ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF ASSAY FOR CARVEDILOL TABLETS BY RP-HPLC, HPTLC AND UV SPECTROSCOPY

Dissertation work submitted to
The TamilNadu Dr. M.G.R. Medical University, Chennai
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MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

Submitted by
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MAY 2012

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This is to certify that the dissertation work entitled "**ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF ASSAY FOR CARVEDILOL TABLETS BY RP-HPLC, HPTLC AND UV SPECTROSCOPY**" is a bonafide work of **Ms. NISHA P.J** carried out in SHASUN PHARMACEUTICALS LIMITED, PUDUCHERRY under my guidance and under the supervision of Mr. SHAIK MOHAMMED NOOR and has completed to my fullest satisfaction for partial fulfillment of the award of degree of **Master of Pharmacy in Pharmaceutical Analysis**, R V S college of Pharmaceutical Sciences, Sulur, Coimbatore, which is affiliated to The Tamilnadu Dr. M.G.R Medical University, Chennai. It is to certify that the part or whole of the work has not been submitted either to this university or any other university. This work is original and confidential.

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EXTERNAL EXAMINER

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Date:

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ABBREVIATIONS

ACN	:	Acetonitrile
g	:	gram
HPLC	:	High performance liquid chromatography
pH	:	Hydrogen ion concentration
ICH	:	International conference on harmonization
mg	:	Milligram
ml	:	Milliliter
µg	:	Microgram
µg/ml	:	Microgram per milliliter
µg	:	Microgram
nm	:	Nanometer
PA	:	Purity Angle
PDA	:	Photo diode array
TH	:	Purity threshold
RSD	:	Relative standard deviation
k	:	Retention time
SD	:	Standard deviation

UV	:	Ultra violet
V/v	:	Volume by Volume

INTRODUCTION

Quality can be defined as the character, which defines the grade of excellence. A good quality drug is something, which will meet the established product specifications, can be safely bought and confidently used for the purpose for which it is intended.[1] To get a good quality drug ,the manufacturing for making a drug should have quality built into it.

Analytical chemistry is the science that seeks ever improved means of measuring the chemical composition of natural and artificial materials. Analytical chemistry is a sub-discipline of chemistry that has the broad mission of understanding the chemical composition of all matter and developing the tools to elucidate such compositions.[2]

TYPES:

Traditionally, analytical chemistry has been split into two main types, qualitative and quantitative.

QUALITATIVE:

Qualitative analysis seeks to establish the presence of a given element, given functional group or inorganic or organic compound in a sample.

QUANTITATIVE:

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

MODERN ANALYTICAL CHEMISTRY:

Modern analytical chemistry is dominated by instrumental analysis. There are so many different types of instruments today that it can seem like a confusing array of acronyms rather than a unified field of study. Most modern analytical chemistry is categorized by different analytical methods.

Analytical methods:-

- Spectrophotometry and colorimetry.

  UV-visible spectroscopy.
- Chromatography and Electrophoresis.

Commonly used methods are,

- High Performance Liquid Chromatography (HPLC).
- High Performance Thin Layer Chromatography (HPTLC).
- Gas chromatography (GC).
- Gas chromatography-Mass spectroscopy (GC-MS).
- Liquid chromatography-Mass spectroscopy (LC-MS).

SPECTROPHOTOMETRIC METHODS:

Spectrophotometry is generally preferred by industries as the cost of the equipment is less and the maintenance problems are minimal. The method of analysis based on measuring the absorption of a monochromatic light by colourless compounds in the near ultraviolet path of spectrum (200-380nm).[3]The photometric methods of analysis are based on the Bouger-Lambert Beer's Law, which establishes that the absorbance of a solution is directly proportional to the concentration of the analyte .The fundamental principle of operation of spectrophotometer covering UV region consists in that light of definite interval of wavelength passes through a cell with solvent and falls on to photoelectric cell that transforms the radiant energy into electrical energy measured by galvanometer.

The important applications are

- Identification of much type of organic, inorganic molecules and ions.
- Quantitative determination of many biological, organic and inorganic species.
- Quantitative determination of mixtures of analytes.
- Monitoring and identification of chromatographic of effluents.
- Determination of equilibrium constants.
- Determination of stoichiometry and chemical reaction.
- Monitoring of environmental and industrial process.
- Monitoring of reaction rates.

INTRODUCTION TO ULTRA-VIOLET SPECTROPHOTOMETRY

Molecular absorption in the ultraviolet and visible region of the spectrum is dependent on the electronic structure of the molecule. Absorption of energy is quantized, resulting in the elevation of electrons from orbital in the ground state to higher energy orbital in the excited state.[4]

The wavelength range of UV radiation starts at the blue end of the visible light and ends at $2000A^0$.The ultraviolet region is subdivided into two spectral regions.

1. The region between $2000\text{-}4000A^0$ is known as near ultraviolet region.

2. The region below $2000A^0$ is called the far or vacuum ultraviolet region.

Wavelengths in the ultraviolet region are usually expressed in nanometres ($1nm=10^{-7}cm$) or angstroms (A^0) ,($1A^0=10^{-8}cm$) occasionally absorption is reported in wave numbers ($v=cm^{-1}$).

White light is made up of a large number of individual waves of varying wavelength. This is shown by passing a beam of light through a prism. When a band of colour, is formed in which each colour corresponds to waves of particular wavelength. Complete spectrum of electromagnetic radiation extends from the ultra-short wave region of the cosmic rays at one end to that of radio wave at the other.

Wavelength:

Distance between any two consecutive parts of the wave for example from the crest of one wave to that of the next. Its symbol is λ (lambda).

Wave number:

This is defined as the reciprocal of the wavelength expressed in cm^{-1} i.e. the number of waves per unit cm. Its units are cm^{-1}.

Frequency:

This is number of waves passing a point in one second i.e. the number of cycles per second.

Its symbol is letter nu.

Atomic Absorption:

The excitation of electrons in atomic orbital to higher energy states may be induced by electromagnetic radiation of the energy. If the radiation exactly matches to difference between the upper and lower energy states.

Atomic absorption Spectrophotometry is the technique for quantitative determination of elements which is based on the measurement of absorption of monochromatic light by ground state atoms.

Molecular Absorption[5]:

The total energy of a molecule is the sum of electronic, vibrational and rotational energies. The relative energies required to induce electronic, vibrational, rotational transitions between the quantized energy levels are approximately 10,000:100:1.

At room temperature the energy of most molecules in the ground electronic (E_E) and vibration (E_{vib}) states .As a result certain electrons within molecules undergo a transition to an excite energy state and this is normally accompanied by an increase in the vibrational and rotational energies of the molecule.

$$\Delta E = (E_E + E_{vib} + E_{rot})\ \text{upper} - (E_E + E_{vib} + E_{rot})\ \text{lower}$$

The technique of ultraviolet–visible spectrophotometry is one of the most frequently employed in pharmaceutical analysis. It involves the measurement of the amount of ultraviolet (190-380) or visible (380-800nm) radiation absorbed by a substance in solution. Instruments which measure the ratio or a function of the ratio of the intensity of two beams of light in the ultraviolet visible region are called UV-visible spectrophotometers.

When a beam of light is passed through a transparent cell containing a solution of an absorbing substance, reduction of the intensity of the light may occur. This is due to:

a. Reflection at the inner and outer surfaces of the cell.

b. Scatter by particles in the solution.

c. Absorption of light by molecules in the solution.

The reflections at the cell surface can be compensated by a reference cell containing the solvent only, and scatter may be eliminated by filtration of the solution. The intensity of light absorbed is then given by

$$I_{absorbed}=I_0-I_T$$

Where I_0 is the original intensity incident on the cell and I_T is the reduced intensity transmitted from the cell. The transmittance (T) is the ratio I_T/I_0 and the % transmittance (%T) is given by

$$\%T = 100I_T/I_0$$

The Beer-Lambert relationship is a relationship of transmittance and absorbance with the concentration of the substance in a solution.

$$A = abc = \log I_0 I = \log I/T$$

Where 'a' is called absorptivity, which is constant. The name and "a" value of a depend on the units of concentration. Where "c" is in moles/litre, the constant is called molar absorptivity and has the symbol E. The equation therefore takes the form.

$$A = \square bc$$

The molar absorptivity at a specified wavelength of a substance in solution is the absorbance at the wavelength of a1mol/L solution in a 1 cm cell. The unit of therefore are 1 $mol^{-1}cm\text{-}^{1}$.

Another one is the specific absorbance which is the absorbance of a 1g/100ml (1%w/v) solution in a 1 cm cell.

$$A = A^{1\%}_{1cm}bc$$

Where c is concentration in g/100ml and b is the band length in cm. The units of $A^{1\%}_{1cm}$

$dlg^{-1}cm^{-1}$.

A simple easily derived equation allows interconversation of $\square$ and $A^{1\%}_{1cm}$ values.

$$\square = A^{1\%}_{1cm} \times \text{molecular weight}/10$$

QUANTITATIVE SPECTROPHOTOMETRIC ASSAY [6]

Assay of single component samples

a) **Single standard or Direct Comparison Method:**

In this method the absorbance of a standard solution of known concentration and a sample solution is measured .The concentration of unknown can be calculated using the formula.

$$C_2 = C_1 x A_2 / A_1$$

A_1, A_2 = Absorbance of standard and sample

C_1 and C_2 = Concentration of standard and sample

b) **Calibration curve method or multiple standard method:**

A calibration curve is plotted using –concentration vs. absorbance value of five or more standard solution. A straight line is drawn either through maximum number of points or in such a way that there is equal magnitude of positive and negative errors that is line of best fit. From the absorbance of the sample solution and using the calibration curve, the concentration of the drug, amount and percentage purity can be calculated.

Assay of substances in multi compound sample:

The basis of all the spectrophotometric techniques for multi compound samples is the property that all samples show the property of ----

1. Absorbance which is the sum of absorbance of the individual components.

2. The measured absorbance is the difference between the total absorbance of the solution in the sample cell and that of the solution in the reference (blank) cell.

Simultaneous equation method:

If a sample contains two drugs (X and Y) each of which absorbs at the λ max of the other, it may be possible to determine both drugs by the simultaneous equation method (Vierodt's method).

When both substances contribute to the absorption at each wavelength, the calculation is as follows:

Let component a have E^1_1 values of a_1 at λ_1 and a_2 at λ_2

Let component b have E^1_1 values of $b_1$at λ_1 and b_2 at λ_2

The extinction of a solution of the mixture is determined at λ_1 and λ_2 and the E^1_1 values are calculated-s_1 and s_2 at λ_1 and λ_2 respectively.

Then

$$100\ S_1 = a_1x + b_1y$$

And

$$100\ S_2 = a_2x + b_2y$$

Where

X = concentration (as %w/w) of component a

Y = concentration (as %w/w) of component b

Solving the simultaneous equation for x and y.

$$X = 100 \left[\frac{b_1S_2 - b_2S_2}{b_1a_2 - b_2a_1} \right]$$

$$Y = 100 \left[\frac{a_1S_2 - a_2S_1}{a_1b_2 - a_2b_1} \right]$$

It should be confirmed that the extinctions of the two substances are additive and the wavelengths selected.

Using the above equations, concentration of individual components in a mixture can be determined.

MEANING OF CHROMATOGRAPHY AND ITS TYPE

Chromatography is a method used for separating organic and inorganic compounds so that they can be analysed and studied. Chromatography is a great physical method for observing mixtures and solvents. The word chromatography means colour separation where chroma means colour and graphy means separation. Chromatography is based on different migration. Solutes with a greater affinity for the mobile phase will spend more time in this phase than solutes that prefer the stationary phase. As the solutes move through the stationary phase the different components are going to be absorbed and are going to stop moving with mobile phase .Thus they are separated. This is called as chromatographic development.

THE DIFFERENT TYPE OF CHROMATOGRAPHY [7]

1. **Adsorption chromatography:**

Adsorption chromatography is probably one of the oldest types of chromatography around. It utilises a mobile liquid or gaseous phase that is absorbed on to the surface of a stationary solid phase. The equilibrium between the mobile and stationary phase accounts for the separation of different solutes.

2. **Partition chromatography:**

This form of chromatography is based on thin film formed on the surface of a solid support by a liquid stationary phase .Solutes equilibrates between the mobile phase and the stationary liquid.

3. **Ion exchange chromatography:**

In this type of chromatography, the use of a resin (the stationary solid phase) is used to covalently attach anions or cations to it. Solute ions of the opposite charge in the mobile liquid phase are attracted to the resin by electrostatic forces.

4. **Molecular exclusion chromatography:**

Also known as gel permeation or gel filtration, this type of chromatography lacks an attractive interaction between stationary phase and solute. The liquid or gaseous phase passes through a porous gel, which separates the molecule according to its size. The pores are normally small and exclude the larger solute molecule, but allow smaller molecule to enter

the gel, causing them to flow through a larger volume. This causes the larger molecules to pass through the column at a faster rate than the smaller ones.

5. **Affinity chromatography:**

This is the most selective type of chromatography employed. It utilises the specific interaction between one kind of solute molecule and a second molecule that is immobilised on a stationary phase. For example the immobilised molecule may be an antibody to some specific protein. When solute containing a mixture of protein is passed by this molecule, only the specific protein is reacted to this antibody, it to the binding stationary phase. This protein is later extracted by changing the ionic strength or PH.

High performance liquid chromatography:

HPLC is able to separate macromolecules and ionic species labile natural products, polymeric materials, and a wide variety of other high –molecular weight poly functional group. HPLC is the fastest growing analytical technique for the analysis of the drugs. It's simplicity, high specificity, and wide range of sensitivity makes it ideal for the analysis of many drugs in both dosage forms and biological fluids .In this ,the separation is about 100 times faster than the conventional liquid chromatography due to packing of particles in the range of 3-10µm.Modern LC uses very small particles for packing. The small particle size results in more rapid approach to the distribution equilibrium and consequently smaller plate height, so that a given length of column includes large number of plates which makes the column efficient and the peak narrow. But close packing of these small particles reduces the flow rate of the mobile phase through the packed bed (the packing said to develop high back pressure) and in order to achieve a reasonable flow rate it is necessary to apply pressure to the mobile phase. So the designation, put forth as high pressure liquid chromatography. Thus HPLC is having advantages of improved resolution, faster separation, improved accuracy, precision and sensitivity.

According to the phases involved, HPLC can be classified into several types which is as follows

1. Normal phase chromatography (NPC)
2. Reverse –Phase chromatography (RPC)
3. Liquid –solid chromatography or adsorption HPLC

4. Liquid-liquid chromatography or partition HPLC
5. Ion exchange chromatography or ion exchange HPLC
6. Size exclusion or gel permeation or steric exclusion HPLC
7. Ion pair HPLC
8. Affinity HPLC

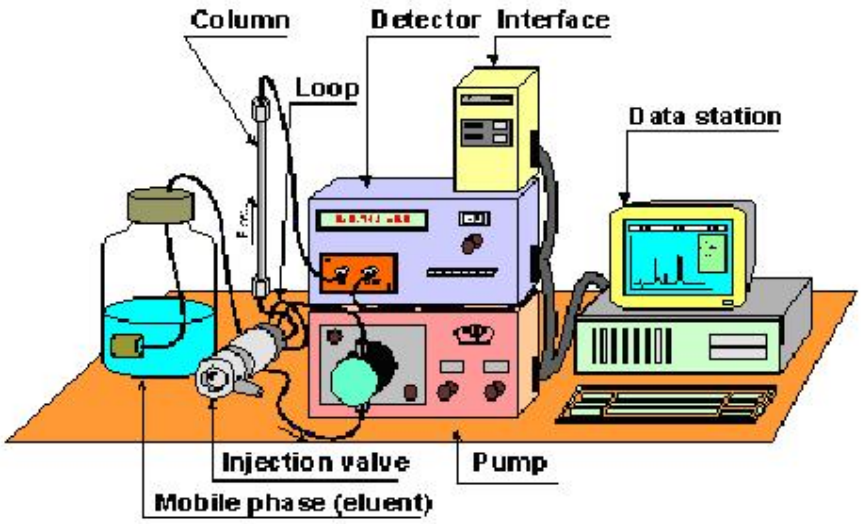
Normal Phase Chromatography (NPC):

In normal phase chromatograph, the stationary phase is more polar than the mobile phase, and the mobile phase is a mixture of organic solvents without added water (eg.Isopropane with hexane) and the column packing is either an inorganic adsorbent(silica) or a polar bonded phase (cyano, diol,amino) on a silica support. Sample retention in normal phase chromatography increases as the polarity of the mobile phase decreases. They are eluted in the order of increasing polarities.

Reverse phase chromatography:

In reverse phase chromatography, the stationary phase is less polar than the mobile phase and the mobile phase is a mixture of organic and aqueous phase. Reverse phase chromatography is typically more convenient and rugged than the other forms of liquid chromatography and is more likely to result in a satisfactory final separation. High Performance RPC columns are efficient stable and reproducible. In this the solutes are eluted in the order of their decreasing polarities. These are prepared by treating the surface silanol group of site with an organic chloro silane reagent.

Basic principle of HPLC [8]



High performance liquid chromatography (HPLC) is a separation technique utilizing differences in distribution of compounds to two phases, called stationary phase and mobile phase. The stationary phase designates a thin layer created on the surface of fine particles and the mobile phase designates the liquid flowing over the particles. Under a certain dynamic condition, each component in a sample has difference distribution equilibrium depending on the solubility in the phase and or molecule size. As a result, components move at different speed over the stationary phase and thereby separated from each other. The column is a stainless steel or resin tube, which is packed with spherical solid particles. Mobile phase is constantly fed into the column inlet at a constant rate by a liquid pump. A sample is injected from the sample injector, located near the column inlet. The injected sample enters the column with mobile phase and the components in the sample migrates through it, passing between the stationary and mobile phase. Compound move in the column only when it is in mobile phase. Compounds that tend to be distributed in the stationary phase migrate slower. In this way,each component is separated on the column and sequentially elute from the outlet. Each component eluting from the column is detected by a detector to the outlet of the column .When the separation process is monitored by the recorders starting at the time of sample is injected, a graph is obtained. This graph is called chromatogram. The time required for a compound to elute (called retention time) and the relationship between compound concentration (amount) and peak area depend on the characteristic of the compound.

Retention is therefore used as an index for qualitative determination and peak surface area as index for quantitative determination. There are two modes of elution process

a) Isocratic elution

b) Gradient elution

Selectivity of HPLC –method development

Most of the drug can be analyzed by HPLC because of several advantages and ease of automation and eliminates tedious extraction and isolation procedures. Some advantages are:-

1. Speed (analysis can be accomplished in 20 minutes or less).
2. Greater sensitivity (various detectors can be employed).
3. Improved resolution.
4. Reliable columns (wide variety of stationary phase).
5. Ideal for substance of low volatility.
6. Easy sample recovery, handling, and maintenance.
7. Easy programming of the numerous functions in each module.
8. Time programmable operation sequence, such as initiating operation of detector lamp and isocratic pump to obtain stable base line and equilibrated column before the work day begins.
9. Excellent reproducibility of retention time.
10. An injection volume variable from 0.1 to100 micro litres without any hardware modification.
11. The flexibility of data analysis.
12. Suitable to avoid any interference from impurity.
13. Suitable for preparative liquid chromatography on a much large scale.

Initial conditions for the development [9]

If HPLC is chosen for the separation, the next step is to classify the sample as regular or special. We define regular samples as typical mixtures of small molecules (<2000 Da), that can be separated using more or less standardized starting conditions. Regular samples can be further classified as neutral or ionic. If the sample is neutral, buffers or additives are generally not required in the mobile phase. Ionic samples require the addition of a buffer to the mobile phase. For basic or cationic sample , "less acidic" reversed phase columns are recommended and amine addition for the mobile phase is beneficial.

A good method of development strategies should require as many experimental runs as necessary to achieve the desired final result.

Nature of the sample:

Before the development, we need to review what is known about the sample. The goals of the separation should also be defined at this point. The chemical composition of the sample can provide valuable clues for the best choice of initial conditions and HPLC separation.

Important information concerning sample composition and properties.

1. Number of compounds present
2. Chemical structures (functionality) of compounds
3. Molecular weights of compounds
4. pka values of compounds
5. UV spectra of compounds
6. Concentration range of compounds in samples of interest
7. Samples solubility

Sample Pretreatment and Detection:

1. Solutions ready for injection

2. Solutions that requires dilution, buffering, addition of an internal standard or other volumetric manipulation.

3. Solid that must first be dissolved or extracted

4. Samples that requires, sample pretreatment to remove interference and/or protect the column or equipment from damage.

Best results are often obtained when the composition of the simple solvent is close to that of the mobile phase, since this minimizes baseline upset and other problems. Some samples require a partial separation (pretreatment) prior to HPLC, because of the need to resolve interferences, concentrate sample analytes, or eliminate "column killers". Before the first sample is injected during HPLC method development, we must be reasonably sure that the detector selected will sense all sample components of interest. For this various types of detectors are used.

The aim of sample preparation is that the sample aliquot should be (1) relatively free of interferences,(2) will not damage the column and (3) is compatible with intended HPLC method, that is sample solvent will dissolve in the mobile phase without affecting sample retention or resolution. A sample pretreatment procedure should provide quantitative recovery of analytes, involve a minimum number of steps and be easily automated. Quantitative recovery of each analyte enhances sensitivity and assay precision, although this does not mean that all the analyte present in the original sample must be included in the final injected sample. If recovery is less than 100% the sample pretreatment must be reproducible.

Derivatization:

Derivatization involves a chemical reaction between analyte and a reagent to change the chemical and physical properties of an analyte.

The four main uses of derivatization in HPLC are to

1. Improve detectability

2. Change the molecular structure or polarity of analyte for better chromatography

3. Change the matrix for better separations

4. Stabilize a sensitive analyte

Ideally, a derivatization reaction should be rapid, quantitative and produce minimal by products. Excess reagent should not interfere with the analysis or should be removed easily from the matrix.

HPLC INSTRUMENTATION [10,11]

The general instrumentation for HPLC incorporates the following components

- There is a solvent reservoir for the mobile phase
- The mobile phase must be delivered to the column by some type of pump. The pumping system must be pulse-free or else have pulse damper to avoid generating baseline instability in the detector.
- Sampling valves are used to inject the sample in the flowing mobile phase just at the head of the separation column. Samples should be dissolved in a portion of the mobile phase to eliminate an unnecessary peak.
- A head of the separation column there may be a guard column or an in-line filter to prevent contamination of the main column by small particulate.
- To measure column inlet pressure a pressure gauge is inserted in front of the separation column.
- The separation column contains the packing needed to accomplish the desired HPLC separation. These may be silica's for adsorption chromatography, bonded phase for liquid-liquid chromatography, ion exchange functional groups bonded to the stationary support for ion exchange chromatography, gels of specific porosity for exclusion chromatography, or some other unique packing for a particular separation method
- A detector with some type of data handling device completes the basic instrumentation.

Detectors:

The sensitivity of universal detector for HPLC has not been devised yet. Thus it is necessary to select a detector on the basis of the problem.

Buffers in Reversed-Phase Liquid Chromatography:

Selection of a buffered aqueous mobile phase for reversed-phase liquid chromatography (RPC) may seen intimidating, but with an understanding of the fundamental effect of pH

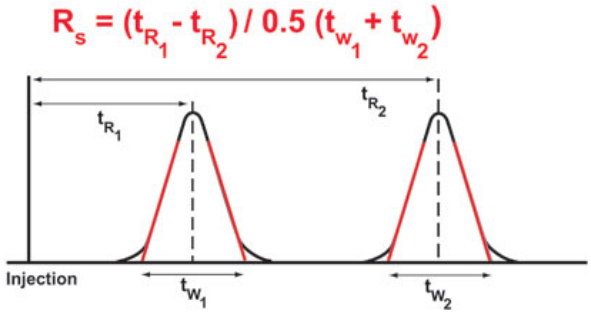
on retention of ionic analytes and checking some properties of the buffer options, a logical and reasonable choice can be derived. This selection may need to be revised by consideration of certain factors, such solubility or limitations of compatibility with the means of detection. Practical aspects of buffer preparation should also be observed for reproducible and trouble free operations.

The pH of the mobile phase will dramatically affect the retention of ionic analytes within 1.5 pH units if their PK_a Thus, it can be understood why control of mobile phase pH has great utility in method development and is critical for functional groups reproducible separations of ionic analytes. Obviously, if the analyte is not ionic mobile phase pH wont directly affect its retention.

Chromatographic parameters:

Resolution:

Chromatographers measure the quality of separation by resolution Rs of adjacent bands



Where t_{w1} and t_{w2} are obtained from the intersection of the tangents with the baseline. For a symmetrical Gaussian peak the tangents are drawn at 0.6times the peak height.

Capacity factor:

It is the measure of how well the sample molecule is retained by the column during an isocratic separation. It is affected by solvent composition, separation aging and temperature of separation.

$$\mathbf{K_1 = t_r - t_0/t_0}$$

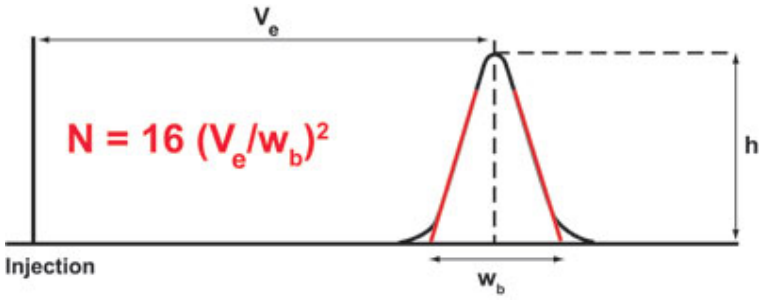
t_r = Band separation time

t_0 = column dead volume

Column efficiency:

It is called as number of theoretical plates. It measures the band spreading of a peak. When band spread is smaller, the number of theoretical plate is higher. It indicates a good column and system performance.

Column performance can be defined in terms of values of N



Where:

N = Number of theoretical plates

V_e = elution volume, retention time or retention distance (mL, sec, or cm)

h = peak height

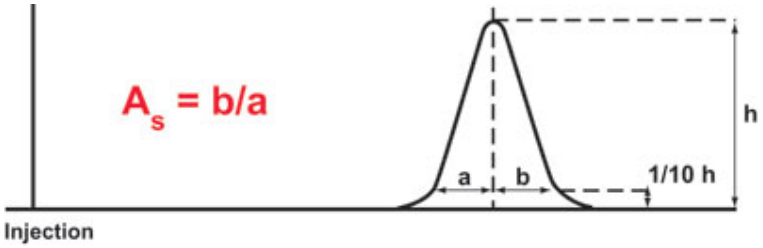
w_b = width of the peak at the base line (ml, sec, or cm)

Peak Asymmetry and Peak tailing:

Peak Asymmetry:

Peak with poor symmetry can result in

- Inaccurate plate number and resolution measurement
- Imprecise quantitation
- Degraded resolution and undetected minor bands in the peak tail
- Poor retention reproducibility



Where:

A_s = peak asymmetry factor

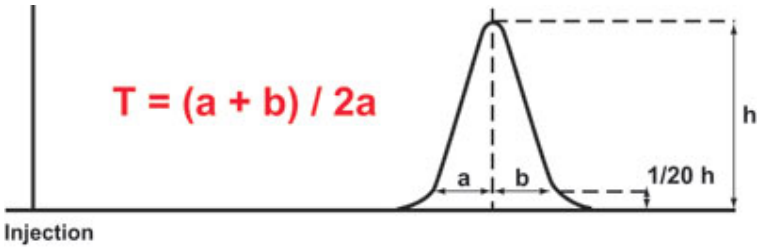
b = distance from the point at peak midpoint to the tailing edge

(measured at 10% of peak height)

a = distance from the leading edge of peak to the midpoint

(measured at 10% of peak height)

Peak tailing:



Where:

T = tailing factor (measured at 5% of peak height)

b = distance the point from at peak midpoint to the tailing edge

a = distance from the leading edge of the peak to the midpoint

Increased peak asymmetry value, $A_{S>}$ 1.5 the sign that the column should be changed

Selectivity:

It measures relative retention of two components. Selectivity is the function of chromatographic surface (column), melting point and temperature.

$$\alpha = K_2/K_1 = V_2\text{-}V_0/V_1\text{-}V_0$$

HPTLC METHOD DEVELOPMENT [12, 13]

HPTLC- High Performance Thin Layer Chromatography is a sophisticated and automated form of TLC.

Main difference of HPTLC and TLC- Particle and pore size of sorbents. The other differences are:

Parameters	**HPTLC**	**TLC**
Layer of sorbent	100µm	250µm
Efficiency	High due to smaller particle size generated	Less
Separations	3-5cm	10-15cm
Analysis time	Shorter migration distance and the analysis time is greatly reduced	Slower
Solid support	Wide choice of stationary phases like silica gel for normal phase and C8, C18 for reversed phase modes	Silica gel, Alumina and Kiesulghur
Development chamber	New type that require less amount of mobile phase	More amount
Sample spotting	Auto-sampler	Manual spotting
Scanning	Use Camag TLC scanner of UV/Visible/Fluorescence scanner scans the entire chromatogram qualitatively and quantitatively and the scanner is an advanced type of densitometer	Not possible

Features of HPTLC [14-16]

1. Simultaneous processing of sample and standard-better analytical precision and accuracy less need for internal standard.

2. Several analysts work simultaneously

3. Lower analysis time and less cost per analysis

4. Low maintenance cost

5. Simple sample preparation- handle samples

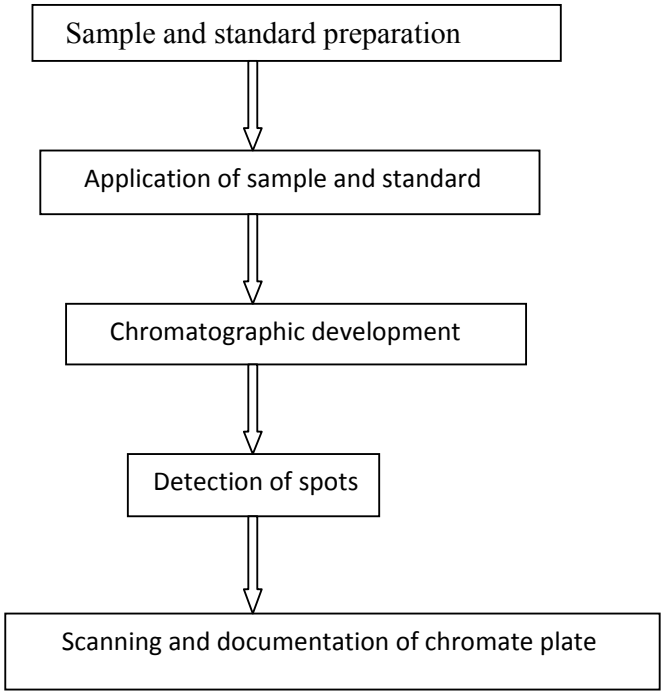
6 .No prior treatment for solvents like filtration and degassing

7. Low mobile phase consumption per sample

8. No interference from previous analysis – fresh stationary and mobile phases for each analysis – no contamination

9. Visual detection possible open system

10. Non UV absorbing compounds detected by post- chromatographic derivatisation



Selection of chromatographic layer:

- Precoated plates – different support materials –different sorbents available
- 80% of analysis –silica gel GF . Basic substances, alkaloids and steroids-aluminium oxide
- Amino acids, dipeptides, sugars and alkaloids-cellulose
- Non-polar substances, fatty acids, carotenoids, cholesterol-RP2,RP8 and

Preservatives, barbiturates, analgesic and phenothiazines- Hybrid plates-RPWF254$_s$

Activation of pre-coated plates:

- Freshly open box of plates do not require activation
- Plates exposed to high humidity or kept on hand for long time to be activated
- By placing in an oven at 110-120^{0}c for 30' prior to spotting
- Aluminium sheets should be in between two glass plates and placing in an oven at110-120^{0}c for 15 minutes.

Application of sample and standard:

- Usual concentration range is 0.1µg/µl
- Above this causes poor separation
- Linomat IV(automatic applicator)-nitrogen gas sprays sample and standard from syringe on TLC plates as bands
- Band wise application-better separation- high response to densitometer
- Selection of mobile phase
- Trial and error
- One' own experience and literature
- Normal phase
- Stationary phase is polar
- Mobile phase is non polar
- Non –polar compounds eluted first because of lower affinity with stationary phase
- Polar compounds retained because of higher affinity with the stationary phase
- Reversed phase
- Stationary phase is non polar
- Mobile phase is polar

- Polar compounds eluted first because of lower affinity with stationary phase
- Non-polar compounds retained because of higher affinity with the stationary phase
- 3-4 component mobile phase should be avoided
- Multi component mobile phase once used not recommended for further use and solvent composition is expressed by volumes (v/v) and sum of volumes is usually100
- Twin trough chambers are used only 10-15 ml of mobile phase is required
- Components of mobile phase should be mixed introduced into the twin- trough chamber

Pre conditioning (Chamber saturation):

- Unsaturated chamber causes high Rf values
- Saturated chamber by lining with filter paper for 30 minutes prior to development – uniform distribution of solvent vapours-less solvent for the sample to travel-lower Rf values.

Chromatographic development and drying:

After development, remove the plate and mobile is removed from the plate- to avoid contamination of lab atmosphere. Dry vacuum desiccator-avoid hair-drier-essential oil components may evaporate

Detection and visualization:

- Detection under UV light is first choice- non destructive
- Spots of fluorescent compounds can be seen at 254 nm (short wavelength) or at 366nm (long wavelength)
- Spots of non-fluorescent compounds can be seen-fluorescent stationary is used-silica gel GF
- Non UV absorbing compounds ilike ethambutol, dicyclomine etc –dipping the plates in 0.1% iodine solution
- When individual compound does not respond to UV –derivetisation required for detection

Quantification:

- Sample and standard should be chromatographed on same plate- after development chromatogram is scanned
- Camag TLC scanner III scan the chromatogram in reflectance or in transmittance mode by absorbance or by fluorescent mode-scanning speed is selectable upto 100mm/s-spectra recording is fast-36 tracks with upto 100 peak windows can be evaluated
- Calibration of single and multiple levels with linear or non linear regressions are possible. When target values are to be verified such as stability testing and dissolution profile single level calibration is suitable
- Statistics such as RSD or CI report automatically
- Concentration of analyte in the sample is calculated by considering the sample initially taken and dilution factors

Documentation:

- E-Merck introduced plates with imprinted identification code- supplier name. Item number, batch number and individual plate number-Avoid manipulation of data at any stage –coding automatically get recorded during photo documentation

METHOD VALIDATION AND ITS TYPE[17]

Validation is a documented program that provides a high degree of assurance that a facility or operation will consistenly produce product meeting a predetermined specifications.

Why Validate?

Economical reasons:

- Rapid and reliable up
- Robust process
- Reduction in rejections/rework/recalls
- Reduce testing
- Rapid introduction
- To increase the productivity
- To target and reduce the number of controls

- To reduce product cost

Method Validation[18-20]

According to method, validation can be defined as "Establishing documented evidence which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its pre determined specification and quality characteristics.

Method validation is an integral part of the method development; it is the process of demonstrating that analytical procedures are suitable for their intended use and that they support the identity, quality, purity and potency of the drug substances and drug products. Simply, method validation is the process of proving that an analytical method is acceptable for its intended purpose.

Method validation, however, is generally a one –time process performed after the method has been developed to demonstrate that the method is scientifically sound and that it serves the intended analytical purpose.

All the variables of the method should be considered, including sampling procedure, sample preparation, chromatographic separation, and detection and data evaluation. For chromatographic methods used in analytical applications there is more consistency in validation practice with key analytical parameters including

Selectivity:

Ability of the developed analytical method to detect analyte quantitatively in the presence of other components, which are expected to be present in the sample matrix or other related substances. Results are expressed as resolution. If the expected impurities or related substances are available, they should be analyzed along with the analyte or sample to check the system suitability, retention factor, tailing factor and resolution etc.

Linearity:

It is the ability of the method to elicit test results that are directly proportional to analyte concentration within a given range. It is generally reported as variance of slope of regression line. It is determined by series of three to six injections of five or more standards.

Range:

It is interval between the upper and lower levels of analyte, which is studied. The range is normally expressed in the same units as the test results obtained by the analytical method. The ICH guidelines specify a minimum of five concentration levels.

Precision:

It is a measure of degree or repeatability of an analytical method under normal operation and it is normally expressed as % of relative standard deviation (% RSD).

$$\% \text{ RSD} = 100S/X$$

Where, S= Standard deviation, X = Mean

It is determined at three levels.

Repeatability:

It is obtained when analysis is carried out in one laboratory by one operator using one piece of equipment over relatively short time span at least 5 or 6 determinations of three different matrices at 2 or 3 different concentrations.

Intermediate Precision:

It is determined by comparing the results of a method run within a single laboratory over a number of weeks. A method intermediate precision may reflect discrepancies in results obtained by different operators, from different instruments, with standards and reagents from different suppliers, with column of different batches.

Reproducibility:

It represents the precision obtained between laboratories. The objective to verify that the method will provide the same results in different laboratories. It is determined by analyzing aliquots from homogenous lots in different laboratories with different analysts with specified parameters of method.

Accuracy:

It is the measure of how close the experimental value to the true value. Accuracy studies, for drug substance and drug product are recommended to be performed at 50%, 100%, and 150% levels of label claim. Three replicates of each concentration should be there and the mean is an estimate of accuracy.

Limit of Detection (LOD):

It is defined as the lowest concentration of an analyte in a sample that can be detected but not quantified. LOD is expressed as a concentration at a specified signal to noise ratio. In chromatography detection limit is injected amount that result in a peak with a height atleast twice or thrice as high as baseline noise level. S/N 2/1 or 3/1.

Limit of quantification (LOQ):

It is defined as lowest concentration of analyte in asample that can be determined with acceptable precision and accuracy under stated operational conditions of the method. LOQ is expressed as a concentration at aspecified signal to noise ratio. In chromatography detection limit is the injected amount that results in a peak with height ten times as high as base line noise level.

$$S/N = 10/1$$

Specificity:

Specificity is the ability to measure accurately and specifically the analyte of interest in the presence of other components that may be expected to be present in the sample matrix. It is a measure of a degree of interference from such things as other active ingredients, excipients, impurities, and degradation products, ensuring that a peak responses due to a single component only. i.e. that no co- elutions exist. Specificity is measured and documented in a separation by the resolution, plate count (efficiency), and tailing factor.

Ruggedness:

Ruggedness is the degree of reproducibility of the results obtained under a variety of conditions, expressed as % RSD. These conditions include different laboratories,analysts, instruments, reagents, days, etc. In guideline on definitions and terminology, the ICH did not

address ruggedness specifically. However, as ICH chose instead to cover the topic of ruggedness as part of precision, as discussed previously.

Robustness:

Robustness is the capacity of a method to remain unaffected by small deliberate variation in method parameters. The robustness of a method is evaluated by varying method parameters such as percent organic,pH, ionic strength, temperature etc;and determining the effect (if any) on the results of the method.As documented in the ICH guidelines, robustness should be considered early in the development of a method. In addition, if the results of a method or other measurements are susceptible to variations in method parameters, these parameters should be adequately controlled and a precautionary statement included in the method documentation.

System suitability testing:

System suitability tests are an integral part of chromatographic methods. These tests are used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. System suitability tests are based on the concept that the equipment, electronics, analytical operations, and samples constitute an integral system that can be evaluated as a whole. System suitability is the checking of a system to ensure system performance before or during the analysis of unknowns. Parameters such as plate count, tailing factors, resolution and reproducibility (%RSD retention time and area for six repetitions) are determined and compared against the specifications set for the method.

LITERATURE REVIEW

Suddhasattya Dey et al[21] develop HPLC method for the estimation of carvedilol in bulk and pharmaceutical dosage form. The specification of the chromatographic system, column 4.6mm×25cm, 5 micron, with mobile phase containing phosphate buffer: acetonitrile, P^H 2, wavelength 240nm, flow rate 1ml/min and the oven temperature 55°C. Retention time was 6.63. A linear response was observed between the concentration rangeof 806-1202µg/ml with a regression co-efficient of 0.99.

Navaneet Verma et al[22] reported a procedure for simultaneous estimation of carvedilol in its dosage form. The UV absorbance maxima of the drug at 285 nm. The drug obeyed Beer's law in the concentration range of 4-36µg/ml with molar absorptivity of 12.6 $\times 10^3$ l/mol. Cm in methanol.

Satish A Patel et al[23] developed UV spectrophotometric method for the determination of carvedilol in tablet formulation. Simple UV spectrophotometric method is based on determination of carvedilol in 0.1 N HCL at 241.2 nm. Linearity was obtained in the concentration range of 1- 12µg/ml.

M. Imran et al[24] developed a new and rapid stability indicating ultraviolet spectroscopic methods for the estimation of ezetimibe and carvedilol in pure form and in their respective formulations. The linearity range for ezetimibe and carvedilol obtained as 2-50µg/ml and 2-20µg/ml respectively.

Ivan Savic et al[25] developed a method to select an appropriate packaging and analysis of its influence on stability of tablets containing carvedilol. After radiation the content was estimated using a validated hplc – method. Retention time was 4.5 detected at 240 nm.

T.E.G.K Murthy et al[26] reported a development of scriminatory method for dissolution of carvedilol marketed formulations .In the study four dissolution media with different agitation speeds were employed. An agitation speed of 100 rpm showed more drug release profile than 50 and 75 rpm.

Ramesh Gannu et al[27] developed a HPLC analytical method for carvedilol in human serum. The method employs a liquid-liquid extraction for isolation and sample concentration followed by reversed phase liquid chromatography analysis using ultraviolet detection at 238nm. Serum samples containing the carvedilol and internal standard and amitriptyline were

eluted through a C_8 kromasil KR 100 5C8 column . Retention time of carvedilol was 6.10 min.

L. J Patel et al[28] developed reverse phase high performance liquid chromatography and high performance thin layer chromatography for estimation of carvedilol in bulk drug and pharmaceutical formulations. For HPLC method Lichrospher 100-c_{18}(200×4.6mm), 5µ column, isocratic mode, with mobile phase containing 50M potassium di hydrogen phosphate buffer: actonitrile : methanol (60:50:10) was used. The retention time was 4.56 .The linearity lies over 1-35µg/ml for HPLC. For HPTLC a CAMAG HPTLC system comprising of Linomat v automatic sample applicator , Hamilton syringe, Camag TLC scanner-3Camag Win CAT software with stationary phase precoated silica gel $60F_{254}$ and mobile phase consisting of ethyl acetate: toluene: methanol. The detection of spot carried out at 242 nm. The R_f value was 0.65. The linearity lies over 50 – 300 ng/spot for HPTLC.

CH. Ajay. Babu[29] developed a high performance liquid chromatography method for the determination of the carvedilol in human plasma. The method utilizes the liquid-liquid extraction with n-hexane ethyl acetate (3:1v/v). Samples were analysed by using phenomine Gemini C_{18} column with UV detection at 241nm.

Laila Ei Sayed Abdel Fattah et al[30] reported a spectrofluorimetric determination of carvedilol in dosage form and spiked human plasma through derivatization with 1-dimethylamino-naphthalene-5-sulphonyl chloride. The fluorescence concentration plot was rectilinear over the range of 5.0-8.0ng/ml with a lower detection limit of 1.90ng/ml.

Olga Galanopoulou et al[31] reported HPLC analysis, isolation and identification of a new degradation product in carvedilol tablets. The separation was achieved with an X-terra C_{18} column using acetonitrile - phosphate buffer pH2.5 as mobile phase.

Sarath Chandiran et al[32] reported a simultaneous quantification of carvedilol and its metabolite in human plasma by using High-throughput liquid chromatography - tandem mass spectrometric method .The method was linear over a concentration range of 0.1 to 250 ng/ml with a limit of quantification of 0.1ng/ml.

R. K. Jat et al[33] developed a sensitive and rapid extractive spectrophotometer method for the assay of carvedilol in bulk drug and tablets The complex formed between carvedilol and bromophenol blue in an acidic medium shows maximum absorbance at 414nm. Linearity lies in the range of 5-20µg

.

F. Behn et al[34] developed high performance liquid chromatography for the determination of the β receptor blocker .Carvedilol in small volumes of the plasma from paediatric patients. Analysis of the extracts was performed on a spherisorb C_6 column with a mobile phase of 65% acetonitrile and 35% potassium acetate buffer and fluorescence detection. Carvedilol and internal standard showed recoveries of 87.0% and 97.7 % respectively.

J.Stojanovic et al[35] developed a reverse phase high performance liquid chromatographic method for separation of carvedilol and its impurities from Karvileks tablets. The best separation was achieved on a chromolit RP 8 e column. Use of acetonitrile : water (45 : 55) v/v adjusted to pH 2.5 with formic acid as mobile phase. UV detection was performed at 280nm.

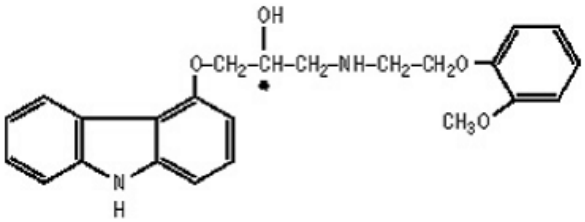
DRUG PROFILE: [36, 37]

CARVEDIOL

INTRODUCTION:

Carvedilol tablets are indicated for the treatment of mild to severe chronic heart failure of ischemic or cardio myopathic origin .usually in addition to diuretics ACE inhibitors and digitalis . They can be used alone or in combination with other antihypertensive agents especially thiazide type diuretics should not be given to patients with severe hepatic impairment .It is a non selective β-adrenergic blocking agent with α-1 blocking activity .Carvedilol has much greater antioxidant activity than other commonly used β blockers .Tablet containing inactive ingredients as colloidal silicon dioxide, crospovidone, hypromellose, lactose monohydrate, magnesium stearate, polyethylene glycol, polysorbate, povidone, and titanium dioxide.

CHEMICAL STRUCTURE:



CHEMICAL NAME :

(±)-[3-(9H-carbazol-4-yloxy)-2 hydroxy propyl] [2-(2-methoxy phenoxy) ethyl] amine.

MOLECULAR FORMULA:

$C_{24}H_{26}N_2O_4$

MOLECULAR WEIGHT:

406.5

CHARECTERESTICS:

White or off –white crystalline powder.

SOLUBILITY:

Insoluble in water, sparingly soluble in 95% ethanol and isopropanol, slightly soluble in ethyl ether soluble in methanol, methylene chloride, freely soluble in dimethyl sulfoxide.

MECHANISM OF ACTION:

Carvedilol is a racemic mixture in which non-selective β- adreno receptor blocking activity is present in the S(-) enantiomers and α_1 adrenergic blocking activity is present in both R(+) and S(-) enantiomers at equal potency. Carvedilol has no intrinsic sympathomimetic activity.

PHARMACOKINETICS:

Bioavailability : 25-35 %

Protein binding : 98%

Metabolism : Hepatic.

Half life : 7-10 hrs

Excretion : Renal 16%, Faecal 60%.

THERAPEUTIC USES:

- Antihypertensive agents.
- Adrenergic β- antagonist.
- Adrenergic α-antagonist.
- Vasodilator agents.

DOSAGE:

Patients weighing < 85 kg (187 lb) - 25mg twice daily.

Patient weighing > 85kg (187lb) -50mg twice daily.

HOW TO USE:

25 mg taken twice daily for two weeks .This dosage is same regardless of the age of weight of the patient. It should be swallowed as a whole tablet and should not crushed, and chewed .Carvedilol should be taken with food to slow the rate of absorption and reduce the incidence

of orthostatic effects .Patient should be observed in the office for one hour after initial dose is given.

STORAGE:

Store in a close, cool and light resistant container.

BRAND NAMES:

Cardivas, Coreg, Carvil, Karvileks.

AIM AND OBJECTIVE

The literature review reveals that several works have been carried out for the quantification of Carvedilol either individually or combination with other drugs and for the analysis of Carvedilol in biological fluids; including HPLC, Capillary electrophoresis, flourimetry, chemiluminescence, dissolution test. In these some methods are only for the estimation of drugs from biological fluids.

The present study describes the development of a new rapid, simple, sensitive and reproducible RP-HPLC, HPTLC, UV Spectrophotometry method for the analysis of Carvedilol that offer certain advantages in its simplicity and sensitivity and applicable in routine analysis. It also describes the development of validation work as per ICH guidelines recommended by the Food and Drug Administration (FDA) of the United States.

PLAN OF WORK

Aimed to develop analytical method and validation for Carvedilol in a tablet dosage form. The plan of the proposed work includes the following steps.

- The extensive survey of literature for Carvedilol regarding their characteristics and analytical methods. This forms the basis for the development of methods.
- To undertake solubility studies for the analyte Carvedilol.
- Selection of suitable solvent for quantitative extraction of analyte present in the formulations.
- Selection of suitable stationary phase and mobile phase.
- Selection of detection of wavelength.
- Develop initial conditions for HPLC, HPTLC,UV, methods.
- Optimization of the HPLC, HPTLC, UV, methods.
- Analytical method validation of the developed HPLC, HPTLC, and UV methods as per the ICH guidelines.

LIST OF INSTRUMENTS USED

S.NO	INSTRUMENT
1	SHIMADZU UV-1700 spectrophotometer
2.	SHIMADZU HPLC with UV detector
3	YMC pack pro C18 column (100 × 4.6mm, 5µ)
4	CAMAG HPTLC instrument
5	CAMAG TLC SCANNER 3
6	CAMAG LINNOMATE V AUTOMAIC SAMPLE APPLICATOR
7	Twin-trough chamber (10 × 10 cm)
8	Ultra Sonicator
9.	Electronic balance (2mg-200gm) (Sartorius)
10.	pH Analyser

LIST OF CHEMICALS AND SOLVENTS

S.NO	CHEMICALS AND SOLVENTS	MANUFACTURER
1.	Carvedilol working standard	USP standards
2.	Acetonitrile (HPLC grade)	Rankem
3.	Methanol (HPLC grade)	Fischer scientific
4.	Potassium di hydrogen phosphate	Merck
5.	Formic acid	Merck
6.	Milli - Q/HPLC water	Merck
7	Toluene	Merck
8	Chloroform	Merck

METHOD DEVELOPMENT FOR ASSAY OF CARVEDILOL TABLETS BY RP-HPLC METHOD

Selection of chromatographic method:

Proper selection of chromatographic method depends on the nature of the drug, molecular weight, and solubility. Since carvedilol is polar in nature, reverse phase chromatography has been used.

Selection of mobile phase:

Various mobile phases were tried in different ratios for selection of mobile phase. The drug carvedilol was injected with different mobile phase at different ratios with different flow rates till a sharp peak without any interference peak containing spectrum was obtained. The different mobile phase were containing either one or the combinations of two or three of following solvents, acetonitrile, water, methanol, tetrahydrofuran.

Tried at different ratios no favourable results obtained. But the mobile phase containing potassium di hydrogen phosphate buffer : acetonitrile in the ratio 60:40 gave acceptable peak with retention time 2.97 min.

Separation using acetonitrile and water

A mobile phase consisting acetonitrile and water at different ratios were tried to achieve the separation. But it was found that carvedilol peak was merging.

Separation using methanol and water

Next trial was done by using mobile phase consisting methanol and water. Although different ratios were tried tailing of the peak occurred.

Separation using tetrahydrofuran, acetonitrile and water

Tetrahydrofuran, acetonitrile, water composition as mobile phase were tried at different ratios peak shape was not found to be good.

Separation using potassium di hydrogen phosphate buffer and acetonitrile.

Potassium di hydrogen phosphate buffer, acetonitrile composition as mobile phase were tried at different ratios. With this good symmetrical peaks were obtained. As our aim was to

get good symmetrical peaks, this mobile phase system was selected for further study. Fig 2(1) – 2(4).

METHOD OPTIMIZATION

INITIAL CHROMATOGRAPHIC CONDITION

Column	-	YMC pack pro C_{18} (100×4.6mm)5µm
Column temperature	-	Ambient
Flow rate	-	1ml/min
Pump mode	-	Isocratic
Injection volume	-	10µl
Run time	-	8 min
P^H	-	3
Mobile phase	-	Phosphate Buffer : ACN

Effect of ratio of mobile phase

After confirming the mobile phase, change in the ratio of mobile phase was done for the optimization of the peak. The ratio of 50: 50, 70: 30, 65: 35 were tried fig 2(5)- 2(7).

In that case 60:40 v/v shows good retention time and resolution. The peak shown in figure 2(11).

Effect of flow rate

After confirming the ratio of mobile phase, flow rate of the mobile phase was changed, at 0.8ml/min it shows increased retention time the flow rate of 1.2ml/min resulted in fronting of the peak. It shows fig 2(8)- 2(9). The flow rate of 1ml/min has given a good result. It is shown in fig. 2(11).

Selection of column

The literature review showed the usage of C_{18} column for the determination of carvedilol. Mostly C_{18} column is used for analytical purpose and the column selected was YMC pack pro

C_{18} column. The column with different dimensions were tried but that showing shifting of retention time. It shows fig 2(10). YMC pack pro C_{18} (100 × 4.6mm, 5µ) column shows good result. Fig 2(11).

Selection of detector wavelength

The sensitivity of the HPLC method that uses UV detector depends upon the proper selection of wavelength. An ideal wavelength is one that gives maximum absorbance and good response for the drug to be detected.

UV detector as the most popular as they can be detect a broad range of compounds and have a fair degree of selectivity for some analytes and is useful for many HPLC applications. For the detection, the analyte chemical structure should consist suitable chromophore such as aromatic ring and the solvent using should be UV grade or non UV absorbing solvents.

A UV spectrum of carvedilol was recorded. From this spectrum λ_{max} at 242 nm was selected for the proposed study.

Figure1(1) acetonitrile: water.

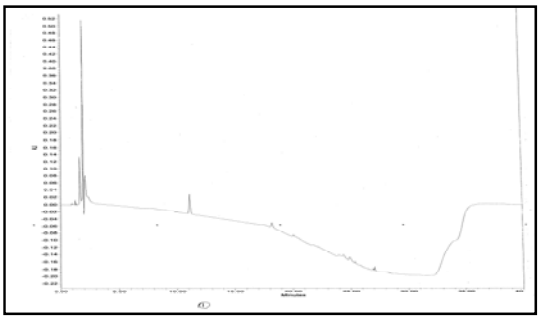


Figure 1(2) methanol:water

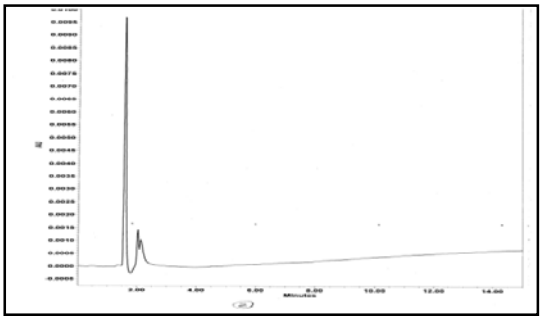


Figure 1(3) tetrahydrofuran: acetonitrile: water

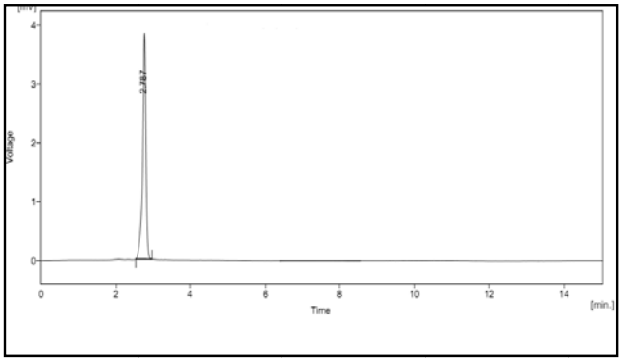


Figure 1(4) buffer: acetonitrile

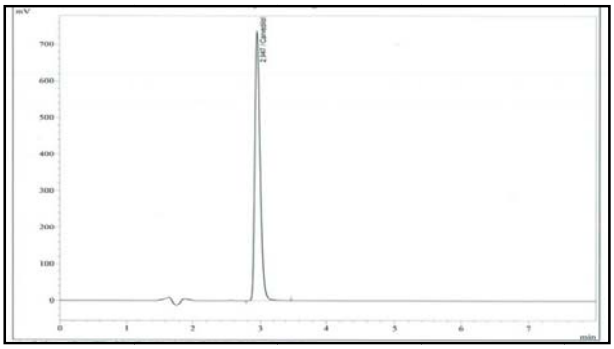


Figure 1(5) buffer: acetonitrile at 50:50 ratio

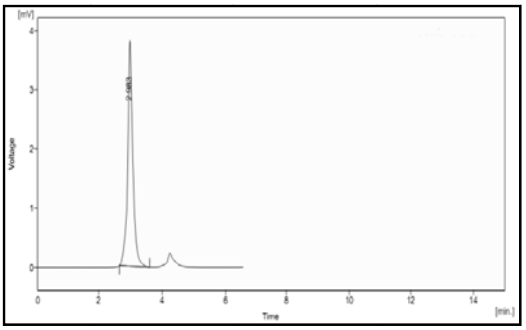


Figure 1(6) buffer: acetonitrile at 70:30 ratio.

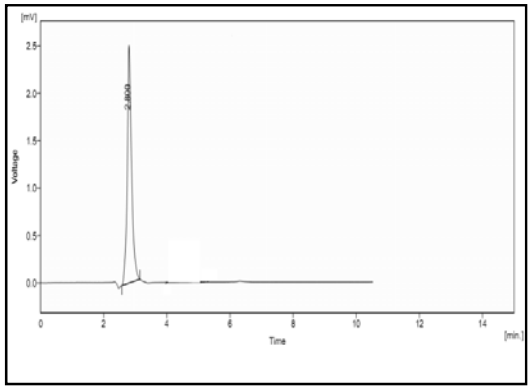


Figure 1(7) buffer :acetonitrile at 65:35 ratio.

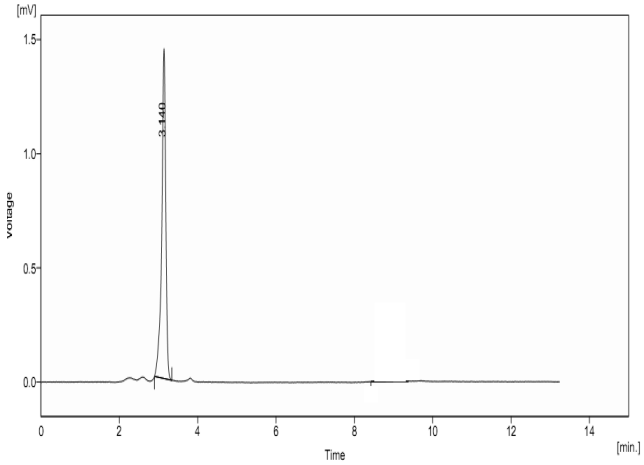


Figure 1(8) flow rate 0.8ml.

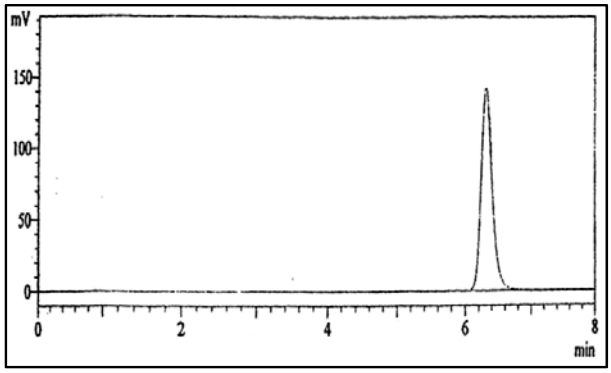


Figure 1(9) shows flow rate 1.2 ml.

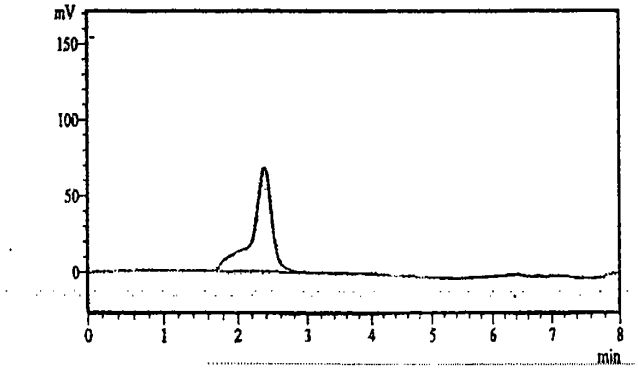


Figure 1(10) shows column C_{18} (250× 4.6 mm)

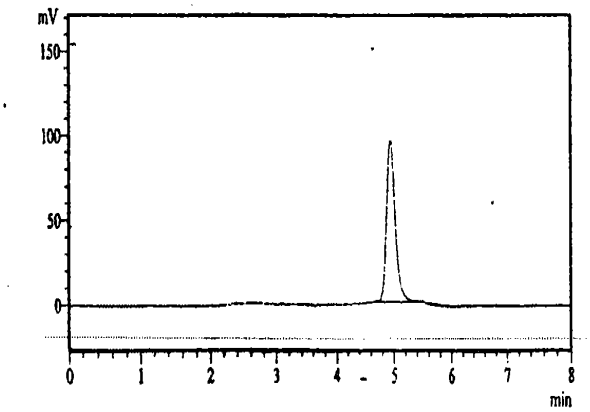
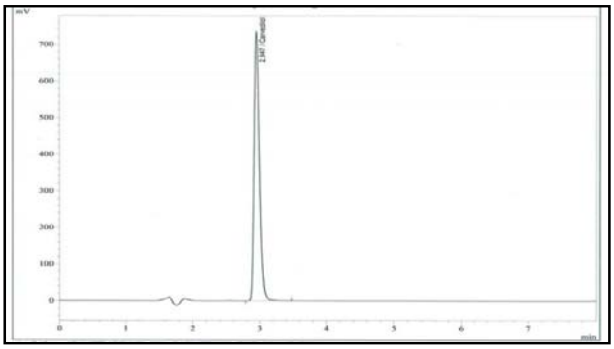


Figure 1(11) shows good symmetric peak.



ASSAY BY HPLC

Reagents	**Grade**
Potassium di hydrogen phosphate	AR
Formic acid	AR
Acetonitrile	HPLC
Water	HPLC/MILLI-Q

Chromatographic conditions

Column	:	YMC pack pro c18(100×4.6mm,5µ)
Column temperature	:	Ambient
Flow rate	:	1.0ml/min
Wavelength	:	242nm
Injection volume	:	10µl
Run time	:	8min
P^H	:	3
Mobile phase	:	Phosphate Buffer : ACN (60 : 40)

SOLUTION PREPARATIONS :

PREPARATION OF BUFFER SOLUTION:

Dissolve about 6.80g of potassium di hydrogen phosphate in1000 ml of HPLC/ Milli – Q water.

Adjusts the pH to 3.0 ± 0.05 with formic acid.

PREPARATION OF MOBILE PHASE:

Prepare a mixture of buffer and acetonitrile in the ratio of 60:40 Filter through 0.45µ Membrane filter and degas.

PREPARATION OF STANDARD SOLUTION:

Weigh accurately about 62.5mg of carvedilol reference /working standard in to a 100ml Volumetric flask, add 30ml of methanol sonicate to dissolve . Then make up to the volume With methanol . Pipette out 10ml of this solution into a100ml volumetric flask and dilute up to the mark with mobile phase. Filter through 0.45µ membrane filter.

Prepare the standard solution in duplicate calculate the similarity factor for standard-I and Standard -II solutions by using the following formula.

$$\frac{\text{Area of standard solution-1}}{\text{Area of standard solution-2 (1}^{st}\text{ injection)}} \times \frac{\text{weight of STD (in mg) solution-2}}{\text{weight of STD (in mg) solution-1}}$$

Note: similarity factor for both the standard solutions should be between 0.98 to 1.02

PREPARATION OF SAMPLE SOLUTIONS:

For 25 mg tablets:

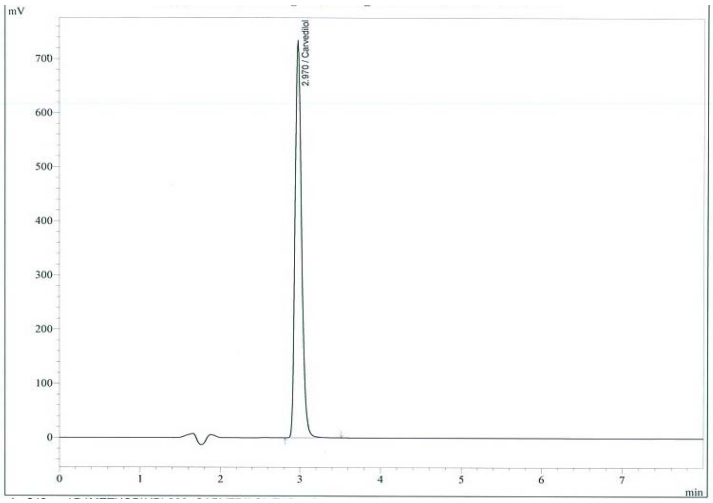
Weigh and transfer 5 tablets into a100ml volumetric flask. Add 50ml of methanol and sonicate to dissolve then make up to the volume with methanol, pipette out 5ml of this solution into a 100ml volumetric flask and dilute up to the mark with mobile phase. Filter through 0.45µ membrane filter.

PROCEDURE:

Inject 10µl portions of blank, standard solution and sample solutions into the chromatograph and record the chromatograms. Record the peak responses for the major peaks.

TABLE 1.1 INJECTION SEQUENCE:

S.NO	DESCRIPTION	NUMBER OF INJECTION
1	Blank	1
2	Std solution-1	1
3	Std solution-II	1
4	Std solution-II	4
5	Sample solution	2
6	Std solution	1



Name of Drug	Retention Time	Area	Theoretical Plate	Tailing Factor
Carvedilol	2.96	4121905	5350.4	1.40

EVALUATION OF SYSTEM SUITABILITY:

1. The relative standard deviation for five replicate injections should not be more than 2.0%

2. Tailing factor for carvedilol peak should be not more than 2.5

3. Theoretical plate for carvedilol peak should be not less than 1500

VALIDATION PROGRAMME FOR ASSAY

TABLE 1.2 VALIDATION PARAMETERS

S.NO	PARAMETERS
1	System suitability
2	Specificity
3	Accuracy/Recovery
4	Linearity/and range
5	Precision a) Repeatability b) Intermediate precision c) System precision d) Method precision
6	Robustness
7	Solution stability

SYSTEM SUITABILITY:

Five replicate injections of the standard solutions were injected the percentage RSD for the peak area and tailing factor for carvedilol were calculated.

ACCEPTANCE CRITERIA:

a) % RSD for standard injections should be not more than 2.0%
b) Tailing factor for carvedilol peak should be not more than 2

TABLE 1.3 SYSTEM SUITABILITY

No of injection	Area	Tailing factor
1	4121144	1.514
2	4111957	1.521
3	4112953	1.520
4	4135077	1.525
5	4118577	1.516
Average	4119942	1.519
SD	9286.85	
%RSD	0.23	

RESULT:

a) % RSD for standard injections is 0.23%

b) Tailing factor for carvedilol peak is 1.5

SPECIFICITY:

Blank, placebo, standard, sample solution injected into HPLC system. There was no interference from the blank and placebo at the retention time of carvedilol peak. Peak purity reveals that carvedilol peak was homogeneous and there were no co-eluting peaks at the retention time of carvedilol peak.

PLACEBO PREPARATION:

Weighed and transferred 1.812 gm of placebo into 100ml volumetric flask, 50 ml of methanol added and sonicated to dissolve. Then made up to the volume with methanol. Pipette out 5 ml of this solution into a 100 ml volumetric flask and diluted up to the mark with mobile phase. Filtered through 0.45µ membrane filter and injected into the chromatogram.

ACCEPTANCE CRITERIA*:*

i) All individual peaks should be well separated.

ii) The interference of carvedilol peak from the other peaks should be nil.

iii) The purity of carvedilol peak should be NLT 0.99.

RESULT:

i. All individual peaks are well separated.

ii. The interference of carvedilol peak from the other peaks is nil.

iii. The purity of carvedilol peak is 0.99.

ACCURACY/RECOVERY:

Known amount of carvedilol spiked with placebo at about 80%,100%, and 120%of working concentration in triplicate and analysed as per testing procedure .The percentage recovery was calculated from the amount found and actual amount added.

Figure1(12) accuracy at 80%

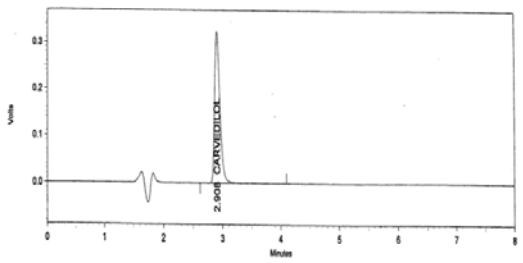


Figure1(13) accuracy at 100%.

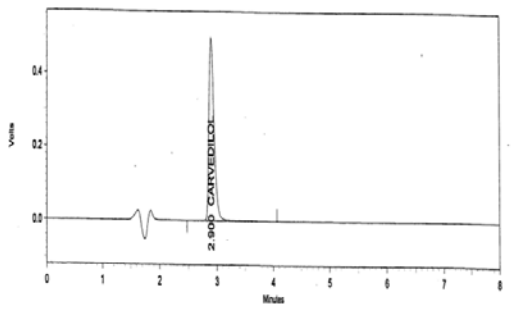


Figure 1 (14) shows accuracy at 120%.

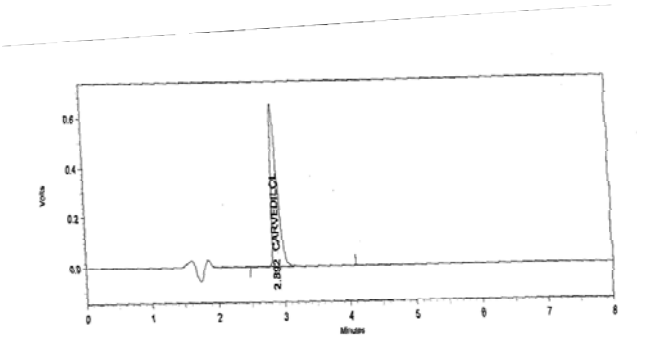


TABLE 1.4 ACCURACY.

LEVEL	**Amount found in µg**	**Actual amount added in µg**	**%recovery**	**Mean**	**%RSD**
Level-1 80%	19.80 19.80 19.78	19.72 19.72 19.72	100.4 100.4 100.3	100.4	0.07
Level-2 100%	24.71 24.69 24.70	24.64 24.64 24.64	100.3 100.2 100.2	100.2	0.07
Level-3 120%	29.76 29.77 29.81	29.58 29.58 29.58	100.6 100.6 100.8	101.7	0.12

ACCEPTANCE CRITERIA:

a) The % recovery should be in between 95% and 105%.

b) The %RSD for all recovery values should be not more than 2%.

RESULT:

a) The % recovery is in between 98.1% and102.7%.

b) The % RSD for all recovery values is 0.64%.

CONCLUSION:

The analytical method meets the acceptance criteria for accuracy study. Hence the method is accurate for the determination of assay of carvedilol tablets.

LINEARITY AND RANGE

The linearity of an analytical procedure is its ability (within a given range)to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

The range of the analytical procedure is the interval between the upper and lower concentration (amount) of analyte in the sample (including the concentrations). For which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity.

PROCEDURE:

PREPARATION OF STANDARD STOCK SOLUTION:

Weigh accurately and transfer about 62.52 mg of carvedilol into a 100 ml volumetric flask. 50 ml methanol added and sonicated to dissolve, then make upto the volume with methanol. Pipette out 10 ml of this solution into a 100 ml volumetric flask and diluted upto the mark with mobile phase. Filtered through 0.45µ membrane filter.

PREPARATION OF SAMPLE SOLUTION:

Transfer the accurately weighed samples 15.62,31.25, 46.88, 62.50, 78.13, 93.75 mg respectively into individual 100 ml flask . 50 ml methanol added and sonicated to dissolve, then make up to the volume with methanol. Pipette out 10 ml of this solution into a 100 ml volumetric flask and diluted up to the mark with mobile phase. Filtered through 0.45 µ membrane filter.

Inject 10µl of blank solution and each linearity level standard solutions into the chromatographic system and measure the peak area .

The linearity of carvedilol was performed in the range of 15.62µg/ml to 93.75µg/ml (25% - 150 % of working concentration). A graph was plotted with concentration in µg/ml on x axis and peak area on y axis. Slope, y intercept, correlation coefficient (r value), were determined.

ACCEPTANCE CRITERIA:

The correlation coefficient should be not less than 0.99.

Figure 1 (15) linearity at 50%

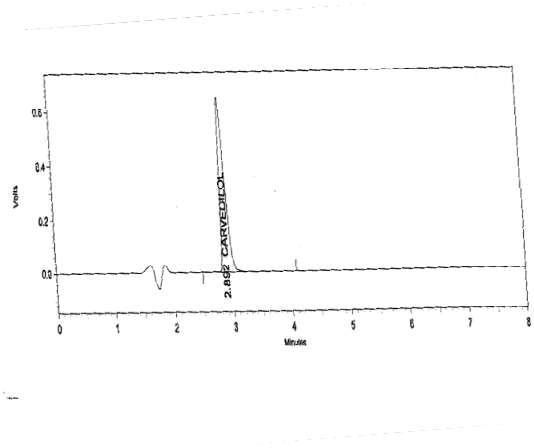


Figure 1 (16) linearity at 100%

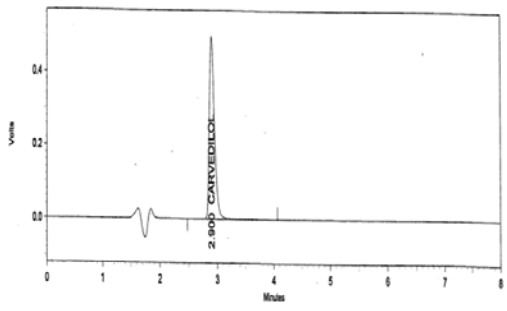


Figure 1 (17) linearity at 150%

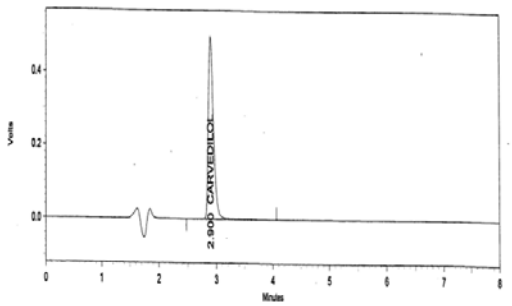
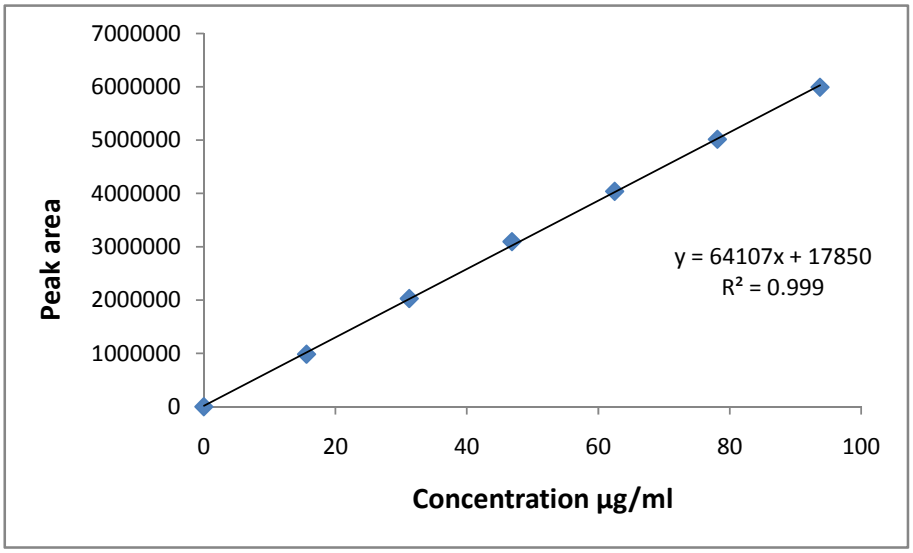


TABLE 1.5 LINEARITY.

LEVEL	CONCENTRATION IN µg/ml	PEAK AREA
25%	15.62	985504
50%	31.25	2030094
75%	46.88	3096294
100%	62.50	4039967
125%	78.13	5016528
150%	93.75	5992094

Calibration Curve for Carvedilol



RESULT:

The correlation coefficient is 0.999.

PRECISION:

a)REPEATABILITY:

Prepare the standard and sample solutions. Inject standard and sample preparations and record the chromatograms .Calculate the % content of carvedilol.

ACCEPTANCE CRITERIA

% content of carvedilol should be in between 90.0 to 110.0

% RSD should be not more than 2.0%.

TABLE 1.6 REPEATABILITY.

SAMPLE	**Wt(mg)**	**AREA**	**% ASSAY**
1	1798.25	4123717	97.51
2	1812.02	4117537	96.62
3	1825.02	4174580	97.26
4	1820.13	4152250	97.00
5	1835.14	4175261	96.74
6	1841.13	4193482	96.85
		Average	96.99
		SD	0.33
		% RSD	0.34

INTERMEDIATE PRECISION (RUGGEDNESS):

Ruggedness of the method was verified by analysing the six samples of same batch which was used for method precision as per testing procedure .This study was performed by different analyst using different instrument and different column on different day. calculated the percentage assay and percentage relative standard deviation for six assay results.

ACCEPTANCE CRITERIA:

a) Content of carvedilol should be in between 90.0 to 110.0.

b) The %RSD between the average results of two analysts should be not more than 2.0%

RESULT:

The % RSD between the average results of two analysts is 0.77

TABLE **1.7 RUGGEDNESS.**

Sample No	**Percentage of assay(w/w)**	
	Analyst-I	**Analyst-II**
1	99.4	99.0
2	99.9	97.0
3	100.5	97.4
4	99.6	97.1
5	99.4	98.9
6	100.6	98.7
Average	99.9	98.0
%RSD	0.54	1.0

RESULT: Percentage relative standard deviation value indicates an acceptable level of ruggedness of the analytical method for the determination of assay of carvedilol tablets.

SYSTEM PRECISION: Five replicate injections of standard solution were injected .The mean and percentage RSD for the peak areas of carvedilol were calculated .

ACCEPTANCE CRITERIA:

The percentage RSD for peak areas of carvedilol is not more than 2.0.

FIG1 (18) SYSTEM PRECISION

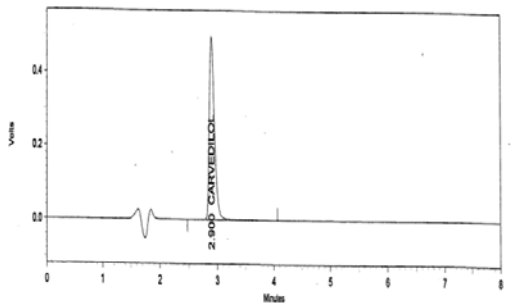


Fig 1(19) SYSTEM PRECISION

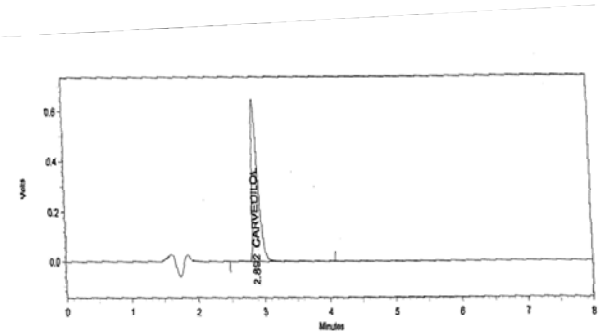


Fig1 (20) SYSTEM PRECISION

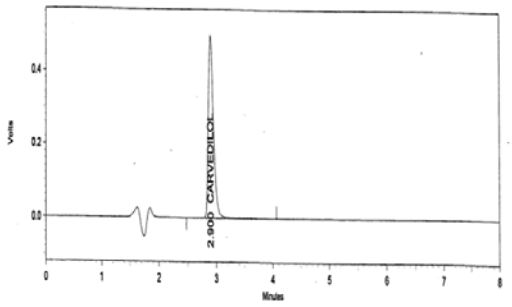


TABLE 1.8 PRECISION .

Sr.No.	**Peak areas**
1	4200402
2	4186308
3	4199120
4	4184070
5	4203797
Mean	4194739
% RSD	0.2

CONCLUSION:

Percentage RSD values indicates an acceptable level of precision of the analytical method for the assay of carvedilol tablets.

METHOD PRESCISION:

Six samples of 25mg tablets were analysed as per test method . The percentage of assay and percentage RSD of six results were calculated.

ACCEPTANCE CRITERIA:

The percentage RSD for percentage assay from six samples is not more than 2.0.

TABLE 1.9 METHOD PRECISION.

Sr. No	**Percentage assay**
1	99.0
2	97.0
3	97.4
4	97.1
5	98.9
6	98.7
mean	98.0
% RSD	1.0

CONCLUSION:

Percentage RSD values indicates an acceptable level of precision of the analytical method for the assay of carvedilol tablets.

ROBUSTNESS:

Carry out the analysis, mentioned under deliberately modified conditions mentioned in the table

TABLE 1.10 CONDITIONS OF ROBUSTNESS.

S.NO	PARAMETERS	NORMAL CONDTION	HIGHER SIDE	LOWER SIDE
1	p^H	3.0	3.2	2.8
2	Flow rate	1.0ml/min	1.2ml/min	0.8ml/min
3	wavelength	242nm	244nm	240nm

ACCEPTANCE CRITERIA:

% Content of carvedilol should be in between 90.0 to110.0.

% RSD should be not more than 2.0%.

PROCEDURE:

Injected all the standard and sample preparations and recorded the chromatograms.
Calculated the % content of carvedilol.

TABLE 1.11 ROBUSTNESS.

SAMPLE TYPE	**%ASSAY**
Flow 0.8ml(spl-1)	100.5
Flow0.8ml(spl-2)	100.4
Flow1.2ml(spl-1)	100.8
Flow1.2ml(spl-2)	100.2
PH 2.8(spl-1)	100.5
PH 2.8(spl-2)	100.1
PH3.2(spl-1)	100.2
PH 3.2(spl-2)	100.0
240nm(spl-1)	100.2
240nm(spl-2)	100.2
244nm(spl-1)	100.3
244nm(spl-2)	100.0
Average	100.3
SD	0.23
% RSD	0.23

RESULT:

% Content of carvedilol is 100.3%.

% RSD is 0.23%.

SOLUTION STABILITY:

Stability of analytical solution was verified by analyzing the standard and filtered sample solution initially and also at different time intervals as mentioned below by storing in sample compartment of HPLC instrument at ambient condition. Calculated the cumulative percentage RSD for peak areas of carvedilol for both the sample and standard solution.

ACCEPTANCE CRITERIA:

The cumulative percentage RSD for peak area is not more than 2.0

Figure 1(21) solution stability at 8 hour

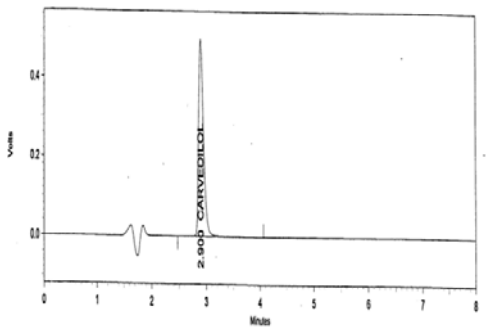


Figure 1(22) solution stability at 16 hour

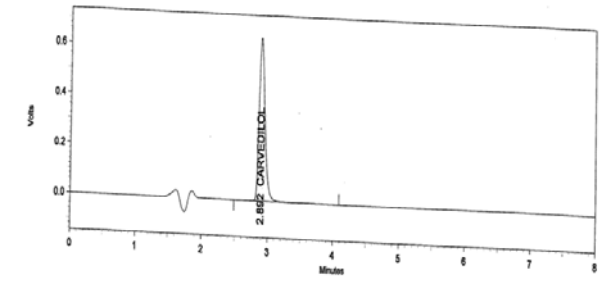
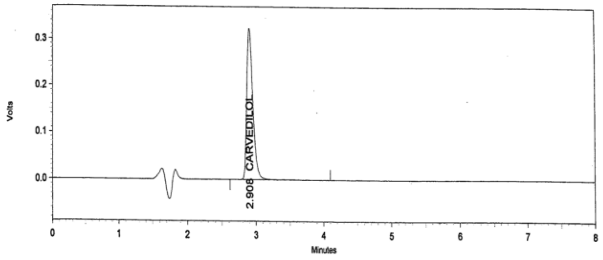


Figure 1(23) solution stability at 24 hours



STANDARD SOLUTION:

TABLE 1.12 SOLUTION STABILITY.

TIME(HOURS)	PEAK AREA	CUMULATIVE % RSD
Initial	4211010	
4	4290684	1.3
8	4240415	0.9
12	4310783	1.1
16	4399875	1.7
20	4352040	1.6
24	4338045	1.5

SOLUTION STABILITY OF SAMPLE :

TABLE1.13 SOLUTION STABILITY OF SAMPLE SOLUTION.

TIME(HOURS)	PEAK AREA	CUMULATIVE % RSD
Initial	4172514	-
4	4112804	1.0
8	4177426	0.9
12	4218648	1.0
16	4232835	1.1
20	4205063	1.0
24	4228953	1.0

CONCLUSION:

The standard and filtered sample solution stable up to 24 hours when stored at ambient condition.

SUMMARY &CONCLUSION FOR ASSAY:

RESULT: The Result of the above study is tabulated as below. The test parameters meet the acceptance criteria .The method employed in this analysis of carvedilol tablets –Assay is validated.

TABLE 2.14 SUMMARY &CONCLUSION FOR ASSAY:

S.NO	TEST	ACCEPTANCE CRITERIA	RESULT
1	System suitability	a. %RSD for std injection is NMT 2.0% b. Tailing factor for carvedilol peak is NMT 2.5	0.23 1.5
2	Specificity	a. All individual peaks should be well separated. b. The interference of carvedilol peak (main peak)from the other peaks should be Nil. c. The carvedilol purity is NLT 0.99	Complies
3	Accuracy	a. The % recovery is in between 95% and 105%. b. The %RSD for all recovery values should be NMT 2%.	98.1% and 102.7% 0.64%
4	Linearity	The correlation co efficient should be NLT 0.99	0.99
5	Precision	a. %Content of carvedilol should be in between 90.0 to 110.0 (Analyst 1). b. %Content of carvedilol should be in between 90.0 to 110.0(Analyst 2) c. %RSD not more than 2.0%(Analyst 1) d. %RSD not more than 2.0% (Analyst 2) e. The %RSD between the average results of two analysts should be not more than 2.0%	99.9% 98.0% 0.54% 1% 0.77%

6	Robustness	a. % Content of carvedilol should be in between 90.0 to 110.0 b. %RSD not more than 2.0%	100.3% 0.23%
7	Solution stability	a.% RSD not more than 2.0%	0.83% for standard 0.64% for sample

METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF CARVEDILOL IN TABLET DOSAGE FORM BY HPTLC

METHOD DEVELOPMENT

1. **Selection of Mobile Phase:**

The selection of mobile phase was done by trial and error method in which several mobile phases were tried. The best mobile phase was selected based on the R_f value of the drugs. The mobile phase tried were listed below,

1. Chloroform : Ethanol
2. Toluene: Ethyl Acetate: Methanol: Acetic
3. Chloroform : toluene
4. dichloromethane : methanol: toluene
5. chloroform: methanol: toluene

2. **Fixed Mobile Phase:**

For carvedilol, chloroform: methanol: toluene (1.5: 3: 3.5) was selected as mobile Phase.

3. **Activation of Pre-Coated Plates:**

Activated by placing in oven at110-120ºc for 30 minutes after sample spotting

4. **Sample Preparation:**

Preparation of Standard Stock Solution:

The standard stock solutions of 1000 µg/ml of carvedilol were prepared by weighing 100mg of carvedilol dissolved with methanol in 100 ml volumetric flask and made up to the volume.

5. Application of Sample:

Generally 0.1-0.5µl is most satisfactory for HPTLC application of the sample & standard as a band gives better separation, equal R_f values, and less spot broadening .The sample is applied a series of 0.1µl, 0.2µl, 0.3µl, 0.4µl & 0.5µl Standard of carvedilol solution were loaded as 4mm band length in the 6 x 10 Silica gel $60F_{254}$ TLC plate using 100µl Hamilton syringe and CAMAG-LINOMAT-5 instrument.

6. Spot Development :

The Sample and standards loaded plate was kept in Twin trough chamber 10 x 10cm with respective mobile phase up to 15min for Chamber saturation. After completion of chamber saturation, the plate was kept in mobile phase for development up to 90mm.

7. Photo-Documentation:

The developed plate was dried by hot-air to evaporate solvents from the plate and the plate was kept in Photo-documentation chamber. The images of developed plate were captured at white light, UV 254nm and UV 366nm using CAMAG-REPROSTAR-3 instrument.

8. Scanning:

The developed plate was scanned in UV 254nm wavelength for carvedilol using CAMAG-TLC SCANNER-3 instrument. The Baseline display, Peak densitogram & Peak table of each track were obtained. The carvedilol content present in the loaded sample was evaluated by Peak assignment with 5 level carvedilol standards.

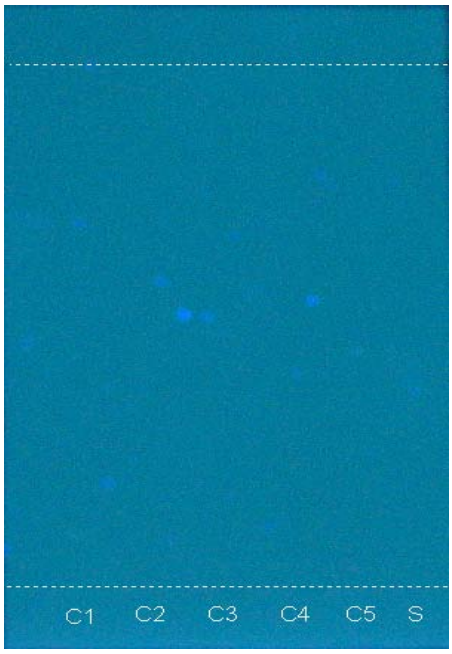
9. Spectrum scanning:

The assigned peaks of standards and sample were scanned in spectrum of UV region (200nm-400nm) and found the λmax value of carvedilol.

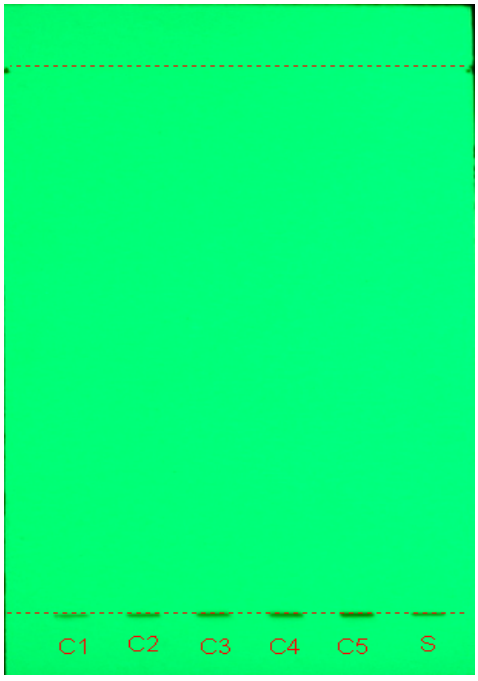
CHROMATOGRAMS

Daylight before mobile phase run

UV 366nm before mobile phase run

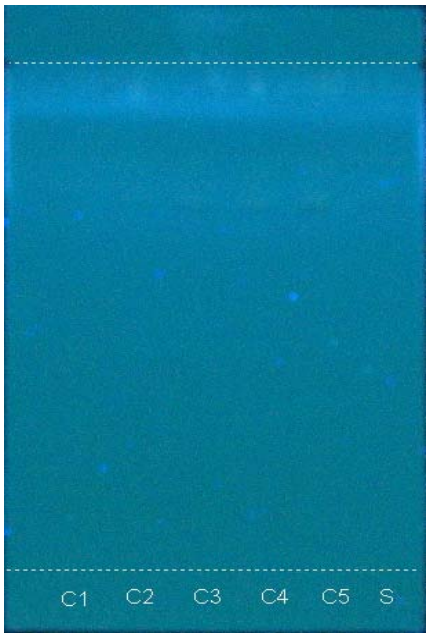


UV 254 nm before mobile phase run

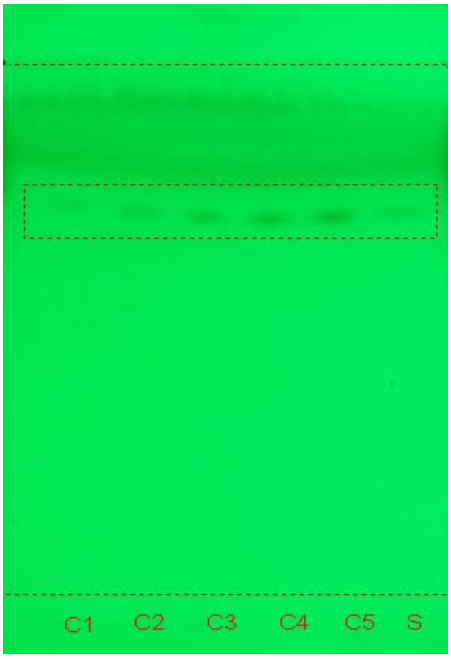


Daylight after mobile phase run

UV 366nm after mobile phase run



UV 254 nm after mobile phase run



10. Linearity:

Aliquots of 0.1-0.5 µg/spot of standard solution of carvedilol is applied on the plate and then developed and detected as earlier method. The calibration curves constructed with the concentration range must obey Beer's law. The linearity was evaluated by regression analysis. The linearity of the drug was determined by calibration curves and the linearity based on the area observed for carvedilol. The regression co-efficient value for carvedilol is 0.999 respectively. The results are summarized in table 1.

TABLE 2.1 LINEARITY OF CARVEDILOL

SL.No	Standard concentration µg/ spot	Peak Area
1	0.1	756.35
2	0.2	1387.96
3	0.3	2081.94
4	0.4	2775.92
5	0.5	3469.9

CALIBRATION CURVE FOR CARVEDILOL

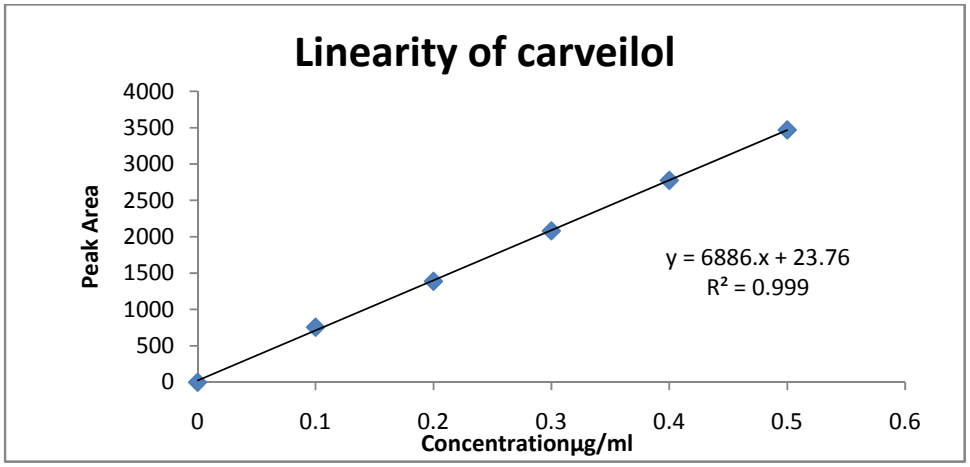


Table 2 -2 Calibration Parameters for CARVEDILOL

Parameters	CARVEDILOL
Linearity Range(µg/Spot)	0.1 – 0.5
Slope	6886
Intercept	23.76
Regression Co-Efficient	0.999

Fig 2.1 Carvedilol Densitogram-0. 1µg

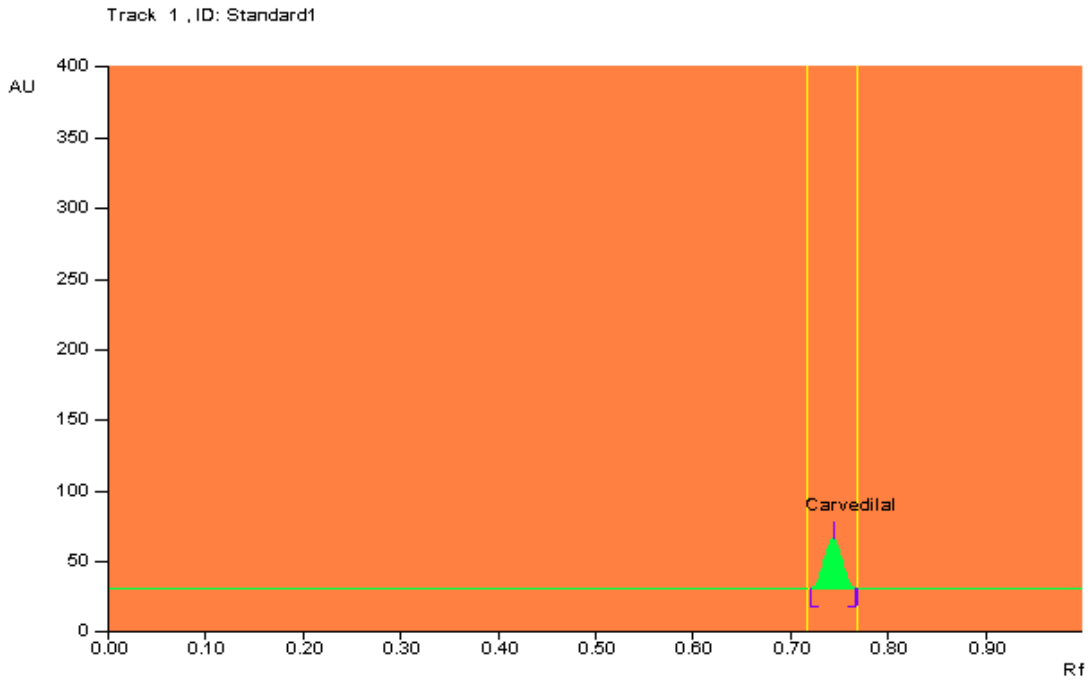


Fig 2.2 Carvedilol Densitogram-0. 2µg

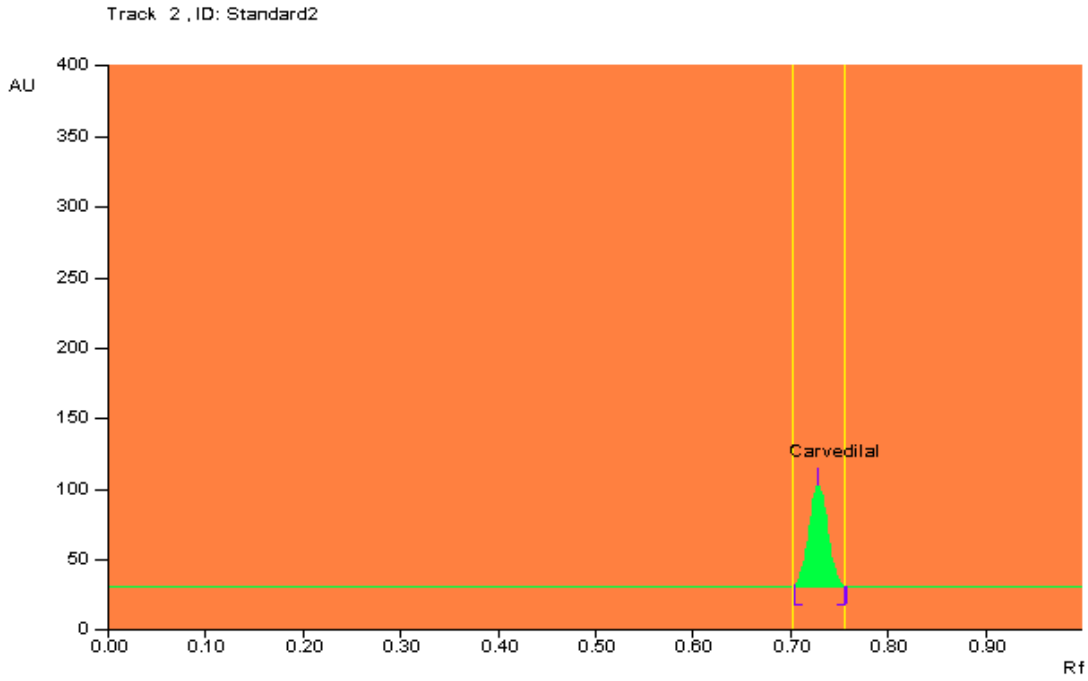


Fig 2.3 Carvedilol Densitogram-0. 3µg

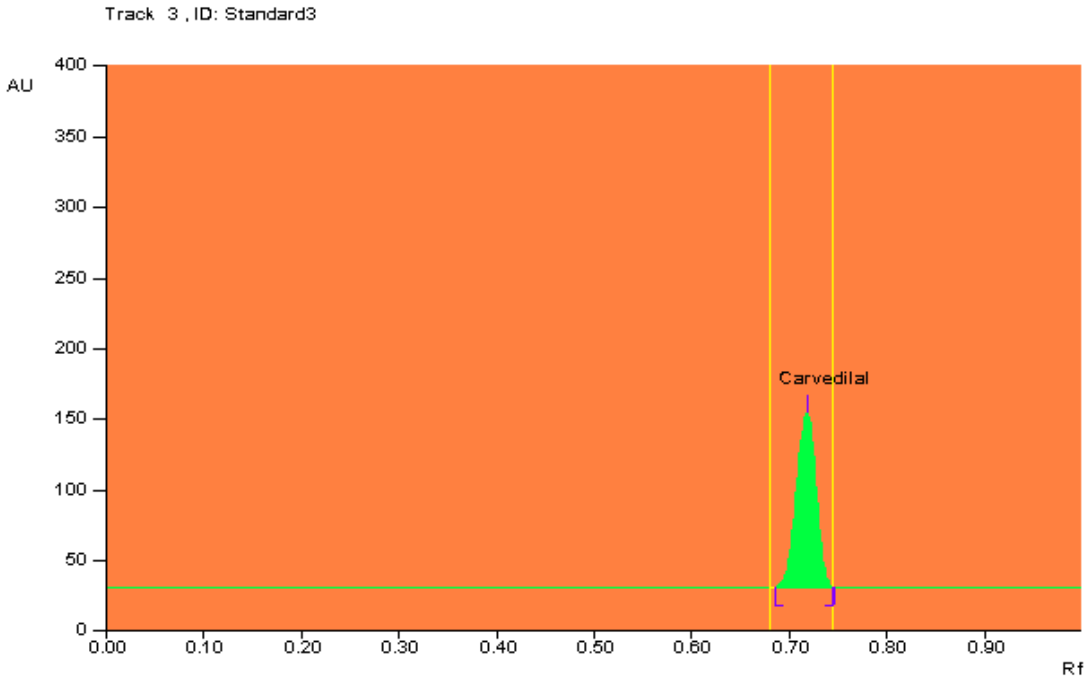


Fig 2.4 Carvedilol Densitogram-0.4µg

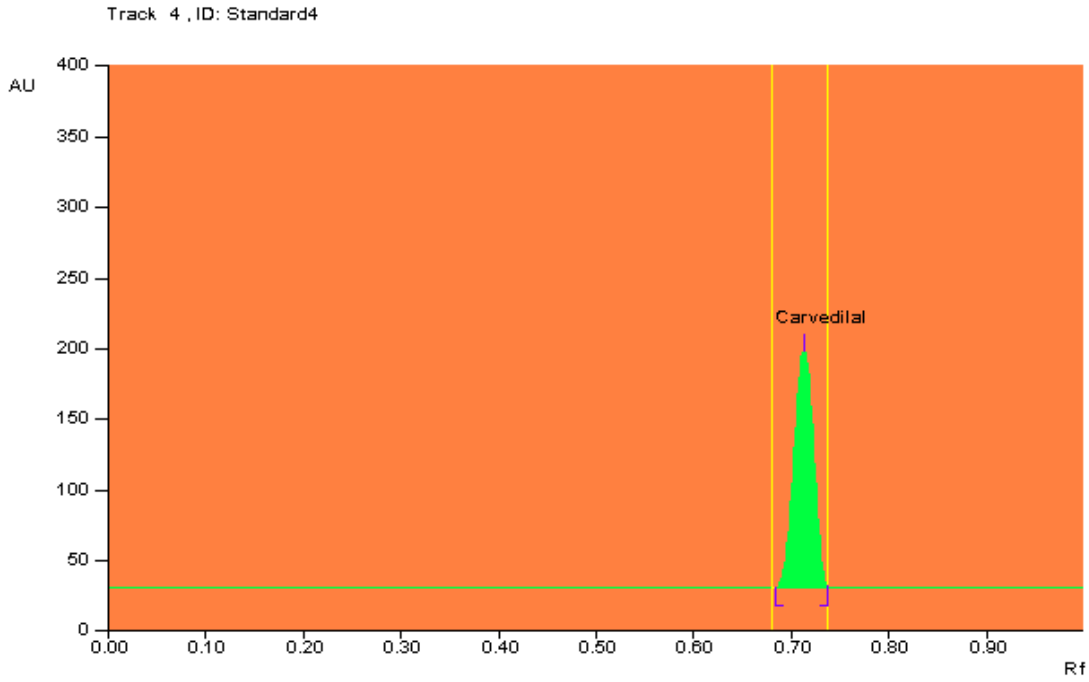
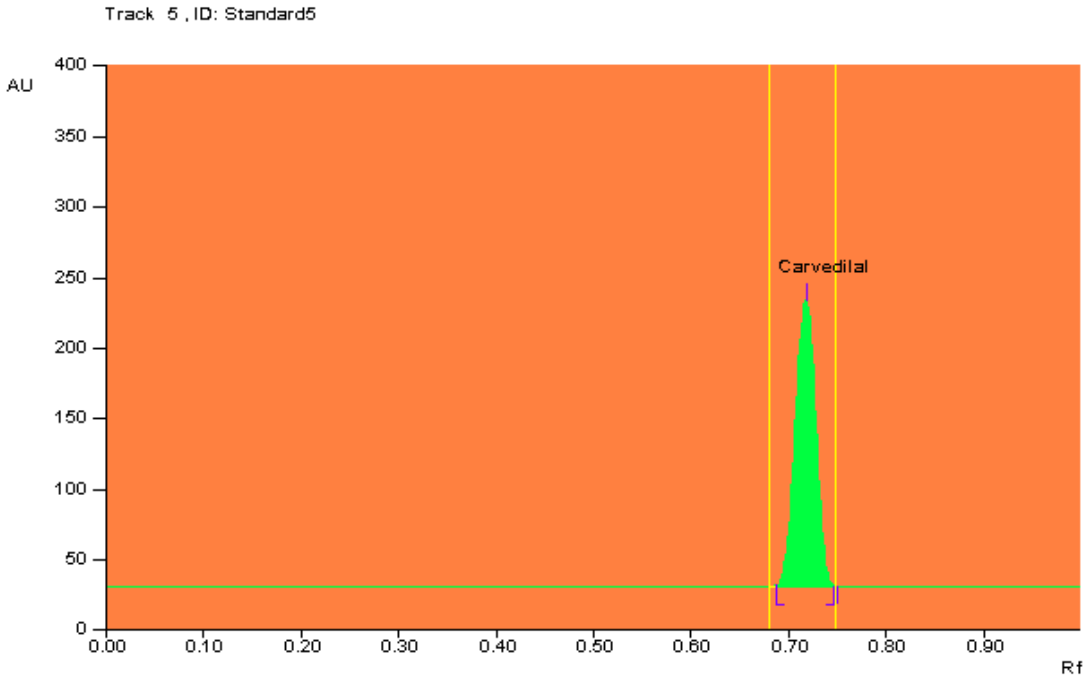


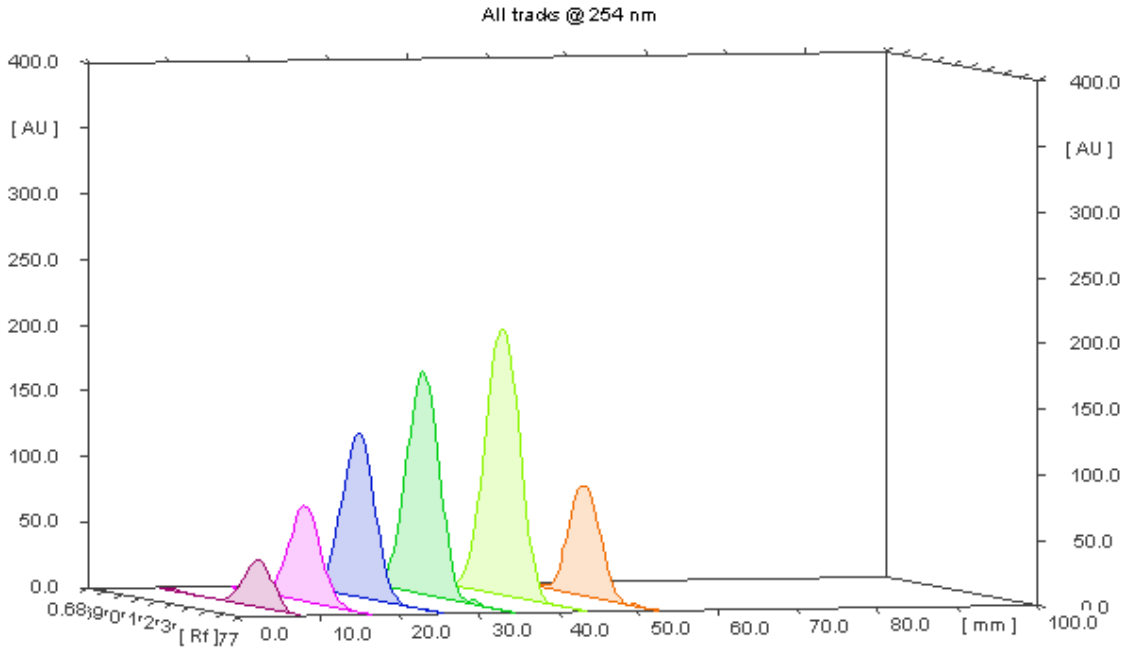
Fig 2.5 Carvedilol Densitogram – 0.5µg



OVERLINE SPECTRA

All colours except orange – Standard Carvedilol ; orange mark - Sample

3D display of Peak densitogram – All tracks (Carvedilol standards and sample) @ 254nm



ANALYSIS OF FORMULATION:

Preparation of Sample Solution:

Twenty tablets were powdered and weighed equivalent to 100 mg of carvedilol which is transferred in to a100ml volumetric flask and extracted with methanol the extract was filtered through Whatman filter paper No.41 and residue washed with methanol and made up to 100ml with methanol. Aliquot of 0.3µl solution of tablet formulation were applied and plate was developed with mobile phase.

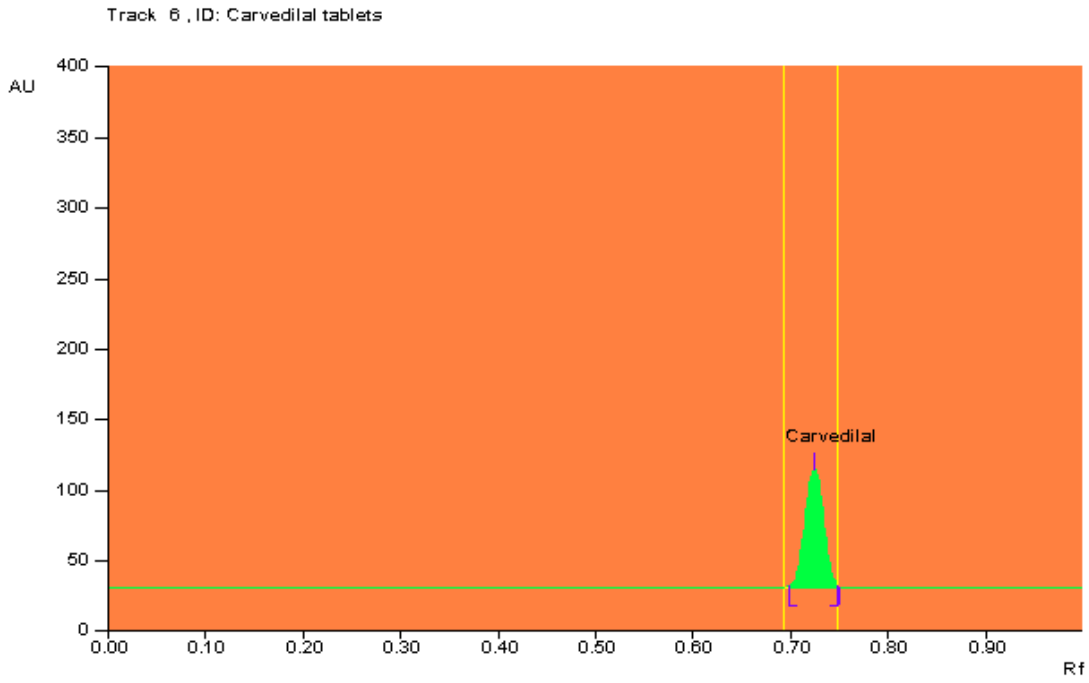
Assay:

The sample solutions were spotted along with the standard to check the specificity. Spotted 0.3µl of sample solution allowed to develop in appropriate mobile phase and detect the spots as described earlier. From the peak area recorded the amount of the drug in the formulation was determined.

Table 2-3 Analysis of Formulations

S.No.	Drug	Label Claim(mg)	Amount found (mg)	Assay %RSD
1.	Carvedilol	25	24.87	99.5

Fig 2.6 Carvedilol densitogram -Sample



METHOD VALIDATION:

1. Linearity:

Aliquots of 0.1-0.5 µg/spot of standard solution of Carvedilol is applied on the plate with the help of micro liter syringe using an automatic sample applicator. The plates were developed, dried and scanned densitometrically at 254 nm. The drug peak-area was calculated for each concentration level and a graph was plotted of drug concentration against the peak area and shown in (Fig.3). Calibration parameters are given in table 2 : 5

Table 2-4 Linearity of Carvedilol

SL.No	Standard concentration µg/ spot	Peak Area	Peak Height
1	0.1	756.35	35.8
2	0.2	1387.96	72.4
3	0.3	2081.94	123.9
4	0.4	2775.92	168.8
5	0.5	3469.9	203.4

CALIBRATION CURVE OF CARVEDILOL

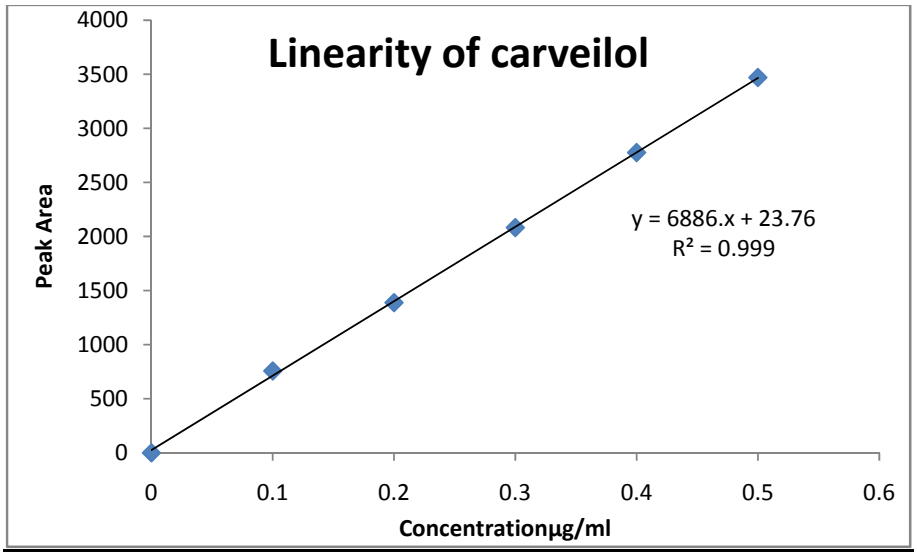


Table 2 -5 Calibration Parameters for Carvedilol

Parameters	Carvedilol
Linearity Range(µg/Spot)	0.1-0.5
Slope	6886
Intercept	23.76
Regression Co-Efficient	0.999

2. Accuracy:

Accuracy of the developed method was confirmed by doing a recovery study as per ICH guidelines at three different concentration levels (80%, 100% and 120%) by replicate analysis (n=3). Standard drug solutions were added to a preanalyzed sample solution, and then percentage of drug content was calculated. The results of the accuracy study are reported in Table **5**. From the recovery study, it was clear that the method is very accurate for quantitative estimation of Carvedilol in tablet dosage form because all the statistical results were within the acceptance range (i.e., % RSD <2.0).

Table 2 -6 Recovery studies for Carvedilol (n=3)

Label claim (mg / tablet)	Recovery level (%)	Amount added (mg)	Amount recovered (mg) ± % RSD	% Recovery
25	80	20	19.96±0.42	99.82
25	100	25	24.65±0.85	98.67
25	120	30	30.45±0.59	101.5

3. Precision:

The precision of the method (system reproducibility) was assessed by spotting 0.3µl of drug solution six times on a TLC plate, followed by development of plate and recording the peak area for 6 spots. The % RSD for peak area values of carvedilol was found to be 0.58. The results were shown in Table-7.

The method reproducibility (intra-day precision) was determined by analyzing standard solution in the concentration range of 0.1 µg/spot to 0.5 µg/spot of drug for 3 times on the same day and inter-day precision was determined by analysing corresponding standards daily for 3 day over a period of one week. The intra-day and inter-day coefficients

of variation (%RSD) are in range of 0.13 to .36 and 0.30 to 0.56, respectively. The results were shown in Table- 7a, 7b.

Table 2 – 7 Precision of Carvedilol

S.No	**Concentration (µg/ spot)**	**Peak Area**
1.	0.3	2081.94
2.	0.3	2111.40
3.	0.3	2090.52
4.	0.3	2075.36
5.	0.3	2087.25
6.	0.3	2092.17
Mean	-	2089.77
Percentage Relative Standard Deviation	-	0.58

Table 2 – 7a Intra-day Precision of Carvedilol

S.No	Concentration (µg / spot)	Area	Mean	Standard Deviation	% RSD
1.	0.1	756.35			
2.	0.1	761.05	757.3	3.37	0.44
3.	0.1	754.50			
1.	0.3	2080.50			
2.	0.3	2083.72	2080.82	2.74	0.13
3.	0.3	2078.25			
1.	0.5	3469.50			
2.	0.5	3465.64	3470.32	5.13	0.14
3.	0.5	3475.82			

Table 2 – 7b Inter-day Precision of Carvedilol

S.No	Concentration (µg / spot)	Area	Mean	Standard Deviation	% RSD
1.	0.1	757.98			
2.	0.1	754.50	758.28	3.93	0.52
3.	0.1	762.35			
1.	0.3	2081.94			
2.	0.3	2090.50	2083.56	6.28	0.30
3.	0.3	2078.25			

DISCUSSION:

The proposed HPTLC analytical method for the quantification of carvedilol in tablet formulation is simple, accurate, rapid and can be employed for the routine analysis. Once the R_f value of the sample is determined, it requires only simple calculation. This method can be applied for the substances which obey Beer's law. The low standard deviation and good percentage recovery indicated the reproducibility and accuracy of the method.

DEVELOPMENT OF UV SPECTROSCOPIC METHOD FOR THE QUANTIFICATION OF CARVEDILOL

Carvedilol contains chromophore system which can absorb UV radiation which is the basis of developing an UV method for its estimation.

AIM:

To perform UV spectrophotometric analysis of carvedilol in bulk and pharmaceutical formulations.

3.1 PRACTICAL REQUIREMENT :

UV spectrophotometric analysis of carvedilol in bulk and pharmaceutical formulations was performed using a Shimadzu UV- 1700 double beam UV visible spectrophotometer with 1cm quartz cell.

Name of the balance : Sartorius- weighing range 20mg-200gm

Name of the formulation : Commercially available tablets of carvedilol were procured from the local market and quantification performed.

3.2 : Reagent used : Methanol [A.R. Grade] Merck.

SELECTION OF SOLVENTS:

The UV spectrum of carvedilol was recorded in various solvents .The spectral pattern and absorbance maxima of carvedilol were thoroughly analysed. It was found that significant spectra of carvedilol appeared in methanol and this solvent was selected for determining carvedilol content in formulation by UV spectroscopic method. Stock solution of carvedilol was prepared by dissolving 100mg of drug in 100ml of methanol to obtain the concentration of 1000µg/ml. It was further diluted to obtain concentration ranging from 1-5µg/ml.

2**. SELECTION OF WAVELENGTH:**

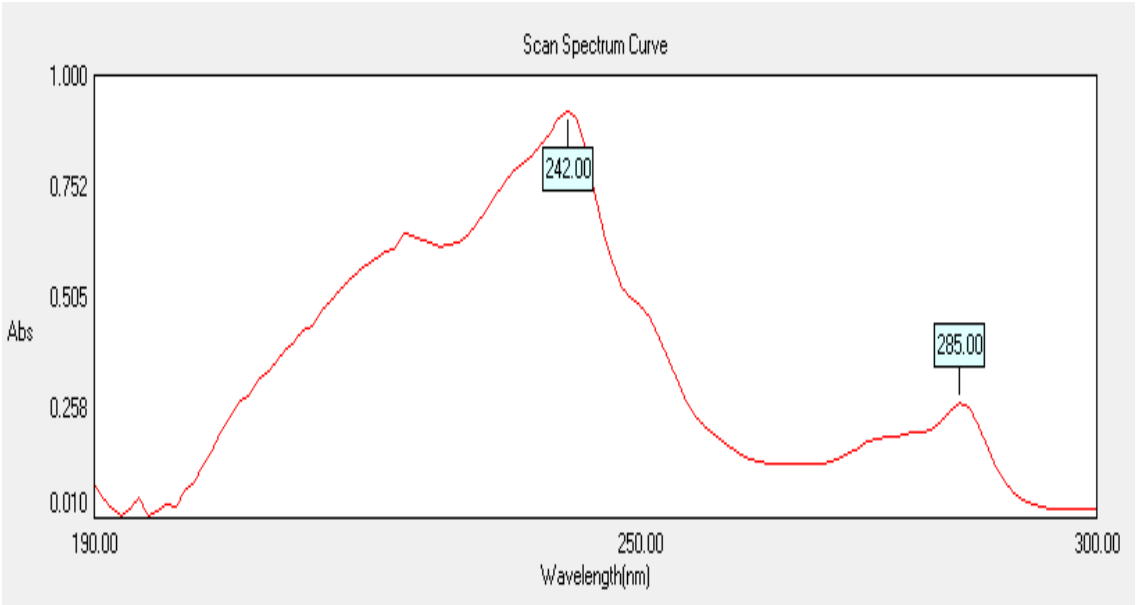
The stock solution was suitably diluted with methanol, so as to contain 10µg/ml of carvedilol. This solution was scanned in the UV region and found that carvedilol exhibited maximum absorbance at about 242 nm .Hence 242 nm was selected for the proposed study.

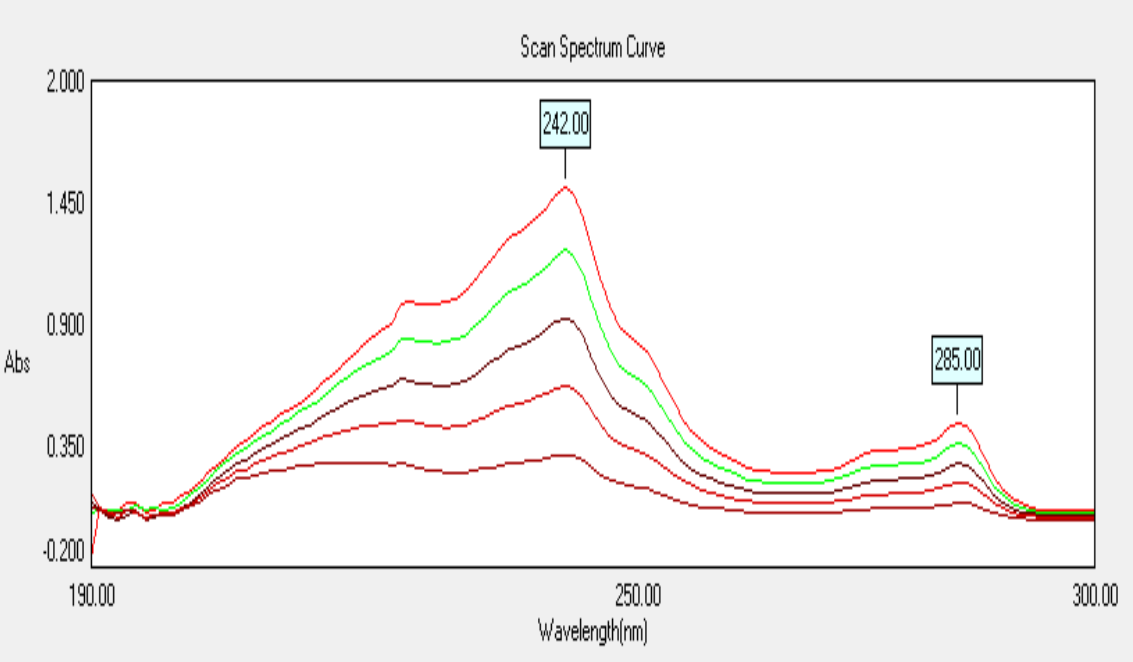
PREPARATION OF STANDARD CURVE :

Adequate dilutions were made from stock solution to get a concentration ranging from 1-5µg/ml for carvedilol using methanol. Absorbance of these solutions were measured at 242nm (Table3.1) .The measured absorbance was plotted against concentration .From the graph it was found that the Beer's law concentration for carvedilol lies between 1 -5µg .The overlain spectra and the calibration graph are shown in fig (1).

Table3.1 Absorbance Of Carvedilol

Concentration (µg/ml)	Absorbance
1	0.113
2	0.220
3	0.323
4	0.433
5	0.521





QUANTIFICATION OF CARVEDILOL IN FORMULATION :

The average weight of 20 tablets of carvedilol was weighed .An aliquot quantity equivalent to 100mg of carvedilol was weighed and transfered to 100ml volumetric flask. The contents were shaken with methanol, so as to dissolve the active ingredients and filtered .Then it was made upto the volume .It was further diluted to obtain the stock solution with a concentration 50µg/ml. From this solution 3ml was taken and diluted to 50 ml to get a concentration of 3µg/ml .The resultant solution was scanned in the wavelength range of 200-400nm and the absorbance was measured. The concentration of drug was determined by single point standardization method and results are shown in table1.2

$$C_{test} = \frac{A_{test} \times C_{stnd}}{A_{stnd}}$$

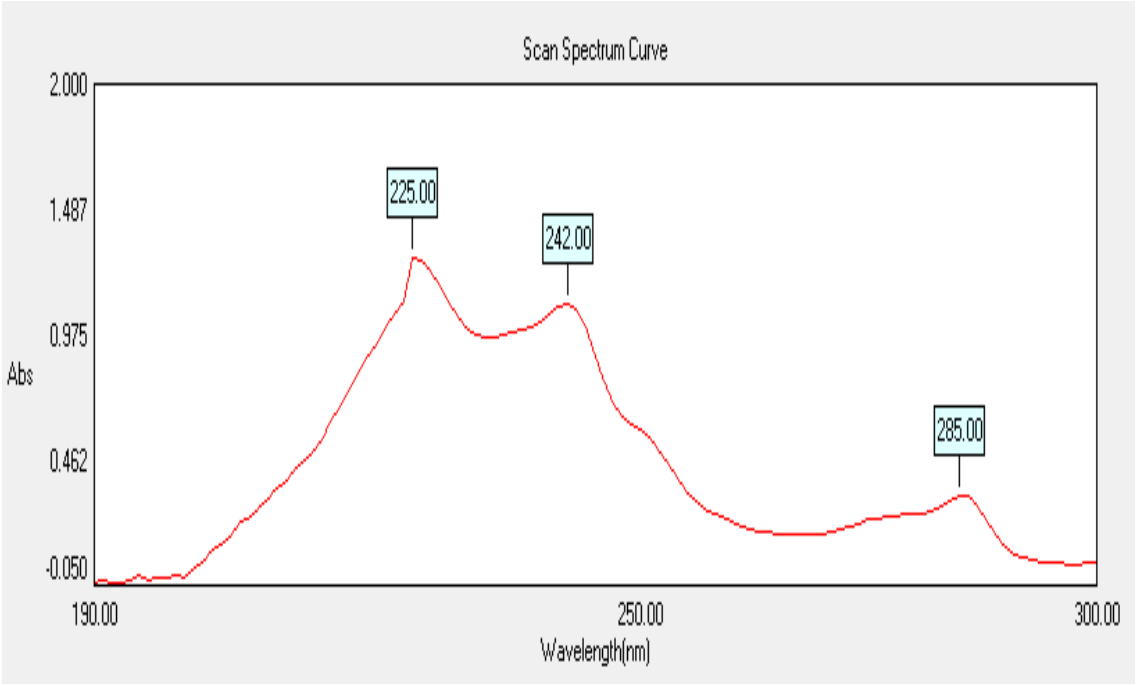


Table 3.2 Analysis of carvedilol formulation

Drug	Amount mg/tab	%Label claim	%RSD
	Label	Found	
Carvedilol	25 mg	25.43 25.4 25.38 25.45 25.43 25.4	0.051

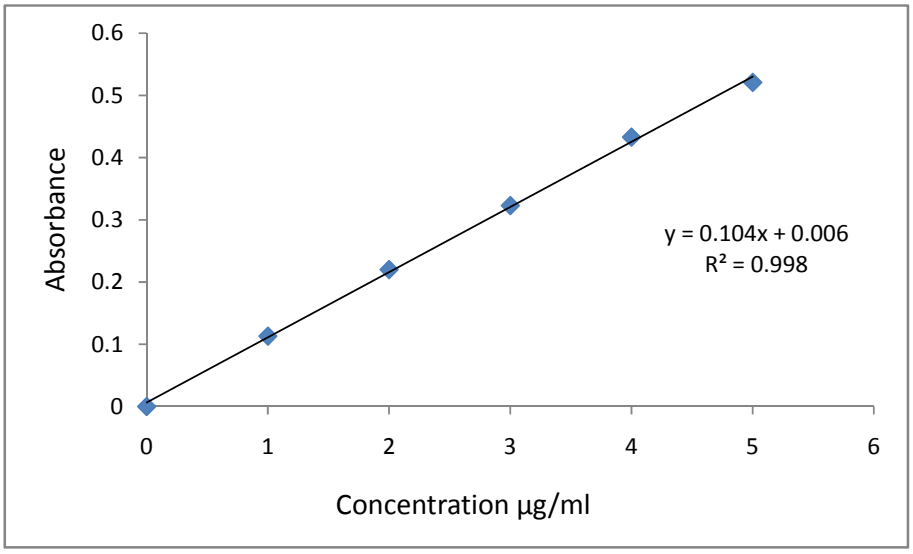
VALIDATION OF THE METHOD

The developed method was validated in terms of linearity, accuracy and stability studies.

1) Linearity

Carvedilol was found to be linear in a concentration range of 1-5µg/ml. The absorbance of this solution were measured at 242 nm and a calibration graph was plotted using absorbances versus concentration.The correlation co-efficient value was found to be 0.998.

CALIBRATION CURVE OF CARVEDILOL



2) Accuracy

The accuracy,specificity, suitability and validity of the present method were satisfied by conducting percentage recovery studies. A known quantity of the drug was added to the pre-analyzed sample formulation at 50% and 100% levels. The percentage recovery and standard deviation were calculated.(Table 3).

The % recovery was calculated by using the following formula

$$\%\text{Recovery} = \frac{\text{Amount of drug found after addition of standard drug} - \text{Amount of drug found in sample before addition of standard drug}}{\text{Amount of standard drug added}} \times 100$$

TABLE 3.3 RECOVERY STUDIES

Drug	Level	Amount found in µg	Actual amount Added in µg	%Recovery	% RSD
Carvedilol	50%	12.30	12.22	100.6	0.12
		12.28	12.22	100.4	
		12.30	12.22	100.6	
	100%	24.71	24.64	100.3	0.06
		24.69	24.64	100.2	
		24.70	24.64	100.2	

3**) Stability**

The drug solution was found to be stable for about three hours at room temperature. Stability data reported in table 4.

TABLE 3.4 STABILITY DATA

Concentration in µg/ml	Time (min)	Absorbance
1	0	0.113
	30	0.110
	60	0.109
	90	0.117
	120	0.112
	150	0.107
	180	0.108

TABLE 3. 5 REPEATABILITY STUDIES

Concentration in µg/ml	Absorbance	%RSD
1	0.117	0.93
	0.118	
	0.119	
	0.117	
	0.117	
	0.116	
5	0.516	0.47
	0.513	
	0.511	
	0.510	
	0.515	
	0.511	

TABLE 3. 6: INTER-DAY PRECISION

Concentration µg/ml)	Day	Absorbance	%RSD
1	1	0.116	1.02
	2	0.117	
	3	0.115	
	4	0.115	
	5	0.116	
	6	0.114	
5	1	0.512	0.40
	2	0.513	
	3	0.512	
	4	0.516	
	5	0.510	
	6	0.513	

TABLE 3. 7 INTRA- DAY PRECISION

Concentration µg/ml	6 times in a day	Absorbance	%RSD
1	1	0.115	1.47
	2	0.113	
	3	0.111	
	4	0.114	
	5	0.115	
	6	0.112	
5	1	0.513	0.45
	2	0.515	
	3	0.510	
	4	0.512	
	5	0.515	
	6	0.516	

DISCUSSION

The proposed UV analytical method for the quantification of carvedilol in tablet formulation is simple, accurate, and rapid and can be employed for the routine analysis. Once the absorbance of the sample is determined ,it requires only simple calculation .This method can be applied for the substances which obey Beer's law. The low standard deviation and good percentage recovery indicated the reproducibility and accuracy of the method.

RESULTS AND DISCUSSION

Validated analytical methods are aimed for the estimation of carvedilol in formulation. Simple, precise, rapid, accurate methods were developed for the estimation of carvedilol in formulation by following methods.

Estimation of carvedilol by RP-HPLC.

Estimation of carvedilol by HPTLC.

Estimation of carvedilol by UV-spectroscopy.

In RP-HPLC method, a wavelength of 242 nm was selected and the mobile phase which consist potassium di hydrogen phosphate buffer : acetonitrile, in the ratio of (60:40). pH 3 adjusted with formic acid at a flow rate of 1ml/min were found to be optimum condition for analysis. The retention time was found to be 2.9 with optimized conditions.

Carvedilol showed the linearity in the range of 15.62 -93.75µg/ml. Where the peak shape was symmetrical and a good correlation coefficient value was obtained.

The percentage label claim and recovery at three different levels, 80%, 100%, 120%, level was carried out. The suitability of the method was thus proved. Precision of the method was studied by making repeated injection of the same sample and standard deviation was determined. Inter day and intra day precision was also carried out and % RSD was calculated.

In HPTLC during the stage of method development different mobile phase were tried and mobile phase comprising of ethyl chloroform: methanol : toluene in the proportion of 1.5: 3: 3.5 v/v/v for carvedilol , were found to be better and produced the R_f value of 0.72 for carvedilol.

The linearity of drug was determined by calibration curve and the linearity based on the area observed in the range of 0.1 – 0.5 µg/ ml.

The regression coefficient value for carvedilol is 0.999. Interday precision of the drugs was studied. No interference with the additives of the formulation was reported .Recovery studies were carried out for the accuracy parameter and were reported .

The validated method was applied for the analysis of tablet containing 25 mg carvedilol drug as the label claim. The method developed was simple. It has showed a good peak and good R_f values.

In case of UV-spectroscopic method solubility is the important parameter. Solubility parameter was studied and methanol was selected as the solvent, since it gave a maximum absorbance and a good spectral pattern when compared with other solvents. The linearity was found to be in the range of 1 -5 µg/ml at the maximum absorbance of 242 nm. The marketed formulation was extracted and diluted to get the concentration in the linearity range. The solution was scanned and measured at 242 nm. Percentage recovery, linearity, stability studies were also carried out. The above method gave a satisfactory recovery values and found to be stable, linear, hence it can be used for routine analysis of the drug formulation.

These methods, RP-HPLC, HPTLC, UV spectroscopy were found to be sensitive, precise, and accurate. However these three methods can be used for the routine analysis of carvedilol from formulation.

CONCLUSION

In order to develop a RP-HPLC, HPTLC, UV effective most of the effect should be spent in method development and optimization as this will improve the final method performance. A well developed method should be easy to validate. A method should be developed with the goal to analyse rapidly, the preclinical samples, formulations and commercial samples.

Review of literature on drug strongly indicates that there is few method available for determination and validation of carvedilol in bulk and pharmaceutical dosage forms . Keeping in this mind we developed methods for determination and validation of carvedilol in bulk and pharmaceutical dosage forms by RP- HPLC, HPTLC, UV methods with some improvements than the existing methods.

The analytical procedure described for assay was specific, linear, precise, accurate, and system suitable for determination of carvedilol in bulk and pharmaceutical dosage forms.

The observations of the validation parameters such as accuracy, precision, specificity, linearity, shows that the developed methods can be employed for routine analysis of bulk and tablets form of carvedilol. The result obtained from the validation parameters met the ICH and USP requirement as well as obeys BEER'S law.

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