RP-HPLC METHOD DEVELOPMENT AND VALIDATION, STABILITY STUDIES OF SILDENAFIL CITRATE IN SOFT GELATIN CAPSULE DOSAGE FORM

A Dissertation Submitted to

THE TAMILNADU Dr.M.G.R MEDICAL UNIVERSITY

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In Partial fulfillment for the award of the degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

Submitted By

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EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled **"RP-HPLC METHOD DEVELOPMENT AND VALIDATION, STABILITY STUDIES OF SILDENAFIL CITRATE IN SOFT GELATIN CAPSULE DOSAGE FORM",** submitted by the student bearing **Reg. No: 261530206** to "**The Tamil Nadu Dr. M.G.R. Medical University – Chennai**" , in partial fulfillment for the award of Degree of **Master of Pharmacy** in **Pharmaceutical Analysis** was evaluated by us during the examination held on……..…….

Internal Examiner **External Examiner**

CERTIFICATE

This is to certify that the work embodied in this dissertation "**RP-HPLC METHOD DEVELOPMENT AND VALIDATION, STABILITY STUDIES OF SILDENAFIL CITRATE IN SOFT GELATIN CAPSULE DOSAGE FORM",** submitted to **"The Tamil Nadu Dr. M.G.R. Medical University**", Chennai, was carried out by **MS. KEERTHANA RANI.K [Reg.No: 261530206],** for the Partial fulfillment of degree of **MASTER OF PHARMACY** in **PHARMACEUTICAL ANALYSIS** under my guidance and supervision during the academic year 2015-2017

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DECLARATION

The work presented in this dissertation entitled "**RP-HPLC METHOD DEVELOPMENT AND VALIDATION, STABILITY STUDIES OF SILDENAFIL CITRATE IN SOFT GELATIN CAPSULE DOSAGE FORM**" was carried out by me, under the direct supervision of **Dr. I. CAROLIN NIMILA, M.Pharm., Ph.D.,** Assistant Professor of Pharmaceutical Analysis, J.K.K. Nattaraja College of Pharmacy, Kumarapalayam.

I further declare that this work is original and this dissertation has not been submitted previously for the award of any other degree, diploma, associate ship and fellowship or any other similar title. The information furnished in this dissertation is genuine to the best of my knowledge.

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CONTENTS

LIST OF ABBREVIATIONS USED

B.P	-	British Pharmacopeia
^{0}C	-	Degree Centigrade
FDA	-	Food and Drug Administration
GC	-	Gas Chromatography
HPLC	-	High Performance Liquid Chromatography
HPTLC	-	High Performance Thin Layer Chromatography
ICH	-	International Conference Of Harmonization
I.P	-	Indian Pharmacopeia
IR	-	Infrared Spectroscopy
IUPAC	-	International Union of Pure and Applied Chemistry
LC	-	Liquid Chromatography
LC-MS	-	Liquid Chromatography-Mass Spectroscopy
LOD	-	Limit of detection
LOQ	-	Limit of quantitation
NMT	-	Not more than
Nm	-	Nanometer
NLT	-	Not less than
M	-	Molar
Max	-	Maximum
mg	-	Milligram
Min	-	Minute
Ml	-	milli litres
Mm	-	millimetre
M.W	-	Molecular weight
ODS	-	Octyl decyl silane
Ppm	-	Parts Per Million
RSD	-	Relative Standard Deviation

RP-HPLC	-	Reverse Phase High Performance Liquid Chromatography
S.D	-	Standard Deviation
TLC	-	Thin Layer Chromatography
t_R	-	Retention time
µg	-	Microgram
µl	-	Microlitre
µg	-	Microgram
US FDA	-	United States Food and Drug Administration
US NF	-	United States National Formulary
USP	-	United States Pharmacopeia
UV	-	Ultra-Violet
% v/v	-	Percentage volume per volume
Wt	-	Weight
%w/w	-	Percentage weight per weight
ʎ	-	Lambda

1. INTRODUCTION

Pharmaceutical Analysis deals not only with medicaments (drugs and formulations), but also with their precursors i.e. with the raw material whose degree of purity, which in turn decides the quality of medicaments. The quality of a drug is determined, after establishing its authenticity, which is carried by testing its purity and the quality of the pure substance in the drug and its formulations.[1-7]

Analytical chemistry has got a pivot role to play in determination of quality of medicines as quality is important and essential aspect to be considered in every product or service. Quality is more vital in medicines as it is related to life. A compromise in pharmaceutical quality is nothing but playing with the life of consumer. Application of analytical chemistry in pharmacy is termed as pharmaceutical analysis. The terms quality, quality control, quality assuarance, total quality management are correlated. The ultimate goal of all these is to provide a product with good quality, safety, efficiency, purity, strength and identity.

General terms associated with chemical analysis

Analytical technique

It is the fundamental scientific phenomenon that is proved useful for providing information on the composition of substances.

Analytical method

The method is the specific application of technique to solve the analytical problem.

- **Protocol**

The most specific description of a method is known as protocol.

- **Procedure**

A procedure is written instructions for carrying out a method.

The "standard methods" developed by the ASTM (American Society for Testing Materials) and the AOAC (Association of Official Analytical Chemists). It only provides the general steps to be followed.

Pharmaceutical analysis is broadly classified into two branches, namely qualitative analysis and quantitative analysis.

A. **Qualitative analysis**: It deals with the identification and establishment of the identity of analyte among elements or compounds present in the sample.

B. **Quantitative analysis**: It deals with the quanification of the analyte present in the matter of sample.

The determination of the quality and of quantity of the sample is done the application of analytical methods.

Analytical methods are classified into two types

- Instrumental methods.
- Non-instrumental methods.

CHROMATOGRAPHY

The term Chromatography (in Greek, khromatos means colour and Graphos

means written) means colourwriting**.** The term chromatography and its principles were discovered in 1903 by ***Mikhiltswett***. Chromatography is the separation of mixture of components into individual components by using a stationary phase and a mobile phase.

Chromatography is method of separating mixtures and identifying their components i.e. it's a separation method that exploits the differences in partitioning behaviour of analyse between a mobile phase and a stationary phase to separate components in a mixture. Components of a mixture may be interacting with the stationary phase based on charge (ion-ion-interactions, ion-dipole-interactions), Vander Waal's forces, relative solubility or adsorption (hydrophobic interactions, specific affinity).

Chromatography may be preparative or analytical. Preparative chromatography seeks to separate the components of a mixture for further use (and is thus a form of purification). Analytical chromatography normally operates with smaller amounts of material and seeks to measure the relative proportions of analytes in a mixture. The two are not mutually exclusive.

Classification of chromatographic methods

- Gas – solid chromatography.
- Gas – liquid chromatography.
- Solid – liquid chromatography.
- Liquid- liquid chromatography.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC):

High-performance liquid chromatography is a chromatographic technique used to separate the components in a mixture, to identify each component, and to quantify each component .The method involves a liquid sample being passed over a solid adsorbent material packed into a column using a flow of liquid solvent. Each analyte in the sample interacts slightly differently with the adsorbent material, thus retarding the flow of the analytes. If the interaction is weak, and the analytes flow off the column in a short amount of time, and if the interaction is strong, then the elution time is long.

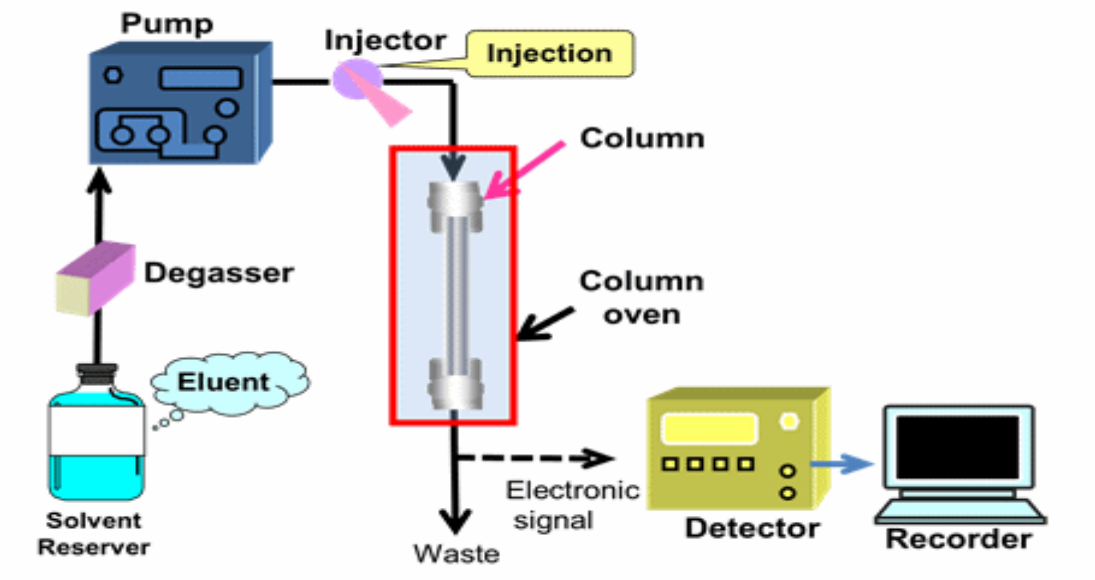
Chromatography may be defined as a method of separating a mixture of components into individual components through equilibrium distribution between two phases**.**

The HPLC method was considered the choice of estimation, since this method is the most powerful of all chromatographic and other separative methods. The HPLC method has enabled analytical chemist to attain great success in solving his analytical problems. The HPLC is the method of choice in the field of analytical chemistry, since this method is specific, robust, linear, precise, and accurate and the limit of detection is low and also it offers the following advantages.

The schematic representation of an HPLC instrument typically includes a sampler, pumps, and a detector. The sampler brings the sample mixture into the mobile phase stream which carries it into the column. The pumps deliver the desired flow and composition of the mobile phase through the column. The detector generates a signal proportional to the amount of sample component emerging from

the column, hence allowing for quantitative analysis of the sample components .A digital microprocessor and user software control the HPLC instrument and provide data analysis. Some models of mechanical pumps in a HPLC instrument can mix multiple solvents together in ratios changing in time, generating a composition gradient in the mobile phase. Various detectors are in common use, such as UV/Vis, photodiode array (PDA) or Refractive index (RI).

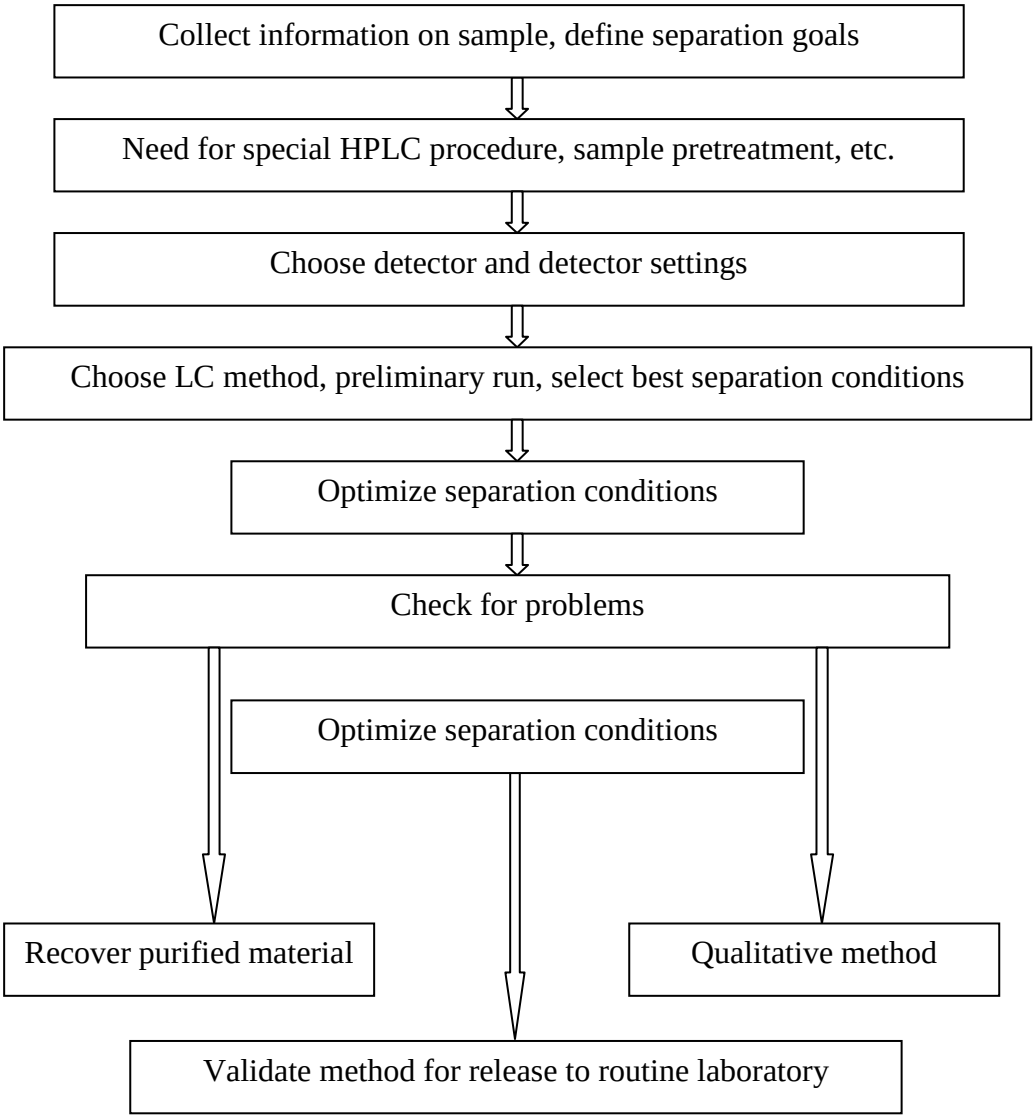
HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)



SCHEMATIC REPRESENTATION OF HPLC SYSTEM

INTRODUCTION TO HPLC METHOD DEVELOPMENT

Method development has following steps:



High-performance liquid chromatography (HPLC) is the fastest growing analytical technique for analysis of drugs. Its simplicity, high specificity and wide range of sensitivity make it ideal for the analysis of many drugs in both dosage forms and biological fluids.

The HPLC offers the following advantages.

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

PERFORMANCE CALCULATIONS SYSTEM SUITABILITY PARAMETERS

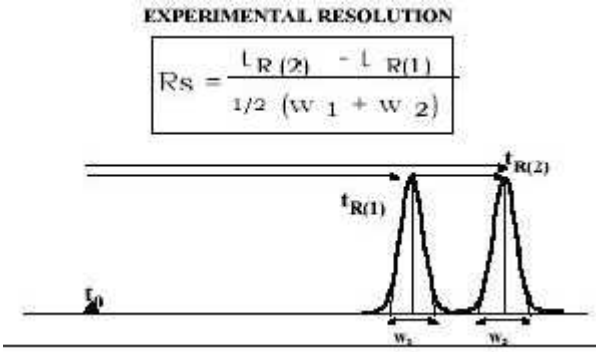
1) Retention time (t_R)
2) Resolution (R_S)
3) Capacity factor ($k`$)
4) Selectivity (α)
5) Number of Theoretical plates (N)
6) HETP
7) Asymmetry factor
8) Tailing factor

1) Retention time

Chromatographic retention is to measure the time between the injection point and maximum of the detector response for correspondent compound. This parameter called “retention time” is inversely proportional to the eluent flow rate.

2) Resolution

The goal of most HPLC analyses is the separation of one or more analytes in the sample from all other components present. Resolution (Rs) is a measure of the degree of separation of two adjacent analytes. Resolution of two adjacent band is defined as the distance between band peaks divided by the average band width. Retention and band width are measured in units of time.



Where,

Rt_1 and Rt_2 are the retention times of components 1 and 2

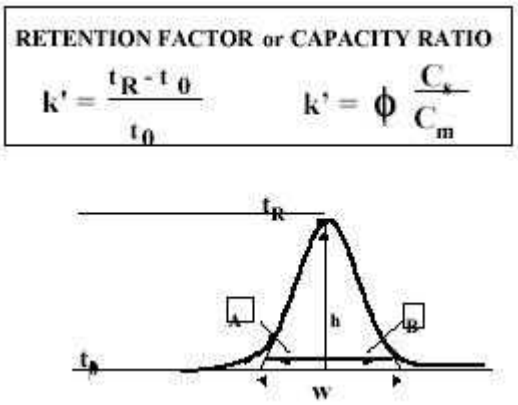
W_1 and W_2 are peak widths of components 1 and 2.

$R_S \geq 2.0$ is a desirable target for method development.

3) Capacity factor (K’)

It is a measure of a sample peak in the chromatogram being specific for a

given compound, a parameter which specifies of a substance to be separated.



The retention factor k is given by the equation

$k = (t_R - t_0) / t_0$

Where,

't_R' is the band retention time

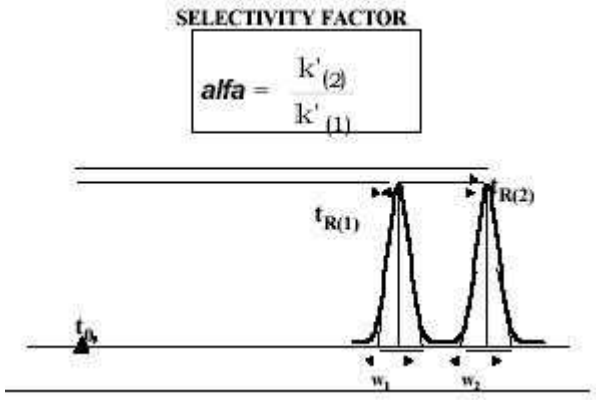
't_0' is the column dead time.

4) Selectivity (α)

The selectivity α is a measure of relative retention of two components in a mixture. The ideal value of selectivity is 2. It can be calculated by using the following formula.

$$\frac{V_2 - V_0}{V_1 - V_0}$$

Where, V_0 is the void volume of the column and V_2 and V_1 are the retention volumes of the second and the first peak, respectively.



COLUMN EFFICIENCY & PLATE NUMBER

The number of theoretical plates or plate number is a measure of column efficiency. An efficient column produces sharp peaks and can separate many sample components in a relatively short time. Theoretical plates (N) are defined as the square of the ratio of the retention time divided by the standard deviation of the peak (σ).

$$N = \left(\frac{t_R}{\sigma}\right)^2 = \left(\frac{4t_R}{w_b}\right)^2 = 16\left(\frac{t_R}{w_b}\right)^2.$$

Another way to express efficiency of column is by calculating height equivalents of theoretical plates (HETP).

H = L/N

Where H = HETP; L = Length of the column; N = number of theoretical plates. Lower the HETP, higher is the efficiency of the column, i.e., higher the theoretical plates more efficient the column.

7) Peak asymmetry

Peak asymmetry factor as can be used as a criterion of column performance. The peak half width, divided by the corresponding front half width, a gives the asymmetry factor. $A \quad \underline{b}^{s} \quad a$

Where,

'b', is the distance at 50% peak height between leading edge to the perpendicular drawn from the peak maxima

'a', is the width of the peak at half the peak height.

8) Tailing factor

The tailing factor T, a measure of peak symmetry, is unity for perfectly symmetrical peaks and its value increases as tailing becomes more pronounced.

In some cases, values less than unity may be observed. As peak asymmetry increases, integration, hence precision becomes less reliable.

According to USP (2000) Peak-tailing factor can be calculated by using the formula

$T = W_{0.05}/2\ f$

Where,

'$W_{0.05}$' is the width of the peak at 5% height and

'f' is the distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 50% of the peak height from the base line.

MOBILE PHASE:

Mobile phases used for HPLC typically are mixtures of organic solvents and water or aqueous buffers. The following points should also be considered when choosing a mobile phase.

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

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

DETECTORS:

Detectors used depend upon the property of the compounds to be separated. Optical detectors are most frequently used.

- UV-Ultraviolet Detector
- RI – Refractive Index (Universal analyte detector)
- FD – Fluorescence detector
- MS – Mass Spectroscopic detector
- PDA - Photo Diode Array Detector
- Conductivity Detector
- Amperometric Detector

HPLC METHOD DEVELOPMENT:

A good method development strategy should require only as many experimental runs as are necessary to achieve the desired final result. Finally method development should be as simple as possible and it should allow the use of sophisticated tools such as computer modelling. During initial method development, a set of initial conditions (detector, column, mobile phase) is selected to obtain the first "scouting" chromatograms of the sample. In most cases, these are based on reversed-phase separations on a C_{18} column with UV detection.

SELECTION OF CHROMATOGRAPHIC MODE:

Reversed-Phase chromatography (RPC), the most common mode for small-organic molecules. Note that ionisable compounds (acids and bases) are often separated by RPC with buffered mobile phases (to keep the analytes in a non-ionized state) or with ion-pairing reagents. In reverse phase mode, the mobile phase is comparatively more polar than the stationary phase. For the separation of polar or moderately polar compounds, the most preferred mode is reverse phase. The nature of the analyte is the primary factor in the selection of the mode of separation .A second factor is the nature of the matrix

SAMPLE PREPARATION:

Samples occur in various forms

- Solutions ready for injection

- Solutions that require dilution, buffering, addition of an internal standard or other volumetric manipulation

- Solids must be dissolved or extracted

- Samples that require pre-treatment to remove interferences and/or protect the column or equipment from damage.

CHOICE OF THE COLUMN:

Selection of the column is the first and the most important step in method development. Some of the important parameters to be considered while selecting chromatographic column.

- Length and diameter of the column
- Packing material
- Shape of the particles
- Size of the particles

SELECTION OF SOLVENT DELIVERY SYSTEM:

Chromatographic separation with isocratic elution i.e. all constituents of the mobile phase is mixed and pumped together as a single solvent, is always preferable however, gradient elution is powerful tool in achieving separation between closely eluting compounds or compounds having widely differing in polarities.

SELECTION OF MOBILE PHASE:

The primary objective in selection and optimization of mobile phase is to achieve optimum separation of all impurities and degradants from each other and from analyte peak.

In liquid chromatography, the solute retention is governed by the solute distribution factor, which reflects the different interactions of the solute-stationary phase, solute-mobile phase, and mobile phase-stationary phase. For a given stationary phase, the nature and the composition of which has to be judiciously selected in order to get appropriate and required solute retention.

- Buffer and its strength
- pH of the buffer or pH of the mobile phase
- Mobile phase composition

SELECTION OF FLOW RATE

Generally flow rate shall not be more than 2.0 ml/min. The flow rate shall be selected based on the following data.

- Retention time
- Column back pressure
- Resolution between the peaks
- Peak symmetries

The flow rate which gives least retention times, good peak symmetries, least back pressures and better separation will be selected.

PERFORMANCE CALCULATIONS SYSTEM SUITABILITY PARAMETERS

1) Retention time (t_R)
2) Resolution (R_S)
3) Capacity factor (*k`*)
4) Selectivity (α)
5) Number of Theoretical plates (N)
6) HETP
7) Asymmetry factor
8) Tailing factor

ANALYTICAL METHOD VALIDATION

Method validation can be defined as per ICH as, "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".

SPECIFICITY/SELECTIVITY

The terms selectivity and specificity are often used interchangeably. According to ICH, the term specific generally refers to a method that produces distinguishable responses for a single analyse of 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.

LINEARITY:

Linearity of an analytical procedure is its ability (with in a given range) to obtain test results which are directly proportional the concentration (amount) of analyte in the sample. It may be demonstrated directly on the drug substance (by dilution of a standard stock solution) and/or separate weighing of synthetic mixtures of the drug product components, using the proposed procedure. Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares.

RANGE:

Range is the interval between the upper and lower concentration of the analyte in the sample for which it has a suitable level of precision, accuracy and linearity**.**

ACCURACY:

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

PRECISION:

Precision is the measure of how close the data values are to each other for a series of measurements under the same analytical conditions obtained from multiple sampling of the same homogeneous sample. Precision may be considered at three levels.

LIMIT OF DETECTION:

Limit of detection is the lowest concentration of analyte in a sample which can be detected, but not necessarily quantitated, as an exact value under the stated experimental conditions.

LIMIT OF QUANTIFICATION:

Limit of quantification is the lowest concentration of analyte in a sample which can be quantitatively determined with acceptable precision and accuracy under the stated experimental conditions. Several approaches for determining the quantification limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

RUGGEDNESS:

Ruggedness is the degree of reproducibility of results obtained under a variety of conditions, such as different laboratories, analysts, instruments, environmental conditions, operators and materials. Ruggedness is determined by the analysis of aliquots from homogeneous lots in different laboratories.

ROBUSTNESS:

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

Examples of typical variations are

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

2. LITERATURE REVIEW [8-21]

Ashvin dudhrejiya et al 2014 had developed a simple, precise, sensitive and reproducible, rapid stability indicating RP-HPLC method for the simultaneous estimation of Sildenafil citrate and Fluoxetine by forced degradation studies has been developed. The proposed HPLC method utilizes the Shimadzu HPLC system on a Hiber R C-18 columns using mobile phase consisting of Acetonitrile : Potassium Dihydrogen Phosphate buffer with 25 mM Triethylamine pH adjusted to 4 with o-phosphoric acid (50:50 v/v) in an gradoamt e;itopm ,pde at a f;pw rate pf 1 ml/min, at 23°C with a load of 20□L. The detection was carried out at 223 nm. The retention time of Sildenafil citrate and Fluoxetine was found to be around 3.68 min and 5.31 min, respectively.[8]

Prasanna Reddy et al 2010 have been developed a simple, selective, accurate reverse phase-high Performance Liquid Chromatographic (RP-HPLC) method he was developed and validated for the analysis of Sildenafil Citrate in pharmaceutical formulations (tablet dosage). The mobile phase was used for acetonitrile/phosphate buffer (70:30, v/v, pH 7.0) at a flow rate of 0.8 ml/m with UV detection at 228 nm. The retention time was 4.087. The proposed method is applicable to stability studies and routine analysis of sildenafil citrate in pharmaceutical formulations.[10]

Kalyani.K et al 2015 has propossed a novel reverse phase high performance liquid chromatographic(RP-HPLC) method she has been developed and validated for simultaneous estimation of Sildenafil Citrate Dapoxetine Hydrochloride in pure and marketed formulations. mobile phase cosmposition of 0.1% orthophosphoric

acid: Acetonitrile in the ratio of 50:50 and PH adjusted to 5.0±0.1 with sodium hydroxide of the flow rate was 1.0 ml/min and the effluent was monitored at 287nm. The retenion time of Sildenafil Citrate was found to be 3.14 min and for Dapoxetine Hydrochrloride 4 min. The method was linear from the concentration of 12.5 -62.5 µg/ml and 7.5-37.5 µg/ml for the estimation of Sildenafil Citrate and Dapoxetine Hydrochloride. The method was validated according to the guidelines of international conference on harmonization (ICH) and was successfully applied in the estimation and commercial formulations.[12]

Nief R. Ahmed et al 2012 had done a simple, precise, rapid and accurate reversed–phase high performance liquid chromatographic method he has been developed for the determination of sildenafil citrate in a pure form, pharmaceutical formulations and industrial effluent samples with dilute. The method was validated for its linearity, precision and accuracy. The proposed method was successfully applied to the determination of sildenafil citrate in tablets and industrial effluent samples phosphoric acid as a mobile phase.[13]

Sushma Chilukuri et al 2016 have been developed a simple, fast, accurate, precise, rugged, sensitive and linear Reverse Phase High Performance Liquid Chromatographic (RP-HPLC) method for simultaneous quantitative estimation of Dapoxetine hydrochloride and Sildenafil citrate in capsules and validate as per ICH guidelines. The optimized method uses a reverse phase column, Phenomenex Luna C_{18} (250 X 4.6 mm;5µ), a mobile phase of triethylammonium phosphate buffer (pH 3.0):acetonitrile in the proportion of 50:50 v/v, flow rate of 1ml/min and a detection wavelength of 232nm using a UV detector. A simple, fast, accurate, precise, linear and rugged RP-HPLC method was developed for simultaneous quantitative

estimation of Dapoxetine hydrochloride and Sildenafil citrate in capsules and validated as per ICH guidelines. Hence this method can be used for the routine analysis of Dapoxetine hydrochloride and Sildenafil citrate in capsules in various pharmaceutical industries.[55]

Prasanna reddy.B et al 2008 had done a simple, selective, accurate reverse phase-high performance liquid chromatographic (RP-HPLC) method he was developed and validated for the analysis of sildenafil citrate in pharmaceutical formulations. Chromatographic separation achieved isocratically on a C_{18} column (Use Inertsil $C_{18,}$ 5µ, 150 mm x 4.6 mm) utilizing a mobile phase of acetonitrile/phosphate buffer (70:30, v/v, pH 7.0) at a flow rate of 0.8 mL/m with UV detection at 228 nm. The retention time was 4.087. The method is accurate precise and linear within range 0.1-30 µg/mL (R2=0.999) concentration and was successfully used in monitoring left over drug. The detection limit of sildenafil citrate at a signal-to-noise ratio of 3 was 1.80 ng/mL in human plasma while quantification limit in human serum was 5.60 ng/mL. The proposed method is applicable to stability studies and routine analysis of sildenafil citrate in pharmaceutical formulations as well as in human plasma samples[11]

Vinod Pawar et al 2012 a Simple, sensitive, specific, spectrophotometric method has been developed for the detection of Sildenafil citrate in pure form and Pharmaceutical formulations. The optimum condition for the analysis of the drug was established.

Sildenafil citrate exhibiting absorption at 228nm and obeyed beers law in the concentration range 10 to 50µg/ml. The correlation coefficient was found to be

0.9998 and slope of line was found to be 0.07877. The percent S.D. for intra assay precision of the method was found to be 1.9976% whereas Inter assay precision was found to be 0.8332%. The sample solution was stable up to 24 hours. The assay results were found to be in good agreement with label claim. The degradation study was checked at different conditions like with acid, alkali, dry heat, oxidative and photolytic. All the results found in degradation study were satisfactory. So this proposed method was simple sensitive, precise, quick and useful for routine quality control.[15]

Shokry.E et al 2010 two simple, accurate, sensitive and reproducible methods have been developed and subsequent validated for the determination of Sildenafil citrate (SC) in presence of its impurities "1-methyl-4-nitro-3-n-propyl-5pyrozole Carboxamide (MNC), 4-amino-1-methyl-3-n-propyl-5-pyrazole Carboxamide (AMP), 4-(2-ethoxy benzoyl amino)-1-methyl-3-n-propyl-5-pyrazole Carboxamide (EMC) and 5-(2-ethoxyphenyl)-1-methyl-3-n-propyl pyrazole [4,3-D] pyramidine-7-1(EMP)" as stability-indicating studies. In the spectrophotometric method, zero crossing technique was adopted for determination of the investigated drug in presence of impurities, by the use of derivative and derivative ratio techniques, respectively. While, the second method was isocratic reversedphase (RP) stability-indicating high-performance liquid chromatographic method, which was adopted for determination of Sildenafil citrate in presence of its impurities. The chromatographic separation was achieved isocratically by using a mobile phase of water and acetonitrile in a ratio of 40:60 V/V containing 50 mm triethylamine. The analysis was carried out using waters c18 (4.6 x 250 mm, 10 μm) at flow rate of 1.0 ml.min-1 and the UV detection at 245 nm. All the proposed methods were validated

according to the International Conference on Harmonization (ICH) guidelines reference and successfully applied for determination of Sildenafil citrate in pure form, in laboratory prepared mixtures and in pharmaceutical preparations. The obtained results were statistically compared to the reported method of analysis for Sildenafil citrate and no significant differences were found.[16]

Thangabalan B et al 2011 had done a simple, safe and sensitive method of spectroscopic determination of sildenafil citrate in UV region was developed using 8 M urea solution as hydrotropic solubilizing agent. Sildenafil citrate showed λ-max at 293 nm and beer's law was obeyed in the concentration range of 10 – 50 μg/ml. The results of analysis have been validated statistically and by recovery studies.[17]

Kalaichelvi .R et al 2012 have been developed a simple, sensitive, accurate, precise and eco-friendly UV spectrosphotometric method for sildenafil citrate was developed. In the present investigation, a 2.0 M sodium benzoate solution was employed as hydrotropic solubilizing agent to solubilize poorly water-soluble drug sildenafil citrate for its spectrophotometric analysis. Sildenafil citrate showed λ-max at 306 nm and beer's law was obeyed in the concentration range of 10 – 50 μg/ml. The results of analysis have been validated statistically and by recovery studies.[18]

Avani P et al 2015 was worked a simple, accurate, precise, reproducible and cost effective first derivative UV Spectrophotometric method developed and validated for the simultaneous estimation of Sildenafil Citrate and Aspirin in bulk as well as tablet dosage form. The solution of both the drugs and tablet were prepared in Methanol. First derivative quantitative determination of these drugs was

performed at 255 nm for Sildenafil Citrate at zero crossing point (ZCP) of Aspirin and at 291nm for Aspirin at zero crossing point (ZCP) of Sildenafil citrate. This method obeys Beer-Lambert's law in concentration range of 5-30 µg/mL and 10-80 µg/mL for Sildenafil Citrate and Aspirin respectively. Co-efficient of correlation were found to be 0.998 for both the drugs. The % RSD were not more than 2.0 % which indicates good intermediate precision. The values LOD and LOQ were 0.588µg/mL and 1.782µg/ml for Sildenafil Citrate and 1.08µg/ml and 3.27µg/ml for Aspirin respectively. Percentage estimation of Sildenafil Citrate and Aspirin in tablet dosage form were found to be 101.00 % and 99.47% respectively.[19]

Anusha. G et al 2012 a simple, sensitive, accurate, precise and eco-friendly UV spectrosphotometric method for sildenafil citrate was developed. In the present investigation, a 2.0 M sodium benzoate solution was employed as hydrotropic solubilizing agent to solubilize poorly water-soluble drug sildenafil citrate for its spectrophotometric analysis. Sildenafil citrate showed λ-max at 306 nm and beer's law was obeyed in the concentration range of 10 – 50 µg/ml. The results of analysis have been validated statistically and by recovery studies.[23]

Anuruddha P et al 2012 was described a One simple, economical, precise and accurate method is described for the simultaneous determination of Dapoxetine and Sildenafil in combined tablet dosage form. The method is Absorption Corrected Method. The amplitudes at 237.54 nm and 325.92 nm in the Absorption Corrected Method were selected to determine DPT and SL, respectively in combined formulation. The method was validated by following the analytical performance parameters suggested by the International Conference on Harmonization (ICH). All validation parameters were within the acceptable range. Under experimental

conditions described, calibration curve, assay of tablets and recovery studies were performed. A critical evaluation of proposed method was performed by statistical analysis of data where slope, intercept, correlation coefficient is shown in Table (1). As per the ICH guidelines, the method validation parameters checked. Beer's law is obeyed in the concentration range of 5-25 μg/ml for DPT and 8-40 μg/ml for SL by the given method. [12]

Yogesh Babu et al 2014 has been developed and validated a simple, rapid, selective and reproducible RPHPLC method for the Analysis of Tadalafil in Bulk, tablets and *In-Vitro* Dissolution Samples. Analysis was performed on an Agilent, EclipseC18 column (150 mm × 4.6 mm, 5 μm) with the mobile phase consisting of ammonium acetate (10 mM):methanol (35:65v/v) at a flow rate of 1.0 mL/min. UV detection was performed at 280 nm and the retention time for TDL was 4.6 minutes. The dissolution media composed of 0.5% SLS, using USP II (Paddle) Dissolution apparatus, at 50 RPM and temperature maintained 37.0 ± 0.5° C. The method was validated according to ICH guidelines. The method was validated for specificity, accuracy, precision, ruggedness, limit of quantification, limit of detection and linearity. The system suitability parameters, such as , tailing factor, theoretical plate and relative standard deviation (RSD) for assay of five standard replicates, were well within the limits. The results of the studies showed that the proposed RP-HPLC method is simple, rapid, precise and accurate. Hence the method can be used for the routine Dissolution Profiling of Tadalafil as well as its assay in bulk and tablets .[20]

Awofisayo. O.S et al 2010 was done a work on the development of basic tests for sildenafil citrate (drug substance) and sildenafil citrate tablet (dosage form). The Basic Tests encompass physical observation, melting characteristics, colour and

other test reactions for the drug substance and the dosage form. These tests are supplemented with thin layer chromatography. The test tube identification reactions were carried out to demonstrate the presence of the phenyl ether and sulphonamide functional groups as well as the N substituted piperazine and pyrimidine rings on the molecule. The presence of sulphur and nitrogen in the drug moiety were also confirmed. The Rf values obtained from the chromatograms using three different solvent systems were found to be approximately same for sildenafil in the extracted form and the powdered tablet. A summary of test result for sildenafil citrate and sildenafil citrate tablet is presented that could be of use in quick verification of the identity of the drug.[21]

Graziano.S et al 2014 Sildenafil citrate (Viagra®) is a vasoactive agent available worldwide since 1998 for the treatment of male erectile dysfunction. It is a selective phosphodiesterase type 5-enzyme inhibitor able to potentiate the downstream effects of nitric oxide on smooth muscle relaxation and vasodilation through its effects on the cyclic guanosine monophosphate (c-GMP) pathway in the erectile tissue of the penis. When sildenafil is orally administered, it is rapidly absorbed with a maximum plasma concentration achieved within 1 h and has a terminal half-life of between 3 to 6 h. The drug is extensively and rapidly metabolized by the liver, primarily by the CYP3A4 enzyme. Although the drug is well tolerated, specific adverse events have been observed, like flushing, headaches, dyspepsia, and visual disturbances. Liver toxicity related to sildenafil consumption has been considered a very rare event. However, in the last decade, some cases of sildenafil-associated hepatotoxicity have been reported. Furthermore, some hepatic intoxications have been reported after the intake of “natural” or “herbal” aphrodisiac supplements sold through Internet, sex

shops, social media, and by word-of-mouth found to contain sildenafil and other phosphodiesterase type 5 (PDE-5) inhibitors. Studies investigating a possible link between sildenafil use and liver damage are limited, and the underlying mechanism responsible for hepatotoxicity is still missing. Studies in animals evidence that the hematopoietic function of the liver may have severely been affected as a result of a probable toxic effect of sildenafil. Here, the studies reporting liver toxicity by sildenafil in humans and in animals are reported and discussed.[22]

3. AIM AND OBJECTIVE OF THE WORK

The drug analysis plays an important role in all aspects regarding the drug right the development to the therapeutic use of the drug. Industries manufacturing pharmaceutical must ensure that the raw material used and the final product obtained meets the required specification to fulfill this purpose they rely upon quantitative chemical analysis.

The Literature survey indicates that a very few methods were developed for the estimation of sildenafil citrate by RP-HPLC and UV in bulk and tablet dosage form. So an attempt was made to develop a new RP-HPLC method for sildenafil citrate in soft gelatin capsules which is more reliable, economical and flexible.

The objective of the present work is to develop a new method of estimation for sildenafil citrate in soft gelatin capsules by RP-HPLC method. So an attempt was made to develop and validate a simple, precise, accurate, linear and rapid RP-HPLC method as per ICH guidelines for the estimation of sildenafil citrate and to apply the developed method to determine the validation of compounds.

4. PLAN OF WORK

1. Literature survey.

2. Selection of drug sample.

3. Procurement of drug &other chemicals

4. UV-Visible spectrophotometry- wavelength selection

5. HPLC method development

- Selection of preliminary HPLC conditions
- Selection of mobile phase
- Selection on column
- Analysis of laboratory mixture (standard)

The steps in method development will be as follows-

- To obtain thorough knowledge of Practical RP-HPLC method.
- To establish the initial chromatographic conditions for method.
- To perform assay for sildenafil citrate.
- To validate the developed method a per the Q2 specifications of ICH guidelines.

1) Specificity

2) Linearity and Range

3) Precision

4) Accuracy

5) Ruggedness

6) Limit of detection

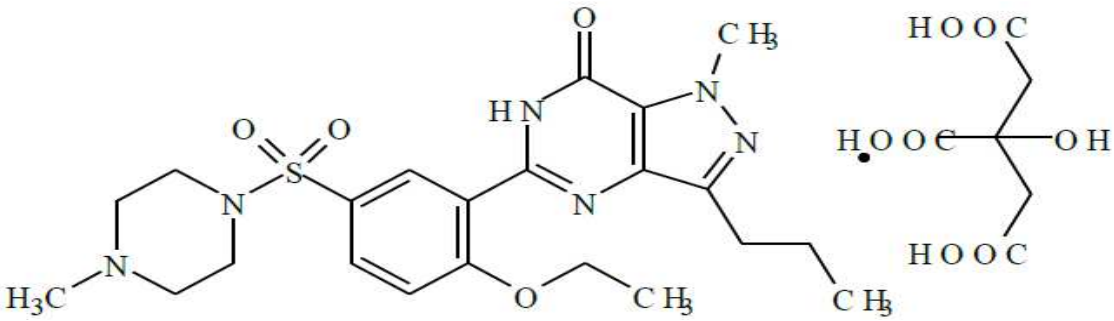
7) Limit of quantitation

8) Selectivity

5. DRUG PROFILE

Name : sildenafil citrate[24-32]

Chemical structure



Chemical Formula : $C_{28}H_{38}N_6O_{11}S$

IUPAC name : 5 -[2-ethoxy-5-(4 methyl piperazinylsulphonyl) Phenyl]-1-methyl-3-n-propyl-1,6-dihydro 7H-pyrazolo[4,3-d] pyrimidin-7-one citrate

Molecular weight : 474.5764g/mol

Description : Blue-coloured oval-shaped soft gelatine capsule

Solubility : Soluble in water, ethanol and methanol

Melting point : 187ºC to 189ºC

Drug category : phosphodiesterase type 5 inhibitor

Mechanism of action : When sexual stimulation causes local release of ON Inhibitor of PDE5 by sildenafil citrate increased levels of sildenafil causes increased levels of Cgmp in the corpus cavernosum. Sildenafil at recommeded doses has no effect in the absence of stimulation.

Pharmacokinetics

Bioavailability	:	Approximately 41% bioavailability orally
Metabolism	:	Sildenafil appears to be completely metabolized in the liver to 16 metabolites. Its metabolism is mediated mainly by cytochrome P450 microsomal isozymes 3A4 (major route) and 2C9 (minor route).
Half life	:	Approximately 4 h
Excretion	:	About 80% of an oral dose is metabolized and excreted in the feces, and about 13% is excreted in the urine.
Dosage form	:	Capsule
Route	:	Oral
Medical uses	:	Sildenafil is used to treat high blood pressure in the lungs (pulmonary hypertension). It works by relaxing and widening the blood vessels in your lungs which allows the blood to flow more easily. Decreasing high blood pressure in the lungs allows your heart and lungs to work better and improves your ability to exercise.
ADVERSE EFFECTS	**:**	Dizziness, lightheadedness, headache, flushing, stomach upset, nosebleeds, trouble sleeping, or swollen hands/ankles/feet (edema) may occur. Vision changes such as increased sensitivity to light, blurred vision, or trouble telling blue and green colors apart may also occur. If any of these effects persist or worsen, tell your doctor or pharmacist promptly.

6. MATERIALS AND INSTRUMENT USED

CHEMICAL AND SOLVENTS USED:

S. No.	Name	Grade	Make	Lot Number	Purity
01	Ammonium Acetate – AR grade	AR	Rankem	P14G102236	97.5%
02	Acetonitrile - HPLC grade	HPLC	Finar	140181013EO	99.80
03	Potassium Hydroxide	AR	Rankem	J038H10	85.0%
04	HPLC water	AN WATER			

COLUMN USED:

S. No.	Make	Column Name	ID	Serial No.
01	Waters Reliant	C18, 15 cm x 4.6mm, 5 µm	AD/LCCN/079	281I3416413863
02	Waters Reliant	C18, 15 cm x 4.6mm, 5 µm	AD/LCCN/050	276I3400214059

SAMPLE USED:

Product name	Batch number	Packing details	Mfg. Date	Exp. Date
Sildenafil Citrate Soft gelatin Capsules 50 mg	SCC - 501	Blister pack	10/2015	09/2018

WORKING STANDARD, PLACEBO USED:

S.No.	Name	WS code	Purity	Valid Up to
01	Sildenafil Citrate Working Standard	CP2WS/058/001	99.02 %	June-2016
02	Placebo	SCC-501P	Not Applicable	

INSTRUMENTS USED:

S.No.	Name	Make & Model	ID	Date of Calibration	Due date of Calibration
01	Analytical Weighing Balance	Mettler Toledo XS105	AD/INS/001	Daily calibration	
02	HPLC	Agilent 1260 & 1290 series	AD/INS/030	07.10.2015	02.04.2016
			AD/INS/031	07.10.2015	02.04.2016
03	Sonicator	PCI India	AD/INS/016	Not Applicable	
04	pH Meter	Hanna	AD/INS/035	Daily calibration	

7. METHOD DEVELOPMENT AND VALIDATION

SOLUBILITY:

According to literature review collected sildenafil citrate are freely soluble in water, methanol, ethanol, DMSO and DMF. The solubility was checked and finally ammonium acetate buffer was chosen for present work.

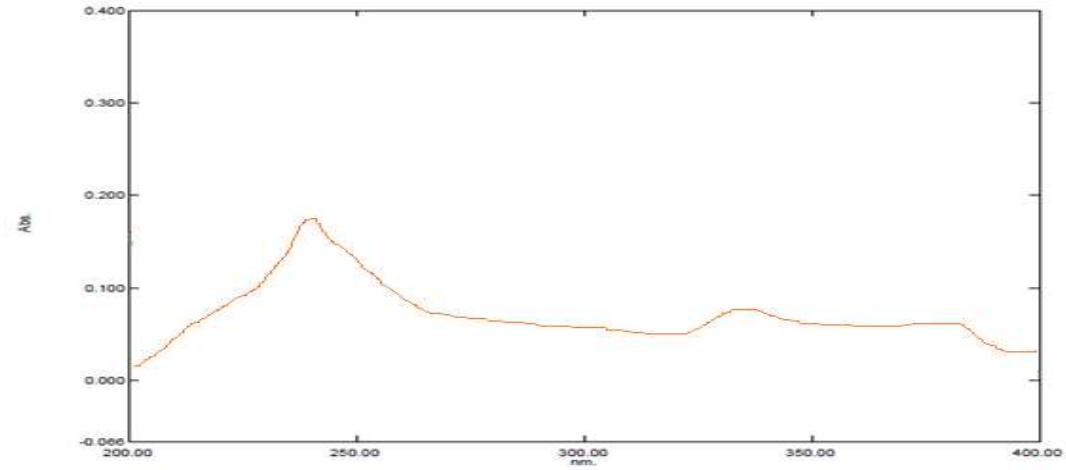
SELECTION OF CHROMATOGRAPHIC METHOD FOR SEPARATION:

Selection of the method depends upon the nature of sample (ionic/ ionisable/ neutral), its molecular weight and solubility. Most of the drugs are polar in nature and hence reversed phase HPLC is preferred over the normal phase HPLC method. The drug combinations concerned for the present study are also polar. Hence reversed phase HPLC was selected for separation of the drug combination because of its suitability.

SELECTION OF DETECTION OF WAVELENGTH (λ_{max}):

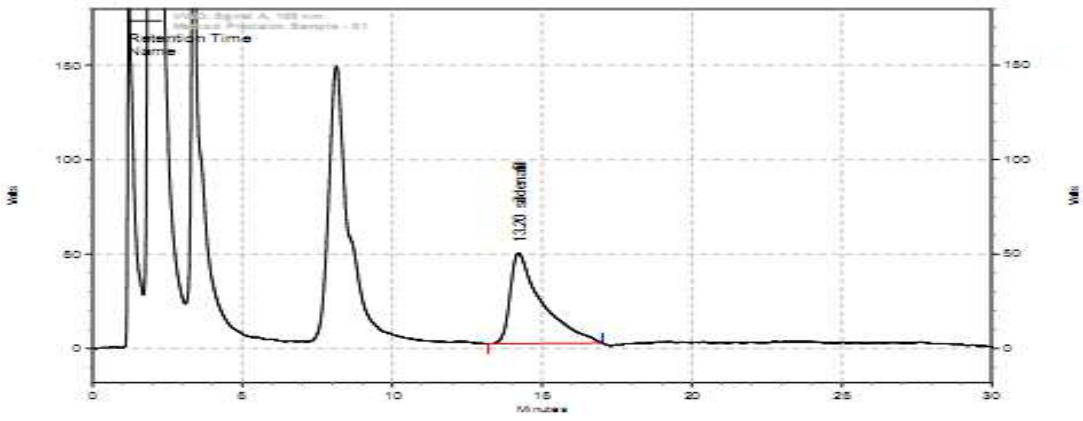
Selection of detection of wavelength is a critical step in the analytical method. The spectrum of diluted solutions was scanned over the range of 200 – 400 nm in spectrum mode.

UV SPECTRA OF SILDENAFIL CITRATE



TRIAL 1

Column	:	waters reliant C_{18}, 15 cm x 4.6 mm, 5 µm
Flow Rate	:	1.0 mL/minute
Pump mode	:	Isocratic
Detector wavelength	:	240 nm
Injection volume	:	20 µL
Column Temperature	:	25.0˚C
Mobile phase composition	:	Phosphate buffer: Acetonitrile 1:1% v/v

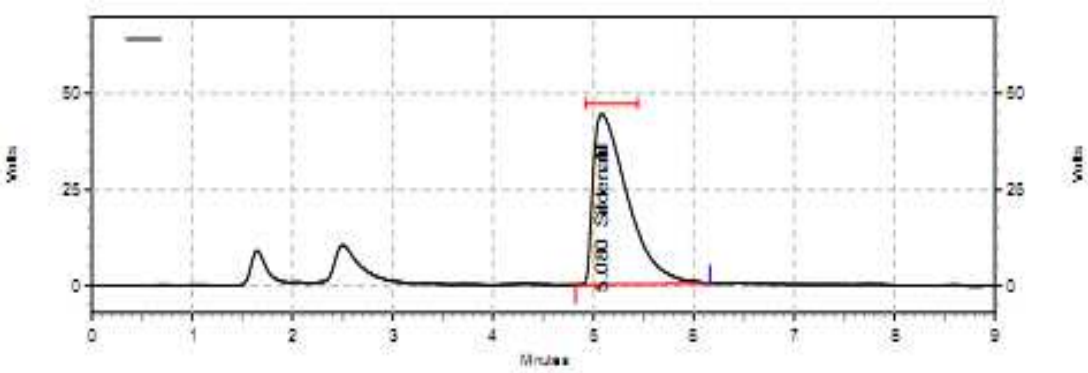


VWD: Signal A, 195 nm
Results

Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	sildenafil	13.203	69726042	1065	2.44

TRIAL 2

Column	:	C18, 15 cm x 4.6 mm, 5 µm
Flow Rate	:	1.0 mL/minute
Pump mode	:	Isocratic
Detector wavelength	:	240 nm
Injection volume	:	20 µL
Column Temperature	:	25.0˚C
Mobile phase composition	:	Ammonium acetate: Acetonitrile 1.5:0.5% v/v

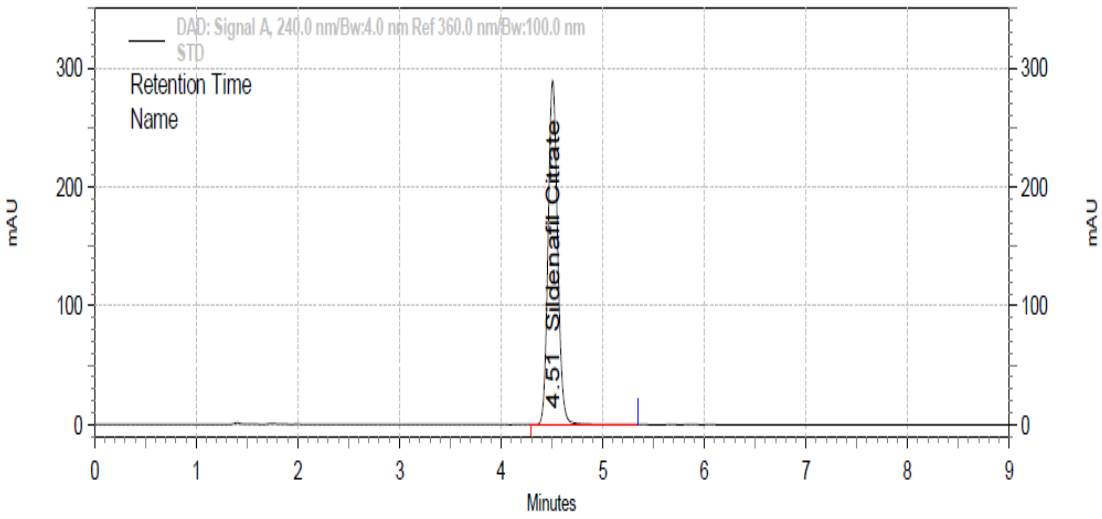


Results

Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
Sildenafil	5.080	142883755	1064	2.88

Optimized Chromatographic conditions:

Column	:	C18, 15 cm x 4.6 mm, 5 µm
Flow Rate	:	1.0 mL/minute
Pump mode	:	Isocratic
Detector wavelength	:	240 nm
Injection volume	:	20 µL
Column Temperature	:	25.0˚C
Mobile phase composition	:	Ammonium acetate buffer : Acetonitrile 1:1% v/v



Methodology adopted

- Mobile phase selection
- Preparation of buffer solution
- Preparation of mobile phase
- Preparation of diluent
- Preparation of standard stock solution

- Preparation of standard solution
- Preparation of sample solution
- Setting the instrumental parameters before performing the analysis for
    1. Detector
    2. Pump
- Development of chromatogram and determination of retention time.

Selection of mobile phase:

The mobile phase system consisting of ammonium acetate and acetonitrile in the ratio of 1:1v/v was found to be effective in the separation of sildenafil citrate in pure form as well as in dosage forms.

MOBILE PHASE PREPARATION:

Preparation of 5M Potassium hydroxide solution:

Weigh and dissolve 28.0 g of Potassium hydroxide in a 100 mL volumetric flask and dilute to volume with purified water.

Preparation of 0.2M Ammonium acetate Buffer:

Weigh and dissolve 15.42 g of ammonium acetate in a 1000 mL volumetric flask, and dilute up to the volume with purified water. Adjust the pH to 7.00 with 5M Potassium hydroxide solution.

Preparation of Mobile Phase:

Prepare a mixture 500 volume of Ammonium acetate Buffer and 500 volume of Acetonitrile, mix well. Filter the solution through 0.45 µm nylon filter and sonicate for 10 minutes.

Preparation of Diluent:

Mobile phase

Procedure for preparation of analytical solution:

Preparation of standard solution (50.0 mcg /mL of Sildenafil Citrate):

Weigh accurately 50 mg of Sildenafil Citrate working standard and transfer into 100 mL volumetric flask, add 60 mL of mobile phase and sonicate for 5 minutes to dissolve. Cool and dilute up to the volume with mobile phase and mix well. Transfer 5 mL of the above solution through pipette into 50 mL volumetric flask and dilute up to the volume with mobile phase . Filter the solution through 0.45 µm nylon filter and collect the solution in an HPLC vial after discarding about the first 2 mL of the filtrate.

Preparation of sample solution for 50 mg (50.0 mcg/mL of Sildenafil Citrate):

Accurately weigh and transfer 5 soft gelatin capsules (equivalent to 250 mg of Sildenafil Citrate) into 250 mL volumetric flask. Add about 25 mL of water and sonicate for 10 minutes to disperse the capsules shell. Add about 120 mL of mobile phase and sonicate for 15 minutes to dissolve. Cool and dilute up to the volume with mobile phases and mix well. Transfer 5 mL of the above solution through pipette

into 100 mL volumetric flask and dilute up to the volume with mobile phases and mix. Filter the solution through 0.45 µm nylon filter and collect the solution in an HPLC vial after discarding about the first 2 mL of the filtrate.

METHOD VALIDATION

Validation of analytical method is a process to establish that the performance characteristics of the developed method meet the requirement of the intended analytical application.[34-53]

SYSTEM SUITABILITY PARAMETERS

System suitability is the test to ensure that the methods can generate results of acceptable accuracy and precision.

Standard solutions of ranitidine Hcl were prepared as per test method and five replicate injections were made to study system suitability parameters.

Parameters studied Tailing Factor.

Number of theoretical plates (N). Retention time.

Relative standard deviation.

Typical analytical parameters used in method validation include

1. Specificity
2. Linearity and Range
3. Precision
4. Accuracy

5. Ruggedness
6. Limit of detection
7. Limit of quantitation
8. Selectivity

SPECIFICITY

The specificity of the method can be defined as the ability to measure accurately the concentration of an analyte in the presence of all other sample materials.

Procedure:

Inject the blank, placebo, standard and sample preparations based on "**Injection sequence"** detailed below and measure the corresponding area**.**

Injection sequence:

Particulars	Number of Injection
Blank	1
Placebo preparation	1
Standard preparation	5
Sample preparation	2
Bracketing standard	1

Acceptance criteria:

No any peak should be obtained in the retention time of Sildenafil Citrate from the blank and placebo chromatograms.

LINEARITY AND RANGE

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

Ability to obtain test results which are directly proportional to the concentration of analyte. For an establishment of the linearity 60%, 80%, 100%, 120%, and 160% of standard and sample concentrations shall be used.

Preparation of Linearity from standard:

Standard stock solution (500 mcg/mL of Sildenafil Citrate):

Weigh accurately about 50 mg of Sildenafil Citrate working standard and transfer into 100 mL volumetric flask, add 60 mL of mobile phase and sonicate for 5 minutes to dissolve. Cool and dilute up to the volume with mobile phase and mix well.

60 % Linearity standard concentration (30.0 mcg/mL of Sildenafil Citrate):

Transfer 3.0 mL of above standard stock solution through pipette into a 50 mL volumetric flask and dilute up to the volume with mobile phase and mix. Filter the

solution through 0.45 µm nylon membrane filter and collect the solution in an HPLC vial after discarding about the first 2 mL of the filtrate.

80 % Linearity standard concentration (40.0 mcg/mL of Sildenafil Citrate):

Transfer 4.0 mL of above standard stock solution through pipette into a 50 mL volumetric flask and dilute up to the volume with mobile phase and mix. Filter the solution through 0.45 µm nylon membrane filter and collect the solution in an HPLC vial after discarding about first 2 mL of filtrate.

100 % Linearity standard concentration (50.0 mcg/mL of Sildenafil Citrate):

Transfer 5.0 mL of above standard stock solution through pipette into a 50 mL volumetric flask and dilute up to the volume with mobile phase and mix. Filter the solution through 0.45 µm nylon membrane filter and collect the solution in an HPLC vial after discarding about the first 2 mL of the filtrate.

120 % Linearity standard concentration (60.0 mcg/mL of Sildenafil Citrate):

Transfer 6.0 mL of above standard stock solution through pipette into a 50 mL volumetric flask and dilute up to the volume with mobile phase and mix. Filter the solution through 0.45 µm nylon membrane filter and collect the solution in an HPLC vial after discarding about the first 2 mL of the filtrate.

160 % Linearity standard concentration (80.0 mcg/mL of Sildenafil Citrate):

Transfer 8.0 mL of above standard stock solution through pipette into a 50 mL volumetric flask and dilute up to the volume with mobile phase and mix. Filter the

solution through 0.45 µm nylon membrane filter and collect the solution in an HPLC vial after discarding about the first 2 mL of the filtrate.

Preparation of Linearity solution from Sample:

Sample stock solution (1000 mcg/mL of Sildenafil Citrate):

Accurately weigh and transfer 5 soft gelatin capsules of Sildenafil Citrate sample (equivalent to 250 mg of Sildenafil Citrate) into 250 mL volumetric flask. Add about 25 mL of water and sonicate for 10 minutes to disperse the capsules shell. Add about 120 mL of mobile phase and sonicate for 15 minutes to dissolve. Cool and dilute up to the volume with mobile phases and mix well.

60 % Linearity sample concentration (30.0 mcg/mL of Sildenafil Citrate):

Transfer 3.0 mL of above Sample stock solution through pipette into a 100 mL volumetric flask and dilute up to the volume with mobile phase and mix. Filter the solution through 0.45 µm nylon membrane filter and collect the solution in an HPLC vial after discarding about the first 2 mL of the filtrate.

80 % Linearity sample concentration (40.0 mcg/mL of Sildenafil Citrate):

Transfer 4.0 mL of above Sample stock solution through pipette into a 100 mL volumetric flask and dilute up to the volume with mobile phase and mix. Filter the solution through 0.45 µm nylon membrane filter and collect the solution in an HPLC vial after discarding about the first 2 mL of the filtrate.

100 % Linearity sample concentration (50.0 mcg/mL of Sildenafil Citrate):

Transfer 5.0 mL of above Sample stock solution through pipette into a 100 mL volumetric flask and dilute up to the volume with mobile phase and mix. Filter the solution through 0.45 µm nylon membrane filter and collect the solution in an HPLC vial after discarding about the first 2 mL of the filtrate.

120 % Linearity sample concentration (60.0 mcg/mL of Sildenafil Citrate):

Transfer 6.0 mL of above Sample stock solution through pipette into a 100 mL volumetric flask and dilute up to the volume with mobile phase and mix. Filter the solution through 0.45 µm nylon membrane filter and collect the solution in an HPLC vial after discarding about the first 2 mL of the filtrate.

160 % Linearity sample concentration (80.0 mcg/mL of Sildenafil Citrate):

Transfer 8.0 mL of above Sample stock solution through pipette into a 100 mL volumetric flask and dilute up to the volume with mobile phase and mix. Filter the solution through 0.45 µm nylon membrane filter and collect the solution in an HPLC vial after discarding about the first 2 mL of the filtrate.

Injection sequence:

Different Linearity Standard and Sample preparations	Number of Injection
Blank	1
Standard preparation	5
60 % Linearity standard and sample concentration	2
80 % Linearity standard and sample concentration	2
100 % Linearity standard and sample concentration	2
120 % Linearity standard and sample concentration	2
160 % Linearity standard and sample concentration	2
Bracketing Standard	1

Acceptance criteria:

- Correlation co-efficient between concentrations and its area should be more than 0.998.

PRECISION

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements under the prescribed conditions.

SYSTEM PRECISION:

Determines the closeness of agreement of the same homogenous standard preparations under the prescribe conditions.

Procedure:

Inject the blank, standard preparations based on "**Injection sequence"**

detailed below and measure the corresponding area**.**

Injection sequence:

Particulars	Number of Injection
Blank	1
Standard preparation	5

System Suitability requirement:

Report the system suitability requirement **A to C** as mentioned in the method of analysis in the step no.: 5.0

Acceptance criteria:

- The Tailing factor of the peak due to Sildenafil Citrate obtained from five replicates standard solution injections should be not more than 2.0.
- Theoretical plates of the peak obtained for five replicates standard solution injections of Sildenafil Citrate should be not less than 2000.
- The relative standard deviation of the area obtained for Five replicate standard solution of Sildenafil Citrate should be not more than 2.0%.
- The relative standard deviation of the retention time obtained for five replicate standard solution of Sildenafil Citrate should be not more than 1.0%.

A) SYSTEM PRECISION

METHOD PRECISION (REPEATABILITY):

Determines the closeness of agreement of the same homogenous sample under the prescribe conditions. Performing assay of sildenafil citrate Soft gelatin Capsules 50 mg a minimum of six sample preparations from a single batch shall be made and analyze separately.

Procedure:

Inject the blank, standard and sample preparations based on "Injection sequence" detailed below and measure the corresponding area.

Injection sequence:

Particulars	Number of Injection
Blank	1
Standard preparation	5
Sample preparations – 1,2,3,4,5,6	2
Bracketing standard	1

Acceptance criteria:

- Assay obtained for each six sample preparations should be between 90.0 and 110.0%.
- The RSD obtained for the assay results for six sample preparations should be not more than 2.0 %.

METHOD PRECISION (REPEATABILITY):

The method precision was determined by preparing six sample solutions of Sildenafil Citrate from Sildenafil Citrate Soft gelatin Capsules 50 mg as per the procedure included in the protocol.

ACCURACY

Accuracy is defined as closeness of measured value to the true value The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

Preparation of analytical solution:

Preparation of standard solution (50.0 mcg /mL of Sildenafil Citrate):

Weigh accurately about 50 mg of Sildenafil Citrate working standard and transfer into 100 mL volumetric flask, add 60 mL of mobile phase and sonicate for 5 minutes to dissolve. Cool and dilute up to the volume with mobile phase and mix well. Transfer 5 mL of the above solution through pipette into 50 mL volumetric flask and dilute up to the volume with mobile phase and mix well. Filter the solution through 0.45 µm nylon filter and collect the solution in an HPLC vial after discarding about the first 2 mL of the filtrate.

Placebo spiked with 50 % standard preparation (25.0 mcg/mL of Sildenafil Citrate):

Weigh accurately 1 Sildenafil Citrate soft gelatin capsules 100 mg Placebo and 50 mg of Sildenafil Citrate Working standard and transfer into 200 mL volumetric flask. Add about 25 mL of water and sonicate for 10 minutes to disperse the capsules shell. Add about 100 mL of mobile phase and sonicate for 15 minutes to dissolve. Cool and dilute up to the volume with mobile phase and mix well. Transfer 5 mL of the above solution through pipette into 50 mL volumetric flask and dilute up to the volume with mobile phase and mix. Filter the solution through 0.45 µm nylon filter and collect the solution in an HPLC vial after discarding about first 2 mL of filtrate. Prepare the sample preparation in triplicate.

Placebo spiked with 100 % standard preparation (50.0 mcg/mL of Sildenafil Citrate):

Weigh accurately 1 Sildenafil Citrate soft gelatin capsules 100 mg Placebo and 100 mg of Sildenafil Citrate Working standard and transfer into 200 mL volumetric flask. Add about 25 mL of water and sonicate for 10 minutes to disperse the capsules shell. Add about 100 mL of mobile phase and sonicate for 15 minutes to dissolve. Cool and dilute up to the volume with mobile phase and mix well. Transfer 5 mL of the above solution through pipette into 50 mL volumetric flask and dilute up to the volume with mobile phase and mix. Filter the solution through 0.45 µm nylon filter and collect the solution in an HPLC vial after discarding about first 2 mL of filtrate. Prepare the sample preparation in triplicate.

Placebo spiked with 150 % standard preparation (75.0 mcg/mL of Sildenafil Citrate):

Weigh accurately 1 Sildenafil Citrate soft gelatin capsules 100 mg Placebo and 150 mg of Sildenafil Citrate Working standard and transfer into 200 mL volumetric flask. Add about 25 mL of water and sonicate for 10 minutes to disperse the capsules shell. Add about 100 mL of mobile phase and sonicate for 15 minutes to dissolve. Cool and dilute up to the volume with mobile phase and mix well. Transfer 5 mL of the above solution through pipette into 50 mL volumetric flask and dilute up to the volume with mobile phase and mix. Filter the solution through 0.45 µm nylon filter and collect the solution in an HPLC vial after discarding about first 2 mL of filtrate. Prepare the sample preparation in triplicate.

Procedure:

Inject the blank, standard and placebo spiked standard preparations based on "**Injection sequence"** detailed below and measure the corresponding area**.**

Injection sequence Table:

Particulars	**No. of injections**
Blank	1
Standard preparation	5
Placebo spiked standard solution 50% - preparations 1, 2 & 3	Each 3
Placebo spiked standard solution 100% - preparations 1, 2 & 3	Each 3
Placebo spiked standard solution 150% - preparations 1, 2 & 3	Each 3
Bracketing Standard	1

Calculations for Sildenafil Citrate accuracy

Step 1: Standard added in mg (Working standard solution)

$$\frac{\text{Standard wt in mg}}{100} \times \frac{5}{50}$$

Step 2: Placebo spiked standard added in mg at 100%

$$\frac{\text{Wt of spiked standard in mg}}{200} \times \frac{5}{50}$$

Step 3: Placebo spiked standard recovered in mg

$$\frac{\text{Placebo spiked standard area X standard added in mg (Working standard solution)}}{\text{Average area of working standard solution}}$$

Step 4: Recovery in percentage

$$\frac{\text{Placebo spiked standard recovered in mg}}{\text{Placebo spiked standard added in mg}} \times 100$$

System Suitability requirement:

Report the system suitability requirement **A, B and C** as mentioned in the method of analysis in the step No.: 5.0.

Acceptance criteria:

In each concentration, the sildenafil citrate working standard spiked with placebo should be recovered between 98.0 % and 102.0 %.

ROBUSTNESS:

The Robustness for the analytical procedure expresses a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during analysis

Procedure:

Working standard solutions and working sample solution were prepared by different analyst on different days. Solutions were injected as per the test method and chromatograms were recorded.

Inject the blank, standard and sample solution preparations based on "**Injection sequence"** detailed below and measure the corresponding area**.**

Injection sequence:

Particulars	Number of Injection
Blank	1
Standard preparation	5
Sample preparations – 1,2,3,4,5,6	2
Bracketing standard	1

Acceptance criteria:

- Assay obtained for each six sample preparations should be between 90.0 and 110.0 %.
- The RSD obtained for the assay results for six sample preparations should be not more than 2.0 %.

a. INTERMEDIATE PRECISION-(RUGGEDNESS) :

The Intermediate precision was determined by preparing six sample solutions of Sildenafil Citrate Soft gelatin Capsules 50 mg by a different analyst.

Robustness

Robustness of an analytical method is measure of its capacity to remain unaffected by small but deliberate variation in method parameters. It provides information about the reliability of method.

DETERMINATION:

The robustness of an analytical method is determined by analysis of aliquots of homogenous lots by differing physical parameters like flow rate and column temperature.

Change in flow rate plus (1.2 mL/minute):

For Chromatographic conditions, follow the method of analysis given in the step No.: 5.0 except by changing the flow to 1.2 mL / minute instead of 1.0 mL / minute.

Change in flow rate minus (0.8 mL/minute):

For Chromatographic conditions, follow the method of analysis given in the step No.: 5.0 except by changing the flow to 0.8 mL / minute instead of 1.0 mL / minute.

Change in wavelength plus (242 nm):

For Chromatographic conditions, follow the method of analysis given in the step No.: 5.0 except by changing the wavelength to 242 nm instead of 240 nm.

Change in wavelength minus (238 nm):

For Chromatographic conditions, follow the method of analysis given in the step No.: 5.0 except by changing the wavelength to 238 nm instead of 240 nm.

System Suitability requirement:

Report the system suitability requirement **A, B and C** as mentioned in the method of analysis given in the step No.: 5.0.

Acceptance criteria:

- Assay obtained for each robustness parameter should be between 90.0 and 110.0%.
- The combined RSD obtained for the assay result of an each robustness parameter and six assay results of method precision should be not more than 2.0 %.

ROBUSTNESS

A deliberate plus and minus changes in the analytical method parameters such as in wavelength, and flow rate was altered and the assay analytical study done as per the protocol.

STABILITY OF ANALYTICAL SOLUTIONS:

Solutions to be used in the analytical method should be analyzed for the study of their stability. This study should be performed by injecting standard solution and sample solution at probable time points, and minimum not less than 24 hours shall be studied.

Note:

- Maintain the chromatographic conditions and solutions preparation as per the procedure given in method of analysis which is described under section No.:5.0 and injections sequence for solution stability study is given in below table.
- The maximum time for inject able usage of standard and sample solution is absolutely depends on the **X Hours** of solutions stability studied.

Injection sequence:

Table No. 24

Particulars	Number of Injections
Blank (0 Hour)	1
Standard preparation (0 Hour)	1
Sample preparation (0 Hour)	1
Blank (X Hours)	1
Standard preparation (X Hours)	1
Sample preparation (X Hours)	1

Acceptance criteria:

Cumulative % RSD for area obtained between initial time point and various probable intervals time points should be not more than 2.0 %.

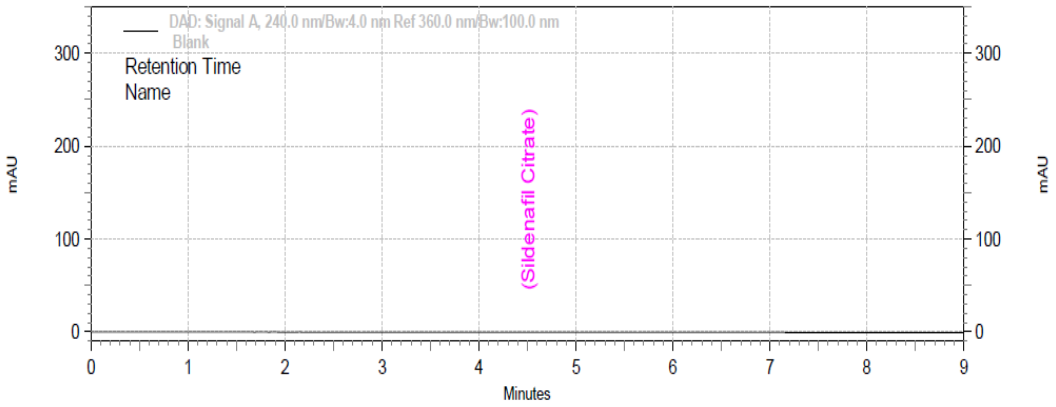
STABILITY OF ANALYTICAL SOLUTIONS:

Standard and sample solutions to be used in the analytical method are scrutinized for their solution's stability. This study was performed by injecting standard and sample solution for the period of 24 hours.

8. CHROMOTOGRAMS

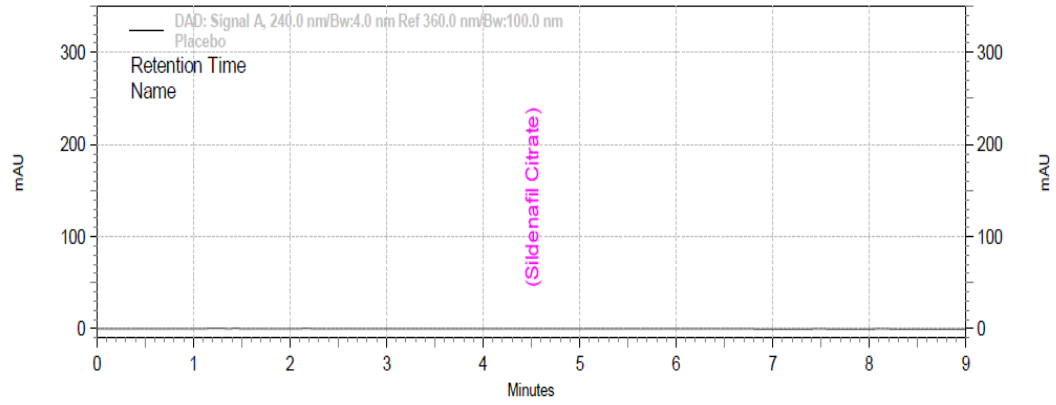
SPECIFICITY (blank , standard & sample)

Chromatogram No : 8.1 Blank



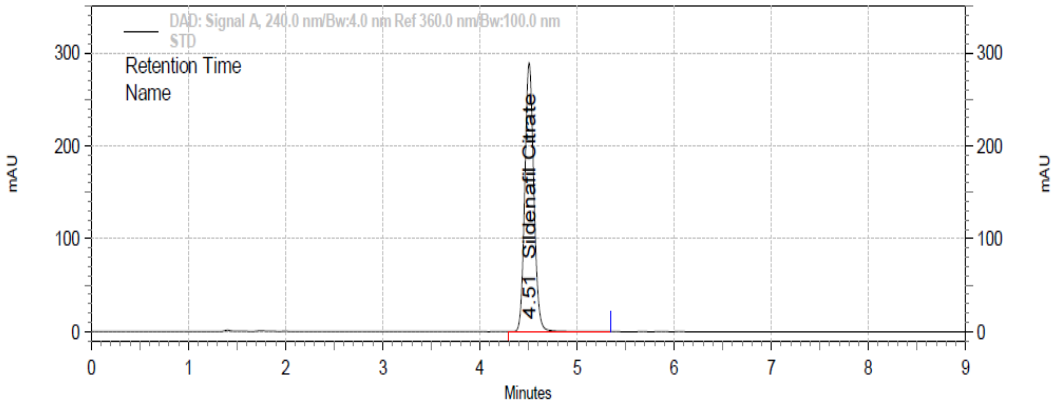
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry

Chromatogram No : 8.2 placebo



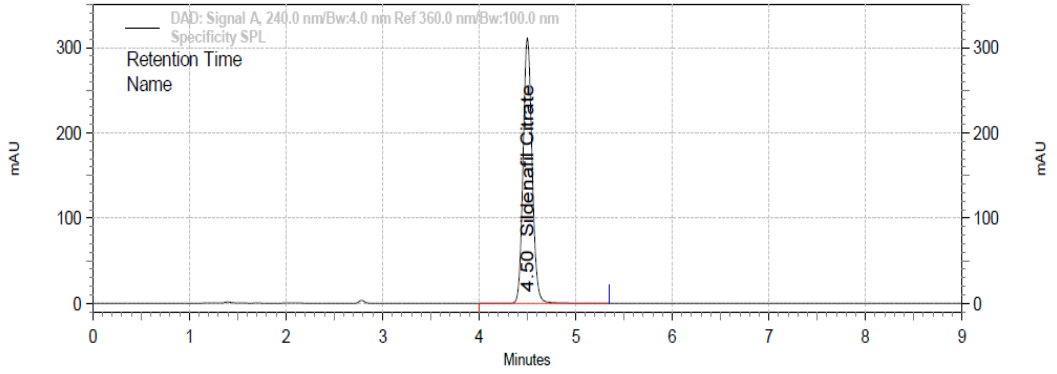
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry

Chromatogram No : 8.3 Standard



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	4.507	251710820	11325	1.12
Totals			251710820		

Chromatogram No : 8.4 Sample

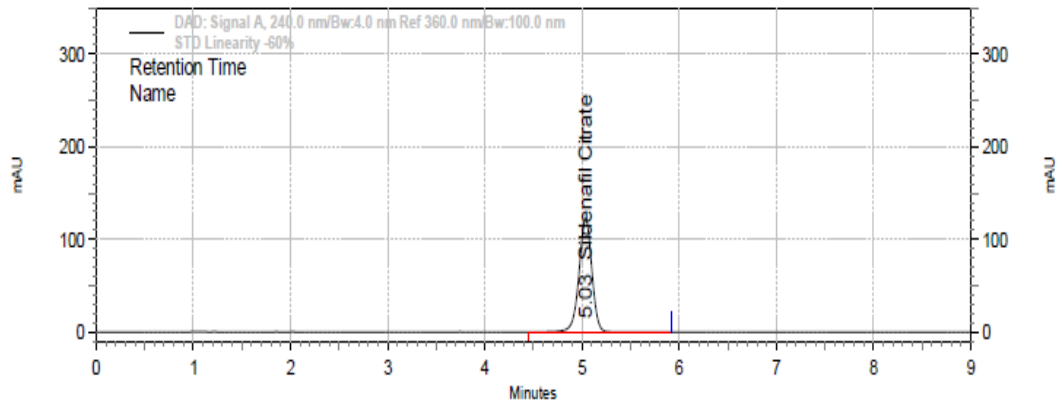


Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	4.500	270393401	11311	1.11
Totals			270393401		

LINEARITY

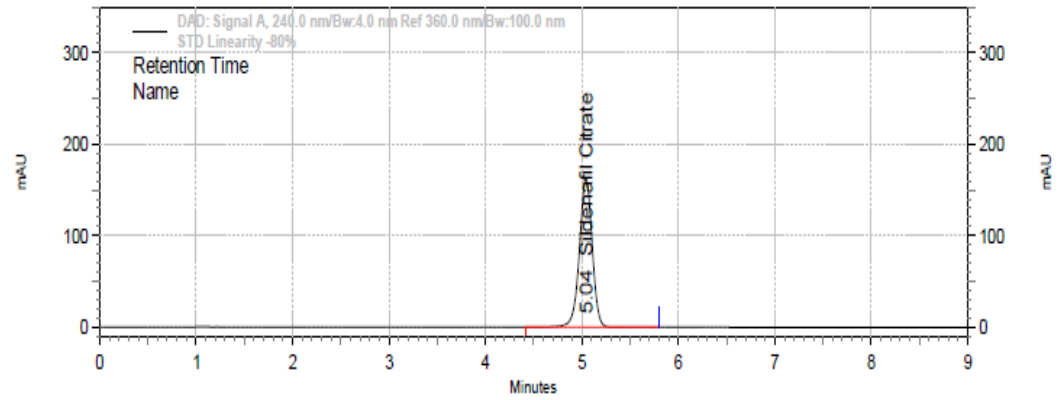
Standard

Chromatogram No:8.5 Linearity 60%



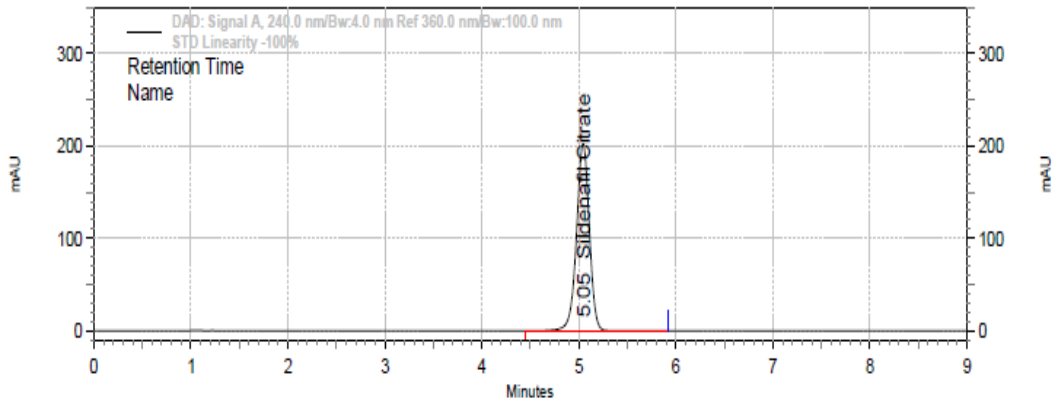
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.033	151677294	6993	0.98
Totals			151677294		

Chromatogram No:8.6 linearity 80%



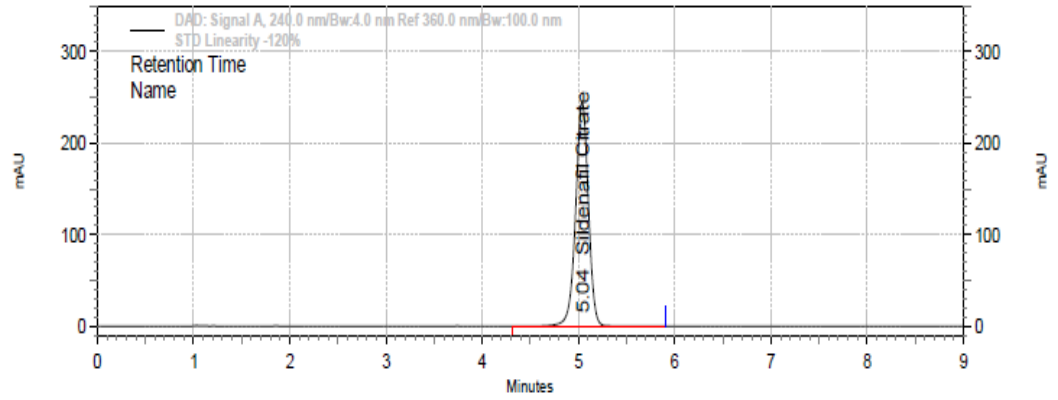
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.040	201279288	6960	0.97
Totals			201279288		

Chromatogram No : 8.7 Linearity 100%



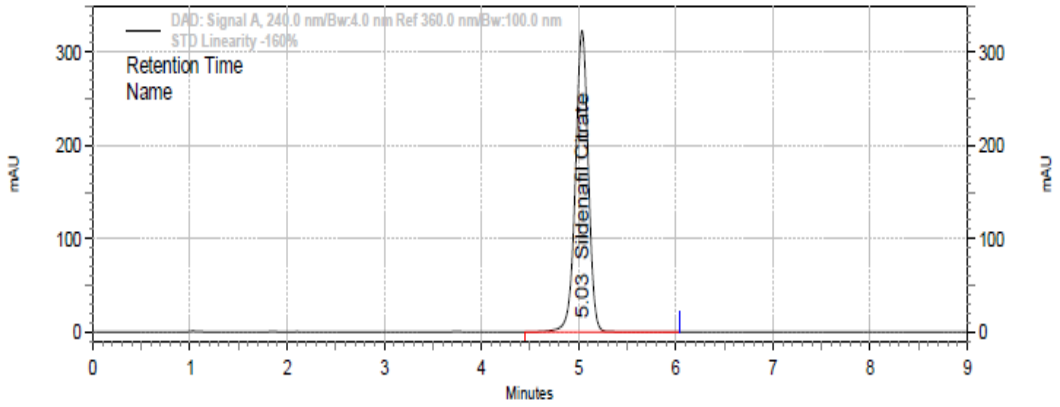
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.047	250885878	7005	0.94
Totals			250885878		

Chromatogram No : 8.8 Linearity 120%



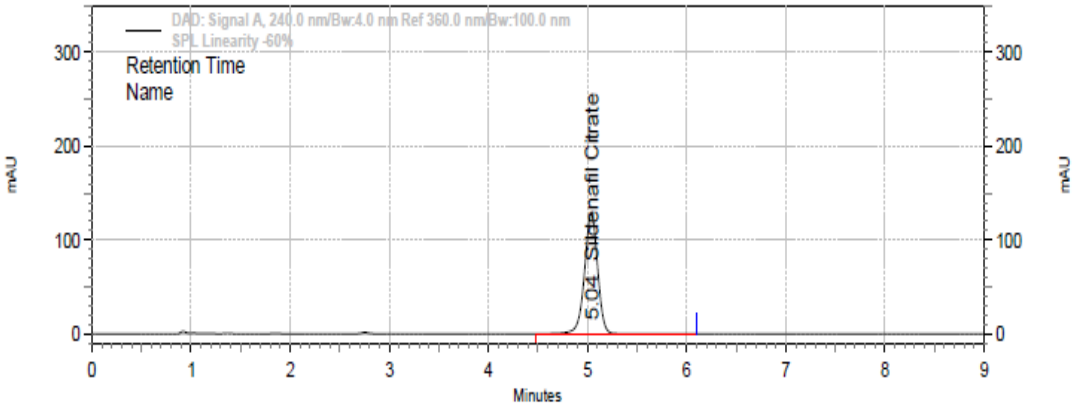
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.040	303794785	6935	0.95
Totals			303794785		

Chromatogram No : 8.9 Linearity 160% Standard



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.033	399892519	6994	0.97
Totals			399892519		

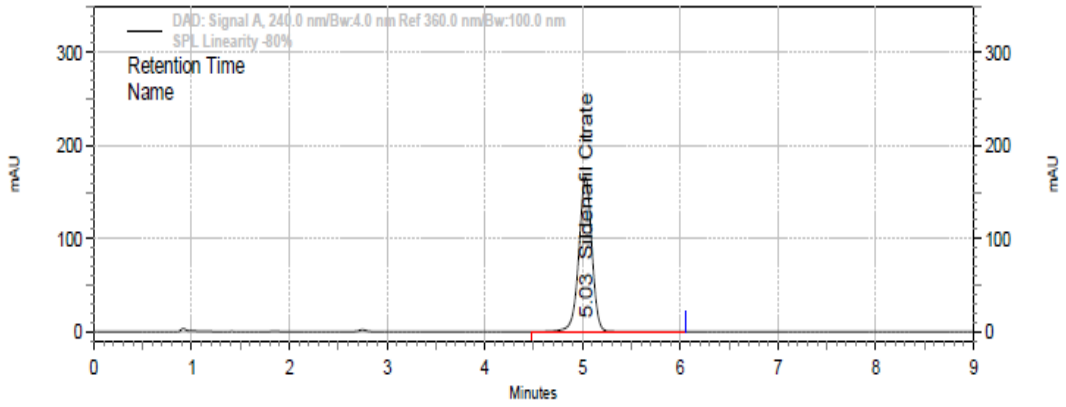
Chromatogram No. : 8.10 Linearity 160%



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.040	158469512	6980	0.94
Totals			158469512		

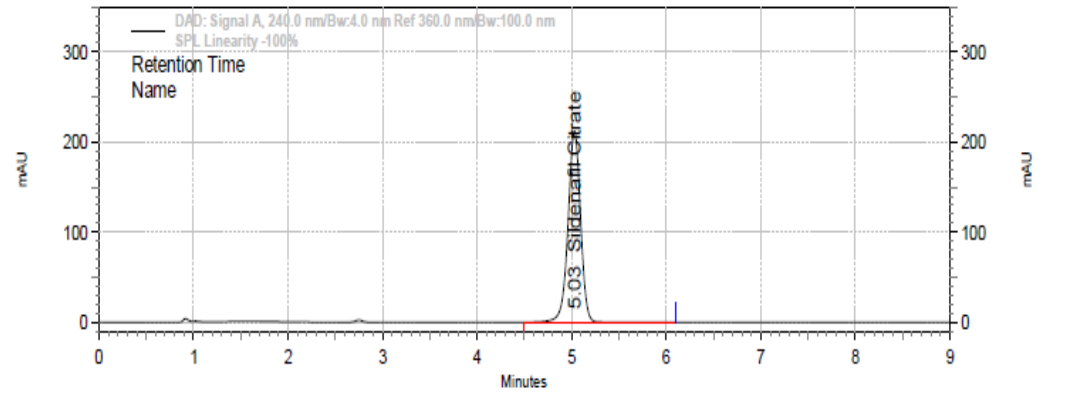
Sample

Chromatogram No : 8.11 Linearity 60%



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.033	205672985	6946	0.94
Totals			205672985		

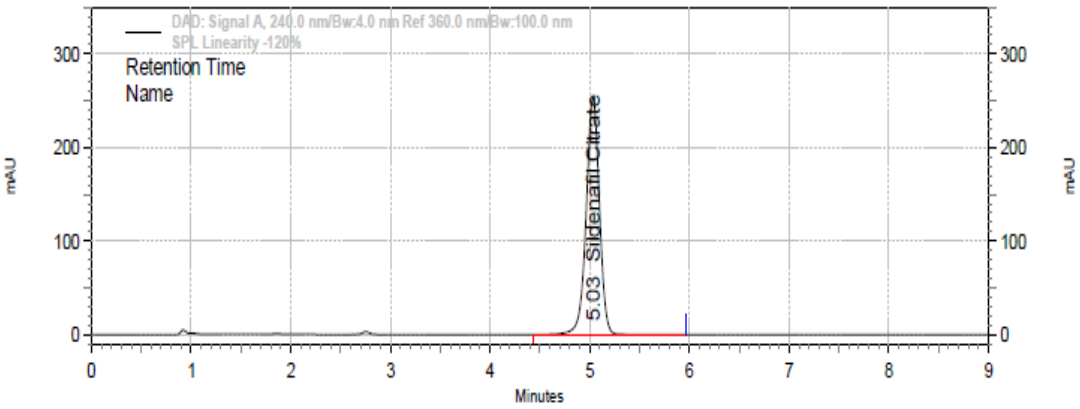
Chromatogram No. : 8.12 Linearity 100%



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.027	262694385	6981	0.97
Totals			262694385		

Chromatogram No : 8.13

Linearity 120%

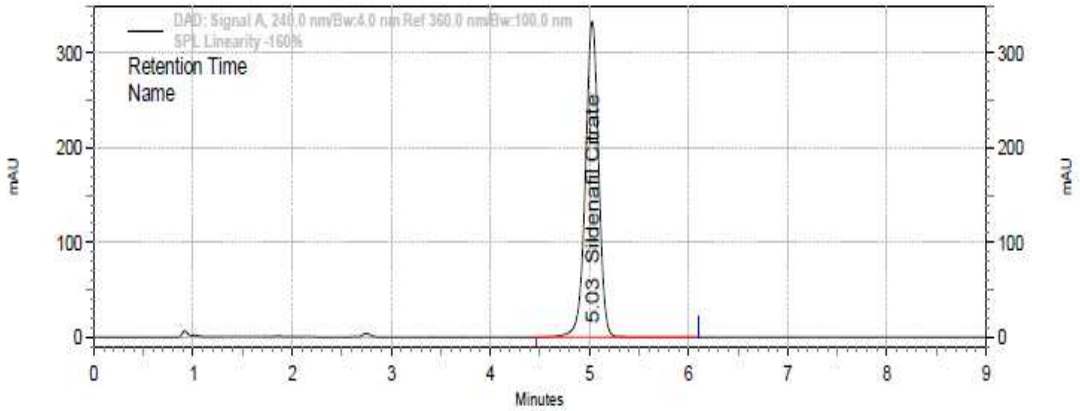


Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.033	313842623	6925	0.95

Totals			313842623

Chromatogram No : 8.14

Linearity 160% Sample

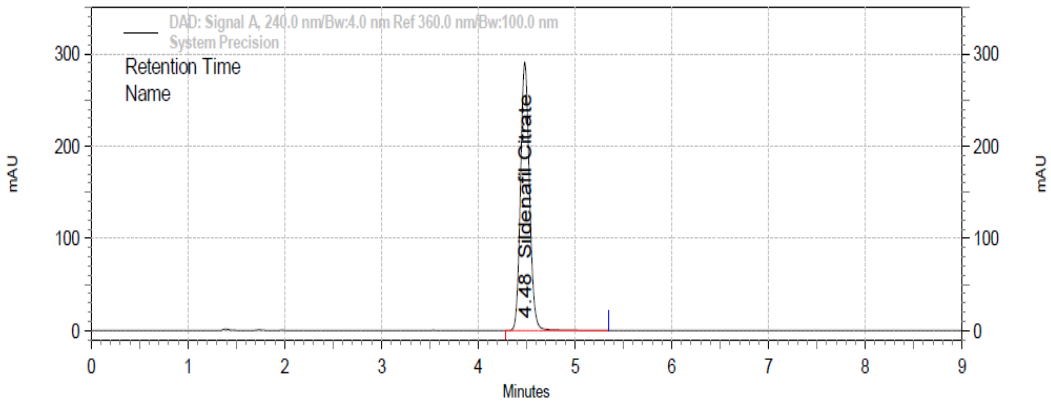


Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.027	413117111	6923	0.96

Totals			413117111

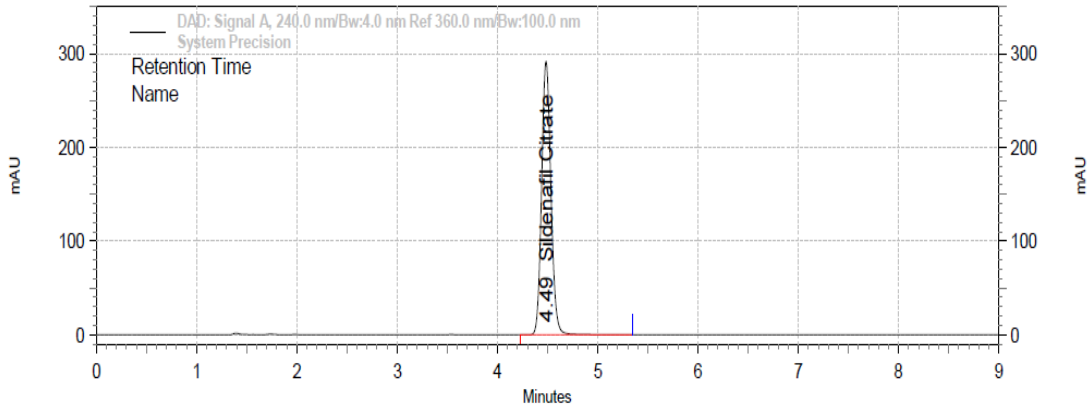
SYSTEM PRECISION

Chromatogram No : 8.15



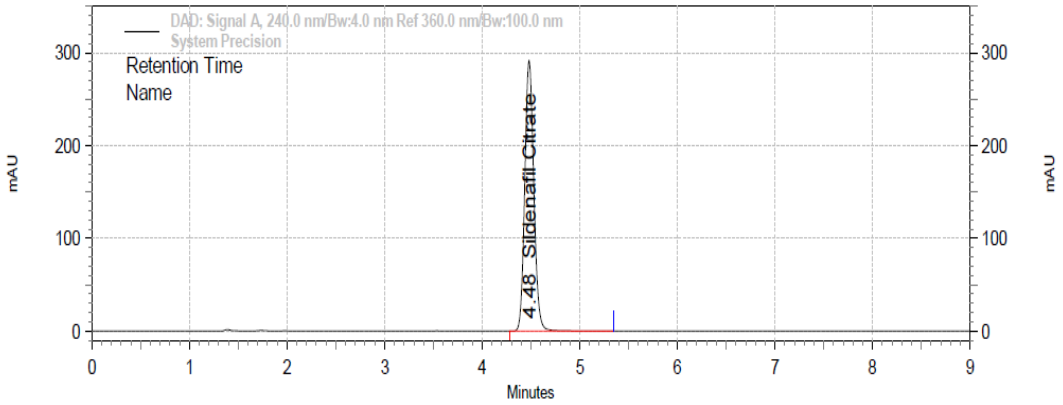
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	4.480	252184542	11339	1.13
Totals			252184542		

Chromatogram No : 8.16



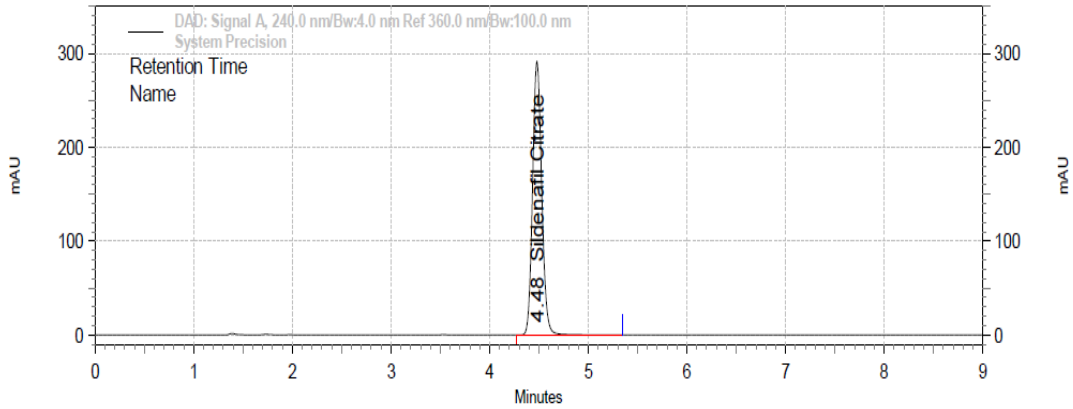
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	4.487	252489103	11336	1.10
Totals			252489103		

Chromatogram No : 8.17



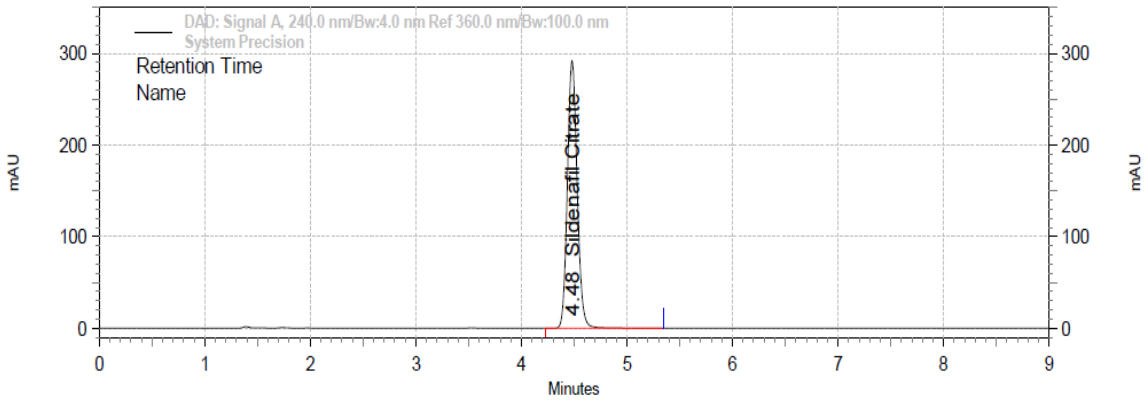
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	4.480	252063502	11352	1.12
Totals			252063502		

Chromatogram No : 8.18



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	4.480	251865543	11350	1.11
Totals			251865543		

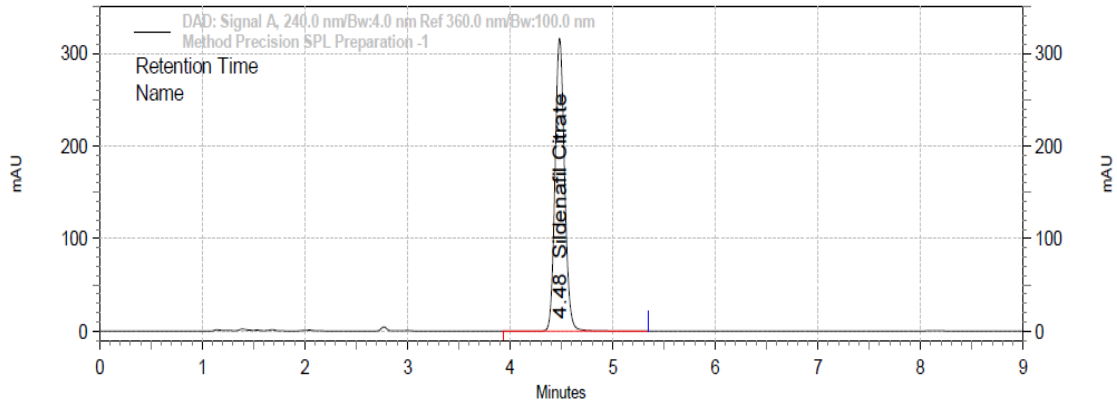
Chromatogram No : 8.19



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	4.480	252233194	11342	1.12
Totals			252233194		

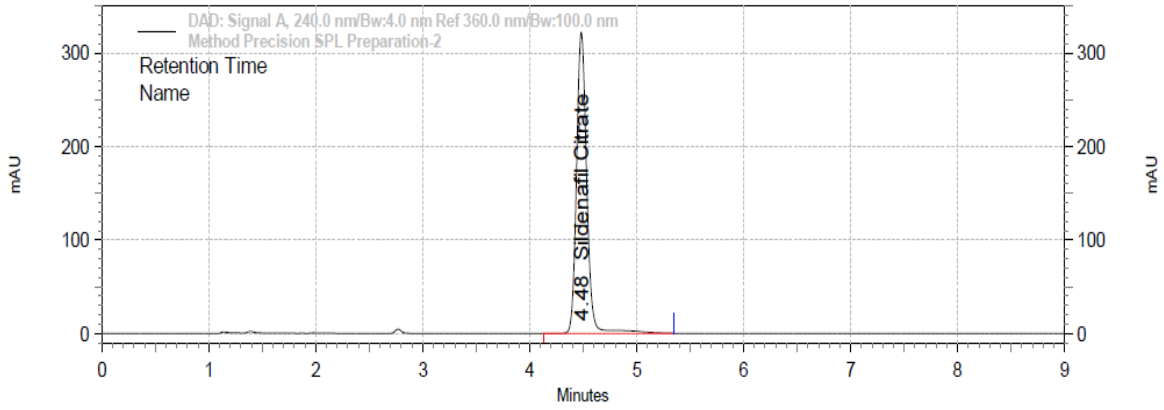
METHOD PRECISION

Chromatogram No : 8.20 Sample



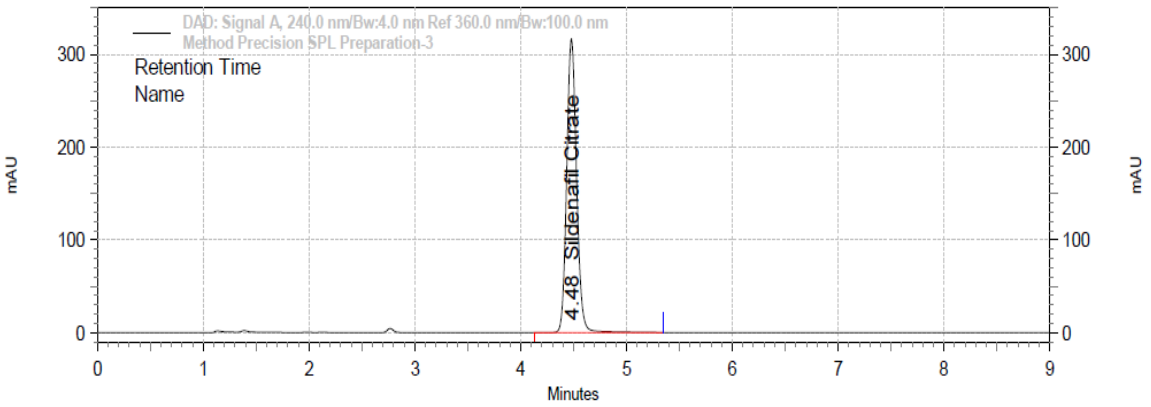
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	4.480	273811226	11314	1.15
Totals			273811226		

Chromatogram No : 8.21



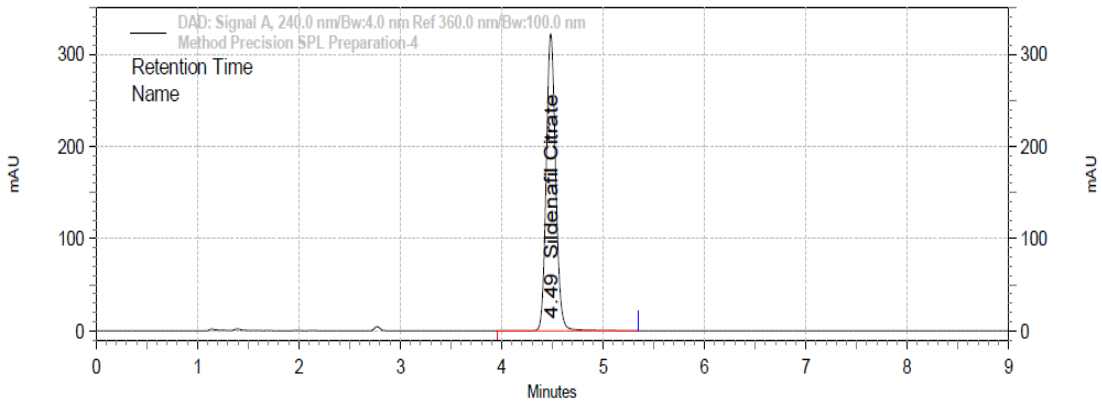
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	4.480	278804156	11302	1.15
Totals			278804156		

Chromatogram No : 8.22



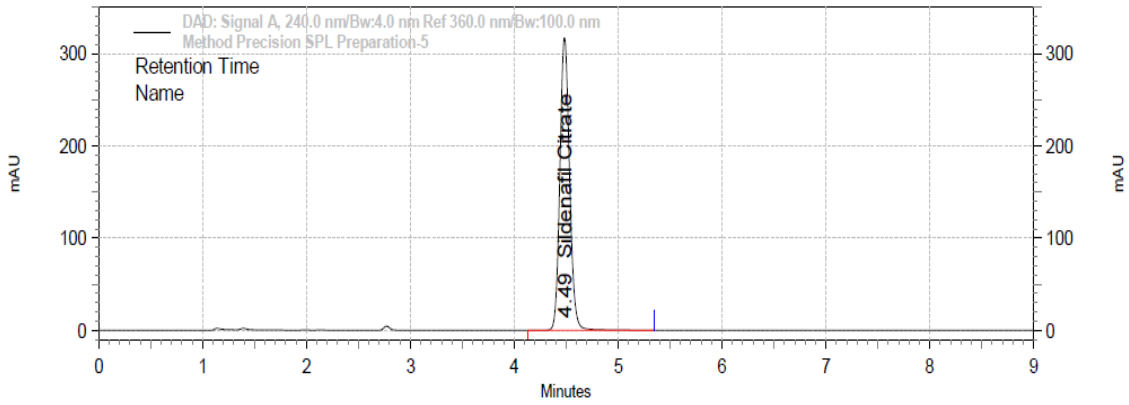
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	4.480	275977625	11344	1.11
Totals			275977625		

Chromatogram No : 8.23



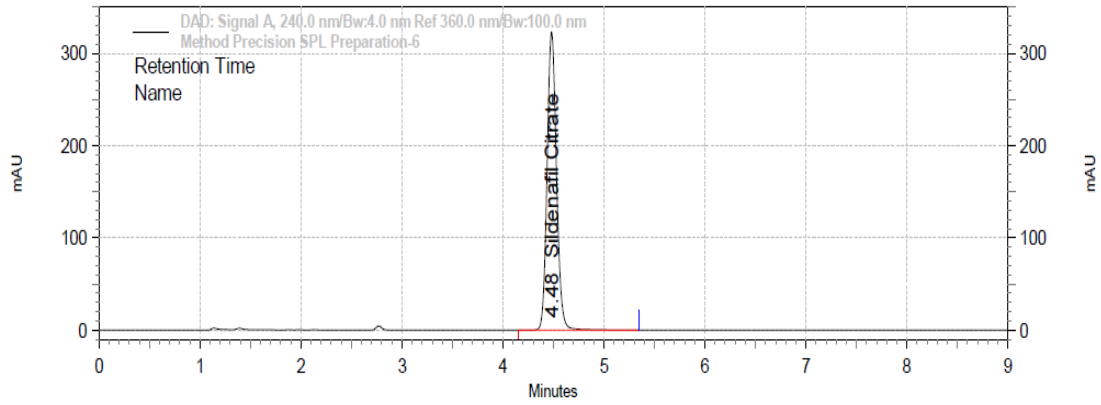
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	4.487	279908440	11354	1.09
Totals			279908440		

Chromatogram No : 8.24



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	4.487	275057250	11363	1.08
Totals			275057250		

Chromatogram No : 8.25

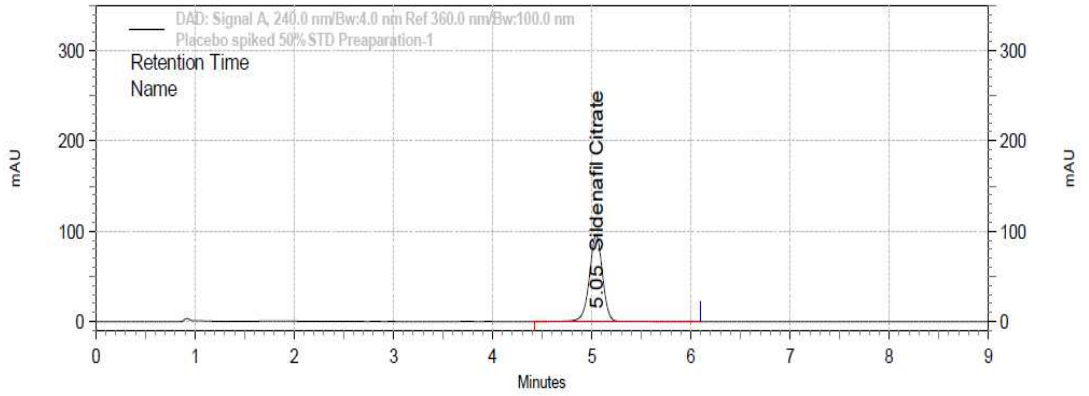


Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	4.480	280056030	11370	1.14
Totals			280056030		

ACCURACY

Chromatogram No : 8.26

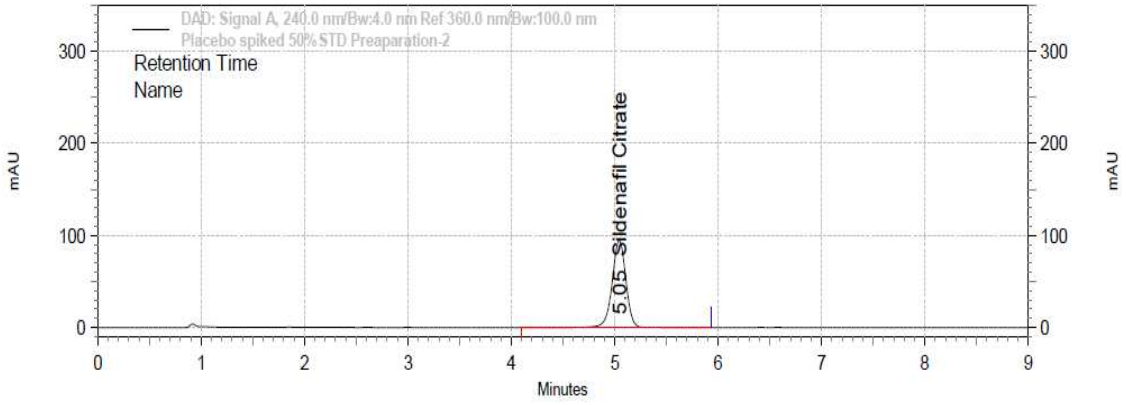
Accuracy 50% Preparation 1



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.047	118429857	6974	0.95
Totals			118429857		

Chromatogram No : 8.27

Accuracy 50% Preparation 2

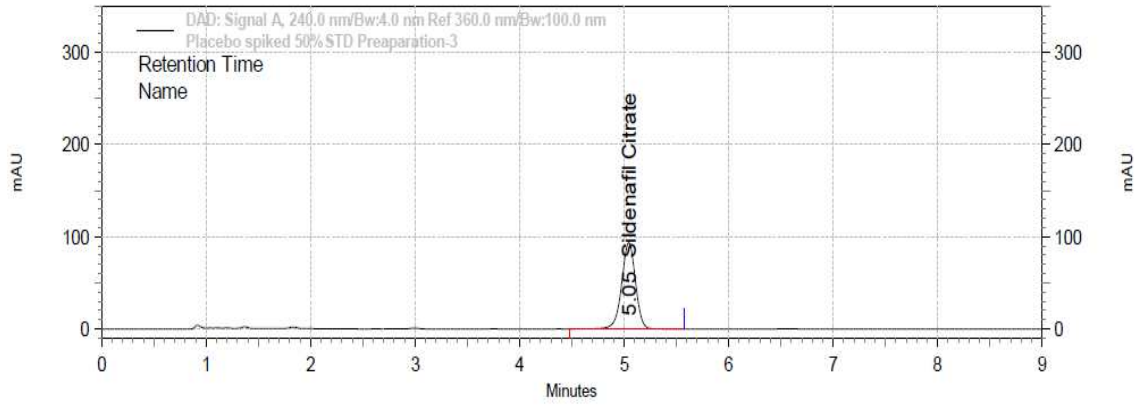


Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.047	117476643	7001	0.94

Totals				
			117476643	

Chromatogram No : 8.28

Accuracy 50% Preparation 3

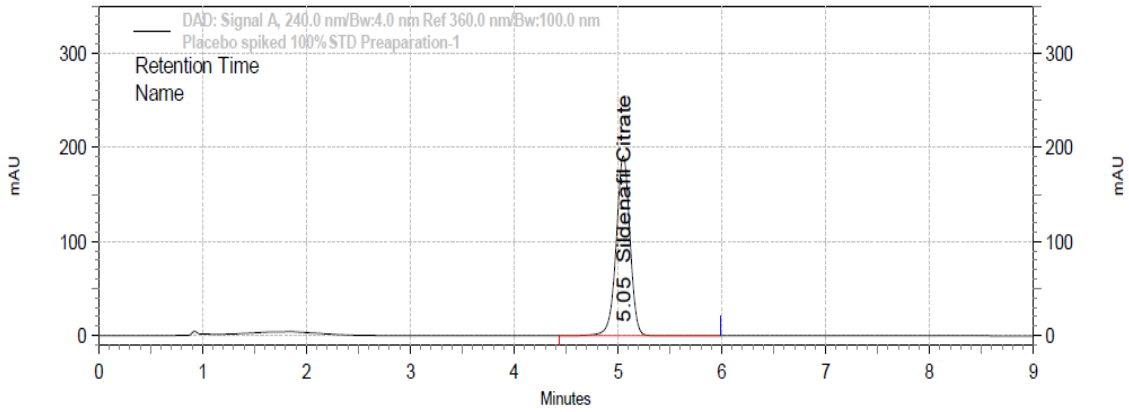


Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.047	116063376	6986	0.94

Totals				
			116063376	

Chromatogram No : 8.29

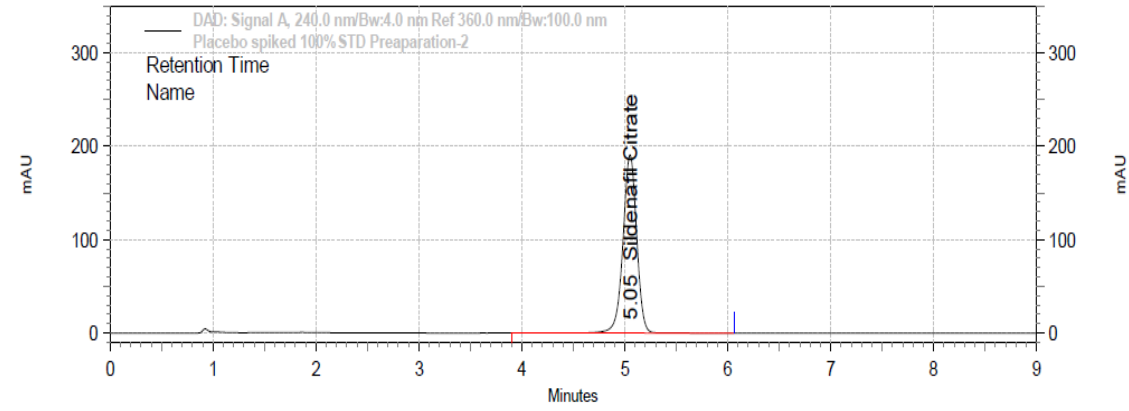
Accuracy100% Preparation 1



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.053	236560722	7000	0.94
Totals			236560722		

Chromatogram No : 8.30

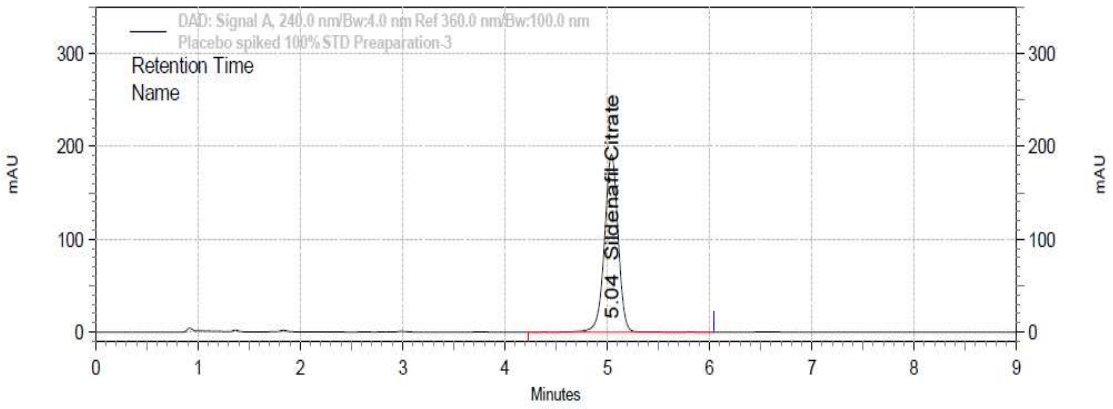
Accuracy 100% Preparation



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.053	236432216	7008	0.94
Totals			236432216		

Chromatogram No : 8.31

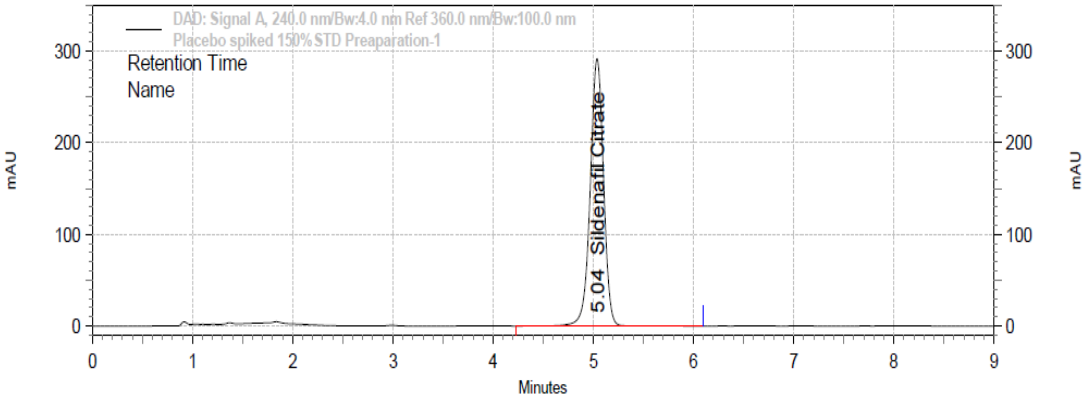
Accuracy 100% Preparation 3



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.040	237435669	6987	0.97
Totals			237435669		

Chromatogram No : 8.32

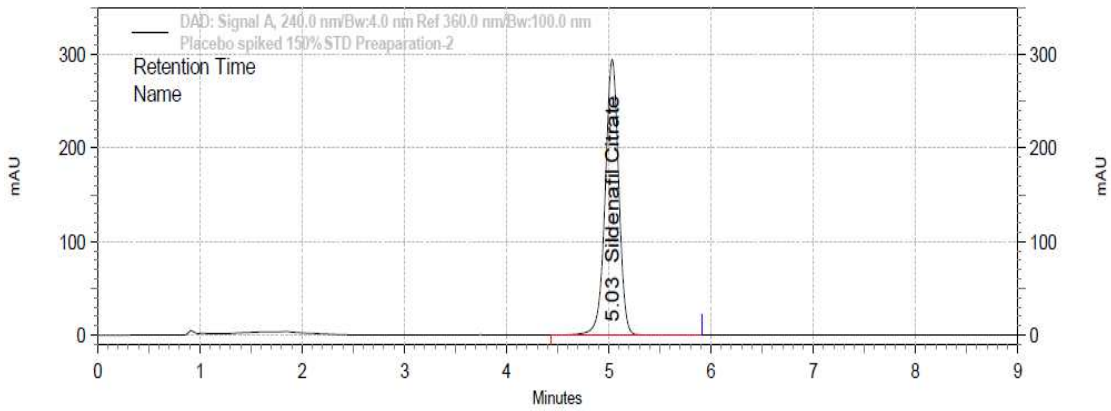
Accuracy 150% Preparation 1



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.040	361355923	6965	0.95
Totals			361355923		

Chromatogram No : 8.33

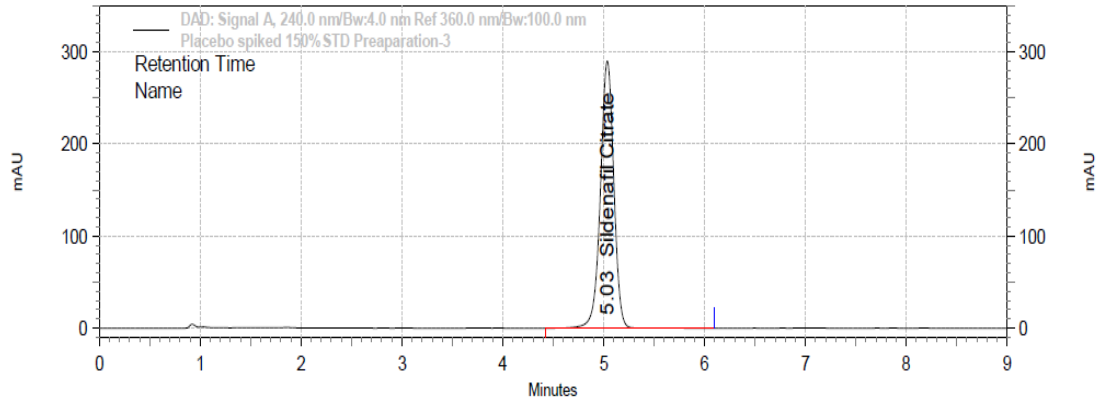
Accuracy 150% preparation 2



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.033	363760994	6958	0.96
Totals			363760994		

Chromatogram No : 8.34

Accuracy 150% Preparation 3

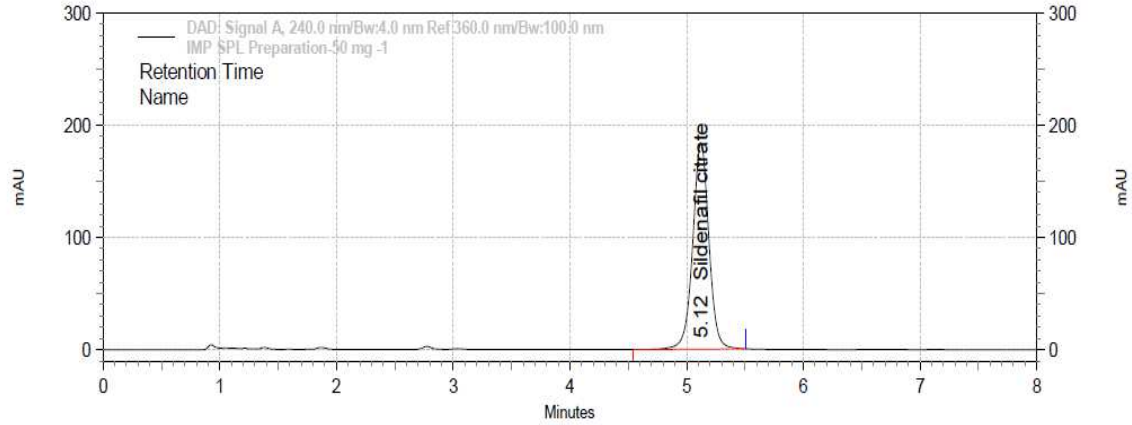


Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.033	359228288	6984	0.98
Totals			359228288		

Chromatogram No : 8.35

RUGGEDNESS (Inter mediate precision)

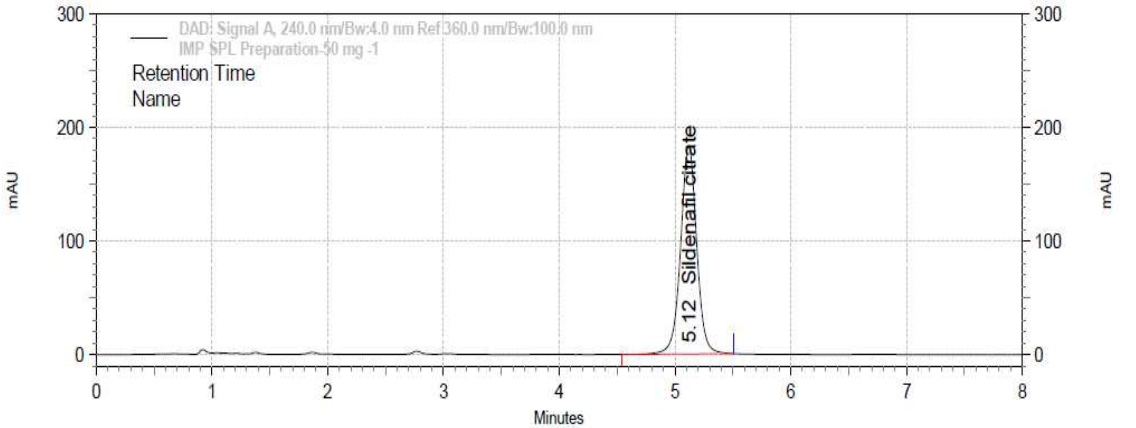
Sample50mg Preparation 1



Peak Number	Name	Area	Retention Time	Asymmetry	Theoretical plates (USP)
1	Sildenafil citrate	247554814	5.120	1.01	6758
Totals		247554814			

Chromatogram No : 8.36

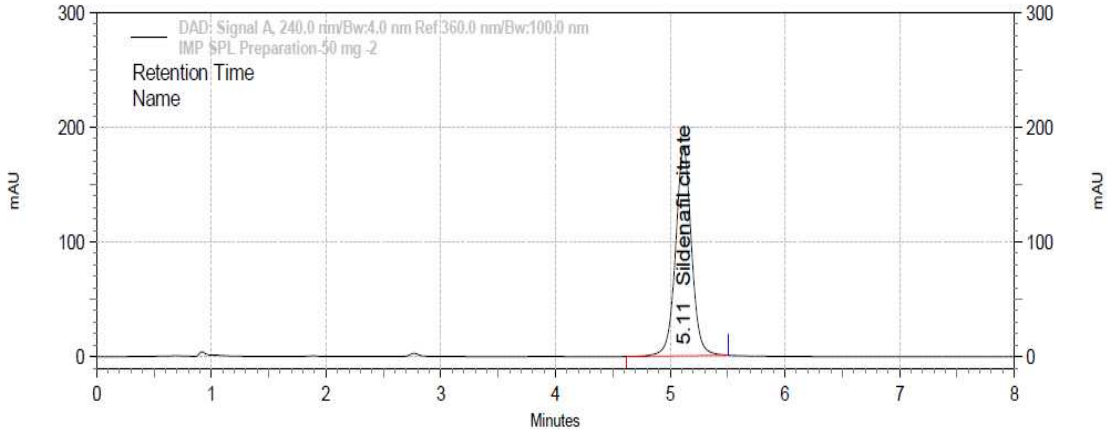
Sample 50 Mg Preparation 2



Peak Number	Name	Area	Retention Time	Asymmetry	Theoretical plates (USP)
1	Sildenafil citrate	247931835	5.120	0.99	6723
Totals		247931835			

Chromatogram No : 8.37

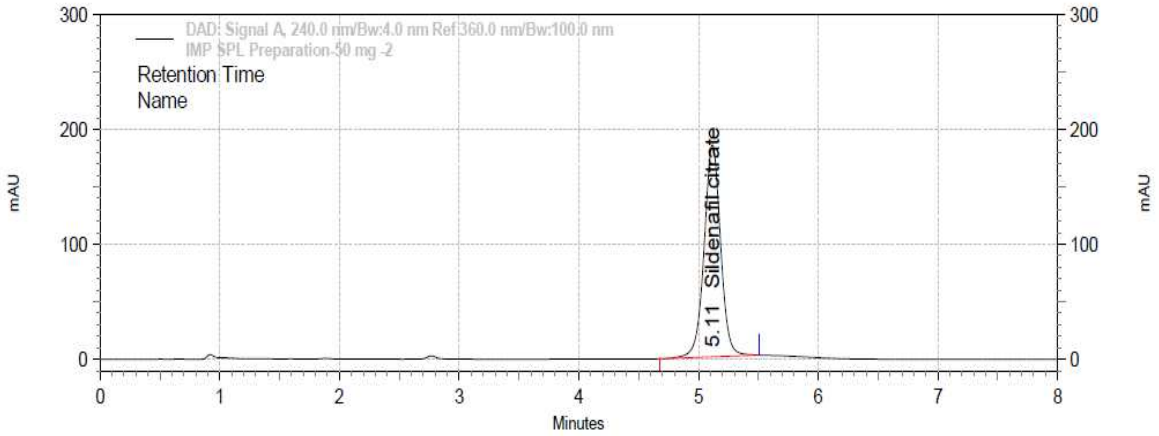
Sample 50 mg Preparation 2



Peak Number	Name	Area	Retention Time	Asymmetry	Theoretical plates (USP)
1	Sildenafil citrate	245984367	5.113	1.04	6693
Totals		245984367			

Chromatogram No : 8.38

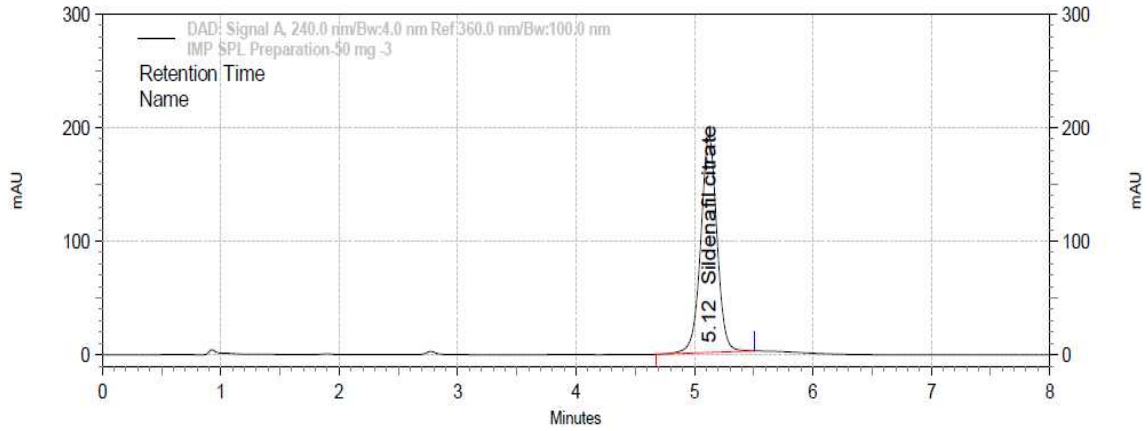
Sample 50mg Preparation 2



Peak Number	Name	Area	Retention Time	Asymmetry	Theoretical plates (USP)
1	Sildenafil citrate	247002518	5.113	1.02	6776
Totals		247002518			

Chromatogram No : 8.39

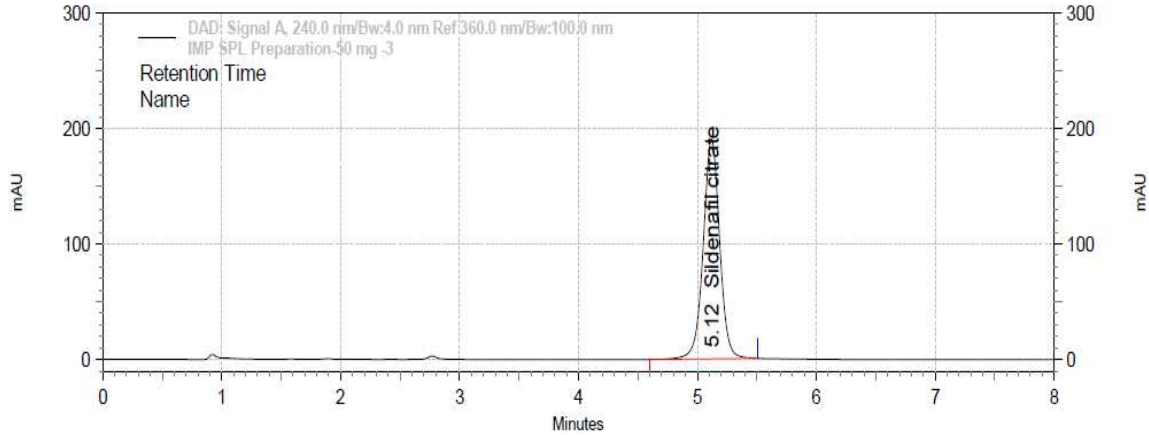
Sample 50 mg reparation 3



Peak Number	Name	Area	Retention Time	Asymmetry	Theoretical plates (USP)
1	Sildenafil citrate	253501297	5.120	1.01	6809
Totals		253501297			

Chromatogram No : 8.40

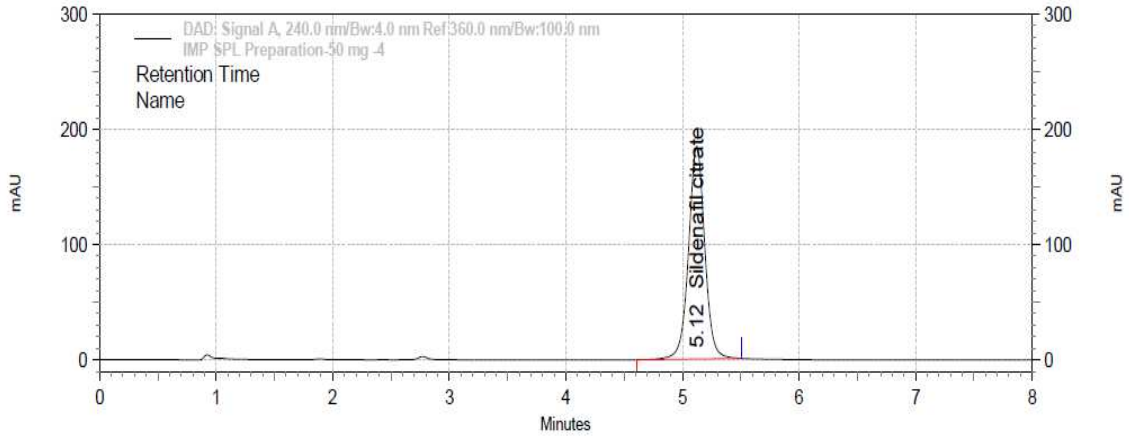
Sample 50mg preparation 3



Peak Number	Name	Area	Retention Time	Asymmetry	Theoretical plates (USP)
1	Sildenafil citrate	255339321	5.120	0.99	6759
Totals		255339321			

Chromatogram No : 8.41

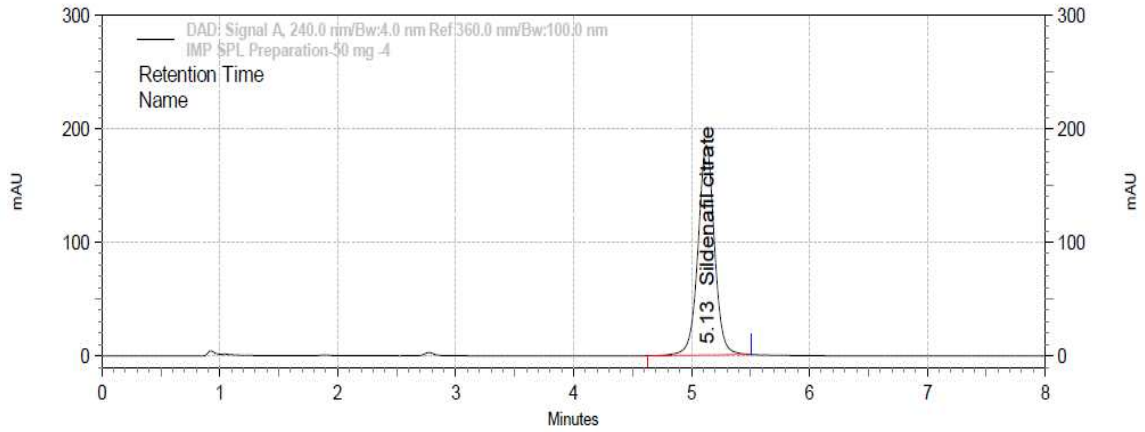
Sample 50 mg Preparation 4



Peak Number	Name	Area	Retention Time	Asymmetry	Theoretical plates (USP)
1	Sildenafil citrate	249292157	5.120	1.02	6734
Totals		249292157			

Chromatogram No : 8.42

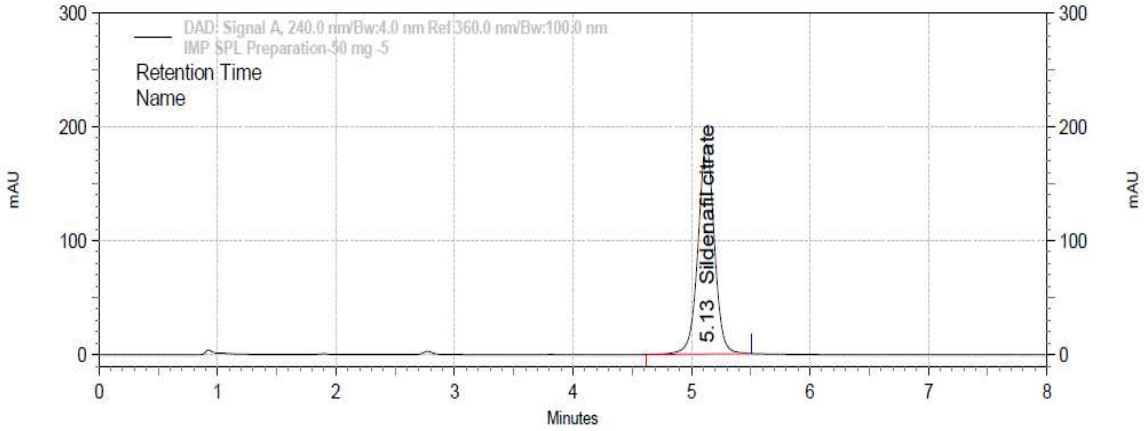
Sample 50mg Preparation 4



Peak Number	Name	Area	Retention Time	Asymmetry	Theoretical plates (USP)
1	Sildenafil citrate	250075032	5.127	1.00	6717
Totals		250075032			

Chromatogram No. : 8.43

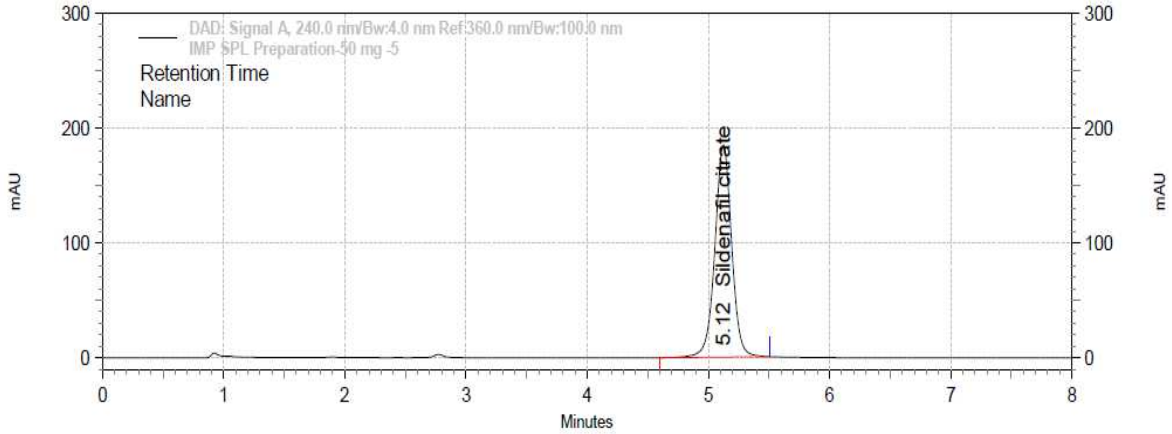
Sample 50 mg Preparation 5



Peak Number	Name	Area	Retention Time	Asymmetry	Theoretical plates (USP)
1	Sildenafil citrate	247967600	5.127	0.99	6751
Totals		247967600			

Chromatogram No : 8.44

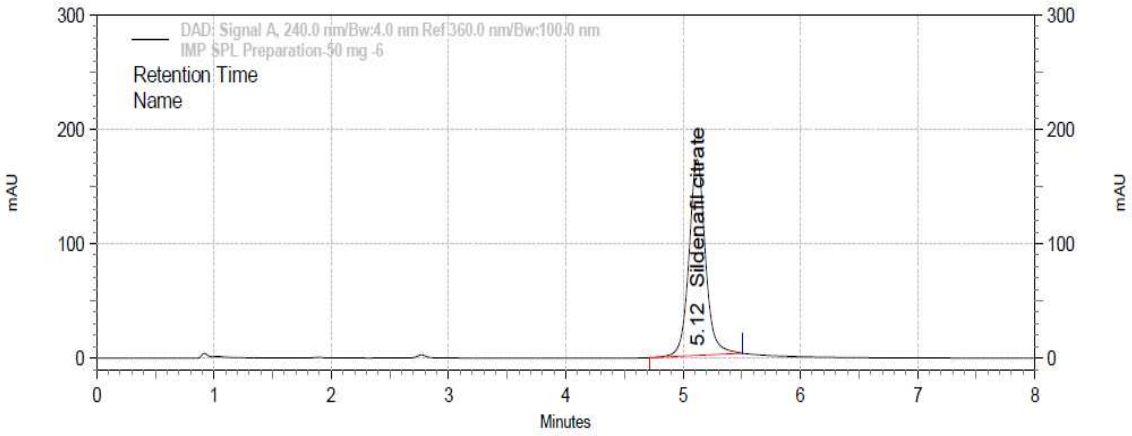
Sample 50mg Preparation 5



Peak Number	Name	Area	Retention Time	Asymmetry	Theoretical plates (USP)
1	Sildenafil citrate	248895826	5.120	1.02	6749
Totals		248895826			

Chromatogram No : 8.45

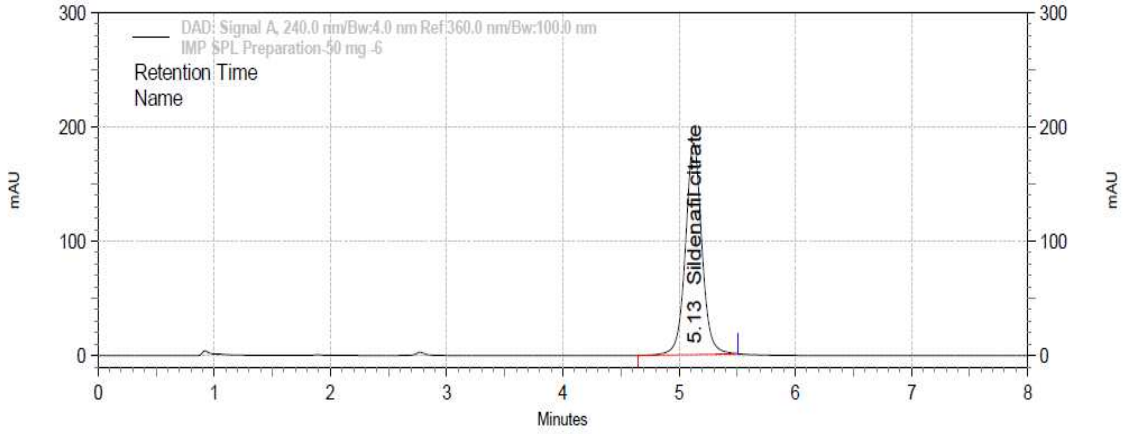
Sample 50mg Preparation 6



Peak Number	Name	Area	Retention Time	Asymmetry	Theoretical plates (USP)
1	Sildenafil citrate	253210685	5.120	1.08	6836
Totals		253210685			

Chromatogram No : 8.46

Sample 50mg Preparation 6



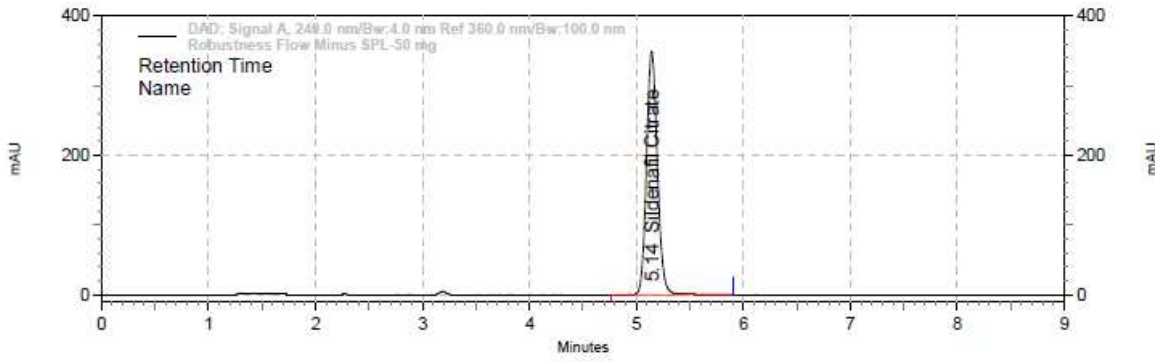
Peak Number	Name	Area	Retention Time	Asymmetry	Theoretical plates (USP)
1	Sildenafil citrate	253399230	5.127	1.03	6632
Totals		253399230			

ROBUSTNESS

Chromatogram No : 8.47

Sample

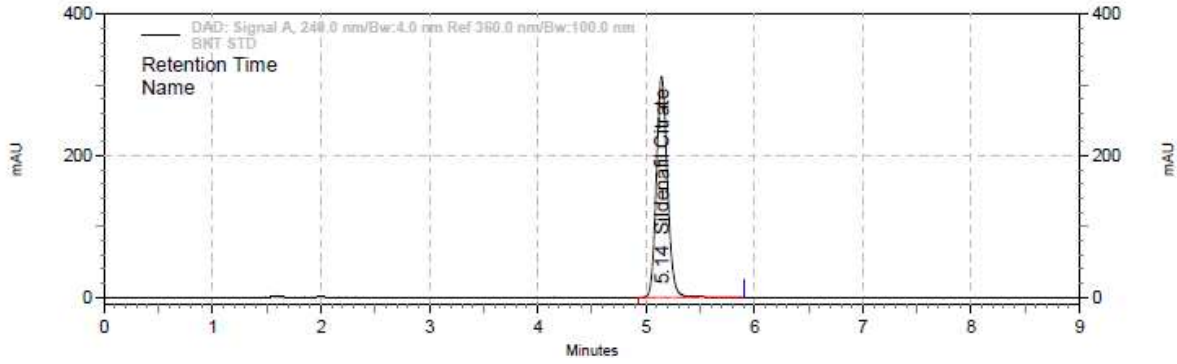
Change in flow rate (0.8 ml)-1



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.140	322155507	12538	1.16
Totals			322155507		

Sample Chromatogram No : 8.48

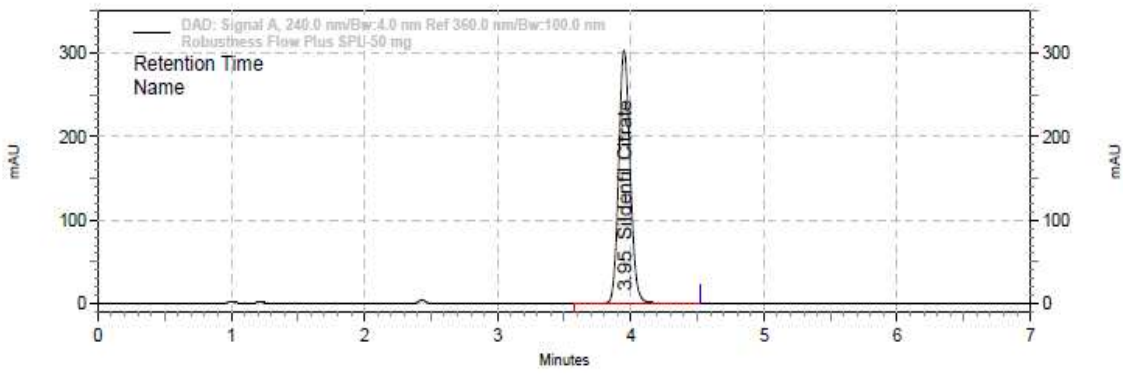
Change in Flow Rate (0.8ml)-2



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.140	294315284	12529	1.17
Totals			294315284		

Chromatogram No : 8.49 Flow Rate Plus

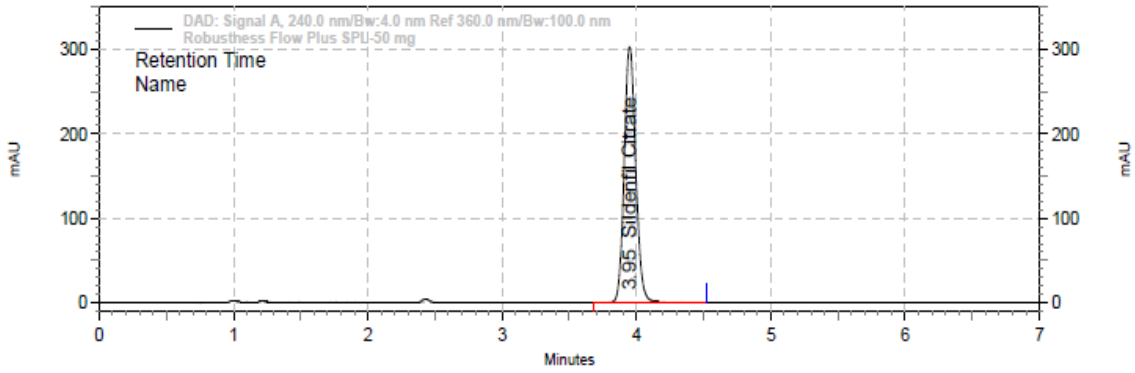
Sample Change in Flow Rate (1.2ml)



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenfil Citrate	3.953	241651646	10348	1.08
Totals			241651646		

Chromatogram No : 8.50

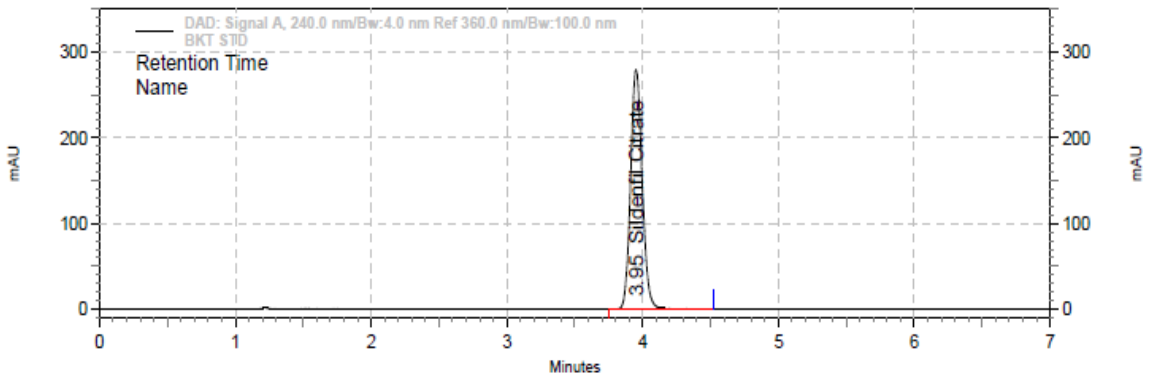
Change in flow rate (1.2ml)



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenfil Citrate	3.947	241697149	10330	1.16
Totals			241697149		

Chromatogram No : 8.51

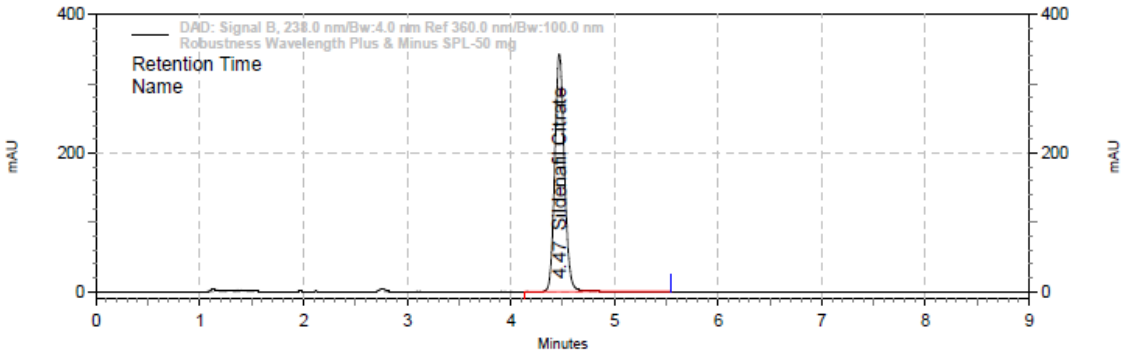
Change in flow rate (1.2ml)



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenfil Citrate	3.953	222273622	10314	1.10
Totals			222273622		

Chromatogram No : 8.52

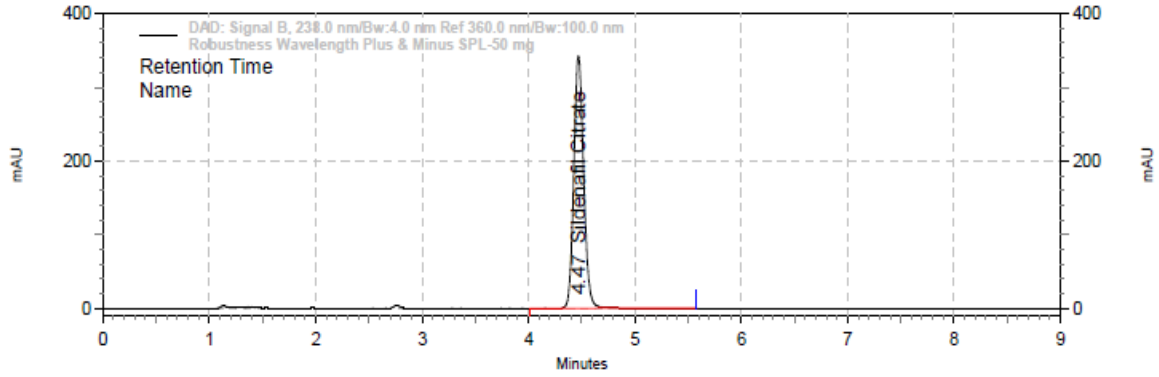
Sample change in wave length (238nm)



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	4.467	295600275	11328	1.14
Totals			295600275		

Chromatogram No : 8.53

Sample change in wave length (238nm)

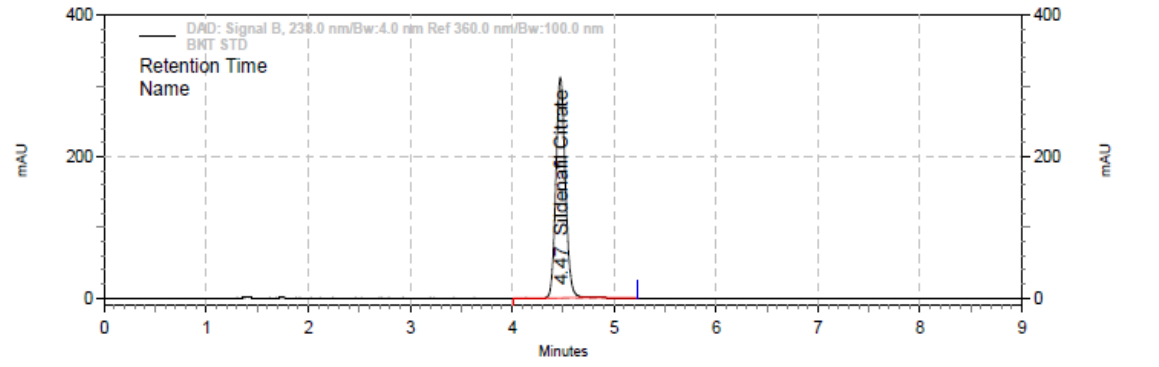


Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	4.467	295662195	11295	1.16

Totals			295662195	

Chromatogram No : 8.54

Sample change in wave length(238nm)

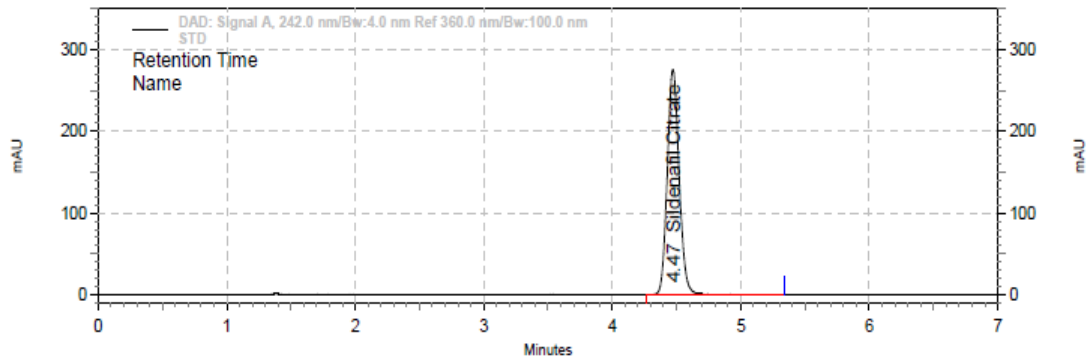


Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	4.473	269197200	11339	1.12

Totals			269197200	

Chromatogram No : 8.55

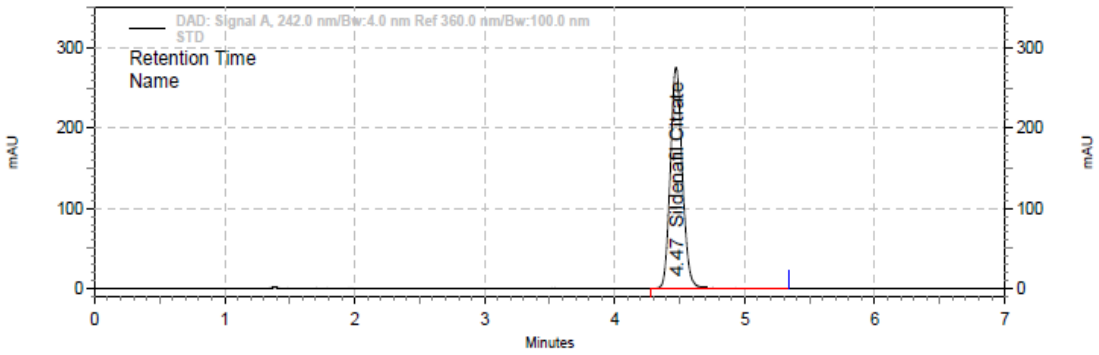
Robusness wavelength plus (242 nm) Sample replicate No 1



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	4.473	237446646	11357	1.11
Totals			237446646		

Chromatogram No : 8.56

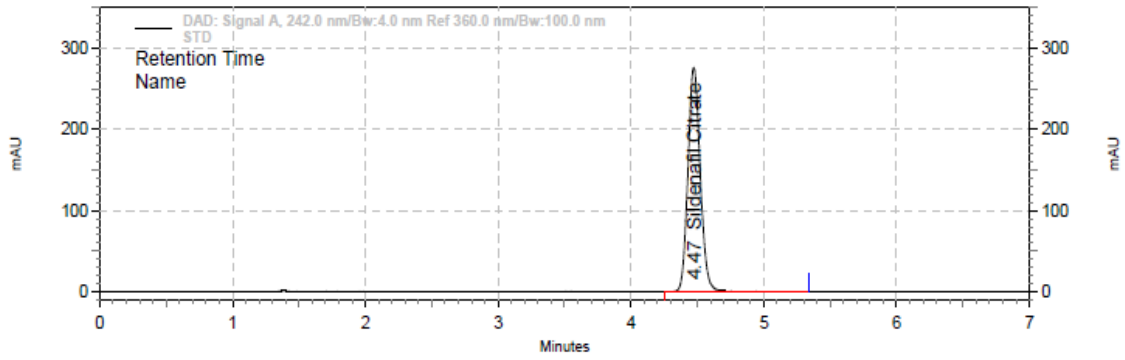
Sample Replicate No. 2



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	4.473	237615481	11334	1.11
Totals			237615481		

Chromatogram No : 8.57

Sample Replicate No. 3

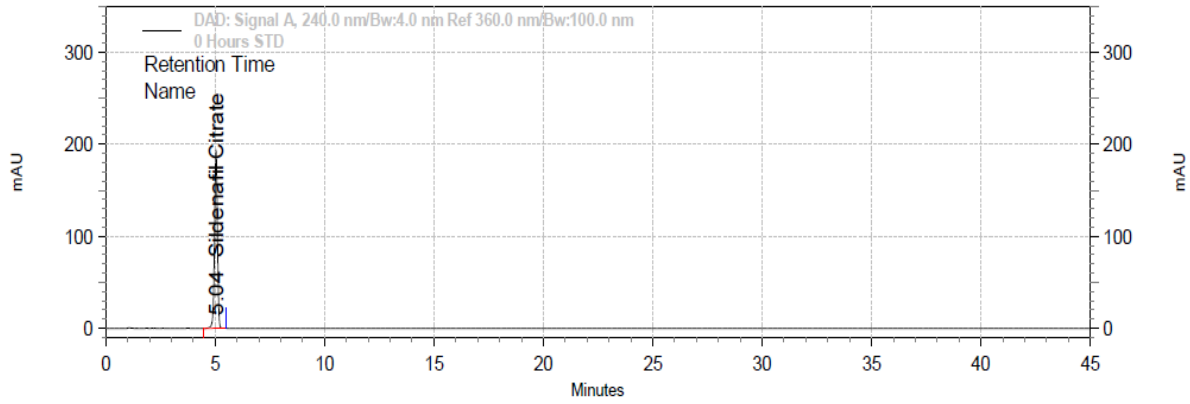


Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	4.473	237827551	11353	1.13
Totals			237827551		

STABILITY STUDY:

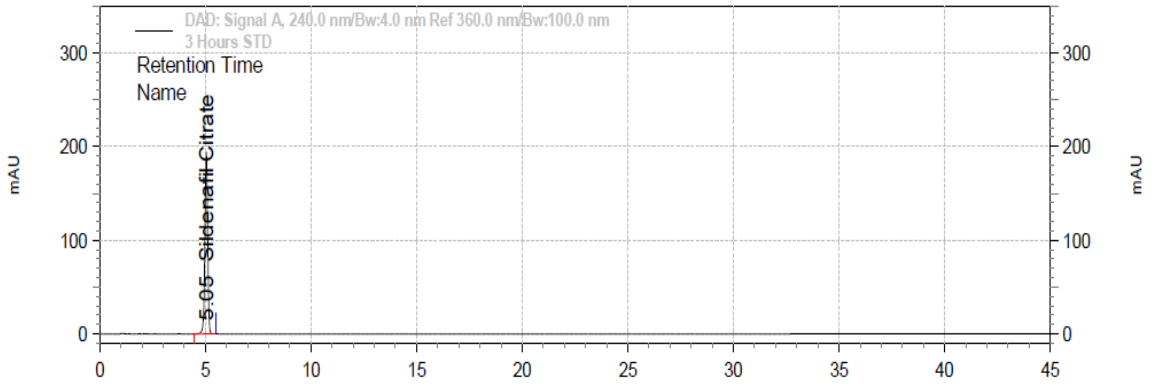
STANDARD 0, 3, 6, 9, 12, 15, 18, 21, 24, HOURS

Chromatogram No : 8.58



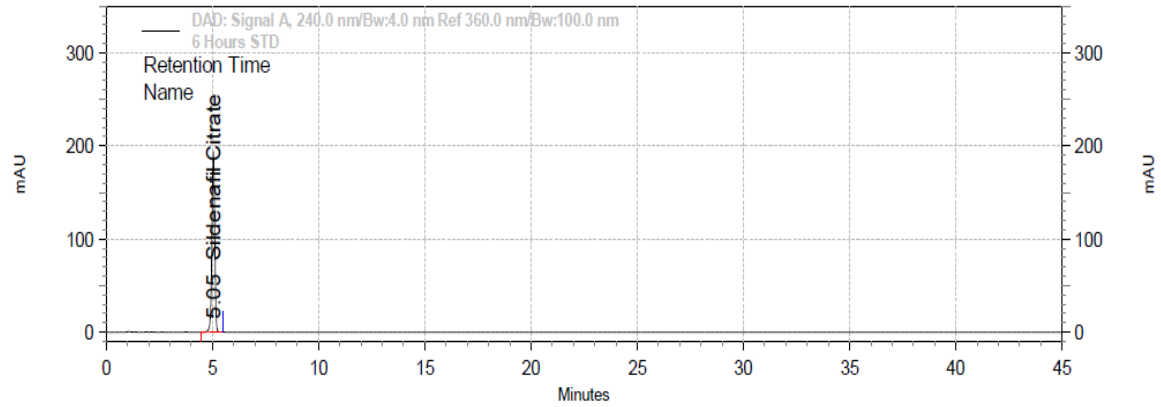
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.040	240467922	6903	0.95
Totals			240467922		

Chromatogram No : 8.59



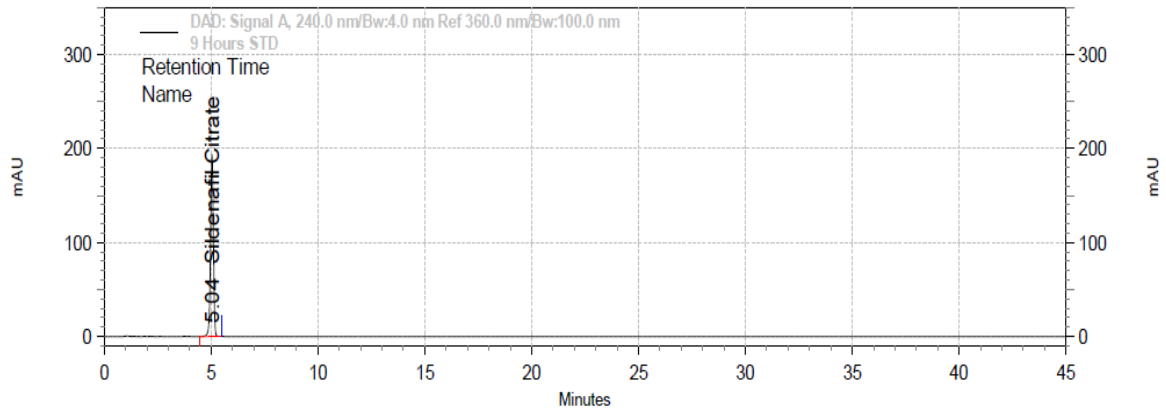
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.047	240670302	6918	0.95
Totals			240670302		

Chromatogram No : 8.60



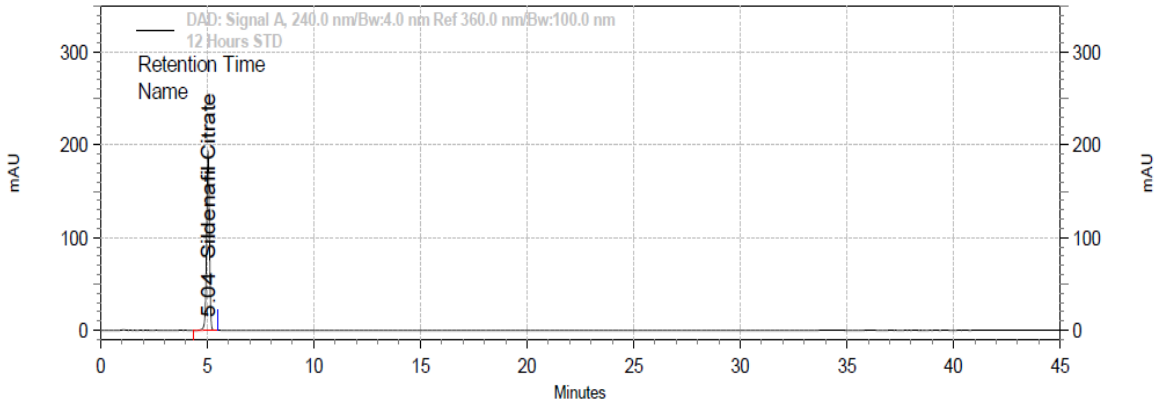
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.047	240602034	6940	0.94
Totals			240602034		

Chromatogram No : 8.61



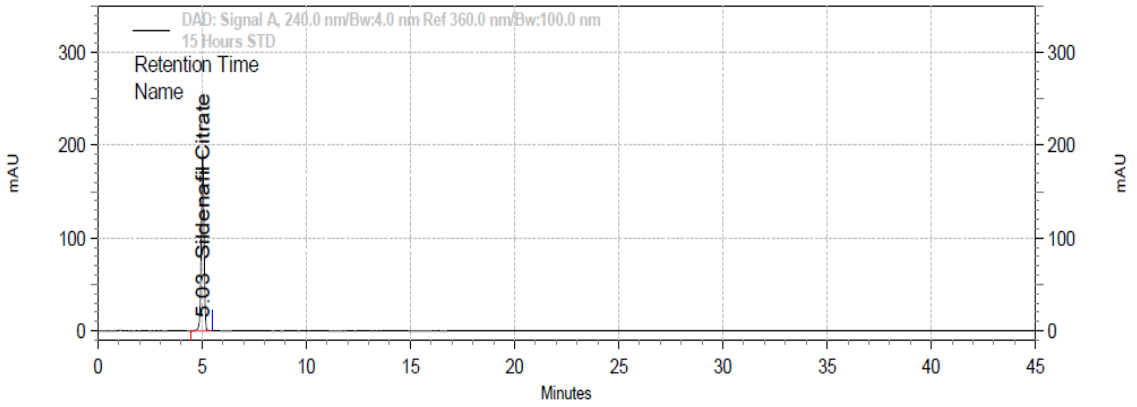
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.040	240773026	6923	0.98
Totals			240773026		

Chromatogram No : 8.62



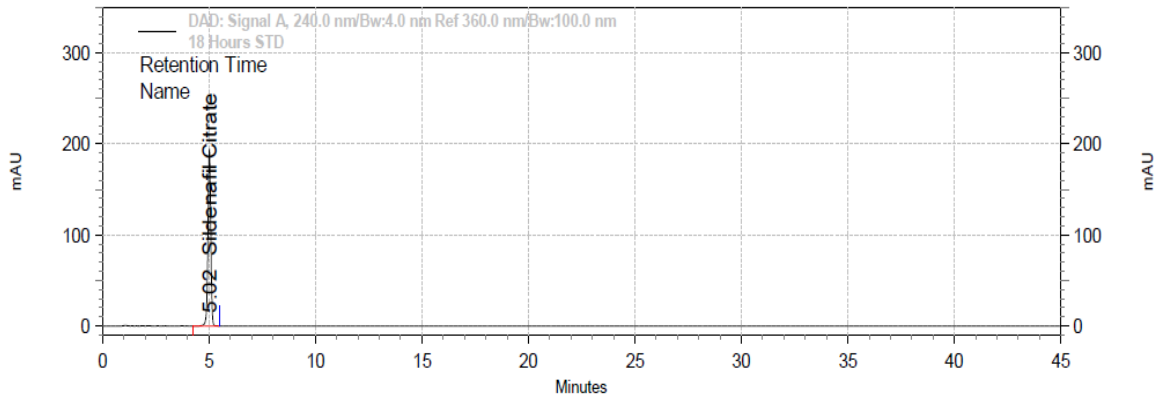
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.040	240744700	6929	0.97
Totals			240744700		

Chromatogram No : 8.63



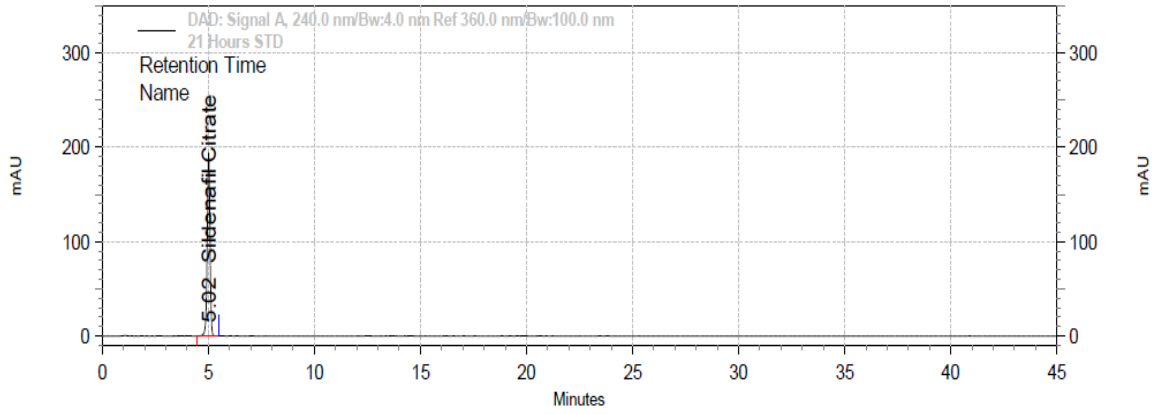
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.033	240913809	6909	0.96
Totals			240913809		

Chromatogram No : 8.64



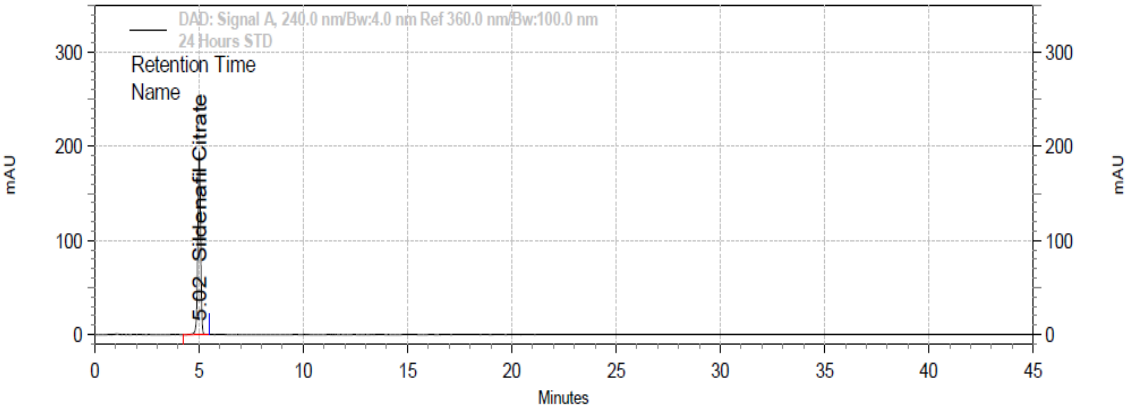
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.020	240411477	6889	0.96
Totals			240411477		

Chromatogram No : 8.65



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.020	239949634	6929	0.94
Totals			239949634		

Chromatogram No : 8.66

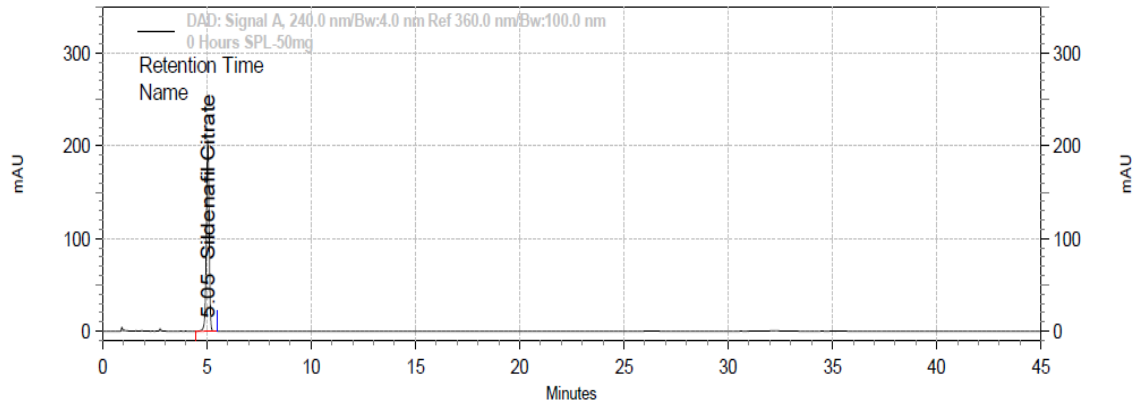


Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.020	240330489	6938	0.94
Totals			240330489		

STABILITY STUDY:

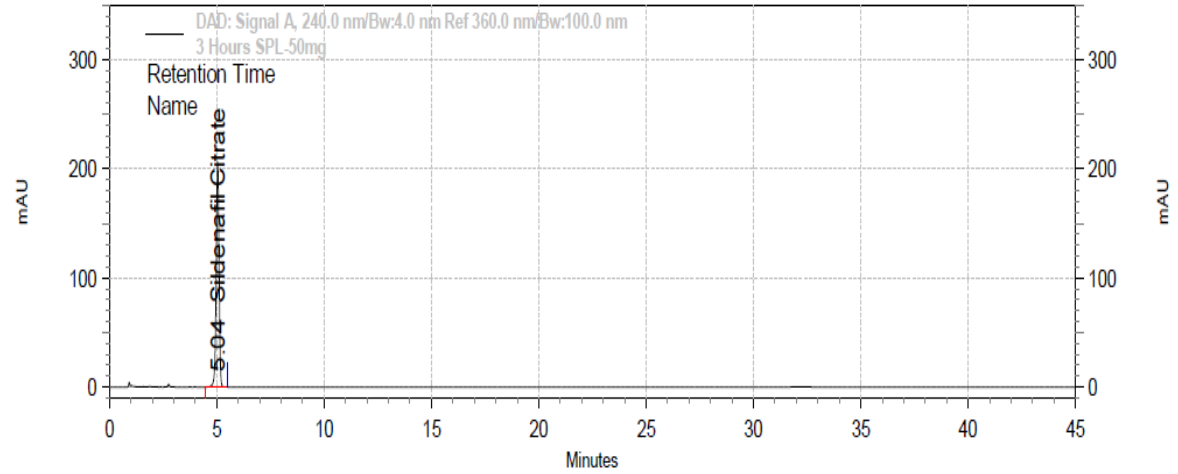
SAMPLE 0, 3, 6, 9, 12, 15, 18, 21, 24 HOURS

Chromatogram No : 8.67



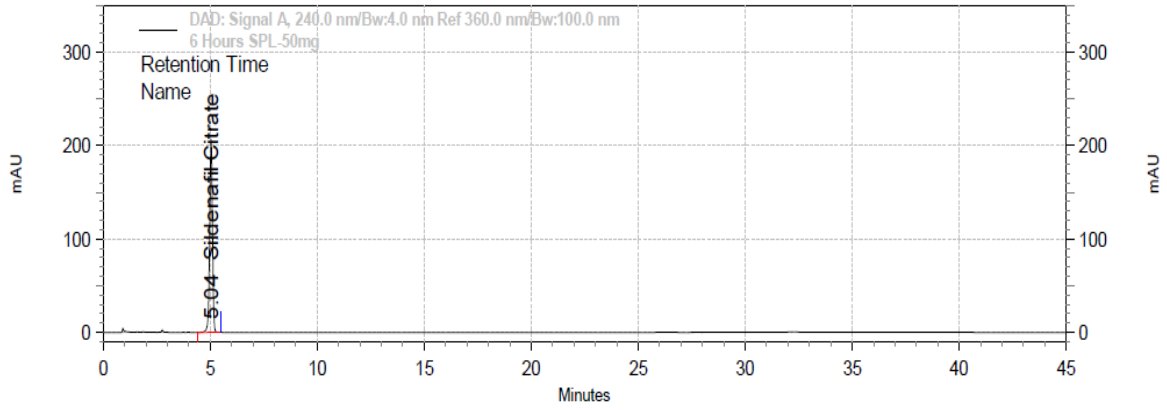
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.047	256661227	6906	0.95
Totals			256661227		

Chromatogram No : 8.68



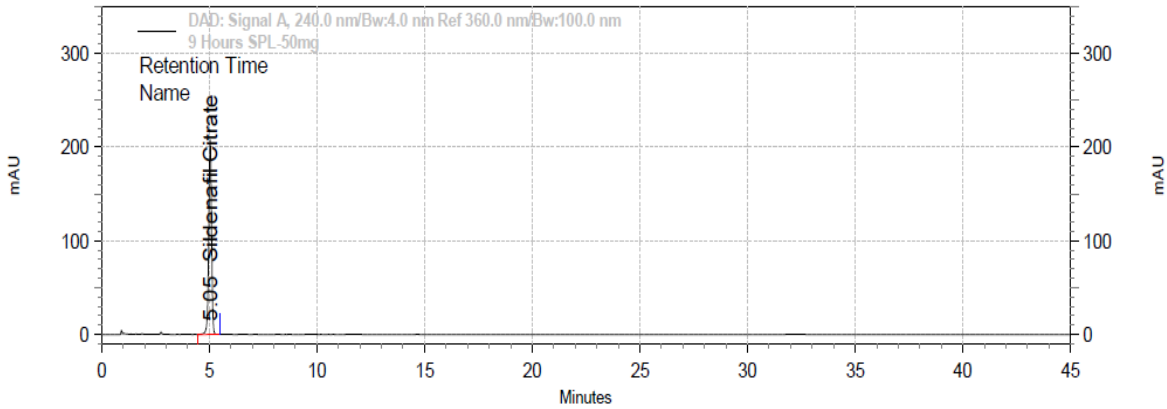
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.040	256401938	6947	0.97
Totals			256401938		

Chromatogram No : 8.69



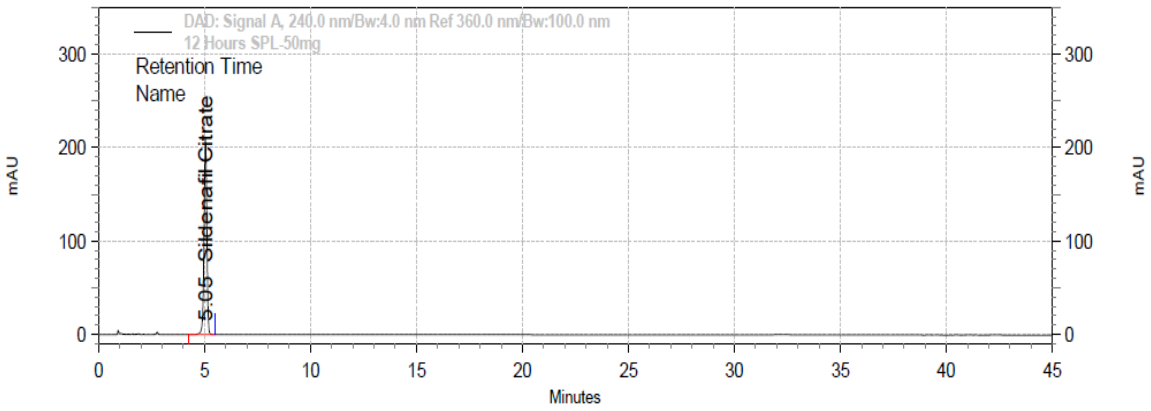
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.040	256749674	6908	0.97
Totals			256749674		

Chromatogram No : 8.70



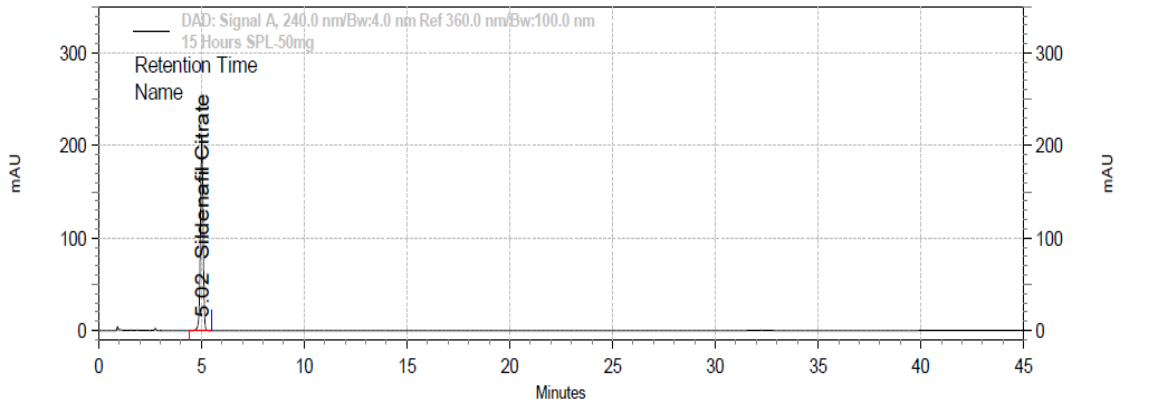
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.047	257485261	6951	0.94
Totals			257485261		

Chromatogram No : 8.71



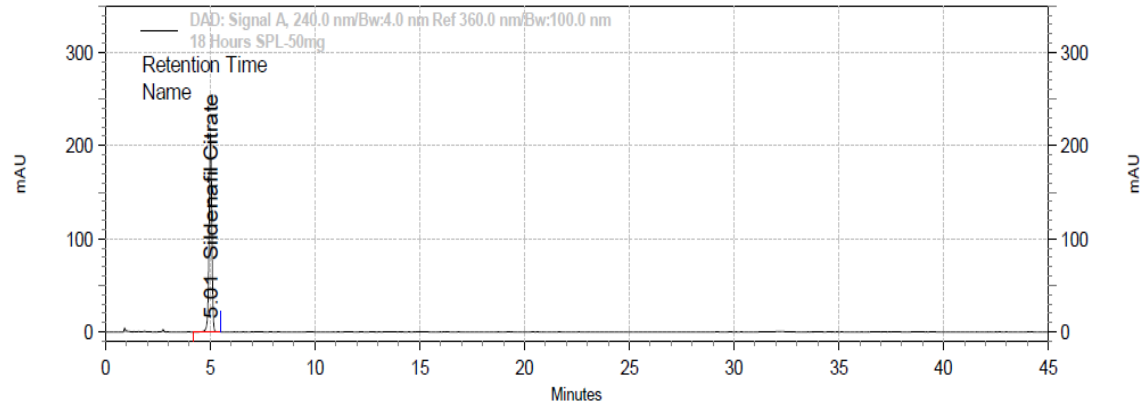
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.047	260634009	6936	0.96
Totals			260634009		

Chromatogram No : 8.72



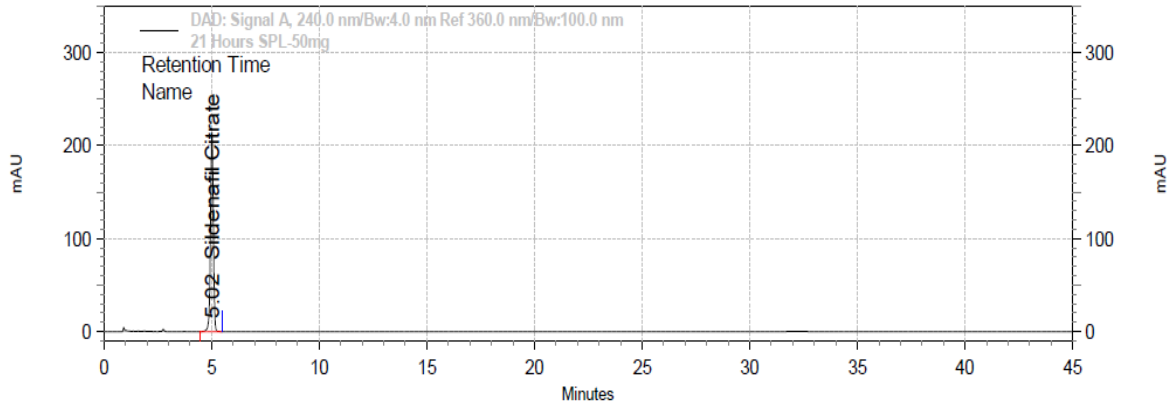
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.020	261251360	6923	0.97
Totals			261251360		

Chromatogram No : 8.73



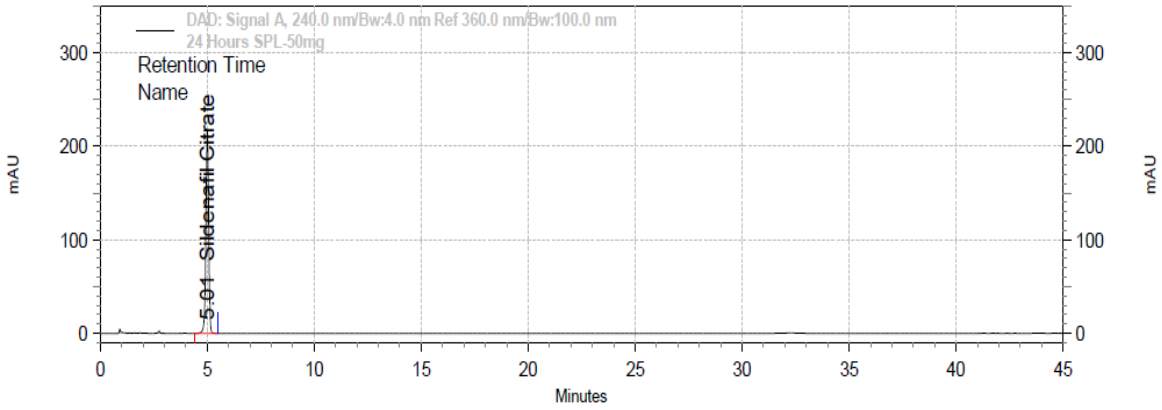
Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.013	262832813	6935	0.97
Totals			262832813		

Chromatogram No : 8.74



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.020	264082144	6933	0.96
Totals			264082144		

Chromatogram No : 8.75



Peak Number	Name	Retention Time	Area	Theoretical plates (USP)	Asymmetry
1	Sildenafil Citrate	5.007	267271980	6911	0.95
Totals			267271980		

9. RESULTS AND DISCUSSION

A new isocratic reverse-phase high performance liquid chromatographic at was developed for quantitative determination of sildenafil citrate soft gelatin capsule dosage forms. The mobile phase was used ammonium acetate and acetonitile in the proportion of 1:1 v/v. The chromatographic method was performed on C_{18}, 15 cm×4.6 mm,5µm column at a flow rate of 1.0 ml/min. Column Temperature was set a 25ºC and injection volume was 20µl.

Estimation of sildenafil citrate soft gelatin capsule dosage form dosage forms by RP-HPLC method was carried out using optimized chromatographic conditions. The standard and sample solutions were prepared. The chromatograms were recorded. The peak area ratio of standard and sample solutions was calculated. The capsule shows percentage purity values ranging from 99.36% to 100.52% for sildenafil citrate respectively.

The method was validated according to the Q2 specifications of the ICH guidelines. The validated parameters were system suitability, Precision, Accuracy, Specificity, linearity, Ruggedness Robustness and. LOD, LOQ.

The resulting chromatograms exhibited retention time at 4.5 min for sildenafil citrate soft gelatin caplules. The number of theoretical plates were more than 2000, for sildenafil citrate it was 11325 respecitively. The tailing factor was less than 2 that is 1.12 for sildenafil citrate. Hence all the system suitability parameters were with the specified limits.

Specificity

The specificity of the method was established by injecting blank, placebo and standard. It was observed that there was no interference caused due to the placebo. The chromatogram were shown in **chromatogram no : 8.1 – 8.4.**

S.No.	Name of the solution	Results obtained	Acceptance criteria
01	Blank	No peak found in the retention time of sildenafil citrate	No any peak should be found in the retention time of sildenafil citrate
02	Placebo	No peak found in the retention time of sildenafil citrate	No any peak should be found in the retention time of sildenafil citrate

System suitability result passes and the results obtained for specificity are found within the acceptance criteria. The obtained results proved that there will not be blank and placebo interference in the sildenafil citrate soft gelatin capsule peak by this assay method.

Linearity

The linearity for the drugs from concentration range of 30 - 60 and 80 µg/ml was established by constructing the calibration curve with concentration on x-axis and peak area on y-axis with the correlation coefficient of 0.999 for the drug which were within specified limits. The results were shown in **Table no: 7.1 ,7.2 and Figure no 7.1,7.2.** The chromatogram were shown in **chromatogram no: 8.5-8.14.**

Table No 7.1: Linearity data obtained from 60 % to 160 % for Standard

Linearity level Concentration in %	Concentration of Sildenafil Citrate mcg/mL)	Standard area -1	Standard area-2	Average area
60	30	151677294	151861664	151769479
80	40	201279288	202136838	201708063
100	50	250885878	251145548	251015713
120	60	303794785	304201777	303998281
160	80	399892519	399849420	399870970

Figure No 7.1: Calibration curve of standard sildenafil citrate

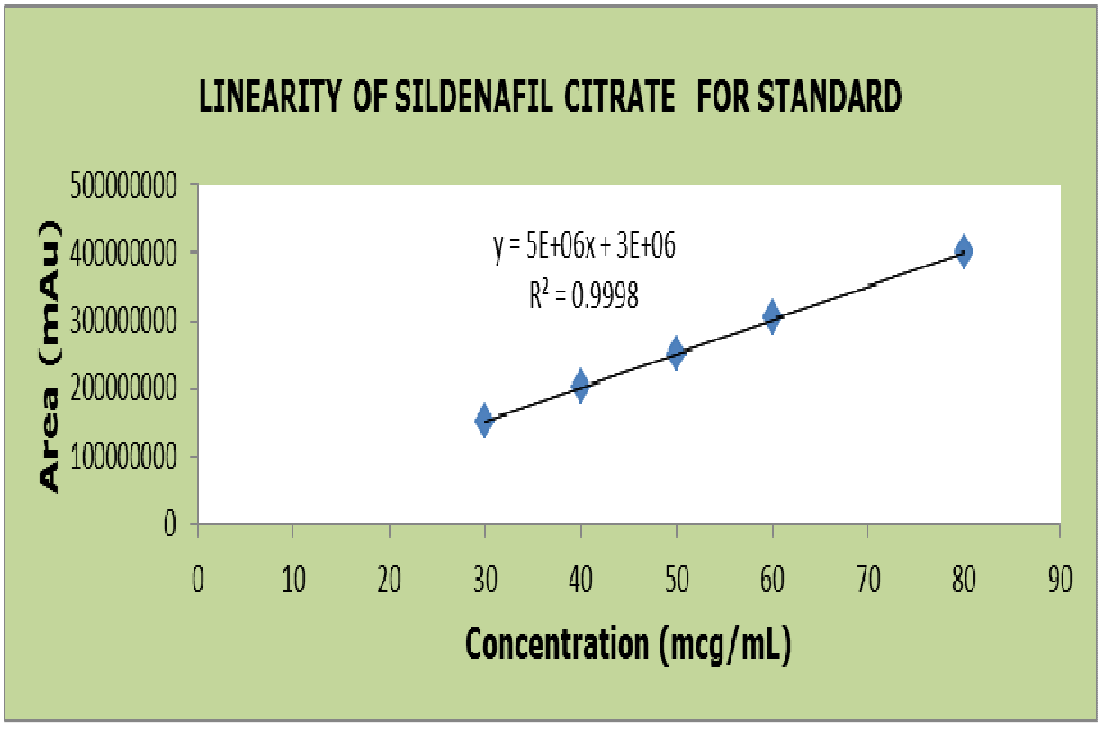
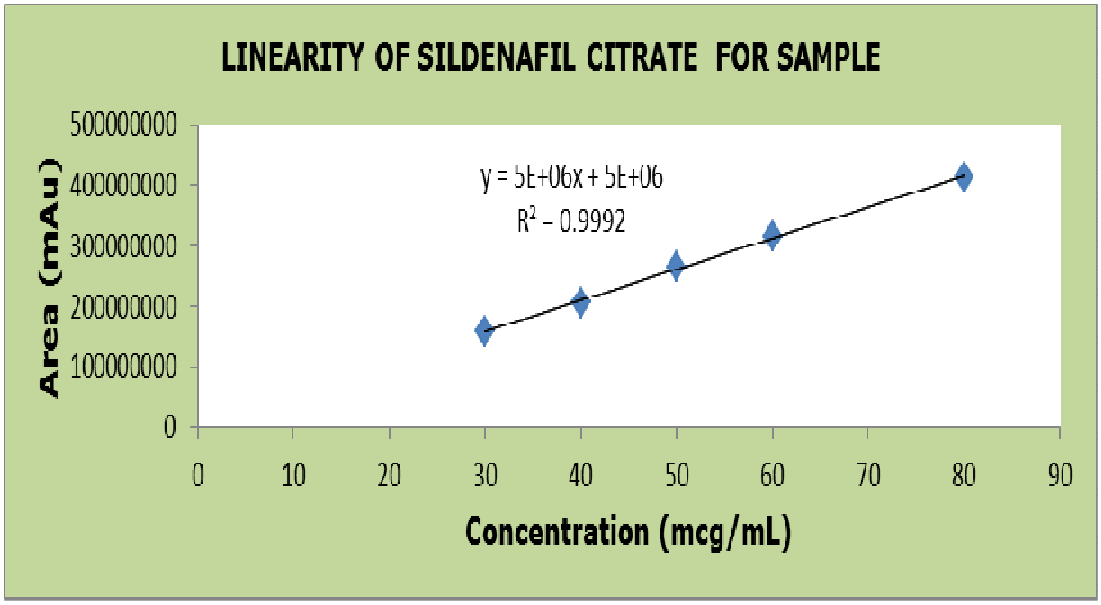


Table no 7.2: Linearity data obtained from 60% to 160% for Sample

Linearity level Concentration in %	Concentration of Sildenafil Citrate (mcg/mL)	Sample area -1	Sample area-2	Average area
60	30	158469512	159881630	159175571
80	40	205672985	206225062	205949024
100	50	262694385	265295848	263995117
120	60	313842623	315675130	314758877
160	80	413117111	413207957	413162534

Figure No 7.2: Calibration curve of sample sildenafil citrate



System suitability result passes and the results obtained for linearity are found within the acceptance criteria. Hence it is concluded that the range of concentrations, 60 % to 160 % with respect to 100 % working concentration for assay method is linear for sildenafil citrate soft gelatin capsule.

PRECISION

System precision was determined by preparing the standard solution at working concentration and analysis was carried for six replicate injections. The percentage relative standard deviation (% RSD) was calculated for the peak areas of sildenafil citrate which was found to be 0.091 respectively which were within the acceptance criteria of not more than 2.0%. The percentage relative standard deviation (% RSD) was calculated for the peak retention time of sildenafil citrate soft gelatin which was found to be 0.003 respectively which were within the acceptance criteria of not more than 1.0%. The results were presented in shown in **Table no 7.3 and Chromatogram No. 8.15 – 8.19.** All the system suitability parameters were well within the desirable limits, it indicates that the prescribed method is suitable to perform the estimation of sildenafil citrate Soft gelatin Capsules Further there was no deviation in the given method

Table No 7.3: System precision (standard solution)

S. No.	Sildenafil Citrate Retention time	Sildenafil Citrate area	Tailing factor	Theoretical plates
01	4.480	252184542	1.13	11339
02	4.487	252489103	1.10	11336
03	4.480	252063502	1.12	11352
04	4.480	251865543	1.11	11350
05	4.480	252233194	1.12	11342
Mean	**4.481**	**252167177**	**1.12**	**11344**
Std dev	**0.003**	**229143.90**	**NMT 2.0**	**NLT 2000**
(RSD %)	**0.07**	**0.091**		
Limit	**NMT 1.0 %**	**NMT 2.0 %**		

Method precision

Method precision was determined by preparing six different samples solutions from the capsules of same batch at working concentration and analysis was carried out for six replicate injections. The percentage relative standard deviation (% RSD) was calculated for the peak areas of sildenafil citrate which was found to be 1.006 for the drug which is within the acceptance criteria of not more than 2.0%. These results give the assurance of method repeatability. The results were presented in **Table no: 7.4** and shown in **Chromatogram No. 8.20 – 8.25.**

Table No 7.4: Method precision results

No. of Sample preparation	Samples Area			Results obtained		Acceptance criteria
	Sample -1	Sample -2	Average	Amount of drug present in mg	Percentage of drug	
01	273811226	273874982	273843104	53.62	107.24	90.0% - 110.0%
02	278804156	279439194	279121675	54.65	109.30	
03	275977625	275569282	275773454	53.99	107.98	
04	279908440	280739967	280324204	54.88	109.76	
05	275057250	275230784	275144017	53.87	107.74	
06	280151042	280056030	280103536	54.84	109.68	
				Mean	**108.62**	
				Std. Dev	**1.093**	
				RSD	**1.006**	**NMT 2.0 %**

System suitability parameters were well within the prescribed limits which revealed that the prescribed procedure is capable to perform Method precision using sample preparation. All the performed samples showed results between 90.0 % and 110.0 %.The Method precision parameter complies as per In-House specification.

Accuracy

Accuracy of the method was determined by performing recovery studies at 50%, 100%, 150%. Percentage recovery sildenafil citrate were found to be % of recovered within the range of 99.40 , 99.36 and 100.52 respectively which were within the acceptance criteria of 98 - 102% respectively. The result of accuracy were presented in **Table No: 7.5** and shown in **Chromatogram No. 8.26-8.34.**

Table No. 7.5: Accuracy Results

Accuracy Level in %	Sildenafil Citrate added in mg	Sildenafil Citrate Recovered in mg	% Recovered	Mean of % Recovered	Acceptance criteria
50	0.024895	0.024741	**99.38**	**99.40**	**98.0 % - 102.0 %**
	0.024675	0.024614	**99.75**		
	0.024645	0.024414	**99.06**		
100	0.049795	0.049570	**99.55**	**99.36**	
	0.050035	0.049681	**99.29**		
	0.050060	0.049677	**99.23**		
150	0.075125	0.075456	**100.44**	**100.52**	
	0.075510	0.076233	**100.96**		
	0.075055	0.075180	**100.17**		

System suitability result passes and the results obtained for accuracy are found within the acceptance criteria. Hence, it is concluded that the assay method for sildenafil citrate Soft gelatin Capsules 50 mg is proficient to recover between 50 % and 150 % of sildenafil citrate drug material when spiked in placebo.

Ruggedness:

Ruggedness was determined by performing the same assay by different analyst on the different days and the results were checked for the reproducibility. The %RSD was found to be 1.267 sildenafil citrate which were within the acceptance range of not more than 2 %. The results of ruggedness were shown in **Table No: 7.6** and **Chromatogram No. 8.35-8.46.**

Table No 7.6: Intermediate Precision Results

No. of Sample preparation	Samples Area			Results obtained		Acceptance criteria
	Sample -1	Sample -2	Average	Amount of drug present in mg	Percentage of drug	
01	247554814	247931835	247743325	53.20	106.40	90.0% - 110.0%
02	245984367	247002518	246493443	52.93	105.86	
03	253501297	255339321	254420309	54.63	109.26	
04	249292157	250075032	249683595	53.62	107.24	
05	247967600	248895826	248431713	53.35	106.70	
06	253210685	253399230	253304958	54.39	108.78	
				Mean	**107.37**	
				Std. Dev	**1.360**	
				RSD	**1.267**	**NMT 2.0 %**

System suitability result passes and the results obtained for Intermediate precision are found within the acceptance criteria. Combined Intermediate precision results and Method precision results are meets the prescribed limit as per In-House specification. Hence, it is concluded that the assay method is capable to generate, repeatable assay results for sildenafil citrate Soft gelatin Capsules 50 mg in multiple preparations of a unique batch, besides by a different analyst

LOD and LOQ:

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) was determined by injecting progressively low concentrations of the standard solutions using the developed RP-HPLC method. The LOD is the smallest concentration of the analyte that gives a measurable response (signal to noise ratio of 3). The detection limit (LOD) was found to be 0.71µg/ml for sildenafil citrate respectively. The LOQ is the smallest concentration of the analyst that given a measurable quantity (singnal to noise ratio of 10). The quantitation limit (LOQ) was found to be 2.15 µg/ml for sildenafil citrate respectively.

Robustness:

The Robustness of the method was established changing the parameters like wave length and flow rate. The changes in system suitability parameters were within the limits which ensures that the method developed can withstand slight changes in the experimental conditions and produce results with good reproducibility and repeatability. Results were presented in **Table no: 7.7-7.8** and the chromatogram were shown in **Chromatogram No. 8.47 – 8.57.**

Table No 7.7: System suitability results

S.No.	Parameter Name	Sildenafil Citrate Results		
		Tailing factor	Theoretical Plates	Area (RSD)
01	Wavelength Plus (242 nm)	1.12	11346	0.113
02	Wavelength Minus (238 nm)	1.12	11345	0.101
03	Flow rate Plus (1.2 mL)	1.12	10330	0.134
04	Flow rate Minus (0.8 mL)	1.13	12516	0.238
Acceptance criteria		**NMT 2.0**	**NLT 2000**	**NMT 2.0%**

Table No 7.8 : Robustness results

S.No.	Parameter Name	Sildenafil Citrate		Acceptance criteria
		Drug Obtained in mg	Drug Obtained in %	
01	Wavelength Plus (242 nm)	54.78	109.56	**90.0% - 110.0%**
02	Wavelength Minus (238 nm)	54.75	109.50	
03	Flow rate Plus (1.2 mL)	54.14	108.28	
04	Flow rate Minus (0.8 mL)	54.65	109.30	

System suitability result passes in all the deliberately changed methods and the results obtained for all deliberately changed methods are found within the acceptance criteria. Combined deliberately changed methods results and Method precision results are well within the desirable limit. It is concluded that the deliberately changed assay methods results are remains unaffected in small

variations which confirmed that all the methods are proficient to estimate sildenafil citrate Soft gelatin Capsules 50 mg.

Stability Study

Standard and sample solutions to be used in the analytical method are scrutinized for their solution's stability. This study was performed by injecting standard and sample solution for the period of 24 hours.

Stability Studies was carried out at 0, 3, 6, 9, 12,15,18,21 hours and 24 hours time lapse of solutions preparation. The %RSD for sildenafil citrate standard were found to be within the 0.059% and 0.120% and The %RSD for sildenafil citrate sample were found to be within the 0.071% and 1.483% which were with the acceptance criteria of not more than 2%. The results of stability were shown in **Table no: 7.9,7.10** and chromatogram were shown in **Chromatogram No. 8.58 – 8.75.**

Table No 7.9: System suitability results and Cumulative % RSD results obtained for Stability of standard solution

S. No.	Time point	Standard Solution area	Results obtained		
			Cumulative % RSD	Tailing factor	Theoretical plate
01	0th hour	240467922	NA	0.95	6903
02	3rd hour	240670302	0.059	0.95	6918
03	6th hour	240602034	0.043	0.94	6940
04	9th hour	240773026	0.053	0.98	6923
05	12th hour	240744700	0.051	0.97	6929
06	15th hour	240913809	0.064	0.96	6909
07	18th hour	240411477	0.073	0.96	6889
08	21st hour	239949634	0.124	0.94	6929
09	24th hour	240330489	0.120	0.94	6938
		Limit	**NMT 2.0 %**	**NMT 2.0**	**NLT 2000**

Table No 7.10: System suitability results and Cumulative % RSD results obtained for Stability of sample solution

S. No.	Time point	Sample Solution area	Results obtained		
			Cumulative % RSD	Tailing factor	Theoretical plate
01	0th hour	256661227	NA	0.95	6906
02	3rd hour	256401938	0.071	0.97	6947
03	6th hour	256749674	0.070	0.97	6908
04	9th hour	257485261	0.181	0.94	6951
05	12th hour	260634009	0.680	0.96	6936
06	15th hour	261251360	0.839	0.97	6923
07	18th hour	262832813	1.020	0.97	6935
08	21st hour	264082144	1.181	0.96	6933
09	24th hour	267271980	1.483	0.95	6911
		Limit	**NMT 2.0 %**	**NMT 2.0**	**NLT 2000**

System suitability result passes and the results obtained for stability of standard solution and sample solution are found within the acceptance criteria for the minimum period of 24 hours study.

10. SUMMARY AND CONCLUSION

A RP-HPLC method is developed and validated as per ICH guidelines for sildenafil citrate soft gelatin capsule dosage form.

This validation of statistical parameter is found that the analytical procedure for **Sildenafil Citrate Soft gelatin Capsules 50 mg** meets system suitability requirements and acceptance criteria for Specificity, Linearity, Precision, Accuracy, Robustness, Ruggedness parameters and Solution stability.

Considering all the fact the following parameter were finally fixed for this method

Equipment	:	High performance liquid chromatography(agilent 1260)
Mobile phase	:	Ammonium acetate :Acetonitrile
Column	:	waters reliant C_{18}, 15 cm x 4.6 mm, 5 µm
Flow Rate	:	1.0 mL/minute
Pump mode	:	Isocratic
Detector wavelength	:	240 nm
Injection volume	:	20 µL
Column Temperature	:	25.0˚C

The proposed method was found to be rapid, accurate, precise, specific, robust, rugged and economical. The mobile phase is simple to prepare and economical. The sample recoveries in all formulations were in good agreement with their respective label claims and they suggested non-interference of formulation excipients in the estimation. This method is also having an advantage

than reported method that the retention time of the drugs is below 5 mins and the drugs can be assayed with the short time. Thus the method is not time consuming and can be used in laboratories for the routine analysis of combination drugs.

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