytes .The analytes should have no interference from other extraneous components and be well resolved from them.

1.4.4.3.4 Purity Test: To ensure that all the analytical procedures performed allow an accurate statement of the content of impurity of the content of impurity of an analyte i.e. related substances test, heavy metals, residual solvents etc.

1.4.4.5 Assay: To provide an exact result, this allows an accurate statement on the content or potency of the analyte in a sample

1.4.4.4 Detection Limit [10]

It is the lowest amount of analyte in a sample that can be detected, but not necessarily quantities as an exact value, under the stated experimental conditions. The detection limit is usually expressed as the concentration of analyte (percentage parts per million) in the sample.

1.4.4.4.1 Determination of detection limit

For instrumental and non- instrumental methods detection limit is generally determined by the analysis of samples with known concentration of analyte. Establishing the minimum level at which the analyte can be reliably detected.

LOD = 3 * SD / slope of calibration curve

SD = Standard deviation of intercepts

1.4.4.5 Quantification Limit

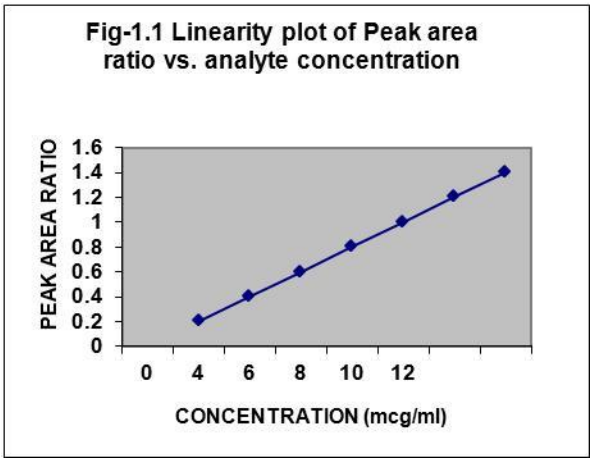
It is the lowest amount of analyte in a sample that can be determined with Acceptable precision and accuracy under the stated experimental conditions. Quantification limit is expressed as the concentration of analyte (e.g. - % ppm) in the sample.

1.7.4.6 Linearity and Range [11]

The linearity of an analytical procedure is its ability to obtain test results that are directly proportional to concentration of analyte in samples. The range of an analytical is the intervals between the upper and lower concentration (amounts) of analyte in the sample for which it has been demonstrated which it has been demonstrated that the analytical procedure has a suitable level of precision accuracy and linearity.

1.4.4.6.1Determination of Linearity and Range

These characteristics are determined by application of the procedure to a series of Samples having analyte concentration spanning the claimed range of procedure. When the relationship between response and concentration is not linear, standardization may be providing by means of a calibration curve. The ICH recommends that for the establishment of linearity a minimum of five concentrations normally used.



1.4.4.7 Ruggedness

Degree of reproducibility of test results obtained by the analysis of the same samples under a variety of condition such as different laboratories, different analysts, different instruments etc., normally expressed as the lack of influence on test results of operational and environmental variable of the analytical method. Ruggedness is a measurement of reproducibility of test results under the variation in condition normally expected from laboratory to laboratory and from analyst to analyst. Degree of representative of test results is then determined as a function of the assay variable.

1.4.4.8 Robustness

Robustness of an analytical method is measure of its capacity to remain unaffected small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Testing varying some or all condition:

-Column temperature

-PH of buffer in mobile phase

-Reagents and flow rate

System Suitability

System suitability tests are based on the concept that the equipment, electronics, analytical operations and samples constitute an integral system that can be evaluated as a whole. According to USP system suitability are an integral part of chromatographic methods. These tests verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. One consequence of the evaluation of robustness

Resolution (R_s)

Ability of a column to separate chromatographic peaks, Resolution can be improved by increasing column length, decreasing particle size, increasing temperature, changing the eluent or stationary phase. It can also be expressed in terms of the separation of the apex of two peaks divided by the tangential width average of the peaks.

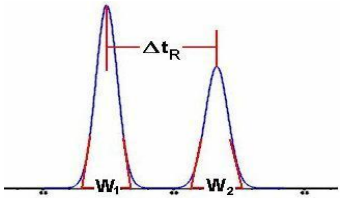


Fig-02: Resolution

$R_s = \Delta t_R / W_1/2 + W_2/2;$

Where $\Delta t_R = t_2 - t_1$

For reliable quantization, well-separated peaks are essential for quantization.

Recommendations

Rs of > 2 between the peak of interest and the closest potential interfering peak (impurity, excipient, degradation product, internal standard, etc.) are desirable.

Statistical procedures and representative calculations [11]

The consistency and suitability of the developed method are substantiated through the statistical analysis like standard deviation, relative standard deviation and theoretical plates per meter.

Relative Standard Deviation = $\square^{/x}i \times 100$

Molar extinction coefficient (mol^{-1} cm^{-1}) = A/C × L

Where, A= Absorbance of drug, C= concentration of drug, L= Path length

Sandell, s sensitivity ($\mu g/cm^2$/0.001 absorbance units) = C/A×0.001

Where, C= concentration of drug, A= Absorbance of drug.

Formula for calculations of related substance:

Calculate the % impurities using following formula

$$\%\ \text{Impurity Known} = \frac{AK \times WS \times 5 \times 1 \times P \times 100}{AS \times 50 \times 10 \times 100 \times LC} \times \frac{\text{weight per ml of the test sample}}{RRF}$$

$$\%\ \text{Impurity Unknown} = \frac{AU \times WS \times 5 \times 1 \times P \times 100}{AS \times 50 \times 50 \times 10 \times 100 \times LC} \times \text{weight per ml of the test sample}$$

Where,

AK : Area of the known impurity obtained in test chromatogram

AU : Area of the known impurity obtained in test chromatogram

AS : Average area of Bimatoprost peak in standard chromatogram

WS : Weight of Bimatoprost working standard taken in mg

P : Potency of Bimatoprost working standard

LC : Labeled claim in mg per ml

RRF : Relative response factor

HPLC Method Validation[13]

Everyday many chromatographers face the need to develop a HPLC separation whereas individual approaches may exhibit considerable diversity; method development often follows the series of steps summarized in the following fig:

Table. NO.1.1: Steps involved in HPLC method validation [1]

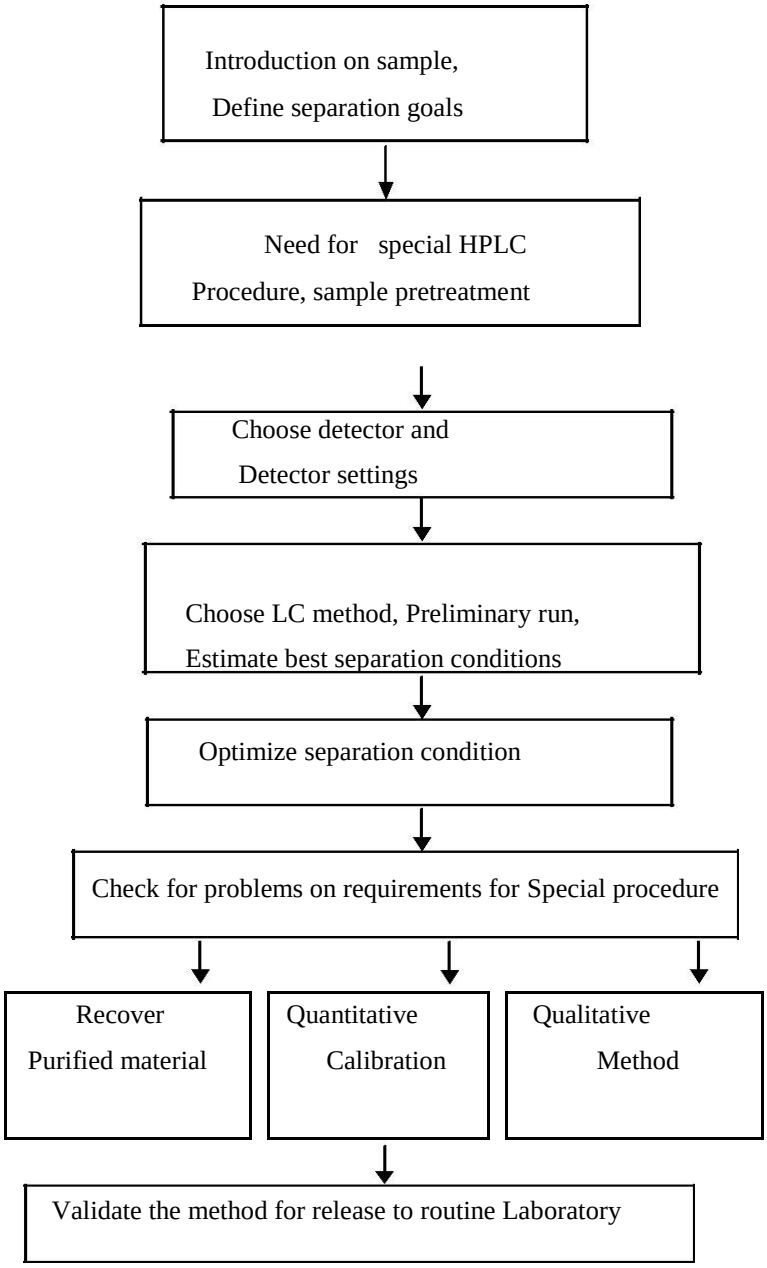


Table No.1.2: System Suitability Parameters and their recommended limits[13]

Parameter	Recommendation
Capacity Factor (K')	The peak should be well-resolved from other peaks and the void volume, generally K' > 2
Repeatability	RSD ≤ 1% N ≥ 5 is desirable
Relative Retention	Not essential as the resolution is stated.
Resolution(R_s)	R_s of > 2 between the peak of interest and the closest eluting potential interferent
Tailing Factor(T)	T ≤ 2
Theoretical Plates(N)	In general should be > 2000.

DRUG PROFILE

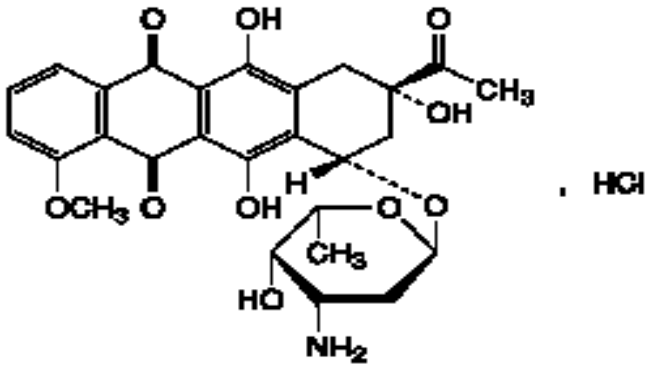
2 .1Daunorubicin:

2.1.1 Molecular Formula: $C_{27}H_{30}ClNO_{10}$

2.1.2 Molecular Weight: 564.0

Daunorubicin (as hydrochloride) 20 mg/10 mL injection

The structural formula is presented below:



2.1.3 Description:

Daunorubicin hydrochloride occurs as a hygroscopic, crystalline, orange-red powder, freely soluble in water and in methanol, slightly soluble in alcohol and practically insoluble in acetone.

Daunorubicin Injection is a sterile, isotonic, preservative free solution containing daunorubicin hydrochloride 2.14 mg/mL (equivalent to 2 mg/mL daunorubicin) and sodium chloride in Water for Injections.

2.1.4 Mechanism of Action:

Daunorubicin is an antineoplastic antibiotic which is structurally related to doxorubicin. The drug appears to act by inhibiting DNA and DNA-dependent RNA synthesis by forming a complex with DNA with intercalation between base pairs and uncoiling of the helix. Daunorubicin may also inhibit polymerase activity, affect regulation of gene expression, and be involved in free radical damage to DNA.

2.1.5 Pharmacology:

Class of Drug: Cytotoxic anthracycline antibiotic.

2.1.5.1 Pharmacodynamics:

Daunorubicin is an antineoplastic antibiotic which is structurally related to doxorubicin. The drug appears to act by inhibiting DNA and DNA-dependent RNA synthesis by forming a complex with DNA with intercalation between base pairs and uncoiling of the helix. Daunorubicin may also inhibit polymerase activity, affect regulation of gene expression, and be involved in free radical damage to DNA. The drug is not cell cycle-phase specific although maximum cytotoxic activity occurs in the S phase. Daunorubicin also has antibacterial and immunosuppressive properties.

2.1.5.2 Pharmacokinetics:

2.1.5.2.1 Distribution

Daunorubicin is rapidly and widely distributed in tissues, with highest levels in the heart, kidneys, liver, lungs and spleen. It binds inside the cells to cellular components, mainly nucleic acids. Daunorubicin does not cross the blood-brain barrier but appears to cross the placenta. It is not known if daunorubicin is present in breast milk.

2.1.5.2.2 Metabolism

Daunorubicin is extensively metabolized in the liver and other tissues, mainly by cytoplasmic aldo-keto reductases, producing daunorubicinol, the major metabolite, which has antineoplastic activity. Approximately 40% of the drug in the plasma is present as daunorubicinol within 30 minutes and 60% in 4 hours after a dose of daunorubicin. Additional metabolism by reductive cleavage of the glycosidic bond produces aglycones, which have little or no cytotoxic activity and are demethylated and conjugated with sulphate and glucuronide by microsomal enzymes. Daunorubicin metabolism may be altered in patients with impaired hepatic function.

2.1.5.2.3 Elimination

Following rapid IV administration, total plasma concentrations of daunorubicin and its metabolites decline in a triphasic manner and plasma concentrations of unchanged daunorubicin decline in a biphasic manner. The plasma half-life of daunorubicin averages 45 minutes in the initial phase and 18.5 hours in the terminal phase. By 1 hour after administration of daunorubicin, the predominant form of the drug in plasma is the metabolite daunorubicinol, which has as average terminal plasma half-life of 26.7 hours. Daunorubicin and its metabolites are excreted in the urine and bile, with urinary excretion accounting for 14-23% of the dose. Most urinary excretion of daunorubicin occurs within 3 days. After the first 24 hours, the drug is excreted in urine mainly as daunorubicinol. An estimated 40%of a dose is eliminated by biliary excretion.

2.1.6 Indications:

Daunorubicin Injection is indicated for the treatment of the following:

• Acute lymphocytic (lymphoblastic) leukemia: Daunorubicin is usually reserved for use in cases shown to be resistant to other drugs. However, combined treatment with daunorubicin, vincristine and a steroid has been used in the early stages of this disease.

• Acute myeloblastic leukemia: Daunorubicin has been used in all stages, alone or in combination with other cytotoxic agents (e.g. cytarabine).

• Disseminated solid tumors: Daunorubicin has been investigated for use in these tumors and found to be effective in some cases of disseminated neuroblastoma and rhabdomyosarcoma.

2.1.6.1 Contraindications:

Daunorubicin Injection is contraindicated in:

• Patients with persistent myelosuppression; or marked myelosuppression induced by previous treatment with other cytotoxic agents or radiotherapy.

• Patients with impaired cardiac function. (Including myocardial insufficiency, recent myocardial infarction and severe arrhythmias).

• Patients who have previously received the full cumulative dose of daunorubicin and/or doxorubicin. And/or other anthracyclines and/or anthracenediones (see Precautions, Cardiac Toxicity).

• Patients with known hypersensitivity to daunorubicin. or any other component of the product, other anthracyclines, or anthracenediones.

• Patients with severe infections.

• Patients with severe hepatic (Child-Pugh Grade C [total score 10-15]) or renal function impairment (GFR <10 mL/min or serum creatinine >7.9 mg/dL).

• Patients who are pregnant.

2.1.6.2 Warnings and precautions:

Daunorubicin should be administered only under the supervision of a physician who is experienced in the use of cancer chemotherapeutic agents**.** Initial treatment with daunorubicin requires close observation of the patient and extensive laboratory monitoring. It is recommended, therefore, that patients be hospitalized at least during the first phase of treatment. Blood counts and monitoring of parameters of renal and liver function should be performed prior to each treatment with daunorubicin. Administration of myelosuppressive drugs such as daunorubicin may lead to an increased frequency of infections and hemorrhagic complications. These

complications are potentially fatal therefore patients should be instructed to notify the physician if fever, sore throat, or unusual bruising or bleeding occurs. Patients should recover from acute toxicities of prior cytotoxic treatment (such as stomatitis, neutropenia, thrombocytopenia, and generalized infections) before beginning treatment with Daunorubicin.

EXPERIMENTAL WORK

3.1. Preliminary Analysis of Drug

Daunorubicin are official in Pharmacopoeia, 2010.Hence, Preliminary analysis of Daunorubicin were performed according to IP, 2010

3.1.1. Description

The sample of Daunorubicin was observed for its colour.

3.1.2. Solubility

The sample of Daunorubicin was taken in test tubes and observed for solubility in various solvents like water, methanol, alcohol, chloroform.

3.1.3. Identification Test

By Infrared spectroscopy:

Standard Preparation: Weigh accurately about 2mg of Daunorubicin WS, finely grind and disperse in 300 mg of potassium bromide. Compress the powder between two suitable cells.

Test Preparation: Weigh accurately about 2mg of substance being examined, finely grind and disperse in 300 mg of potassium bromide. Compress the powder between two suitable cells.

Procedure: Record the spectra of the test preparation and standard preparation. The infrared spectrum of test should concordant with the infrared spectrum of standard spectrum of Daunorubicin.**(Fig no.1.1)**

3.1.4. PH

Procedure: Weigh accurately about of 10mg Daunorubicin drug dissolved in 10 ml of water for injection**.**

Control limit: Between 4.5 and 6.5

Instruments Used

Electronic Weighing Balance (Sartorius – TE 214 S)

Lab India Picu pH meter (version 2.13)

Shimadzu IRAffinity-1s (model FTIR-8400s)

3.2 HPLC:

3.2.1 Selection of Analytical Technique

HPLC was selected as analytical technique for estimation of Daunorubicin

Instruments:

The analysis of the drug was carried out on Youngline (S.K.) Gradient System UV Detector. Equiped with Reverse Phase (Cosmosil) C18 column (4.6mm x 250mm; 5µm), a SP930D pump, a 20µl injection loop and UV730D Absorbance detector and running autochro-3000 software.

a) Selection of stationary phase:

The column used in this method C_{18} Cosmosil the configuration of the column is 4.6 x 250 mm, particle size 5 µm. C18 column gives high non polar retentivity, symmetric peak shape, highly reproducible and stable ideal for HPLC method.

b) Solubility Studies:

This study was carried out to find an ideal solvent in which drugs are completely soluble. Various solvents were tried for checking solubility of Daunorubicin From solubility studies it was concluded that of Daunorubicin is poorly soluble in Methanol and water however it is soluble in Water PH adjusted 0.1%Orthophosphoric Acid, Buffer pH 2.7.

c) Chromatographic conditions:

The following chromatographic conditions were established by trial and error and Were kept constant throughout the experimentation.

Table No. 1.3: Chromatographic conditions (HPLC) details used during

1	HPLC	Younglin (S.K)Gradient System UV Detector
2	Software	Autochro -3000
3	Column	(Cosmosil)) C18 column (4.6mm x 250mm)
4	Particle size packing	5 µm

5	Stationary phase	C18 (Cosmosil)
6	Mobile Phase	Acetonitrile: Water(0.1% OPA) 95 : 5
7	Detection Wavelength	230 nm
8	Flow rate	1.0 ml/min
9	Temperature	Ambient
10	Sample size	20 µl
11	pH	2.7
12	Run Time	15min
13	Filter paper	0.45 µm

Method Development.

3.2.2 UV-VIS Spectrophotometer: UV-VIS Spectrophotometer was selected as analytical technique for estimation of Daunorubicin. UV absorbance range of 200-400nm.

Instrument:

Analytical Technologies® Limited UV-VIS Spectrophotometer is double beam, high Speed scanning spectrophotometer, The instrument needs about 1minute for initialization. The light source used is Deuterium lamp of spectrophotometer, a computer is attached which helps in data processing and manipulation Quartz cuvette with path length 1cm was used.

Study on the selection of UV spectrum use in uv-vis spectrometer of Daunorubicin:

Accurately weigh and transfer 10mg Daunorubicin working standard into 10 ml volumetric flask as about dilute Methanol prepared in completely and make volume up to the mark with the same solvent to get 1000µg/ml standard (stock solution) and 15 min sonicate to dissolve it and from the resulting solution 0.5ml was transferred to 10 ml volumetric flask and the volume was made up to the mark with Water**. (Fig No: 1.2)**

3.2.3 Study on the chromatographic conditions of Daunorubicin:

Accurately weigh and transfer 10mg Daunorubicin working standard into 10 ml volumetric flask as about dilute Methanol prepared in completely and make volume up to the mark with the same solvent to get 1000µg/ml standard (stock solution) and 15 min sonicate to dissolve it and from the resulting solution 0.1ml was transferred to 10 ml volumetric flask and the volume was made up to the mark with mobile phase Acetonitrile: water (0.1%OPA) solvent. The resulting 10µg/ml of solution was subjected to chromatographic analyses using mobile phases of different strengths with chromatographic conditions mentioned below:

- Analytical column : Cosmosil C18 Column (250mm x 4.6mm)
- partical size : 5µm
- Injection volume : 20µl
- Flow rate : 1 ml/min
- Detection : 230 nm
- Run Time : 15 mi

3.3 Method Development of HPLC:

Following Mobile phase were tried:

3.3.1 List of Mobile Phase:

Table no.1.4 List of Mobile Phases

Sr.No.	Mobile Phase
1	Methanol + 0.1% (OPA)Water ,(90+10% v/v)
2	Methanol+0.1%(OPA)Water, (80+20% v/v)
3	Methanol (100% v/v)
4	Acetonitrile+0.1% (OPA)Water(95+05%v/v)

3.3.2 Preparation of Stock Standard Solution :

Standard Solution Stock I : (Daunorubicin) Accurately weight and transfer 10mg Daunorubicin working standard into 10 ml volumetric flask as about diluents Methanol completely and make volume up to the mark with the same solvent to get 1000µg/ml standard (stock solution) and 15 min sonicate to dissolve it and the resulting stock solution 0.1ml was

transferred to 10 ml volumetric flask and the volume was made up to the mark with mobile phase Acetonitrile : Water(0.1%OPA)Water, prepared in (95mlACN : 5ml WATER v/v)solvent .Result as shown as; **(Figure No.1.7),(Table No : 1.4)**

3.3.3 Validation

The developed method was validated as per ICH guidelines.

3.3.4. Analytical Method validation

Analytical method validation was carried out as per ICH method validation guidelines Q2 (R1).

3.4 Linearity:

Linearity of an analytical method is its ability to elicit test results that are directly or by a well defined mathematical transformation, proportional to the concentration of analyte in samples within a given range, The Result are shown in; **(Table No 1.5)**

3.4.1. Determination:

The linearity of the analytical method is determined by mathematical treatment of test results obtained by analysis of samples with analyte concentrations across the claimed range. Area is plotted graphically as a function of analyte concentration. Percentage curve fittings are calculated**.** The Result are shown in ;**(Table No.1.7 and Table No. 1.8) ;(Fig No.7.8 and Fig No. 7.9)**

3.4.2 Preparation of standard stock solution for linearity:

Average weight of vial sample (equivalent to 10 mg of **Daunorubicin** were weighed and transferred to 10 ml volumetric flask & diluents was added to make up the volume. Sonicated for 10 min with occasional swirling. 0.1 ml of this solution diluted up to 10 ml volumetric flask with diluents was added to make up the volume.

Table no.1.5 Preparation of standard stock solution for linearity

Sr. no.	Concentration (µg/ml) Daunorubicin
1	10
2	20
3	30
4	40
5	50

3.4.3 Precision:

Precision of an analytical method is the degree of agreement among Individual test results when the procedure is applied repeatedly to multiple Samplings of a homogenous sample. Precision of an analytical method is usually expressed as standard deviation or relative standard deviation. Also, the results obtained were subjected to one way ANOVA and within-day mean square and between-day mean square was determined and compared using F-test. **(Fig No: 7.10)**

3.4.4 Intra-day precision:

Sample solutions containing 10 mg of Daunorubicin three different concentration (10µg/ml, 30µg/ml, 50µg/ml) Daunorubicin were analyzed three times on the same day and %R.S.D was calculated. The Result is shown in; **(Table No.7.9) & (Fig No: 7.10)**

3.4.4..1 Inter-day precision:

Sample solutions containing 10 mg of Daunorubicin three different concentration (10µg/ml, 30µg/ml, 50µg/ml) Daunorubicin were analyzed three times on the next day and %R.S.D was calculated. The Result are shown in ;**(Table No.7.9) & (Fig No: 7.11)**

3.4.4.2 Repeatability:

Precision of the system was determined with the sample. Six replicates of sample solution containing 10mg of Daunorubicin were injected and peak areas were measured and %RSD was calculated. is was repeated for five times :result are shown in;**(Table No : 7.10)& (Fig No :7.12,7.13)**

3.4.4.3 Application of proposed method for analysis of IV for inj. formulation:

Average weight of lyophilized vial sample (equivalent to 10 mg of Daunorubicin were weighed and transferred to 10ml volumetric flask & diluents (MEOH) was added to make up the volume. Sonicated for 10 min with occasional swirling. The above solution was filtered through 0.45µm membrane filter 0.1 ml of this solution diluted up to 10 ml with diluents. (MEOH)

3.4.4.4 Accuracy (recovery):

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. Accuracy may often the expressed as percent recovery by the assay of known added amounts of analyte. The accuracy of an analytical method is determined by applying the

method to analyzed samples, to which known amounts of analyte have been added. The accuracy is calculated from the test results as the

Percentage of analyte recovered by the assay, The Result is shown in; **(Table No: 7.11, 7.12)**

Preparation of standard stock solution:

10 mg of Daunorubicin working standards were weighed and transferred to 10 mL volumetric flask & diluents (MEOH) was added to make up the volume 0.3 ml of this solution diluted up to 10 ml with diluents (MEOH)

3.5 Analysis of marketed formulation (Assay)

To determine the content of Daunorubicin in marketed formulation (20 vials were weighed, and average weight 398 mg was calculated. Powder is equivalent to 10.3 mg of Daunorubicin was weighed and transferred it 10ml volumetric flask and Methanol was added to make up the volume. Sonicated for 15 min. 0.1 ml of this solution diluted upto 10 ml volumetric flask with mobile phase was added to make up the volume. The resulting solution was injected in HPLC and drug peak area was noted**. (Fig No: 7.23)**.

Regression equation was generated using peak areas of standard solutions. Using the regression equation and peak area of the sample the amount of Daunorubicin in the sample was calculated. The amount of Daunorubicin per vial was obtained from the regression equation of the calibration curve as described in analysis of l vial formulation are shown in (**Table No.7.14, 7.15).**

RESULT AND DISCUSSION

4.1. Preliminary Studies on Daunorubicin

4.1.1. Description:

The sample of Daunorubicin was observed for orange red crystalline powder.

4.1.2 Solubility:

The drug was found to be

- Freely soluble in Water and in Methanol
- Slightly soluble in alcohol
- Very slightly soluble in Chloroform

4.1.3 Identification Test:

The infrared spectrum of test should concordant with the infrared spectrum of standard spectrum of Daunorubicin was taken in the range of 500- 2000 nm.

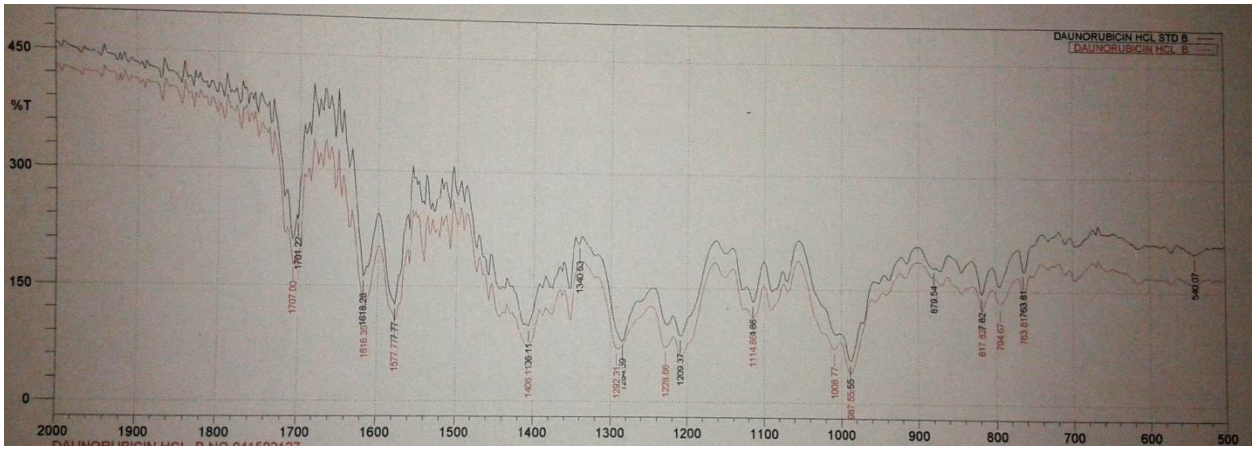


Fig. 1.1 IR of Daunorubicin

The standard and test preparation of Daunorubicin was compaired to each of ranges matched.

4.1.4 PH:

The sample of Daunorubicin observed PH was 5.86

4.1.5 UV- Spectroscopy:

UV absorption of 10 µg/ml solution of Daunorubicin in methanol was generated and absorbance was taken in the range of 200-400 nm.λmax

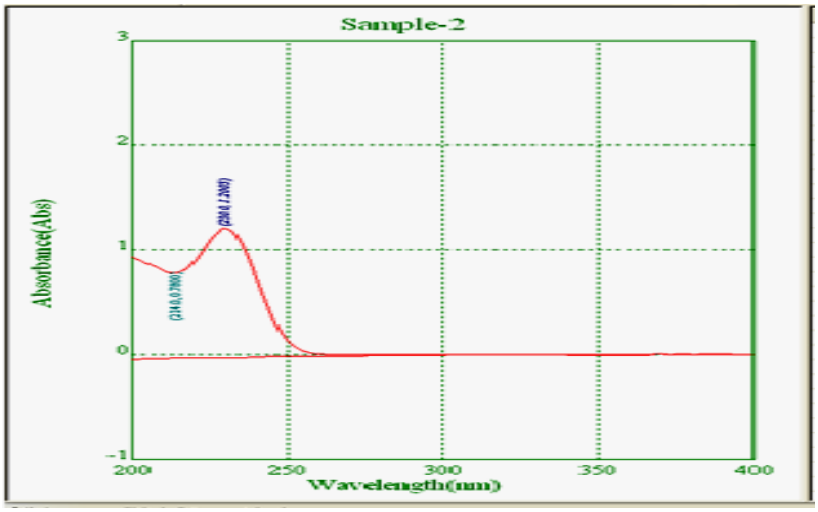


Fig. 1.2. UV-Spectrum of Daunorubicin

Standard solutions were scanned in the range of 200-400nm ,against 10 ml methanol and volume make with water solvent system as reference Daunorubicin in water was found to be selected wavelength is 230 nm **(Figure No:7.2)**

4.1.6. Studies on the chromatographic behavior of Daunorubicin:

After the selection of suitable mobile phase, it was then optimized for its reproducibility, sensitivity & accuracy. The optimized parameters for selected method are as below.

Table. No 1.6: Different Trials of Chromatographic Condition

Sr. No.	Column used	Mobile phase, Flow Rate and Wavelength	Inj. Vol.	Observation	Conclusion
1	Cosmosil C18 Column (250mm x 4.6mm)	Methanol + 0.1% (OPA)Water ,(90+10% v/v) PH 2.7, 230nm, Flow rate 1.0 ml/min	20 µl	Sharp Peaks were not obtained	Hence rejected
2	Cosmosil C18 Column (250mm x 4.6mm)	Methanol+0.1%(OPA)Water, (80+20% v/v) PH 2.7, 230nm, Flow rate 1.0 ml/min	20 µl	Sharp Peaks were not obtained	Hence rejected
3	Cosmosil C18 Column (250mm x 4.6mm)	Methanol (100% v/v) PH 2.7, 230nm, Flow rate 1.0 ml/min	20 µl	Sharp Peaks were not obtained	Hence rejected
4	Cosmosil C18 Column (250mm x	Acetonitrile+0.1% (OPA)Water(95+05%v/v) PH 2.7, 230nm, Flow rate 1.0 ml/min	20 µl	Sharp and well resolved Peaks were	Hence selected

	4.6mm)			obtained	

Thus, from the above, it has been observed that, using mobile phase of ACN+0.1% (OPA)water,(95:05 % v/v),PH 2.7,230nm, Flow rate 20µl gave adequate retention at 4.3167 min with good peak shape (Theoretical plates: Daunorubicin 7591.4)

Chromatogram of Trial 1:

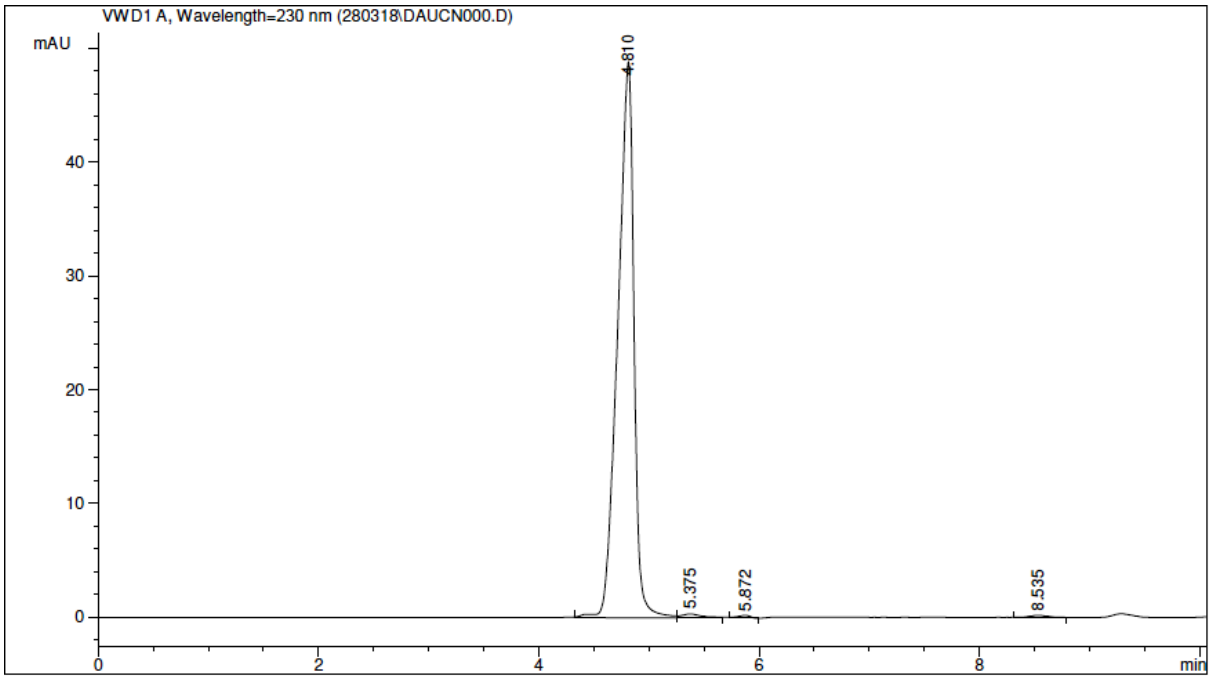


Fig No (1.3) 7.3: Chromatogram of Trial 1

Table No 1.7: Result for Chromatogram of Trial 1

Sr.No.	RT[min]	Area[mAU*s]	TP	TF	Resolution
1	4.810	498.70273	5314	1.61	-
2	5.375	4.82289	3356	0.60	1.78
3	5.872	2.04282	9984	1.68	1.64
4	8.535	2.07752	12292	0.91	9.79
Sum		507.64596			

Section A-Research paper
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Chromatogram of Trial 2:

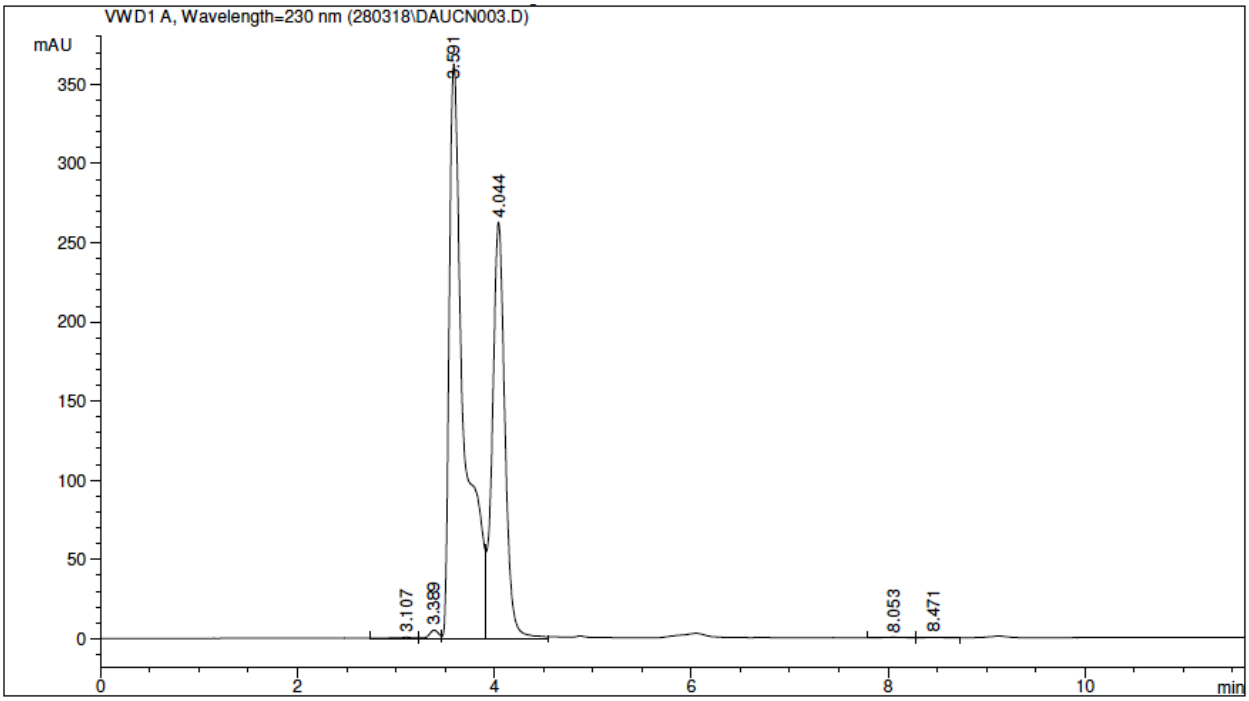

Fig No (1.4) 7.4: Chromatogram of Trial 2

Table No 1.8: Result for Chromatogram of Trial 2

No	RT[min]	Area[mAU*s]	TP	TF	Resolution
1	3.107	6.03437	5677	1.64	-
2	3.389	29.81501	6607	1.12	1.70
3	3.591	3826.64819	4757	0.39	1.07
4	4.044	2330.49561	5547	0.91	2.13
5	8.053	4.01285	8123	0.86	13.93
6	8.471	2.56205	10910	1.10	1.22
Sum		6199.56808			

Chromatogram of Trial 3:

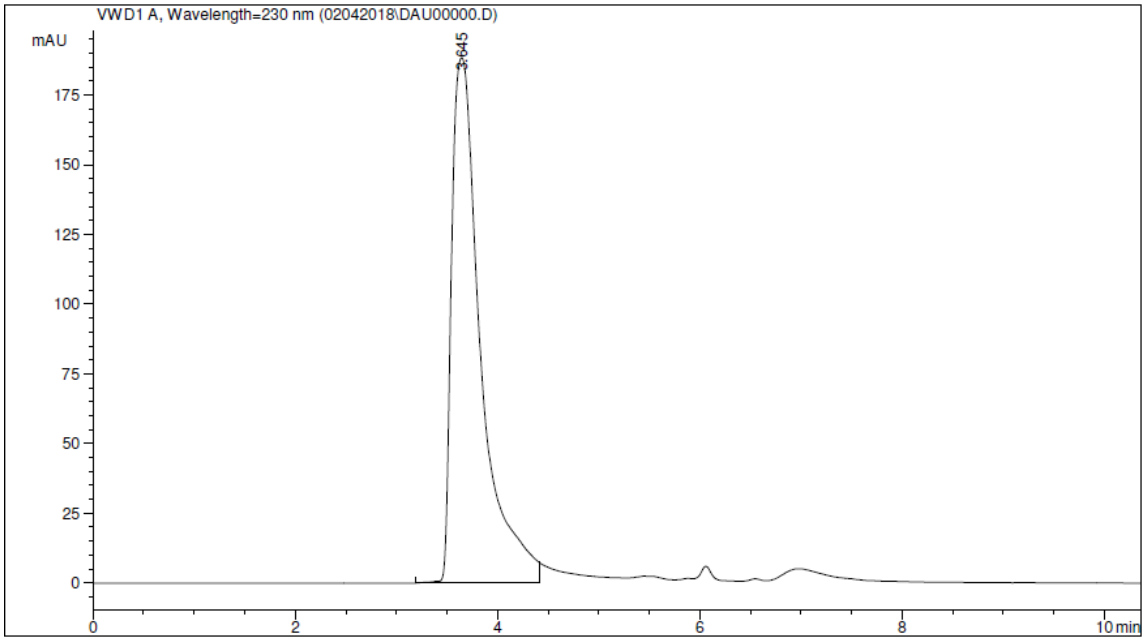

Fig No (1.5) 7.5: Chromatogram of Trial 3

Table No 1.9: Result for Chromatogram of Trial 3

No	RT[min]	Area[mAU*s]	TP	TF	Resolution
1	3.645	3765.00708	979	0.46	-

Chromatogram of Final graph 4:

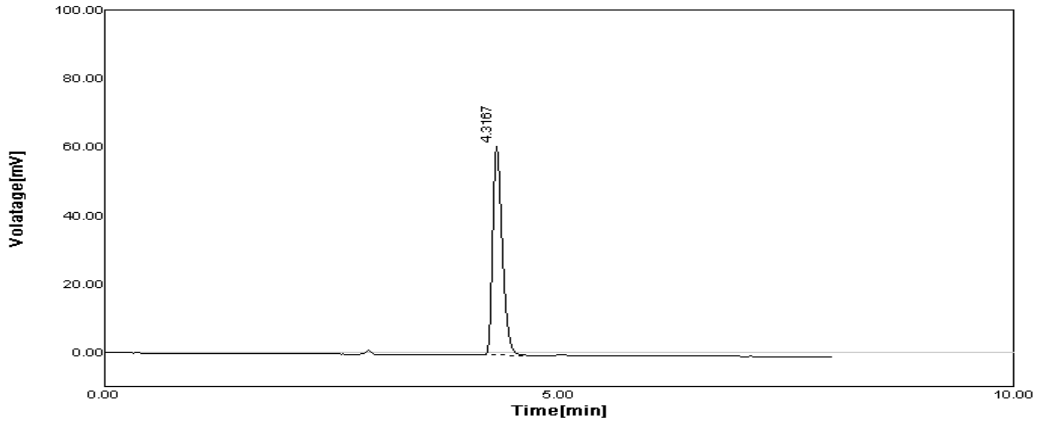

Fig No (1.6) 7.6: Chromatogram of Final graph

Table No.2: Result for Chromatogram of Trial 4

No.	RT[min]	Area[mAU*s]	Area %	TP	TF	Resolution
1	4.3167	448.6774	100.00	7591.4	1.3333	0.0

The final chromatographic conditions selected were as follow:

- Analytical column : Cosmosil C18 Column (250mm x 4.6mm, 5µm packing size).
- Injection volume : 20µl
- Flow rate : 1 ml/min
- Mobile phase : Acetonitrile : water(0.1 % OPA) (95: 5% V/V)
- Detection : 230 nm
- Run Time : 15 min

Preparation of Standard chromatogram of Daunorubicin:

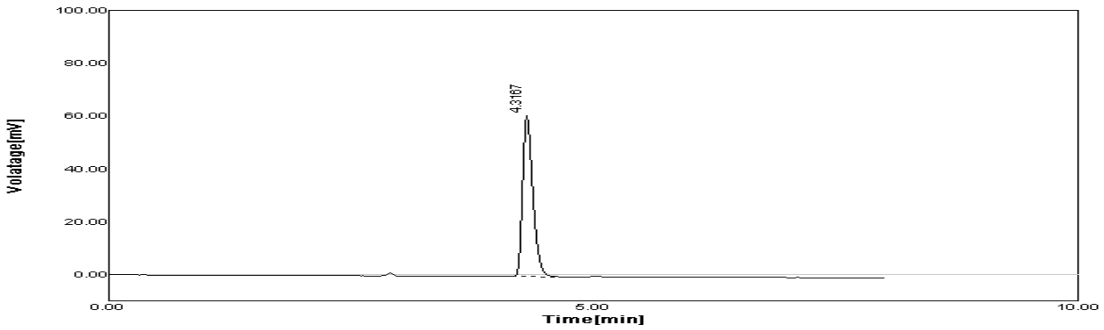

Fig No.(1.7) Chromatogram of standard Daunorubicin

Table No.2.1: Result for standard Chromatogram of Daunorubicin

No.	RT[min]	Area[mAU*s]	Area%	TP	TF	Resolution
1	4.3167	448.6774	100.00	7591.4	1.3333	0000

5. Analytical of Method Validation:

5.1 Linearity:

From Daunorubicin standard stock solution, different working standard solution (10-50µg/ml) were prepared in mobile phase 20 µl of sample solution was injected into the chromatographic system using mixed volume loop injector Chromatograms were recorded. The area for each conce ntration was recorded (**Table No. 7.7)** The Calibration curves are shown in **[Fig. No.7.9]**

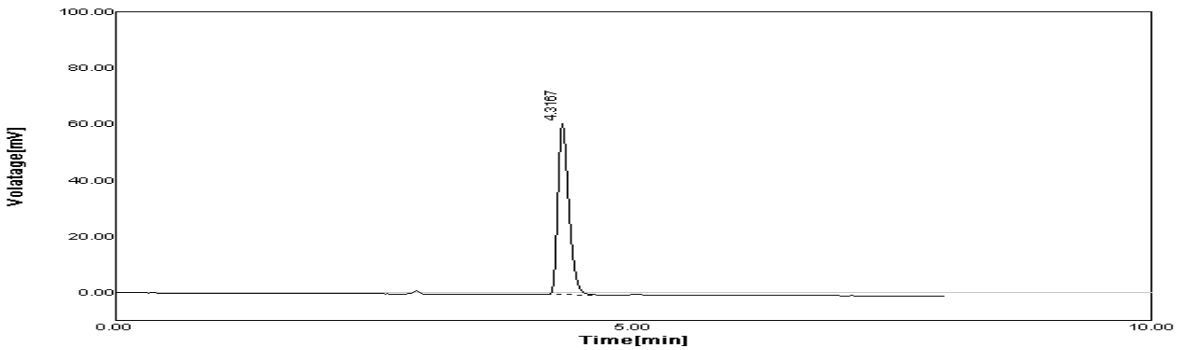

Fig. No.(1.8) 7.8.Chromatogram of linearity

Table No 2.2. Linearity of Daunorubicin

Sr. No.	Concentration µg/ml	Area Daunorubicin
1	10	447.5196
2	20	829.3618
3	30	1252.134
4	40	1641.563
5	50	2001.386

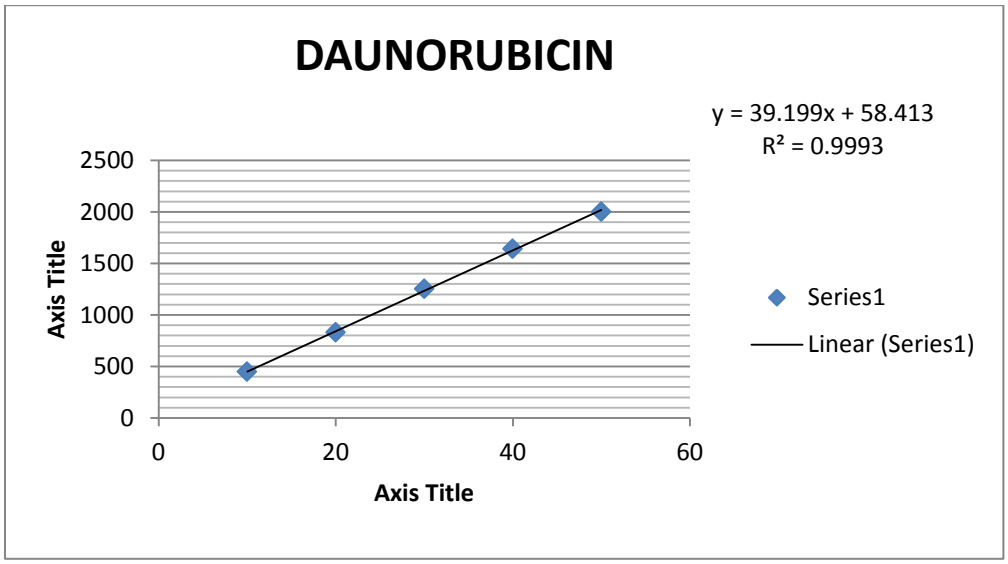

Fig. No. (1.9) 7.9. Calibration curve of Daunorubicin

Table No 7.8. Regression equation data for Daunorubicin

Regression Equation Data Y=mx+c	
Slope(m)	39.19
Intercept(c)	58.41
Correlation Coefficient	0.999

Linearity of of Daunorubicin was observed in the range of 10-50µg/ml . Detection wavelength used was 230 nm**.(Fig No. 7.7)**

The calibration curve yielded correlation coefficient (r^2) 0.999 for Daunorubicin respectively.(**Table No. 7.8)**

***7.2.2. Precision*:-**

The method was established by analyzing various replicates standards of Daunorubicin. All the solution was analyzed thrice in order to record any intra-day & inter-day variation in the result that concluded. The result obtained for intraday and interday is shown in

(Table No. 7.9) respectively.

Chromatogram of Precision:

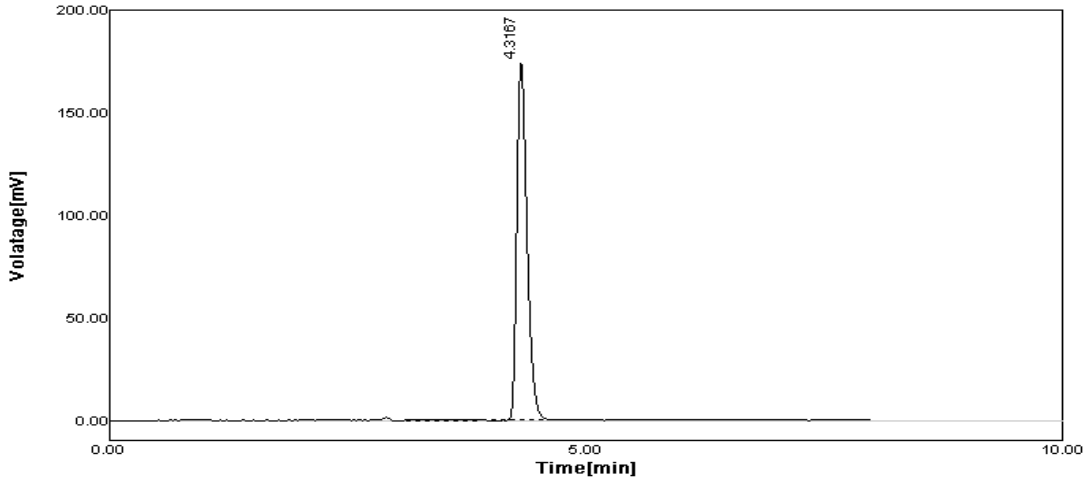

Fig No.7.10:Chromatogram Intra-day precision

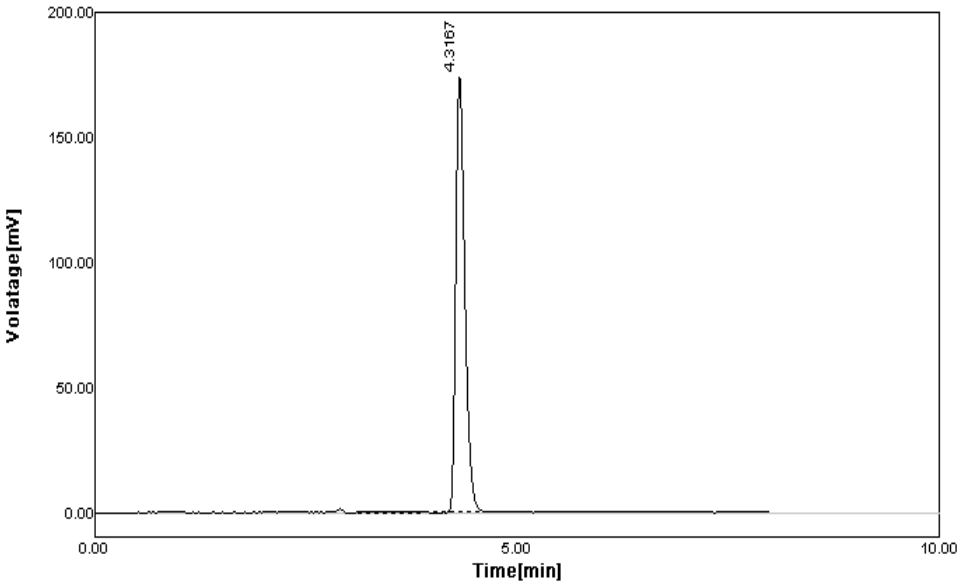

Fig No.7.11:Chromatogram Inter-day precision

Table No .7.9: Result of Intra day and Inter day Precision for Daunorubicin

Conc^{n}(µg/ml)	**Intraday Precision**			**Interday Precision**		
	Mean± SD	**%Amt Found**	**%RSD**	**Mean± SD**	**%Amt Found**	**%RSD**
10	448.29±2.72	99.40	0.61	447.69±0.61	99.30	0.14
30	1242.51±7.59	100.70	0.61	1239.16±1.43	100.40	0.12
50	2049.56±7.52	101.60	0.37	2050.66±7.76	101.66	0.38

***Mean of each 2 reading**

Intra day and Inter day Precision for Daunorubicin which shows the high precision %amount in between 98% to 100% indicates to analytical method that conclusion

7.2.3.System suitability parameters:(Repetability)

To ascertain the resolution and reproducibility of the proposed chromatographic system for estimation of Daunorubicin system suitability parameters were studied. The result shown in below **(Table No.7.10)**

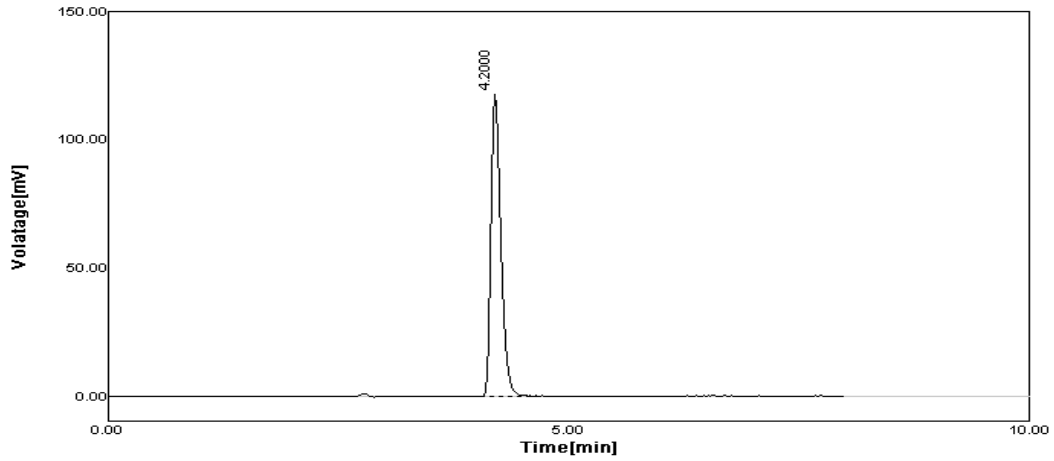

Fig No.7.12: Chromatogram of System suitability No- 1

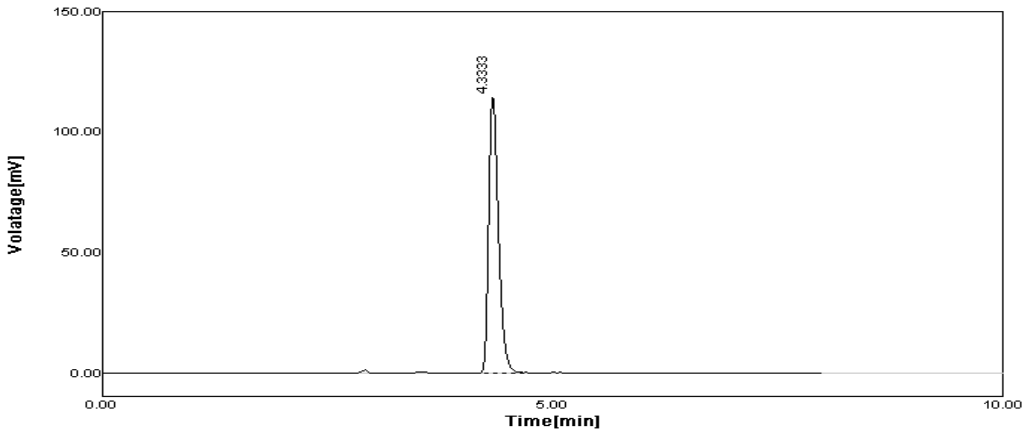

Fig No 7.13: Chromatogram of System suitability No- 2

Table No.7.10: Reapetability studies on Daunorubicin

Sr.No.	**Concentration of Daunorubicin (mg/ml)**	**Peak area**	**Amount found (mg)**	**% Amount found**
1	20	828.1954	19.65	98.25
2	20	829.3467	19.67	98.35
		Mean	19.66	
		SD	0.81	
		%RSD	0.10	

Reapetability studies Daunorubicin was found to be ,The %RSD was less than 2, which shows high percentage amount found in between 98% to 102% indicates the analytical method that concluded .

7.2.4. Accuracy:-

Recovery studies were performed to validate the accuracy of developed method. To pre analyzed tablet solution, a definite concentration of standard drug (80%, 100%, and 120%) was added and

then its recovery was analyzed **(Table No.7.11).** Statistical validation of recovery studies shown in **(Table No. 7.12)**

Accuracy 80%

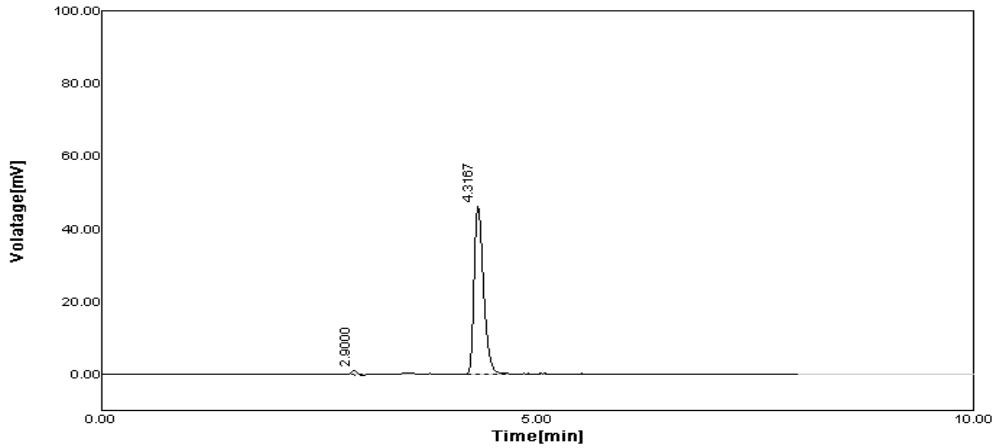

Fig.7.14. Chromatogram of Accuracy 80%

Accuracy 100%

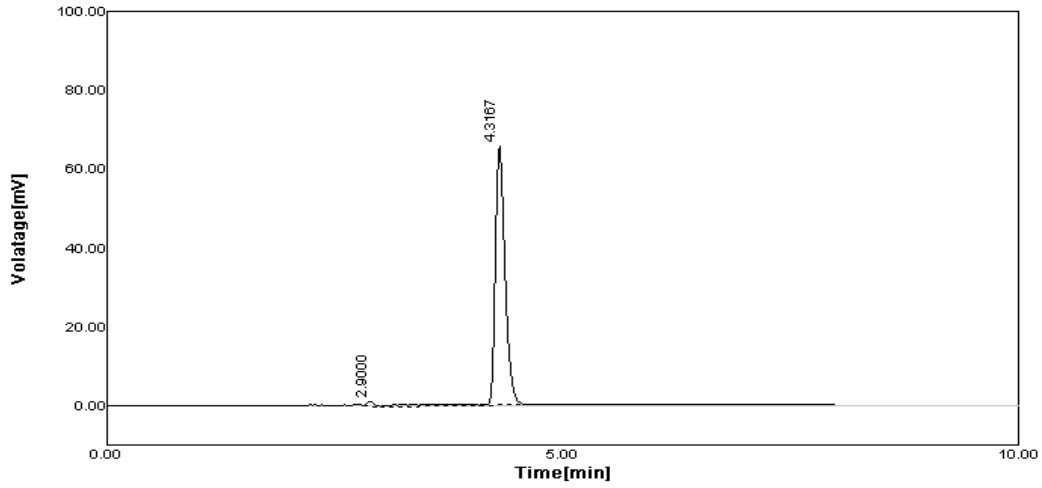

Fig.7.15. Chromatogram of Accuracy 100%

Accuracy 120%

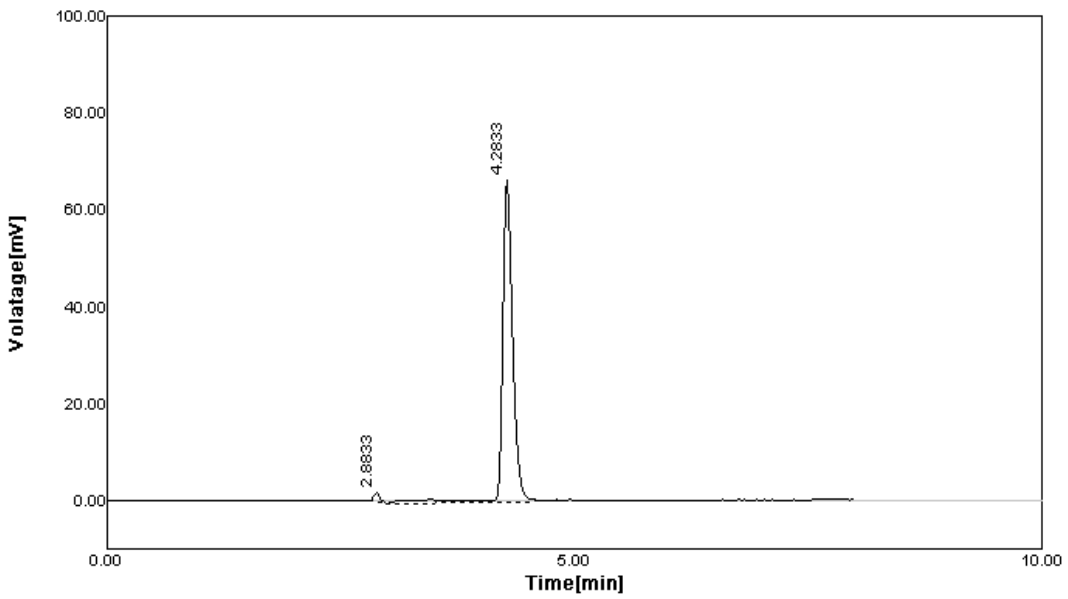

Fig.7.16. Chromatogram of Accuracy 120%

Table no .7.11. Result of Recovery data for Daunorubicin

Level (%)	**Amt. taken (µg/ml)**	**Amt. Added (µg/ml)**	**Absorbance Mean* ± S.D.**	**Amt. recovered Mean *±S.D.**	**%Recovery Mean *± S.D.**
80%	10	8	18.03±0.12	8.03±0.12	100.38±1.55
100%	10	10	20.21±0.13	20.58±0.18	100.03±1.78
120%	10	12	22.32±0.21	12.32±0.21	101.58±1.07

***mean of each 2 reading.**

Table no. 7.12. Statistical Validation of Recovery Studies Daunorubicin

Level of Recovery (%)	**Drug**	**Mean % Recovery**	**Standard Deviation***	**% RSD**
80	Daunorubicin	100.38	1.55	1.54
100	Daunorubicin	100.03	1.78	1.78
120	Daunorubicin	101.58	1.07	1.05

***Denotes average of two determinations.**

Accuracy of method is ascertained by recovery studies performed at different levels of concentrations (80%, 100% and 120%). The % recovery was found to be within 100-101% (Table No. 7.11,7.12)

7.2.5. Robustness:

The Robustness of a method is its ability to remain unaffected by small deliberate changes in parameters. To evaluate the robustness of the proposed method, small but deliberate variations in the optimized method parameters were done. The effect of changes in mobile phase composition and flow rate ,wavelength on retention time and tailing factor of drug peak was studied.

The mobile phase composition was changed in(± 1 ml/min^{-1}**)** proportion and the flow rate was varied by of optimized chromatographic condition. The results of robustness studies are shown in **(Table No.7.13**).Robustness parameters were also found satisfactory; hence the analytical method would be concluded.

7.2.5.1. Flow Rate Change 0.9 ml

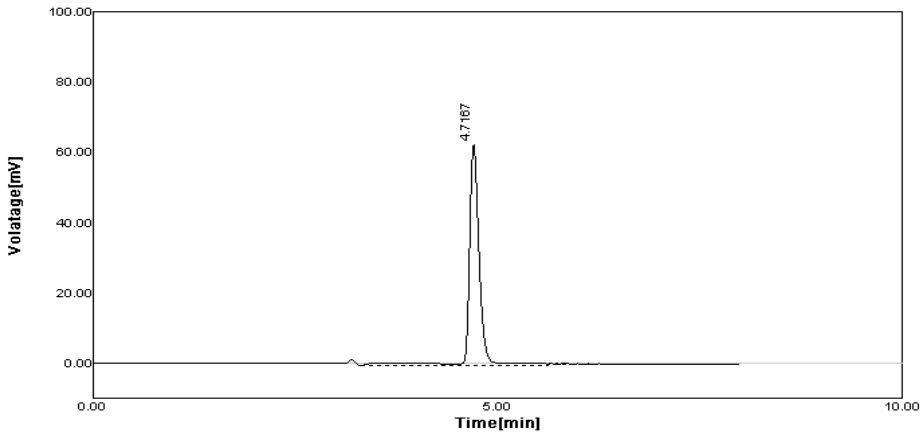

Fig No.7.17. Chromatogram of Flow rate change 0.9ml

7.2.5.2. Flow Rate Change 1.0 ml

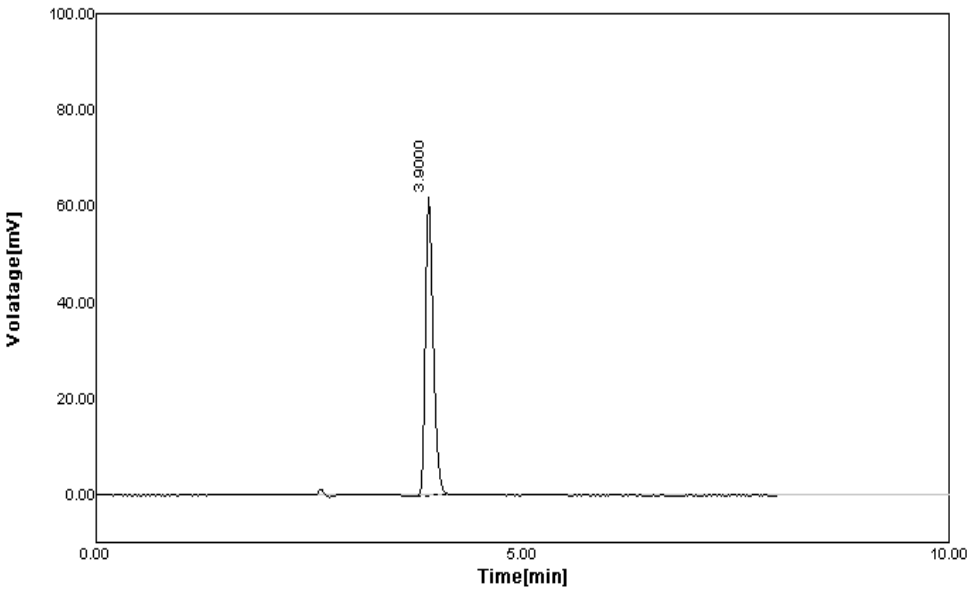

Fig No 7.18. Chromatogram of Flow rate change 1.0 ml

7.2.5.3. Mobile phase composition Change : 94ml ACN + 0.1%(OPA) 06ml Water

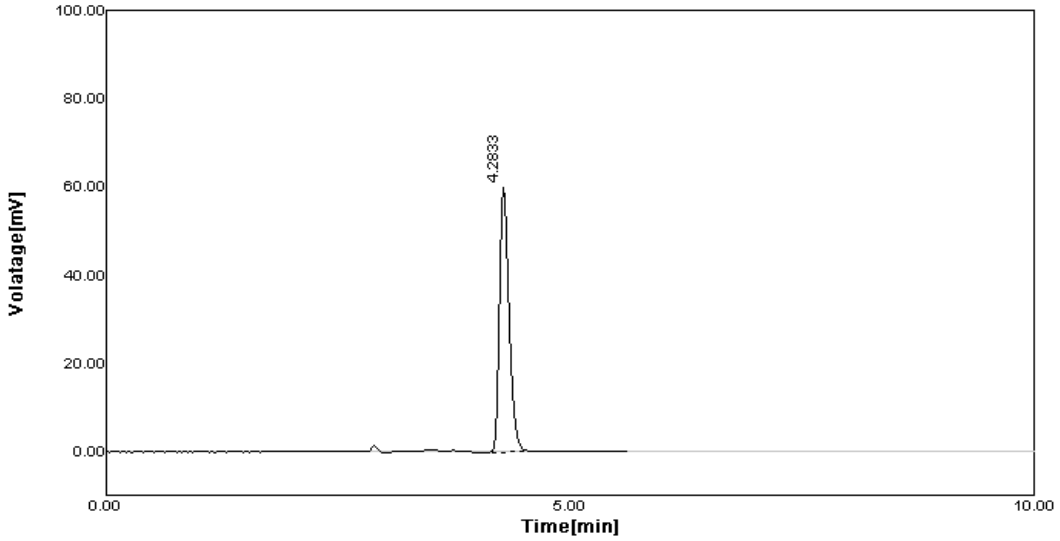

Fig No .7.19. Chromatogram of Mobile phase composition change 94 ml ACN + 0.1%(OPA) 06 ml Water

7.2.5.4 Mobile phase composition Change: 96 ml ACN+ 0.1%(OPA)04 ml Water

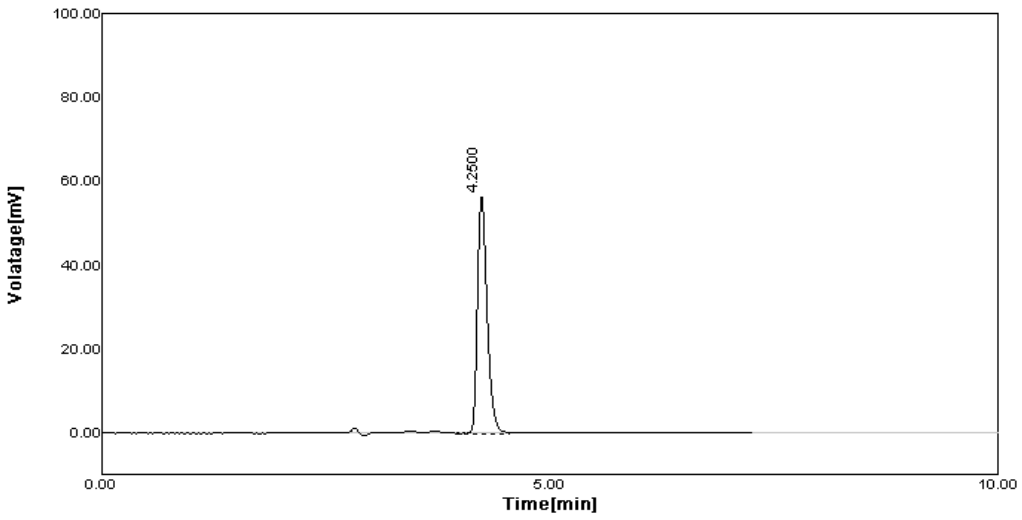

Fig No.7.20 Chromatogram of Mobile phase composition change 96 ml ACN+ 0.1% (OPA) 04 ml Water

7.2.5.5Wavelength Change 229 nm

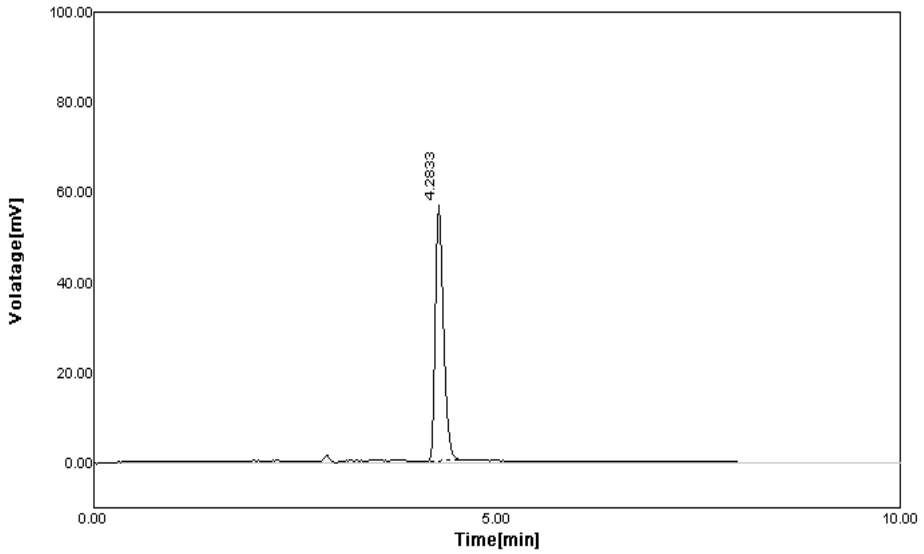

Fig.No 7.21: Chromatogram of comp change wavelength change 229nm

7.2.5.6 Wavelength Change 231 nm

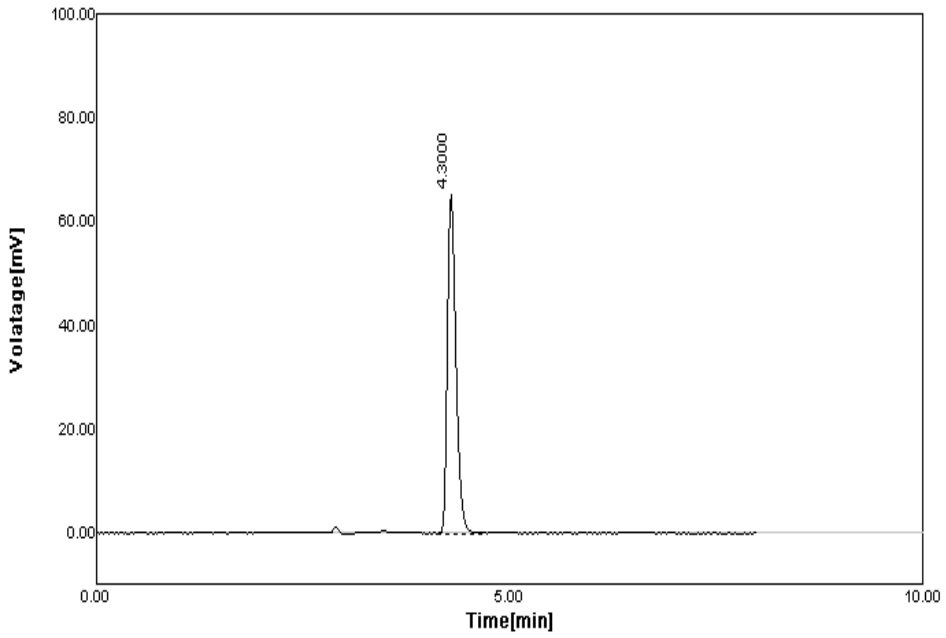

Fig.No 7.22:. Chromatogram of comp change wavelength change 231 nm

Table No.7.13 Robustness Study With Different Condition

Parameters	**Conc.(µg/ml)**	**Amount of detected(mean ±SD)**	**%RSD**
Flow rate change(0.9ml)	**10**	**461.07±1.20**	**0.26**
Flow rate change(1.1 ml)	**10**	**470.16±0.65**	**0.14**
Mobilephase composition(94ml+06ml)Acetonitrile + 0.1% (OPA)water	**10**	**432.6±0.81**	**0.19**
Mobilephase composition(96ml+04ml)Acetonitrile + 0.1% (OPA)water	**10**	**430.21±1.63**	**0.38**
Wavelength change 229nm	**10**	**401.6±0.68**	**0.17**
Wavelength change 231 nm	**10**	**488.76±6.30**	**1.29**

Robustness Study of Daunorubicin :

The changes were did flow rate (±1 ml/ min^{-1}),PH of mobile phase composition ,and Wavelength .%RSD for peak area was calculated which should be less than 2%.the result shown in analytical method that concluded.(**Table No.7.13)**

7.2.5.6. Limit Detection

The LOD is the lowest limit that can be detected. Based on the S.D. deviation of the response and the slope The limit of detection (LOD) may be expressed as:

LOD= 3.3xAvd.SD/Slope

3.3x2.59/39.19=0.21

where, SD = Standard deviation of Y intercept

7.2.5.7. Limit Quantification

The LOQ is the lowest concentration that can be quantitatively measured. Based on the S.D. deviation of the response and the slope,

The quantitation limit (LOQ) may be expressed as:

LOQ = 10 (SD)/ S

=10 X 2.59 / 39.19

= 0.66

where, SD = Standard deviation Y intercept

S = Slope

The LOQ of Daunorubicin was found to be 0.66 (µg/mL) analytical method that concluded.

7.3. Analysis of formulation:-

Procedure:

Weigh 20 Daunorubicin vials and calculated the average weigh 300.45 mg accurately weigh and transfer the sample equivalent to 20.03 mg Daunorubicin into 25 ml volumetric flask. Add about 20 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with diluent. Mix well and filter through 0.45 µm filter. Further pipette 0.3ml of the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluents.. The simple chromatogram of test Daunorubicin Shown in **(Fig No:7.23)** The amounts of Daunorubicin per vial were calculated by extrapolating the value of area from the calibration curve. Analysis procedure was repeated five times with lyophilised formulation. Assay for %Lable claim for %RSD Calculated, Result was shown in **(Table No. 7.14 & 7.15)**

Brand Name : Daunorubicin (NAPROD LIFE SCIENCES PVT. LTD)

Total weight of 20 vial wt. . = 6.009 gms

Avg Weight =0.30045 gms./vial

Eq. wt for 20 mg= 20 X 300.45 /300 =20.03mg

Take 20.03 mg in 10 ml Methanol sonicate 10 min

i.e. 2000 µgm/ml ----- STOCK -II

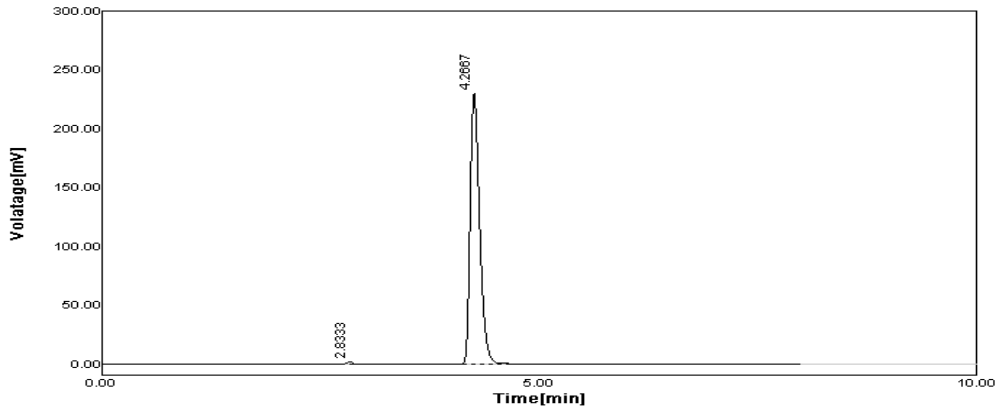

Fig No.7.23: Chromatogram for Marketed Formulation

Table No.7.14 . Analysis of marketed formulation

Sr.no	**Amount present in mg**	**Area(I)**	**Amount found in mg**	**% Label claim**
	DAUNORUBICIN	**DAUNORUBICIN**	**DAUNORUBICIN**	**DAUNORUBICIN**
1	40	1669.595	41.12	102.80
2	40	1627.124	40.02	100.05
	Mean	1648.36	40.57	101.425
	SD	30.03	0.78	0.27
	%RSD	1.82	1.96	0.27

Analysis of marketed formulation were also %Lable Claim was found to be 100%- 102% Satisfactory are concluded.(Table No.7.14).

Formulation Assay for % Lable Claim

Table No.7.15 : Formulation for %Lable claim

Sample	**Label claimed**	**%Label claimed.± SD**	**%RSD**
Daunorubicin	Daunorubicin=20 mg	101.425±0.27	0.27

Formulation Assay for %Lable claim for were also was found to be 101.425% and %RSD are less than 2 satisfactory result that concluded. **(Table No:7.15)**

SUMMARY AND CONCLUSIONS:

A Daunorubicin is clinically used in the anthracycline antibiotics, which very effective in anticancer drugs with proved activity in acute lymphomas and wide range of carcinomas. The present work deals with the Development and validation of RP-HPLC method for determination of Daunorubicin by pure for injection (IV)dosage form

8.1Summary of RP-HPLC method:

Attempts were made to develop RP-HPLC method for of Daunorubicin for injection. For the RP - HPLC method,Younglin (S.K) GradientSystem UV Detector and C_{18} col umn with 250mm x4.6 mm i.d and 5µm particle size Acetonitrile : Water 0.1 % OPA (95:05v/v) pH 2.7 was used as the mobile phase for the method. The detection wavelength was 230 nm and flow rate was 1 ml/min. In the developed method, the retention time of Daunorubicin were found to be 4.3167 min

The developed method was validated according to the ICH guidelines. The linearity, precision, robustness was within the limits as specified by the ICH guidelines. Hence the method was found to be simple, accurate, precise, economic and reproducible.

So, it is worthwhile that, the proposed methods can be successfully utilized for the routine quality control analysis Daunorubicin in bulk drug as well as in formulations.

8.2Conclusion:

Simple, rapid, accurate and precise RP-HPLC have been developed and validated for the routine analysis of Daunorubicin in API and for injection dosage forms. Both methods are suitable for the simultaneous determination of Daunorubicin in Single-component formulations without interference of each other. The developed methods are recommended for routine and quality control analysis of the investigated drugs in two component pharmaceutical preparations. The amount found from the proposed methods was in good agreement with the label claim of the formulation. Also the value of standard deviation and coefficient of variation calculated were satisfactorily low, indicating the suitability of the proposed methods for the routine estimation of for injection dosage forms.

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