ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF DENAVERINE HYDROCHLORIDE IN BULK AND INJECTABLE PHARMACEUTICAL DOSAGE FORM

A Dissertation submitted to
THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY
CHENNAI - 600 032

In partial fulfillment of the requirements for the award of the Degree of
MASTER OF PHARMACY
IN
BRANCH III – PHARMACEUTICAL ANALYSIS
Submitted by

J. IJAZ AHAMED

REGISTRATION No.: 261930151

Under the guidance of

Prof. Dr. G. KRISHNAMOORTHY

Vice Principal & Head of the Department

Department of Pharmaceutical Chemistry including Pharmaceutical Analysis

PERIYAR COLLEGE OF PHARMACEUTICAL SCIENCES
TIRUCHIRAPPALLI - 620 021.
(An ISO 9001: 2015 Certified Institution)

OCTOBER– 2021

Prof. Dr. G. Krishnamoorthy,

Vice Principal & Head of the Department

Department of Pharmaceutical Chemistry Including Pharmaceutical Analysis

Periyar College of Pharmaceutical Sciences

Tiruchirappalli - 620 021.

CERTIFICATE

This is to certify that the dissertation entitled **"ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF DENAVERINE HYDROCHLORIDE IN BULK AND INJECTABLE PHARMACEUTICAL DOSAGE FORM"** submitted in partial   fulfillment of the requirements for the award of the degree of **Master of Pharmacy in Pharmaceutical Analysis** by The Tamil Nadu Dr. M.G.R. Medical University, Chennai is a bonafide work carried out at **Periyar College of Pharmaceutical Sciences, Tiruchirappalli** - 620 021, by **Reg. No.: 261930151** under my guidance and supervision during the academic year of 2020 - 2021.

Date: **(Prof. Dr. G. Krishnamoorthy)**

Place: Tiruchirappalli.

Prof. Dr. R. Senthamarai, M. Pharm., Ph. D.,
Principal
Periyar College of Pharmaceutical Sciences
Tiruchirappalli – 620 021.

<div align="center">

CERTIFICATE

</div>

This is to certify that the dissertation entitled **"ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF DENAVERINE HYDROCHLORIDE IN BULK AND INJECTABLE PHARMACEUTICAL DOSAGE FORM"** submitted by **Reg. No.: 261930151** in partial fulfillment of the requirements for the award of the degree of **Master of Pharmacy in Pharmaceutical Analysis** by The Tamil Nadu Dr. M.G.R. Medical University, Chennai is a bonafide record of work done by her during the academic year of 2020 – 2021 under guidance of **Prof. Dr. G. KRISHNAMOORTHY,** Vice Principal & Head, Department of Pharmaceutical Chemistry Including Pharmaceutical Analysis, Periyar College of Pharmaceutical Sciences, Tiruchirappalli - 620 021.

Date: **(Prof. Dr. R. Senthamarai)**

Place: Tiruchirappalli.

ACKNOWLEDGEMENT

I also express my heartfelt thanks to the **Librarian** and **Assistant Librarian** for providing materials for my research work.

I wish to acknowledge my batchmates for their support throughout my dissertation work.

I also thank to my junior and friends for their timely help for completion of my project work.

Finally, I take privilege to remember my beloved parents and all other family members for their help, moral support and boundless enthusiasm, which helped me to complete this work successfully.

Reg. No.: 261930151

LIST OF ABBREVIATION

API   : Active Pharmaceutical Ingredient

AR   : Analytical Reagent

EMR   : Electro Magnetic Resonance

ESI   : Electro Spray Ionization

FDA   : Food and Drug Administration

Fig   : Figure

GLC   : Gas Liquid Chromatography

Hcl   : Hydrochloric acid

HPLC   : High Performance Liquid Chromatography

hrs   : Hours

ICH   : International Conference on Harmonization

IR   : Infra-Red

IS   : Internal Standard

IUPAC   : International Union of Pure and Applied Chemistry

L   : Litre

LOD   : Limit of Detection

LOQ   : Limit of Quantitation

Mg   : Milli gram

mL   : Milli litre

mm   : Milli meter

Mm	**: Milli Mole**
min	**: Minute**
ng	**: Nano gram**
nm	**: Nano meter**
NMR	**: Nuclear Magnetic Resonance**
RP- HPLC	**: Reverse Phase High Performance Liquid Chromatography**
Rt	**: Retention time**
RSD	**: Relative Standard Deviation**
SD	**: Standard Deviation**
UV	**: Ultra Violet**
VIS	**: Visible**
v/v	**: Volume by volume**
µg	**: Micro gram**

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

47	**Precision study -5 in Phosphate Buffer pH 3.5: Acetonitrile (30: 70%v/v)**
48	**Intermediate precision-1 in Phosphate Buffer pH 3.5: Acetonitrile (30: 70%v/v)**
49	**Intermediate precision-2 in Phosphate Buffer pH 3.5: Acetonitrile (30: 70%v/v)**
50	**Intermediate precision-3 in Phosphate Buffer pH 3.5: Acetonitrile (30: 70%v/v)**
51	**Intermediate precision-4 in Phosphate Buffer pH 3.5: Acetonitrile (30: 70%v/v)**
52	**Intermediate precision-5 in Phosphate Buffer pH 3.5: Acetonitrile (30: 70%v/v)**
53	**Robustness-Less Flow in Phosphate Buffer pH 3.5: Acetonitrile (30: 70%v/v)**
54	**Robustness-More Flow in Phosphate Buffer pH 3.5: Acetonitrile (30: 70%v/v)**
55	**Robustness- Less Organic in Phosphate Buffer pH 3.5: Acetonitrile (30: 70%v/v)**
56	**Robustness- More Organic in Phosphate Buffer pH 3.5: Acetonitrile (30: 70%v/v)**

Chapter-1

INTRODUCTION

1. INTRODUCTION

1.1 Analytical chemistry

Analytical chemistry[1] is often described as the area of chemistry responsible for characterizing the composition of matter, both qualitatively (what is present) and quantitatively (how much is present). Analytical chemistry is not a separate branch of chemistry, but simply the application of analytical data.

Pharmaceutical analysis[2] is the branch of chemistry involved in separating, identifying and determining the relative amounts of the components making up a sample of matter. It is mainly involved in the qualitative identification or detection of compounds and quantitative measurements of the substances present in bulk and pharmaceutical preparation.

Pharmaceutical analysis is a branch of practical chemistry that involves a series of process for identification, determination, quantification and purification of a substance, separation of the components of a solution or mixture, or determination of structure of chemical compounds.

The technique[3] employed in quantitative analysis is based upon the quantitative performance of suitable chemical reactions and either measuring the amount of reagent needed to complete the reaction or ascertaining the amount of reaction product obtained.

Quality[4] is important in every product or service but it is vital in medicine as it involveslife. Unlike ordinary consumer goods there can be no "second quality" in drugs.

Quality control is a concept, which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different stages of production.

The number of new drugs is constantly growing. This requires new methods for controlling their quality. Modern pharmaceutical analysis must need the following requirements.

1. The analysis should take a minimal time.

2. The accuracy of the analysis should meet the demands of Pharmacopoeia.

3. The analysis should be economical.

4. The selected method should be precise and selective.

These requirements are met by the Physico-chemical methods of analysis, a merit of which is their universal nature that can be employed for analyzing organic compounds with a diverse structure. Of them, Visible Spectrophotometry is generally preferred especially by small scale industries as the cost of the equipment is less and the maintenance problems are minimal.

The substance may be a single compound or a mixture of compounds and it may be in any of the dosage form. The substance used as pharmaceuticals are animals, plants, microorganisms, minerals and various synthetic products.

The sample to be analysed is called as analyse and on the basis of size of sample, they can be classified as macro (0.1 g or more), semi micro (0.01 g to 0.1 g), micro (0.001 g to 0.01 g), sub micro (0.0001 g to 0.001 g), ultramicro (below 10-4 g), trace analysis (100 to 10000 ppm). Among all, the semi micro analysis is widely used.

1.1.1. TYPES

There are main two types of chemical analysis.[5-15]

> 1. Qualitative (identification)
> 2. Quantitative (estimation)

1. Qualitative analysis is performed to establish composition of natural/synthetic substances. These tests are performed to indicate whether the substance or compound is present in the sample or not. Various qualitative tests are detection of evolved gas, formation of precipitates, limit tests, colour change reactions, melting point and boiling point test etc.

2. Quantitative analytical techniques are mainly used to quantify any compound or substance in the sample. These techniques are based in (a) the quantitative performance of suitable chemical reaction and either measuring the amount of reagent added to complete the reaction or measuring the amount of reaction product obtained, (b) the characteristic movement of a substance through a defined medium under controlled conditions, (c) electrical measurement, (d) measurement of some spectroscopic properties of the compound.

Various types of Qualitative analysis:

1. Chemical methods

      a) Volumetric or titrimetric methods

      b) Gravimetric methods

      c) Gasometric analysis

2. Electrical methods

3. Instrumental methods

      a. Spectroscopic techniques

          i. Ultraviolet and Visible spectrophotometry

          ii. Fluorescence and Phosphorescence spectrophotometry

          iii. Atomic spectrometry (emission and absorption)

          iv. Infrared spectrophotometry

          v. Raman spectroscopy

          vi. X-ray spectroscopy

          vii. Nuclear Magnetic Resonance spectroscopy

          viii. Electron Spin Resonance spectroscopy

      b. Electrochemical techniques

          i. Potentiometry (pH and ion selective electrodes)

          ii. Voltammetry

          iii. Electrogravimetry

          iv. Conductance techniques

      c. Chromatographic techniques

          i. Gas chromatography

          ii. High-performance liquid chromatographic techniques

      d. Miscellaneous techniques

          i. Thermal analysis

          ii. Mass spectrometry

      e. Hyphenated techniques

          i. GC-MS (gas chromatography-mass spectrometry)

          ii. ICP-MS (inductively coupled plasma-mass spectrometry)

          iii. GC-IR (gas chromatography-infrared spectroscopy)

          iv. MS-MS (mass spectrometry-mass spectrometry)

4. **Biological and microbiological**

```
                              METHODS OF ANALYSIS

              INSTRUMENTAL                                    NON-
                                                         INSTRUMENTAL

Electrical    Spectroscopic   Chromatographic   Other        Biological          Chemical
methods       methods         techniques        techniques   methods             Methods

Potentiometry  Ultra Violet       GC             RIA          Animal Assay    Titrimetry    Gravinmetry
               Spectroscopy

Conductomet    visible            HPLC           Flow         Microbiologic     i)Acid-base
ry             Spectroscopy                      Injection    al Assay          titrations

Polarography   Infra Red          TLC            ELISA                          Complexomet
               Spectroscopy                                                     ric titrations

Amperometry    Atomic             HPTLC                                         Precipitation
               Absorption                                                       titrations
               Spectroscopy

Voltametry     Atomic             PC                                            Oxidation
               Emission                                                         reduction
               Spectroscopy                                                     titrations

               nuclear                                                          Non aqueous
               magnetic                                                         titrations
               resonance
               spectroscopy

               Flame
               Emission
               Spectroscopy

               Spectrofluro
               metry

               Spectrophoto
               metry

               Colorimetry

               Nephelometry
```

Figure 1: Various types of Qualitative analysis

1. Chemical methods

a) Titrimetric or volumetric method

It involves reaction of substance to be determined with an appropriate reagent as a standard solution, and volume of solution required to complete the reaction is determined. Volumetric methods require simple and less apparatus and they are susceptible of high accuracy.

Various types of titrimetric methods are:

i)   Acid-base titrations (neutralization reactions)

ii) Complexometric titrations

iii) Precipitation titrations

iv) Oxidation reduction titrations

v)   Non aqueous titrations

b) Gravimetric methods

In gravimetric analysis, a substance to be determined is converted into an insoluble precipitate in the purest form, which is then collected and weighed. It is the time-consuming process.

In electrogravimetry, electrolysis of the sample is carried out on the electrodes is weighed after drying.

Thermogravimetry (TG) records the change in weight, differential thermal analysis (DTA) records the difference in temperature between test substance and an inert reference material, differential scanning calorimetry (DSC) records the energy needed to establish a zero-temperature difference between a test substance and reference material.

c) Gasometric analysis

Gasometry involves measurement of the volume of gas evolved or absorbed in a chemical reaction.

Some of the gases which are analysed by Gasometry are CO_2, N_2O, cyclopropane, amyl nitrate, ethylene, nitrogen, helium etc.

2. Electrical methods

Electrical methods of analysis involve the measurement of electric current, voltage or resistance in relation to the concentration of some species in the solution. Electrical methods of analysis include:

- (a) Potentiometry
- (b) Conductometry
- (c) Polarography
- (d) Voltametry
- (e) Amperometry

Potentiometry measures electrical potential of an electrode in equilibrium with an ion to be determined.

Conductometry measures electrical conductivity of an electrode with a reference electrode while Polarography, Voltametry and Amperometry measures electrical current at a micro-electrode.

3. Instrumental methods of analysis

Instrumental method involves measurement of some physical properties of the compound or a substance. These methods are employed for determination of minor or trace concentration of element in the sample.

Instrumental methods are preferred due to their selectivity, high speed, accuracy and simplicity of analysis. Any change in the properties of the system are detected by measurement of absorbance, specific rotation, refractive index, migration difference, charge to mass ratio.

Spectroscopic methods of analysis depend upon measurement of the amount of radiant energy of a particular wavelength emitted by the sample.

Methods which include absorption of radiation are ultra violet, visible, infra-red, atomic absorption, nuclear magnetic resonance spectroscopy

Emission methods involve heating or electrical treatment of the sample so that the atoms are raised to the excited state to emit the energy and the intensity of this energy is measured. Emission methods include emission spectroscopy, flame photometry, fluorimetry.

Absorption spectrophotometry is the measurement of the absorption of electromagnetic radiation of definite and narrow wavelength range by molecules and atoms of a chemical

substance. Techniques frequently employed in pharmaceutical analysis include atomic, fluorescence, infrared, ultra-violet and visible spectrophotometry.

Atomic spectrophotometry is the measurement of the intensity of emission or absorption of light at particular wavelength by the atomic vapours of certain metals, generated by introducing into flame solutions containing ions of such metals.

Fluorescence spectrophotometry is the measurement of the intensity of emission of fluorescent light emitted by a chemical substance while it is being exposed to ultra-violet, visible, or other electromagnetic radiation.

The wavelength range available for these measurements extends from the wavelength of the ultraviolet through the infra - red. For convenience of reference, this special range is roughly divided into the ultraviolet (185 nm to 380 nm), the visible (380 nm to 780 nm), the near Infra-red (780 nm to 3000 nm), and the far infrared (3000 nm to 4000 nm).

The infrared absorption spectrum is unique for any given chemical compound with the exception of optical isomers, which have identical spectra. However, polymorphism may sometimes show differences in the infrared spectrum of a given compound in the solid state. Because of the large numbers of maxima in an infrared absorption spectrum, it is sometimes impossible to measure qualitative composition without prior separation.

For many pharmaceutical substances, measurements can be made in the **ultra-violet and visible regions of the spectrum** with greater accuracy and sensitivity than in the infrared. When solutions are observed in 1-cm cells, concentrations of about 10 mg of substance per ml may produce enough absorbance in the ultra-violet and visible region. In the infrared and near infrared regions, concentrations of 1 to 10 mg or up to 100 mg per mg/mL respectively may be needed to produce sufficient absorption; for these spectral ranges, cell lengths of 0.01 mm and upwards up to 3 mm are commonly used.

The ultra-violet and visible spectra of substances generally do not have a high degree of specificity. Nevertheless, they are suitable for quantitative assays and for many substances they are useful as additional means of identification.

Chromatographic techniques

Chromatography is the collective term for a family of laboratory techniques for the separation of mixtures. It involves passing a mixture dissolved in a "mobile phase" through a stationary phase, which separates the analyte to be measured from other molecules in the mixture and allows it to be isolated. 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.

Various chromatographic techniques are Paper chromatography, Thin layer chromatography, Gas chromatography, High performance liquid chromatography, High pressure thin layer chromatography.

Mass spectrometry involves vaporization of material using a high vaccum and the vapour is bombarded by a high energy electron beam. Vapour molecules undergo fragmentation to produce ions of varying size. These ions are differentiated by accelerating them in electrical field and then deflecting them in a magnetic field. Each kind of ion gives a peak in the mass spectrum.

HYPHENATED TECHNIQUES

Many techniques combine two or more analytical methods sometimes called "hyphenated" methods. Hyphenated Techniques combine chromatographic and spectral methods to exploit the advantages of both.
Examples of this include

- GC-MS (Gas Chromatography-Mass Spectroscopy)
- LC-MS (Liquid Chromatography- Mass Spectroscopy)
- ICP-MS (Inductively-Coupled Plasma - Mass Spectrometry).
- MS-MS (Mass Spectroscopy- Mass Spectroscopy)
- LC-MS-MS
- ICP-AES (Inductively-Coupled Plasma-Atomic Emission Spectroscopy).

4. Biological and microbiological methods

Biological methods are used when potency of a drug or its derivative cannot be properly determined by any physical or chemical methods. They are called bio-assays.

Microbiological methods are used to observe potency of antibiotic or anti- microbial agents. In antimicrobial assay, inhibition of growth of bacteria of the sample is compared with that of the standard antibiotic. These methods include cup plate method and turbidimetric analysis.

ADVANTAGES OF INSTRUMENTAL METHODS

1. A small amount of a sample is needed for analysis.

2. Determination by instrumental method is considerably fast.

3. Complex mixture can be analyzed either with or without their separation.

4. Sufficient reliability and accuracy of results are obtained by instrumental method.

5. When non-instrumental method is not possible, instrumental method is the only answer to the problem.

1.2 ULTRAVIOLET SPECTROPHOTOMETRY

Ultraviolet spectroscopy [16-26] is concerned with the study of absorption of UV radiation which ranges from 200mm to 400nm. Compounds which are coloured. absorb radiation from 400nm-800nm. But compounds which are colourless absorb radiation in the UV region. In both UV as well as visible spectroscopy, only the valence electrons absorb the energy. thereby the molecule undergoes transition from Ground state to excited state. This absorption is characteristic and depends on the nature of electrons present. The intensity of absorption depends on the concentration and path length as given by Beer-Lambert's law.

The types of electrons present in any molecule may be conveniently classified as

1. 'σ' electrons: These are the ones present in saturated compounds. Such electrons do not absorb near UVbut absorb vacuum UV radiation (<200nm).

2. 'π' electrons: These electrons are present in unsaturated compounds (eg) double or triple bonds (eg) >C=C<. -C=C

3 'n' electrons: These are non-bonded electrons which are not involved in any bonding (e.g.) lone pair of electrons like in S, O, N & Halogens (X).

PRINCIPLE

Any molecule has either n, or a or a combination of these electrons. These bonding (σ& π) and non-bonding (n) electrons absorb the characteristic radiation and undergoes transition from ground state to excited state by the characteristic absorption peaks, the nature of the electrons present and hence the molecular structure can be elucidated.

1.2.1. ELECTRONIC TRANSITIONS.

It was stated earlier that σ, n and π electrons are present in a molecule and can be excited from the ground state by the absorption of UV radiation. The various transitions are **n- π*, π → π*, n→ σ* and σ → σ*.** The different energy states associated with such transitions can be given by the diagram.
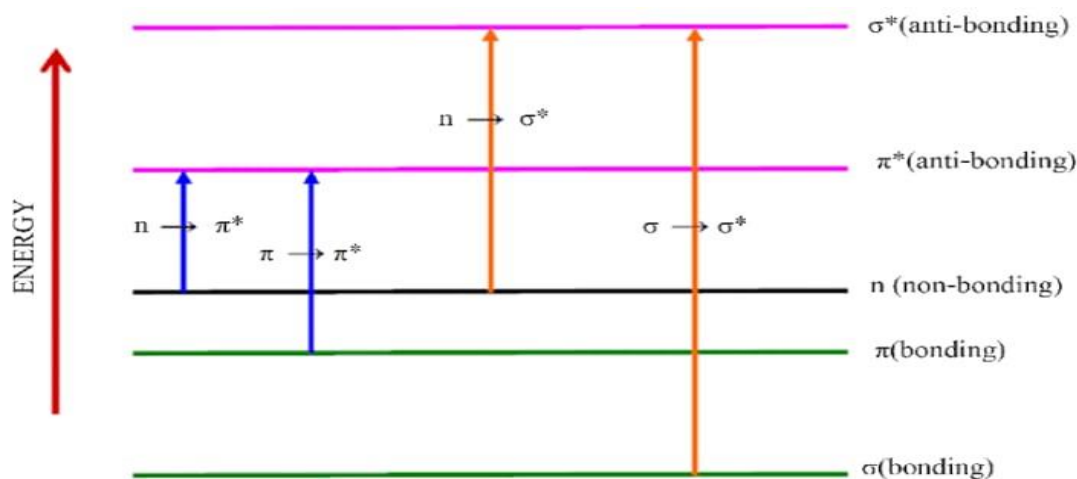
Figure 2: Electronic Transitions

TYPES OF ELECTRONIC TRANSITIONS

1. n → π*: Of all the types of transition, n→ π* transition requires the lowest energy longer wavelength). The peaks due to this transition is also called as R-bands. This type of peak can be seen in compounds where 'n' electron (present in S. O. N or halogen) is present in a compound containing double bond nitro compounds, etc. or triple bond (eg) aldehydes or ketones,

2. π → π*: This type of transition gives rise to B. E & K bands.

The energy requirement of this transition is between n→ σ* and n→ π*. But extended conjugation (addition of more double/triple bonds) and alkyl substituents shifts the λ max towards longer (Bathochromic shift). Also, trans isomer of olefin absorbs at longer λ with more intensity than Cis isomer. (Bathochromic shift and hyperchromic effect). Extended conjugation (and alkyl substitution) shifts λ max to such an extent that the λ max falls in the colorimetric region (e.g.) Plant pigments like ß-carotene. lycopene. etc. The λ max of some chromophores and other systems are given below.

3. n→ σ*

This transition occurs in saturated compounds, with hetero atom(s) like S, O, N or Halogens. It requires lesser energy when compared to σ → σ* transition. Normally the peaks due to this transition occurs from 180nm to 250nm. As these peaks are observed at the lower end of the U spectrum. It can be called as end absorption.

4. σ → σ*: Of all the electronic transitions, this type of transition requires the highest energy.

This is observed with saturated compounds (especially hydrocarbons). The peaks do not appear in UV region, but occur in vacuum UV or for UV region. i.e. 125-135nm. Some of the compounds with such transition are Methane (122nm), Ethane (135nm), Propane (135mm) and Cyclopropane (190nm). Since UV spectrophotometers are operated above 200nm, saturated hydrocarbons like cyclohexane (195nm) can be used as nonpolar solvents, as It does not give solvent peak.

Beer's Law

When a monochromatic radiation is passed through a solution, the decrease in the intensity of radiation with thickness of the solution is directly proportional to the intensity of the incident light as well as concentration of the solution.

Let   I -   be the intensity of incident radiation.

   x -   be the thickness of the solution.

   C -   be the concentration of the solution.

Beer's Law

$$A = E.C.l$$

$$T = I/I_O \text{ or } -\log T = \log I/I_O = A$$

From the equation it is seen that the absorbance which is also called as optical density (OD) of a solution in a container of fixed path length is directly proportional to the concentration of a solution.

Lambert's Law

When a monochromatic radiation is passed through a solution, the decrease in the intensity of radiation with thickness of the solution is directly proportional to the intensity of the incident light.

Let   I - be the intensity of incident radiation.

         x - be the thickness of the solution.

The expression of Beer-Lambert law is

$$\mathbf{A = \log (I/I_o) = Ecl}$$

Where, A   =   Absorbance

   I_0 =   Intensity of light incident upon sample cell

   I   =   Intensity of light leaving sample cell

   c   =   Molar concentration of solute

   l   =   Length of sample cell (cm)

   E   =   Molar absorptivity

1.2.2. INSTRUMENTATION

COMPONENTS OF SPECTROPHOTOMETER

- Source
- Wavelength selectors
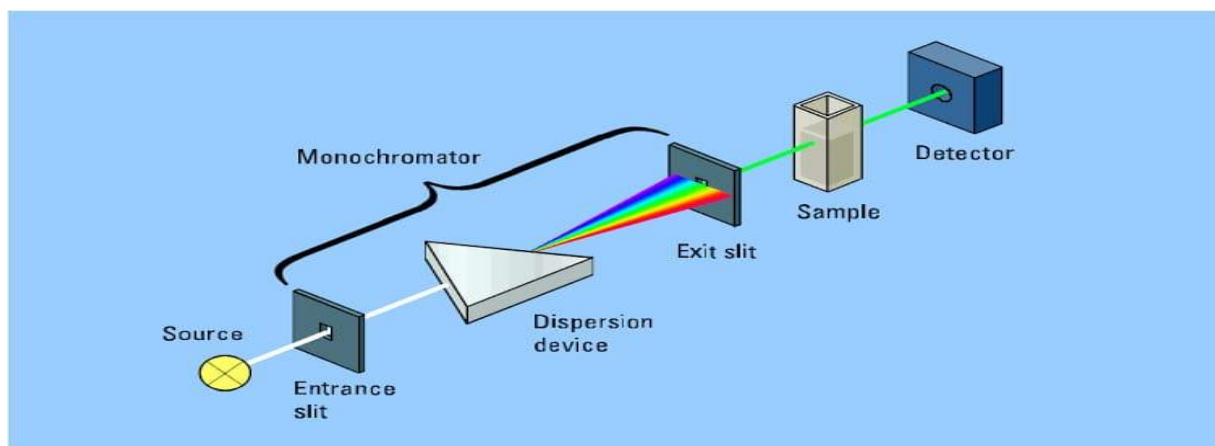- Sample containers
- Detector
- Recorder

Figure 3: Instrumentation of UV Spectrophotometer

1.2.2.1. RADIATION SOURCE

It is important that the power of the radiation source does not change abruptly over its wavelength range. The electrical excitation of deuterium or hydrogen at low pressure produces a continuous UV spectrum. Both Deuterium and Hydrogen lamps emit radiation in the range 160 - 375 nm.

Problem

- o Due to evaporation of tungsten life period decreases
- o It is overcome by using tungsten-halogen lamp
- o Halogen gas prevents evaporation of tungsten
- o For ultra violet region

Hydrogen discharge lamp

It consists of two electrode contain in deuterium filled silica envelop. UV-Vis spectro-photometer have both deuterium & tungsten lamps. Selection of lamp is made by moving lamp mounting or mirror to cause the light fall on monochromator.

Deuterium lamp

Radiation emitted is 3-5 times more than the hydrogen discharge lamps.

Xenon discharge lamp

Xenon stored under pressure in 10-30 atmosphere.

1.2.2.2. Wavelength selectors

- Converts polychromatic light to monochromatic light
- Filters and Monochromators are used for this purposes

FILTERS

It is frequently necessary to filter or remove wide bands of radiation from a signal.

- Filters isolate a wider band than the monochromators.
- There are two types:

1. Absorption Filters

These filters have a bandwidth that ranges from 30-250μm

- The absorption filters consist of coloured glass or a dye suspended in gelatin and sandwiched between the two glass plates
- The coloured glass filter has the advantage of greater thermal stability
- Each instrument is provided with a set of 12 filters to cover the range from 390-700μm

- A narrow spectral band can be obtained by coupling cut off filters with other filters but this combination decreases the intensity of light

2. Interference Filters

- Based on interference phenomenon at desired. wavelength thus permitting rejection of unwanted radiation by selective reflection and producing narrow band
- It consists of a dielectric layer (eg: CaF_2) between two parallel silver films which is sandwiched by glass plate
- It has a bandpass of 100-150Å and a peak transmittance of 40-60%.

Monochromators

- It is used to disperse the heterochromic radiation into its component wavelength and to permit the isolation of desired portion of the spectrum.
- It consists of an entrance slit, an exit slit and a dispersing device either a prism or grating.
- Materials of construction should be selected with care to suit the range in which it has to work.
- For e.g.:
  - Quartz for ultraviolet
  - Normal glass for visual range
  - Alkali halides for IR region
- Gratings are cheaper than prism

1. PRISM

- Made up of glass, quartz or fused silica
- Quartz or fused silica is the choice of material of UV spectrum
- When white light is passed through the glass prism, dispersion of polychromatic light in rainbow occurs. Now by the rotation of the prism different wavelengths of the spectrum can be made to pass 25 through in exit slit on the sample
- The effective wavelength depends on the dispersive power of prism material and the optical angle of the prism
- There are two types of mounting in an instrument:
  1. Cornu type (refractive)
  2. Littrow type (reflective)

CORNU TYPE:

It has an optical angle of 60° and it is adjusted such that on rotation the emerging light is allowed to fall on exit slit.

LITTROW TYPE:

It has optical angle 30° and its one surface is aluminized with reflected light back to pass through prism and to emerge on the same side of the light source i.e. Light doesn't pass through the prism on other side.

2. GRATING MONOCHROMATOR

Gratings are of two types.

        1. Diffraction grating

        2. Transmission grating

1. Diffraction Grating

More refined dispersion of light is obtained by means of diffraction gratings. These consists of large no.of parallel lines (grooves) about 15000-30000/inch is ruled on 29 highly polished surface of aluminum. To make the surface reflective, a deposit of aluminum is made on the surface. In order to minimize to greater amounts of scattered radiation and appearance of unwanted radiation of other spectral orders, the gratings are blazed to concentrate the radiation into a single order.

2. Transmission Grating

It is similar to diffraction grating but refraction takes place instead of reflection. Refraction produces reinforcement. This occurs when radiation transmitted through grating reinforces with the partially refracted radiation.

1.2.2.3. SAMPLE CONTAINERS

A variety of sample cells available for UV region. The choice of sample cell is based on

        a) the path length, shape, size

        b) the transmission characteristics at the desired wavelength

        c) the relative expense

The cell holding the sample should be transparent to the wavelength region to be recorded. Quartz or fused silica cuvettes are required for spectroscopy in the UV region. Silicate

glasses can be used for the manufacture of cuvettes for use between 350 and 2000 nm. The thickness of the cell is generally 1 cm. cells may be rectangular in shape or cylindrical with flat ends.

1.2.2.3. DETECTORS

Device which converts light energy into electrical signals, that are displayed on readout devices.

The transmitted radiation falls on the detector which determines the intensity of radiation absorbed by sample. The followed types of detectors are employed in instrumentation of absorption spectrophotometer.

1. Barrier layer cell/Photovoltaic cell

2. Photomultiplier tube

3. Phototubes/Photoemissive tube

1. BARRIER LAYER CELL/ PHOTOVOLTAIC CELL

- The detector has a thin film metallic layer coated with silver or gold and act as another electrode

- It also has a metal base plate which act as another electrode

- These 2 layers are separated by a semiconductor layer of selenium

- This creates a potential difference between two electrodes & causes the flow of current

- When it is connected to galvanometer, a flow of current observed which is proportional to the intensity and wavelength of light falling on it

- When light radiation falls on selenium layer, electrons become mobile and are taken up by transparent metal layer
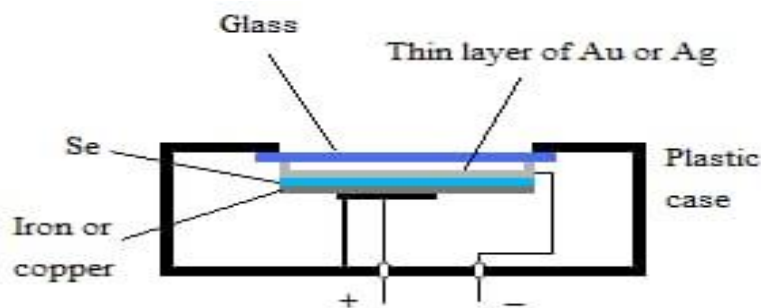

Figure 4: Barrier Layer Cell

2. PHOTOMULTIPLIER TUBES

- The principle employed in this detector is that, multiplication of photoelectrons by secondary emission of electrons
- In a vaccum tube a primary photo-cathode is fixed which receives radiation from the sample
- Some eight to 10 dynodes are fixed each with increasing potential of 75-100V higher than proceeding one
- Photomultiplier is extremely sensitive to light and is best suited where weaker or low radiation is received
- Near the last dynode is fixed an anode or electron collector electrode

3. PHOTOTUBES/PHOTOEMISSIVE TUBES

- It Consists of an evacuated glass tube with a photocathode and collector anode
- The surface of photocathode is coated with a layer of elements like cesium, silver oxide or mixture of them
- When radiant energy falls on photosensitive cathode, electrons are attracted to anode causing current to flow
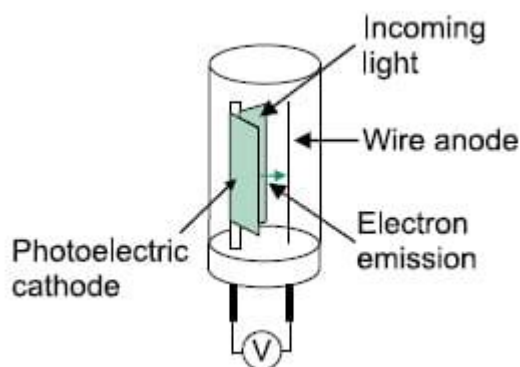- More sensitive compared to barrier layer cell and therefore widely used

Figure 5: phototubes

1.2.3. INSTRUMENT DESIGN

Depending upon the monochromators (filters or dispersing device) used to isolate and transmit a narrow beam of radiant energy from the incident light determines whether the instrument is classified as Photometer or a Spectrophotometer. Spectrophotometers used here detects the percentage transmittance of light radiation, when light of certain intensity & frequency range is passed through the sample. Both can be a single beam or double beam optical system.

SINGLE BEAM SPECTROPHOTOMETER

Light from the source is carried through lens and/or through aperture to pass through a suitable filter. The type of filter to be used is governed by the colour of the solution.

The sample solution to be analysed is placed in cuvettes. After passing through the solution, the light strikes the surface of detector (barrier-layer cell or phototube) and produces electrical current.

The output of current is measured by the deflection of needle of light-spot galvanometer or micro ammeter. This meter is calibrated in terms of transmittance as well as optical density. The readings of solution of both standard and unknown are recorded in optical density units after adjusting instrument to a reagent blank.
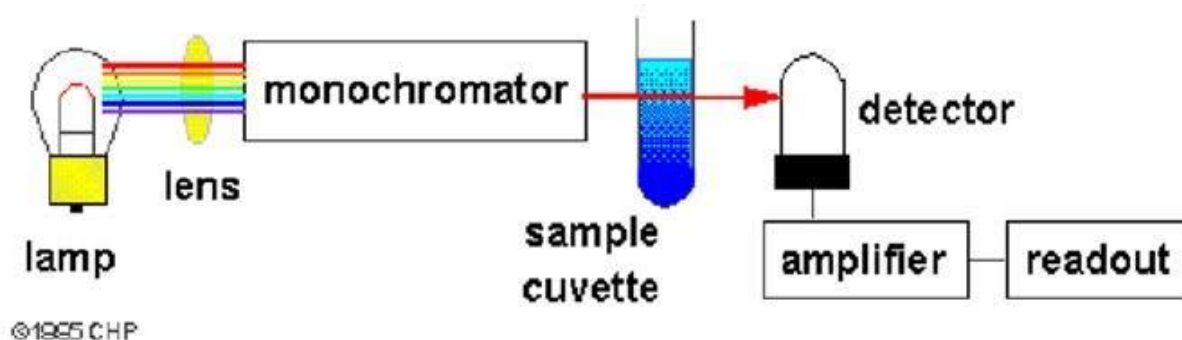
Figure 6: Single Beam Spectrophotometer

DOUBLE BEAM UV-VIS SPECTROPHOTOMETER

Double beam instrument is the one in which two beams are formed in the space by a U shaped mirror called as beam splitter or beam chopper.

Chopper is a device consisting of a circular disc. One third of the disc is opaque and one third is transparent, remaining one third is mirrored. It splits the monochromatic beam of light into two beams of equal intensities.
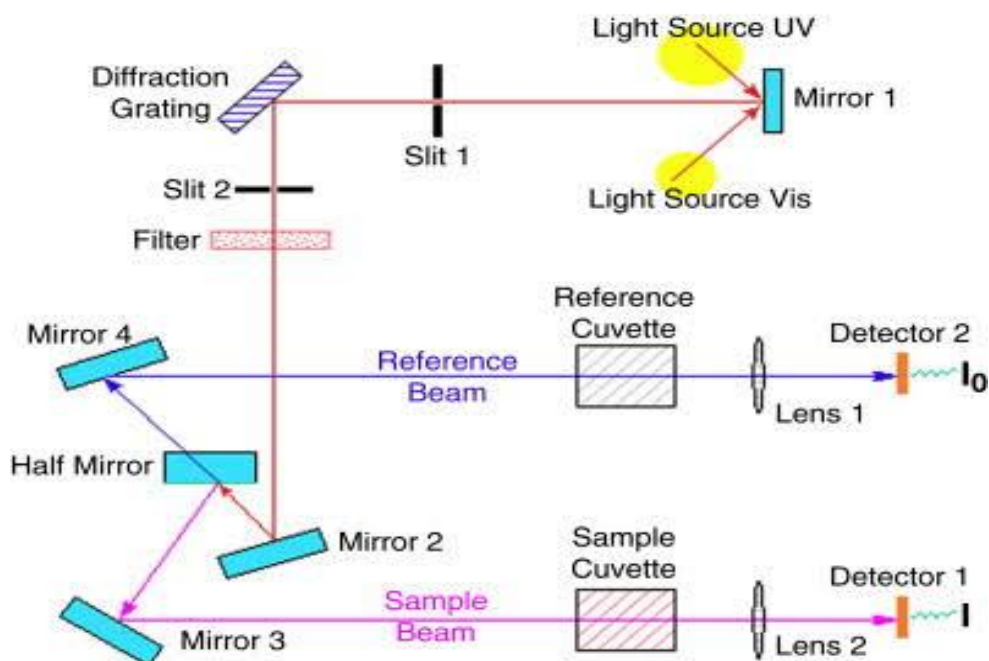
Figure 7: Double Beam Spectrophotometer

ADVANTAGES

Single beam

- ✓ Simple in construction
- ✓ Easy to handle
- ✓ Economical

Double beam

- ✓ It facilitates rapid scanning over wide à region.
- ✓ Fluctuations due to radiation source are minimised.
- ✓ It doesn't require adjustment of the transmittance at 0% and 100% at each wavelength.
- ✓ It gives ratio of intensities of sample & reference beams simultaneously.

DISADVANTAGES

Single beam

- ✓ Any fluctuation in the intensity of radiation sources affects the absorbance.
- ✓ Continuous spectrum is not obtained.

Double beam

- ✓ Construction is complicated.
- ✓ Instrument is expensive.

1.3 Infrared Spectroscopy (IR)

Infrared (IR) spectroscopy [27-32] or vibrational spectroscopy is an analytical technique that takes advantage of the vibrational transitions of a molecule.

It is one of the most common and widely used spectroscopic techniques employed mainly by inorganic and organic chemists due to its usefulness in determining structures of compounds and identifying them.

The method or technique of infrared spectroscopy is conducted with an instrument called an infrared spectrometer (or **spectrophotometer**) to produce an infrared spectrum.
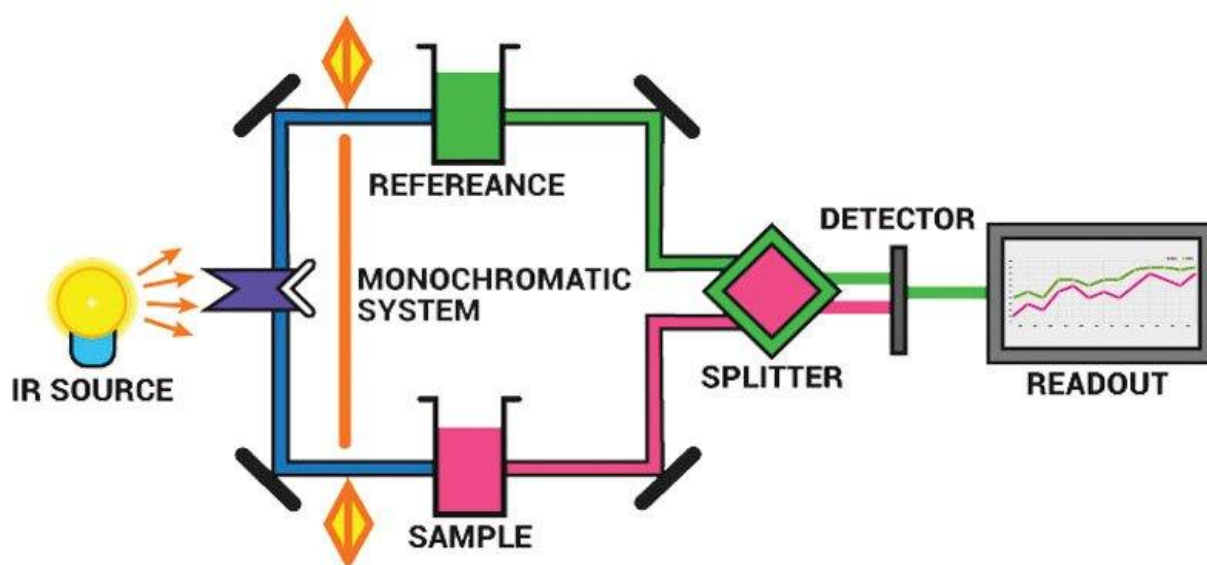

Figure 8: Infrared Spectroscopy (IR)

Principle of Infrared Spectroscopy (IR)

- Infrared Spectroscopy is the analysis of infrared light interacting with a molecule
- The portion of the infrared region most useful for analysis of organic compounds have a wavelength range from 2,500 to 16,000 nm, with a corresponding frequency range from 1.9*1013 to 1.2*1014 Hz
- Photon energies associated with this part of the infrared (from 1 to 15 kcal/mole) are not large enough to excite electrons, but may induce vibrational excitation of covalently bonded atoms and groups
- It is known that in addition to the facile rotation of groups about single bonds, molecules experience a wide variety of vibrational motions, characteristic of their component atoms

- Consequently, virtually all organic compounds will absorb infrared radiation that corresponds in energy to these vibrations

- Infrared spectrometers, similar in principle to other spectrometer, permit chemists to obtain absorption spectra of compounds that are a unique reflection of their molecular structure

- The fundamental measurement obtained in infrared spectroscopy is an infrared spectrum, which is a plot of measured infrared intensity versus wavelength (or frequency) of light

- IR Spectroscopy measures the vibrations of atoms, and based on this it is possible to determine the functional groups

- Generally, stronger bonds and light atoms will vibrate at a high stretching frequency (wavenumber)

1.3.1. FOURIER TRANSFORM INFRARED SPECTROPHOTOMETER (INTERFEROMETER)

FTIR is of great importance in infrared spectroscopy. FTIR spectrometer simultaneously collects spectral data in a wide spectral range. This confers a significant advantage over a dispersive spectrophotometer which measures intensity over a narrow range of wavelengths at a time.

Fourier transform infrared (FTIR) spectroscopy is a measurement technique that allows one to record infrared spectra. Infrared light is guided through an interferometer and then through the sample (or vice versa). A moving mirror inside the apparatus alters the distribution of infrared light that passes through the interferometer. The signal directly recorded, called an "interferogram", represents light output as a function of mirror position. A data processing technique called Fourier transform turns this raw data into the desired result (the sample's spectrum): Light output as wavelength (or equivalently, wavenumber).

1.3.2. INTERPRETATION OF SPECTRA

The interpretation of infrared spectra involves the correlation of absorption bands in the spectrum of an unknown compound with the known absorption frequencies for types of bonds.

4000-1450 cm^{-1} portion of the IR spectrum, sometimes called the functional group re-

gion. The segment of the IR spectrum below 1450 cm^{-1} is called the fingerprint region. The fingerprint region to the right of 1300 cm^{-1} is particularly useful in this type of compound identification. The fingerprint region contains many absorptions. The complex pattern of peaks in this region are unique for each compound, so they can be used (like human fingerprints) to confirm that an unknown is, in fact, the suspect compound.

An infrared spectrum of a molecule is like a fingerprint, and the nature of the molecule can be determined from its spectrum. Spectra are interpreted with the aid of 'Structure Correlation Charts', which correlate absorbance bands with bonds, and therefore functional groups, in the molecule. While it's usually not possible to determine a connection table from the infrared spectrum alone, especially for complex molecules, the infrared spectrum can give important clues about the properties of a molecule.

Every molecule will have its own characteristic spectrum. The bands that appear depend on the types of bonds and the structure of the molecule. Study the sample spectra, noting similarities and differences, and relate these to structure and bonding within the molecules. A portion of the spectrum where % transmittance drops to a low value then rises back to near 100% is called a "band". A band is associated with a particular vibration within the molecule. The width of a band is described as broad or narrow based on how large a range of frequencies it covers. The efficiencies for the different vibrations determine how "intense" or strong the absorption bands are. A band is described as strong, medium, or weak depending on its depth.

IR spectra can be used to identify molecules by recording the spectrum for an unknown and comparing this to a library or data base of spectra of known compounds. Computerized spectra data bases and digitized spectra are used routinely in this way in research, medicine, and a number of other fields.

1.3.3. APPLICATIONS OF IR SPECTROSCOPY

Infrared spectroscopy is widely used in industry as well as in research. It is a simple and reliable technique for measurement, quality control and dynamic measurement. It is also employed in forensic analysis in civil and criminal analysis.

Some of the major applications of IR spectroscopy are as follows:

1. Identification of functional group and structure elucidation

Entire IR region is divided into group frequency region and fingerprint region. Range of group frequency is 4000-1500 cm^{-1} while that of finger print region is 1500-400 cm^{-1}.

In group frequency region, the peaks corresponding to different functional groups can be observed. According to corresponding peaks, functional group can be determined.

Each atom of the molecule is connected by bond and each bond requires different IR region so characteristic peaks are observed. This region of IR spectrum is called as finger print region of the molecule. It can be determined by characteristic peaks.

2. Identification of substances

IR spectroscopy is used to establish whether a given sample of an organic substance is identical with another or not. This is because large number of absorption bands is observed in the IR spectra of organic molecules and the probability that any two compounds will produce identical spectra is almost zero. So, if two compounds have identical IR spectra then both of them must be samples of the same substances.

IR spectra of two enantiomeric compound are identical. So, IR spectroscopy fails to distinguish between enantiomers.

For example, an IR spectrum of benzaldehyde is observed as follows.

C-H stretching of aromatic ring	- 3080 cm^{-1}
C-H stretching of aldehyde	- 2860 cm^{-1} and 2775 cm^{-1}
C=O stretching of an aromatic aldehyde	- 1700 cm^{-1}
C=C stretching of an aromatic ring	- 1595 cm^{-1}
C-H bending	- 745 cm^{-1} and 685 cm^{-1}

No other compound then benzaldehyde produces same IR spectra as shown above.

3. Studying the progress of the reaction

Progress of chemical reaction can be determined by examining the small portion of the reaction mixture withdrawn from time to time. The rate of disappearance of a characteristic absorption band of the reactant group and/or the rate of appearance of the characteristic absorption band of the product group due to formation of product is observed.

4. Detection of impurities

IR spectrum of the test sample to be determined is compared with the standard compound. If any additional peaks are observed in the IR spectrum, then it is due to impurities present in the compound.

5. Quantitative analysis

The quantity of the substance can be determined either in pure form or as a mixture of two or more compounds. In this, characteristic peak corresponding to the drug substance is chosen and log I0/It of peaks for standard and test sample is compared. This is called base line technique to determine the quantity of the substance.

1.4 High-Performance Liquid Chromatography (HPLC)

- High performance liquid chromatography [33-44] or commonly known as HPLC is an analytical technique used to separate, identify or quantify each component in a mixture
- The mixture is separated using the basic principle of **column chromatography** and then identified and quantified by spectroscopy
- In the 1960s the column chromatography LC with its low-pressure suitable glass columns was further developed to the HPLC with its high-pressure adapted metal columns.
- HPLC is thus basically a highly improved form of column liquid chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres

1.4.1. Principle

- The purification takes place in a separation column between a stationary and a mobile phase.
- The stationary phase is a granular material with very small porous particles in a separation column.
- The mobile phase, on the other hand, is a solvent or solvent mixture which is forced at high pressure through the separation column.
- Via a valve with a connected sample loop, i.e. a small tube or a capillary made of stainless steel, the sample is injected into the mobile phase flow from the pump to the separation column using a syringe.

- Subsequently, the individual components of the sample migrate through the column at different rates because they are retained to a varying degree by interactions with the stationary phase.

- After leaving the column, the individual substances are detected by a suitable detector and passed on as a signal to the HPLC software on the computer.

- At the end of this operation/run, a chromatogram in the HPLC software on the computer is obtained

- The chromatogram allows the identification and quantification of the different substances
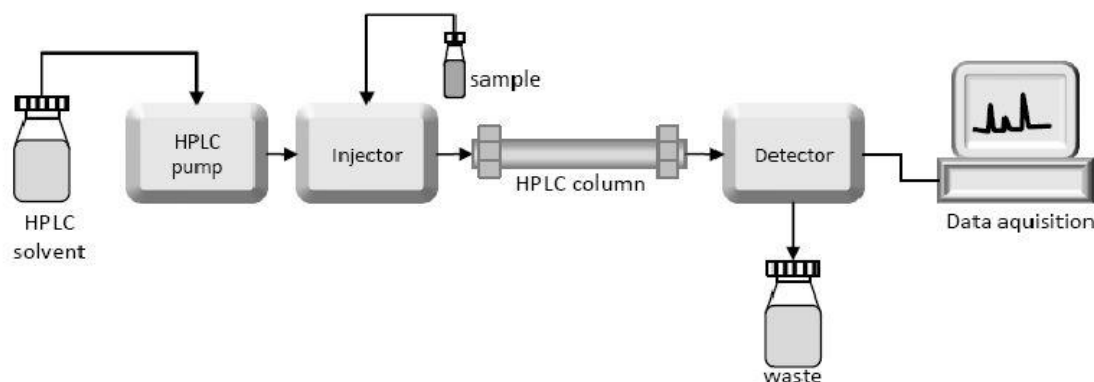
Instrumentations

Figure 9: Instrumentation of HPLC

1. The Pump

- The development of HPLC led to the development of the pump system

- The pump is positioned in the most upper stream of the liquid chromatography system and generates a flow of eluent from the solvent reservoir into the system

- High-pressure generation is a "standard" requirement of pumps besides which, it should also to be able to provide a consistent pressure at any condition and a controllable and reproducible flow rate

- Most pumps used in current LC systems generate the flow by back-and-forth motion of a motor-driven piston (reciprocating pumps). Because of this piston motion, it produces "pulses"

2. Injector

- An injector is placed next to the pump

- The simplest method is to use a syringe, and the sample is introduced to the flow of eluent

- The most widely used injection method is based on sampling loops

- The use of the autosampler (auto-injector) system is also widely used that allows repeated injections in a set scheduled-timing

3. Column

- The separation is performed inside the column
- The recent columns are often prepared in a stainless steel housing, instead of glass columns
- The packing material generally used is silica or polymer gels compared to calcium carbonate

  The eluent used for LC varies from acidic to basic solvents
- Most column housing is made of stainless steel since stainless is tolerant towards a large variety of solvents

4. Detector

- Separation of analytes is performed inside the column, whereas a detector is used to observe the obtained separation
- The composition of the eluent is consistent when no analyte is present. While the presence of analyte changes the composition of the eluent. What detector does is to measure these differences
- This difference is monitored as a form of an electronic signal. There are different types of detectors available

Based on the application, the detectors classified into

- Bulk property detectors
- Solute property detectors.

Bulk Property Detectors

Compare an overall change in physical property of mobile phase with or without an eluting solute. These types of detectors tend to be relatively low sensitive and require temperature control.

e.g. Refractive index detector

Solute Property Detectors

They respond to a physical property of the solute that is not exhibited by the pure mobile phase.These detectors are more sensitive, detect the sample in nanograms quantity.

e.g. Uv visible detector, Electrochemical detector, Fluorescence detector.

5. Recorder

- The change in eluent detected by a detector is in the form of an electronic signal, and thus it is still not visible to our eyes

- In older days, the pen (paper)-chart recorder was popularly used. Nowadays, a computer-based data processor (integrator) is more common

- There are various types of data processors; from a simple system consisting of the in-built printer and word processor while those with software that are specifically designed for an LC system which not only data acquisition but features like peak-fitting, baseline correction, automatic concentration calculation, molecular weight determination, etc

Degasser

- The eluent used for LC analysis may contain gases such as oxygen that are non-visible to our eyes

- When gas is present in the eluent, this is detected as noise and causes an unstable baseline

- Degasser uses special polymer membrane tubing to remove gases

- The numerous very small pores on the surface of the polymer tube allow the air to go through while preventing any liquid to go through the pore

Column Heater

The LC separation is often largely influenced by the column temperature.

- In order to obtain repeatable results, it is important to keep consistent temperature conditions

- Also, for some analysis, such as sugar and organic acid, better resolutions can be obtained at elevated temperatures (50 to 80°C)

- Thus, columns are generally kept inside the column oven (column heater)

1..4.3. Types of HPLC

1. **Normal phase:**

Column packing is polar (e.g silica) and the mobile phase is non-polar. It is used for water-sensitive compounds, geometric isomers, cis-trans isomers, and chiral compounds.

2. **Reverse phase:**

The column packing is non-polar (e.g C18), the mobile phase is water miscible solvent (e.g methanol). It can be used for polar, non-polar, ionizable and ionic samples.

3. **Ion exchange:**

Column packing contains ionic groups and the mobile phase is buffer. It is used to separate anions and cations.

4. **Size exclusion:**

Molecules diffuse into pores of a porous medium and are separated according to their relative size to the pore size. Large molecules elute first and smaller molecules elute later.

1.4.4. Applications

The HPLC has developed into a universally applicable method so that it finds its use in almost all areas of chemistry, biochemistry, and pharmacy.

- Analysis of drugs
- Analysis of synthetic polymers
- Analysis of pollutants in environmental analytics
- Determination of drugs in biological matrices
- Isolation of valuable products
- Product purity and quality control of industrial products and fine chemicals
- Separation and purification of biopolymers such as enzymes or nucleic acids
- Water purification
- Pre-concentration of trace components
- Ligand-exchange chromatography
- Ion-exchange chromatography of proteins
- High-pH anion-exchange chromatography of carbohydrates and oligosaccharides

Advantages

1. Speed
2. Efficiency
3. Accuracy
4. Versatile and extremely precise when it comes to identifying and quantifying chemical components.

Limitations

1. **Cost:** Despite its advantages, HPLC can be costly, requiring large quantities of expensive organics.

2. **Complexity**

3. HPLC does have **low sensitivity** for certain compounds, and some cannot be detected as they are irreversibly adsorbed.

4. Volatile substances are better separated by gas chromatography.

1.4.5. PARAMETERS USED IN HPLC

1. Retention time
2. Retention volume
3. Separation factor
4. Resolution
5. Height Equivalent to a Theoretical Plate (HETP)
6. Asymmetry factor

1. Retention time:

Retention time is the difference in time between the point of injection and appearance of peak maxima. It is also defined as time required for 50% of a component to be eluted from a column. It is measured in minutes and seconds.

2. Retention volume:

Retention volume is the volume of carrier gas required to elute 50% of the component from the column. It is the product of retention time and flow rate.

Retention volume = Retention time x flow rate

3. Resolution:

Resolution is the measure of extent of separation of 2 components and the base line separation achieved.

$$Rs = 2\ (Rt_1 - Rt_2)/w_1 + w_2$$

4. Height Equivalent to a Theoretical Plate (HETP):

A theoretical plate is an imaginary or hypothetical unit of a column where distribution of solute between stationary phase and mobile phase has attained equilibrium. It can also be called as a functional unit of the column.

5. Asymmetry factor:

A chromatographic peak should be symmetrical about its centre and said to follow Gaussian distribution. But in practice due to some factors, the peak is not symmetrical and shows tailing or fronting.

Fronting is due to saturation of stationary phase and can be avoided by using less quantity of sample.

Tailing is due to more active adsorption sites and can be eliminated by support pretreatment.

Asymmetry factor (0.95 to 1.05) can be calculated by AF = b/a (b, a calculated by 5% or 10% of the peak height).

1.5 ANALYTICAL METHOD VALIDATION PARAMETERS [45-52]

1. Accuracy

The accuracy of analytical method is the closeness of the test results obtained by that method to the true value. The accuracy of an analytical method should be established across its range. Accuracy is often expressed as percent recovery by the assay of known, added amounts of analyte.

Determination

The accuracy of an analytical method may be determined by applying that method of synthetic mixture of drug product component to which known amount of analyte have been added. The accuracy is then calculated from the test result as the percentage of analyte recovered by the assay.

2. Precision

The precision of an analytical method is the degree of agreement among individual test result when the method is applied repeatedly to multiple samplings of homogenous samples. The precision of an analytical method is usually expressed as the standard deviation or relative standard deviation precisions may be measure of either the degree of reproducibility or of repeatability of the analytical method under normal operating condition.

Reproducibility refers to use of the analytical procedure in different laboratories. Repeatability refers to the use of the analytical procedure within a laboratory over a short period of time using the same analysts with the same equipment.

Determination

The precision of an analytical method is determined by assaying a sufficient number of aliquots of a homogenous sample to be able to calculate statistically valid estimate of standard deviation or relative standard deviation.

3. Specificity

The specificity of an analytical method is its ability to assess unequivocally the analyte in the presence of component that may be expected to be present in the sample matrix. Specificity may often be expressed as the degree of bias of test results obtained by analysis of sample containing added impurities, degradation products and related chemical compound or place to ingredients when compared to test result from sample without added substances. Specificity is a measure of the degree of interference (or absence there of) in the analysis of complex sample mixture.

Determination

The specificity of an analytical method is determined by comparing test result, from the analysis of samples containing impurities degradation products or placebo ingredients, with those obtained from the analysis of sample without added impurities, degradation products or placebo ingredients.

4. Limit of detection (LOD)

It is the lowest amount of an analyte in a sample that can be detected, but not necessarily quantitated under the stated experimental conditions. It is usually expressed as the concentration of analyte in percentage or parts per million in the sample.

Determination

Determination of limit of detection of an analytical method will vary depending on whether it is instrumental or a non- instrumental procedure for instrumental method some investigators determine the signal concentration of analyte with those of blank sample and establish the minimum level at which the analyte can be reliably decade. A signal to noise ratio of 2:1 generally accepted for non- instrumental method limit of detections.

Generally accepted for non-instrumental method the limit of detection is determined by the analysis of samples with known concentration of analyte and by establishing the minimum level at which the analyte can be reliably detected.

5. Limit of Quantitation (LOQ)

Limit of quantitation is a parameter of quantitative assay for low levels of compounds in sample matrices, such as impurities in bulk drug substances and degradation products in finished pharmaceuticals. It is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under stated experimental conditions. It is expressed as the concentration of analyte in the sample.

Determination

Determination of limit of quantitation of an analytical method may vary depending on whether it is an instrumental or a non-instrumental procedure. For instrumental procedure, a common approach is to measure the magnitude of analytical background response by analyzing a number of blank samples and calculating the standard deviation of this response. The standard deviation multiplied by factor, usually 10 provides an estimate of the limit of quantitation. For non-instrumental methods, the limit of quantitation is generally determined by the analysis of sample with known concentration of analysis and by establishing the minimum level at which the analyte can detected with acceptable accuracy and precision.

6. Linearity and Range

Linearity

The Linearity of an analytical method is its ability to elicit test result that are directly or by a well-defined mathematical transformation proportional to the concentration of analyte in samples within a given range. Linearity is usually expressed by visual examination of a plot of signals as function of analyte concentration.

Range

The range of an analytical method is the interval between the upper and lower levels of analyte that have been demonstrated to be determined with precision, accuracy and linearity using the method. The range is normally expressed in the same units as test results (i.e. percent or part per million) obtained by the analytical method.

Determination of Linearity and Range

The linearity of an analytical method is determined by mathematical treatment of test result obtained by analysis of sample with analyte concentrations across the claimed range of the method. The slope of the regression line of test result versus analyte concentration and the Y - Intercept is a measure of the potential assay bias.

The range of the method in validated by verifying that the analytical method provides acceptable precision, accuracy and linearity when applied to sample containing analyte at the extremes of the range as well as within the range.

7. Ruggedness

The ruggedness of an analytical method is the degree of reproducibility of test result obtained by the analysis of the same samples under a variety of normal test conditions such as different laboratories, different analyst, different instruments, different lots of reagents, different elapsed assay times, different assay temperature, different days etc., Ruggedness is normally expressed as the case of influence on test results of operational and environmental variables on the analytical method. Ruggedness is a measure of reproducibility of test results under normal expected operational conditions from laboratory to laboratory and from analyst to analyst.

Determination

The ruggedness of an analytical method is determined by analysis of aliquots from homogenous lots in the different laboratories, by different analysts using operation and environmental conditions that may differ but are still within the specified parameters of the assay.

The degree of reproducibility of test results is then determined as a function of assay variables. This reproducibility be compared to the precision of the assay under normal conditions to obtain a measure of the ruggedness of the analytical method.

8. Robustness

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

Chapter-2

DRUG PROFILE

2. DRUG PROFILE

ANTISPASMODIC DRUGS

An antispasmodic (synonym: spasmolytic) [52-58] is a pharmaceutical drug or other agent that suppresses muscle spasms.

Smooth Muscle Spasm

One type of antispasmodics is used for smooth muscle relaxation, especially in tubular organs of the gastrointestinal tract. The effect is to prevent spasms of the stomach, intestine or urinary bladder. Both dicyclomine and hyoscyamine are antispasmodic due to their anticholinergic action. Both of these drugs have general side effects and can worsen gastroesophageal reflux disease.

Mebeverine is a muscolotropic spasmolytic with a strong and selective action on the smooth muscle spasm of the gastrointestinal tract, particularly of the colon. It does not have the acetylecholine side effect commonly seen in an anticholinergic antispasmodic.

Papaverine is an opium alkaloid used to treat visceral spasms, erectile dysfunction and investigated as antipsychotic drug due to its potency to inhibit phosphodiesterase PDE10A.

Peppermint oil has been traditionally used as an antispasmodic, and a review of studies on the topic found that it "could be efficacious for symptom relief in IBS" (as an antispasmodic) although more carefully controlled studies are needed. A later study showed it is an effective antispasmodic when test-applied topically to the intestine during endoscopy.

Bamboo shoots have been used for gastrointestinal and antispasmodic symptoms. Anisotropine, atropine, clidinium bromide are also the most commonly used modern antispasmodics.

Skeletal muscle spasm

Pharmacotherapy may be used for acute musculoskeletal conditions when physical therapy is unavailable or has not been fully successful. Another class of antispasmodics for such treatment includes cyclobenzaprine, carisoprodol, diazepam, orphenadrine, and tizanidine.

Meprobamate is another effective antispasmodic which was first introduced for clinical usage in 1955 mainly as an anxiolytic and soon afterward became a blockbuster psychotropic drug. While clinical usage of meprobamate has largely become obsolete since the development of benzodiazepines due to its liability for developing physical dependence and

severe toxicity during instances of acute overdose, it is still manufactured and available by prescription. Carisoprodol is similar to meprobamate as they both belong to the carbamate drug class and meprobamate is a clinically significant active metabolite of carisoprodol, although carisoprodol itself possesses additional antispasmodic properties which are distinct from its metabolites. Effectiveness has not been clearly shown for metaxalone, methocarbamol, chlorzoxazone, baclofen, or dantrolene. Applicable conditions include acute back or neck pain, or pain after an injury.

Spasm may also be seen in movement disorders featuring spasticity in neurologic conditions such as cerebral palsy, multiple sclerosis, and spinal cord disease. Medications are commonly used for spastic movement disorders, but research has not shown functional benefit for some drugs. Some studies have shown that medications have been effective in decreasing spasticity, but that this has not been accompanied by functional benefits. Medications such as baclofen, tizanidine, and dantrolene have been used.

DENAVERINE HYDROCHLORIDE

Denaverine hydrochloride is a muscle relaxant. It was developed and patented in Germany in 1974. Under the brand name Sensiblex, Denaverine hydrochloride is used in Veterinary medicine as a muscle relaxant for the myometrium of cows and dogs during parturition. Now, the drug is in trial with human plasma to treat urogenital and gastrointestinal spasms under the brand Spasmalgan.

Denaverine hydrochloride, is a neurotropic–musculotropic spasmolytic with analgesic effect. It's used to treat gastrointestinal and urogenital smooth muscle spasms, as well as postoperative abdominal pain and obstetrics. Despite the fact that denaverine hydrochloride has been used successfully in therapy for over 30 years, there was little information on its biotransformation in humans.

Mechanism of action:

Denaverine inhibits the enzyme phosphodiesterase. A phosphodiesterase inhibitor is a drug that inhibits the inactivation of the intracellular second messengers cyclic Adenosine MonoPhosphate (cAMP) and cyclic Guanosine MonoPhosphate (cGMP) by one or more of the five subtypes of the enzyme PhosphoDiEsterase (PDE). It has anticholinergic properties. Anticholinergics (anticholinergic agents) are a class of drugs that prevent the neurotransmitter acetylcholine (ACh) from acting at synapses in the central and peripheral nervous systems.

PHARMACOLOGICAL OR IMMUNOLOGICAL PROPERTIES

Pharmacotherapeutic group:

- Genitourinary system
- sex hormones
- other gynaecological

Pharmacodynamic properties

Denaverine hydrochloride is a spasmolytic agent with a relaxant effect on smooth muscle. It has a relaxing effect on the uterus *sub partu* and increases the distensibility of the soft-tissue of the birth canal. Following intramuscular injection, the spasmolytic effect commences within 15 to 30 minutes and lasts for several hours.

Pharmacokinetic properties

Denaverine is excreted rapidly from the treated animals.

Metabolism: N-monodemethyl denaverine

Bioavailability: 30%

half -life: 3 hours

Storage: Store in a cool and dry place and at 0-4°C for short term (days to weeks) or -66°C for long term (months to years).

Shelf Life : 2 years

Indications for use, specifying the target species

Cows, heifers:

- Promotes dilation of the soft tissues of the birth canal in cases where the birth canal is insufficiently opened.
- Regulates uterine contractions in animals with hypertonic muscular contractions of the uterus.

Heifers: Promotes dilation of the soft tissues of the birth canal to facilitate parturition.

Drug Interactions:

Interaction with other medicinal products and other forms of interaction

The product should not be mixed with other veterinary medicinal products. In the case of additional administration of oxytocin or its analogues, the dose of this active substance must be carefully selected because denaverine may amplify its effects.

Toxicity:

- convulsions or effect on seizure threshold
- lungs, thorax, or respiration, dyspnea

Adverse reactions

- Increased restlessness.
- swellings at the injection site.
- absent or insufficient effectiveness necessitating further obstetric measures.

Overdose

- In case of overdose or intravenous application, anticholinergic effects, e.g. increased heart and decreased respiration rate may occur.

Contraindications

- Do not administer in cases of mechanical obstetrical disorders.
- Do not use in cases of hypersensitivity to the active substance or to any of the excipients.

DRUG CHEMISTRY

Drug Name   **: Denaverine hydrochloride**

Molecular Structure:
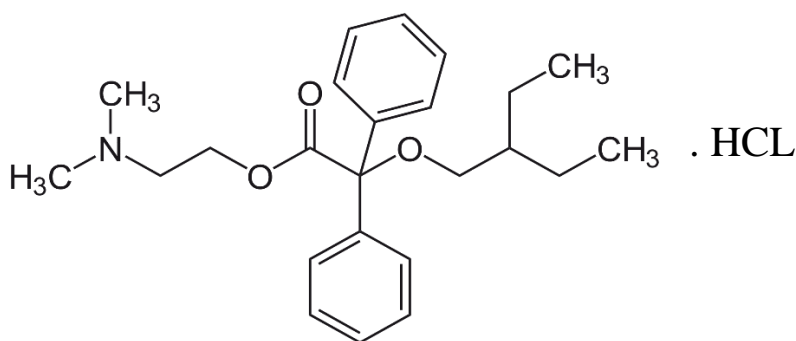
Chemical Name   **:** 2-dimethylaminoethyl2-(2-ethylbutoxy)-2,2diphenylacetate

                                    hydrochloride

Molecular formula   **: $C_{24}H_{34}ClNO_3$**

Molecular weight   **:** 420 g.mol^{-1}

Melting Point   **:** 140-142 °C

Boiling Point   **:** 489.9°Cat760mmHg

Category   **:** Antispasmodic Drug

Appearance   **:** White powder

Chapter-3

LITERATURE REVIEW

3. LITERATURE REVIEW

- **K Lange *et al.,2019* performed influence of denaverine hydrochloride on calving ease in Holstein-Friesian heifers[59]**

In a randomized, controlled, and blinded trial, we measured the effect of denaverine hydrochloride on physical and physiological calving parameters. Eighty-three Holstein-Friesian heifers were included in the analysis. The concentration of cortisol was measured in serum before and after parturition. There was no effect of treatment group on calving modality duration of calving, and cortisol concentration. The area under the curve of pulling force × time (n = 44), Also, duration of calving assistance was numerically shorter in the treatment group compared with the placebo group. The results provide evidence that calving ease can be influenced by denaverine hydrochloride during calving assistance.

- **Katrin Lange *et al.,2018* performed effect of denaverine hydrochloride application theifers on the APGAR score and lactate concentration in newborn calves[60]**

A total of 83 calvings with 38 female and 45 male calves were included in the study. Thirty minutes after onset of stage 2 of calving, 400 mg DNH or placebo (0.9% NaCl) were administered subcutaneously. Directly after parturition, vitality of calves was evaluated using a modified APGAR score. Additionally, lactate concentration in blood from Vena auricular is was measured with a handheld measuring device (lactate scout). No effect of treatment was observed on APGAR score and lactate concentration.

- **Robert Zobel, *et al.,2014* performed denaverine hydrochloride and carbetocin increased welfare during and after parturition and enhanced subsequent fertility in cattle[61]**

Altogether 200 animals (100 cows and 100 heifers) of the Simmental breed were divided into 2 groups: treatment (n = 100) and control (n = 100). Animals in the treatment group received denaverine hydrochloride and carbetocin during delivery over a maximum of 4 waiting periods (30 min each), whereas control animals experienced the same waiting periods but received no treatment. The treatment increased the number of animals with the birth canal dilated by more than 25 cm, and halved the need for any assistance at parturition. The treatment protocol had an effect throughout the entire puerperal period, as treated animals conceived with fewer artificial inseminations (1.3 vs. 1.6 artificial inseminations/pregnancy) and sooner (67 vs. 78 d open) compared with control animals. Denaverine hydrochloride and carbetocin administered

in combination during parturition affected the progression and ease of calving, and thus the welfare of cows in labor and subsequently.

- **Jinmin Miao** *et al.,2013* **performed ortho acylation of benzoic acids with α-oxocarboxylic acids[62]**

Palladium-catalyzed chemoselective decarboxylative cross coupling of benzoic acids with α-oxocarboxylic acids was realized via an arene sp(2) C-H functionalization process. This work represents the first example of transition-metal-catalyzed cross-coupling reactions with two acids acting in different roles. The synthetic utility of this method was confirmed by the synthesis of pitofenone, an antispasmodic used in the combined drug Spasmalgon

- **J Künzel** *et al.,2011* **performed myometrial response to neurotropic and musculotropic spasmolytic drugs in an extracorporeal perfusion model of swine uteri[63]**

To compare the effects of neurotropic and musculotropic spasmolytic drugs in isolated swine uterus specimens, 80 swine uteri were perfused using an established model for preserving a viable organ that responds to oxytocic hormones and spasmolytic drugs. Initiation of rhythmic uterine contractions and recording of spontaneous rhythmic contractions, spasmolytic drugs were administered at various concentrations. The musculotropic relaxant denaverine in particular showed significant results ($P \leq 0.05$) for all dosages and parameters investigated. In terms of muscle physiology, musculotropic agents comparison with neurotropic or musculoneurotropic spasmolytic drugs for inhibiting contractions. Experiments with pethidine also promoting rapid directed sperm transport. Denaverine and pethidine in particular may in the future be able to play an important role in improving the pregnancy rate after IVF

- *B. Kilian et al.,2008* **developed liquid chromatography–tandem mass spectrometric (LC–MS–MS) method for the determination of the neurotropic–musculotropic spasmolytic agent Denaverine Hcl and five of its metabolites in urine.[64]**

In a first step -glucuronidase was used to cleave glucuronides in the human urine. After that samples containing Denaverine Hcl and its phase I metabolites were extracted using solid phase extraction method. The analytes were measured employing the multiple reaction-monitoring mode (MRM). The linear dynamic range for Denaverine Hcl and its five metabolites determination was demonstrated from lower limit of quantification (8.0 ng/ml) to at least 500 ng/ml. The presented method is suitable for pharmacokinetic or toxicokinetic studies.

- **Alexander Staab *et al.,*2003 performed pharmacokinetics and bioavailability of denaverine hydrochloride in healthy subjects following intravenous, oral and rectal single doses[65]**

The objectives of this clinical trial were to determine the basic pharmacokinetic parameters of denaverine after intravenous administration, to assess the feasibility of using the oral route of administration and to characterise the bioavailability of the suppository formulation. To achieve this, healthy subjects received 50 mg denaverine hydrochloride intravenously, orally and rectally in aqueous solutions and rectally as suppository in an open, randomised crossover design. Total body clearance, volume of distribution at steady-state and half-life of denaverine are 5.7 ml/min per kg, 7.1 l /kg and 33.8 h, respectively. The absolute bioavailability after oral administration of an aqueous solution is 37%. First-pass metabolism leading to the formation of *N*-mono dimethyl denaverine was found to be one reason for the incomplete bioavailability after oral administration. Rectal administration of an aqueous solution of denaverine hydrochloride resulted in a decreased rate (median of *C* ratios: 26%, difference in median *t* values: 1.9 h) and extent (31%) of bioavailability compared to oral c_{max} administration. Using the suppository formulation led to a further reduction in rate (median of *C* ratios: 30%, difference in median *t* max values: 3 h) and extent (42%) of bioavailability compared to the rectal solution.

- **A.*Staab et al*., 2001 developed reversed-phase high-performance liquid chromatographic method for the simultaneous determination of Denaverine Hcl and its N-monodemethyl metabolite (MD 6) in human plasma.[66]**

The assay involves the extraction with an n-heptane–2-propanol mixture (9:1, v/v) followed by back extraction into 12.5% (w/w) phosphoric acid. The analytes of interest and the internal standard were separated on a Superspher RP8 column using a mobile phase of acetonitrile– 0.12 M NH_4 H_2 PO_4 –tetrahydrofuran (24:17.2:1, v/v), adjusted to pH 3 with 85% (w/w) phosphoric acid. Ultraviolet detection was used at wavelength of 220 nm. The retention times of MD 6, Denaverine Hcl and the internal standard were 5.1, 6.3 and 10.2 min, respectively. linear range of 2.5–150 µg/ml for Denaverine Hcl and MD 6. Extraction recoveries ranged from 44 to 49% and from 42 to 47%, respectively. The stability of Denaverine Hcl and MD 6 in plasma was demonstrated after 24 h storage at room temperature, after three freeze–thaw cycles and after 7 months frozen storage below 220^0C. The stability of processed samples in the autosampler at room temperature was confirmed after 24 h storage.

- **A. Staab *et al.,1994* developed hplc determination of denaverine and propiverine in human plasma[67]**

In order to characterize the pharmacokinetic properties of denaverine and propiverine as well as to determine their absolute and relative bio availabilities specific, sensitive and rapid (10 minutes' runtime) isocratic HPLC methods for the quantitation of the compounds in human plasma were developed. the analytes were extracted into a mixture of n-heptane and 2-propanol (9:1 v/v) under alkaline conditions (pH 11) subsequently reextracted from the organic phase with 12.5 % phosphoric acid. Extraction recovery was determined as 50 % for denaverine and 75 % for propiverine. Chromatographic separation with acetonitrile: 0.12 M ammonium dihydrogenphosphate buffer (1:1.4 v/v for denaverine and 1:2 v/v for propiverine; adjusted to pH 3 with 85 % phosphoric acid) on a RP-8 column and UV detection (220 nm) allows the determination of denaverine or propiverine with lower limits of quantitation of 5 ng/ml (calculated as hydrochlorides). For both methods calibration curves from 5 to 150 ng/ml were validated. Correlation coefficients of the linear calibration curves were 0.994 or better, obtained during pre-study validation experiments and analysis of study.

- **V Bredow.,*1992* performed use of tramadol versus pethidine versus denaverine suppositories in labor a contribution to noninvasive therapy of labor pain[68]**

In a prospective study on at all 49 women under labour the clinical effect of the noninvasive rectal application of Tramadol, Pethidin, and Denaverin has been compared. The first dosage was 100 mg of all substances. Around the half of the women said that analgetic effect was good or very good. On only every fifth it was sufficient or not enough. The effect was at near the same in all treatment groups. Because of a low incidence of maternal side effects, the absence of side effects on the newborn, and near the same results on the analgetic effect of parenteral application in other studies, tramadol suppositories can be recommended for obstetrical analgesia

- **B Gober *et al.,1988* performed biotransformation of denaverin (Spasmalgan) in the rat[69]**

After oral application of denaverine hydrochloride to rats (200-250 mg/kg) 12 metabolites have been detectecd in urine. Besides the unchanged drug, 8 metabolites were identified by MS as 2,2-diphenyl-(2-dimethylaminoethyl) acetate (3), diphenylacetic (5) and benzilic acid (6); methyl-and ethyl [2-(2-ethylbutoxy)-2,2-diphenyl] acetate (7, 11), methylbenzilate (10), N-demethyl-1 (12) and 3,3-diphenyl-morpholin-2-one (13). 6 and the metabonate 13 represent

the main metabolic products. Compounds 7 and 11 indicate the metabolic pathway about an alkoxybenzilic acid (4). Phenols resp. conjugates were not detected.

- **H H Borchert *et al.,*1985 performed metabolic interactions of denaverine[70]**

Owing to enzyme induction, the pretreatment of female Wistar rats with denaverine (Spasmalgan) produces a dose-dependent shortening of the hexobarbital sleeping time, corresponding with an increase in aminophenazon-N- and p-nitroanisol-O-demethylation, cytochrome P-450 concentration and NADPH-cytochrome-c-reductase activity in the 9000 g supernatant of liver homogenates and the relative liver weight, but no change in p-nitrophenetol-O-dealkylation and p-nitrophenol-glucuronidation. Our data suggest that denaverine is a phenobarbital-type microsomal enzyme inducer and justify the expectation of drug interactions in case of repeated application of denaverine in the framework of a combined therapy.

Chapter-4

OBJECTIVES

4. Aim and Objective

Aim of this study involves Analytical Method Development and Validation of Denaverine Hydrochloride as per ICH Guidelines

Objective(s) of the Project:

- To develop an analytical method that is eco-friendly.
- To develop an analytical method that can be used for routine analysis in the laboratories.
- The solvent should be readily available, economical and of analytical grade.
- Infrared spectroscopy to be carried out for the identification of chemical compounds.
- UV Spectroscopy to find out the λ max, amount, percentage of purity.
- To develop methods and to validate with various parameter like accurate, Linearity, Precision, Accuracy, Robustness, Limit of detection and Limit of quantitation.
- To compare the recovery studies.
- To compare the results of the bulk drug and its pharmaceutical dosage form.

Chapter-5

PLAN OF WORK

5. Plan of Work

❖ **Preliminary studies**

- Determination of solubility of the drug by using various solvents
- Determination of melting point

❖ **Infrared Spectroscopy**

- Identification of functional group by using FTIR

❖ **Ultraviolet Spectroscopy**

- Selection of suitable solvent system
- Find out the concentration of drug
- Determination of λ max /absorbance
- Find out amount and percentage purity of the drug
- Recovery studies
- Validate the methods according to ICH guidelines

❖ **High Performance Liquid Chromatography**

- Selection of suitable solvent system
- Selection of Column
- Selection of Column particle size
- Optimized chromatographic condition
- Determination of flow rate and injection volume
- Detector Selection
- Recovery studies
- Validate the methods according to ICH guidelines

Chapter-6

MATERIALS AND METHODS

6. MATERIALS AND METHODS

6.1 PRELIMINARY STUDIES

Solubility Studies

A small quantity of bulk drug of Denaverine hydrochloride is dissolved in different solvents like water, methanol, ethanol, acetonitrile, and buffer solutions of different pH.

Melting Point

Sample is taken to 3/4th height of capillary tube. capillary tube is placed in the sample holder. The temperature at which the drug present in capillary tube melts is noted.

Table 1: Instrument used in Melting Point

Name of the instrument	Make	Model
Melting Point apparatus	Scientific	MP-D

6.2 INFRARED SPECTROSCOPY

Chemicals and Reagents

Pure sample of denaverine hydrochloride was gift from Nebulae Hitech laboratory, Chennai. Mortar & pestle, potassium bromide used were of analytical grade.

Table 2: Instrument used in Infrared Spectroscopy

Name of the instrument	Make	Model	Software
Analytical balance	Wensar	Semi micro	-
IR Spectrophotometer	Thermoscientific	Nicolet is 5	OMNIC

Sample preparation

KBr pressed pellet technique was used.

A homogeneous mixture will give the best results excessive grinding of the potassium bromide is not required.

Added about 1% of sample, mixed and grind with potassium bromide (KBr) to form a very fine powder. This powder was then compressed into a thin pellet. The thin transparent KBr pellet was analyzed by FTIR.

6.3 UV SPECTROPHOTOMETRIC METHOD

Chemicals and Reagents:

All the chemicals used were of analytical grade. An analytically pure sample of Denaverine hydrochloride was procured as gift sample from Nebulae Hitech laboratory, Chennai. Sensiblex 40mg/mL were purchased from local market.

Instrumentation

Table 3: Instrument used in **UV Spectrophotometric Method**

S.No.	Name of the Instrument	make	model	software
1.	Analytical balance	Wensar	Semi micro	-
2.	Sonicator	PCI analysis	-	-
3.	UV/VIS Spectrophotometer	Shimadzu	UV-1700	UV Probe

Table 4: Shimadzu UV-1700 UV/VIS Spectrophotometer Specifications

Shimadzu UV-1700 UV/VIS Spectrophotometer Specifications	
Light source	20 W halogen lamp, Deuterium lamp. Light source position automatic adjustment mechanism.
Monochromator	Aberration-correcting concave holographic grating
Detector	Silicon Photodiode
Stray Light	0.04% or less (220 nm: NaI 10 g/ L) 0.04% or less (340 nm: $NaNo_2$ 50 g/ L)
Wavelength range	190-1100 nm
Spectral Band Width	1 nm or less (190 to 900 nm)
Wavelength Accuracy	± 0.5 nm automatic wavelength calibration mechanism
Recording range	Absorbance: -3.99~3.99 Abs,Transmittance: -399~399%
Photometric Accuracy	± 0.004 Abs (at 1.0 Abs), ±0.002 Abs (at 0.5 Abs)
Operating Temperature/Humidity	Temperature range: 15 to 35° C Humidity range: 35 to 80% (15 to below 30° C) 35 to 70% (30 to 35° C)

Preparation of Solution

Standard Solution Preparation

Accurately weighed and transferred 10 mg of Denaverine working standard into a 10 mL volumetric flask. About 7 mL of methanol was added and sonicate to dissolve it completely. Then the solution was made volume up to the mark with the same solvent (1000 µg/mL). Further 1.0 mL of the Denaverine stock solution was pipette out into a 10 mL volumetric flask and dilute up to the mark with solvent (100µg/mL). Further 0.9 mL of the Denaverine stock solution was pipette out into a 10 mL volumetric flask and dilute up to the mark with solvent (9µg/mL).

Sample Solution Preparation

0.25 mL of injection (Sensiblex 40mg/mL) was measured accurately and transfered the sample (equivalent to 10 mg of Denaverine hydrochloride) into a 10 mL volumetric flask. 7 mL of methanol was added and sonicate to dissolve it completely. Then it was made volume up to the mark with same solvent (1000 µg/mL). The solution was mixed well and filter through 0.45 µm filter. Further pipette 1 mL of the Denaverine solution into a 10 mL volumetric flask and dilute up to the mark with solvent (100µg/mL). Further pipette 0.9 mL of the Denaverine stock solution into a 10 mL volumetric flask and dilute up to the mark with solvent (9 µg/mL). The sample solution (9 µg/mL) absorbance was measured against blank at 221nm. Percentage purity was calculated by using the following formula.

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

Where:

AT	=	Absorbance of Denaverine obtained with test preparation.
AS	=	Absorbance of Denaverine obtained with standard preparation.
WS	=	Weight of working standard taken in mg
WT	=	Weight of sample taken in mg
DS	=	Dilution of Standard solution
DT	=	Dilution of sample solution
P	=	Percentage purity of working standard

Method development and validation

Linearity:

From the stock solution, the aliquots of stock solution of Denaverine (1000 µg/mL) further dilution make up to (0.3 to 1.5 mL of 1000 µg/mL) and transferred in to 100 mL volumetric flask and made up to the mark with solvent. The absorbance of the solution of different concentration were measured at 221 nm against blank. The calibration curve was plotted using Concentration Vs Absorbance. The curve obtained was linear with concentration range 3-15 µg/mL.

Recovery:

0.25mLof injection (Sensiblex 40mg/mL) was measured accurately and transferred the sample (equivalent to 10 mg of Denaverine hydrochloride) into three separate 10 mL volumetric flask. Then 5mg, 10mg and 15mg (50%, 100%, 150%) of standard were accurately weighed and added. 7 mL of methanol was added and sonicate to dissolve it completely. Then the solution was made volume up to the mark with the same solvent. 0.9 mL was pipette out from each flask and transferred to separate 100 mL volumetric flask. Then the solution was made volume up to the mark with the same solvent. Absorbance was measured at 221 nm against blank. The amount of drug recovered was calculated by using slope and intercept values.

Precision:

Repeatability and intermediate precision studies were done to the precision of the method. Repeatability studies were done by consequently measuring the absorbance of standard solution (9µg/mL). These solutions were prepared in duplicate and absorbance were measured at 221 nm against blank and calculate the % RSD.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ were estimated from the set of s calibration curves used to determine method linearity.

LOD= 3.3 σ /S and LOQ=10* σ /S

Where, σ= - the standard deviation of y-intercepts of regression lines.

S - the slope of the calibration curve.

Statistical Formula

Mean

The Mean is computed by adding all of the numbers in the data together and dividing by the number elements contain in the data set.

Mean = {Sum of Observation} ÷ {Total numbers of Observations}.

Standard Deviation

A standard deviation (or σ) is a measure of how dispersed the data is in relation to the mean. The precision of an analytical method is usually expressed as standard deviation.

Standard deviation calculated statistically using following formula

$$s = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}}$$

Where

$\bar{x}$ = Arithmetic mean

x = Observed value

$x - \bar{x}$ = Deviation of the value from the mean

n = Number of observation

Percentage Relative Standard Deviation

Percent relative standard deviation (%RSD) is one such tool. By formula, it is the standard deviation of a data set divided by the average of the data set multiplied by 100.

$$RSD = \frac{Standard\ Deviation}{Mean} \times 100$$

Regression equation

Regression is the estimation or prediction of unknown values of the variable from known values of variables of another variable.

The regression equation is given as

$$Y = mx + c$$

Where,

Y = The variable taken here as absorbance

x = The variable taken here as concentration

c = Intercept

m = Slope

$$\text{Slope } m = \frac{N\Sigma xy - \Sigma X.\Sigma.y}{NEX^2 - (Ex)^2}$$

$$\text{Intercept } c = \frac{\Sigma y. \Sigma x^2 - \Sigma X. \Sigma Y}{NEX^2 - (Ex)^2}$$

From the formula

m = 0.0040

C = 0.0252

6.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Instrumentation:

Table 5: Instrument used in HPLC

S.No.	Name of the Instrument	Make	Model	Software
1.	Analytical balance	Wensar	Semi micro	-
2.	Sonicator	PCI analysis	-	-
3.	pH meter	ELICO	L1610	-
3.	HPLC	Shimadzu Prominence	HPLC iLc2030	LAB SOLUTON

Table 6: SHIMADZU Prominence HPLC iLC2030 Plus

SHIMADZU Prominence HPLC iLC2030 Plus	
PUMP	P4000
AUTO SAMPLER	AS 3000
UV –VIS DETECTOR	UV 2000
VACUME DEGASSER	SCM 1000
COLUMN	Symmetry C_{18} (4.6 x 150mm, 5 μm, Make: XTerra)
SAMPLE COOLER	UP TO 2- 4° C
SOFTWARE	LAB SOLUTON

Chemicals and Reagents:

All the chemicals used were of analytical grade. An analytically pure sample of Denaverine hydrochloride was procured as gift sample from Nebulae Hitech laboratory, Chennai. Sensiblex 40mg/mL were purchased from local market.

Preparation of Phosphate buffer

7.0 grams of Potassium di hydrogen Phosphate was weighed and transferred into a 1000 mL beaker, dissolve and diluted to 1000 mL with HPLC water. The pH to 3.5 was adjusted with ortho phosphoric acid.

Preparation of mobile phase

Phosphate buffer 300 mL (30%) and 700 mL of Acetonitrile HPLC (70%) was mixed well and degassing in ultrasonic water bath for 5 minutes. Filter through 0.45 μ filter under vacuum filtration.

Chromatographic Conditions

Mode of operation	:	Isocratic
Instrument	:	HPLC Waters
Detector	:	UV detector
Column	:	Symmetry C_{18} (4.6 x 150mm, 5 μm, Make: XTerra)
Temperature	:	Ambient
Flow rate	:	0.6 mL/min
Wave length	:	306 nm
Runtime	:	5 min
Sample size	:	20 μL
Mobile Phase	:	Phosphate buffer: Acetonitrile (30:70 %v/v)

Standard Solution Preparation

Accurately weigh and transfer 10 mg of Denaverine working standard into a 10 mL volumetric flask add about 7 mL of mobile phase and sonicate to dissolve it completely and make volume up to the mark with the same solvent (1000 μg/mL). Further 0.3 mL was pipette out (1 mg/mL) of the above stock solution into a 10 mL volumetric flask and dilute up to the

mark with mobile phase (30 µg/mL). Then the solution was mixed well and filter through 0.45 µm filter.

Sample Solution Preparation

0.25 mLof injection (Sensiblex 40mg/mL) was measured accurately and transfer the sample (equivalent to 10 mg of Denaverine hydrochloride) into a 10 mL volumetric flask. 7 mL of mobile phase was added and sonicate to dissolve it completely. Then it was made volume up to the mark with mobile phase (1000 µg/mL). The solution was mixed well and filter through 0.45 µm filter. Further 0.3 mL was pipette out of the above stock solution into a 10 mL volumetric flask and dilute up to the mark with mobile phase (30 µg/mL). Mix well and filter through 0.45µm filter.

Method development and validation

The method was validated in accordance with ICH guidelines. The parameters assessed were System Suitability Parameters, Linearity, Accuracy, Precision, Specificity, Assay, Limit of Detection (LOD), Limit of Quantification (LOQ), Robustness, Ruggedness and Stability of the solution.

Linearity

From the stock standard (1000 µg/mL), the aliquots (0.1 to 0.5 mL of 1000 µg/mL) solution taken 0.1, 0.2, 0.3, 0.4, 0.5 mL were taken in a separate 10 mL volumetric flasks and made up to 10 mL with mobile phase (10–50 µg/mL). This solution can inject into the chromatographic system and record the Chromatogram. The calibration graph was plotted with peak area in the Y axis and concentration of standard solution in the X axis.

Recovery

0.25 mL of injection (Sensiblex 40mg/mL) was measured accurately and transferred the sample (equivalent to 10 mg of Denaverine hydrochloride) into three separate 10 mL volumetric flask. Then 5mg, 10mg and 15mg (50%, 100%, 150%) of standard were accurately weighed and added. 7 mL of mobile phase was added and sonicate to dissolve it completely. Then the solution was made volume up to the mark with the same. 0.3 mL was pipette out from each flask and transferred to separate 10 mL volumetric flask. Then the solution was made volume up to the mark with the same. 20µL solution was injected in to chromatographic system and the chromatogram was recorded.

Precision

Repeatability and intermediate precision studies were done to the precision of the method. Repeatability studies were done by consequently measuring the absorbance of standard solution. These solutions were prepared in duplicate and absorbance were measured at 306 nm against blank and calculate the % RSD.

Robustness

For demonstrating the robustness of the developed method, experimental conditions were purposely altered and evaluated. The method must be robust enough to withstand such slight changes and allow routine analysis of the sample. For this present study mobile phase and flow rate has slightly changed and the assay was checked.

Chapter-7

RESULTS AND DISCUSSION

7. RESULTS AND DISCUSSION

7.1 Preliminary Studies

Solubility Studies

A small quantity of bulk drug of Denaverine hydrochloride were dissolved in different solvents.

Table 7: Solubility of the drug

Solvents used	Approximate volume of solvent in milliliters per gram of solute	Solubility status
Methanol	1-10 parts	Freely soluble
Phosphate Buffer pH 3.5	10-30 parts	soluble
Water	30-100 parts	Sparingly soluble
Acetonitrile	30-100 parts	Sparingly soluble
Ethanol	100-1000 parts	Slightly soluble
Chloroform	≥10000 parts	Practically Insoluble

Melting Point

The melting point of denaverine hydrochloride - **142^0c**

Table 8: Melting Point

Drug name	Standard value	Melting Point
Denaverine hydrochloride	$140\text{-}142^0c$	142^0c

7.2 INFRERED SPECTROSCOPY
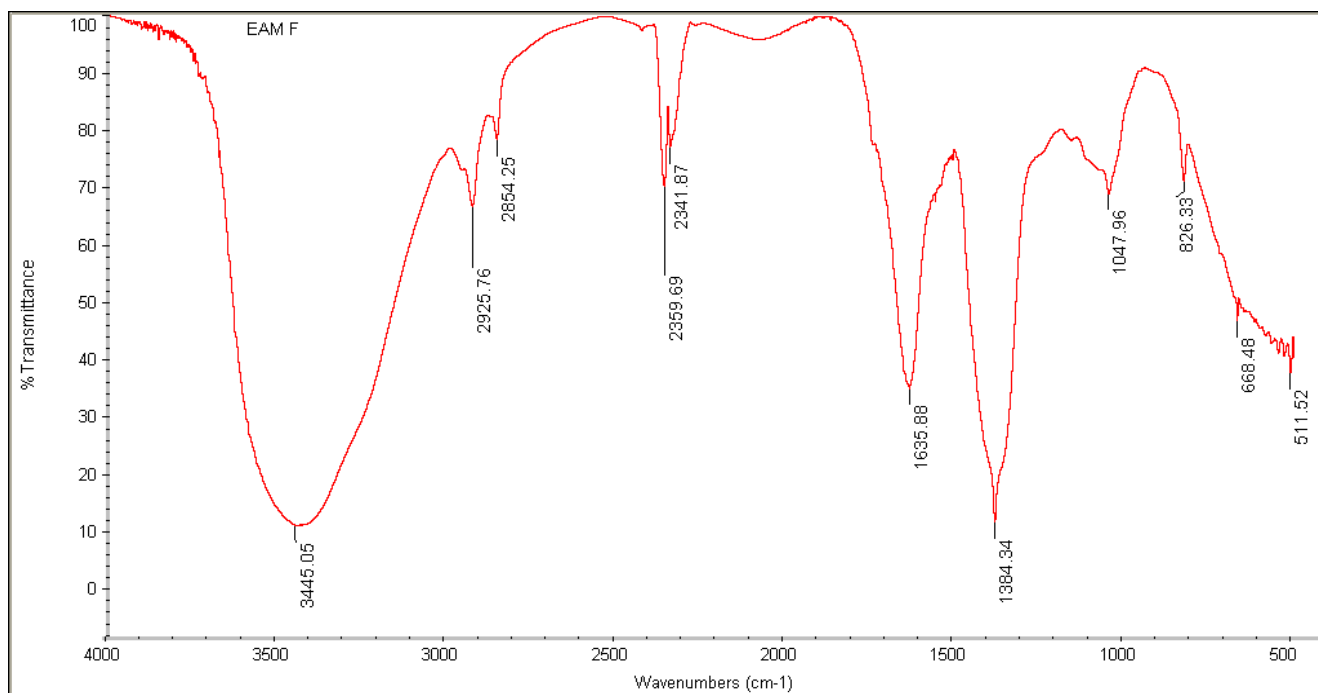
Figure 10: IR Spectrum of Denaverine hydrochloride

Table 9: IR Interpretation of Denaverine hydrochloride

WAVE NUMBER (cm⁻¹)	FUNCTIONAL GROUP	COMPOUND CLASS
3345.05	OH Stretching	Alcohol (intermolecular bonded)
2925.76	N-H Stretching	Amine
2854.20	C-C Stretching	Alkane
2359.69	C=O Stretching	Aromatic ring
2341.87	C=O Stretching	Ketone
1635.88	C=C Stretching	Alkene
1384.34	C-H Bending	Alkane
1047.96	C-N Stretching	Amine
826.33	C=C Bending	Alkene
668.48	C=C Bending	Alkene

7.3 UV SPECTROPHOTOMETRIC METHOD

- λ Max was observed at 221nm
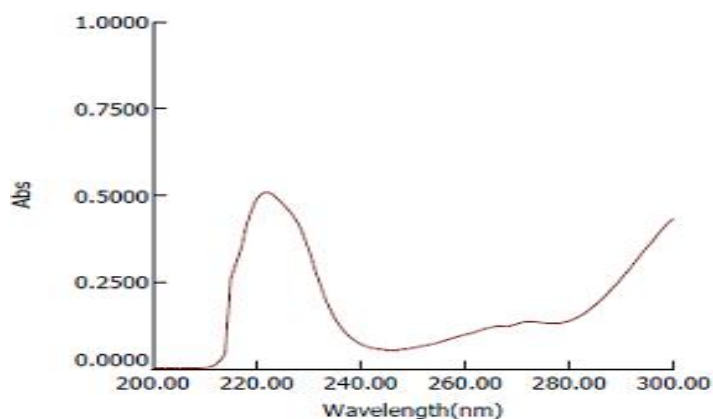
Figure 11: UV Spectrum of Denaverine hydrochloride in Methanol (9 µg/mL)

Table 10: Absorption Spectral Data

Wavelength (nm)	Absorbance
217	0.442
218	0.458
219	0.476
220	0.489
221	**0.495**
222	0.482
223	0.463
224	0.449
225	0.426
226	0.406

Table 11:   Beer's law plot reading of Denaverine Hcl at 221nm

S. No	Concentration (μg/mL)	Absorbance
1	3	0.155
2	6	0.328
3	9	0.495
4	12	0.663
5	15	0.840

Calibration curve for Denaverine

$y = 0.0561x - 0.0073$
$R^2 = 0.9997$
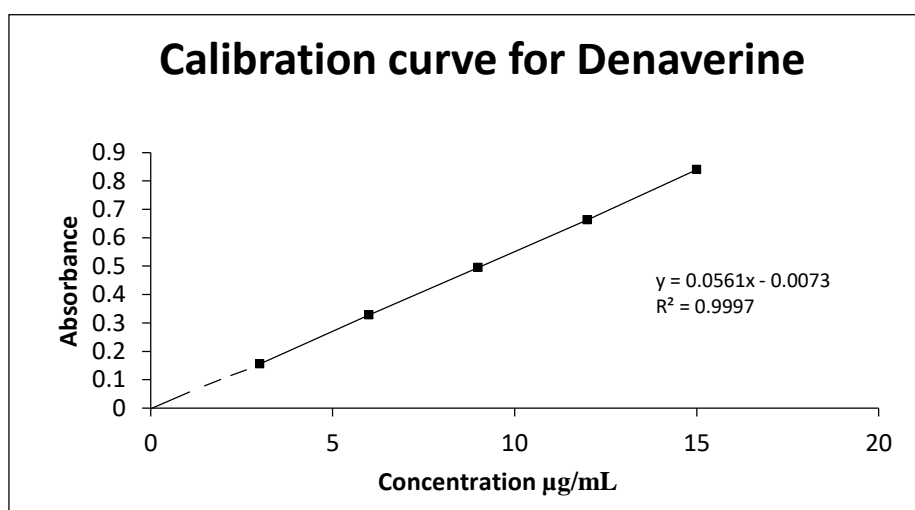
Figure 12: Calibration curve for Denaverine Hcl in Methanol

Quantification of Formulation (Assay)

The percentage of Denaverine in marketed formulation (Injection) was calculated from the calibration curve of Denaverine. %Assay was found to be 99.95% as shown in Table 12.

Calculation:

Each mL contains denaverine $= 40mg$

Average content of 10 ampoules $= 10$ mL

Average content of each ampoule $= 1$ mL

Volume equivalent to 10mg of denaverine $= \frac{Avg.wt}{Label\ claim} \times equivalent\ weight\ factor$

$= \frac{1}{0.04} \times 0.01$

$= 0.25$ mL

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

$$= \frac{0.490}{0.495} \times \frac{0.01}{10} \times \frac{1}{10} \times \frac{0.9}{10} \times \frac{10}{0.25} \times \frac{10}{1} \times \frac{10}{0.9} \times \frac{99.5}{100} \times \frac{1}{0.04} \times 100$$

$= 98.98$ %v/v

Table 12: Results of Quantification of formulation (Assay)

S.No	Standard Absorbance	Sample Absorbance	Percentage purity (%)	Average * Percentage (%)	SD	%RSD
1	0.495	0.490	98.98			
2	0.482	0.485	100.62			
3	0.484	0.488	100.82			
4	0.480	0.481	99.79	99.95	0.7610	0.7613
5	0.470	0.468	99.57			

*mean of five readings.

Recovery:

The percentage recovery study for Denaverine hydrochloride was found to be 98.8-100.53.
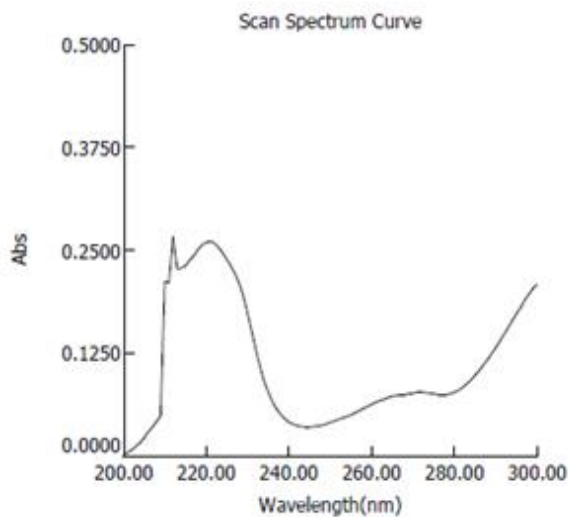
Figure 13: Recovery Spectrum (Level-I)

of Denaverine Hcl in Methanol
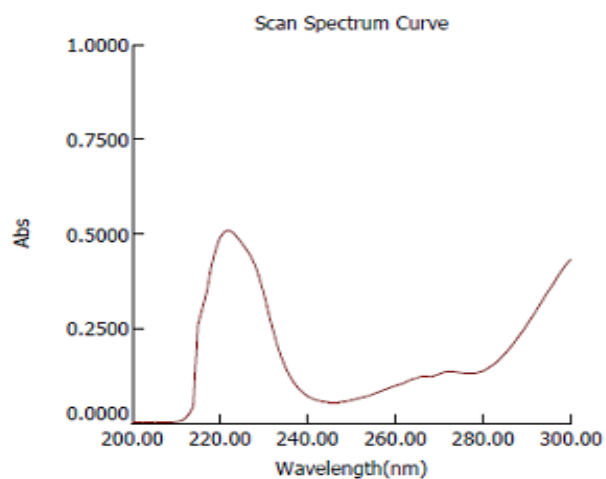
Figure 14: Recovery Spectrum (Level-II)

of Denaverine Hcl in Methanol
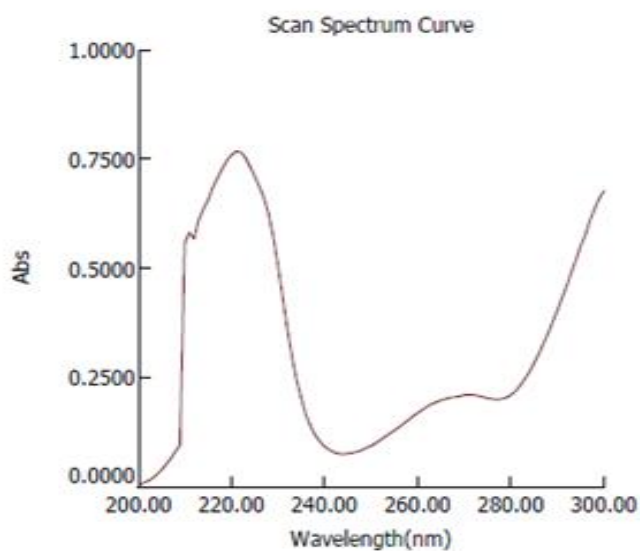
Figure 15: Recovery Spectrum (Level-III) of Denaverine Hcl in Methanol

Table 13: Results of Recovery Data

% Concentration	Absorbance	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery *
50%	0.261	5.1	5.12	100.53%	
100%	0.508	10.1	9.98	98.8%	100.0%
150%	0.769	15.0	15.10	100.7%	

*mean of three readings.

Linearity:

The Linearity for Denaverine hydrochloride was found to be concentration range 3-15 µg/mL with Correlation Coefficient 0.9997. LOD and LOQ were found to be 0.216 µg/mL and 0.655 µg/mL. Calibration data and %RSD is shown in table 14 and curve is shown in figure 16-20.
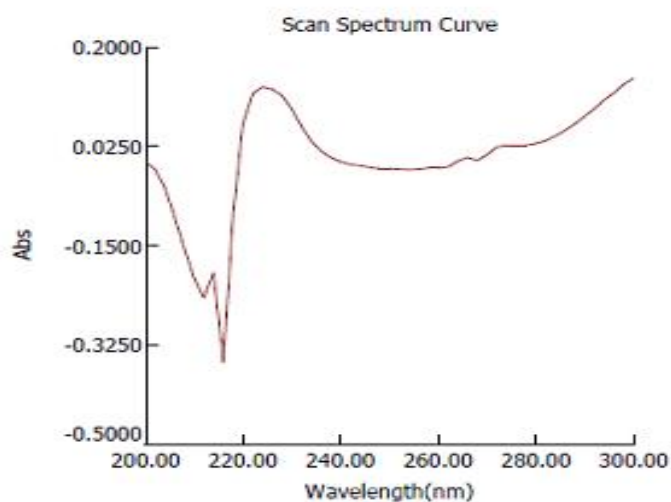
Figure 16: Linearity Spectrum (3µg/mL) of Denaverine Hcl in Methanol
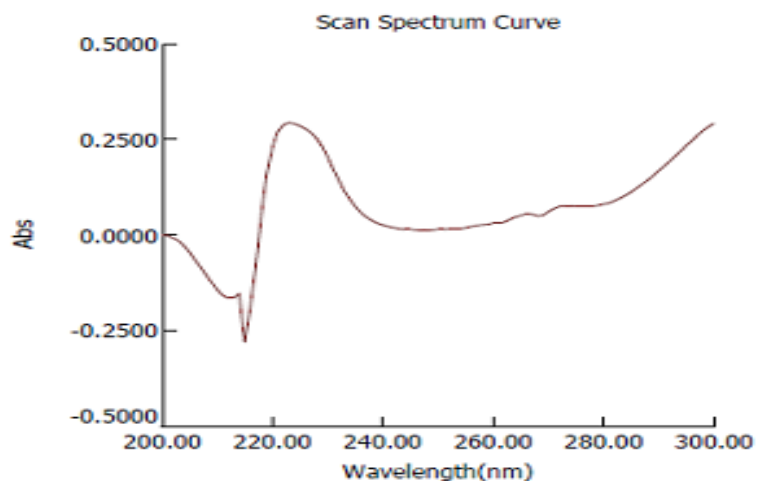
Figure 17: Linearity Spectrum (6µg/mL) of Denaverine Hcl in Methanol
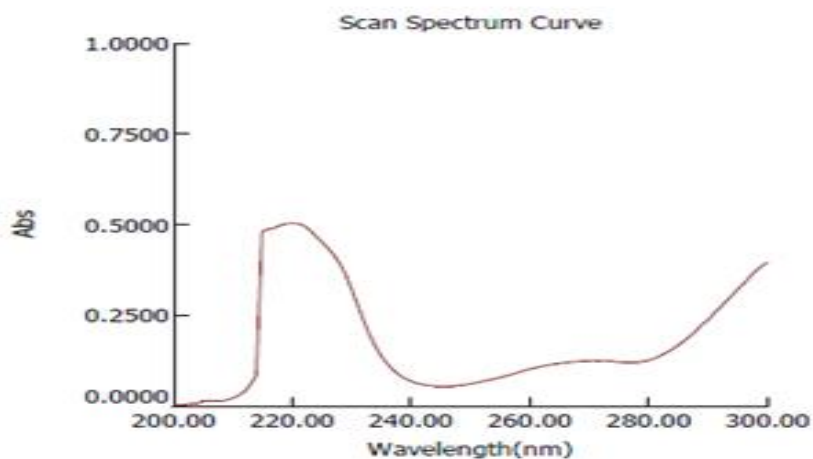
Figure 18: Linearity Spectrum (9µg/mL) of Denaverine Hcl in Methanol
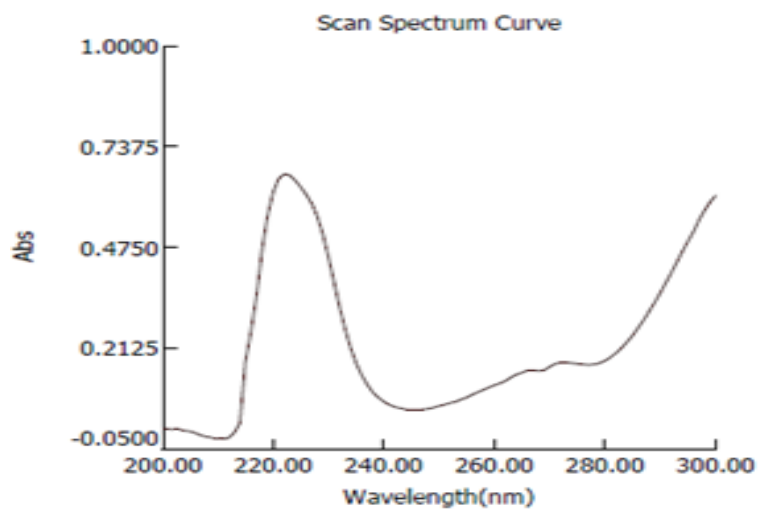
Figure 19: Linearity Spectrum (12µg/mL) of Denaverine Hcl in Methanol
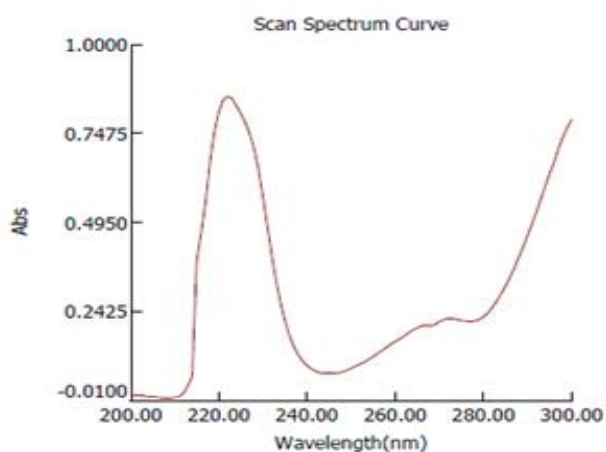
Figure 20: Linearity Spectrum (15μg/mL) of Denaverine Hcl in Methanol

Table 14: Results of Linearity

S. No	Concentration	Absorbance	Correlation Coefficient	LOD	LOQ	Slope	Intercept
1	3μg/mL	0.155					
2	6μg/mL	0.328					
3	9μg/mL	0.495	0.9997	0.216	0.655	0.056	-0.007
4	12μg/mL	0.663					
5	15μg/mL	0.840					

Precision:

% RSD was found to be 0.16

Scan Spectrum Curve
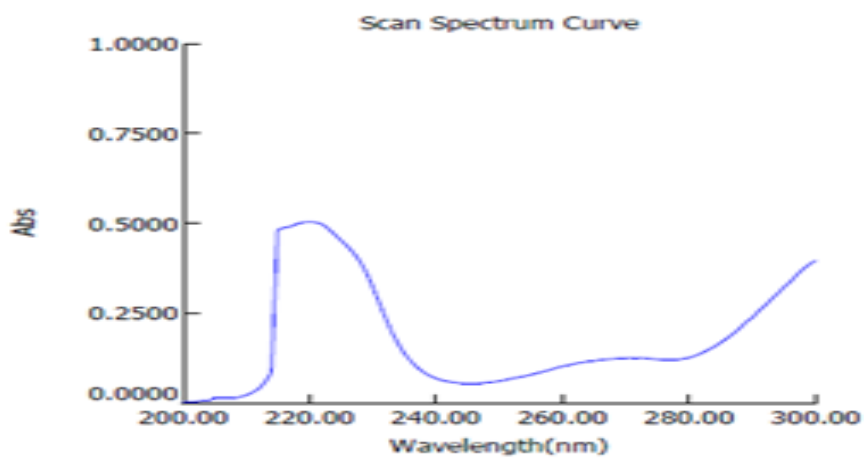
Figure 21: Precision of Denaverine Hcl in Methanol

Table 15: Results of Precision Data

S. No	Absorbance	Average Absorbance *	SD	%RSD
1	0.511			
2	0.511			
3	0.512	0.5118	0.0008	0.16
4	0.512			
5	0.513			

*mean of five readings.

Intermediate precision:

% RSD was found to be 0.222
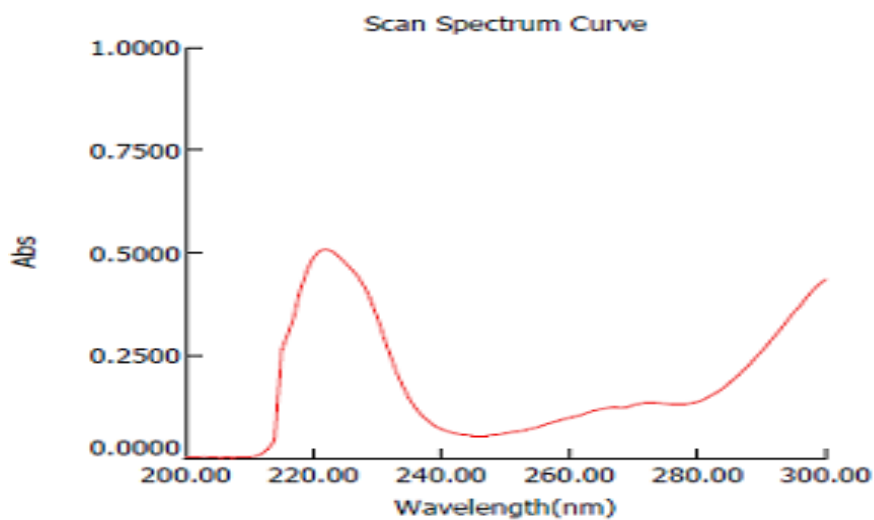
Figure 22: Intermediate Precision Spectrum of Denaverine Hcl in Methanol

Table 16: Results of Intermediate Precision Data

S. No	Absorbance	Average Absorbance *	SD	%RSD
1	0.510			
2	0.511			
3	0.513	0.511	0.0011	0.222
4	0.511			
5	0.512			

*mean of five readings

7.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY
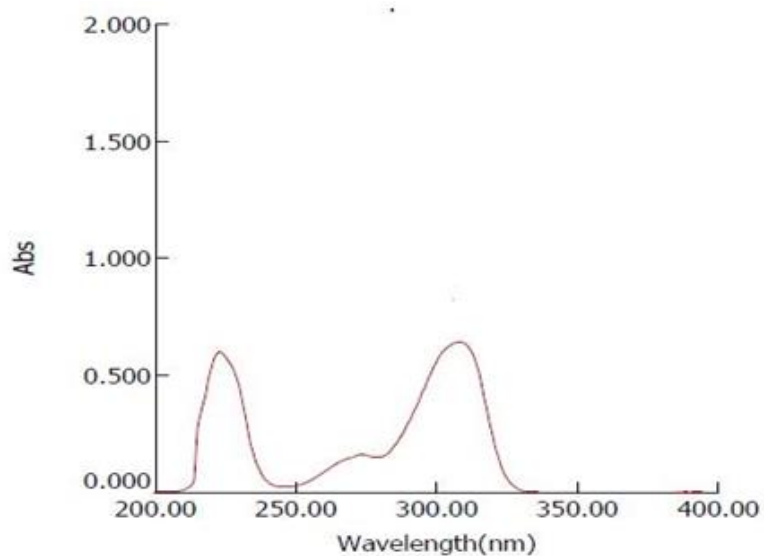
Figure 23: Spectrum showing UV Detector in HPLC of Denaverine [Phosphate Buffer pH 3.5: Acetonitrile (30:70 %v/v)]
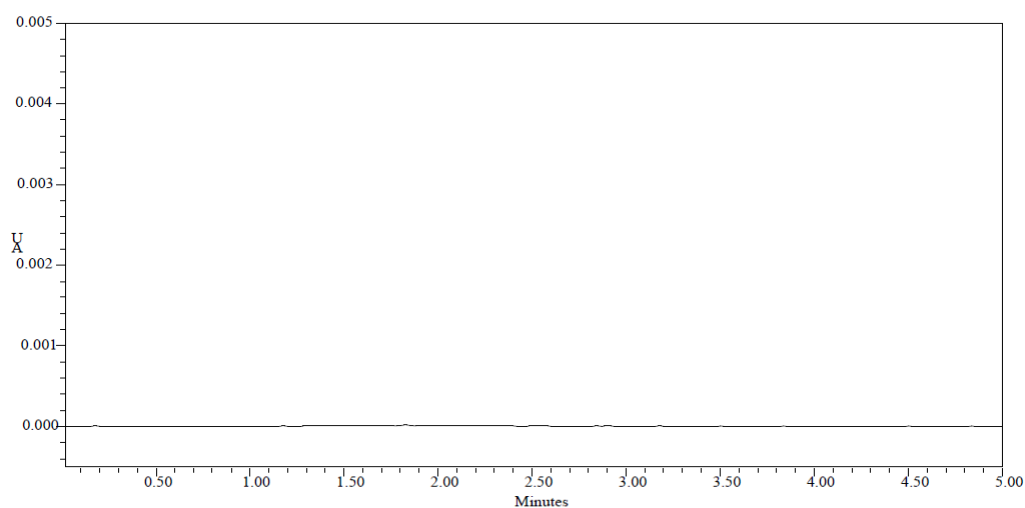
	Baseline Noise (mV)
1	0.048

Figure 24: Baseline correction

Initial Separation Condition

Mode of operation	:	Isocratic
Instrument	:	HPLC Waters
Detector	:	UV detector
Column	:	Symmetry C_{18} (4.6 x 150mm, 5 μm, Make: XTerra)
Temperature	:	Ambient
Flow rate	:	0.6 ml/min
Wave length	:	306 nm
Runtime	:	5 min
Sample size	:	20 μL
Mobile Phase	:	Water: Methanol (50:50 % v/v)
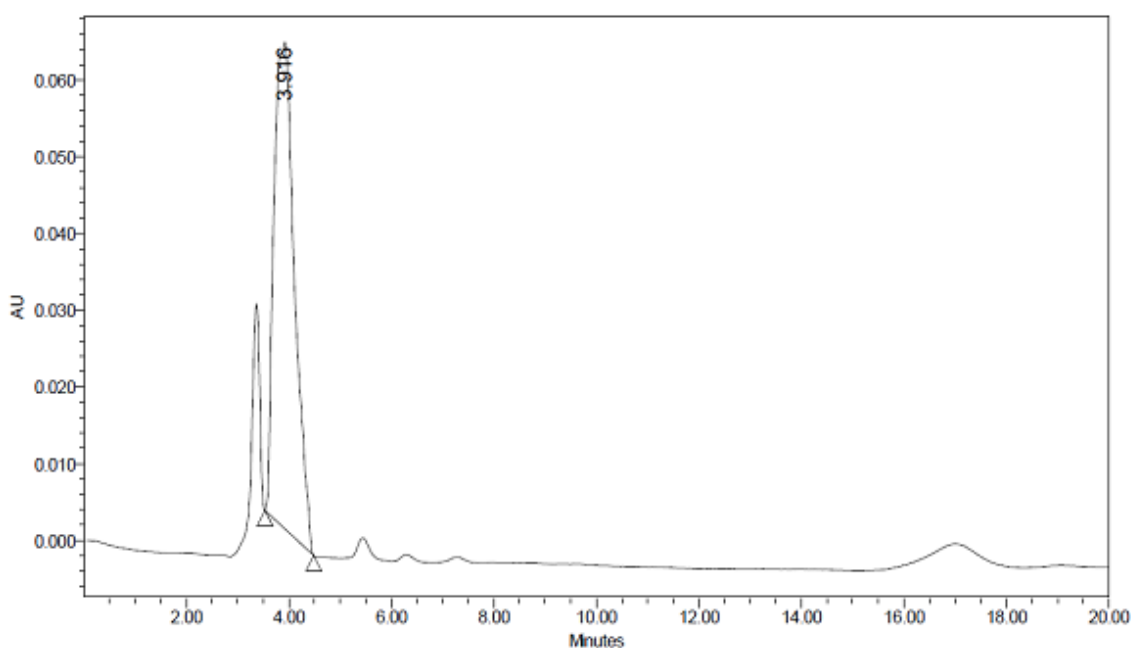
RT	Area	Height	USP Plate Count	USP Tailing
3.916	1760477	63310	480.7	1.3

Figure 25: Trial 1-Water: Methanol (50: 50 %v/v)
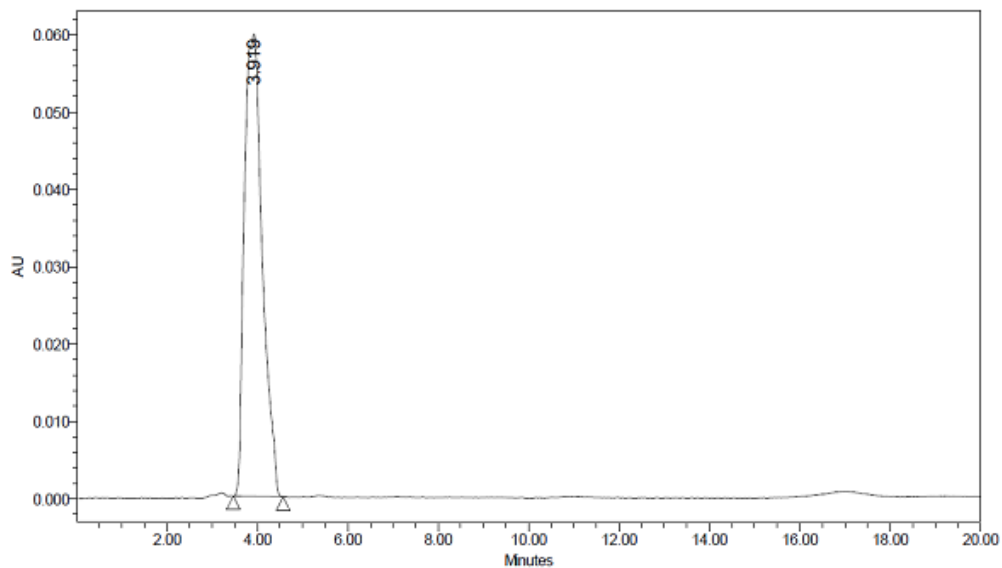
RT	Area	Height	USP Plate Count	USP Tailing
3.919	1650713	59758	509.6	1.2

Figure 26: Trial 2-Water: Acetonitrile (40: 60 %v/v)
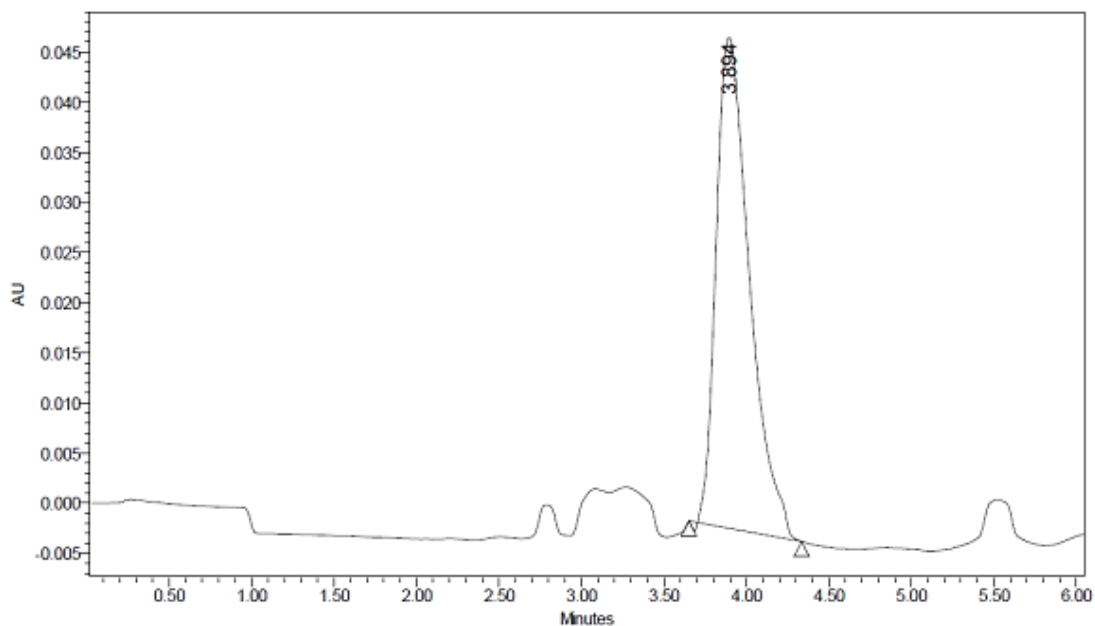
RT	Area	Height	USP Plate Count	USP Tailing
3.894	691820	49018	1780.6	1.6

Figure 27: Trial 3-Phosphate Buffer pH3: Methanol (50: 50%v/v)
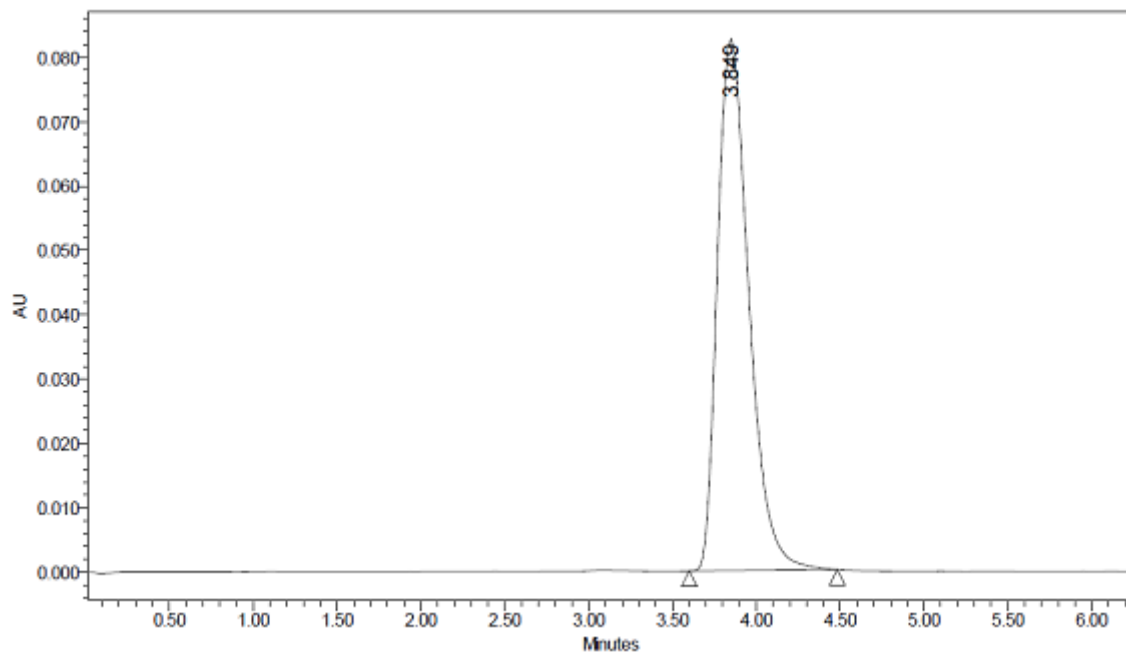
RT	Area	Height	USP Plate Count	USP Tailing
3.849	1075343	82659	2050.8	1.4

Figure 28: Trial 4- Phosphate Buffer pH3.5: Acetonitrile (40: 60%v/v)
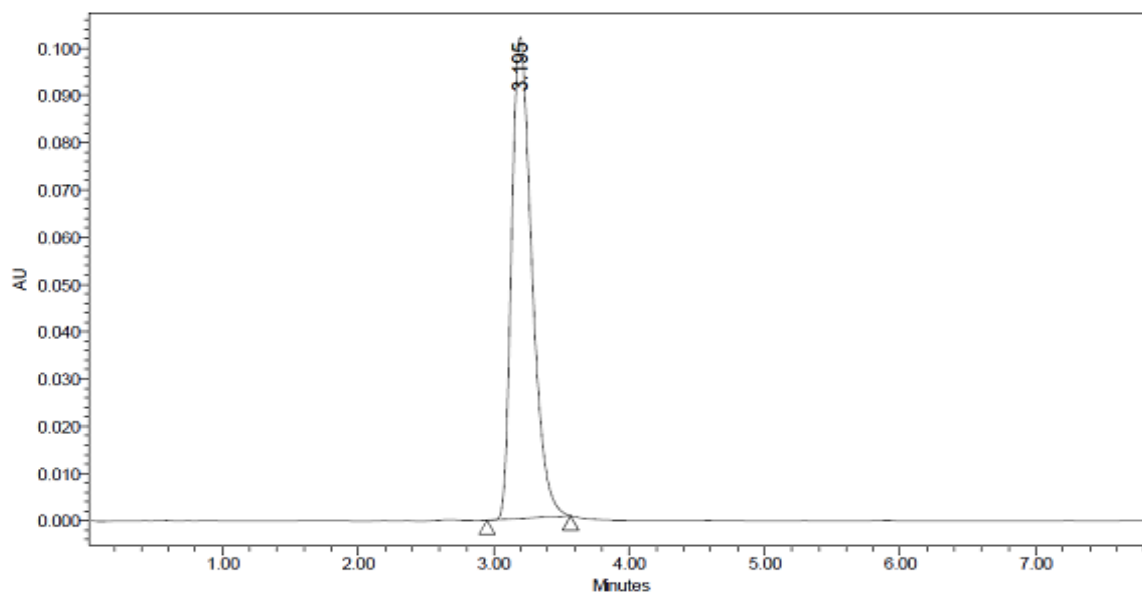
	RT	Area	Height	USP Plate Count	USP Tailing
1	3.195	1058299	102124	2178.3	1.4

Figure 29: Optimized Chromatogram Phosphate Buffer pH3.5: Acetonitrile (30: 70%v/v)

System suitability parameters:

The system suitability studies carried out as specified in ICH guidelines and USP. The parameters like tailing factor, number of theoretical plate were calculated.

Table 17: System suitability

Parameter	Denaverine
Tailing factor	1.6
No of Theoretical plate	2951
Retention time	3.2

Linearity:

From the stock standard (1000 µg/mL), the aliquots (0.1 to 0.5 ml of 1000 µg/mL) solution taken 0.1, 0.2, 0.3, 0.4, 0.5 ml were taken in a separate 10 ml volumetric flasks and made up to 10 mL with mobile phase (10–50 µg/mL). This solution can inject into the chromatographic system and record the Chromatogram. The calibration graph was plotted with peak area in the Y axis and concentration of standard solution in the X axis.
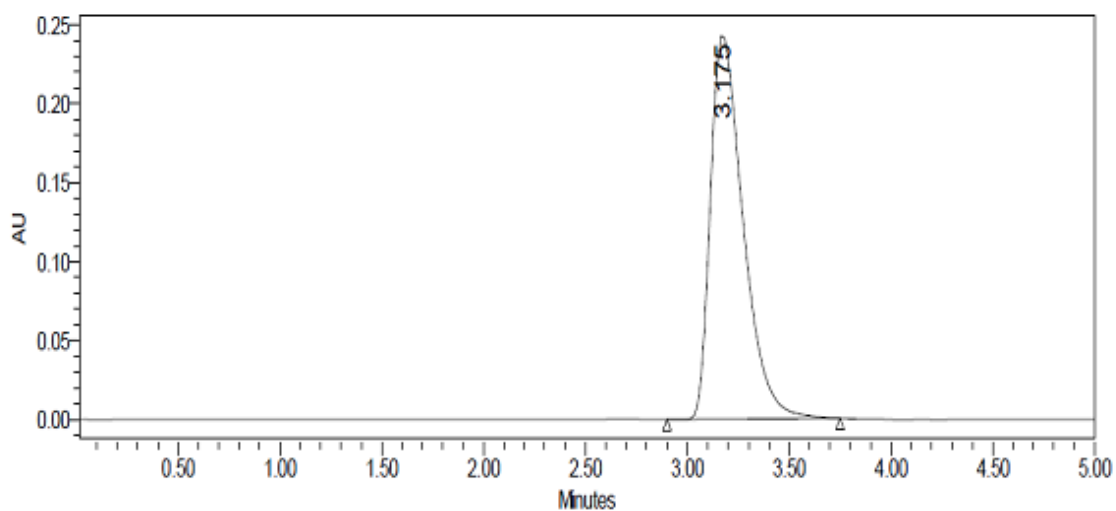
Figure 30: Linearity Chromatogram (10µg/mL) in Phosphate Buffer pH 3.5: Acetonitrile (30: 70%v/v)
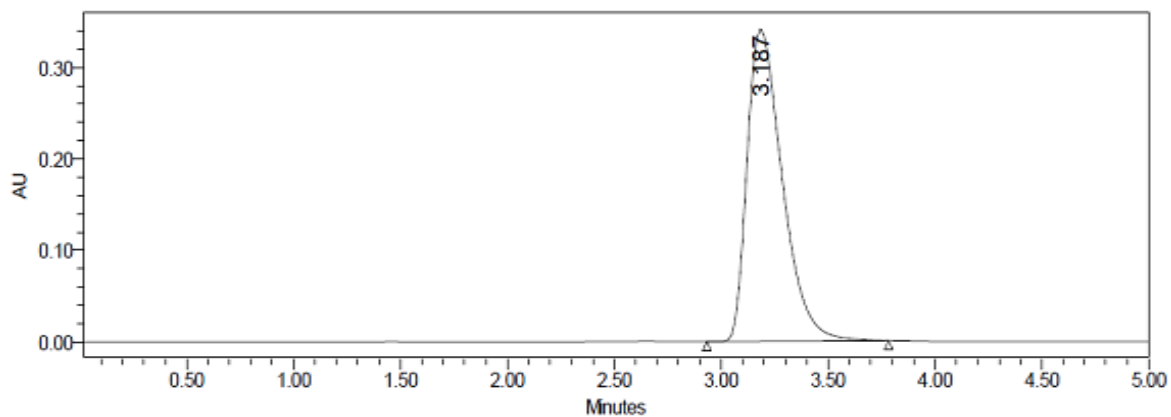
Figure 31: Linearity Chromatogram (20µg/mL) in Phosphate Buffer pH 3.5: Acetonitrile (30: 70%v/v)
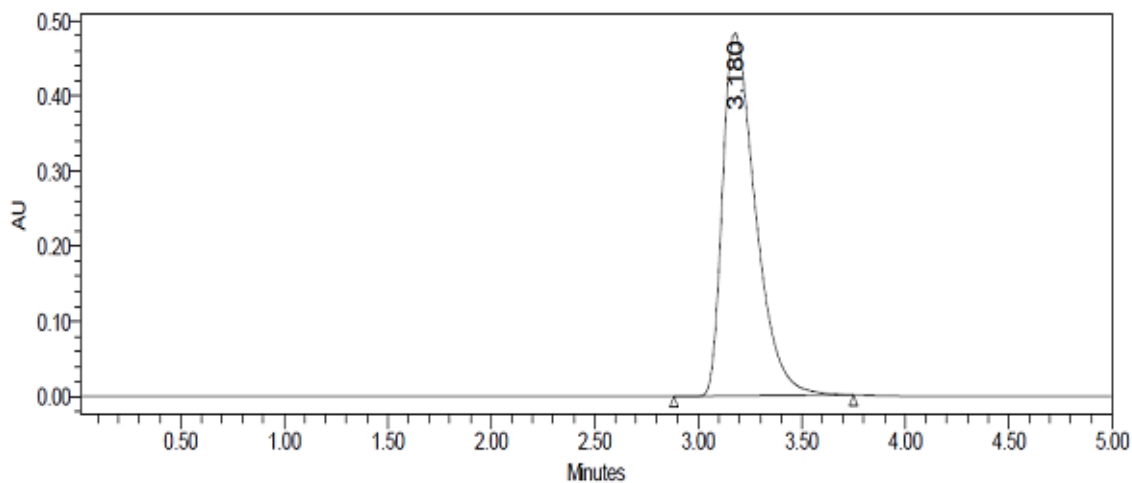
Figure 32: Linearity Chromatogram (30µg/mL) in Phosphate Buffer pH 3.5: Acetonitrile (30: 70%v/v)
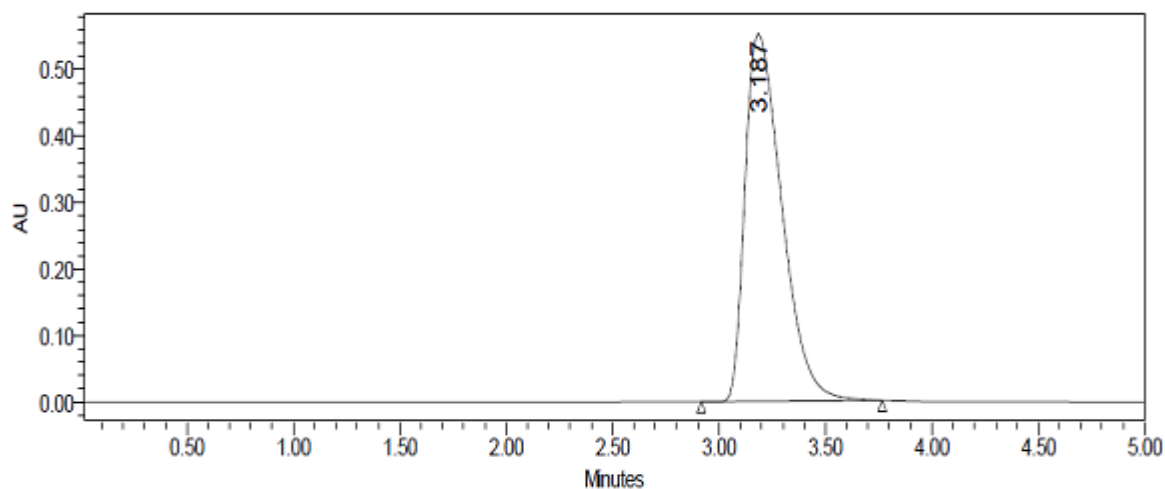
Figure 33: Linearity Chromatogram (40µg/mL) in Phosphate Buffer pH 3.5: Acetonitrile (30: 70%v/v)
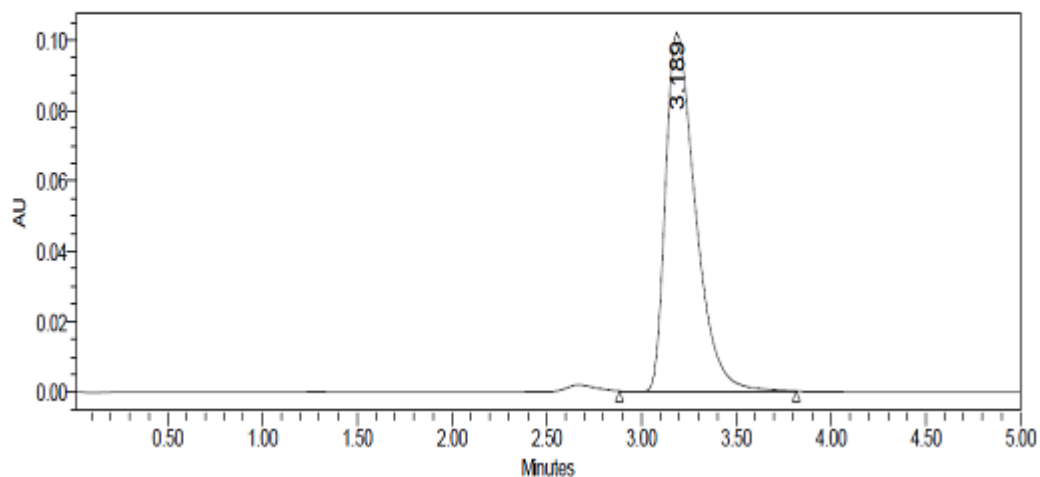
Figure 34: Linearity Chromatogram (50µg/mL) in Phosphate Buffer pH 3.5: Acetonitrile (30: 70%v/v)

RT	Area	Height (µV)
3.189	1113634	102352
3.175	2712792	244484
3.187	3908404	342239
3.180	5328851	484112
3.187	6652686	555333

Table 18: Results of Linearity

S.No	Concentration (µg/ml)	Peak Area	LOD	LOQ
1	10	1323634		
2	20	2712792		
3	30	3998490	0.014	0.0465
4	40	5328851		
5	50	6652686		
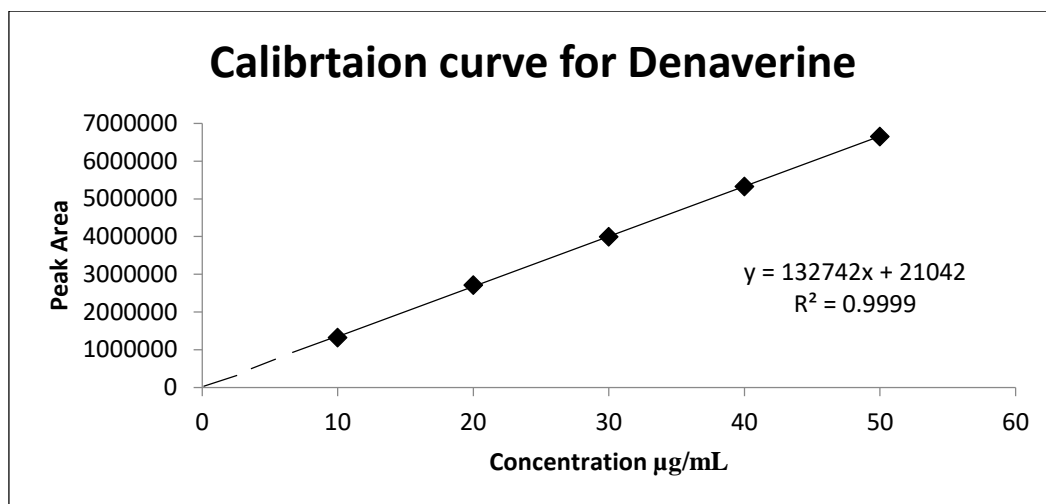
Figure 35: Calibration curve for Denaverine in Phosphate Buffer pH 3.5: Acetonitrile (30: 70%v/v)

LOD
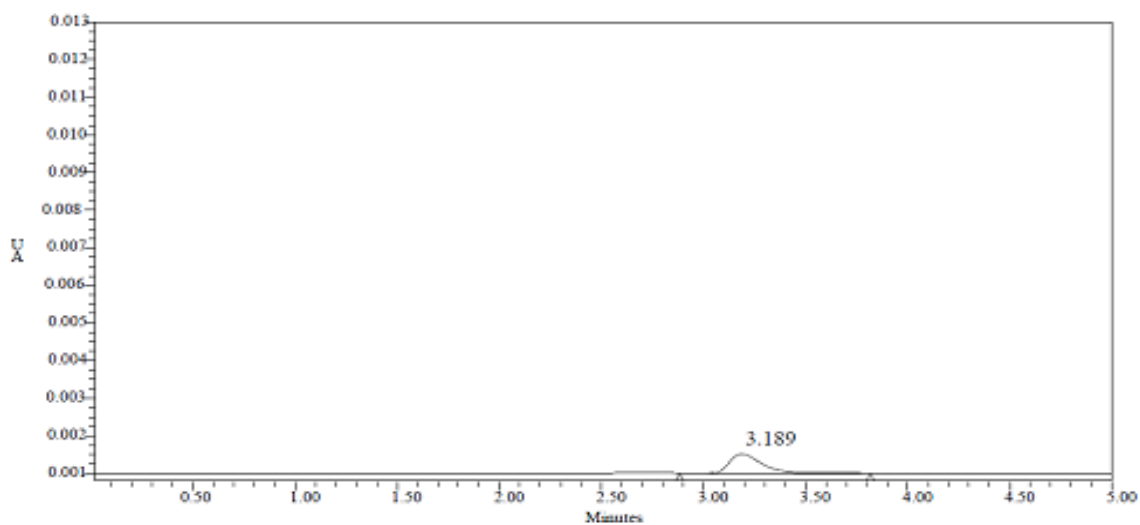
Retention Time (min)	Area (µV*sec)	Height (µV)
3.189	1545	142

Figure 36: LOD Chromatogram

LOQ
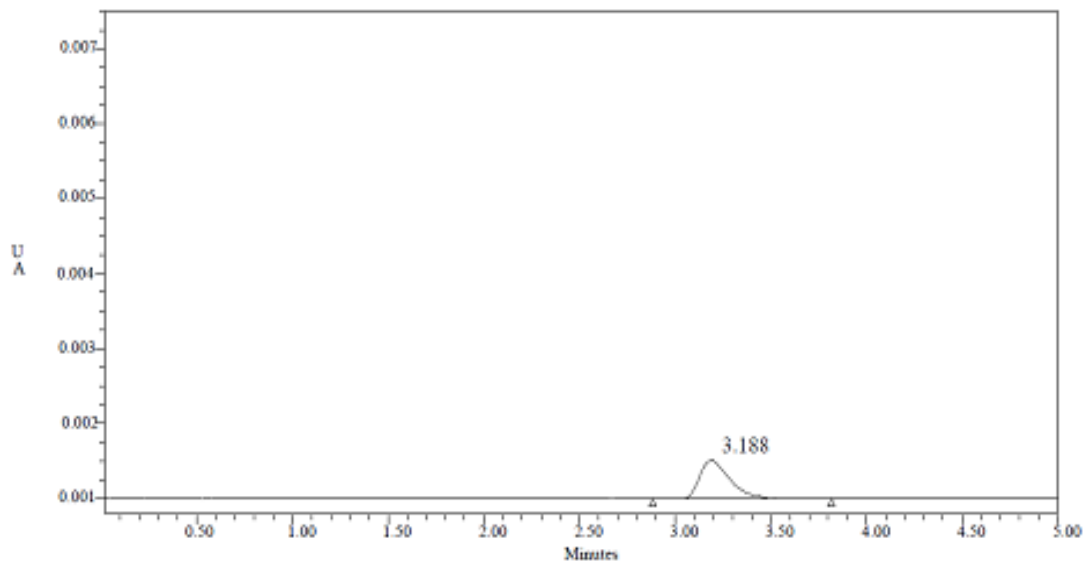
Retention Time (min)	Area (μV*sec)	Height (μV)
3.188	5277	485

Figure 37: LOQ Chromatogram

Quantification of Formulation
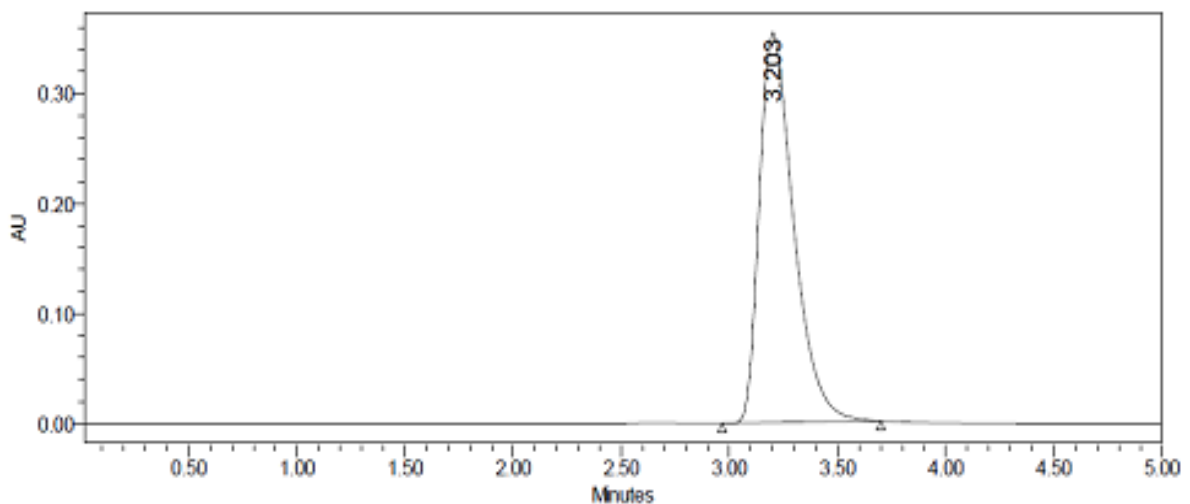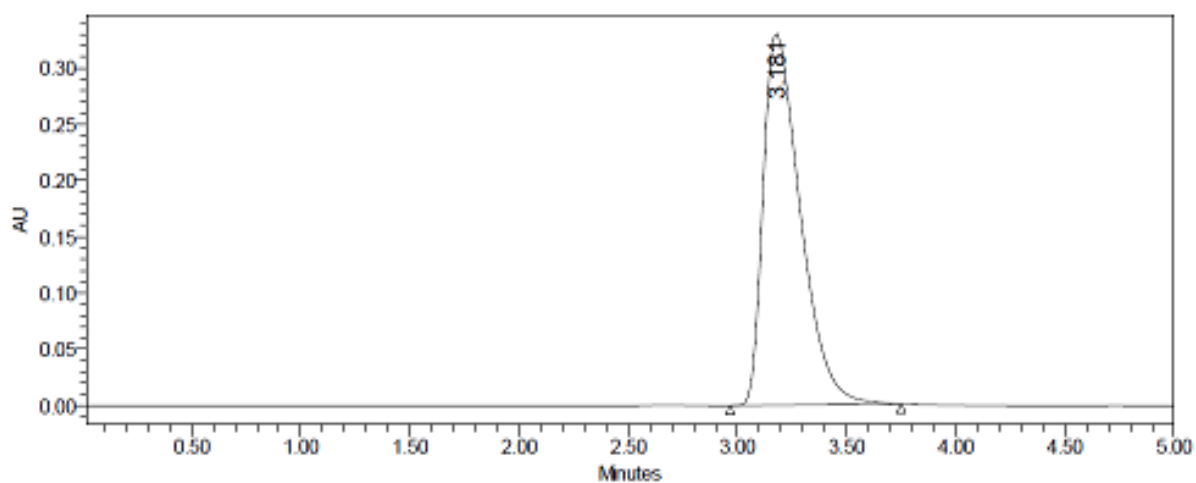
RT	Injection	Area
3.203	1	3912105
3.181	2	3907203

Figure 38: Sample Chromatogram
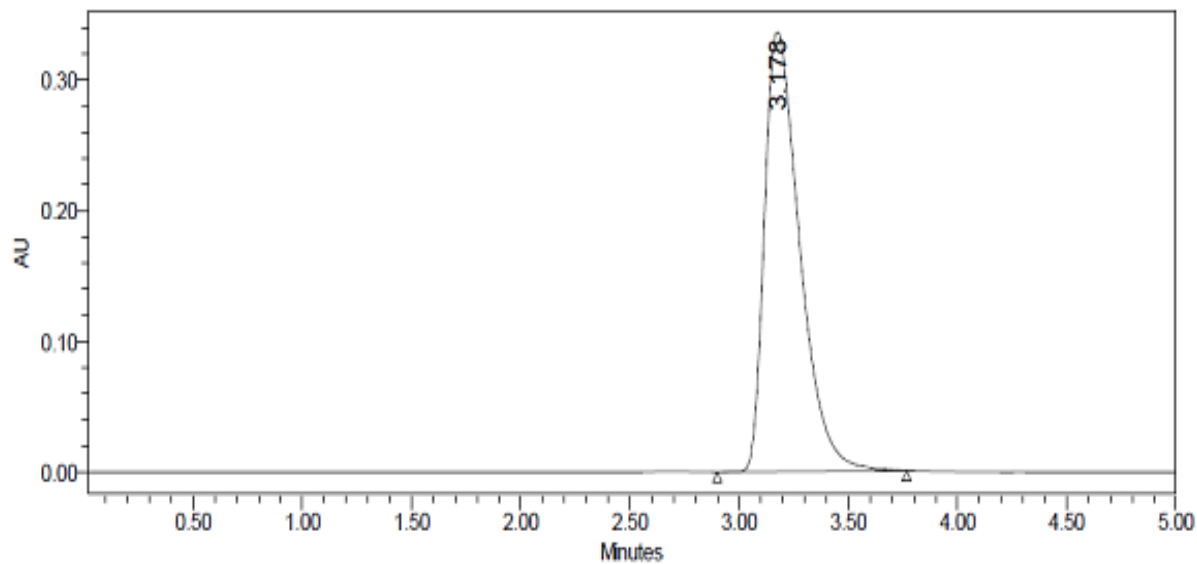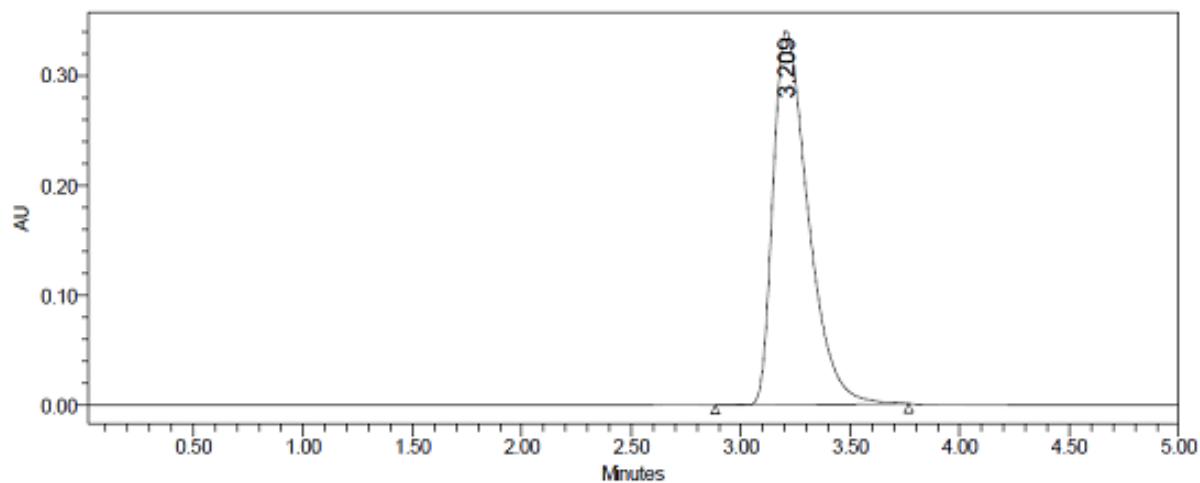
RT	Injection	Area
3.178	1	3775295
3.209	2	3778402

Figure 39: Standard Chromatogram

Table 19: Results of Quantification of formulation (Assay)

S.No	Standard Peak area	Sample Peak Area	Percentage purity (%)	Average Percentage (%)*	SD	%RSD
1	3775295	3912105	101.65	101.52	0.1767	0.1740
2	3778402	3907203	101.40			

*mean of two readings

Recovery:
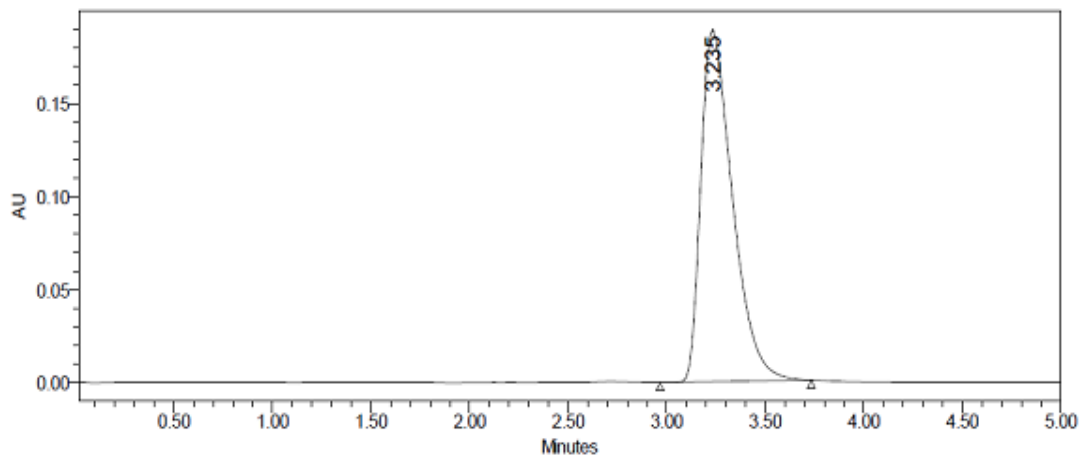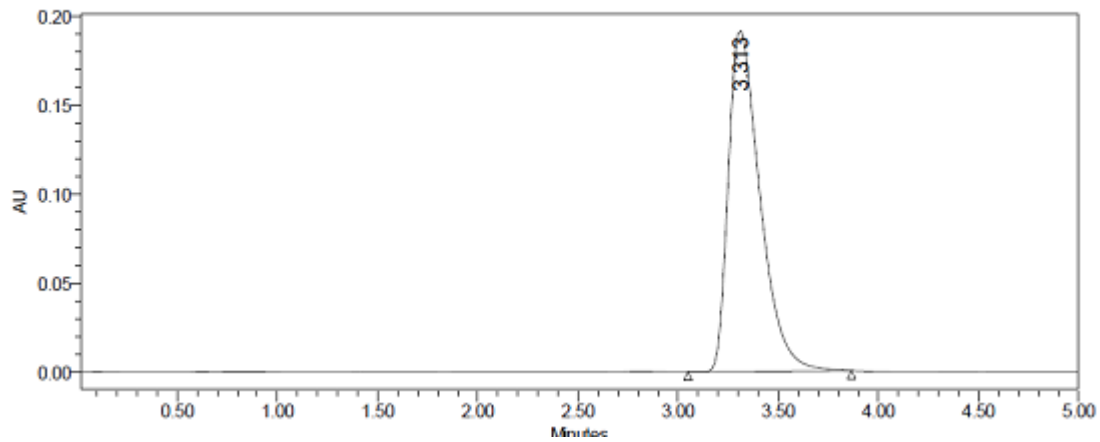
RT	Injection	Area
3.313	1	2108215
3.184	2	2109521
3.235	3	2114103

Figure 40: Recovery (Level-I) in Phosphate Buffer pH 3.5: Acetonitrile (30: 70%v/v)
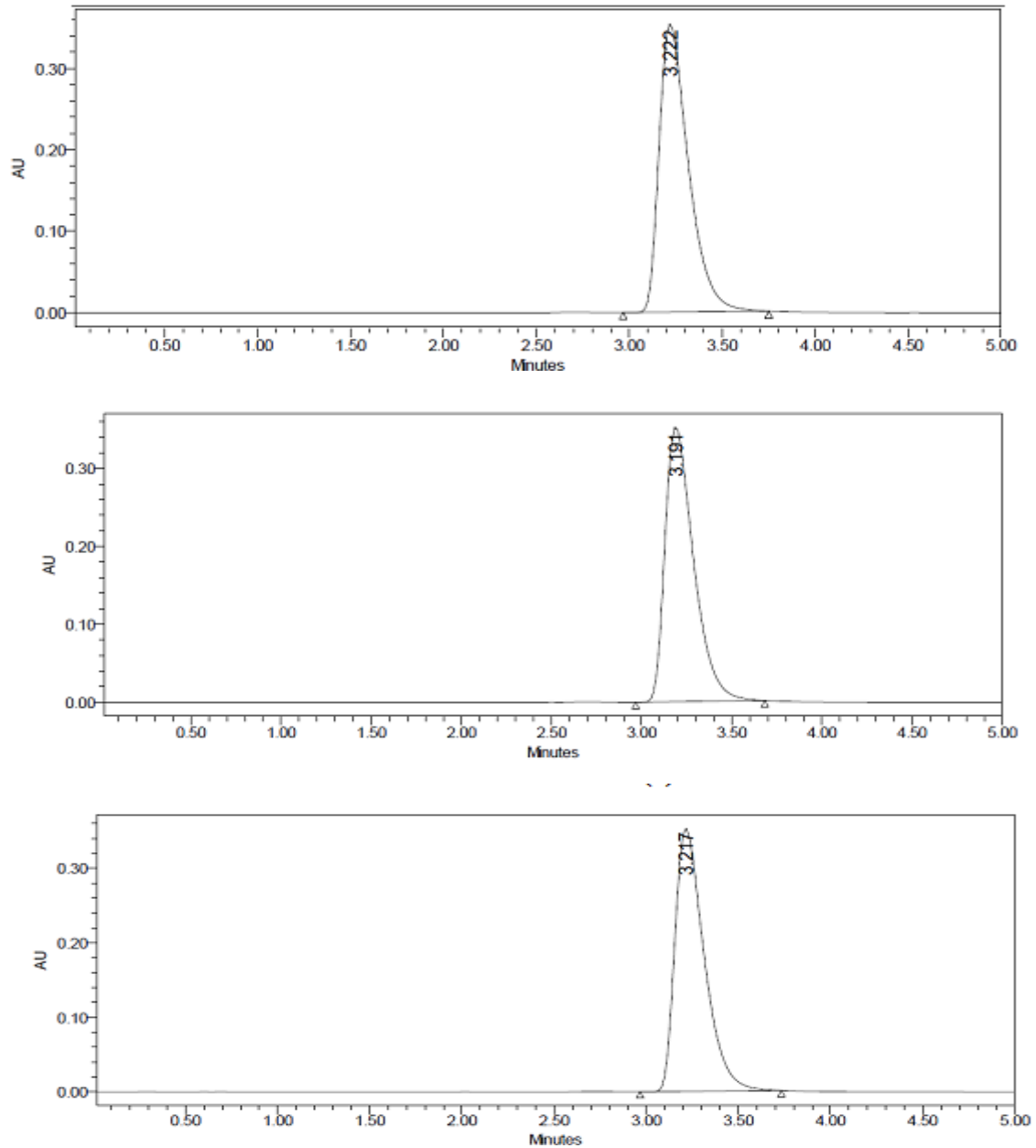
RT	Injection	Area
3.222	1	3875174
3.191	2	3888449
3.217	3	3893469

Figure 41: Recovery (Level-II) in Phosphate Buffer pH 3.5: Acetonitrile (30: 70%v/v)
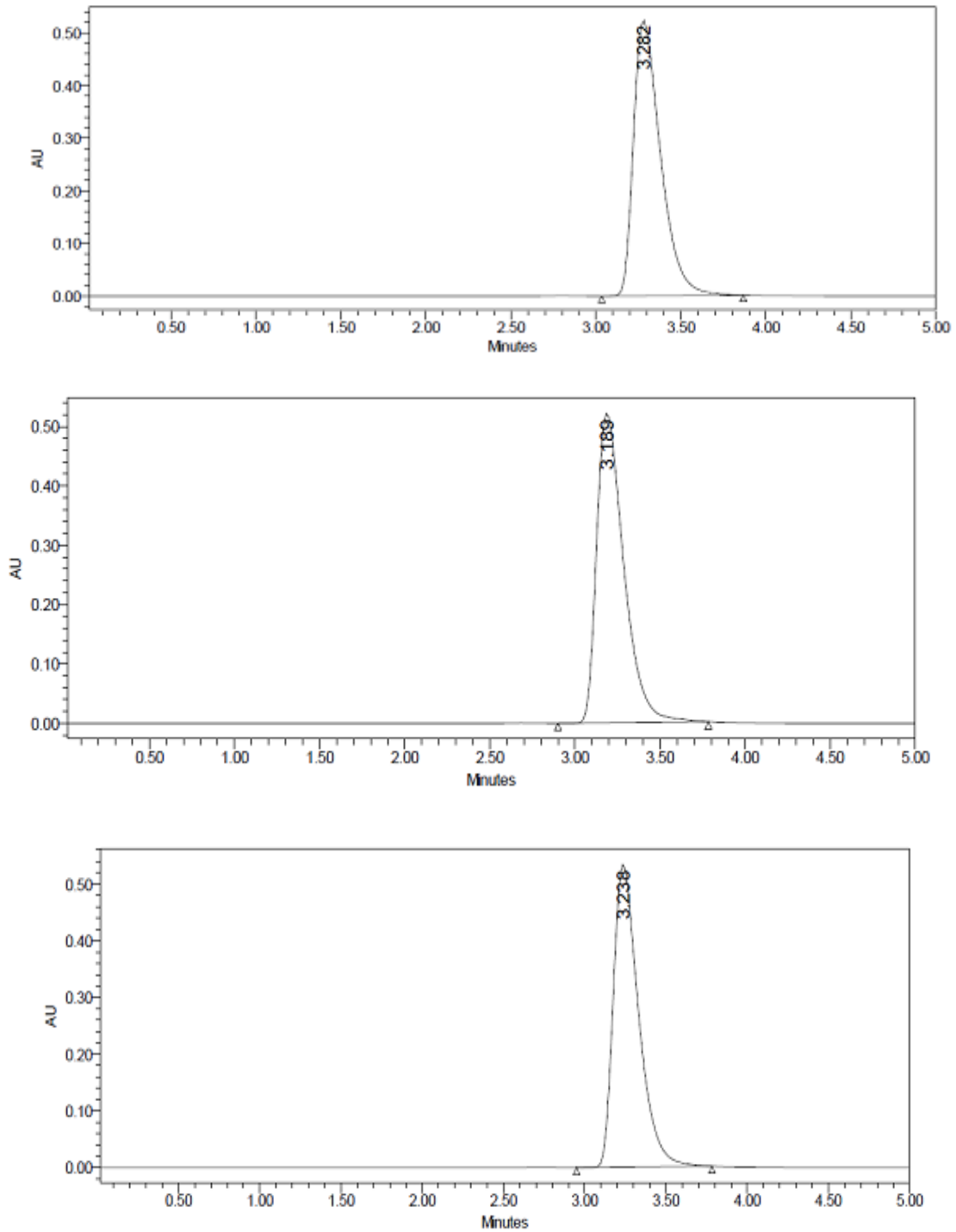
RT	Injection	Area
3.282	1	5781825
3.189	2	5779001
3.238	3	5773680

Figure 42: Recovery (Level-III) in Phosphate Buffer pH 3.5: Acetonitrile (30: 70%v/v)

Table 20: Results of Recovery Data

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery[*]	Mean Recovery
50%	2110613	5.5	5.57	101.3	
100%	3885698	10.1	10.2	101.5	101.3%
150%	5778169	15.1	15.2	101.0	

[*]mean of three readings

Precision:

% RSD was found to be 0.28
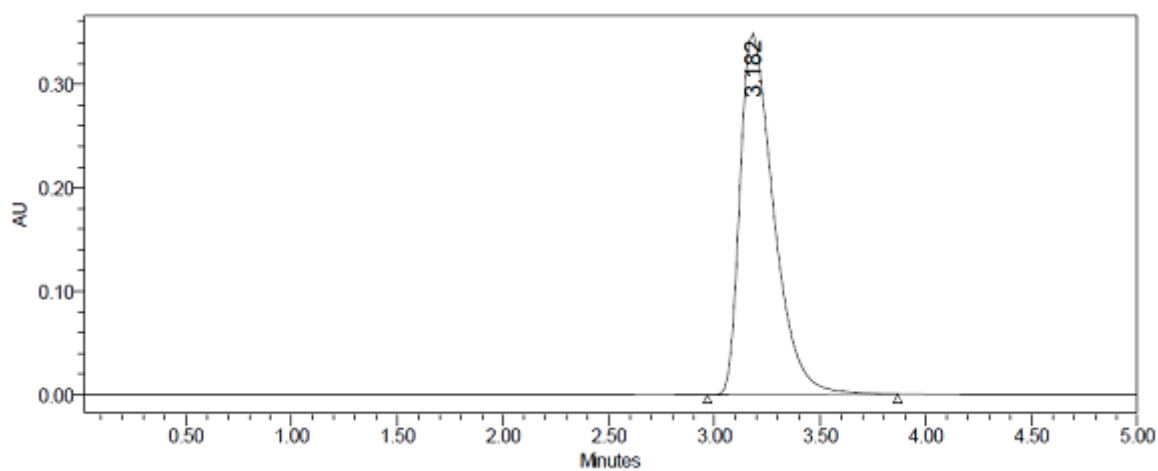
Figure 43: Precision study -1 in Phosphate Buffer pH 3.5: Acetonitrile (30: 70%v/v)
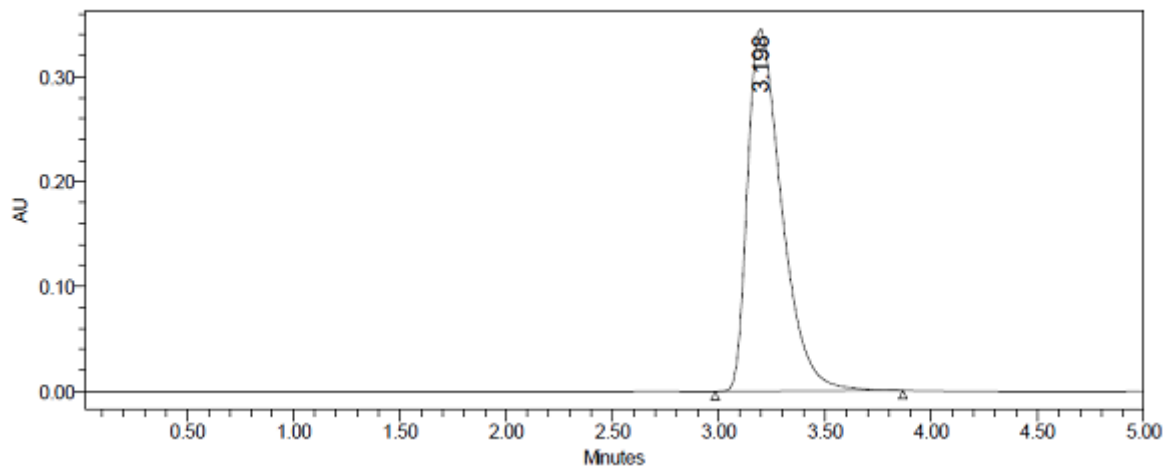
Figure 44: Precision study -2 in Phosphate Buffer pH 3.5: Acetonitrile (30: 70%v/v)
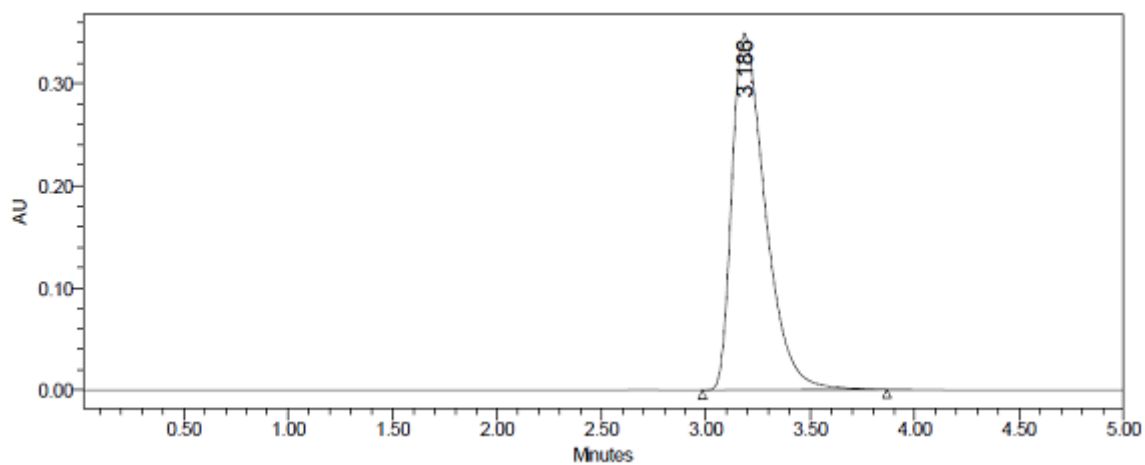
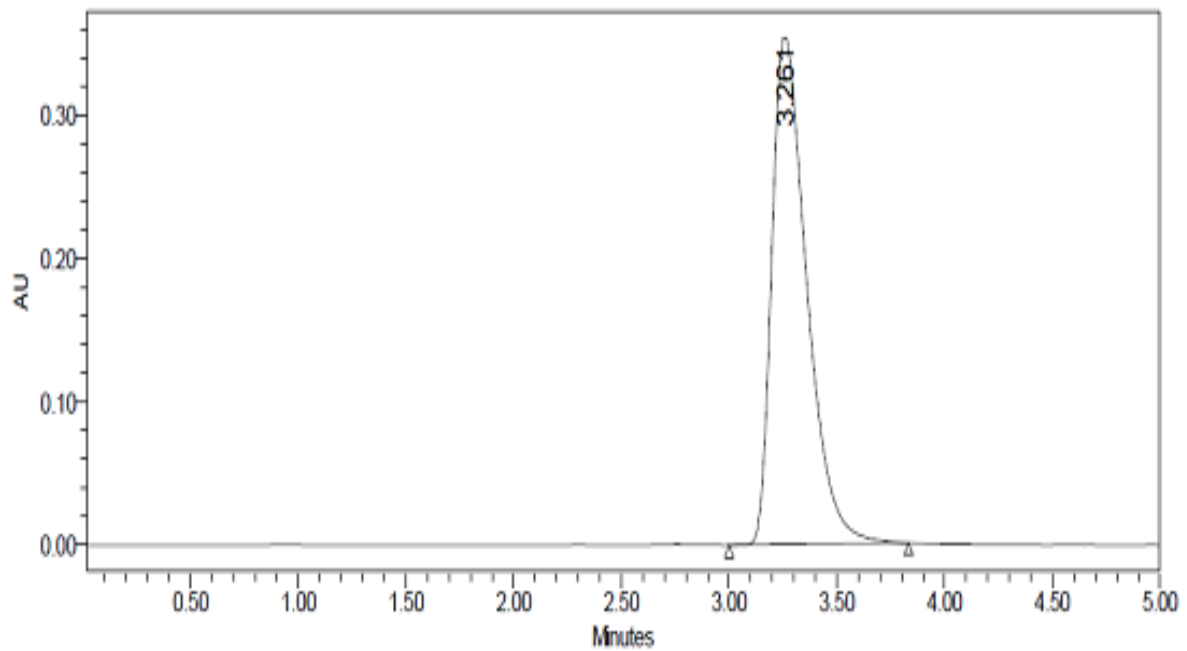**Figure 45: Precision study -3 in Phosphate Buffer pH 3.5: Acetonitrile (30: 70%v/v)**
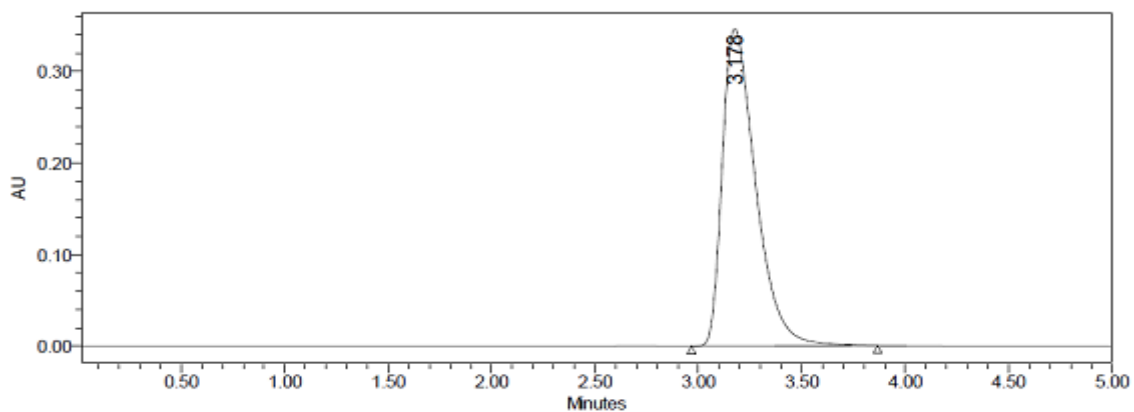
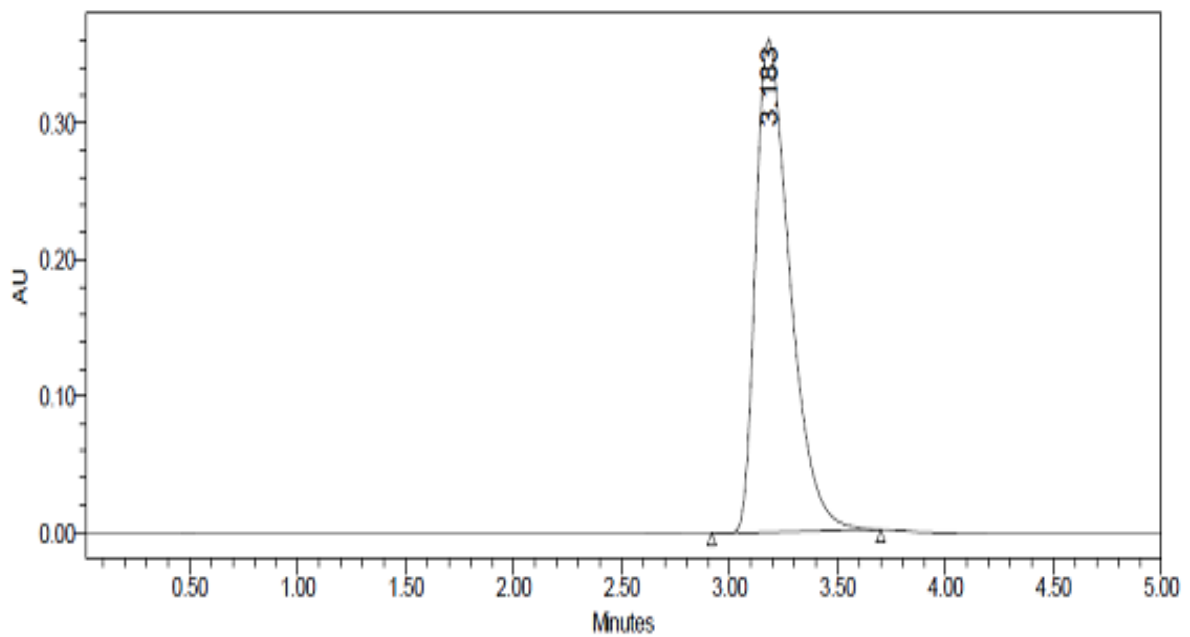**Figure 46: Precision study -4 in Phosphate Buffer pH 3.5: Acetonitrile (30: 70%v/v)**
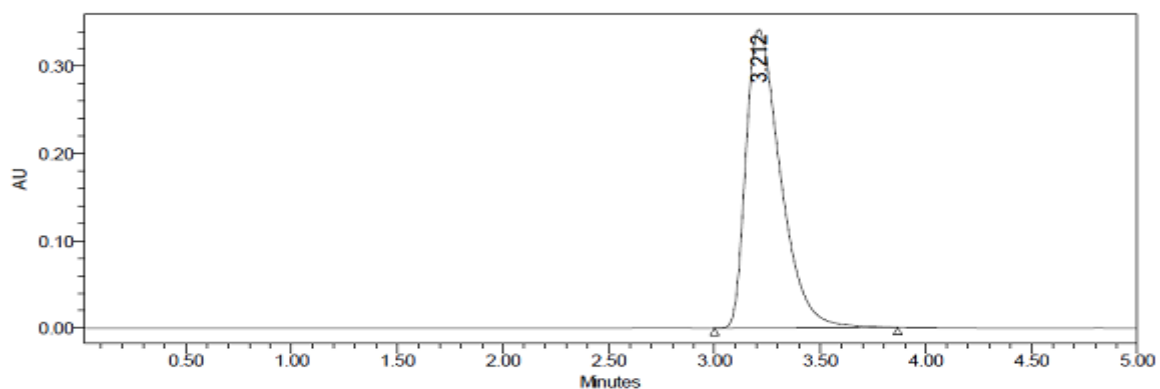
Figure 47: Precision study -5 in Phosphate Buffer pH 3.5: Acetonitrile (30: 70%v/v)

RT	Injection	Area
3.182	1	3855508
3.198	2	3865126
3.186	3	3871273
3.178	4	3878408
3.212	5	3882797

Table 21: Results of Precision Data

S.No	Concentration (µg/mL)	Peak Area	Average[*]	SD	%RSD
1		3855508			
2		3865126			
3	30	3871273	3870622	10815.8	0.28
4		3878408			
5		3882797			

[*]mean of five readings

Intermediate precision
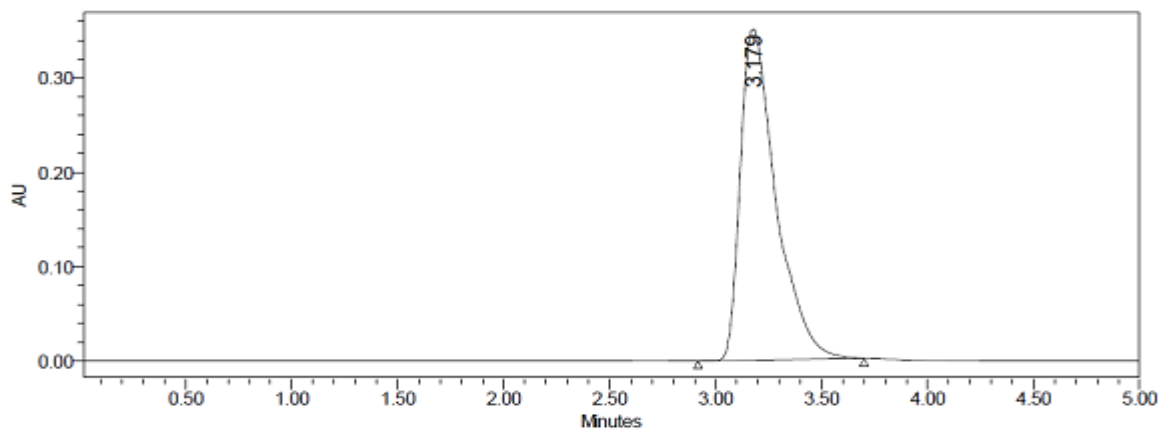
Figure 48: Intermediate precision-1 in Phosphate Buffer pH 3.5: Acetonitrile
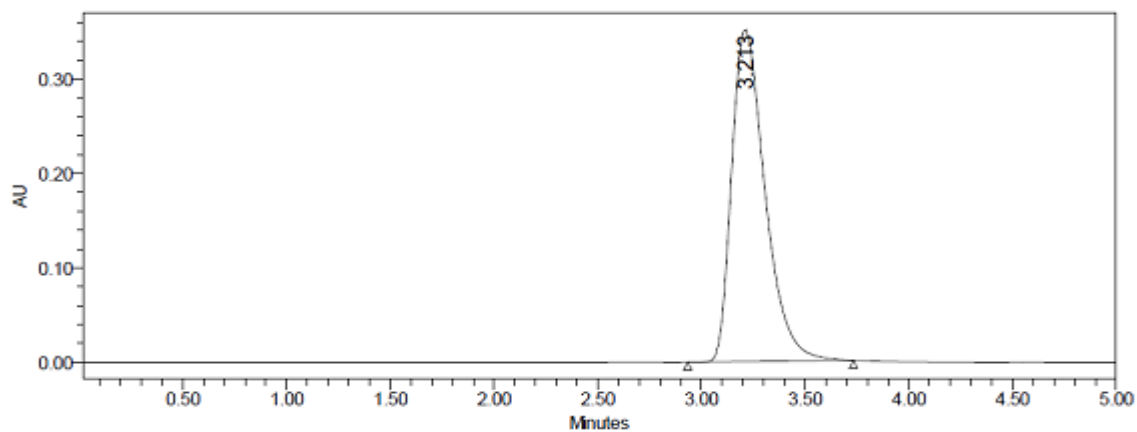
(30: 70%v/v)

Figure 49: Intermediate precision-2 in Phosphate Buffer pH 3.5: Acetonitrile
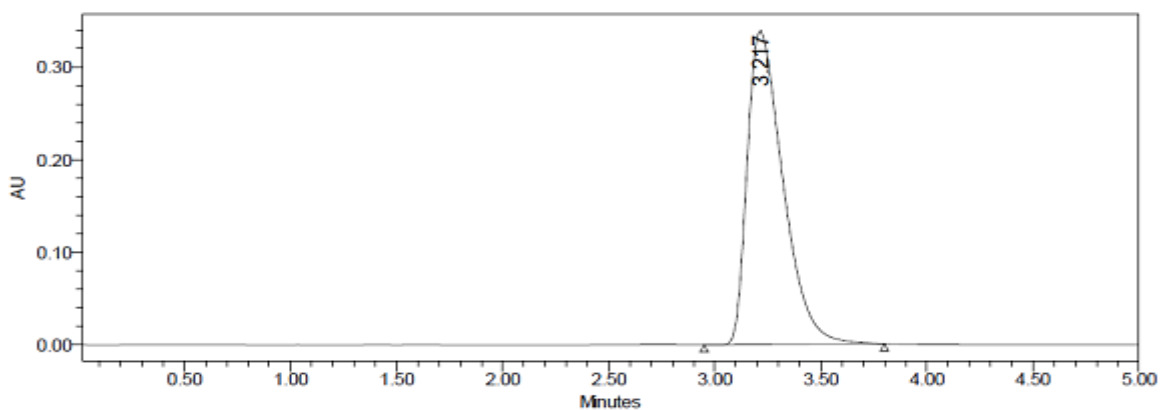
(30: 70%v/v)

Figure 50: Intermediate precision-3 in Phosphate Buffer pH 3.5: Acetonitrile

(30: 70%v/v)

Figure 51: Intermediate precision-4 in Phosphate Buffer pH 3.5: Acetonitrile

(30: 70%v/v)

Figure 52: Intermediate precision-5 in Phosphate Buffer pH 3.5: Acetonitrile

(30: 70%v/v)

RT	Injection	Area
3.179	1	4095410
3.213	2	3935121
3.217	3	3963812
3.261	4	3990300
3.183	5	3976949

Intermediate Precision

Table 22: Results of Intermediate Precision Data

S.No	Concentration (µg/mL)	Peak Area	Average[*]	SD	%RSD
1		4095410			
2		3935121			
3	30	3963812	3992318	61140.1	1.53
4		3990300			
5		3976949			

[*]mean of five readings

Robustness

It shows that there is no change in the values even after making deliberate change in the analytical procedure.
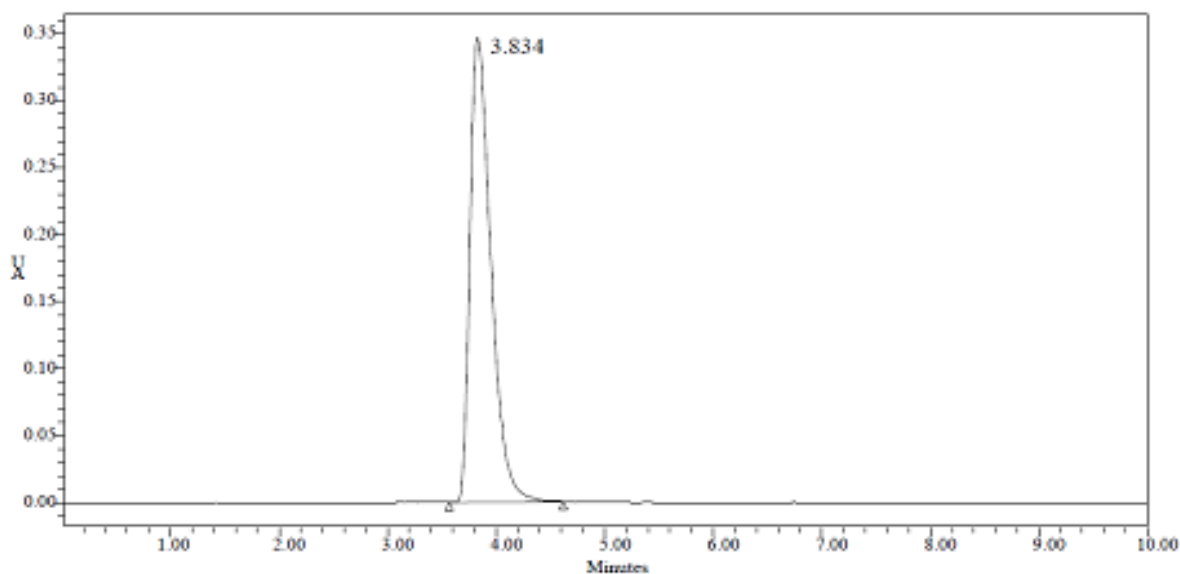
Retention Time (min)	Area (µV*sec)	USP Plate Count	USP Tailing
3.834	4694313	2889.4	1.6

Figure 53: Robustness-Less Flow in Phosphate Buffer pH 3.5: Acetonitrile (30: 70%v/v)
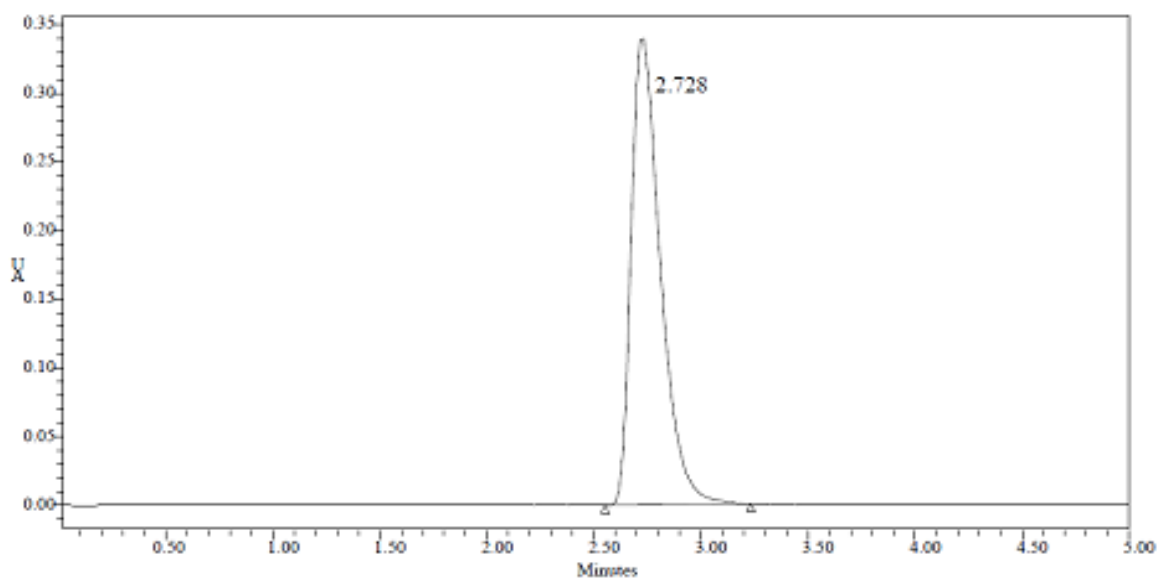
Figure 54: Robustness-More Flow in Phosphate Buffer pH 3.5: Acetonitrile (30:70%v/v)

Retention Time (min)	Area (µV*sec)	USP Plate Count	USP Tailing
2.728	3231666	2961.0	1.5
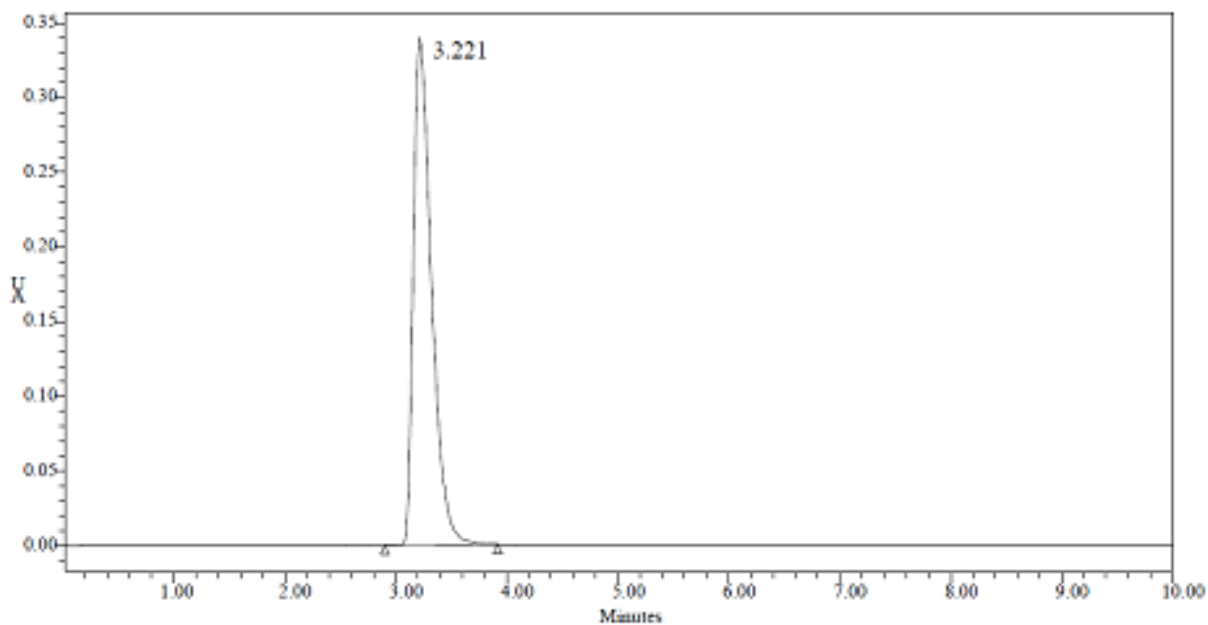
Figure 55: Robustness- Less Organic in Phosphate Buffer pH 3.5:

Acetonitrile (30: 70%v/v)
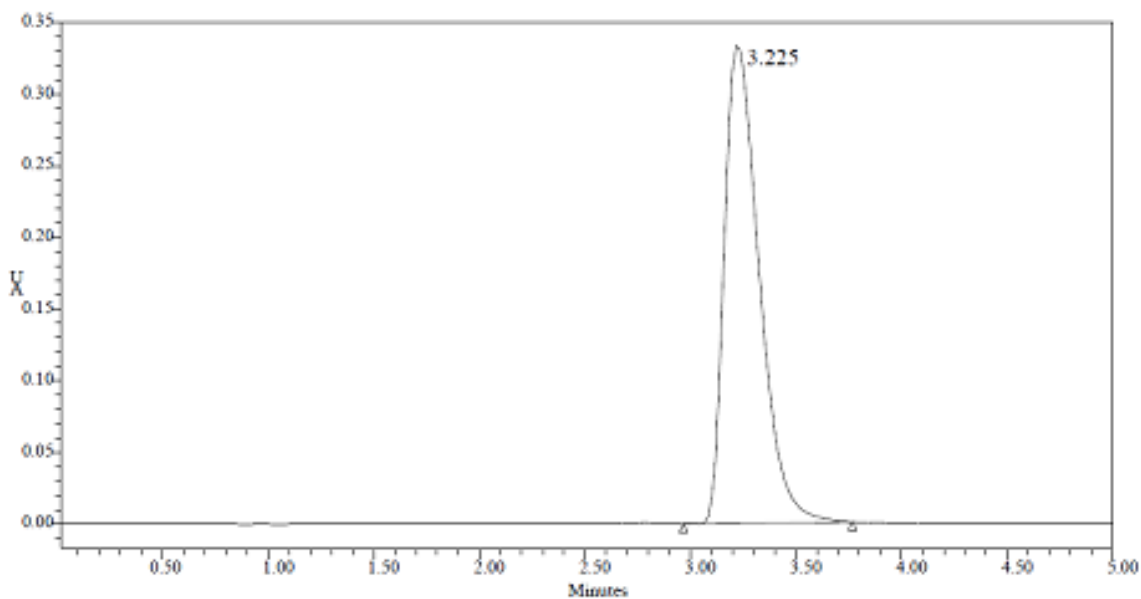
Figure 56: Robustness- More Organic in Phosphate Buffer pH 3.5:

Acetonitrile (30: 70%v/v)

Retention Time (min)	Area (µV*sec)	USP Plate Count	USP Tailing
3.225	3828751	2856.9	1.5

Table 23: Results of Robustness Data

Parameters	Theoretical plate	Tailing factor
Less flow (0.5 ml/min)	2889	1.6
More flow (0.7 ml/min)	2961	1.5
Less organic phase (60 %)	2874	1.6
More organic phase (80%)	2856	1.5

7.5 DISCUSSION

In the present work different analytical methods Viz, UV spectrophotometric method, HPLC method were developed for estimation of denaverine hydrochloride and its injection dosage form.

UV SPECTROPHOTOMETRIC METHOD

Table: 24 Optimum conditions, optical characteristics and statistical data of the regression equation in UV method

S. No.	Parameters	UV method observations
1	Absorption Maxima (nm)	221
2	Beer's range (µg/mL)	3-5
3	Regression equation (Y)* Slope (m) Intercept (c)	Y=mx+c 0.0561 0.0073
4	Correlation coefficient(R^2)	0.9997
5	Accuracy (%RSD)	100.0%
6	Precision (%RSD)	0.16
7	LOD (µg/mL)	0.7610
8	LOQ (µg/mL)	0.7613

*Y= mx + c where x is the concentration of denverine hydrochloride in µg/mL

and Yis the absorbance at the respective λ_{max}.

HPLC METHOD

Table: 25 Optimum conditions, optical characteristics and statistical data of the regression equation by the proposed RP-HPLC

S. No.	Parameters	RP-HPLC method observations
1	Absorption Maxima (nm)	306
2	Beer's range (µg/mL)	10-50
3	Mobile phase [phosphate buffer (v/v; pH: 3.5) : acetonitrile]	30:70
4	Retention time (min)	3.2
5	Regression equation (Y)* Slope (m) Intercept (c)	Y=mx+c132742 21042
6	Correlation coefficient	0.9999
7	Accuracy	101.3%
8	Precision (%RSD)	0.28
9	System suitability	1.6
10	Robustness	1.5
11	LOD (µg/mL)	0.014
12	LOQ (µg/mL)	0.0465

*Y= mx + c where x is the concentration of denverine hydrochloride in µg/mL and Y is the peak area.

Chapter-8

CONCLUSION

8. CONCLUSION

For routine analytical purpose, it is always necessary to establish methods capable of analyzing huge number of samples in a short time period with due accuracy and precision.

In this literature, the determination of Denaverine Hydrochloride in plasma was done by **HPLC and LC-MS**. So, we have planned to develop a new analytical method for Denaverine Hydrochloride Bulk drug and injectable Pharmaceutical dosage forms with sensitivity, accuracy, precision and economy.

In the present investigation, we have developed UV Spectroscopic method, and HPLC method for the quantitative estimation of Denaverine Hydrochloride in bulk drug and injectable pharmaceutical formulations. The **λmax** was determined by scanning **9 µg/mL** solution of drug in the methanol in the range of 190-400 nm and absorbance maxima observed at **221 nm**.

It obeys the beer's law in the concentration range of **3-15 µg/mL** with correlation co-efficient value of **0.9999**. The results are expressed in Tables no. 24 for UV Spectroscopic method and Tables no.25 for RP- HPLC method, are promising.

The results obtained with proposed methods confirm the suitability of these methods for injectable pharmaceutical dosage forms. The other active ingredients and excipients usually present in the pharmaceutical dosage forms did not interfere in this estimation methods.

The accuracy of the methods were confirmed by the recovery studies, by adding known amount of the formulation drug to the pure drug which is already analyzed by this method. The recovery studies for the newly developed UV Spectroscopic and HPLC methods confirms its accuracy and precision. The statistical parameter and the recovery data reveal good accuracy, linearity and precision of the methods. The %RSD for all parameter were found within specified limits.

These methods can be used for the routine analysis for determination of Denaverine Hydrochloride in bulk drug and injectable Pharmaceutical formulations.

Chapter-9

BIBLIOGRAPHY

9. BIBLIOGRAPHY

1. David Harvey., **Modern Analytical Chemistry,** 1st ed. United States of America: Harcourt Brace & Company; 1997, p. 2, 578-80.

2. Sharma BK., **Instrumental Methods of Chemical Analysis,** 13th ed. Meerut: Goel Publisher House; 2000. p.1, 7.

3. Jeffery GH, Bassett J, Mendham J, Denney RC., **Vogel's Text book of Quantitative Chemical Analysis,** 5th ed. New York: John Wily & sons, Inc.; 1989, p.7, 668-9.

4. Sethi PD., **Quantitative Analysis of Drugs in Pharmaceutical Formulations,** 3rd ed. New Delhi: CBS Publishers and Distributors; CBS Publishers and Distributors 1986, p.1- 20.

5. Willard H H., Merritt L., Dean J A., Settle F A**., Instrumental methods of Analysis, 7th edition, CBS Publishers & Distributors,** New Delhi, 1986: 580-600.

6. Chatwal G R., Anand S K., **Instrumental methods of Chemical Analysis,** 5th edition, Himalaya publishing house, 1986, 126-137.

7. Vogel I., **Quantitative Organic Analysis,** 2nd edition, Part-II, CBS Publishers & Distributors, New Delhi, 1987: 241-257.

8. Beckett A H., Stenlake J B., **Practical Pharmaceutical Chemistry,** 4th edition, Part-II, CBS Publishers and Distributors, New Delhi, 270-290.

9. Martindale., **The Complete Drug References,** 2009, 36- 723.

10. Mahinder Singh., **Analytical Chemistry,** Dominant publishers and distributors, New Delhi, 2002: 56-68. 13.

11. Synder K L., Krikland J J., Glajch J L., **Practical HPLC Method Development,** 2nd edition, Wiley Interscience Publication, USA, 1983: 174-188.

12. Aushtosh Kar., **Pharmaceutical Drug Analysis,** 2nd edition, New Age International Publishers, New Delhi, 2005, 85-90.

13. Skoog., West., Holler., Creuch., **Fundamentals of Analytical Chemistry,** 8th edition, Estern Press Pvt.Ltd, Bangalore, 2004:20.

14. Ewing W G., **Instrumental methods of Chemical Analysis,** 5th edition, McGraw-Hill Book company, Singapore, 1985: 41-68.

15. Satinder Ahuja., Stephen Scypinski., **Handbook of Modern Pharmaceutical Analysis, Academic Press,** USA, 2001: 510-520.

16. Gurdeep R. chatwal., **Instrumental methods of chemical analysis,** Page. No 2.116-2.122

17. Y R Shrama., **Elementary organic analysis,** Principles and chemical applications, Page.No 12-14.

18. kasturi A V., **A textbook of pharmaceutical analysis,** Vol 3 10th ed, 169-81

19. Skoog, Holler, Crouch., **Instrumental Analysis.,** Eighth Indian Reprint 2011, Page No: 191, 392-407.

20. http://www2.chemistry.msu.edu/faculty/reusch/VirtTxtJml/Spectrpy/UVVis/spectrum.html

21. http://en.wikipedia.org/wiki/Ultraviolet %E2%80%93visible spectroscopy

22. http://teaching.shu.ac.uk/hwb/chemistry/tut orials/molspec/uvvisab1.html

23. http://www.indiastudychannel.com/resources/146681-Principle-working-and-applications-of-UV-spectroscopy.aspx

24. https://www.slideshare.net/AlexaJacob1/uv-visible-spectroscopy-ppt.

25. https://www.slideshare.net/manishpharm a/application-of-uv-spectroscopy.

26. https://medium.com/@ankur1857/princip   le-of-ultra-violet-uv-spectrophotometer e6a1c435d258.

27. https://chem.libretexts.org

28. https://en.wikipedia.org/wiki/Infrared_spectroscopy

29. https://www2.chemistry.msu.edu/faculty/reusch/virttxtjml/spectrpy/infrared/infrared.html.

30. http://www.wag.caltech.edu/home/jang/ genchem/infrared.html.

31. https://web.vscht.cz/~poustkaj/EN A SFA AU Koplik_Infrared_spectroscopy.pdf

32. https://www.pharmatutor.org/pharmaceuticalanalysis/analytical-aspects-of-infra-red spectroscopy-ir/instrumentation-of-ir spectrophotometry

33. Gurdeep R. Chatwal, Sham K. Anand., **Instrumental Method of Chemical Analysis,** Himalaya Publishing House, 2003, p. 2.624 to 2.638

34. PD Sethi, Quantitative Analysis of Pharmaceutical Preparations Douglas A. Skoog, Instrumental Analysis, Books/Cole,2007, p. 897 to 899

35. Dr. S. Ravi Shankar., **Textbook of Pharmaceutical Analysis,** Rx Publications, 2005, p. 18-1 T0 18-11

36. Robert D. Braun., **Introduction Instrumental Analysis Pharma Book Syndicate,** 2006, p. 860 to 863

37. https://www.shodex.com/en/kouza/a.html

38. https://www.alphacrom.com/en/hplc-basics

39. https://laboratoryinfo.com/hplc

40. https://sciencing.com/disadvantages-advantages-hplc-5911530.html

41. https://www.slideshare.net/krakeshguptha/hplc-26970638

42. https://www.chemguide.co.uk/analysis/chromatography/hplc.html

43. https://www.azom.com/article.aspx?ArticleID=8468

44. https://www.ru.ac.za/media/rhodesuniversity/content/nanotechnology/documents/chromatography%20Augustus.pdf

45. Guidance for Industry. **Analytical Procedures and Methods Validation: Chemistry**, **Manufacturing and Controls Documentation:** Guidance Rockville, MD. US FDA. 2000

46. International Conference on Harmonization (ICH), **Q2 (RI), "Validation of Analytical Procedures: Text and methodology"** 2000.

47. International Conference on Harmonization**, "Q2A: Text on Validation of Analytical Procedures,"** Federal Register, 1995; 60(40): 11260-11262.

48. International Conference on Harmonization, **"Q2B: Validation of Analytical Procedures: Methodology; Availability,"** Federal Register 1997; 62(96): 27463-27467.

49. Validation requirements of the U.S Food and Drug administration, The U.S Pharmacopoeia and International Conference on Harmonization*. J Cromatogr A*. 2003; 987: 57-66.

50. Sidney H. Willing and James R. Stokar; **"Good Manufacturing Practices for Total Quality Control";** Fourth Edition, Revised and expanded; Marcel Dekker Inc. Pg. no 141-143.

51. World Health Organization Geneva "**Quality assurance of Pharmaceutical - A Compendium of guidelines and related materials"**; Pharma Book Syndicate: Volume -1, 119-123.

52. ICH Harmonized Tripartite Guideline **on validation of Analytical Procedures: Q2(R1),** November 2005.

53. H. Ḧuller,W. Scheler, E. Schulz, **Acta Biol. Med.** German. 12 (1964) 682.

54. H. Ḧuller, W. Scheler, H. Oberender, R. Peters, **Acta Biol. Med**. German. 22 (1969) 751.

55. H. Ḧuller, Zbl. **Pharmaz**. 109 (1970) 115.

56. V. Bredow, Zbl. **Gynakol.** 114 (1992) 551.

57. E. Neumayer, **Medicamentum** 16 (1975) 264.

58. B. Vesper, **Medicamentum** 12 (1971) 335.

59. K.Lange, W. Heuwieser, C. Fischer-Tenhagen, Influence of denaverine hydrochloride on calving ease in Holstein-Friesian heifers, **Journal of Dairy Science, volume** 102, issue 6, P5410-5418, 2019.

60. Katrin Lange, Wolfgang, Heuwieser, Carola Fischer-Tenhagen, Effect of denaverine hydrochloride application to heifers on the APGAR score and lactate concentration in newborn calves, 2018; 46(03): 150-153

61. Robert Zobel, Juhani Taponen 2014, denaverine hydrochloride and carbetocin increased welfare during and after parturition and enhanced subsequent fertility in cattle, **Journal of Dairy Science**, volume 97, issue 6, P 3570-3577, 2014

62. Jinmin Miao, Haibo Ge, Palladium-catalyzed chemo selective decarboxylative ortho acylation of benzoic acids with α-oxocarboxylic acids Org Lett, **ACS publication** 15(12):2930-3,2013

63. J Künzel 1, K Geisler, I Hoffmann, A Müller, M W Beckmann, R Dittrich, Myometrial response to neurotropic and musculotropic spasmolytic drugs in an extracorporeal perfusion model of swine uteri, **Reproductive Biomedicine Online** volume 23, issue 1, p132-140, 2011.

64. B. Kilian, R. Oertel , W. Kirch, Determination of denaverine and its metabolites in urine samples by liquid chromatography–tandem mass spectrometry, **Journal of Chromatography A,** 1189 (2008) 339–346

65. Alexander Staab, Barbar S,Schug ,Ve´ronique Larsimont ,Martina Elze , Daniel Thu¨mmler , Ernst Mutschler , Henning Blume, Pharmacokinetics and bioavailability of denaverine hydrochloride in healthy subjects following intravenous, oral and rectal single doses, **European Journal of Pharmaceutical Sciences**, 18 (2003) 121–128.

66. Alexander Staab , Steffen Scheithauer, Hiltrud Fieger-Bu¨schges, Ernst Mutschler, Henning Blume, A validated high-performance liquid chromatographic assay for the simultaneous determination of denaverine and its N-monodemethyl metabolite in human plasma, **Journal of Chromatography B,** 751 (2001) 221–228.

67. A. Staab Scheithauser, E. Mutschler, H. Blume, hplc determina'i1on of denaverine and propiverine in human plasma, **European Journal of Pharmaceutical Sciences**, 2 (1994) 117-194

68. V Bredow, Use of tramadol versus pethidine versus denaverine suppositories in labor--a contribution to noninvasive therapy of labor pain**, Zentralbl Gynakol**, 1992;114(11):551-4.

69. B Göber, H Lisowski, D Friese, P Franke, Biotransformation of denaverin (Spasmalgan) in the rat, **Pharmazie**, 1988 Jul;43(7):493-5.

70. H H Borchert, H Pipping, S Pfeifer, Metabolic interactions of denaverine, **Pharmazie**,1985 Oct;40(10):720-2.

ANNEXURE

ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF ESTIMATION OF DENAVERINE HYDROCHLORIDE IN BULK AND INJECTABLE PHARMACEUTICAL DOSAGE FORM BY UV-SPECTROPHOTOMETRIC METHOD

Dr. G. Krishnamoorthy

Head, Department of Pharmaceutical Analysis, Periyar College of Pharmaceutical Sciences, Tiruchirappalli, Tamil Nadu, India.
E-Mail. Id: vkm292011@hotmail.com

Mr. J. Ijaz Ahamed

Post graduate student, Department of Pharmaceutical Analysis, Periyar College of Pharmaceutical Sciences, Tiruchirappalli, Tamil Nadu, India.
E-Mail. Id: ijazvkr@gmail.com

Miss. S. Ramya

Post graduate student, Department of Pharmaceutical Analysis. Periyar College of Pharmaceutical Sciences, Tiruchirappalli. Tamil Nadu. India.
E-Mail. Id: ramyashanthi225@gmail.com

Mr. M. Sivaganesh

Post graduate student, Department of Pharmaceutical Analysis, Periyar College of Pharmaceutical Sciences, Tiruchirappalli, Tamil Nadu, India.
E-Mail. Id: sivaganesh2761@gmail.com

Dr. R. Senthamarai

Principal. Department of Pharmacognosy, Periyar College of Pharmaceutical Sciences, Tiruchirappalli, Tamil Nadu, India.
E-Mail. Id: periyarcps@gmail.com

Corresponding Author:

Dr. G. Krishnamoorthy, Head, Department of Pharmaceutical Analysis, Periyar College of Pharmaceutical Sciences, Tiruchirappalli, Tamil Nadu, India.
E-Mail. Id: vkm292011@hotmail.com Ph: 9843482447

Abstract:

It has been accomplished the development of a simple and rapid UV-spectrophotometric method to estimate for the evaluation of Denaverine hydrochloride, which is used in the treatment of antispasmodic drug in Veterinary medicine. The present study describes the method developed a simple and reproducible. UV-Visible spectrophotometric method for the quantitative determination of Denaverine hydrochloride. A UV-Visible spectrophotometric method with 1cm quartz cells and distilled Methanol solvent was employed. The proposed method was successfully applied for the estimation of Denaverine hydrochloride in commercial pharmaceutical preparation with UV detection at 221 nm. It obey's Beer law in the concentration range of 3 to 15µg/mL having correlation coefficient of 0.9997, Percentage recovery (100.0%). In terms of linearity, precision, accuracy, recovery, limit of detection (LOD), and limit of quantitation (LOQ), the developed technique was within limit as per ICH guidelines.

Key words: Denaverine hydrochloride, Absorbance Maxima, UV Spectrophotometry.

INTRODUCTION

Denaverine hydrochloride[1-6] is a muscle relaxant. It was developed and patented in Germany in 1974. Under the brand name Sensiblex, Denaverine hydrochloride is used in Veterinary medicine as a muscle relaxant for the myometrium of cows and dogs during parturition. Now,the drug is in trial with human plasma to treat urogenital and gastrointestinal spasms under the brand Spasmalgan.

Denaverine hydrochloride, is a neurotropic–musculotropic spasmolytic with analgesic effect. It's used to treat gastrointestinal and urogenital smooth muscle spasms, as well as postoperative abdominal pain and obstetrics. Despite the fact that denaverine hydrochloride has been used successfully in therapy for over 30 years, there was little information on its biotransformation in humans.

Structure

Fig. 1 Chemical Structure of Denaverine hydrochloride

Chemical name: 2-dimethylaminoethyl2-(2-ethylbutoxy)-2,2diphenylacetate hydrochloride

Mechanisms of Action

Denaverine inhibits the enzyme phosphodiesterase. A phosphodiesterase inhibitor is a drug that inhibits the inactivation of the intracellular second messengers cyclic Adenosine MonoPhosphate (cAMP) and cyclic Guanosine MonoPhosphate (cGMP) by one or more of the five subtypes of the enzyme PhosphoDiEsterase (PDE). It has anticholinergic properties. Anticholinergics (anticholinergic agents) are a class of drugs that prevent the neurotransmitter acetylcholine (ACh) from acting at synapses in the central and peripheral nervous systems.

MATERIALS AND METHODS:

Instrument: A Shimadzu UV-1600 UV/VIS Spectrophotometer was used with 1 cm matched quartz cell.

Materials and Reagents:

All the chemicals used were of analytical grade. An analytically pure sample of Denaverine hydrochloride was procured as gift sample from HITECH LABORATORY,Chennai.

Preparation of Solution

Standard Solution Preparation

Accurately weighed and transfered 10 mg of Denaverine working standard into a 10 mL volumetric flask. About 7 mL of methanol was added and sonicate to dissolve it completely. Then the solution was made volume up to the mark with the same solvent (1000 μg/mL). Further 1.0 mL of the Denaverine stock solution was pipette out into a 10 mL volumetric flask and dilute up to the mark with solvent (100μg/mL). Further 0.9 mL of the Denaverine stock solution was pipette out into a 10 mL volumetric flask and dilute up to the mark with solvent (9μg/mL).

Sample Solution Preparation

0.25mL of injection (Sensiblex 40mg/mL) was measured accurately and transfered the sample (equivalent to 10 mg of Denaverine hydrochloride) into a 10 mL volumetric flask. 7 mL of methanol was added and sonicate to dissolve it completely. Then it was made volume up to the mark with same solvent (1000 μg/mL). The solution was mixed well and filter through 0.45 μm filter. Further pipette 1 mL of the Denaverine solution into a 10 mL volumetric flask and dilute up to the mark with solvent (100μg/mL). Further pipette 0.9 mL of the Denaverine stock solution into a 10 mL volumetric flask and dilute up to the mark with solvent (9 μg/mL).The sample solution (9 μg/mL) absorbance was measured against blank at 221nm. Percentage purity was calculated by using the following formula.

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

Method development and validation

Linearity:

From the stock solution, the aliquots of stock solution of Denaverine (1000 µg/mL) further dilution make up to (0.3 to 1.5 mL of 1000 µg/mL) and transferred in to 100 mL volumetric flask and made up to the mark with solvent. The absorbance of the solution of different concentration were measured at 221 nm against blank. The calibration curve was plotted using Concentration Vs Absorbance. The curve obtained was linear with concentration range 3-15 µg/mL.

Recovery:

0.25mL of injection (Sensiblex 40mg/mL) was measured accurately and transferred the sample (equivalent to 10 mg of Denaverine hydrochloride) into three separate 10 mL volumetric flask. Then 5mg, 10mg and 15mg (50%, 100%, 150%) of standard were accurately weighed and added. 7 mL of methanol was added and sonicate to dissolve it completely. Then the solution was made volume up to the mark with the same solvent. 0.9 mL was pipette out from each flask and transferred to separate 100 mL volumetric flask. Then the solution was made volume up to the mark with the same solvent. Absorbance was measured at 221 nm against blank. The amount of drug recovered was calculated by using slope and intercept values.

Precision:

Repeatability and intermediate precision studies were done to the precision of the method. Repeatability studies were done by consequently measuring the absorbance of standard solution (9µg/mL). These solutions were prepared in duplicate and absorbance were measured at 221 nm against blank and calculate the % RSD.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ were estimated from the set of s calibration curves used to determine method linearity.

LOD= 3.3 σ /S and LOQ=10* σ /S

Where, σ= the standard deviation of y-intercepts of regression lines.

S the slope of the calibration curve.

RESULTS AND DISCUSSION

- λ Max was observed at 221nm
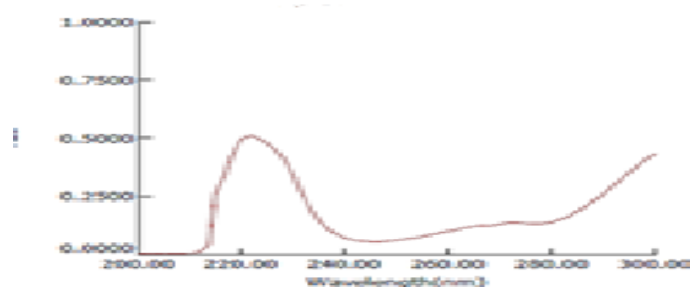
Fig. 2 **Spectrum of Denaverine hydrochloride**

Linearity:

The Linearity for Denaverine hydrochloride was found to be concentration range 3-15 µg/mL with Correlation Coefficient 0.9997. LOD and LOQ were found to be 0.216 µg/mL and 0.655 µg/mL. Calibration data and %RSD is shown in table 1 and curve is shown in figure 8.
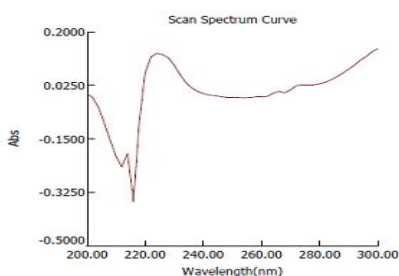
Fig. 3 Linearity Spectrum (3µg/mL)
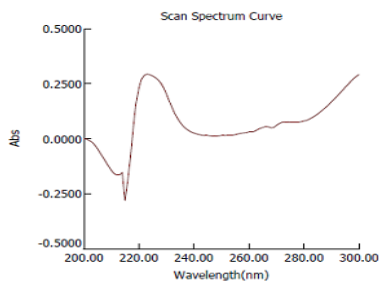
Fig. 4 Linearity Spectrum (6µg/mL)
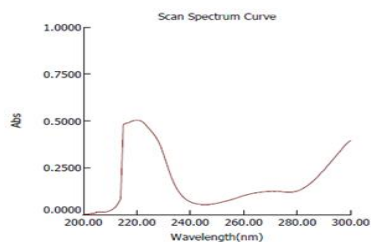
Fig. 5 Linearity (9µg/mL)
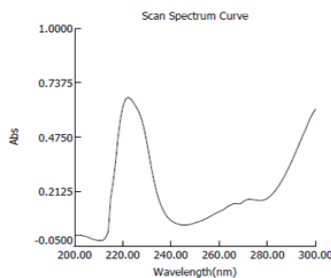
Fig. 6   Linearity (12µg/mL)
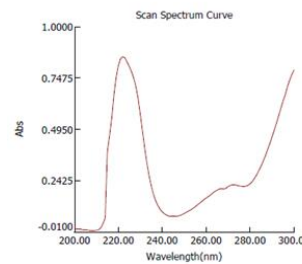
Fig. 7 Linearity (15µg/mL)

Table. 1 Results of Linearity

S. No	Concentration	Average Absorbance	Correlation Coefficient	LOD	LOQ	Slope	Intercept
1	3µg/mL	0.155					
2	6µg/mL	0.328					
3	9µg/mL	0.495	0.9997	0.216	0.655	0.056	-0.007
4	12µg/mL	0.663					
5	15µg/mL	0.840					

Fig. 8 Calibration curve for Denaverine

Quantification of Formulation (Assay)

The percentage of Denaverine in marketed formulation (Injection) was calculated from the calibration curve of Denaverine. %Assay was found to be 99.95% as shown in Table 2

Table. 2 Results of Quantification of formulation (Assay)

S.No	Standard Absorbance	Sample Absorbance	Percentage purity (%)	Average Percentage (%)	SD	%RSD
1	0.495	0.490	98.98			
2	0.482	0.485	100.62			
3	0.484	0.488	100.82	99.95	0.7610	0.7613
4	0.480	0.481	99.79			
5	0.470	0.468	99.57			

*mean of five readings.

Recovery:

The percentage recovery study for Denaverine hydrochloride was found to be 98.8-100.53.

Fig. 8 Recovery Spectrum (50 %) Fig. 9 Recovery Spectrum (100 %) Fig. 10 Recovery Spectrum (150 %)

Table. 3 Results of Recovery Data

% Concentration	Absorbance	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	0.261	5.1	5.12	100.53%	
100%	0.508	10.1	9.98	98.8%	100.0%
150%	0.769	15.0	15.10	100.7%	

*mean of three readings.

Precision:

% RSD was found to be 0.16
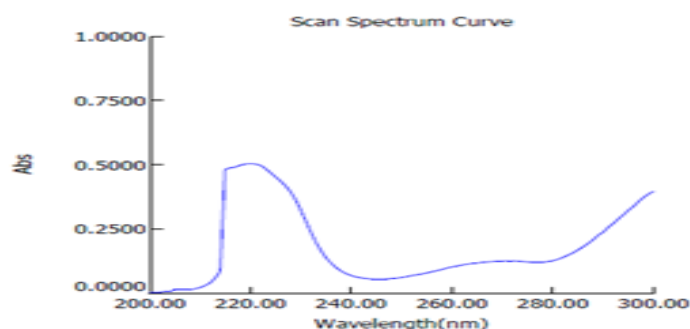
Fig. 11 Precision

Table. 4 Results of Precision Data

S. No	Absorbance	Average Absorbance	SD	%RSD
1	0.511			
2	0.511			
3	0.512	0.5118	0.0008	0.16
4	0.512			
5	0.513			

*mean of five readings.

Intermediate precision:

% RSD was found to be 0.222
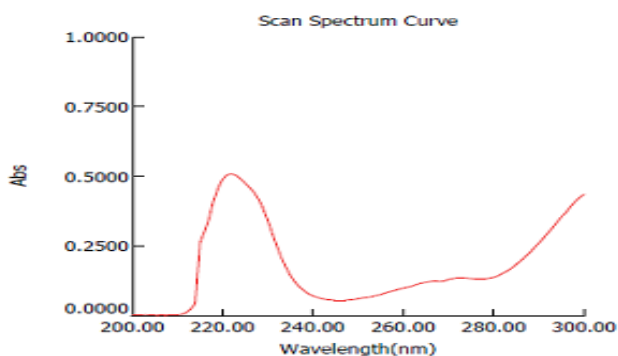
Fig. 12 Intermediate Precision Spectrum

Table. 5 Results of Intermediate Precision Data

S. No	Absorbance	Average Absorbance	SD	%RSD
1	0.510			
2	0.511			
3	0.513	0.511	0.0011	0.222
4	0.511			
5	0.512			

*mean of five readings.

Conclusion

The developed method was found to be simple, sensitive, accurate, precise, reproducible, and can be used for routine quality control analysis of Denaverine Hcl in bulk and pharmaceutical formulation.

ACKNOWLEDGEMENTS

REFERENCES

[1] H. Huller,W. Scheler, E. Schulz, Acta Biol. Med. German. 12 (1964) 682.

[2] H. Huller, W. Scheler, H. Oberender, R. Peters, Acta Biol. Med. German. 22 (1969) 751.

[3] H. H˙uller, Zbl. Pharmaz. 109 (1970) 115.

[4] V. Bredow, Zbl. Gynakol. 114 (1992) 551.

[5] E. Neumayer, Medicamentum 16 (1975) 264.

[6] B. Vesper, Medicamentum 12 (1971) 335.

ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF DENAVERINE HYDROCHLORIDE IN BULK AND INJECTABLE PHARMACEUTICAL DOSAGE FORM BY HPLC METHOD

G. Krishnamoorthy*[1], J. Ijaz Ahamed[1] , R. Senthamarai[1], A.M. Ismail[1]

[1] Department of Pharmaceutical Analysis, Periyar College of Pharmaceutical Sciences,

Tiruchirappalli, Tamil Nadu 620 021

*Corresponding Author

Prof. G. Krishnamoorthy,

Head, Department of Pharmaceutical Analysis,

Periyar College of Pharmaceutical Sciences,

Tiruchirappalli,

Tamil Nadu 620021.

Email: vkm292011@hotmail.com

Ph:9843482447

Abstract:

A new, simple, accurate, precise and rapid Reverse-Phase High Performance Liquid Chromatographic (RP-HPLC) method has been developed and subsequently validated for the estimation of Denaverine HCl in bulk and injection dosage form, which is used in the treatment of antispasmodic drug in Veterinary medicine. The proposed method is based on the separation of the drugs in reversed-phase mode using Symmetry C_{18} Column (4.6 x 150mm, 5 μm, Make : XTerra) The optimized mobile phase was disodium hydrogen phosphate buffer (pH 3.5): Acetonitrile (30:70 % v/v). The flow rate was at 0.6 mL/min and UV detection at 306 nm. The retention time was 3.2 min for Denaverine HCl. The method was validated according to ICH guidelines. It was found to be accurate and reproducible. Linearity was obtained in the concentration range of 10-50μg/mL for Denaverine HCl. Mean percent recovery of samples at each level for both drugs were found to be 101.3 % v/v for Denaverine HCl. In terms of Linearity, Precision, Accuracy, Recovery, Limit Of Detection (LOD), And Limit Of Quantitation (LOQ), the developed technique was within limit as per ICH guidelines. The proposed method can be successfully applied in the quality control of bulk and injectable pharmaceutical dosage forms.

Key words: Denaverine hydrochloride, validation, estimation , HPLC.

INTRODUCTION

Denaverine hydrochloride [1-6] is a muscle relaxant. It was developed and patented in Germany in 1974. Under the brand name Sensiblex, denaverine hydrochloride is used in veterinary medicine as a muscle relaxant for the myometrium of cows and dogs during parturition. Now,the drug is in trial with human plasma to treat urogenital and gastrointestinal spasms under the brand Spasmalgan.

Denaverine hydrochloride, is a neurotropic–musculotropic spasmolytic with analgesic effect. It's used to treat gastrointestinal and urogenital smooth muscle spasms, as well as postoperative abdominal pain and obstetrics. Despite the fact that denaverine hydrochloride has been used successfully in therapy for over 30 years, there was little information on its biotransformation in humans.

Structure:

Fig. 1 Chemical Structure of Denaverine hydrochloride

2-dimethylaminoethyl 2-(2-ethylbutoxy)-2,2 diphenylacetate hydrochloride

Mechanisms of Action:

Denaverine inhibits the enzyme phosphodiesterase. A phosphodiesterase inhibitor is a drug that inhibits the inactivation of the intracellular second messengers cyclic Adenosine MonoPhosphate (cAMP) and cyclic Guanosine MonoPhosphate (cGMP) by one or more of the five subtypes of the enzyme PhosphoDiEsterase (PDE). It has anticholinergic properties. Anticholinergics (anticholinergic agents) are a class of drugs that prevent the neurotransmitter acetylcholine (ACh) from acting at synapses in the central and peripheral nervous systems.

EXPERIMENTAL

MATERIALS AND METHODS

Instrumentation: A Shimadzu Prominence HPLC iLc2030

Materials and Reagents:

All the chemicals used were of analytical grade. An analytically pure sample of Denaverine hydrochloride was procured as gift sample from Nebulae Hitech Laboratory,Chennai.

RP-HPLC method

Preparation of Phosphate buffer

      7.0 grams of Potassium di hydrogen Phosphate was weighed and transferred into a 1000 mL beaker, dissolve and diluted to 1000 mL with HPLC water. The pH to 3.5 was adjusted with ortho phosphoric acid.

Preparation of mobile phase

      Phosphate buffer 300 mL (30%) and 700 mLof Acetonitrile HPLC (70%) was mixed well and degassing in ultrasonic water bath for 5 minutes. Filter through 0.45 μ filter under vacuum filtration.

Chromatographic Conditions

Mode of operation	:	Isocratic
Instrument	:	HPLC Waters
Detector	:	UV detector
Column	:	Symmetry C_{18} (4.6 x 150mm, 5 μm, Make: XTerra)
Temperature	:	Ambient
Flow rate	:	0.6 ml/min
Wave length	:	306 nm
Runtime	:	5 min
Sample size	:	20 μL
Mobile Phase	:	Phosphate buffer: Acetonitrile (30:70 %v/v)

Standard Solution Preparation

      Accurately weigh and transfer 10 mg of Denaverine working standard into a 10 mL volumetric flask add about 7 mL of mobile phase and sonicate to dissolve it completely and

make volume up to the mark with the same solvent (1000 µg/mL). Further 0.3 mL was pipette out (1 mg/ml) of the above stock solution into a 10 mL volumetric flask and dilute up to the mark with mobile phase (30 µg/mL). Then the solution was mixed well and filter through 0.45 µm filter.

Sample Solution Preparation

0.25mLof injection (Sensiblex 40mg/mL) was measured accurately and transfer the sample (equivalent to 10 mg of Denaverine hydrochloride) into a 10 mL volumetric flask.7 mL of mobile phase was added and sonicate to dissolve it completely. Then it was made volume up to the mark with mobile phase (1000 µg/mL). The solution was mixed well and filter through 0.45 µm filter. Further 0.3 mL was pipette out of the above stock solution into a 10 mL volumetric flask and dilute up to the mark with mobile phase (30 µg/mL). Mix well and filter through 0.45µm filter.

Method Validation

The method was validated in accordance with ICH guidelines[7-9]. The parameters such as Linearity, Accuracy, Precision, Specificity, Assay, Limit of Detection (LOD), Limit of Quantification (LOQ), Robustness, Ruggedness and Stability of the solution were assessed as per ICH guidelines.

RESULTS AND DISCUSSION

Chromatographic Conditions
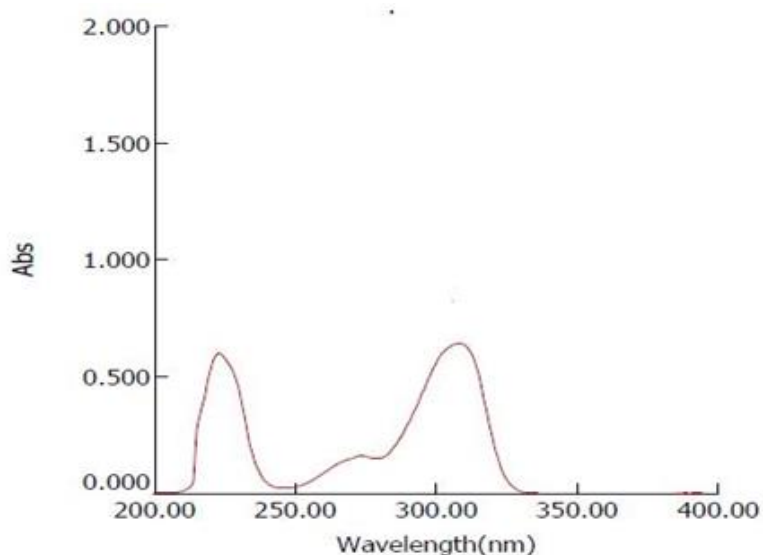
Fig 1: UV Spectrum of Denaverine In Phosphate Buffer pH 3.5:
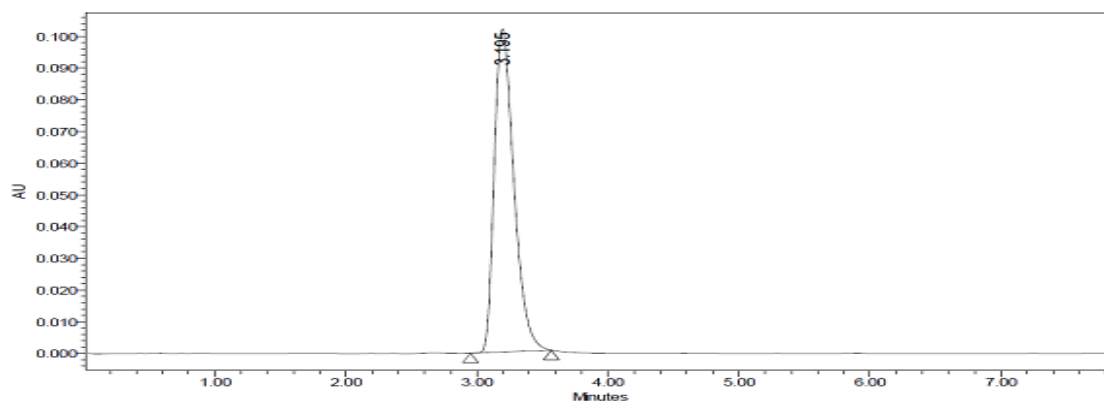
	RT	Area	Height	USP Plate Count	USP Tailing
1	3.195	1058299	102124	2178.3	1.4

Fig 2: Optimized Chromatogram Phosphate Buffer pH3.5: Acetonitrile (30: 70%V/V)

System suitability

The system suitability studies carried out as specified in ICH guidelines and USP. The parameters like tailing factor, number of theoretical plate were calculated.

Table 1: **System suitability**

Parameter	Denaverine
Tailing factor	1.6
No of Theoretical plate	2951
Retention time	3.2

Linearity:

From the stock standard (1000 µg/ml), the aliquots (0.1 to 0.5 mL of 1000 µg/mL) solution taken 0.1, 0.2, 0.3, 0.4, 0.5 mL were taken in a separate 10 mL volumetric flasks and made up to 10 mL with mobile phase (10–50 µg/mL).. This solution can inject into the chromatographic system and record the Chromatogram. The calibration graph was plotted with peak area in the Y axis and concentration of standard solution in the X axis.
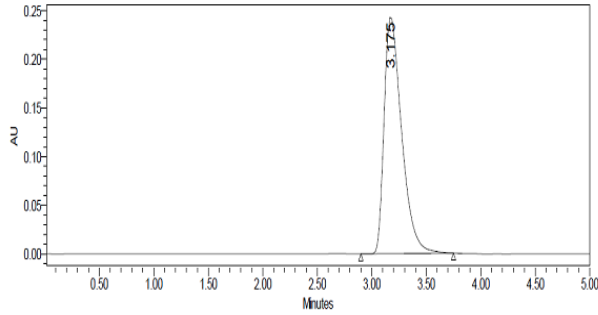
Fig 3: Linearity Chromatogram 10μg/mL
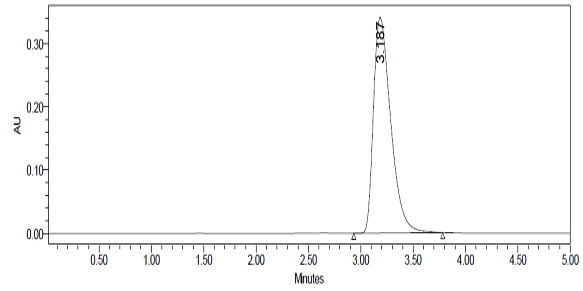
Fig 4: Linearity Chromatogram 20μg/mL
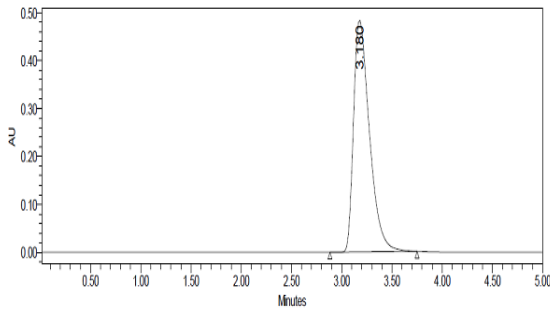
Fig 5: Linearity Chromatogram 30μg/mL
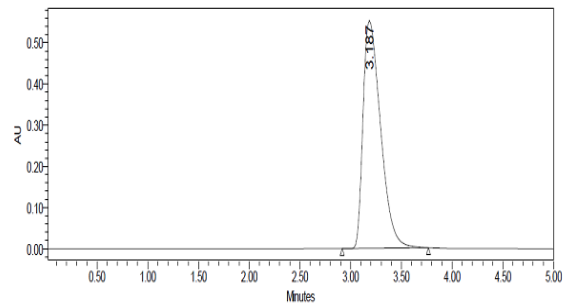
Fig 6: Linearity Chromatogram 40μg/mL
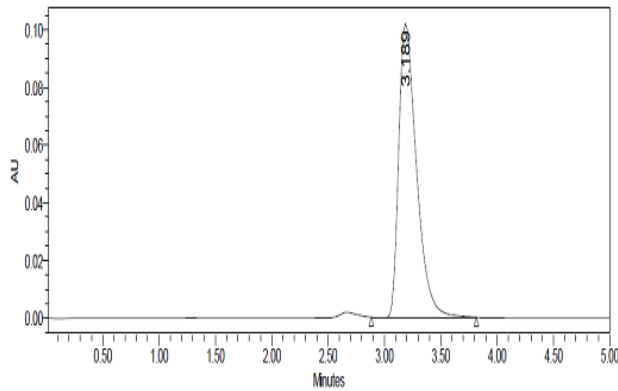
Fig 7: Linearity Chromatogram 50μg/mL

RT	Area	Height (μV)
3.189	1113634	102352
3.175	2712792	244484
3.187	3908404	342239
3.180	5328851	484112
3.187	6652686	555333

linearity -Data

Table. 2 Results of Linearity

S.No	Concentration (μg/ml)	Peak Area	LOD	LOQ
1	10	1323634		
2	20	2712792		
3	30	3998490	0.014	0.0465
4	40	5328851		
5	50	6652686		
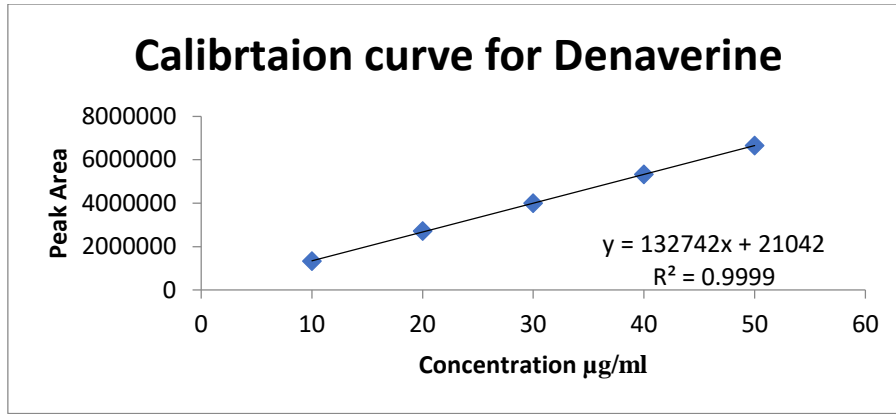
Fig 8: Calibration curve for Denaverine

LOD
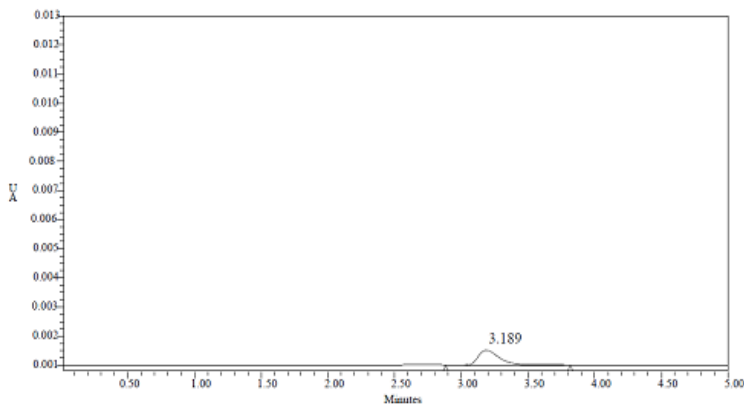
Retention Time (min)	Area (µV*sec)	Height (µV)
3.189	1545	142

Fig 9: LOD Chromatogram

LOQ
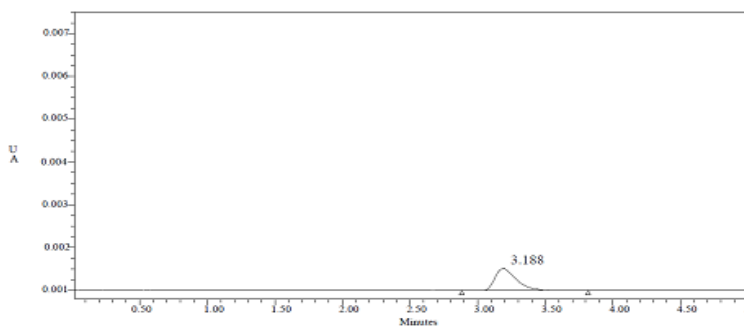
Retention Time (min)	Area (µV*sec)	Height (µV)
3.188	5277	485

Fig 10: LOQ Chromatogram

Quantification of Formulation

Fig 11: Sample Chromatogram

RT	Injection	Area
3.178	1	3775295
3.209	2	3778402
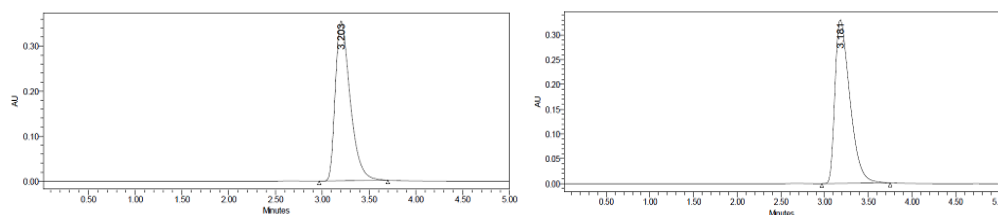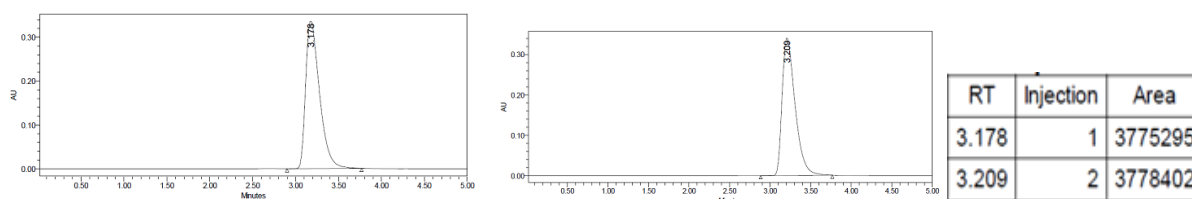
Fig 12: Standard Chromatogram

Table. 3 Results of Assay

S.No	Standard Peak area	Sample Peak Area	Percentage purity (%)	Average Percentage (%)	SD	%RSD
1	3775295	3912105	101.65	101.52	0.1767	0.1740
2	3778402	3907203	101.40			

Recovery:

0.25mL of injection (Sensiblex 40mg/mL) was measured accurately and transferred the sample (equivalent to 10 mg of Denaverine hydrochloride) into three separate 10 mL volumetric flask. Then 5mg, 10mg and 15mg (50%, 100%, 150%) of standard were accurately weighed and added. 7 mL of mobile phase was added and sonicate to dissolve it completely. Then the solution was made volume up to the mark with the same. 0.3 mL was pipette out from each flask and transferred to separate 10 mL volumetric flask. Then the solution was made volume up to the mark with the same. 20µl solution was injected in to chromatographic system and the chromatogram was recorded.

RT	Injection	Area
3.313	1	2108215
3.184	2	2109521
3.235	3	2114103

Fig 13: Recovery-50%

RT	Injection	Area
3.222	1	3875174
3.191	2	3888449
3.217	3	3893469

Fig 14: Recovery -100%

RT	Injection	Area
3.282	1	5781825
3.189	2	5779001
3.238	3	5773680

Fig 15: Recovery-150%

Table. 4 Results of Recovery

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	2110613	5.5	5.57	101.3%	
100%	3885698	10.1	10.2	101.5%	101.3%
150%	5778169	15.1	15.2	101.0%	

Precision:

Repeatability and intermediate precision studies were done to the precision of the method. Repeatability studies were done by consequently measuring the absorbance of standard solution. These solutions were prepared in duplicate and absorbances were measured at 306 nm against blank and calculate the % RSD.
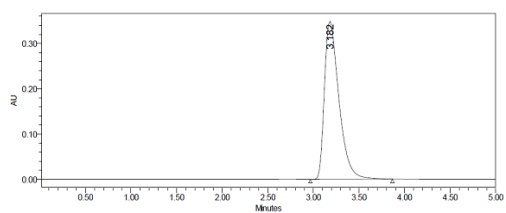
Fig 16: Precision study -1
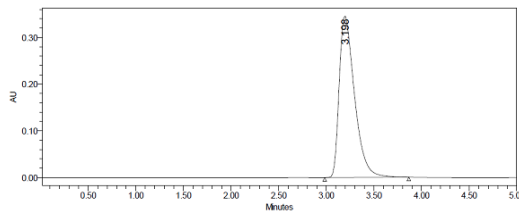
Fig 17: Precision study -2
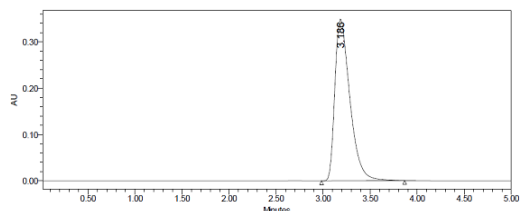
Fig 18:   Precision study -3
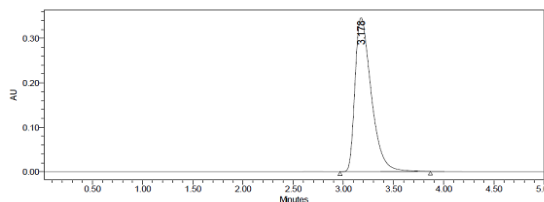
Fig 19: Precision study -4
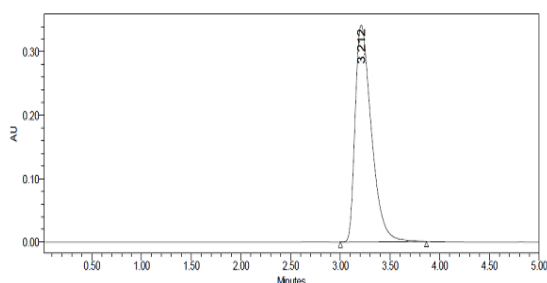
Fig 20: Precision study -5

RT	Injection	Area
3.182	1	3855508
3.198	2	3865126
3.186	3	3871273
3.178	4	3878408
3.212	5	3882797

Table. 5 Results of Precision

S.No	Concentration (µg/ml)	Peak Area	Average	SD	%RSD
1		3855508			
2		3865126			
3	30	3871273	3870622	10815.8	0.28
4		3878408			
5		3882797			

Intermediate precision
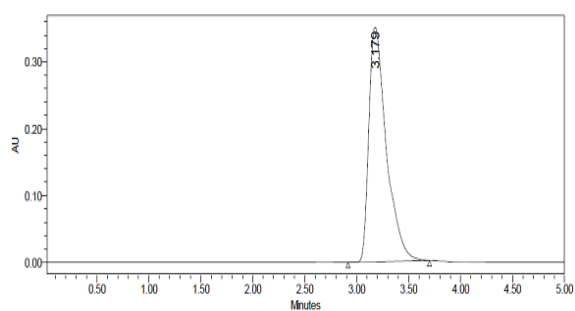
Fig 21: Intermediate precision-1
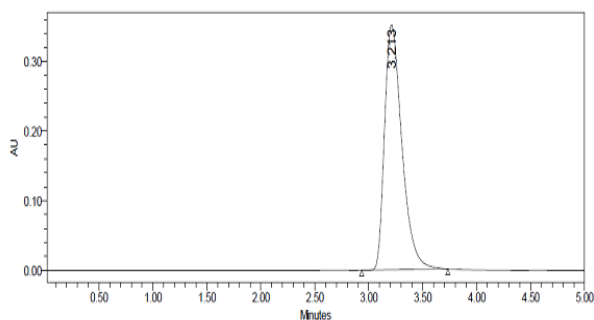
Fig 22: Intermediate precision-2
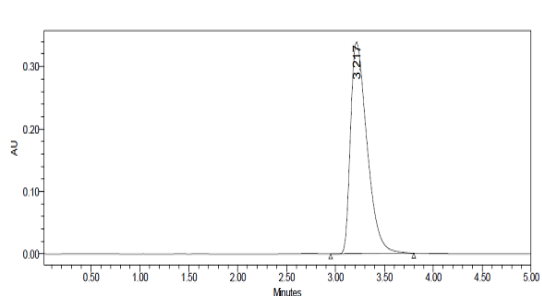
Fig23: Intermediate precision-3
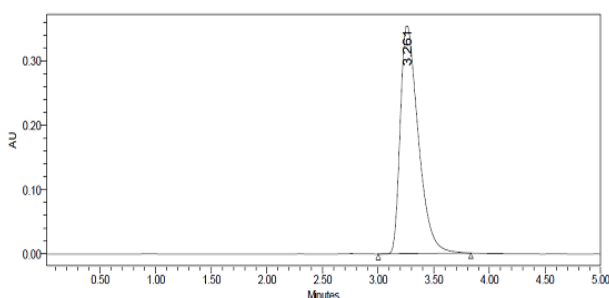
Fig 24: Intermediate precision-4
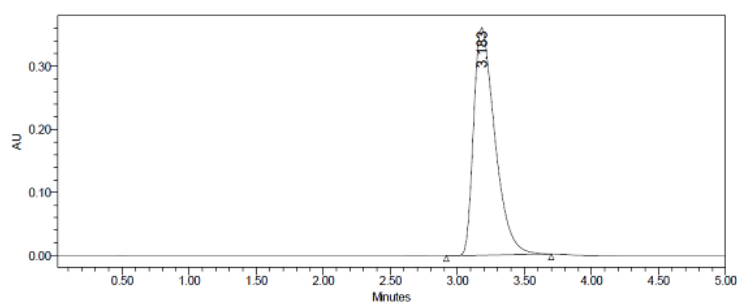
Fig 25: Intermediate precision-5

RT	Injection	Area
3.179	1	4095410
3.213	2	3935121
3.217	3	3963812
3.261	4	3990300
3.183	5	3976949

Table. 6 Results of Intermediate Precision

S.No	Concentration (µg/ml)	Peak Area	Average	SD	%RSD
1		4095410			
2		3935121			
3	30	3963812	3992318	61140.1	1.53
4		3990300			
5		3976949			

Robustness

For demonstrating the robustness of the developed method, experimental conditions were purposely altered and evaluated. The method must be robust enough to withstand such slight changes and allow routine analysis of the sample. For this present study mobile phase and flow rate has slightly changed and the assay was checked.
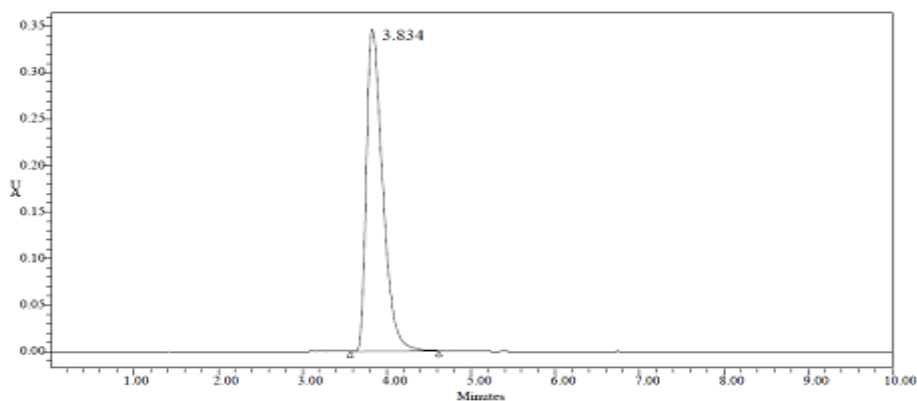

Fig 26: Robustness-Less Flow

Retention Time (min)	Area (µV*sec)	USP Plate Count	USP Tailing
3.834	4694313	2889.4	1.6
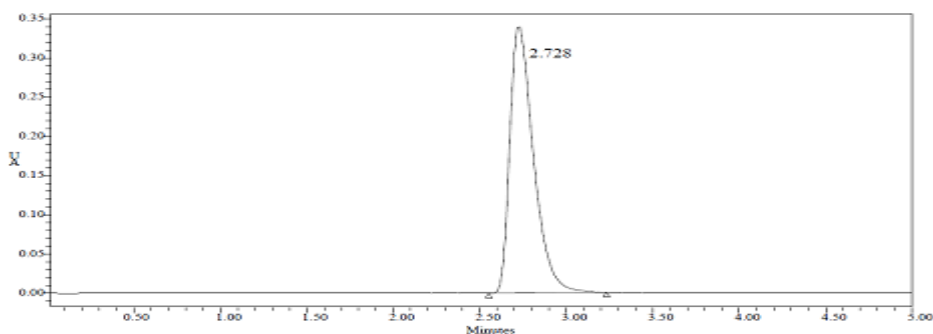
Fig 27: Robustness-More Flow

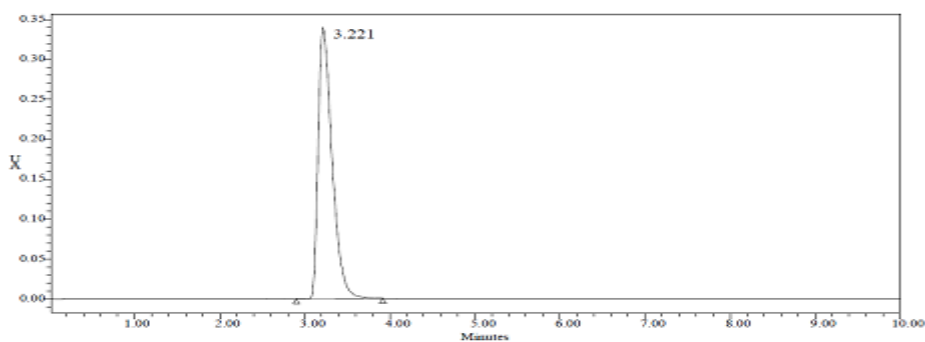| Retention Time (min)	Area (µV*sec)	USP Plate Count	USP Tailing
2.728	3231666	2961.0	1.5

Fig 28: Robustness- Less Organic
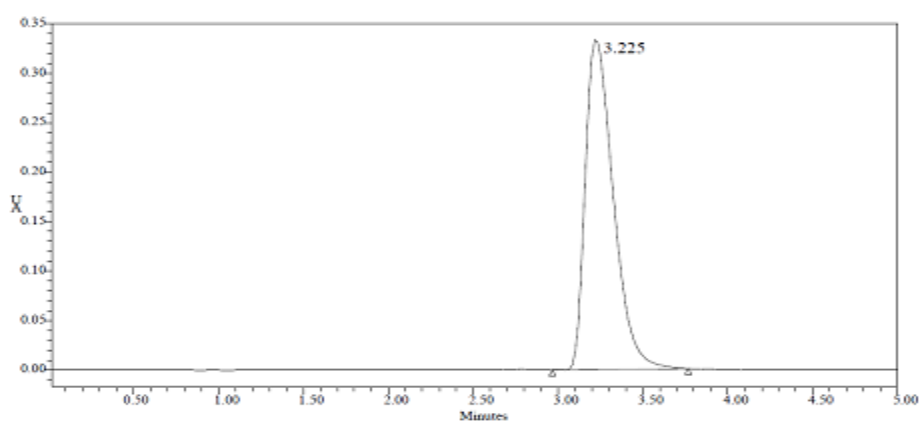
Fig 29: Robustness-More Organic

Retention Time (min)	Area (µV*sec)	USP Plate Count	USP Tailing
3.225	3828751	2856.9	1.5

Table. 7 Results of Robustness

Parameters	Theoretical plate	Tailing factor
Less flow (0.5 ml/min)	2889	1.6
More flow (0.7 ml/min)	2961	1.5
Less organic phase (60 %)	2874	1.6
More organic phase (80%)	2856	1.5

It shows that there is no change in the values even after making deliberate change in the analytical procedure.

Conclusions

The developed method was found to be simple, sensitive, accurate, precise, reproducible, and can be used for routine quality control analysis of Denaverine Hcl in bulk and injectable pharmaceutical formulation.

ACKNOWLEDGEMENTS

REFERENCES

1. H. H˙uller,W. Scheler, E. Schulz, Acta Biol. Med. German. 12 (1964) 682.
2. H. H˙uller, W. Scheler, H. Oberender, R. Peters, Acta Biol. Med. German. 22 (1969) 751.
3. H. H˙uller, Zbl. Pharmaz. 109 (1970) 115.
4. V. Bredow, Zbl. Gynakol. 114 (1992) 551.
5. E. Neumayer, Medicamentum 16 (1975) 264.
6. B. Vesper, Medicamentum 12 (1971) 335.
7. ICH Q2A; Guidelines on validation of analytical procedure; definitions and terminology. Federal Register 1995; 60: 11260.
8. ICH Q2B; Guidelines on validation of analytical procedure; Methodology. Federal register 1996; 60: 27464.
9. http://www.emea.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002662.pdf.