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Development And Validation Of High-Performance Liquid Chromatographic Method For Analysis Of Tadalafil In Marketed Oral Jelly Formulation

Author 1. Suraj Dinkarrao Kaware[1],

Author 2. Prof. (Dr.) Anil P. Dewani[2] ,

Author 3. Prof. (Dr.) Anil V. Chandewar[3],

Author 4. Deepak Mourya[4],

Author 5. Sandeep Bindra[5]

[1]Pataldhamal Wadhwani College of Pharmacy, Yavatmal-445001.

[*]Natural Products and Medicinal Chemistry Division, CSIR-Indian Institute of Integrative Medicine, Jammu- 180001.

Abstract: A simple, precise, and accurate high-performance liquid chromatographic (HPLC) method was developed and validated for the quantitative analysis of tadalafil in a marketed oral jelly formulation. The chromatographic separation was achieved using a reverse-phase C18 column with a mobile phase consisting of acetonitrile and buffer (0.1%OPA) in a suitable ratio, at a flow rate of 1.0 mL/min. Detection was carried out using a UV detector set at 285 nm. The method exhibited good linearity in the concentration range of 80–180 µg/mL, with a correlation coefficient (R^2) greater than 0.999. Method validation was performed in accordance with ICH guidelines, demonstrating acceptable results for accuracy, precision, specificity, robustness, and system suitability parameters. The developed method was successfully applied for the quantification of tadalafil in commercially available oral jelly products, confirming its applicability for quality control in pharmaceutical formulations.

Key words: High-performance liquid chromatographic, Tadalafil, Analytical technique, chromatography.

1. INTRODUCTION

Analytical chemistry deals with methods for determining the chemical composition of samples of matter. Analytical Chemistry plays an important role in the resolution of a chemical compound into its proximate or ultimate parts, determination of its elements or of the foreign substances it may contain. Its application extends to all parts of an industrial society.[1-6]

1.1 HISTORY OF ANALYTICAL CHEMISTRY

Analytical chemistry has been important since the early days of chemistry, providing methods for determining which elements and chemicals are present in the world around us. The first instrumental analysis was flame emissive spectrometry developed by Robert Bunsen and Gustav Kirchhoff who

discovered rubidium (Rb) and caesium (Cs) in 1860. Most of the major developments in analytical chemistry took place after 1900. During late 20th century analytical chemistry found wide application in forensic, environmental, industrial and medical field.

Importance of Analytical Chemistry:

1) It finds numerous applications in various disciplines of chemistry.
2) It finds wide applications in other fields of related sciences.

Analytical chemistry is concerned with chemical characterization of matter, both qualitative and quantitative.

A. **Qualitative analysis** deals with the identification of elements, ions or compounds present in the sample.

B. Quantitative analysis

Quantitative analytical measurement plays a vital role in many research areas in chemistry, biochemistry, biology, geology and other sciences. It deals with the determination of how much amount of one or more constituents are present in the sample.[1-6]

1.2 METHOD DEVELOPMENT

Method development is a challenging and time-consuming process requiring much experience, creativity, logical thinking, and experimentation. With all the software and automated systems available today, method development is still very much a trial-and-error approach, expedited by a logical sequence of generic scouting runs and fine-tuning steps to achieve the requisite resolution and method performance.[7]

1.3 CONSIDERATIONS BEFORE METHOD DEVELOPMENT

Developing and validating new analytical methods is costly and time consuming. Before starting the arduous process, a thorough literature search should be conducted for existing methodologies of the intended analytes or similar compounds. This should include a computerized search of chemical abstracts and other relevant sources such as compendial monographs (USP, EP), journal articles, manufacturer literature, and the Internet. Although this search might not uncover a directly usable method, it often provides a starting point for method development or at least some useful references. [7]

New analytical methods are needed for the following reasons:

- Existing methods are not available (e.g., New Chemical Entity (NCE) for consideration as a new drug candidate).
- Existing methods are not sufficiently reliable, sensitive, or cost effective.
- New instrumentation or technique has better performance (ease of use, rapid turnaround, automation, higher sensitivity).
- An alternate (orthogonal) method is required for regulatory compliance.

1.4 FACTORS AFFECTING THE CHOICE OF ANALYTICAL METHOD:

Analytical techniques have different degrees of sophistication, sensitivity and selectivity, as well as, different cost and time requirements. An important task for the analyst is to select best procedure for a given determination this will require careful consideration of the following criteria:[1-6]

a) The type of analysis required: elemental or molecular, routine or occasional.

b) Problem arising from the nature of the material to be investigated, e.g. radio-active substance, corrosive substance, substances affected by water.
c) Possible interference from components of the material other than those of interes
d) The concentration range to be investigated.
e) The accuracy required.
f) The facilities available, particularly the instrument.
g) The time required to complete the analysis.
h) The amount of analysis of similar type which have to be performed.

1.5 SELECTION OF ANALYTICAL METHOD

First stage in the selection or development of method is to establish what is to be measured and how accurately it should measure. Unless one has series of methods at hand to assess quality of the product, validation program may have limited validity.

The selected method must have the following parameters:[1-6]

a) As simple as possible,
b) Most specific,
c) Most productive, economical and convenient,
d) As accurate and precise as required,
e) Multiple sources of key component (reagents, columns, TLC plates) should be avoided,
f) To be fully optimized before transfer for validation of its characteristics such as accuracy, precision, sensitivity, ruggedness etc.

1.6. CLASSIFICATION OF ANALYTICAL METHODS:

The analytical methods can be broadly classified into two categories:

1.6.1. Classical methods:

For qualitative analysis the separated compounds are treated with reagents that could be recognized by either color, by their boiling or melting points, their solubility in a series of solvents, their optical activities or their refractive indices. For quantitative

analysis, the amount of analyte was determined by gravimetric or titrimetric measurements.[1-6]

Advantages of Classical Methods:

- Procedure is simple and accurate.
- The equipment needed is cheap.
- Methods are based on absolute measurements.
- Specialized training is not required.

Limitations of Classical Methods:

- Chemical environment is critical.
- There is a lack of specificity and versatility.
- Accuracy decreases with decreasing amount.
- Procedure is time consuming.

Types of Classical Methods:

a) **Volumetric Method:** It is based on the determination of a solution of known strength required to complete a chemical reaction with the substance being analyzed.

b) **Gravimetric Method:** In this method of analysis, the assay results generally are obtained either by determining the weight of a substance in the sample, or the weight of some other substance derived from the sample, the equivalent weight of which serves as the basis for calculating the result.

1.6.2. Instrumental Methods:

These methods are based upon the measurement of some physical property of substance using instrument like conductivity, electrode potential, light absorption and emission, mass to charge ratio and fluorescence. These methods are now being used for quantitative analysis of a variety of inorganic, organic and biochemical analyte.

Advantages of Instrumental Methods:

- Small Samples can be used.
- High sensitivity is obtained.
- Measurements obtained are reliable.
- The determination is very fast.

- Complex samples can be handled.

Limitations of Instrumental Methods:

- Skilled person is required.
- The sensitivity and accuracy depend on type of instrument.
- Cost of equipment is high.
- Sizable space is required.

There are many techniques available for the analysis of materials; however, they are all based on the material's interaction with energy. This interaction permits the creation of a signal that is subsequently detected and processed for its information content.

There are many techniques available for the analysis of analytes:

a) Spectroscopic Analysis

Spectroscopy measures the interaction of the material with electromagnetic radiation. Different types are: -

1. Ultraviolet and visible spectrophotometry - Excitation of valence electrons.
2. Infra-red spectroscopy - Excitation of molecular vibrations.
3. Raman spectroscopy - Excitation of molecular vibrations by light scattering.
4. Atomic absorption spectroscopy - Absorption of atomic resonance line.
5. Atomic emission spectroscopy - Light emission from excited electronic states of atoms.
6. X-ray diffraction - Diffraction of X-rays from crystal planes.
7. X-ray fluorescence - Re-emission of X-rays from excited atoms.
8. Fluorimetry and Phosphorimetry - Emission of light energy by electrons.
9. Mass spectroscopy - Ionization and conversion of molecule into fragment ions.
10. NMR spectroscopy - Reorientation of magnetic nuclei in a magnetic field.
11. Nephelometry and Turbidimetry – Intensity measurement of transmitted light as a function of concentration of the dispersed phase.
12. Electron spins resonance spectroscopy - Reorientation of magnetic electrons in a magnetic field.

b) Chromatographic techniques

Separation processes are used to decrease the complexity of material mixtures. The most utilized separation method is chromatography. Following types of techniques are used:

1. Gas chromatography (GC)
2. High performance liquid chromatography (HPLC)
3. Size- exclusion chromatography
4. High-performance thin layer chromatography (HPTLC)
5. Paper chromatography
6. Thin layer chromatography (TLC)
7. Affinity chromatography
8. Ion exchange chromatography

After the isolation of material signal is generated, the signal must be detected and interpreted.

c) Hyphenated Techniques

Combinations of the above techniques are called as "hybrid" or "hyphenated" techniques. Several examples are in popular use today and new hybrid techniques are under development.

1. GC-MS (gas chromatography – mass spectrometry)
2. ICP-MS (inductively coupled plasma-mass spectrometry)
3. GC-IR (gas chromatography-infrared spectroscopy)
4. MS-MS (mass spectrometry-mass spectrometry)

Instrumental methods are sensitive and it needs small amount of sample. Complex mixtures can be analyzed with or without their prior separation with sufficient reliability and accuracy of results.

d) Miscellaneous Techniques

Following types of miscellaneous techniques are used:

a) **Mass Analysis:** Mass spectrometry measures the interaction of charged materials and electric and magnetic fields.

b) **Thermal Analysis:** Calorimetry and thermo gravimetric analysis measure the interaction of material and heat. In order to utilize the techniques available currently, complex material mixtures must be separated into simpler samples for individual analysis.

1.7 SPECTROPHOTOMETRIC METHODS

Absorption spectroscopy is one of the most useful and widely used tools available to the analyte for quantitative analysis. The relation between the concentration of analyte and the amount of light absorbed is the basis of most analytical applications of molecular spectroscopy. This method of analysis is gaining importance due to simple, rapid, precise, highly accurate and less time consuming. Spectrophotometric multi-component analysis can be applied where the spectra of drugs overlaps. It utilizes measurement of the intensity of electromagnetic Radiation emitted or absorbed by the analyte.[8]

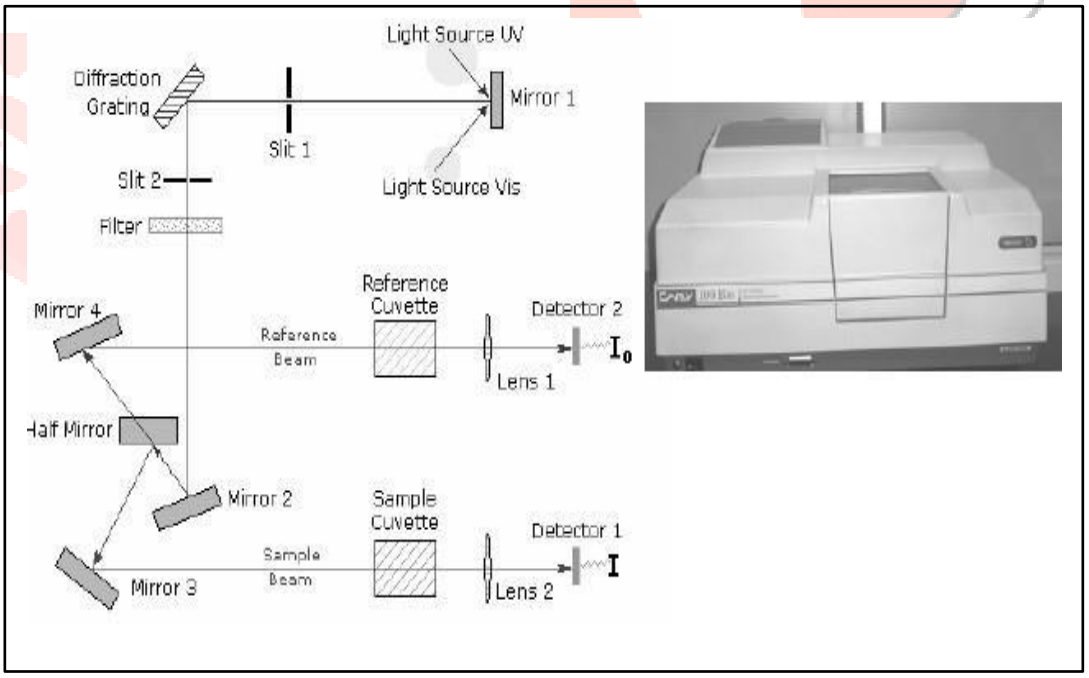


Fig. No. 1: UV-visible spectrophotometer and its flow diagram

1.7.1 Quantitative Spectrophotometric Assay of Medical Substance

The assay of an absorbing substance may be quickly carried out by preparing a solution in a solvent and measuring its absorbance at a suitable wavelength.[9]

1.7.2 Single component analysis:

The analysis of sample containing single component can be carried out using one of the following modes-[10]

a) Using Standard Absorptivity Values:

The absorptivity value A (1%, 1cm) of a standard at selected wavelength (usually) in particular solvent is established and concentration of sample is determined by comparison with standard value.

b) Using Standard Calibration Graph:

In this procedure the absorbance of a number of standard solutions of the reference substance at concentration encompassing the sample are measured and a calibration graph is constructed. The concentration of the analyte in the sample solution is read from the graph as the concentration corresponding to the absorbance of the solution.

c) Single- Or Double-Point Standardization:

The single point involves the measurement of the absorbance of the sample solution of the reference substance. The concentration of the substance in the sample is calculated from the proportional relationship that exists between absorbance and concentration.

$$Ctest = \frac{A_{test}}{A_{std}} \times C_{std}$$

Where

Ctest and Cstd = Concentration of the sample and standard solutions respectively Atest and Astd = Absorbance of the sample and standard solutions respectively

A two-point bracketing standardization is required sometimes due to non – proportional relationship between concentration and absorbance.

The concentration of the analyte is given by equation-

$$C_{test} = \frac{(A_{test} - A_{std\,1})(C_{std\,1} - C_{std2}) + C_{std\,1}(A_{std\,1} - A_{std\,2})}{A_{std\,1} - A_{std\,2}}$$

Where, the subscript Astd1 and Astd2 = more and less concentrated std. respectively.

1.7.3 Multi Component analysis

Analyst frequently encounter a situation where concentration of one or more substances is required in samples is known to contain other absorbing substances, which potentially interfere in the assay. A number of modifications to the simple Spectrophotometric procedure for single-component sample are available to the analyst, which may eliminate certain sources of interference and permit the accurate determination of one or all of the absorbing components. The basis of all Spectrophotometric technique for multicomponent samples is property that at all wavelengths:[10]

- The absorbance of a solution is the sum of absorbance of the individual components; or
- The measured absorbance is the difference between the total absorbance of the solution in the sample cell and that of the solution in the reference (blank) cell.

The determination of substance(s) in multicomponent formulation can be done by one of the following modes-

1. Simultaneous equation method or Vierodt's method.
2. Absorption ratio method or Isobestic point method.
3. Absorption factor method or absorption corrected method.
4. Two-wavelength method.
5. Area under curve method.
6. Derivative Spectrophotometry.
7. Difference spectrometry.
8. Multicomponent method.
9. Geometric correction method.
10. Orthogonal polynomial method.

1.8 CHROMATOGRAPHY

Chromatography is defined as a method of separating a mixture of components in to individual components through equilibrium distribution between two phases.

Chromatography was first invented by M. Tswett, a botanist in 1906 in Warsaw. The method chromatography is named after Greek words chroma and graphos meaning colour and writing respectively.[11]

1.8.1 Principle of Chromatographic Separation

Chromatographic techniques are dynamic process where in a mobile phase transports the sample mixture across or through a stationary phase medium. As the sample comes in contact with the stationary phase interaction occurs. A partitioning or separation of the component in the mixture results from the differential affinity of each component with the stationary phase.[11]

As the separated component emerges or elutes, a detector respond with a signal change that is plotted against time thus producing a chromatogram.

1.8.2 Classification of Chromatography

Chromatographic methods are classified based on mechanism or nature of mobile phase or stationary phase.[12]

A. By state of phases and mechanism:

Table No.1: Classification based on state of phases and mechanism

Mobile Phase	Stationary Phase	Mechanism
Gas	Solid	Adsorption Chromatography
	Liquid	Partition Chromatography
Liquid	Solid	Adsorption Chromatography
	Liquid	Partition Chromatography
Liquid		Electro migration

B. By the polarity of phases:

Table No.2: Classification based on polarity of phases

Polarity Type	Stationary Phase	Mobile Phase
Normal phase chromatography	More polar	Less polar
Reverse phasechromatography	Less polar	More polar

C. By geometry of the separation region:

Table No.3: Classification based on geometry of the separation region

Type	Geometry
Planar chromatography	2-dimensional
Column chromatography	1-dimensional (Tubular)

D. By experimental parameter variation over separation period:

Table No.4: Classification based on experimental parameter variation over separation period

Type	Parameter to control
Isothermal	Column temperature held constant
Temperature programmed	Column temperature changed systematically
Isocratic	Solvent (mobile phase) composition held constant
Solvent programmed	le phase) composition changed systematically

E. By column dimension:

Table No. 5: Classification based on column dimension

Packed column	Capillary Column (Open tubular)
5 - 3m length; solid inert support 'holds' stationary phase and fills the column	100-700 µm i.d.; 10 - 60m length; open tubing, tubing wall 'holds' the stationary phase
	support coated open tubular
Megabore Column	porous layer open tubular
wider capillary columns	wall coated open tubular

1.9. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

1.9.1 Introduction

HPLC is a modern form of liquid chromatography that uses small-particle column through which the mobile phase is pumped at high pressure. This is chromatographic process, where a mixture of analytes is separated into two distinct bands as they migrate down the column filled with stationary phase. HPLC is a dynamic partitioning process of analytes between the flowing liquid and spherical packing particles. HPLC is used either in the liquid-solid adsorption chromatography mode or the liquid-liquid partition

chromatography mode, either normal or reversed phase. Both partition and adsorption chromatography operate on differences in solute polarity since polarity is important in determining both adsorption and solubility.[13-18] As a general rule, highly polar materials are best separated using partition chromatography, while very non polar are separated using adsorption chromatography.

1.9.2 Scope

High-performance liquid chromatography is a versatile analytical technology widely used for the analysis of pharmaceuticals, biomolecules, polymers and many organic and ionic compounds.

1.9.3 Advantages & Limitations of HPLC

A. Advantages:

1. Rapid and precise quantitative analysis.
2. Automated operation
3. High sensitivity detection
4. Quantitative sample recovery
5. Amenable to diverse sample

B. Limitations:

1. High cost
2. Less separation efficiency than capillary GC.[13-18]

1.9.4 Instrumentation of HPLC

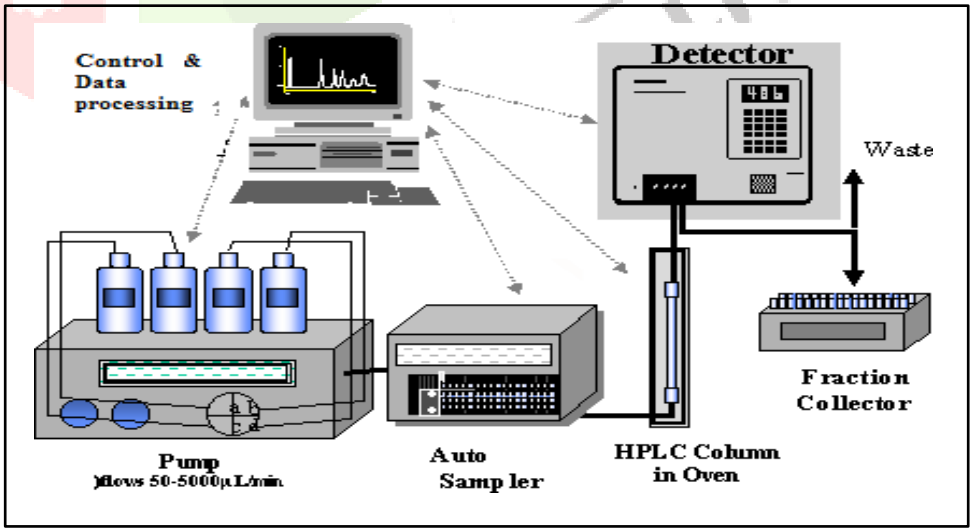


Fig. No. 2: Schematic diagram of HPLC system

1.9.5 Modes of HPLC

1. Normal phase chromatography

The separation by this method is based on adsorption of the analyte on to polar stationary phase. The typical stationary phases employed in normal phase or adsorption chromatography are common porous adsorbents such as silica and alumina that have polar hydroxyl group on their surface. It can be used for separation of non polar compound and isomers as well as for the fractionation of complex sample by function nal groups or sample clean-up.[13,17]

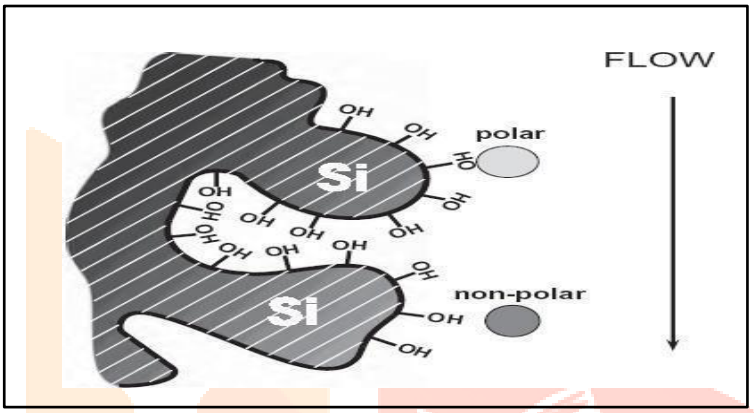


Fig. No.3: Separation modes of Normal phase chromatography

2. Reverse phase chromatography

The separation is based on analyte partition coefficient between a polar mobile phase and (hydrophobic) nonpolar stationary phase. Stationary phase commonly used is permanently bonding hydrophobic group such as octadecyl (C18) bonded group on silica support. It is most popular HPLC mode and it is used in 70 % of all HPLC analysis. It is suitable for analysis of polar (water-soluble), medium polarity and some non-polar analytes.

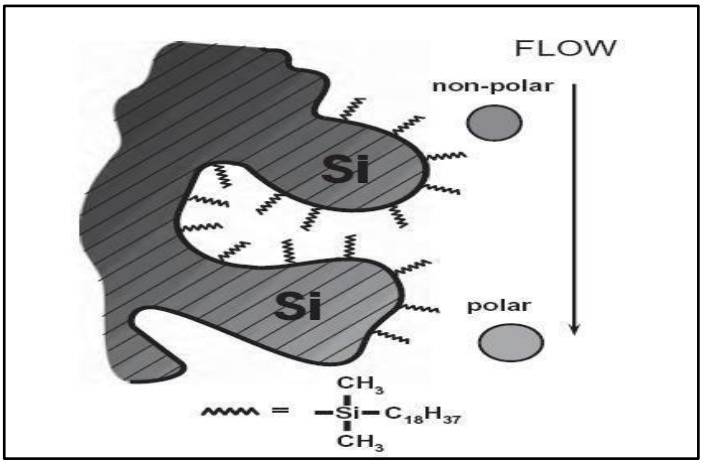


Fig. No. 4: Separation modes of reverse phase chromatography

3. Ion-exchange chromatography

The separation mode is based on exchange of ionic analytes with the counter ion of the ionic groups attached to solid support. Stationary phases are cationic exchange (sulfate) anionic exchange (quaternary ammonium) groups bonded to polymeric or silica materials. The technique is commonly used for analysis of ions and biological components such as amino acids, proteins/peptides and polynucleotides.[19, 20]

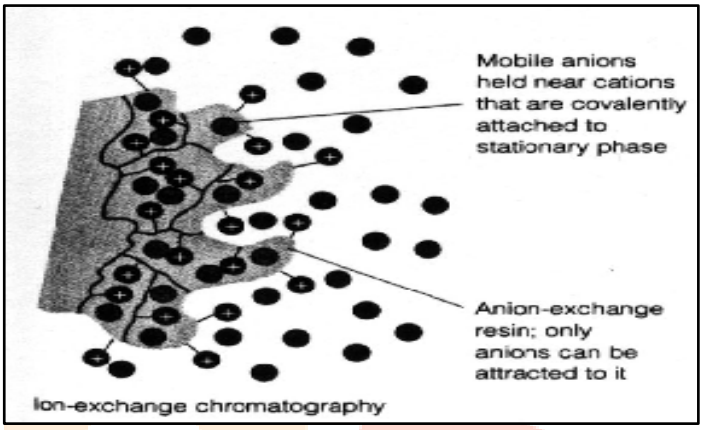


Fig. No. 5: Separation modes of ion exchange chromatography

4. Size Exclusion Chromatography

This is a separation mode based on the analytes molecular size. In this mode large molecule is excluded from the pores and migrates quickly, whereas a small molecule can penetrate the pores and migrate more slowly down the column[14-18, 10, and 20].

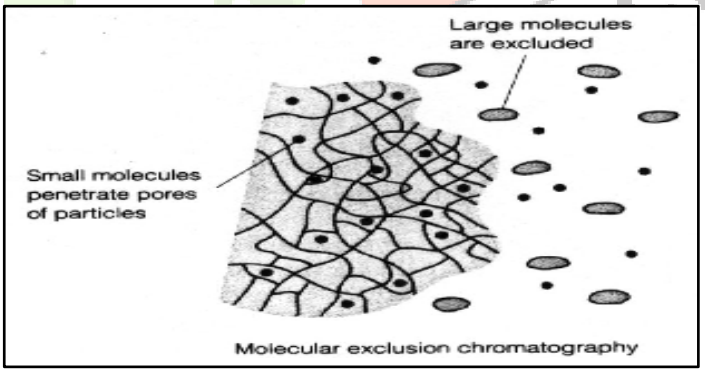


Fig. No. 6: Separation modes of size exclusion chromatography

1.9.6 CRITERIA FOR SELECTING PROPER HPLC METHOD

It is based on nature of the sample i.e. regular and special. Regular samples are typical mixtures of small molecules (< 2000 Da) that can be separated, using more or less standardized conditions. Regular samples can further be classified into neutral or ionic; Ionic samples include acids, bases, amphoteric compounds and organic salts

(ionized strong acids or bases). In neutral samples, generally buffers or additives are not required in mobile phase while acidic or basic compounds need addition of buffer to mobile phase. For basic or cationic samples, reversed phase columns are recommended, and amine additives for mobile phase may be beneficial. In some cases, reverse phase conditions provide insufficient sample retention, suggesting the use of either ion – pair or normal phase HPLC. Alternatively, the sample may be strongly retained with 100 % Acetonitrile as mobile phase, suggesting the use of non – aqueous reversed – phase (NARP) or normal – phase HPLC methods.[21]

Review of International conference on harmonization (ICH) guidelines for impurities in new drug substance and new products, and accompanying guidelines for method validation quickly underscore the usefulness of HPLC for pharmaceutical analysis. It provides- [12]

- Reliable quantitative precision and accuracy
- Linear dynamic range
- Determination of Active Pharmaceutical Ingredient (API) and related substances in the same run using variety of detectors
- Can be performed on fully automated instrumentation
- Excellent reproducibility
- Applicable to wide array of compounds by judicious choice of HPLC column chemistry.

HPLC is in some respects is more versatile than gas chromatography since:

a) It is not limited to volatile and thermally stable sample
b) The choice of mobile phase and stationary phase is wider

HPLC is the method of choice for the analysis of:

- Non-volatile substances (for volatile substances GC is an alternative)
- Substances with high polarity or ionic samples
- Substances with high molecular weight
- Thermally unstable and decomposable substances

1.9.7 Method Development in HPLC

Method development and optimization in liquid chromatography is still an attractive field of research for theoreticians (researchers) and attracts also a lot of interest from practical analysts. Complex mixtures or samples required systematic method development involving accurate modeling of the retention behavior of the

analyte. Among all, the liquid chromatographic methods, the reversed phase systems based on modified silica offers the highest probability of successful results. However, a large number of (system) variables (parameters) affect the selectivity and the resolution.

"Best column, best mobile phase, best detection wavelength, efforts in separation can make a world of difference while developing HPLC method for routine analysis. Determining the ideal combination of these factorsassures faster delivery of desired results- a validated method of separation."

Drug molecule can be viewed as a collection of functional groups. Functional groups determine such characteristics as ionization, solubility, Pka, reactivity, chemical stability. Before proceeding with development of method for a particular sample it is absolutely essential to have detailed information about the sample and separation goal should be clearly defined.[21]

1.9.8 Information about sample:

1. Number of components present in the sample
2. Pka values of different components
3. UV spectra of each analyte
4. Concentration range of each component
5. Solubility behavior
6. Nature of sample (solid, liquid, semisolid)[21]
7. Formula

Table No.6: Separation goal and its remarks in Chromatography[21]

Aim	Remarks
Resolution (Rs)	For precise and accurate quantitative method, Rs should be> 1.5
Separation time	For routine procedure<5-10 min.
Quantitation Pressure	RSD <2.0%
Pressure	<150 bars
Peak height	Narrow peaks for large S/N ratio
Solvent consumption	Minimum per sum is desirable

HPLC method development is based on few basic steps includes[21]

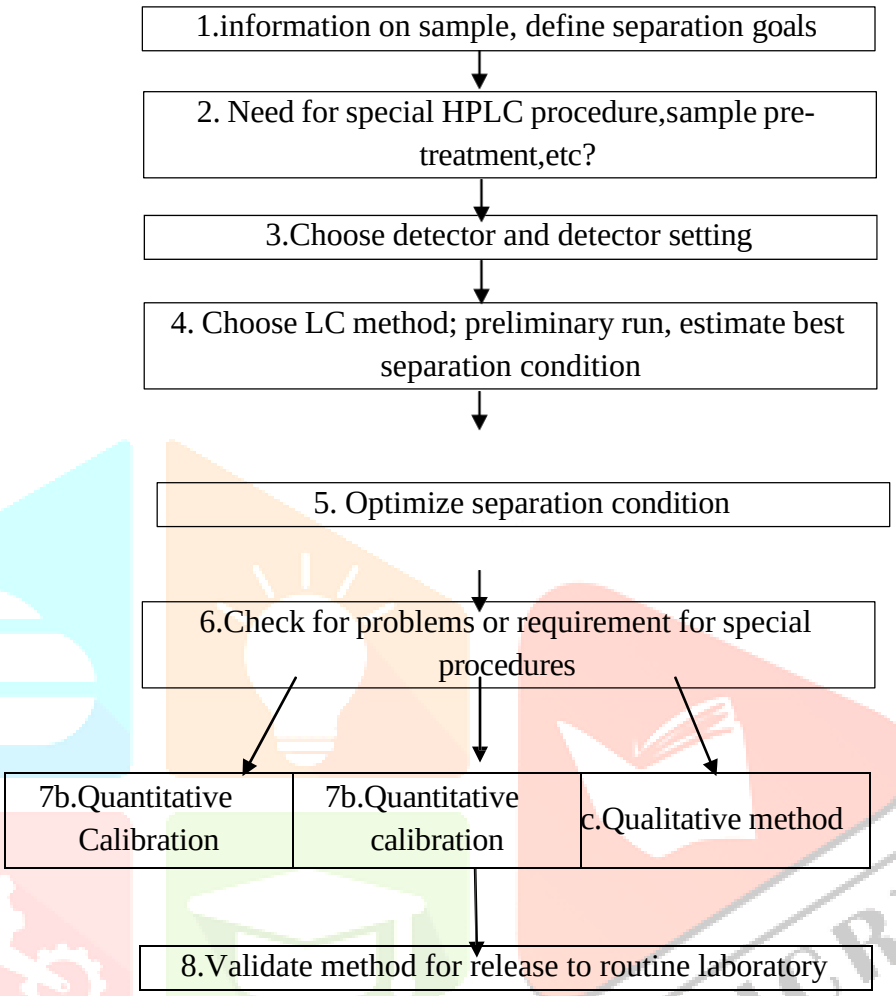


Fig. No. 7: Steps in HPLC method development.

2. SYSTEM SUITABILITY PARAMETERS:

A system suitability test is an integral part of gas and liquid chromatographic method. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The test is based on concept that the equipment, electronic, analytical operation and sample to be analyzed constitute an integral system that can be evaluated as such.

It is the verification of the system to ensure system performance before or during the analysis. Parameter such as plate count, tailing factor, reproducibility and resolution are determined and compared against the specification set for the method. The area under curve (AUC) of five replicate injections should not be more than 2% of relative standard deviation (RSD).[21]

A. Retention Time (Rt)

Retention Time is the time of elution of peak of maximum after injection of compound.

B. Theoretical Plates (N)

It is also called as column efficiency. A column can be considered as being made of large number of theoretical plates where distribution of sample between liquid- liquid or solid-solid phase occurs. The number of theoretical plates in column is given by the relationship

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

Where t_R is the retention time and w is the width at the base of the peak.

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

L = length of column

Theoretical Plates should be more than 2000.

C. Resolution (R)

It is a function of Column efficiency and is specified to ensure that closely eluting compounds are resolved from each other to establish the general resolving power of the system. For the separation of two components in mixture the resolution is determined by equation

$$R = \frac{2(t2 - t1)}{W1 + W2}$$

Where t_2 and t_1 is the retention time of second and first compounds respectively, where as W_2 and W_1 are the corresponding widths at the bases of peak obtained by extrapolating straight sides of the peaks to baselines. R should be more than 2 between peak of interest and the closest eluted potential interferences (impurities, excipients, degradation products or internal standard).

D. Tailing factor (T)

It is the measure of peak symmetry, is unity for perfectly symmetrical peaks and its value increases as tailing become more pronounced.

$$T = \frac{W_{0.05}}{2F}$$

Where, $W_{0.05}$ is the width of peak at 5% height and F is the distance from the peak maximum to the leading edge of the peak height from the baseline. Tailing factor should be less than 2.

E. Capacity Factor (k')

It is calculated by the formula

$$k' = \frac{t}{ta - 1}$$

Where t is the retention time of the drug ta is the retention time of non-retarded component, air with thermal conductivity detection.

F. Selectivity (α)

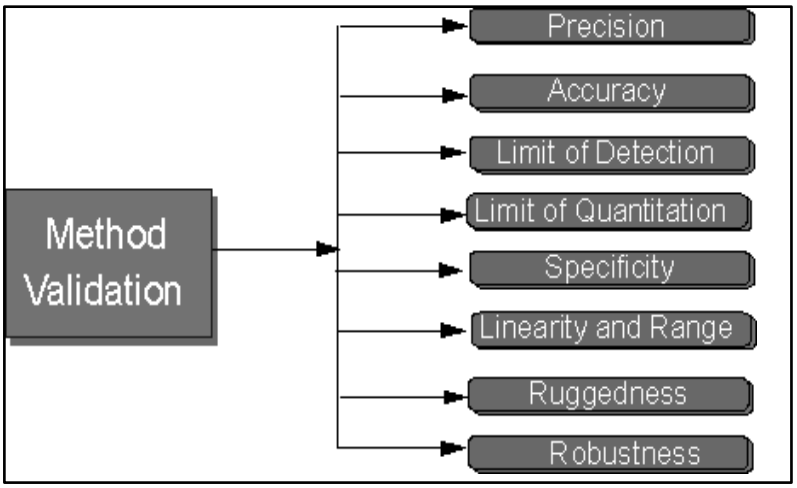
Also known as separation factor, it is a measure of peak spacing and expressed as

$$\alpha = \frac{K'_2}{K'_1}$$

3. VALIDATION OF METHODS

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice.

The USP has published specific guidelines for method validation for compound evaluation.USP defines eight steps for validation: [21]



a) Precision

The precision of analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of homogenous sample. The precision of an analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurement.

b) Accuracy

The accuracy of an analytical method is the closeness of test results, obtained by that method to the true value. The accuracy of an analytical method should be established across its range. In the case of the assay of a drug in the formulated product, accuracy may be determined by application of the analytical method to synthetic mixtures of drug product components to which known amount of analyte have been added within the range of the method. Average recovery should be 99 to 101 % of drug at each level.

c) Limit of Detection

The lowest conc. of the analyte in the sample that the method can detect but not necessarily quantify under the stated experimental conditions simply indicates that the sample is below or above certain level. Limit test prescribed as percentage or as parts per million. The limit of detection will not only depend on the procedure of analysis but also on type of instrument.

d) Limit of Quantitation

The limit of quantitation (LOQ) is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. It is expressed as the conc. of analyte (e.g., percentage, parts per billion) in the sample. The S/N ratio should not less than 10 and RSD ≤ 3%.

e) Specificity

The specificity is the ability to assess unequivocally the analyte of interest in the presence of component that may be expected to be present, such as impurities, degradation products, and matrix components. In case of assay, demonstration of specificity requires that the procedure is unaffected by presence of impurities or excipients. In practice, this can be done by spiking the drug substances or productwith appropriate levels of impurities or excipients, and demonstrating that the assay result is unaffected by the presence of these extraneous materials. If impurities of degradation product standards are unavailable, specificity may be demonstrated by comparing the test result of samples containing impurities of degradation products to second well characterized procedure. These comparisons should include samples stored under relevant stress conditions (e.g., light, heat, humidity, acid or base hydrolysis and oxidation).

f) Linearity and range

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the conc. of analyte in sample within the given range. It should be established across the range of the analytical procedure. Linearity is generally reported as the correlation coefficients, the slope of regression line i.e., $r^2 \geq 0.999$. The range of analytical method is the interval between the upper and lower level of analyte that have been demonstrated to be determined with suitable level of precision, accuracy, and linearity using method written. The range is normally expressed in the same unit as test results (e.g., percent, part per billion).

g) Ruggedness

The ruggedness of analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions such

as different laboratories, different instruments, different lots of reagents, different temperatures, different days, different analysts, etc. It is normally expressed as the lack of influence on test results of proportional and environmental variables of the analytical method. For ruggedness study, the conc. of analyte is measured using different parameters such as.

1. Different operator in same laboratory
2. Different equipment in same laboratory
3. Different source of segment and solution
4. Different laboratory

h) Robustness

The robustness of analytical method is the measure of its capacity, to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Experiments are performed by changing conditions such as temperature (± 5 ^{0}C), buffer pH (± 0.5), and ionic strength of buffers, level of additives to mobile phase. The method must be robust enough to withstand slight changes and allow routine analysis of sample.[21]

Table No. 7: Characteristics to be validated in HPLC

Characteristics	Acceptance Criteria
Accuracy/trueness	Recovery 98-102% (individual) with 80,
Precision	RSD < 2%
Repeatability	RSD < 2%
Intermediate Precision	RSD < 2%
Specificity / Selectivity	No interference
Detection Limit	S/N > 2 or 3
Quantitation Limit	S/N > 10
Linearity	Correlation coefficient r > 0.999
Range	80 –120 %
Sample solution stability	> 24 h or >12 h

3.RATIONALE OF SELECTED WORK

There are very few HPLC methods reported for analysis of Tadalafil. The methods include analysis of Tadalafil in various matrixes such as plasma and pharmaceutical formulations such as tablets, orally disintegration strips. The methods suffer disadvantage of utilizing concentrated buffers like phosphate buffer and acetate buffers. Literatures reveled that there are no methods involved in analysis of recently approved oral jelly formulation of Tadalafil. The work was undertaken to develop a HPLC method involving simple mobile phase for analysis of Tadalafil in oral jelly formulation.

- **Analytical Challenges:**

1. Chemical variability of excipients for oral jelly formulation.
2. Complexity in formulations with excipients of oral jelly formulation.
3. Demand of simple and short method for routine analysis.

4.OBJECTIVE AND PLAN

4.1. OBJECTIVE

Analysis is important in every product but it is vital in medicines as it involves life. The assurance of quality is achieved through analysis of the drug product. Now days, newer pharmaceutical dosage form are evolving for assuring better bioavailability and for getting good therapeutic response from available drugs.

The market survey revealed that **Tadalafil** has been recently came up in market in an oral jelly formulation, the formulation facilitates rapid absorption of Tadalafil compared to its tablet formulation.

Literature survey revealed that there are few analytical methods reported for estimation of Tadalafil singly or in combination also there are no reports for Tadalafil in oral jelly formulation. The available HPLC methods suffer disadvantage of using highly concentrated buffer solutions decreasing the column life.

The present work was undertaken with an objective to develop an accurate, simple, precise and reliable method for estimation of **Tadalafil** in oral jelly formulation.

4.2 PLAN OF WORK

1. Literature survey.
2. Procurement of pure **Tadalafil standard** drug sample and itsmarketed formulation.
3. Trial of instrumental method on pure drug samples which includes following Steps-

Reverse Phase-High Performance Liquid Chromatography (RP-HPLC):

- Selection of column.
- Selection and optimization of mobile phase.
- Selection of chromatographic conditions.
- System suitability parameters study.

1. Analysis of standard laboratory mixture to see feasibility of proposed method.
2. To adopt selected method on marketed formulation.
3. Recovery studies.
4. Validation of proposed method for

- System Suitability
- Linearity
- Accuracy
- Precision
- Robustness

5.0 EXPERIMENTAL WORK

5.1.Material and instruments:

Materials:

The drugs used for the present investigation were obtained from Arrow chem Mumbai.

Details of Pure drug:

Table No. 8: Details of API

Drug	Supplied by	Quantity	Purity (Assay)
Tadalafil	Arrow Chem Mumbai.	10 g	99.9 % w/w

Marketed Preparation:

Table No. 9: Details of marketed Preparation

Brand Name	Mfd by	Content	Quantity
Tastylia® Oral Jelly	Healing Pharma	Tadalafil 20 mg	5 gm sachet

The marketed preparation was obtained from local market and is referred here after in this thesis by the name as such.

Reagents and chemicals:

All reagents and chemicals used were of AR grade and HPLC grade.

Methanol	(HPLC grade).
Acetonitrile	(HPLC grade)
Disodium hydrogen phosphate	(AR grade).
Distilled Water	(HPLC grade).
Triethylamine	(HPLC grade).
Ortho Phosphoric Acid	(HPLC grade).

Instruments:

Table No. 10: Instruments Used

Sr. No	Instruments	Make	Model
1	**UV-Visible Spectrophotometer**	Shimadzu	UV 1900i
2	**HPLC**	Waters 600	996 PDA Detector
3	**pH Meter**	Hanna	-
4	**Balance**	Citizen	CY 104 (Micro Analytical Balance)
5	**Ultra sonicator**	-	1.5 L 50

5.2. Study of Functional Group by Using Infra-Red Spectroscopy:

Tadalafil API: - Accurately weighed 3 mg of Tadalafil API was mixed properly with 300 mg of dried KBr, then carefully triturated in a mortar pestle. Keep this mixture in a die and IR spectrum was taken using the Diffused Attenuated reflectance mode.

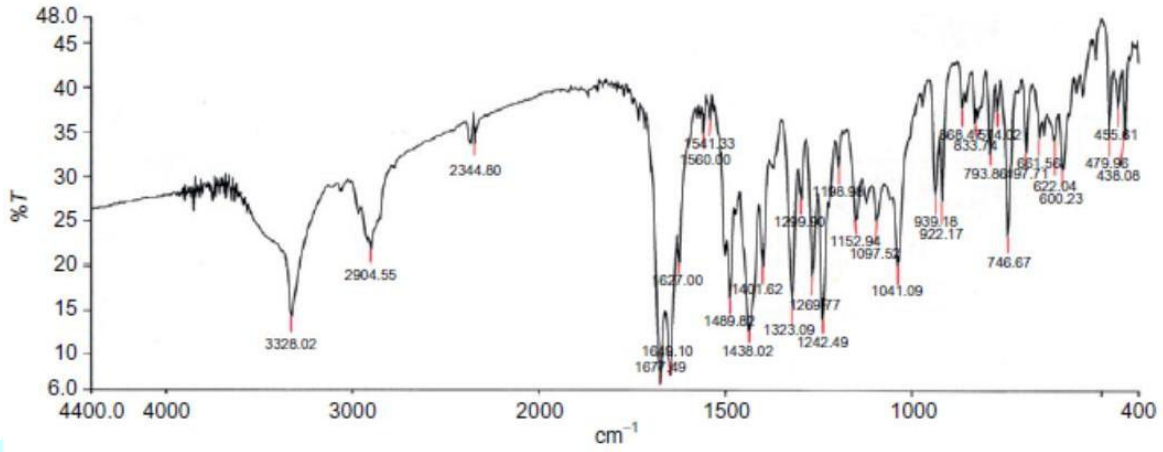


Fig. No. 8: IR Spectra of Tadalafil

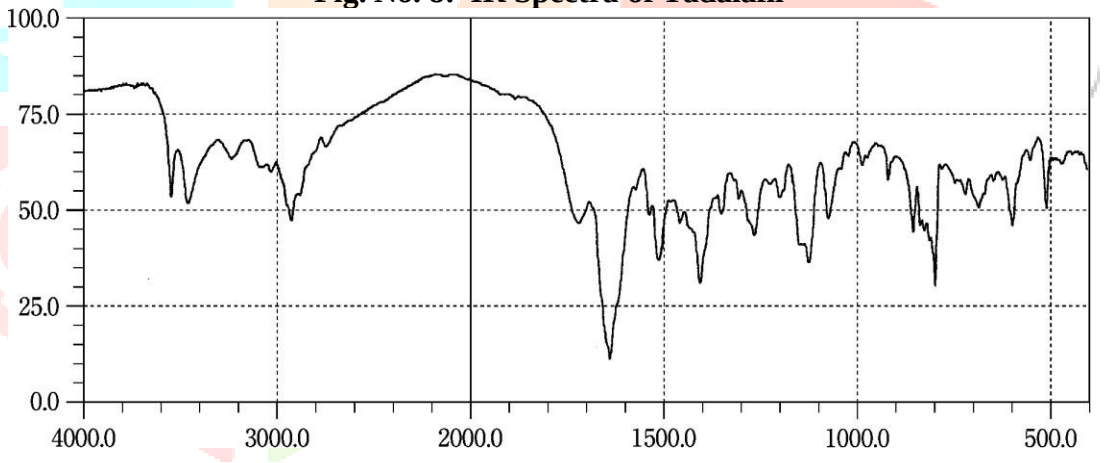


Fig. No. 9: Reference IR Spectra of Tadalafil

Conclusion:

The IR spectra of the given test drugs is matches with the IR spectra of standard drugs.

5.3. Determination of wavelength maxima Tadalafil standard stock solution:

An accurately weighed quantity of Tadalafil (TAD) 5 mg was transferred to the 10 ml volumetric flask and dissolved in HPLC grade ACN. The volume was made up to the mark with the same to make (500 □g/ml).

The aliquot portions of stock standard solutions were diluted appropriately with HPLC grade ACN to obtain concentration 5 □g/ml of TAD. The solutions were scanned in the range of 400–200 nm in 1 cm cell against blank. The UV absorbance spectrum of TAD were recorded and found to be 285nm.

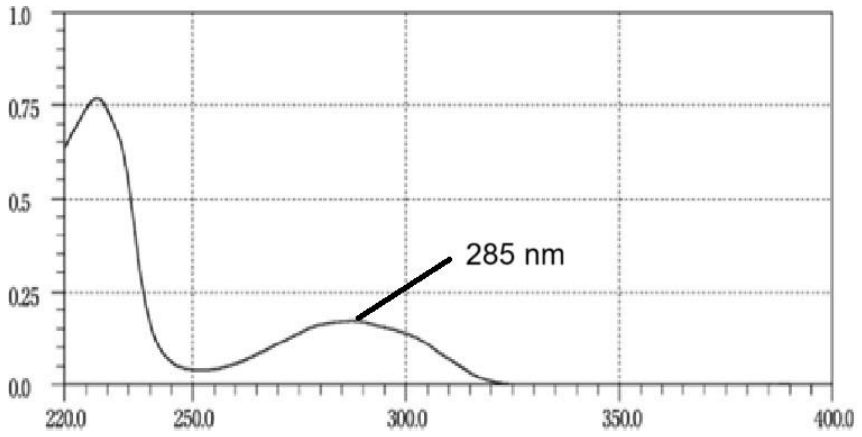


Fig. No. 10: Wavelength Maxima for Tadalafil.

5.4.Development of HPLC method for estimation of Tadalafil

5.4.1.Method Development Strategy:

5.4.1.1 Selection of Common Solvent (Diluents):

ACN of HPLC grade was selected as common solvent for preparation of stock solution and developing spectral characteristics of drugs, further dilutions from stock solutions were made in mobile phase. The selection was made after assessing the solubility of Tadalafil in different solvents i.e Acetonitrile and water.

5.4.1.2 Preparation of standard stock solution:

Accurately weighted TAD 20.0 mg was dissolved in 100ml ACN. This solution was used as standard stock solution.

Preparation of diluent:

ACN of HPLC grade was selected as common solvent for preparation of stock solution and developing spectral characteristics of drugs, further dilutions from stock solutions were made in the Mobile phase.

Procedure:

The mobile phase was allowed to equilibrate with stationary phase until steady baseline was obtained. The standard solution containing TAD was injected in different combinations of solvents, to get a stable peak with good peak characters. Each solution was filtered through Membrane filter (size 0.2µ). To achieve peaks with good symmetry various mobile phase compositions were evaluated to achieve acceptable separation using selected chromatographic conditions. The following chromatographic conditions were established by trial and error and were kept constant throughout the method.

Chromatographic Parameters:

Column:	C18 (Thermo Hypersil gold) /4.6 x 250 mm, 5µ particle size
Flow Rate:	1.0ml/min
Wavelength:	285 nm
Injection volume:	20µl
Column oven Temperature:	Ambient (25^0C)
Run Time:	10 minutes
Mobile Phase:	0.1% OPA and ACN **(**50:50)

Preparation of 0.1% OPA: Dilute 1 ml ortho phosphoric acid in 1000 ml of volumetric flask and makeup the volume upto the mark with HPLC water.

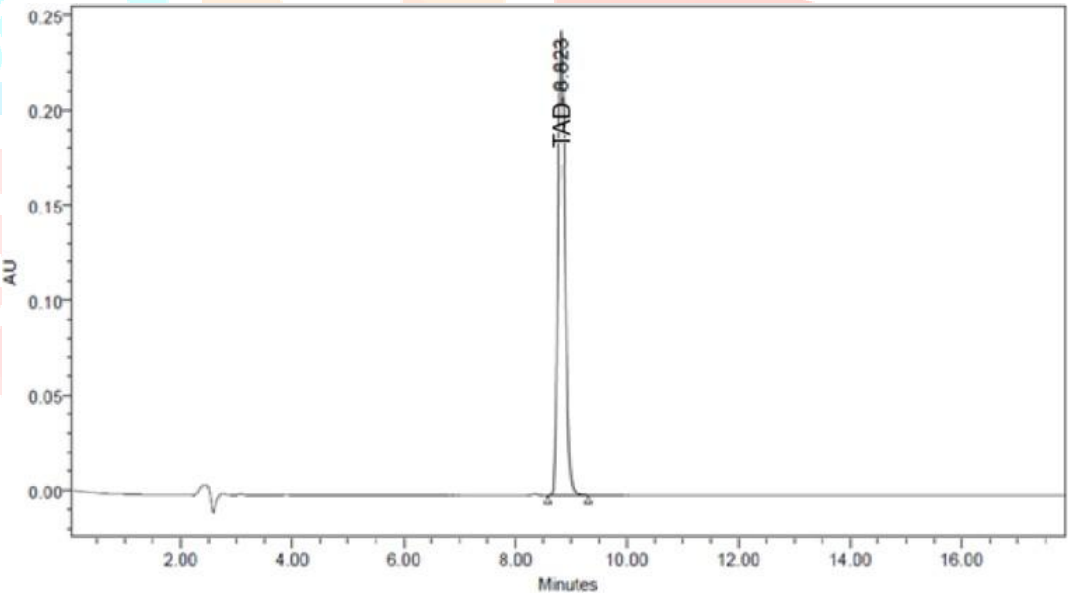


Fig. No. 11: Separation of TAD in selected mobile phase showing retention time at 8.823 min.

5.4.1.3 System suitability studies

System suitability is a pharmacopeial requirement and is used to verify, whether the resolution and reproducibility of the chromatographic system are adequate for analysis to be done. The test was performed by collecting data from 5 replicate injections of standard solutions.

The mobile phase was allowed to equilibrate with the stationary phase until steady baseline was obtained. Standard working solution of TAD was injected five times under optimized chromatographic conditions. System suitability parameters were recorded and reported.

B) Procedure:

Filtered mobile phase was allowed to equilibrate with stationary phase until steady baseline was obtained. A 20 □L std. drug solution was injected which was made in five replicates and the system suitability parameters were recorded.

Table No. 11: Result of System suitability test

Sr.No	Peak area	Retention Time	Symmetry	of theoretical Plates
	TAD	TAD	TAD	TAD
1	750269	8.90	1.10	8526
2	748952	8.92	1.20	8588
3	745896	9.01	1.00	8645
4	752698	8.98	1.25	8600
5	747895	8.99	1.20	8550
Mean	749142	8.96	1.15	8582
S.D	2551.61	0.057	0.1	46.07
%R.S.D.	0.34	0.47	1.5	0.48

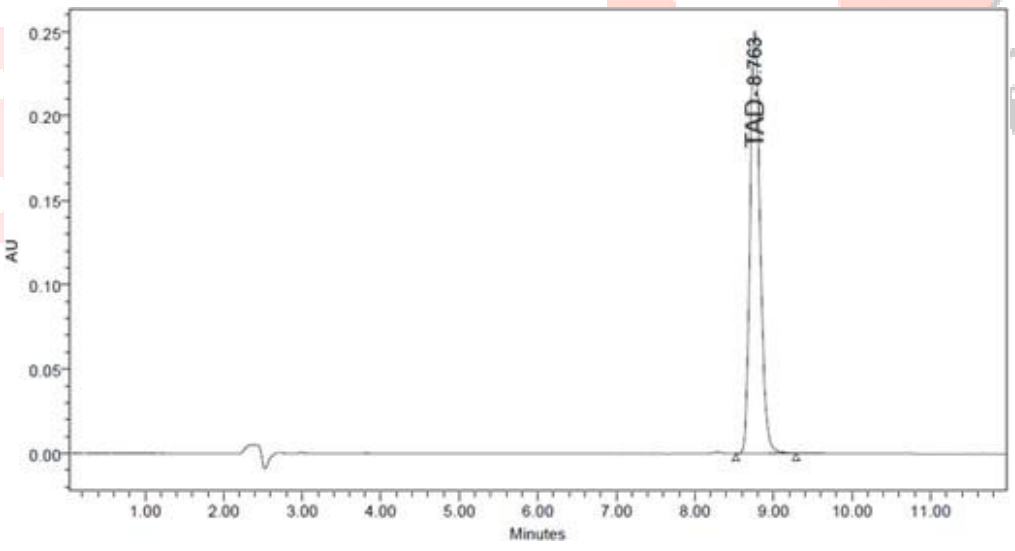


Fig. No 12: Separation of TAD in selected mobile phase showing retention time at 8.763 min.

5.5. Application of proposed method for estimation of TAD in Oral jelly formulation:

Standard stock solution:

a) Preparation of standard solutions:

Tadalafil standard stock solution: Accurately weighed quantity of 20 mg TAD was dissolved in ACN and volume was made up to 100 ml mark by same to obtain 200 µg/ml stock solution.

Tadalafil standard working solution: Pipette out 1 ml from standard stock solution and dilute it with 10 ml ACN to obtain 20 µg/ml of TAD.

Sample solution preparation:

Entire content of Tastylia® Oral Jelly (20 mg) spray was transferred to a 100 ml volumetric flask, the volume was made upto the mark with methanol, the resultant concentration was 200 µg/ml. The whole content was centrifuged at 5000 rpm for 10 min followed by passing through 0.45 µ membrane filter. 1 ml of resultant was transferred to a 10 ml volumetric flask and the volume was made upto the mark with methanol, the concentration of working sample solution was 20 µg/ml.

Procedure:

Equal volume (20□L) of standard and sample solution were injected separately after equilibrium of stationary phase. The chromatograms were recorded and the response
i.e. peak area of major peaks were measured. The content of TAD was calculated by comparing a sample peak with that of standard.

Amount of drug in Oral jelly formulation sample was calculated using following formula-

$$\% \text{ Estimation} = \frac{A_t}{A_S} \times \frac{D_S}{D_t} \times \frac{W_S}{W_t} \times 100$$

Where,

At = Area count for sample solution. As = Area count for standard solution. Ds = Dilution factor for standard.

Dt = Dilution factor for sample. Ws = Weight of standard (mg) Wt = Weight of sample (mg)

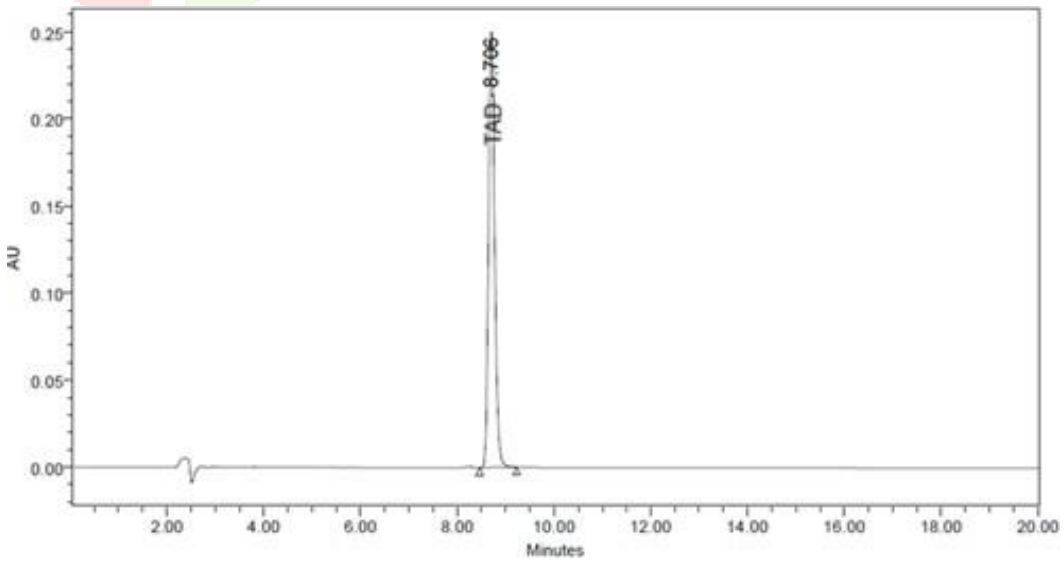


Fig. No.13: Chromatogram of TAD marketed formulation showing retention time 8.706 min.

Brand name: Tastylia® Oral Jelly

Sr.No.		
	Assay (mg)	% Purity
1	19.98	99.99
2	19.95	99.75
3	19.98	99.99
4	19.96	99.80
5	19.98	99.99
Average	19.97	99.90
SD	0.014	0.11
% RSD	0.07	0.11

Table No. 12: Results and statistical data for estimation of TAD in marketed formulation

5.6.Validation parameters:

- **System suitability system precision**
- **Accuracy**
- **Precision**
- **Ruggedness**
- **Robustness**
- **Linearity and range**
- **Specificity**
- **Placebo Interference study**
    - **Accuracy:**

The accuracy placebo were prepared by spiking the standard into the pre- analyzed formulation sample at different concentrations (80%,100% and 120%) and injected each in triplicate. The resultant mix was injected and recovery of standard spiked was calculated.

The % Recovery was then calculated by using formula

% Recovery = A-B x 100/ C

Where- A = Total amount of drug estimated.
B = Amount of drug found on pre analyzed basis.
C = Amount of pure drug added.

Calculate the amount recovered, % recovery, average recovery, % RSD of triplicate Placebo spike sample preparation, overall recovery and overall % RSD. Record the observation into the following table.

	TAD		
	Levels		
	80%	100%	120%
Amt added (µg/ml)	16	20	24
	16	20	24
	16	20	24
Amt taken (µg/ml)	16	20	24
	16	20	24
	16	20	24
Amt recovered (µg/ml)	15.98	19.98	23.98
	15.98	19.98	23.99
	15.99	19.99	23.98
% Recovery	99.88	99.90	99.91
	99.88	99.90	99.95
	99.93	99.99	99.91
Mean % recovery	99.89	99.93	99.92
% RSD	0.48	0.39	0.56

Table No 13: Accuracy studies by standard addition method Acceptance criteria:

1) The % RSD for the triplicate at each spike level shall be NMT 2.0.

2) The overall % RSD for % recovery for all spike levels shall be NMT 2.0.

3) The % recovery at each spike level shall be NLT 98.0 and NMT 102.0 of the added amount.

- **Precision:**

a) System precision

Prepared the standard solution as per test method and inject into the HPLC system in three replicates. Calculate the % RSD for the area responses and record the observations into the following table

Sr. No.	Parameter	Observations	Limits
1	The % RSD of peak area response for three replicate injections of standard	1.217	NMT 2.0
2	Theoretical plates	8557.53	NLT 2000
3	Tailing factor	1.278	NMT 2.0

Table No. 14: Results for System Precision showing system suitability

Where, NMT - Not More Than NLT – Not Less Than

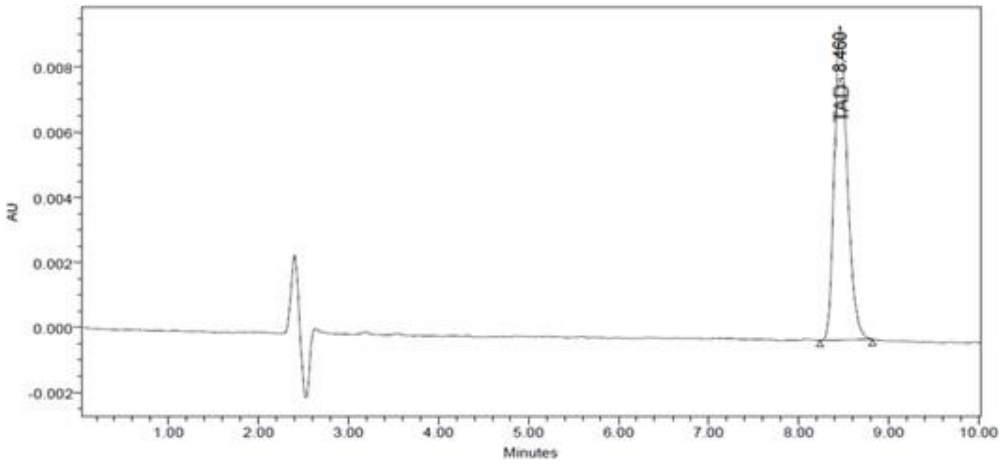


Fig.no 14: Chromatogram System precision Showing Repeatability

Injection No.	Area Response
	TAD
1	750298
2	751600
3	749500
Average	750466
SD	21060
% RSD	0.70

Table No. 15: System precision Showing Repeatability Acceptance criteria:

% RSD for replicate injections shall be NMT 2.0

b) Method precision:

Prepared three samples solutions as per the test method and injected into the HPLC system by following the conditions prescribed in the Test method.

Procedure:

Sample solution was prepared and injected into the HPLC system, the chromatograms were recorded for peak area response for the TAD. The assay and % label claim for TAD was calculated.

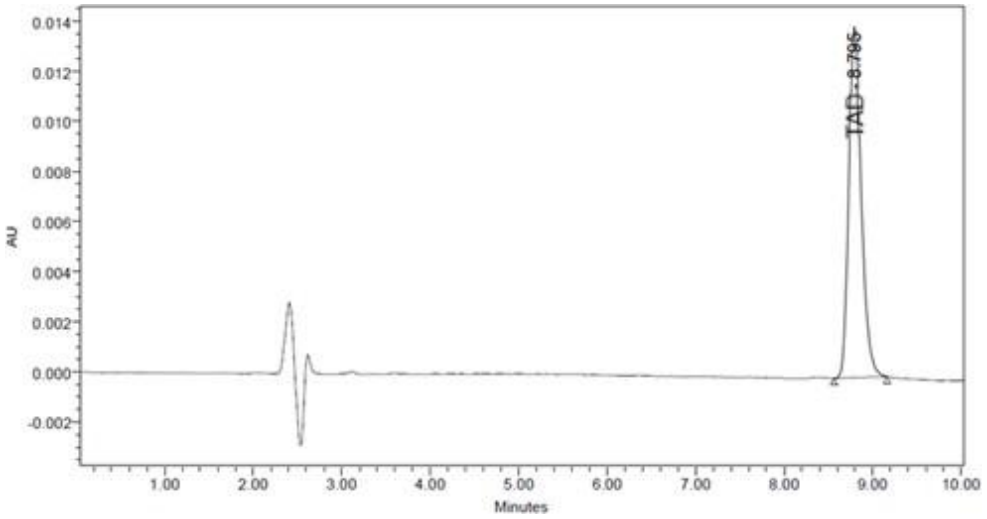


Fig.no 15: Chromatogram of Method precision

Sr.no.	TAD	
	Assay (mg)	Assay % of LC
1	19.99	99.99
2	19.99	99.99
3	19.98	99.95
Average	19.95	99.96
SD	0.70	0.45
% RSD	0.47	0.45

Table No.16: Method Precision Studies Set – I

Acceptance criteria: The% RSD for the three determinations shall be NMT 2.0

- **Ruggedness: Intermediate precision**

Prepared three sample solutions as per the test method. Injected into the different HPLC system (preferably with different manufacturer or same manufacturer with different configuration) by using the different column and by the different analyst at different date.

Sr.No.	TAD	
	Assay (mg)	Assay % of LC
1	19.98	99.95
2	19.97	99.90
3	19.98	99.95
Average	19.98	99.87
SD	0.012	0.824
% RSD	0.83	0.825

Table No. 17: Intermediate precision Studies (Ruggedness) Set – II Acceptance criteria: The % RSD for the three determinations shall be NMT 2.0

Data analysis between method precision and Intermediate precision:

Compared the data obtained in this section verses the data obtained in method precision and evaluate the overall average, overall SD and overall % RSD and recorded the observation into the following table

Sr.no.	% Assay of LC	
	TAD	
	Set – I	Set - II
1	99.99	99.95
2	99.99	99.90
3	99.95	99.95
Average	99.94	
SD	0.036	
% RSD	0.036	

Table No. 18: Intermediate precision (Ruggedness) evaluation of data SET – I: Method Precision data**, SET – II:** Intermediate Precision data

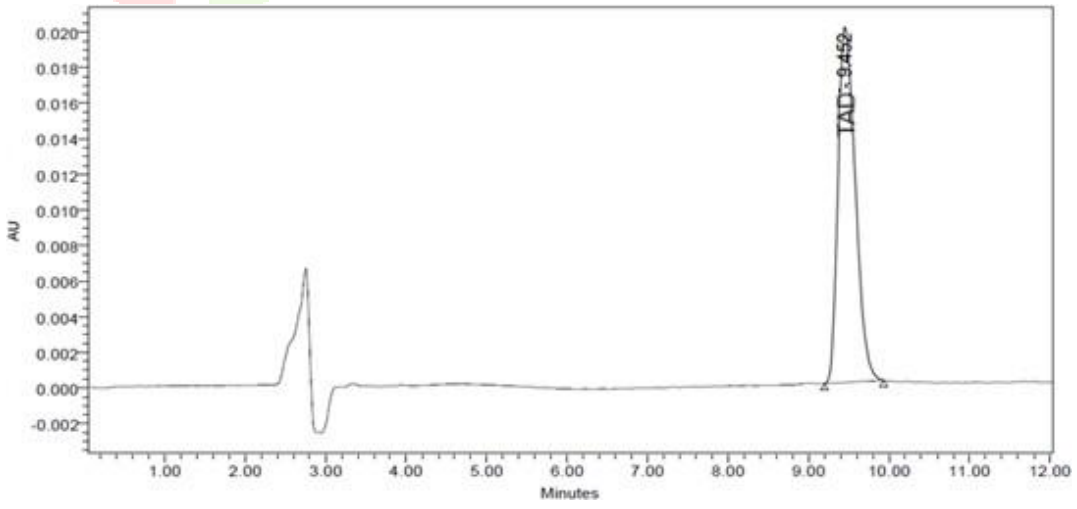
Acceptance criteria: The overall % RSD for the twelve determinations shall be NMT 2.

- **Robustness:**

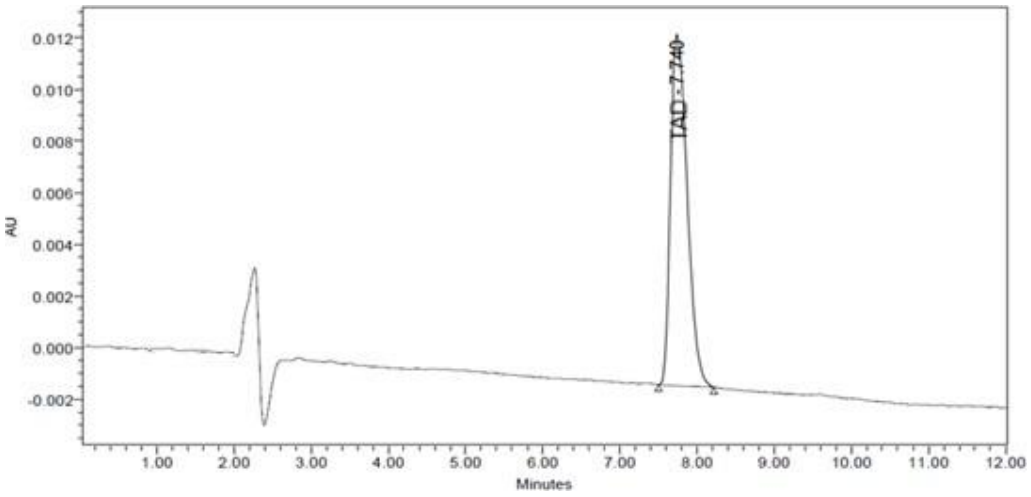
I. Effect of Variation in flow rate of mobile phase by ± 10%:

Prepared the system suitability solution (Standard Preparation) and inject into the HPLC system at –10% flow rate (0.9mL/min) and +10% flow rate (1.1mL/min) when compared with the Test method flow rate.

Procedure: Injected standard solution into the HPLC System in normal conditions and followed by the robust conditions. Measured the peak response for the major peaks.



0.9 ml/min



1.1 ml/min

Fig. No. 16: Chromatograms of Change in Flow Rate

System suitability parameters were recorded and the results are presented in the table below.

Sr. No.	System Suitability parameter		Observations for flow rate			Limits
			Unchanged	0.9 ml	1.1 ml	
1	The % RSD of peak area response for five replicate injections	TAD	1.12	0.82	0.95	NMT 2.0
2	Theoretical plates	TAD	8197.53	8138.7	8557.9	NLT 2000
3	Tailing factor	TAD	1.28	1.91	1.10	NMT 2.0
4	Retention Time (Min)	TAD	8.895	9.54	7.74	

Table No. 19: System suitability of change in Flow Rate

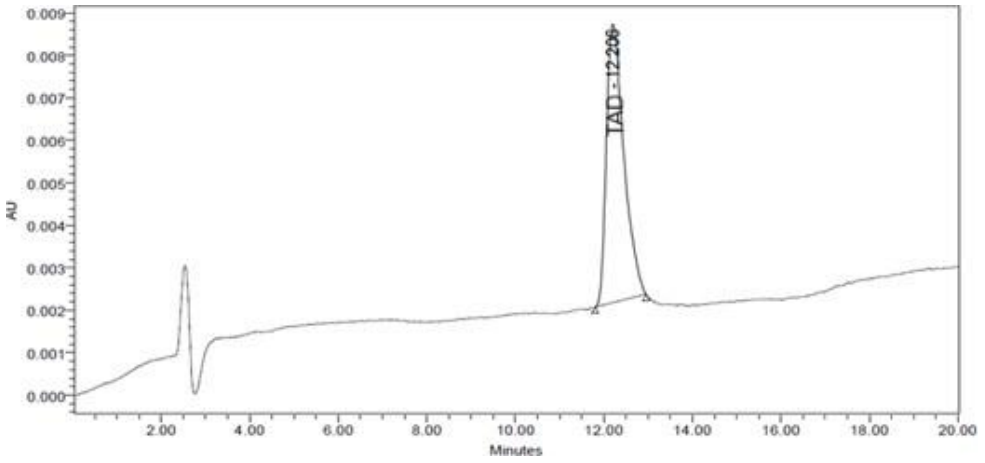
Observation: The allowable variation in flow rate of the method is from 0.9ml/min to 1.1 ml/min

Acceptance criteria: All the system suitability parameters shall pass

II. **Change in organic composition of mobile phase ± 10% (0.1%OPA: ACN)** System suitability dilution was prepared and injected into the HPLC system at –10% and + 10 % ACN (Organic phase) compared with the optimized method mobile phase concentration.

Procedure: Injected standard solution into the HPLC system in normal conditions and followed by the robust conditions. Measure the peak response for the major peaks. Check the system suitability and record the results in the table.

-10% ACN: (0.1%OPA: ACN 55:45)



+10% ACN: (0.1%OPA: ACN 45:55)

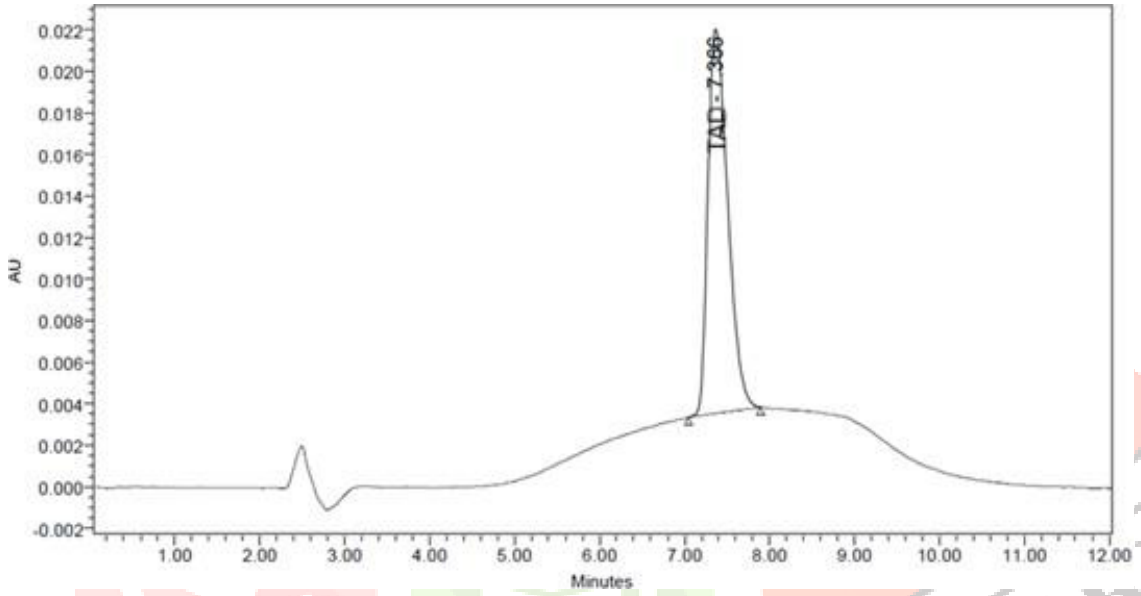


Fig. No. 17: Chromatograms of Change Organic Composition of mobile Phase

Sr. No.	System Suitability parameter		Observations			Limits
			Unchanged	- 10%	+ 10%	
1	The % RSD of peak area response for five replicate injections	TAD	1.027	0.855	0.246	NMT 2.0
2	Theoretical plates	TAD	8197.53	8996	8347.6	NLT 2000
3	Tailing factor	TAD	1.28	1.166	1.08	NMT 2.0
4	Retention Time (Min)	TAD	8.98	12.206	7.360	

Table No. 20: System suitability of change in Organic Composition Observation: The allowable variation in ACN composition of method is from 90% to 110%. **Acceptance criteria:** 1. All the system suitability parameters shall pass.

III. **Effect of Variation in Wavelength by ± 2 units:**

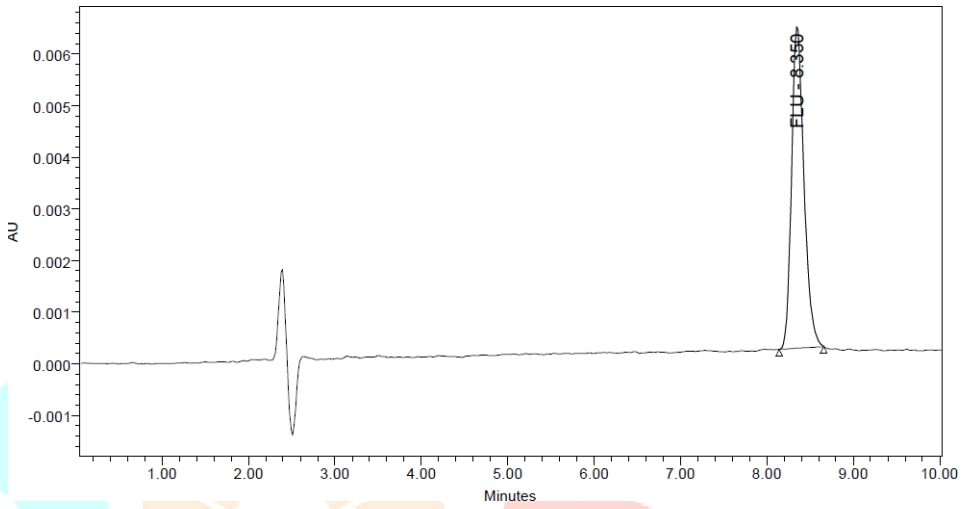
Prepared the system suitability solution (Standard Preparation) and inject into the HPLC system.

Measure the peak area response at different wavelengths at flow rate 1 ml/min.

Procedure:

Injected standard solution into the HPLC System in normal conditions and followed by the robust conditions. Measure the peak response for the major peaks.

At 283nm wavelength



At 287 nm wavelength

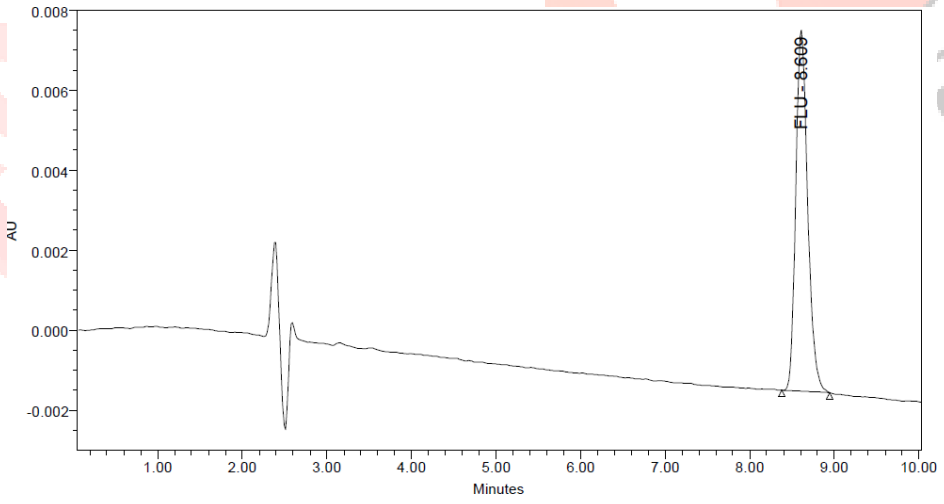


Fig. No. 18: Chromatograms of Change in wavelength.

Sr. No.	System Suitability parameter		Observations for wavelength			Limits
			Unchanged	283nm	287nm	
1	The % RSD of peak area response for five replicate injections	TAD	1.21	0.263	0.241	NMT 2.0
2	Theoretical plates	TAD	8057.53	8987.9	8678.3	NLT 2000
3	Tailing factor	TAD	1.06	1.00	0.94	NMT 2.0

4	Retention Time (Min)	TAD	8.80	8.35	8.60	

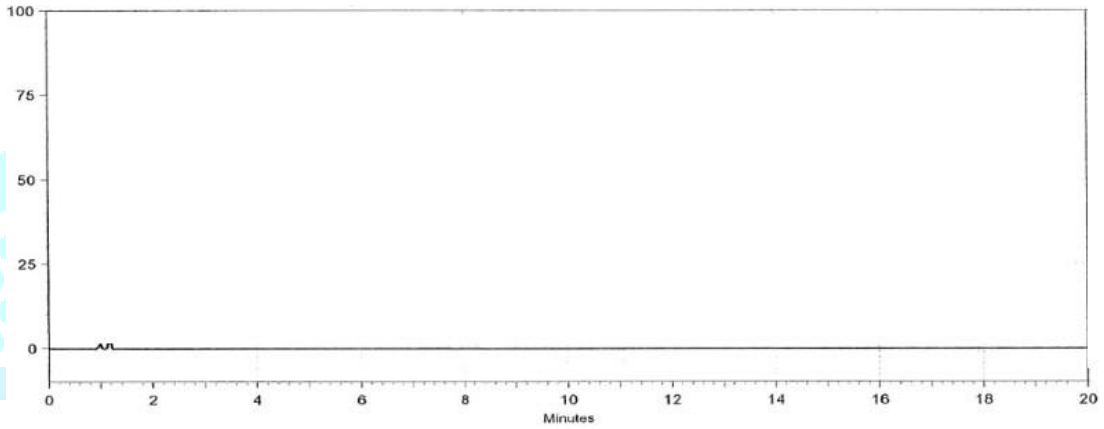
Table No.21: System suitability of change in wavelength

- **Specificity:**

1) Placebo Interference study:

Prepared the placebo solution by weighing equivalent amount of placebo present in the sample to be taken for assay preparation in triplicate, diluted it as per the test method and injected into the HPLC system. Evaluate the % interference from placebo and recorded the observation.

Diluent



Placebo Preparation

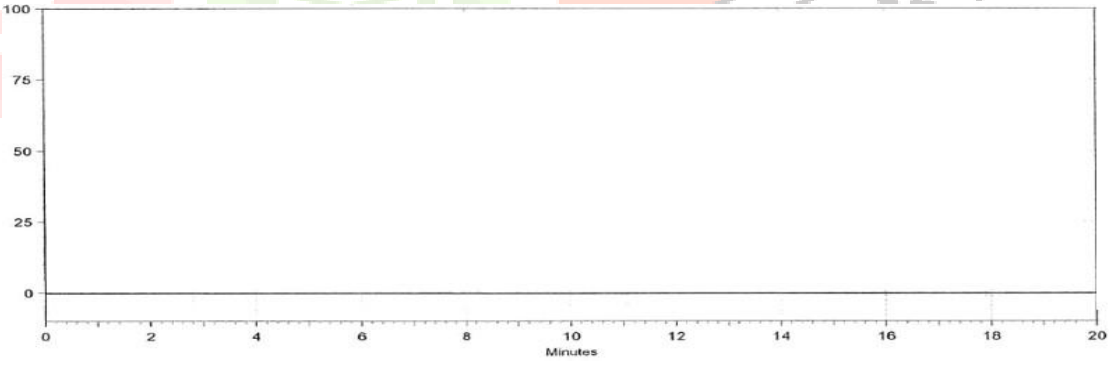


Fig. No. 19: Chromatograms of placebo interference study

Observation	Placebo prep.1	Placebo prep.2	Placebo prep.3
% Interference	No Interference	No Interference	No Interference

Table No. 22: Placebo Interference

Acceptance criteria No interference should observe from placebo at the retention time of TAD.

• **Linearity and range:**

Prepared the series of standard concentrations ranging from 80 % to 180 % of the targeted concentration of TAD. Each of the linearity dilution was injected into the HPLC system with optimized chromatographic parameters.

Procedure:

Separately inject standard preparation and linearity preparations into the HPLC system, record the chromatograms and measure the peak responses for TAD peaks.

The details of mean peak areas for linearity concentrations are presented in following table and plot the graph of concentration verses average area response for TAD, the correlation coefficient and equation of regression were recorded.

Sr. No.	% Level	TAD	
		Conc. (µg/ml)	Mean peak area
1	80	16	601000
2	100	20	749850
3	120	24	900100
4	160	32	1225000
5	180	36	1355600

Table No.23: - Observations of Linearity and range study for TAD

Acceptance criteria: The correlation coefficient shall be NLT 0.99

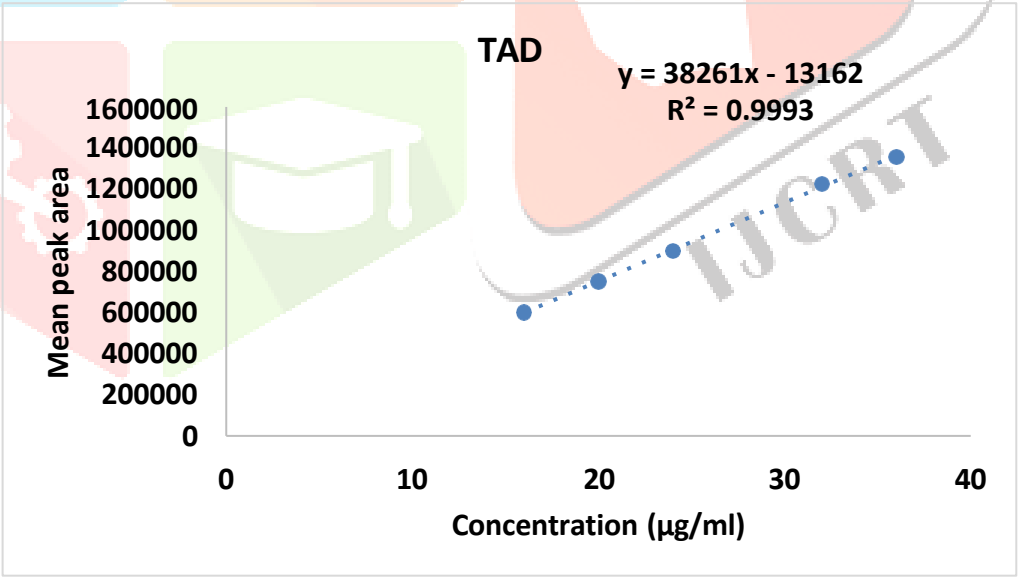


Fig. No. 20: Plot of linearity and range study for TAD

6. RESULT AND DISCUSSION

High Performance Liquid Chromatography which is a highly sophisticated technique, it is used for the determination of active molecules from their formulations. In the present study a HPLC method was developed for analysis of TAD from its oral jelly formulation.

Recently a sublingual spray formulation containing TAD have been introduced in market for treating

erectile dysfunction.

Very few methods are so far reported for estimation of TAD. In the present investigation an attempt has been made to develop a simple HPLC method for estimation of TAD from its formulation. Pure standards of **TAD** were procured from the Arrow chem Mumbai. Percent purity of above-mentioned drug was reported by Supplier Company as follows-

Table No. 24: Details of API

Drug	Supplied by	Quantity	Purity (Assay)
Tadalafil	Arrow Chem Mumbai.	10 g	99.9 % w/w

These were not analyzed in our study and the % purity stated by the suppliers was taken as standard for comparison studies.

6.1. RP-High Performance Liquid Chromatography (HPLC) Method:

HPLC has gained the valuable position in the field of analysis due to ease of performance, specificity, sensitivity and the analysis of sample of complex nature. This technique is commonly used for the quantitative estimation of the drugs from their formulation as well as for studying their metabolites of drugs and their estimation in their biological fluids. This method offers advantages of estimating the constituents for the multi component system. This technique was employed in the present investigation for estimation of TAD in oral jelly formulation. Careful evaluation of various parameters influencing analysis is an important aspect for the development of analytical method. In order to establish RP-HPLC method the following parameters were studied.

6.1.1. HPLC Column Selected:

HPLC Waters 600 system with C_{18} (Thermo Hypersil gold) /4.6 x 250mm, 5µ particle size column and PDA detector were used for the study. The standard and sample solution of TAD were prepared in diluent. Different pure solvents of varying polarity in different proportions were tried as mobile phase for development of the chromatogram.

6.1.2. Mobile Phase selected:

Mobile phase composed of water (0.1 % OPA) and ACN (50:50 % v/v). An isocratic program was developed contributing a total run time of 20 min. The wavelength 285 nm was selected for the evaluation of the chromatogram of drugs. The selection of the wavelength was based on the λ max obtained by scanning of standard solution. This system gave good resolution and optimum retention time with appropriate tailing factor (<2). The mean values of system suitability test result are depicted in Table below. The following chromatographic conditions were established by trial and error and were keptconstant throughout the method.

Table No. 25: Chromatographic Parameters:

Column	C18 (Thermo Hyrmpersil gold) / 4.6 x 250 mm
Flow Rate	1 ml/min
Wavelength	285 nm
Injection volume	20µl
Column oven Temperature	Ambient
Run Time	20 minutes
Mobile Phase	ACN & water (0.1% OPA) in ratio 50:50 % v/v

Mobile phase-preparation

Dilute 1 ml ortho phosphoric acid in 1000 ml of volumetric flask and make up the volume upto the mark with HPLC water.

Preparation of diluent:

ACN of HPLC grade was selected as common solvent for preparation ofstock solution and developing spectral characteristics of drugs, further dilutions from stock solutions were made in the Mobile phase.

Table No. 26: Summary of system suitability of Test results

Sr.No	Peak area	Retention Time	Symmetry	of theoretical Plates
	TAD	TAD	TAD	TAD
1	750269	8.90	1.10	8526
2	748952	8.92	1.20	8588
3	745896	9.01	1.00	8645
4	752698	8.98	1.25	8600
5	747895	8.99	1.20	8550
Mean	749142	8.96	1.15	8582
S.D	2551.61	0.057	0.1	46.07
%R.S.D.	0.34	0.47	1.5	0.48

After establishing the chromatographic conditions, Mix standard and marketed preparation solutions were prepared and analyzed by procedure described under experimental work. It gave accurate, reliable results and was extended for estimation of drugs in marketed Oral jelly formulation.

Amount of drug in Oral jelly was calculated using following formula:

$$\text{Assay (mg/ml)} = \frac{A_t}{A_S} \times \frac{D_S}{D_t} \times \frac{W_S}{W_t} \times \frac{P}{100} \times Wt$$

$$\% \text{ Label claim} = \frac{\text{Assay (mg/ml)} \times 100}{\text{Label claim in mg/ml}}$$

Where,

At =Area count for sample solution. As = Area count for standard solution.

Ds =Dilution factor for standard. Dt=Dilution factor for sample. P=Potency of drug

6.1.3. VALIDATION

Validation of these methods was performed as per the USP guidelines for these following parameters:

Precision:

System Precision

Prepared the standard solution as per test method and injected into the HPLC system in three replicates. It was found that all system suitability parameters are well within the limits.

Method Precision

Replicate estimation of Oral jelly analyzed by the proposed method has yielded quite consistent result indicating repeatability of method. Study showed R.S.D. less than 2.

Table No. 27: Data showing system Precision

Sr. No.	Parameter	Observations	Limits
1	The % RSD of peak area response for three replicate injections of standard	1.217	NMT 2.0
2	Theoretical plates	8557.53	NLT 2000
3	Tailing factor	1.278	NMT 2.0

Table No.28: Method Precision Studies Set – I

Sr.no.	TAD	
	Assay (mg)	Assay (mg)
1	19.99	19.99
2	19.99	19.99
3	19.98	19.98
Average	19.95	1.499
SD	0.70	0.70
% RSD	0.47	0.47

6.1.4. Linearity & Range:

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample. Linearity was carried out for five levels in the range of 80% to 150%. A graph was plotted with concentration on X axis and mean peak areas on Y- axis. The R^2value was found to be 0.999 for TAD. The result show that an excellent correlation exists between concentration and mean peak areas within the concentration range. Thus, the method developed is accurate, precise, specific, & linear. Hence it can be said that, RP-HPLC is the most accurate, precise and reproducible among all methods.

6.1.5. Accuracy:

Accuracy of the proposed method was ascertained from the recovery studies by standard addition method. Recovery results werewell within the range **99-101%.** Thus, the method was found to be accurate.

Table No. 29: Result of Accuracy Studies

	TAD		
	Levels		
	80%	100%	120%
Amt added (µg/ml)	16	20	24
	16	20	24
	16	20	24
Amt taken (µg/ml)	16	20	24
	16	20	24
	16	20	24
Amt recovered (µg/ml)	15.98	19.98	23.98
	15.98	19.98	23.99
	15.99	19.99	23.98
% Recovery	99.88	99.90	99.91

	99.88	99.90	99.95
	99.93	99.99	99.91
Mean % recovery	99.89	99.93	99.92
% RSD	0.48	0.39	0.56

6.1.6. Robustness:

Robustness of the proposed analytical method was evaluated by making deliberate changes in the chromatographic system method parameters, the standard solution and test solutions were injected for each of the changes made to access the Robustness of proposed analytical method.

Following Parameters were covered under robustness parameter.

1. Effect of variation in flow rate of mobile phase by ± 10%
2. Organic phase composition (± 10%)
3. Change in Wavelength by ± 2 units

The results suggested all the system suitability parameters were within limits.

6.1.7. Specificity:

Is the ability to assess unequivocally the analyte in the presence of impurities, degradants, matrix etc. It is evaluated by injecting the blank, placebo and the control sample solution prepared as per the proposed method to check for the interference if any peak at the retention time of TAD. Thus, no interference was foundat the Retention time of TAD.

7. SUMMARY AND CONCLUSION

7.1. SUMMARY

Sublingual spray formulation containing TAD is recently introduced in market to treat erectile dysfunction condition. Literature survey revealed very few methods for the estimation of TAD.

The present study was undertaken with an objective of developing suitable, sensitive and simple analytical RP-HPLC method for estimation of TAD in the oral jelly formulation.

In the developed RP-HPLC method the analyte was resolved using Mobile phase composed of water (0.1% OPA) and ACN in the ratio 50:50 % v/v. A isocratic program was developed contributing a total run time of 20 min. using HPLC auto- sampler system containing PDA detector with EMPOWER Software and C18 (Thermo Hypersil gold) /4.6 x 250 mm, 5µ particle size column, the detection wavelength was 285 nm. The method gave the good resolution and suitable retention time.

The results of analysis in all the method were validated in terms of accuracy, precision, ruggedness, linearity and range. The methods were found to be sensitive, reliable, reproducible, rapid and economic also.

7.2. CONCLUSION

From the results of the study, it can be concluded that the present RP-HPLC technique was successfully used for the estimation of the TAD in the oral jelly formulation.

The method showed good reproducibility, it was accurate, precise, specific, reproducible and sensitive. The analysis of oral jelly formulation of TAD was done by the developed and validated RP-HPLC method.

The RP-HPLC method was also simple, accurate, precise, reproducible and economical too. It may be adopted for routine control analysis of TAD alone and in combined dosage form.

No interference of additives, matrix etc. is encountered in these methods. Further studies on other pharmaceutical formulations would throw more light on these studies.

Suitability of these methods on biological samples needs to be studed.

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