DEVELOPMENT AND VALIDATION OF ANALYTICAL METHODS FOR THE SIMULTANEOUS ESTIMATION OF ATORVASTATIN CALCIUM, ASPIRIN, RAMIPRIL AND METOPROLOL TARTRATE IN BULK AND IN PHARMACEUTICAL DOSAGE FORM BY UV SPECTROSCOPY AND HPTLC

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In partial fulfillment for the award of Degree of

MASTER OF PHARMACY

(Pharmaceutical Analysis)

Submitted by

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(Accredited by "NAAC" with CGPA of 2.74 on a four point scale at "B" Grade)

MELMARUVATHUR – 603 319

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CERTIFICATE

This is to certify that the research work entitled **"DEVELOPMENT AND VALIDATION OF ANALYTICAL METHODS FOR THE SIMULTANEOUS ESTIMATION OF ATORVASTATIN CALCIUM, ASPIRIN, RAMIPRIL AND METOPROLOL TARTRATE IN BULK AND IN PHARMACEUTICAL DOSAGE FORM BY UV SPECTROSCOPY AND HPTLC"** submitted to The Tamil Nadu Dr. M.G.R. Medical University in partial fulfillment for the award of the Degree of Master of Pharmacy (Pharmaceutical Analysis) was carried out by **S. SATHISH KUMAR (Register No. 26106129)** in the Department of Pharmaceutical Analysis under my direct guidance and supervision during the academic year 2011-2012.

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DEDICATED

TO MY

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SYMBOLS AND ABBREVIATIONS

ICH	-	International Conference on Harmonization
λ	-	Lambda
LOD	-	Limit of Detection
LOQ	-	Limit of Quantitation
µg/ ml	-	Microgram Per Millilitre
mg/ tab	-	Milligram Per Tablet
ml	-	Millilitre
mM	-	Milli Mole
nm	-	Nanometer
pH	-	Negative Logarithm of Hydrogen Ion Concentration
%	-	Percentage
% RSD	-	Percentage Relative Standard Deviation
RP - HPLC	-	Reverse Phase -High Performance Liquid Chromatography
HPTLC	-	High Performance Thin Layer Chromatography
SD	-	Standard Deviation
SE	-	Standard Error
UV-VIS	-	Ultraviolet - Visible
USP	-	United States Pharmacopoeia
IP	-	Indian Pharmacopoeia
BP	-	British Pharmacopoeia
IR	-	Infra Red
°C	-	Degree Celsius

Gms	-	Grams
µl	-	Microlitre
rpm	-	Rotations Per Minute
µ	-	Micron
v/v/v/v	-	Volume/Volume/Volume/Volume
min	-	Minute
ml/ min	-	Millilitre/minute
ng/ µl	-	Nanogram/ microlitre
ATR	-	Atorvastatin Calcium
ASP	-	Aspirin
RAM	-	Ramipril
MET	-	Metoprolol Tartrate
hυ	-	Planck's Constant
LC-MS	-	Liquid Chromatography Mass Spectrometry
GC-MS	-	Gas Chromatography Mass Spectrometry
GC	-	Gas Chromatography
CRF	-	Chromatographic Response Factor
2D – LC	-	Two Dimensional Liquid Chromatography
UPLC-TOFMS	-	Ultra Performance Liquid chromatography – Time of Flight Mass Spectrometry
USFDA	-	United States Food and Drug Administration
WHO	-	World Health Organization
GMP	-	Good Manufacturing Practice

GLP	-	Good Laboratory Practice
S/N	-	Signal to Noise ratio
m	-	Slope
c	-	Intercept
LDP	-	Low Density Lipoprotein
HMG – COA	-	Hydroxy Methyl Glutaryl – Co-Enzyme
CYP	-	Cytochrome Phosphate
ACE	-	Angiotensin Converting Enzyme
COX	-	Cyclo Oxygenase
PGH_2	-	Prostaglandin Hydroxy Synthase
ILC	-	Inverse Least Square
PCR	-	Principle Component Regression
PLS	-	Partial Least Square
ODS	-	Octa Decyl Silane
Hg	-	Mercury
CV	-	Cardiovascular
CABG	-	Coronary Artery Byepass Graft
AR	-	Analytical Reagent
NaI	-	Sodium Iodide

INTRODUCTION

1. INTRODUCTION

1.1 INTRODUCTION TO ANALYTICAL CHEMISTRY (Kellner *et al*., 2004)

Analytical chemistry is a metrological discipline that develops, optimizes and applied measurement processes intended to derive quality (bio) chemical information of global and partial type from natural and artificial objects or systems in order to solve analytical problems derived from information needs.

1.1.1 Aims and Objectives

Thus, Analytical chemistry has two main aims are Qualitative (intrinsic) and Quantitative (Extrinsic). The Qualitative aim is the achievement of metrological quality, i.e. ensuring full consistency between the analytical results delivered and the actual value of the measured parameters; in metrological terms, this translates into producing high traceable results subject to very little uncertainty. The Quantitative aim is solving the analytical problems derived from the (bio) chemical information needs posed by a variety of "clients". Example:- Industry and research centers.

The principal objective of analytical chemistry is to obtain as much (bio) chemical information and of as high a quality as possible from objects and systems by using as little material time, and human resources as possible and with minimal costs and risks.

1.1.2 Two Sides of Analytical Chemistry

The multitude of definitions of analytical chemistry issued to date coverage on two key features, namely its basic and applied natures, which is consistent with the two essential aims.

The basic side of analytical chemistry encompasses a variety of research and development activities aimed at expanding and improving its foundations, such activities include the development of new analytical tools and methods for the preparation of reference materials of a high metrological quality, and the establishment of new calibration procedures and chemometric approaches.

The applied side is directly concerned with solving the analytical problems derived from (bio) chemical information needs in a variety of economic and social areas including industry, the environment, food science, pharmaceutical analysis and clinical analysis. The results obtained by analyzing such a variety of samples are used to make social and economic decisions within the frame work of the analytical problem concerned.

1.2 IMPORTANCE OF ANALYSIS (Kellner *et al*., 2004)

Newer analytical methods are developed for the drugs or drug combinations due to the patient must receive the good quality of drugs, the drug or drug combinations may not be official in any pharmacopoeia, a literature search may not reveal an analytical procedure for the drug or it combinations, analytical methods may not be available for the drug combinations due to interference caused by excipients and analytical methods for the quantification of drug or drug combinations from biological fluids may not be available.

The development of newer analytical methods are very importance in many of the fields like research institutions, quality control department in industries, approved testing laboratories, biopharmaceutical and bio equivalence study and clinical pharmacokinetic studies.

1.3 ANALYTICAL ERRORS (Kellner *et al*., 2004)

Analytical information can be classified in to three major groups according to its nearness to the true value. Further classification allows one to establish the so-called "Chemical metrological hierarchy"

Analytical errors can be defined as alterations of the analytical information delivered, i.e. as differences between the true values or held as true and the parameters on the other levels of the metrological hierarchy, and also as differences between results. An analytical error can arise both from an analytical result and analytical process. It can be expressed in absolute or relative terms. There are three main types of analytical errors are random error, systemic error and gross error.

Types of Analytical Errors

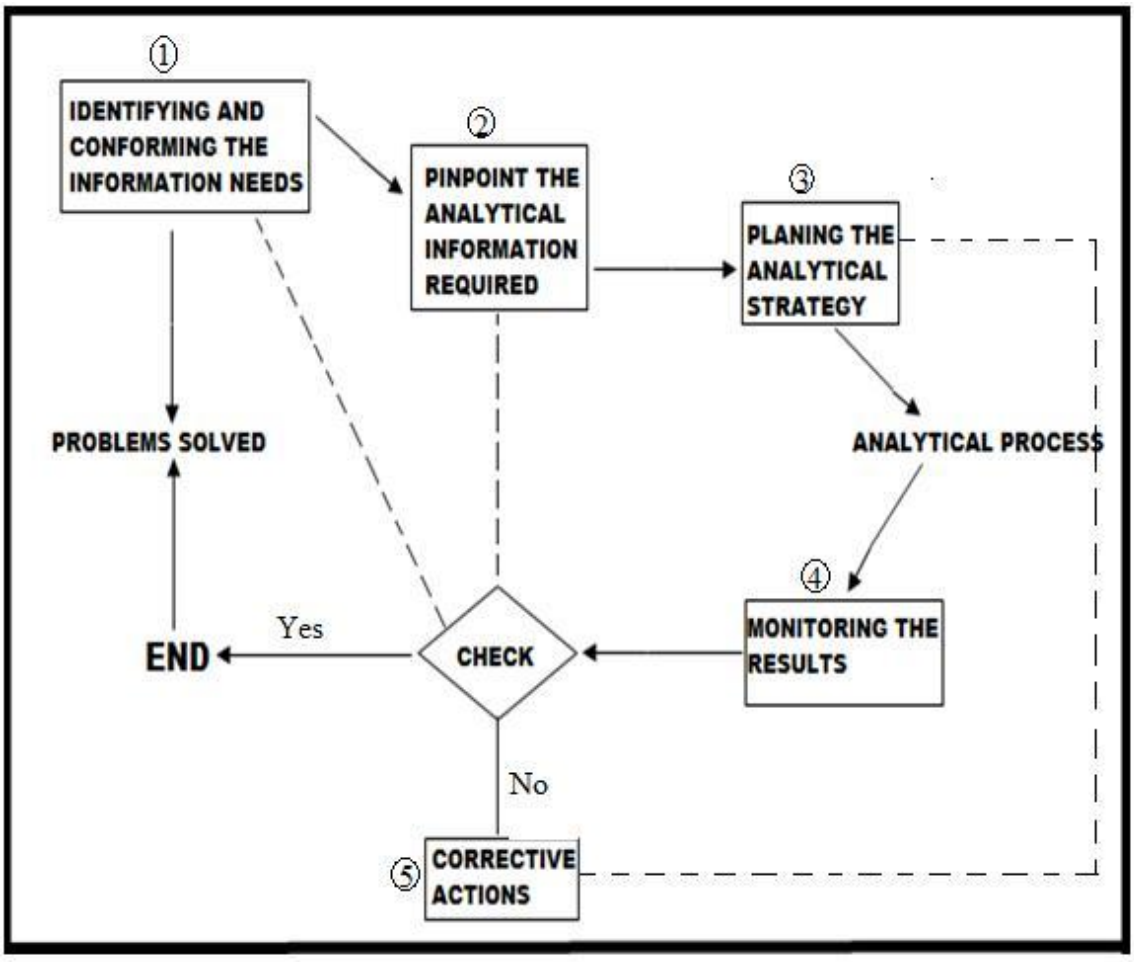
Differences between types of errors	Types of Errors		
	Random	**Systematic**	**Gross**
Source	Indeterminate	Determinate (well defined)	
References for definition	Means of a set ($\bar{x}$, μ')	True value ($x^{\wedge}$) value held as true ($x^{\wedge}$)	
Sign	Unpredictable (+) and (-)	Unique (+) or (-)	
Relative Magnitude (in general terms)	Small	Small	Large
Analytical properties involved	Precision	Accuracy	
		Uncertainty (traceability)	

1.4 QUALITY AND THE ANALYTICAL PROBLEM

The general approach to quality encompasses both the basic and the practical side and their mutual complementary relationships. Analytical chemical quality has two additional components namely: (i) Metrological features, which include capital (accuracy, traceability, uncertainty) and basic analytical properties (precision, sensitivity, selectivity); and (ii) the analytical problem- solving process, which is related to capital and basic properties, and also to productivity- related properties (expeditiousness, cost-effectiveness, personnel-related factors). The basic and applied sides of quality in general and the analytical problem in particular are mutually related.

Properly solving analytical problems in order to ensure client satisfaction is thus an essential ingredient of analytical quality. At present, analytical quality is frequently identified almost soley with the metrological side. It is widely accepted that *"no quality is to be expected unless the objectives are properly defined"*; in fact, the analytical problem provides analytical chemists with such objectives. Also, *"quality is the concern of us all rather than a few"*. It is therefore essential to have analytical laboratories systematically plan and solve analytical problems if integral analytical quality is to be achieved; this entails breaking some traditional barriers of analytical chemistry.

Five General Steps Involving in Planning and Solving Analytical Problems



1.5 ANALYSIS OF DRUGS IN BULK AND DOSAGE FORM

Bulk drugs are obtained by chemical synthesis, biosynthesis isolation from plants or animals or biotechnological sources. Bulk drugs are manufactured in to dosage forms

with the help of additives prior to their use in patients. The purity and the suitability of drugs to be converted into dosage forms are assayed by analytical methods. The evaluation of dosage forms varies with type of dosage form. It includes physical appearance, strength, content uniformity and active ingredients etc. Various components present in dosage forms, including the presence of additives and multiple drug entities such as multi vitamins are some of the challenges encountered during the development of assay methods.

1.6 SAMPLE PRETREATMENT (Lloyd R. Snyder *et al*., 1997)

Drug samples from bulk dosage form need to be extracted with suitable solvent before being taken for analytical purposes. Sample pretreatment ensures such efficient procedures, which provide reproducible and homogeneous drug samples. It will further assure that the sample is (i) relatively free of interference (ii) will not damage the parts of the instruments (iii) will be compatible with assay procedures (such as solvent mobile phase etc.), such as pretreatment include Liquid-liquid extraction, solid phase extraction and membrane filtration. The selection of extraction solvent and the method of extraction are based on the solubility and recovery of the drug.

1.6.1 The Importance of Sample Management (Kellner *et al*., 2004)

Whilst the analytical measurement approach may or may not influence the analytical result, the sample selected always does. The overall variance of the analytical result, s^2_{total}, is the sum of the individual contributory variances. This may be expressed as

$$s^2_{total} = s^2_{process} + s^2_{sampling} + s^2_{analytical}$$

However, the most difficult variance to estimate is the sampling variance. For this reason, it is important to have a structured approach to sampling and its nomenclature. The IUPAC sampling model covers both continuous or bulk goods and discrete or packaged goods. For a commercial perspective, it is extremely important that the sample is representative of the consignment if its value is to be determined on that basis.

1.7 ANALYTICAL METHOD (Douglas A. Skoog, *et al.*, 2006)

The analytical method maybe

1) Qualitative analysis

2) Quantitative analysis

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

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

The analytical methods are of two types

- Classical methods
- Instrumental methods

In classical methods, for qualitative analysis, the analyte was extracted and treated with the reagent specific for a functional group to give a coloured reaction. In quantitative analysis, the amount of the analyte in determined by titrimetric method or by gravimetric method.

The instrumental methods are based on the physical properties of the analyte such as the light absorption or emission, conductivity, mass to charge ratio, fluorescence, adsorption and partition etc. The instrumental methods are basically categorised as follows

- Spectroscopic methods
- Chromatographic methods

- Electro analytical methods
- Thermal methods
- Light scattering methods

The instruments used for the instrumental methods basically consist of a source, system under study and the response. The source may be a light source, electrical source or heat source etc., as required by the instrument. The system under study is the analyte under study or any physical character of the analyte. The response is the unit in which the analytical signal from the analyte which is converted to an output signal for the interpretation.

1.8 SPECTROSCOPIC METHODS

Spectroscopy deals with the interaction of an analyte with electromagnetic radiation. The interaction of the electromagnetic radiation results in absorption or emission radiations. Based on the absorption or emission the spectroscopy is classified into, absorption spectroscopy and emission spectroscopy.

1.8.1 Absorption Spectroscopy

(Gurudeep R. Chatwal, *et al*., 2008; Beckett and Stenlake, *et al*., 2007)

When a beam of electromagnetic radiation is passed through an analyte, certain amount of the radiation is absorbed into the matter. The analyte after absorbing the radiation goes from the ground state to the excited state giving the absorption spectra. The various absorption spectroscopy include the UV-Visible absorption, X-ray absorption, infrared absorption, microwave absorption, radio frequency absorption and atomic absorption, etc.

1.8.1.1 Atomic Absorption

In the atomic absorption spectra the electrons are excited from the lower energy state to a higher energy state by absorption of electromagnetic radiation. The atom absorbs the electromagnetic radiation of energy corresponding to the difference in energy between the higher and lower energy states of the absorbing atom. UV-Visible radiation can excite only the electrons in the outer most orbital, whereas the X-ray has the capacity to excite the electrons located in the inner shell near to the nuclei.

1.8.1.2 Molecular Absorption

The molecular absorption spectra of polyatomic molecules are more complex than the atomic absorption spectra since the number of energy states are higher. The energies associated with a molecule are rotational energy, vibrational energy and electronic energy.

$$E = E_{electronic} + E_{vibrational} + E_{rotational}$$

The molecule absorbs electromagnetic radiation of energy corresponding to the difference in the energy of the ground state molecule and the excited state molecule. The difference in energy ΔE is given by,

$$\Delta E = (E_{electronic} + E_{vibrational} + E_{rotational})_{excited} - (E_{electronic} + E_{vibrational} + E_{rotational})_{ground}$$

The UV-Visible radiations and X-rays have the energy to induce the transition from the ground state to the excited state.

1.8.2 Emission Spectroscopy

Emission spectroscopy is the technique in which the wavelength of the photons emitted by an analyte due to the transition from higher energy level to lower energy on exposure to an electromagnetic radiation was studied. Each analyte emits a specific wavelength of radiation corresponding to the composition of the sample. The energy of the photons emitted is given by

$$E_{photon} = h\upsilon$$

Where E is the energy of the photon, ν is the frequency and h is the Planck's constant. The various instrumental techniques which are based on the measurement of the emitted radiation include flame photometry, fluorimetry, radiochemical methods.

1.8.2.1Atomic Emission

When an analyte is heated, it emits light characteristic of the atom present in it. For example, sodium when heated emits yellow light and potassium emits lilac light. When a metal is heated, the electrons in the outer orbital absorb the heat and goes to a higher energy state. The atom then comes back to the ground state by emitting the photons of light which has energy equal to the difference between the higher energy state and the lower energy state. The instrumental analytical technique, such as the flame emission spectroscopy works on the principle of measuring the photons of energy emitted by a thermally excited atom.

1.8.2.2 Molecular Emission

When a beam of electromagnetic radiation falls on a molecular species, it absorbs the radiation and gets excited. The excited molecules are short lived and it fall back to the ground state immediately. The molecules in the excited sate have higher vibrational energy than that of the ground state. They emit the absorbed energy by the following ways, fluorescence and phosphorescence.

The difference in the energy levels of the absorbed radiation, fluorescence and phosphorescence are as below

$\Delta E_{absorption} > \Delta E_{fluorescence} > \Delta E_{phosphorescence}$

The analytical techniques spectrofluorimetry and phosphorimetry involves the principle of molecular emission.

1.8.3 UV Spectroscopy (Sharma *et al*., 2009; Gurudeep R. Chatwal, *et al*., 2008)

UV-visible spectroscopic methods are based on the type of chromophore/functional group present in the drug moiety. Multi component systems are also easily analysed by means of spectral isolation. Spectroscopic methods are widely used as tools for quantitative analysis, characterization and quality control in the pharmaceutical, agricultural and biomedical fields

The UV spectroscopy is one of the most widely used instrumental analytical techniques for the analysis of pharmaceuticals. The UV region extends from 190 nm to 380 nm. The instrument used to measure the intensity of the UV radiation absorbed or transmitted is known as the UV - Visible spectrophotometers. A molecule can absorb the UV radiation only when the energy of the radiation matches the energy that was required to induce electronic transition in the molecule.

1.8.3.1 Laws of Absorption

When a beam of UV light is allowed to pass through a substance which absorbs the UV light, the intensity of the transmitted light was lesser than the incident light. The reduction of the intensity is may be due to reflections on the surface of the cell, scattering of light by macro molecules and absorption.

The two important laws which govern the UV spectroscopy are the Lambert's law and Beer's law. Lambert's law states that the intensity of the light decreases exponentially with decrease in the thickness of the medium through which it passes.

Beer's law states that the intensity of the light decreases exponentially with increase in the concentration of the absorbing substance.

The two laws where combined to form the Beer-Lambert's law, which is given by the equation

$$A = abc$$

Where, A is the absorbance

a is the absorptivity

b is the path length

c is the concentration.

The absorptivity is defined as, the absorbance of a substance at a specific wavelength of 1 g/100 ml solution in a 1cm cell.

1.8.3.2 Deviation of Beer's Law

When the absorbance is plotted against concentration, a straight line passing through the origin should be obtained. But there is always a deviation from the linear relationship in the plot of absorbance versus concentration. The deviation in the Beer's law may be due to anyone of the following reasons.

The presence of foreign substance affects the light absorption and alters the extinction coefficient.

- Due to dissociation or association of the molecule. Example, benzyl alcohol in chloroform exists as a polymer. The monomer absorbs at lower wavelength and the polymer at higher wavelength.
- Due to presence of foreign substance which absorbs at the same wavelength as the analyte.
- If monochromatic light is not used.
- Due to undesirable radiations falling on the detector.

1.8.3.3 Transitions in Organic Molecules

(Sharma *et al*., 2009; Gurdeep R. Chatwal, *et al*., 2008)

The absorption in the ultraviolet region results in the transition of the valence electron form the ground level to the excited level. The three types of electrons involved in the transition are

σ-electrons: These are involved in the formation of saturated bonds. The energy required for the excitation of the electrons is more than that of the UV radiations. Hence these electrons do not absorb near UV radiation.

π-electrons: These are involved in the formation of unsaturated bonds.

Example: Dienes, trienes and aromatic compounds. It absorbs radiation in near UV region.

n-electrons: These are the lone pair of electrons present in atoms such as oxygen, nitrogen etc., in a molecule. They can be excited by both UV and Visible radiations.

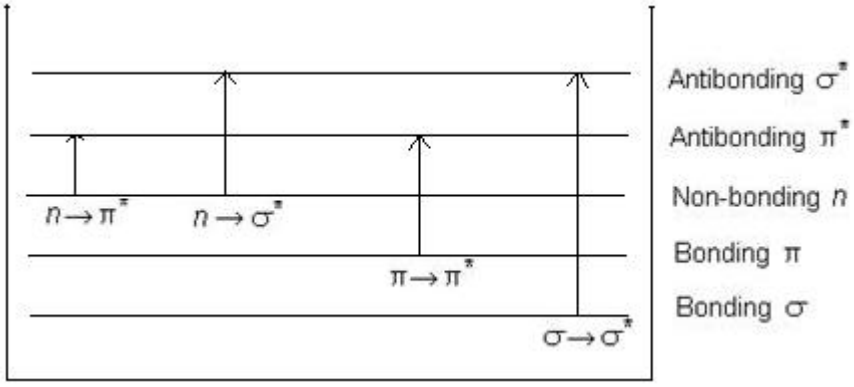
The various types of transitions are

$\sigma \rightarrow \sigma^*$

$n \rightarrow \sigma^*$

$\pi \rightarrow \pi^*$

$n \rightarrow \pi^*$



(http://www.pharmatutor.org/pharma-analysis/analytical-aspects-of-uv-visible-spectroscopy/types-of-electrone-transition.html)

The energy required for the various types of transitions are

$\sigma \rightarrow \sigma^* > n \rightarrow \sigma^* > \pi \rightarrow \pi^* > n \rightarrow \pi^*$

1.8.3.4 σ→σ transitions*

These transitions occur in saturated hydrocarbons with single bonds and no lone pair of electrons. The energy required for this type of transition is very high because of the strong sigma bond formed by the valence electrons. Thus, the transitions occur at very short wavelength. The saturated hydrocarbons such as methane, ethane, propane etc. absorbs at 126 -135 nm region of the UV region. Hence these compounds are used as solvents in UV spectroscopy.

1.8.3.5 n → σ transitions*

Saturated compounds with lone pair of electrons show n→σ* transitions in addition to σ→σ* transitions. The energy required for the n→σ* transition is lesser than the energy required for σ→σ* transitions. The energy required for n→σ* transition, in alkyl halides, decreases with increase in the size of the halogen atom. Alcohols and amines forms hydrogen bonding with the solvent hence require higher energy for the transitions.

*1.8.3.6 π → π * transitions*

These transitions occur in unsaturated compounds containing double or triple bonds and also in aromatic compounds. Lower energy is required for these transitions and hence a longer wavelength causes the excitation of the molecule.

1.8.3.7 n→ π^* transitions

These transitions occur in compound which contains oxygen, nitrogen, sulphur and halogens because of the presence of free lone pair of electrons. These transitions require least amount of energy and hence they occur in UV and Visible region. Saturated carbonyl compounds shows two types of transitions, low energy n→ π^* transitions occurring at longer wavelength and high energy n→ π^* transitions occurring at lower wavelength. The shifts in the absorption of the carbonyl compounds are due to the polarity of the solvent.

1.8.4 Transition Probability (Gurudeep R. Chatwal, *et al.*, 2008)

It is not essential that, when a compound is exposed to UV light, transition of the electron should take place. The probability that an electronic transition should take place depends on the value of extinction coefficient. The transitions are classified as allowed transition and forbidden transition.

1.8.4.1 Allowed Transitions

The transitions having ε_{max} value greater than 10^4 are called allowed transitions. They generally arise due to the π → π * transitions. For example, 1, 3 – butadiene exhibits absorption maximum at 217 nm and has ε_{max} value of 21000 represents allowed transitions.

1.8.4.2 Forbidden Transitions

These transitions have ε_{max} value less than 10^4. They occur due to n→ π^* transitions. Example, saturated carbonyl compound (R-C=O) shows absorption near 290 nm and ε_{max} value less than 100 represent forbidden transitions.

1.8.5 Chromophore

These are groups or structure which is responsible to impart colour to the compound. The presence of chromophore is responsible for the absorption of UV radiation by any compound. The groups include nitro group, amine groups, double bonds, triple bonds, etc.

There are two types of chromophore

- Groups containing π electrons and undergoes π → π * transitions. Example: ethylene, acetylenes
- Groups containing π electrons and n electrons. They undergo two types of transition like π → π * transitions and n→ π^* transitions. Example:- carbonyls, nitriles, azo compounds etc.

1.8.6 Auxochrome

Any groups which do not itself act as a chromophore but its presence brings a shift in the position of absorption maximum. Chromophores are unsaturated whereas the auxochromes are covalently saturated. The auxochromes are of two types

Co-ordinately unsaturated- (NH2, -S- groups containing lone pair of electrons).

Co-ordinately saturated- (NH3+ groups).

1.8.7 Absorption and Intensity shifts

1.8.7.1 Bathochromic shift or Red shift

The shift in the absorption maximum of a compound, due to the presence of certain auxochromes, towards longer wavelength is called as the bathochromic shift.

1.8.7.2 Hypsochromic shift or Blue shift

The shift in the absorption maximum to shorter wavelength is called Hypsochromic shift. The shift is due to solvent effect or removal of conjugation in a molecule.

1.8.7.3 Hyperchromic effect

The increase in intensity of absorption by inclusion of an auxochrome to a system is hyperchromic shift.

1.8.7.4 Hypochromic shift

The decrease in the intensity of absorption is due to the distortion of the geometry of the molecule.

1.8.8 Solvent Effect

The solvent used for the spectral analysis should not interfere in the absorbance of the analyte. It means that the solvent should not have any absorbance in the region under investigation. Based on the polarity of the solvent used the intensity of the absorption changes for a particular analyte. The α, β – unsaturated carbonyl compounds shows two different types of transitions

$n \rightarrow \pi^*$ transition

The increase in polarity moves the absorption maximum to a shorter wavelength. The ground state is more polar when compared to the excited state.

$\pi \rightarrow \pi^*$ transitions

The increase in polarity moves the absorption maximum to longer wavelength. Only lesser energy is required for this transition and hence shows red shift.

1.8.9 Choice of Solvent

There are two important requirements a solvent must satisfy to be used as a solvent in UV spectroscopy.

They are

- It should be transparent throughout the region of UV under investigation
- It should not interact with the solute molecules and should be less polar.

1.8.10 Instrumentation

The components of a UV-Visible spectrophotometer are

- Light source
- Monochromators
- Sample cell
- Detectors

Light source

The various source of light used in the spectrophotometer are tungsten lamp, hydrogen discharge lamp, deuterium lamp, xenon arc lamp and mercury arc lamp. The most commonly used lamp is the deuterium discharge lamp.

Monochromators

The monochromators are used to disperse the light for the required wavelength. The monochromators consists of three units entrance slit, dispersing element and exit slit. The dispersing unit may be filters, prisms or gratings. The fused silica prisms and quartz prisms are commonly used in UV spectrophotometers.

Sample cell

The cell must be transparent throughout the wavelength region of study. The cells are made of fused glass or fused silica or quartz. The glass cells are not used since they absorb in the UV region. Quartz cells are commonly used.

Detectors

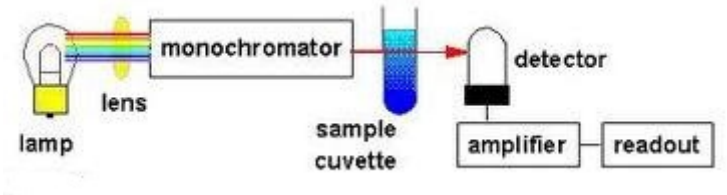
The detectors employed are barrier layer cells, photo emissive tubes, photodiodes and photomultiplier tubes. Photodiodes are commonly used in the instrument.

1.9.12 Spectrophotometers

There are two designs of spectrophotometer. They are single beam spectrophotometer and double beam spectrophotometer.

Single beam system

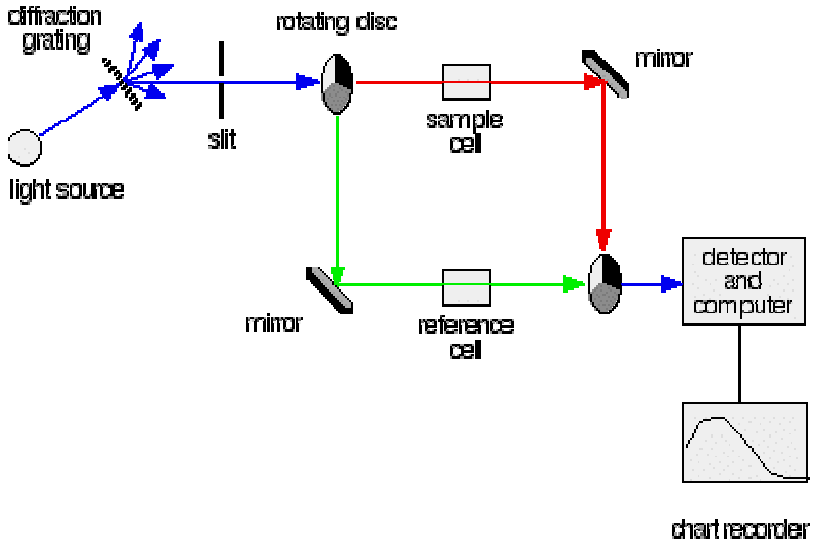
- Light given off from the source
- Lens gathers the light and focuses on the monochromators
- The light of specific wavelength comes out of the monochromator
- Radiation passes through the sample in the sample cell and to the detector
- Detector measures the intensity of the light reaching it.



UV-Visible Single beam spectrophotometer (chemistry.adelaide.edu.au)

Double beam system

- Radiation from source falls on the monochromator
- The radiation of the required wavelength pass out of the exit slit reaches the rotating disc
- Rotating disc splits the beam into two, one passes through the sample cell and the other passes through the reference cell.
- The light beam falls on the detector
- Detector measures the intensity of the light.



Double beam UV-Visible spectrophotometer (Chemguide.co.uk)

1.8.11 Quantitative Analysis of Single Component (Beckett and Stenlake, *et al*., 2007)

The assay of an analyte is done by dissolving the analyte in a suitable solvent and measuring the absorbance of the solution at the required wavelength. The selected wavelength is the absorbance maximum of the analyte in that particular solvent. The concentration of the analyte can be determined by

- Use of absorptivity value
- Use of calibration graph
- Single or double point standardization

1.8.11.1 Absorptivity Value Method

This method is usually followed in official books such as Indian Pharmacopoeia, British Pharmacopoeia etc. The advantage of the method is, the preparation of standard

solutions of reference substance is not required for the calculation of the concentration of the analyte.

1.8.11.2 Calibration Graph Method

In this method, a series of linear concentration solutions of the reference solutions are prepared and the value of absorbance is plotted against the concentration of the reference solution. From the graph the absorbance of the sample solution is plotted and the concentration is found.

1.8.11.3 Single Point or Double Point Standardization

In single point standardization, the standard and the sample solutions are prepared under same identical condition. Also, the standard and the sample concentration are almost equal. Then after the measurement of absorbance the following formula is applied to find the unknown sample concentration

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

Double point standardization is used when there is a linear but non proportional relationship between concentration and absorbance. The concentration of one of the standard is higher and the concentration of other is lower than that of the standard.

$$C_{test} = \frac{(A_{test} - A_{std1})(C_{std1} - C_{std2}) + C_{std1}(A_{std1} - A_{std2})}{A_{std1} - A_{std2}}$$

1.8.12 Assay of Substance in Multi Component Samples

The spectrophotometric assay of drugs rarely involves the measurement of absorbance of sample containing only one absorbing component. The pharmaceutical analyst frequently encounters the situation where the concentration of one or more substances is required in samples known to contain other absorbing substances which potentially interfere in the assay. Alternatively, interference which is difficult to quantify may arise in the analysis of formulations from manufacturing impurities, decomposition products and formulation excipients. Unwanted absorption from these sources is termed irrelevant absorption and, if not removed, imparts a systematic error to the assay of the drug in the sample.

The basis of all the spectrophotometric techniques for multi component sample is the property that at all wavelengths;

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

Multi component analysis is done when the sample contains more than one analyte to be quantified in the sample. In such methods one of the analyte may be taken as interferent and the absorbance of the interferent reduced to find the true absorbance of the analyte. Similarly the absorbance of the other analyte is found by taking the first analyte as the interferent. The various methods used are as follows.

- Simultaneous equation method
- Absorption ratio method
- Geometric correction method
- Orthogonal polynomial method
- Difference spectroscopy
- Area under curve method
- Absorbance ratio method
- Derivative spectrophotometry

Simultaneous Equations method

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

The information required is:

a. The absorptivities of X at λ_1 and λ_2, a_{x1} and a_{x2} respectively.
b. The absorptivities of Y at λ_1 and λ_2, a_{y1} and a_{y2} respectively.
c. The absorbances of the diluted sample at λ_1 and λ_2, A_1 and A_2 respectively.

Let Cx and Cy be the concentrations of X and Y respectively for diluted sample.

Two equations are constructed based upon the fact that at λ_1 and λ_2 the absorbance of the mixture is the sum of individual absorbances of X and Y.

At λ_1

$$A_1 = ax_1bcx + ay_1bcy \qquad (1)$$

At λ_2

$$A_1 = ax_2bc_x + ay_2bc_y \qquad (2)$$

For measurements in 1 cm cells, b = 1

Rearrange eq. (2)

$$c_y = \frac{A_2 - a_{x2}c_x}{a_{y2}}$$

Substituting for c_y in eq. (1) and rearranging gives

$$C_x = \frac{A_2a_{y1} - A_1a_{y2}}{a_{x2}a_{y1} - a_{x1}a_{y2}}$$

And

$$C_y = \frac{A_1a_{x2} - A_2a_{x1}}{a_{x2}a_{y1} - a_{x1}a_{y2}}$$

As an exercise you should derive modified equations containing a symbol (b) for path length, for application in situations where A_1 and A_2 are measured in cells other than 1 cm path length.

Criteria for obtaining maximum precision, based upon absorbance ratios, have been suggested that place limits on the relative concentrations of the components of the mixture. The criteria are that the ratios

$$\frac{A_2/A_1}{a_{x2}/a_{x1}} and \frac{a_{y2}/a_{y1}}{A_2/A_1}$$

Should lie outside the range 0.1 - 2.0 for the precise determination of Y and X respectively, these criteria are satisfied only when the λ_{max} of the two components are reasonably dissimilar. An additional criterion is that the two components do not interact chemically, thereby negating the initial assumption that the total absorbance is the sum of

the individual absorbance. The additive of the absorbance should always be confirmed in the development of a new application of this technique.

Absorbance Ratio Method

The absorbance ratio method is a modification of the simultaneous equations procedure. It depends on the property that, for a substance which obeys Beer's Law at all wavelengths, the ratio of absorbances at any two wavelengths is a constant value independent of concentration or path length. For example, two different dilutions of the same substance give the same absorbance ratio A1/A2, 2.0. In the USP, this ratio is referred to as a Q value. The British Pharmacopoeia also uses a ratio of absorbance at specified wavelengths in certain confirmatory tests of identity.

In the quantitative assay of two components in admixture by the absorbance ratio method, absorbances are measured at two wavelengths one being the λ_{max} of one of the components (λ_2) and the other being a wavelength of equal absorptivity of the two components (λ_1), i.e., an iso-absorptive point. Two equations are constructed as described above for the method of simultaneous equation (eq. (1) and eq.(2)). Their treatment is somewhat different, however, and uses the relationship $a_x = a_{y1}$ at (λ_1). Assume b=1 cm.

$$A_1 = a_{x1}c_x + a_{x1}C_Y \qquad (5)$$

$$\frac{A_2}{A_1} = \frac{a_{x2}c_x + a_{y2}c_y}{a_{x1}c_x + a_{x1}c_y}$$

Divide each term by $c_x + c_y$ and let $F_x = c_x/(c_x + c_y)$ and $F_y = c_y/(c_x + c_y)$ i.e. Fx and Fy are the fractions of X and Y respectively in the mixture:

$$\frac{A_2}{A_1} = \frac{a_{x2}F_x + a_{y2}F_y}{A_{x1}F_x + a_{x1}F_y}$$

But $F_y = 1 - F_x$,

$$\frac{A_2}{A_1} = \frac{F_x a_{x2} - F_x a_{y2} + a_{y2}}{a_{x1}}$$

$$\frac{A_2}{A_1} = \frac{F_x a_{x2}}{a_{x1}} - \frac{F_x a_{y2}}{a_{y1}} + \frac{a_{y2}}{a_{y1}}$$

$$\text{Let } Q_x = \frac{a_{x2}}{a_{x1}}, Q_y \frac{a_{y2}}{a_{y1}} and\ Q_M \frac{A_2}{A_1}$$

$$Q_M = F_x(Q_x - Q_y) + Q_y$$

$$F_x = \frac{Q_M - Q_y}{Q_x - Q_y} \qquad (6)$$

Equation 6 gives the fraction, rather than the concentration of X (and consequently of Y) in the mixture in terms of absorbance ratios. As these are independent of concentration, only approximate, rather than accurate, dilutions of X, Y and the sample mixture are required to determine Q_x, Q_y and Q_M respectively.

$$A_1 = a_{x1} + (c_x + c_Y)$$

$$c_x + c_y = \frac{A_1}{a_{x1}}$$

From eq. (6)

$$\frac{c_x}{f2c_x + c_y} = \frac{Q_m - Q_y}{Q_x - Q_y b7}$$

$$\frac{c_x}{A_1/a_{x1}} = \frac{Q_M - Q_y}{Q_x - Q_y}$$

$$c_x = \frac{Q_m - Q_y}{Q_x - Q_Y} . \frac{A_1}{a_{x1}} \qquad (7)$$

Equation 7 gives the concentration of X in terms of absorbance ratios, the absorbance of the mixture and the absorptivity of the compounds at the iso-absorptive wavelength. Accurate dilutions of the sample solution and of the standard solutions of X and Y are necessary for the accurate measurement of A1 and ax1 respectively.

Geometric Correction Method

A number of mathematical correction procedures have been developed which reduce or eliminate the background irrelevant absorption that may be present in samples of biological origin. The simplest of these procedures is the three-point geometric procedure, which may be applied if the irrelevant absorption is linear at the three wavelengths selected.

$$\text{Corrected absorbance, D} = \frac{y(A2-A3)+ z(A2-A1)}{(Y+Z)(1-r)}$$

Orthogonal Polynomial Method

The technique of orthogonal polynomials is another mathematical correction procedure which involves more complex calculations than the three-point correction procedure. The basis of the method is that an absorption spectrum may be represented in terms of orthogonal functions as follows

$$A(\lambda) = p_0P_0(\lambda) + p_1P_1(\lambda) + p_2P_2(\lambda)\ldots.p_nP_n\,(\lambda)$$

Where, A = Absorbance

λ = Wave length

$P_o\,(\lambda)$, $P_1\,(\lambda)$, $P_2\,(\lambda)\ldots.P_n\,(\lambda)$ represent the polynomial coefficient

Each coefficient is proportional to each other. These polynomials represent a series of fundamental shapes and the contribution that each shape, e.g.P_2 makes to the absorption spectrum is defined by the appropriate coefficient, e.g. p_2 for P_2. The coefficients are proportional to the concentration of the absorbing analyte, and a modified Beer – Lambert equation may be constructed:

$$p_j = \alpha_j bc$$

For example, when b is 1 cm and concentration of the analyte (c), is in g/ dl. When irrelevant absorption so, present in a sample solution, the calculated coefficient (p_i) comprises the coefficients of the analyte and of the irrelevant absorption (Z).

Thus,

$$P_j = \alpha_j c + p_j(Z)$$

Where,

P_j = polynomial coefficient

a_j = proportionality constant

b = path length

c = concentration

With the correct choice of polynomial, number of wavelengths and the wavelength interval, the contribution from the irrelevant absorption may be negligible. In general, a quadratic (P_2) polynomial eliminates linear or almost linear irrelevant absorption and a cubic (P_3) polynomial eliminates parabolic irrelevant absorption.

The segment of the spectrum of the drug between $\lambda_1$and λ_8 shows a minimum around λ_3 and a maximum around λ_5. Its shape may therefore be represented by a cubic polynomial. The irrelevant absorption is a simple parabolic curve which does not contain a cubic contribution. The coefficient (P_3) of the polynomial for each set of eight absorbances (A_1……..A_8) is calculated from:

$$P_3 = [(-7)A_1 + (+5)A_2 + (+7)A_3 + (+3)A_4 + (-3)A_5 + (-7)A_6 + (-5)A_7 + (+7)A_8]$$

Where the factors are those of an eight –point cubic polynomial obtained from standard texts of numerical analysis (e.g. Fischer and Yates, 1953). The contribution of the irrelevant absorption to the coefficient of the polynomial of the sample is eliminated (Table 7.2) by the selection of these parameters, and the concentration of the drug in the sample may be calculate with reference to a standard solution of the drug, from the proportional relationship that exists between the calculated P_3 value and concentration.

The accuracy of the orthogonal functions procedure depends on the correct choice of polynomial order and set of wavelengths. Usually, quadratic or cubic polynomials are selected depending on the shape of the absorption spectra of the drug and the irrelevant absorption. The set of wavelengths is defined by the number of wavelengths, the interval, and the mean wavelength of the set (λm). approximately linear irrelevant absorption is

normally eliminated using six to eight wavelengths, although many more, up to 20, wavelengths may be required if the irrelevant absorption contains high frequency components. The wavelength interval and λm are best obtained from convoluted absorption curve. This is a plot of the coefficient (P_j) for a specified order of polynomial, a specified number of wavelengths and a specified wavelength interval (on the ordinate) against the λm of the set of wavelengths. The optimum set of wavelengths corresponds with a maximum or minimum in the convoluted curve of the analyte and with a coefficient of zero in the convoluted curve of the irrelevant absorption. In favourable circumstances the concentration of an absorbing drug in admixture with another may be calculated if the correct choice of polynomial parameters is made, thereby eliminating the contribution of one drug from polynomial of the mixture. For, example, the selective assay phenobarbitone, combined with phenytoin in a capsule formulation using a six-point quardratic polynomial, has been reported.

The determination of the optimum set of wavelengths is readily accomplished with the aid of a microcomputer. A suitable exercise is to write a program to compute and plot the data for convoluted spectrum.

Difference Spectrophotometry

The selectivity and accuracy of spectrophotometric analysis of samples containing absorbing interferents may be markedly improved by the technique of difference spectrophotometry. The essential feature of a difference spectrophotometric assay is that the measured value is the difference absorbance (A) between two equimolar solutions of the analyte in different chemical forms which exhibit different spectral characteristics.
The criteria for applying difference spectrophotometry to the assay of a substance in the presence of other absorbing substances are that:

- reproducible changes may be induced in the spectrum of the analyte by the addition of one or more reagents
- the absorbance of the interfering substances is not altered by the reagents.

The simplest and most commonly employed technique for altering the spectral properties of the analyte is the adjustment of the pH by means of aqueous solutions of acid, alkali or buffers. The ultraviolet-visible absorption spectra of many substances containing ionisable functional groups, e.g. phenols, aromatic carboxylic acids and amines, are dependent on the state of ionization of the functional groups and consequently on the pH of the solution.

The absorption spectra of equimolar solutions of Phenylephrine, a phenolic sympathomimetic agent, in both 0.1M hydrochloric acid (pH 1) and 0.1M sodium hydroxide (pH 13) are shown in figure. The ionization of the phenolic group in alkaline solution generates an additional n (non-bonded) electron that interacts with the with the ring π electrons to produce a bathochromic shift of the λmax from 271nm in acidic solution to 291 nm and an increase in absorbance at the (hyperchromic effect). The difference absorption spectrum is a plot of the difference in absorbance between the solution at pH 13 and that at pH 1 against wavelength. It may be generated automatically using a double-beam recording spectrophotometer with the solution at pH 13 in the sample cell and the solution at pH 1 in the reference pH 13 in the sample cell and the solution at pH 1 in the reference cell. At 257 and 278 nm both solutions have identical absorbance and consequently exhibit zero difference absorbance. Such wavelengths of equal absorptivity of the two species are called isobestic or iso-absorptive points. Above 278 nm the alkaline solution absorbs more intensely than the acidic solution and the ΔA is therefore positive. Between 257 and 278 nm it has a negative value. The measure value in a quantitative difference spectrophotometric assay is the ΔA at any suitable wavelength measured to the baseline, e.g. ΔA1 at λ1 or amplitude between an adjacent maximum and minimum, e.g. ΔA1 at λ2 and λ1.

At λ_1 $\Delta A = A_{alk} - A_{acid}$

Where Aalk and Aacid are the individual absorbances in 0.1M sodium hydroxide and 0.1M hydrochloric acid solution respectively. If the individual absorbance, and are proportional to the concentration of the analyte and path length, the also obeys the Beer – Lambert Law and a modified equation may be derived

Where Δa is the difference absorptivity of the substance at the wavelength of measurement.

If one or more other absorbing substances is present in the sample which at the analytical wavelength has identical absorbance in the alkaline and acidic solutions, its interference in the spectrophotometric measurement is eliminated. The selectivity of the ΔA procedure depends on the correct choice of the pH values to induce the spectral change of the analyte without altering the absorbance of the interfering components of the sample. The use of 0.1M sodium hydrochloric acid to induce the ΔA of the analyte is convenient and satisfactory when the irrelevant absorption arises from pH intensive substances.

Area Under the Curve Method

From the spectra obtained for calculating the simultaneous equation, the area under the curve were selected at a particular wavelength range for both the drugs were each drug have its absorption. The "X" values of the drugs were determined at the selected AUC range. The "X" value is the ratio of area under the curve at the selected wavelength range with the concentration of the component in mg/ ml. These "X" values were the mean of six independent determinations. A set of two simultaneous equations were obtained by using mean "X" values. And further calculations are carried out to obtain the concentration of each drug present in the sample.

Absorption Correction Method

The method can be used to calculate the concentration of component of interest found in a mixture containing it along some unwanted interfering component. The absorption different between two points on the mixture spectra is directly proportional to the concentration of the component to be determined irrespective of the interfering component. If the identity, concentration and absorptivity of the absorbing interferences are known, it is possible to calculate their contribution to the total absorbance of a mixture. The concentration of the absorbing component of interest is then calculated from the corrected absorbance (total absorbance minus the absorbance of the interfering substance) in a usual way. The data required for the construction of absorbance corrected for interference are

The λmax of the drugs should be found out by using reference standards of the drugs.
The calibration curve is plotted for each drug and linearity range should be found out.
At one wavelength, one of the drugs shows no absorbance. Hence the other drug was calculated without any interference.
The absorbance values of every drug at the two wave lengths should be measured and the absorptivity values should be calculated.
In another wavelength, the absorbance corrected for another drug and the first drug was determined.

Derivative Spectrophotometry

In this method, the normal spectrum is converted into derivative spectrum of higher orders such as first order, second order, etc. The transformations that occur in the derivative spectra are understood by reference to a Gaussian band which represents an ideal absorption band.

The first order derivative spectrum is a plot of the rate of change of absorbance with wavelengths against wavelength, i.e. a plot of the slope of the fundamental spectrum against wavelength or a plot of $dA/d\lambda$ vs λ. At $\lambda 2$ and $\lambda 4$, the maximum positive and maximum negative slope respectively in the D1 spectrum. The λmax at $\lambda 3$ is a wavelength of zero slope and gives $dA/d\lambda = 0$, i.e. a cross-over point, in the D1 spectrum.

The second derivative (D2) spectrum is a plot of the curvature of the D^o spectrum against wavelength. The maximum negative curvature at $\lambda 1$ and $\lambda 5$ the maximum positive curvature in the D^o spectrum gives two small maxima called 'satellite' bands in the D2 spectrum. At $\lambda 2$ and $\lambda 4$ the wavelengths of maximum slope and zero curvature in the D^o spectrum correspond with cross-over points in the D2 spectrum.

In summary, the first derivative spectrum of an absorption band is characterised by a maximum, a minimum, and a cross –over point at the λmax of the absorption band. The second order derivative spectrum is characterised by two satellite maxima and an inverted band of which the minimum corresponds to the λmax of the fundamental band.

The advantages of derivative method are the higher resolution and band width discrimination. This advantage permits the determination of analyte even in the presence of non specific interferents. The higher resolution and band width discrimination

increases with the increase in the order of derivatization. This provides much better finger prints than traditional working spectrum (Hassan *et al*., 2000 and Gindy *et al*., 2000) But the signal to noise ratio decreases with the increase in derivative order.

1.9 Chromatographic Methods

(Gurudeep R. Chatwal, *et al*., 2008; Beckett and Stenlake, *et al*., 2007)

Chromatography is the science which deals with the separation achieved by the distributing the analyte between two phases, a stationary phase and a mobile phase based on the principle of adsorption and partition.

1.9.1 Mechanism

The various mechanisms in the chromatography are

- Adsorption chromatography
- Partition chromatography
- Ion exchange chromatography
- Gel chromatography

1.9.1.1 Adsorption Chromatography

This type of chromatography contains a solid stationary phase and a liquid or gaseous mobile phase. Paper Chromatography, Thin Layer Chromatography, High Performance Liquid Chromatography work based on the principle of adsorption.

1.9.1.2Partition Chromatography

In this the stationary phase is a liquid and the mobile phase maybe a liquid or a gas. Gas – Liquid Chromatography, Liquid – Liquid Chromatography works on the principle of partition.

1.9.1.3 Ion Exchange Chromatography

Solid stationary phase with replaceable ions and ionic liquid mobile phase are features of ion exchange chromatography.

1.9.1.4 Gel Chromatography

An inert gel acts as the stationary phase and a liquid mobile phase are the features of the gel chromatography. They separate the analyte according to their sizes.

1.9.2 Techniques

The various techniques of development of chromatogram are frontal analysis, elution analysis and displacement analysis

1.9.2.1 Frontal Analysis

The sample solution is allowed to pass through the column continuously; the strongly adsorbed solutes are retained in the column whereas the less strongly adsorbed solutes are washed off easily.

1.9.2.2 Elution Analysis

A small amount of the sample is placed at the top of the column and then the eluant is allowed to flow through the column. The solutes elute out of the column based on the partition coefficient of the solutes.

1.9.2.3 Displacement Analysis

The sample solution is introduced at the top of the column and the elution is made by using a solution containing a strongly adsorbing solute. The strongly adsorbed solute displaces the adsorbed analyte from the column. Hence this method is called the displacement analysis and the strongly adsorbing solute is called as the displacing agent.

1.9.3 High Performance Thin Layer Chromatography (Sethi, *et al*., 1996)

High Performance Thin Layer Chromatography is a versatile separation technique and is official in most of the Pharmacopoeias for determining content uniformity, purity profile, assay value and dissolution rates in unlimited number of monographs. It is precisely for these reasons that almost every laboratory today is equipped HPLC system.

However, it cannot be denied that more than often, the systems are working beyond their capacities and mostly dedicated who would like to change a well running stabilized column and prepare fresh solutions only because few assorted samples even though urgent are required to be analyzed.

1.9.3.1 Various Steps Involved in HPTLC

- Selection of HPTLC plates and sorbent.
- Samples preparation including any clean up and
- Pre-chromatographic derivatization.
- Application of sample.
- Development.
- Detection including post-chromatographic derivatization.
- Quantification.
- Documentation.

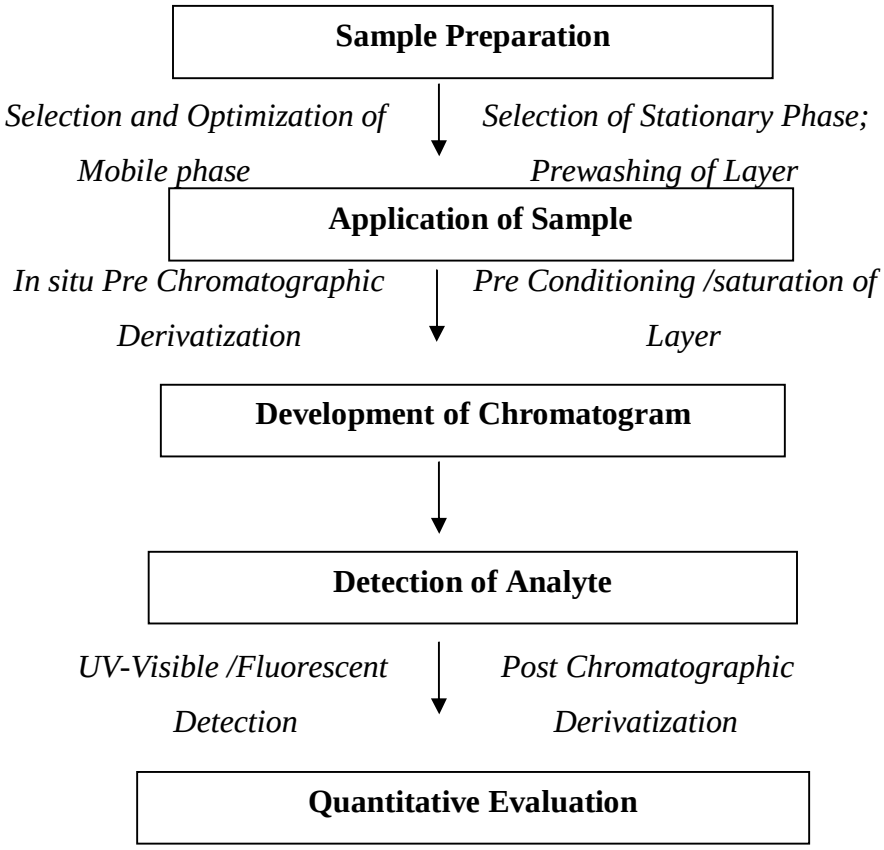
1.9.3.2 Criteria for Identification of an Analyte by HPTLC

- Rf value of an analyte should agree ± 3 % compared to standard material used under similar conditions.
- Visual appearance of the analyte should be indistinguishable from that of standard material.
- Centre of the spot nearest to that due to analyte should be separated from it by at least half the sum of analyte spot diameter.
- For conforming the identity, co-chromatography is mandatory, as a result, only the spot supposed to be due to analyte should be visible and no additional spot appear.
- Whenever spectrum detection is used, maximum absorption wavelength of the sample and standard should be same within limits of resolution of detection system and UV spectra should not be visibly different from that of the standard material.

1.9.3.3 Factors Influencing the HPTLC Separation and Resolution of Spots

- Type of stationary phase, its particle size and activity
- Type of plates
- Layer thickness
- pH of the layer
- Binder in the layer
- Mobile phase
- Solvent purity
- Type and size of developing chamber
- Degree of chamber saturation
- Solvent for the sample preparation
- Sample volume spotted
- Size of initial spot and gradient
- Temperature and relative humidity

Method Development for HPTLC



1.10 Selection of an Analytical Method (Douglas A. Skoog, *et al.*, 2006)

To select an analytical method, it is essential to clarify the nature of analytical problem-the accuracy required, quantity of sample available, concentration range of the analyte, interfering substance, physicochemical properties of sample matrix and number of samples to be analyzed.

1.11 Performance Characteristic of Instruments

The performance characteristic of the instrument such as precision, bias, sensitivity detection limit, dynamic range and selectivity are considered before selecting the method itself. The method should also be fast, easy, convenient and cost effective.

1.12 Development of Analytical Method

Due to advances in high throughput organic synthesis and discovery teams, there is a great increase in the number of analogues introduced for the absorption, distribution, metabolism and excretion properties.

Fixed dose combinations forms the main stay in clinical management of many acute and chronic disease as they offer several advantages over single products with respect to storage, prescribing, dispensing patients, use, consumption and synergistic effect in disease management

1.13 New Applications in Analytical Technique (Regina M.B.O Duarte *et al*., 2012)

A new chromatographic response function (CRF_{2D}) is proposed and tested for the estimation of the quality index of separation in comprehensive two-dimensional liquid chromatography (2D-LC) of complex organic mixtures. This objective function is based on the concept of peak purity for one-dimensional liquid chromatography, which has been redefined for 2D-LC. The new CRF_{2D} also includes other separation quality criteria, namely the number of 2D peaks appearing in the chromatogram and the analysis time. To compute the peak purity for a given 2D peak, three important steps have been tackled in this study: (a) the development of an alternative algorithm for detecting 2D peaks automatically from real experimental 2D-LC data; (b) the application of a mathematical model to fit the obtained chromatographic data; and (c) the estimation of the volume of the overlapping region between two or more 2D peaks. The capability of the new function to qualify the overall separation degree that it is attained under different chromatographic conditions was further assessed through a 2D-LC study of a mixture of compounds.

Screening analysis for detection of selected drugs of abuse and psychoactive medical drugs in whole blood using solid phase extraction and UPLC-TOFMS.

1.14 VALIDATION *(www.askaboutvalidation.com/forum/showthread.php?t=1175)*

The word “Validation” means “Assessment” of validity or action of proving effectiveness.

Validation as defined by different agencies

USFDA- According to this “validation” is the process of establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its predetermined specifications and quality attributes.

WHO- Defines validation as an action of providing any procedure process equipment material, activity or system actually leads to the expected results.

EUROPEON COMMITTEE- Defines validation as an action of providing in a accordance with the principles of GMP that any procedure, process material and activity or system actually leads to expected results

1.14.1 Method Validation

This process consists of establishment of the performance characteristics and the limitations of the method

Method performance parameters are determined using equipment that is

- Within specification
- Working correctly
- Adequately calibrated

Method validation is required when

- New method is been developed
- Revision of already existed methods
- When established method are used in different laborarties and analyst
- Comparison of methods
- When quality control indicates any change in method.

1.14.1.1The Objectives of the Validation

The primary objective of validation is to form a basis for written procedure for production and process control which are designed to assure that the drug products have the identity, quality and purity they purport or are represented to possess.

i. Assurance of quantity.
ii. Government regulation.

1.14.1.2 The Importance of Validation

i. As the quality of product cannot always be assured by routine quality control because of testing of statistically insignificant number of sample, the validation thus should provide adequacy and reliability of a system or product to meet the pre-determined criteria or attributes providing high degree of confidence that the same level of quality is consistently built into each of finished product from batch to batch.

ii. Retrospective validation is useful for trend comparison of results complains to cGMP to cGLP.

iii. For taking appropriate action in case of non-compliance.

1.14.1.3 The Benefits of Validation

- Regulatory compliance
- Minimize rejection and reworking
- Minimize utility costs
- Minimize complaints
- Reduce testing requirements
- More rapid and reliable start-up new equipment
- Easier scale-up from development

1.14.2 Types of Validation (Michael E. Swartz *et al*., 2009)

The validation is divided into different types. They are,

Prospective Validation

This method is employed when historical data of the product is not available or is not sufficient and in process and finished product testing is not adequate to ensure reproducibility or high degree of compliance to product likely attributes.

Retrospective Validation

This provides trend of comparative result (i.e.) review and evaluation of existing information for comparison when historical data is sufficient and readily available.

Concurrent Validation

Based on information generated during implementation of a system for this extensive testing and monitoring are performed as part of initial run of the method.

Re-Validation

Revalidation provides the evidence that changes in a process and are the process environment, introduced either intentionally or unintentionally, do not adversely affect process characteristic and product quality.

There are two basic categories of revalidation. Revalidation in case of known change (including transfer of process from one company to another or from one site to another). Periodic revalidation carried out at scheduled intervals.

1.14.3 Reasons/Purpose of Validation: (Sethi, *et al*., *1996*)

- Setting standards of evaluation procedures.
- Taking appropriate action in case of non-compliance.
- Retrospective validation is useful for trend comparison of results compliance to cGMP/cGLP.
- Closer interaction with pharmacopoeia forum to address analytical problems. Enables scientist to communicate scientifically and effectively on technical matters.

1.14.4 Analytical Validation (Shah *et al*., 1991)

Analytical validation of a pharmaceutical product or of specific ingredients within the product is necessary to ensure its safety/efficacy throughout all phases of its shelf life. Such monitoring is in accordance with the specifications elaborated/validated during the product development.

Analytical method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Method validation is the documented successful evaluation of an analytical method that provides a high level of assurance that such method will consistently yield result that are accurate with in previously established specification.

Analytical testing of a pharmaceutical product is necessary to ensure its purity, stability, safety and efficacy. Analytical method validation is an integral part of the quality control system.

Although a thorough validation cannot rule out all potential problems, the process of method development and validation should address the most common ones.

Analytical method validation ensures that the selective analytical method will give reproducible and reliable results adequate for intended purpose.

1.14.5 Analytical Parameters used in Assay Validation as per ICH Guidelines (Code Q2A; Q2B, ICH Guidelines 1994 and 1996; USP, 1995)

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. A tabular summation of the characteristics applicable to identification, control of impurities and assay procedures is included.

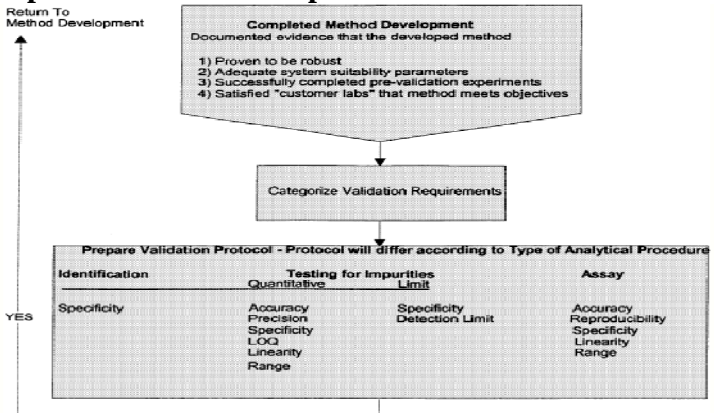
Types of Analytical Procedures to be Validated

The discussion of the validation of analytical procedures is directed to the four most common types of analytical procedures,

Identification Tests

- Quantitative tests for impurities content.
- Limit tests for the control of impurities.
- Quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product.
- A brief description of the types of tests considered in this document is provided below.
- Identification tests are intended to ensure the identity of an analyte in a sample this is normally achieved by comparison of a property of the sample (example spectrum, chromatographic behaviour, chemical reactivity etc.) to that of a reference standard.

Complete Method Development and Validation Parameters



Accuracy

The word accuracy refers to term trueness. It expresses the closeness between the true value or the reference value and the value found in the analysis. True value is the accepted value of the reference value. The accuracy is determined by the recovery studies.

Precision

Precision expresses the degree of scatter between a series of measurement made in multiple sampling from the same homogenous sample. It may be considered fewer than three levels

- Repeatability
- Intermediate precision
- Reproducibility

The precision is expressed as Variance, standard deviation and coefficient of variation for a series of measurements.

The repeatability is confirmed by a minimum of 6 estimations at 100% of test concentrations. The standard deviations should be less than 2.

The intermediate precision is confirmed by inter day and intraday analysis, different instruments and different analyst.

Specificity

Specificity refers to the ability of the method to assess the analyte in the presence of other components like impurities, matrix or degradants, etc. The implications of specificity are

Identification: To ensure identity

Purity tests: To determine the content of impurity

Assay: Content of the analyte in the sample

Limit of Detection

It is the lowest amount of an analyte that can be detected by the analytical procedure but cannot be quantified exactly.

The LOD is performed based on the following parameters

- Based on visual examination
- Based on signal to noise ratio

- Based on the standard deviation and slope value.

The visual examination is done by analysing the sample with known quantity of standard and by establishing the minimum level at which the analyte can be detected.

A signal to noise ratio of 3 o 2.1 is considered as acceptable value for calculating the detection limit.

Based on slope and standard deviation values, Detection limit can be calculated by using the formula,

$$LOD = \frac{3.3\sigma}{S}$$

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve (of the analyte)

Limit of Quantitation

The lowest amount of the analyte which can be quantified by an analytical method with precision and accuracy is the limit of quantification.

Three approaches are made for determining the quantification limit. They are similar to that of determining the detection limit.

- Based on visual examination
- Based on signal to noise ratio
- Based on slope and standard deviation value

$$LOQ = \frac{10\sigma}{S}$$

Where,

σ = the standard deviation of the response.

S = the slope of the calibration curve (of the analyte).

Linearity

Linearity is the ability of an analytical method to obtain results which are directly proportional to the analyte concentration within a given range. The linearity is evaluated as a plot of signals as a function of analyte concentration. The statistical parameters such as the slope, intercept, regression equation and correlation coefficient are calculated. For establishing of linearity, a minimum of 5 concentrations is required.

Range

It is the interval between the lower and upper limit of concentration in a sample for which the analytical method has suitable precision, accuracy and linearity. The minimum range considered for the assay of drug or finished product is form 80 to 120 percent of the test concentration.

Robustness

It is the ability of an analytical method to remain unaltered by small but deliberate variations in various parameters of the method and indicate its reliability. The typical variation includes stability of analytical solutions and extraction time. In case of HPLC, the change in the ratio of mobile phase, flow rate, variation of pH of the solution are done for determining the robustness of the method.

Acceptance criteria of validation

S.No.	Characteristics	Acceptance Criteria
1	Accuracy	Recovery 98-102% with 80,100,120% spiked sample.
2	Precision	RSD < 2%
2a	Repeatability	RSD < 2%
2b	Intermediate Precision	RSD < 2%
3	Specificity/ Selectivity	No interference
4	Detection Limit	S/N > 2 or 3
5	Quantification Limit	S/N > 10
6	Linearity	r > 0.999
7	Range	80-120%

1.15 BASIC STATISTICAL PARAMETERS

(Gupta, *et al.*, 1995; Bolton, *et al.*,2004; Mendham, *et al.*, 1994)

Statistical techniques have been widely used in many diverse areas of scientific investigation. Statistical applications have been recognized as crucial to quality control procedure, test, specification and definitions. Principle of modern analytical techniques and skill in their application are necessary attribute of the successful pharmaceutical analyst, thus does not ensure the satisfactory solution of all the problem that may

encountered. Some auxiliary knowledge methods those can aid the analyst in designing experiment, collecting data, and interpreting the result.

1.15.1 Linear Regression

Linear regression is a statistical technique that defines the functional relationship between two variables by best-fitting straight line. Once a linear relationship has been shown to have a high probability by the value of the correlation coefficient 'r', then the best straight line through the data points has to be estimated. This can often be done be done by visual inspection of the calibration graph, but in many cases it is far more sensible to evaluate the best straight line by linear regression (the method of least squares).

The equation of straight line is

$$y = mx + c$$

Where, y the dependent variable is plotted as result of changing x, the independent variable.

To obtain the regression line 'y on x' the slope 'm' of the line and the intercept 'c' on the y axis are given by the following equation.

$$m = \frac{N\sum xy - (\sum x)(\sum y)}{N\sum x^2 - (\sum x)^2} \quad \text{and} \quad c = \frac{(\sum y)(\sum x^2) - (\sum x)(\sum xy)}{N\sum x^2 - (\sum x)^2}$$

1.15.2 Correlation Coefficient (r)

It is a procedure commonly used to characterize quantitatively the relationship between variable. Correlation is related to linear regression. To establish whether there is a linear relationship between two variables x_1 and y_1, use Pearson's correlation coefficient r.

$$r = \frac{n\sum x_1 y_1 - \sum x_1 y_1}{\{[n\sum {x_1}^2 - (\sum x_1)^2]\,[n\sum {y_1}^2 - (\sum y_1)^2]\}^{1/2}}$$

Where n is the number of data points.

The value of r must lie between +1 and -1, the nearer it is to +1, the greater the probability that a definite linear relationship exists between the variables x and y, values close to +1 indicate positive correlation and values close to -1 indicate negative

correlation values of 'r' that tend towards zero indicate that x and y are not linearly related (they made be related in a non-linear fashion).

1.15.3 Standard Deviation (SD)

It is commonly used in statistics as a measure of precision statistics as a measure of precision and is more meaningful than is the average deviation. It may be thought of as a root-mean-square deviation of values from their average and is expressed mathematically as

$$S = \sqrt{\frac{\sum_{i=1}^{i=n} (x_i - \bar{x})}{N-1}}$$

Where,

S is standard deviation.

If N is large (50 or more) then of course it is immaterial whether the term in the denomination is N -1 or N

Σ = sum

$\bar{x}$ = Mean or arithmetic average.

$x - \bar{x}$ = deviation of a value from the mean.

N = Number of observations.

1.15.4 Percentage Relative Standard Deviation (%RSD)

It is also known as coefficient of variation (CV). It is defined as the standard deviation (SD) expressed as the percentage of mean.

$$CV \text{ or } \% \text{ RSD} = \frac{S.D}{\bar{x}} \times 100$$

Where,

SD = the standard deviation,

$\bar{x}$ = Mean or arithmetic average.

The variance is defined as S^2 and is more important in statistics than S itself. However, the latter is much more commonly used with chemical data.

1.15.5 Standard Error of Mean (SE)

Standard error of mean can be defined as the value obtained by division of standard deviation by square root of number of observations. It is mathematically expressed as

$$\text{S.E.} = \frac{\text{S.D.}}{\sqrt{n}}$$

Where,

SD = Standard deviation.

n = number of observation

1.15.6 Confidence Interval (CI)

A confidence interval gives an estimated range of values which is likely to include a unknown population parameter, the estimated range being calculated from a given set of sample data. A confidence interval with a particular confidence level (95% selected by the user) is intended to give the assurance that, if the statistical model is correct then the interval could deliver the true value.

Confidence interval for a normal population,

$$\bar{Y} \pm \frac{z_{\alpha/2}\ \sigma}{\sqrt{N}}$$

Where

$\bar{Y}$ = Sample mean

$z_{\alpha/2}$ = upper $\alpha/2$ critical value of standard normal distribution

N = Size of sample

σ = Standard deviation

LITERATURE REVIEW

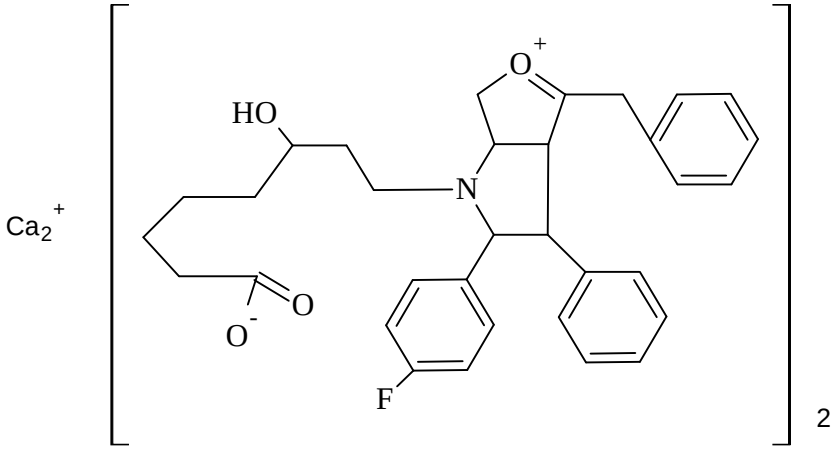
2. LITERATURE REVIEW

2.1 DRUG PROFILE

2.1.1 Atorvastatin Calcium

(IP, 2007; The Merck index, 2006; Martindale, The Extra Pharmacopeia, 1993; Clarke's, 2004; Rang & Dale *et al*.,2008; www.drugs.com/atorvastatin.html)

Molecular Structure



Chemical Name

(βR,8R)-2-(4-fluorophenyl)-α,δ-dihydroxy-5-(1-methylethyl)-3-phenyl-4-(phenylamino)carbonyl]-1H-pyrrole-1-heptanoic acid trihydrate.

Molecular Formulae

$C_{66}H_{68}CaF_2N_4O_{10}.3H_20$

Molecular Weight

1155.36 g/ mol

Category

Antilipemic

Description

A white to off-white, crystalline powder.

Solubility

Freely soluble in methanol; slightly soluble in ethanol; very slightly soluble in acetonitrile; distilled water, phosphate buffer pH 7.4; insoluble in aqueous solution of pH 4 and below.

Identification

1. Melting Point

Standard Value	Observed Value*
159.2º - 160.7ºC	160.ºC

(*Average of six observations)

2. IR spectrum was recorded and shown in figure.1

Storage

Stored protected from light at a temperature not exceeding 30ºC.

pKa value

4.46

Mechanism of Action

Atorvastatin Calcium a synthetic cholesterol-lowering agent, is a medicine called as HMG-CoA (3hydroxy-3-methylglutaryl-coenzyme A) reductase inhibitor. These enzymes are involved in cholesterol biosynthesis by catalyzing the conversion reaction of HMG-CoA to mevalonate. The function of lowering the amount of cholesterol leads to the result in clearing the LDP (low-density lipoprotein) cholesterol in the blood by increased LDL receptors. The Calcium salt of Atorvastatin is used in the treatment of primary hypercholesterolemia and dyslipidemia.

Pharmacokinetics

Absorbtion

Rapidly absorbed; T_{max} is 1 to 2 h. Bioavailability is approximately 14%; low bioavailability is because of pre systemic C1 in G1 mucosa and/or hepatic first-pass

metabolism. Food decreases rate and extent of absorbtion approximately 25% and 9% respectively, but does not alter efficacy.

Distribution

98% bind to plasma protein albumin.

Metabolism

Hepatic and extra hepatic metabolism, including first- pass metabolism and CYP3A4.

Elimination

Eliminated primarily in bile. Less than 2% of dose is recovered in the urine. Plasma $t_{1/2}$ is approximately 14 h.

Adverse Effects

Atorvastatin Calcium are well tolerated: mild unwanted effects include gastrointestinal disturbance, increased plasma concentration of liver enzymes, insomnia and rash. More serious adverse effects are rare but include severe myositis ("rhabdomyolysis") and angio edema.

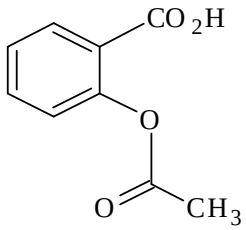
Drug Interactions

The risk of myopathy during treatment with statins is increased with concurrent administration of fibric acid derivatives, lipid-modifying doses of niacin, cyclosporine, or strong CYP 3A4 inhibitors (e.g., clarithromycin, HIV protease inhibitors, and itraconazole).

2.1.2 **Aspirin**

(IP, 2007; USP, 2009; BP 2009; The Merck index, 2006; Martindale, The Extra Pharmacopeia, 1993; Clarke's, 2004; Rang & Dale *et al.*,2008)

Molecular Structure



Chemical Name

2-(Acetyloxy)benzoic acid.

Molecular Formulae

$C_9H_8O_4$

Molecular Weight

180.2 g/ mol

Category

Analgesic; anti pyretic; anti-inflammatory; anti-thrombotic

Description

Colorless or white crystals, white crystalline powder; odorless or almost odourless.

Solubility

Slightly soluble in water; freely soluble in alcohol; soluble in chloroform and ether.

Identification

1. Melting Point

Standard Value	Observed Value*
135ºC	139ºC

(* Average of six observations)

2. IR spectrum was recorded and shown in figure.4

Storage

Stored protected from moisture at a temperature not exceeding 30ºC.

pKa value

3.5

Mechanism of Action

Aspirin acetylates prostaglandin endoperoxide synthase (prostaglandin G/H-synthase) and irreversibly inhibits its cyclooxygenase (COX) activity. The enzyme catalyses the conversion of arachidonic acid to PGH_2. Two isoforms of prostaglandin endo-peroxide synthase exist, PGHS-1 and PGHS-2. PGHS-1 is expressed consecutively in most cell types, including platlets. PGHS-2 is undetectable in most Malian cells but its expression can be induced rapidly in response to mitogenic and inflammatory stimuli.

Pharmacokinetics

Absorbtion

Rapidly and completely absorbed with systemic bioavailability of 80-100% in oral dosage form.

Distribution

99.6% bind to plasma protein albumin.

Metabolism

Metabolized consider hepatic half life 300–650 mg dose: 3.1–3.2 h 1 g dose: 5 h and 2 g dose: 9 h.

Elimination

95% was eliminated through kidneys.

Adverse Effects

Therapeutic doses: some gastric bleeding usually minimal, is common

Large doses: Dizziness, deafness and tinnitus

Toxic doses: Uncompensated respiratory acidosis plus metabolic acidosis, the later seen particularly in children

Aspirin is epidemiologically linked with an encephalitis (Reye's syndrome) if give to children with viral infections.

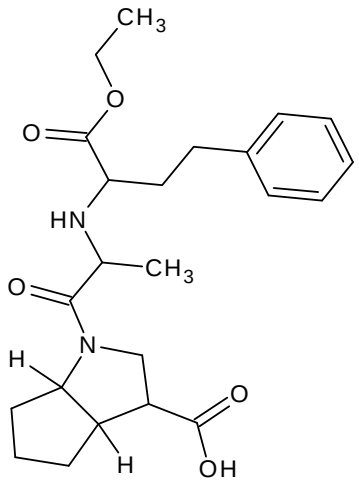
Drug Interactions

Aspirin given concomitantly with warfarin, aspirin can cause a potentially hazardous increase in the risk of bleeding, digoxin, clonidine etc.

2.1.3 Ramipril

(IP, 2007; USP, 2009; BP 2009; The Merck index, 2006; Martindale, The Extra Pharmacopeia, 1993; Clarke's, 2004; www.drugs.com/ramipril.html)

Molecular Structure



Chemical Name

(2S.3aS.6aS)-1-[(S)-N-[(S)-1-Carboxy-3-phenylpropyl]alanyl] octahydrocyclopenta[b]pyrrole-2-carboxylic acid. 1-ethyl ester.

Molecular Formulae

$C_{23}H_{32}N_2O_5$

Molecular Weight

416.5 g/ mol

Category

Anti-hypertensive

Description

White or almost white, crystalline powder.

Solubility

Sparingly soluble in water, freely soluble in methanol.

Identification

1. Melting Point

Standard Value	Observed Value*
106º - 108ºC	108.16ºC

(*Average of six observations)

2. IR spectrum was recorded and shown in figure.2

Storage

Stored at protected from moisture.

pKa value

3.32

Mechanism of Action

Ramipril, the active metabolite, competes with angiotensin I for binding at the angiotensin-converting enzyme; block the conversion of angiotensin I to angiotensin II. As angiotensin II is a vasoconstrictor and a negative-feedback mediator for rennin activity, lower concentrations result in a decrease in blood pressure and an increase in plasma renin. Ramipril may also act on kinase II, an enzyme identical to ACE that degrades the vasodilator bradykinin.

Pharmacokinetics

Absorption

Well absorbed with systemic bioavailability of 90%.

Distribution

99% binds to plasma protein albumin.

Metabolism

Metabolized to a carboxylic acid derivative by cytochrome P450.

Elimination

Excreted unchanged and as active metabolite in urine.

Adverse Effects

- Chronic cough (due to increase the bradykinin level)
- Dizziness
- Pedal edema

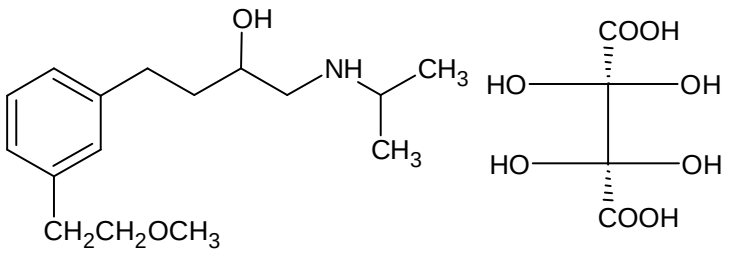
Drug Interactions

Digoxin, penicillin and cyclosporine, etc.

2.1.4 Metoprolol Tartrate

(IP, 2007; BP 2009; The Merck index, 2006; Martindale, The Extra Pharmacopeia, 1993; Clarke's, 2004)

Molecular Structure



Chemical Name

(RS)-1-(isopropylamino)-3-p-(2-methoxyethyl)phenoxypropan-2-ol (2R,3R)-tartrate

Molecular Formulae

$(C_{15}H_{25}NO_3)_2,C_4H_6O_6$

Molecular Weight

684.8 g/mol

Category

Anti hypertensive; anti angina; anti arrhythmic.

Description

A white, crystalline powder or colorless crystals.

Solubility

Very soluble in water, soluble in ethanol and chloroform; practically insoluble in ether.

Identification

1. Melting Point

Standard Value	Observed Value*
120º - 121ºC	120ºC

(*Average of six observations)

2. IR spectrum was recorded and shown in figure.3

Storage

Stored protected from light

pKa value

9.7

Mechanism of Action

Metoprolol is a competitive β_1-selective beta blocker; it blocks β_1 receptors at dosages that are much lower than those necessary to block β_2 receptors in heart and vascular smooth muscles.

Pharmacokinetics

Absorption

Well absorbed with the systemic bioavailability of 50% its increasing while continue the dose and intake with food.

Distribution

12% is bound to human serum albumin and 5-10% is bind to plasma proteins.

Metabolism

Metoprolol is extensively metabolized by the cytochrome P450 enzyme system in the liver.

Elimination

95% of the oral dose was eliminated through urine and 5% of the dose unmodified via kidneys.

Adverse Effects

- Hypotension
- Myocardial infraction
- Dizziness
- Mask the glucose level in diabetic patients

Drug Interactions

Catecholamine-depleting drugs (e.g., reserpine) may have an additive effect when given with beta-blocking agents. Patients treated with Metoprolol Tartrate plus a catecholamine depletor should therefore be closely observed for evidence of hypotension or marked bradycardia, which may produce vertigo, syncope, or postural hypotension.

Both digitalis glycosides and beta blockers slow atrioventricular conduction and decrease heart rate. Concomitant use can increase the risk of bradycardia. General anesthetics, clonidine, digoxin, and CYP2D6 inhibitors.

2.2 REPORTED METHODS

1. Satheesh Kumar Shetty *et al*., (2011), reported **"Stress Degradation Behavior of a Polypill and Development of Stability Indicating UHPLC Method for the Simultaneous Estimation of Aspirin, Atorvastatin, Ramipril and Metoprolol Succinate".** The chromatographic Separations were achieved on a Waters Acquity UPLC BEH C_{18} column 100×2.1mm, 1.7µm stationary phase with simple mobile phase combination buffer consisting of 0.1% Perchloric acid (pH 2.5) and Acetonitrile in the ratio of 80:20% v/v, delivered in a gradient mode and quantification was carried out using ultraviolet detection at 215 nm with a flow rate of 0.6 ml/ min.

2. Kamatchi Sankar A.S. *et al*., (2011), reported **"Simultaneous Estimation of Ramipril, Acetyl salicylic acid and Atorvastatin Calcium by Chemometrics Assisted UV-Spectrophotometric Method in Capsules".** The three different spectrophotometric methods for simultaneous estimation of Ramipril, Aspirin and Atorvastatin Calcium in raw material and in formulations are described. Overlapped data was quantitatively resolved by using chemometric methods *viz.* Inverse Least square (ILS), Principal Component Regression (PCR) and Partial Least Square (PLS). Calibration was constructed using the absorption data matrix corresponding to the concentration data matrix. The linearity range was found to be 1-5, 10-50 and 2-10 µg/ ml for Ramipril, Aspirin and Atorvastatin Calcium, respectively.

3. Shaik Harun Rasheed *et al*., (2010), reported **"A Stability-Indicating LC Method for the Simultaneous Determination of Metoprolol, Atorvastatin and Ramipril in Combined Pharmaceutical Dosage Form".** A chromatographic separation of the three drugs was achieved with a Hypersil C_8,15-cm analytical column using buffer-acetonitrile (55:45 v/v). The buffer used in mobile phase contains 0.02 M Sodium perchlorate in double distilled water. The instrumental settings are flow rate of 1.0 ml/ min, column temperature at ambient, the detector wavelength of 210 nm for Metoprolol, Atorvastatin and Ramipril using an ultra violet detection.

4. Raja Kumar Seshadri *et al*., (2010), reported **"Simultaneous Quantitative Determination of Metoprolol, Atorvastatin and Ramipril in Capsules by a Validated**

Stability-Indicating RP-UPLC Method". The method was developed using Zobrax® XDB-C_{18} (4.6 mm × 50 mm, 1.8 µm) column with a mobile phase consisting of 0.06% ortho phosphoric acid in milli Q® water having an ion pair reagent, 0.0045 sodium lauryl sulphate as buffer, at ratio of buffer: Acetonitrile (50:50 v/v), at 55ºC column temperature with the flow rate of 1.0 ml/ min, ultra violet detection at 210 nm. The retention times were about 1.3, 2.1 and 2.6 min for Metoprolol, Atorvastatin and Ramipril, respectively.

5. Patole S.M. *et al*., (2010), reported **"A Validated HPLC Method for Analysis of Atorvastatin Calcium, Ramipril and Aspirin as the Bulk Drug and in Combined Capsule Dosage Forms".** The separation was carried out on C_{18} column using methanol and acetate buffer (pH 3.1) adjusted with diluted ortho phosphoric acid in the ratio of 70:30 v/v. The detection wavelengths were 210 nm for Ramipril, 245 nm for Atorvastatin Calcium and 254 nm for Aspirin, with the flow rate of 1.0 ml/ min. The retention time of Atorvastatin Calcium was 8.38±0.10 min, Ramipril was 5.62±0.02 min and Aspirin was 3.04±0.15 min.

6. Vijaya Kumar Ravi *et al*., (2008), reported **"LC and LC-MS Methods for the Investigations of Polypills for the Treatment of Cardiovascular Diseases: part 1 Separation of Active Components and Classification of their Interaction/Degradation Products".** Chromatographic was carried out on C_8 column using acetonitrile and phosphate buffer (pH 2.3) as mobile phase in the ratio of 70:30 v/v, with the flow rate of 1.0 ml/ min at 210 nm.

7. Londhe S.V. *et al*., (2011), reported **" Development and Validation of RP-HPLC for Simultaneous Determination of Aspirin, Atorvastatin Calcium and Clopidogrel Bisulphate in Capsules".** The chromatographic separation was carried out on an Intersil ODS analytical column (150×4.6 mm; 5 µm) with a mixture of acetonitrile:phosphate buffer pH 3.0 adjusted with ortho phosphoric acid (50:50, v/v) as mobile phase; at a flow rate of 1.2 ml/ min. UV detection was performed at 235 nm. The retention times were 1.89, 6.6 and 19.8 min. for aspirin, Atorvastatin Calcium and clopidogrel bisulphate, respectively.

8. Ismail *et al*., (2008), reported **"RP-HPLC Method for the Simultaneous Determination of Aspirin, Atorvastatin Calcium and Pioglitazone in Capsule Dosage Form".** The separation was carried out on Zorbax SBCN, 5µm, 4.6 x 250mm, AGILENT column was used for the separation of these compounds. Acetonitrile and phosphate buffer with pH 3.5 (40 %: 60 % v/v) is used as the mobile phase, with the flow rate of 1 ml/ min, 261nm was the detection wavelength for this study.

9. Yogesh B. Zambare *et al*., (2009), reported **"Simultaneous Estimation of Atorvastatin and Ramipril by First Order Derivative Spectrophotometric Method".** The first order derivative spectrophotometric method was developed using methanol as solvent. The wavelengths selected for the analysis were 246 nm and 208 nm for Atorvastatin Calcium and Ramipril, respectively. Beer's law obeyed the concentration range of 10 – 50 µg/ ml for both the drugs.

10. GARG Gopal *et al*., (2007), reported **"Simultaneous Estimation of Ramipril and Metoprolol Tartrate in Combined Dosage Forms".** In simultaneous equation method, the drugs were determined by using the absorbance values of Ramipril and Metoprolol at selected wavelengths, viz. 205 nm and 222.5 nm respectively. The second method depends on the application of area under the curve at selected wavelength range, 200 to 210 nm and 217.5 to 227.5 nm for Ramipril and Metoprolol tartrate, respectively. Third method is based on the determination of graphical absorbance ratio at two selected wavelengths; one being the iso-absorptive point for the drugs (216.5 nm) and the other being the absorption maximum of Metoprolol (222.5 nm). The fourth method is based on the derivative spectrophotometric method at zero crossing wavelengths.

11. Nagaraj *et al*., (2007), reported **"Simultaneous Quantitative Resolution of Atorvastatin Calcium and Fenofibrate in Pharmaceutical Preparation by using Derivative Ratio Spectrophotometry and Chemometric Calibrations".** In ratio spectra derivative spectrophotometry, analytical signals were measured at wavelengths corresponding to either maximums or minimums for both drugs in first derivative spectra of ratio spectra obtained by using either spectrum as divisor. For the remaining four methods using chemometric techniques, namely, classical least squares (CLS), inverse

least squares (ILS), principal component regression (PCR) and partial least squares (PLS), the calibrations were constructed by using the absorption data matrix corresponding to the concentration data matrix, with measurements in the range of 231 - 310 nm (Deltalambda = 1 nm) in their zero-order spectra.

12. Khan M.R. *et al*., (2006), reported **"Simultaneous Spectrophotometric Determination of Atorvastatin Calcium and Amlodipine Besylate in Tablets".** First method employs formation and solving of simultaneous equations using 245 nm and 363 nm as two analytical wavelengths. Second is dual wavelength method, which uses the difference of absorbance value at 259.9 nm and 354 nm for estimation of Atorvastatin Calcium and absorbance at 363 nm for Amlodipine besylate. Fifty percent methanol was used as solvent.

13. Sonawane S.S. *et al.,* (2007), reported **"Simultaneous Spectrophotometric Estimation of Atorvastatin Calcium and Ezetimibe in Tablets".** The method involves, Q- absorbance ratio method, the absorbance measurement at 235.5 nm (iso-absorptive point) and 246.0 nm (λmax of Atorvastatin Calcium), in methanol.

14. Popat B. Mohite *et al*., (2010), reported **"Simultaneous Estimation of Ramipril and Telmisartan in Tablet Dosage Form by Spectrophotometry".** The method based on UVspectrophotometric determination of two drugs, Method A is by using multicomponent method. It involves absorbance measurement at 205.0 nm (λmax of Ramipril) and 291.0 nm (λmax of Telmisartan) in 0.2M H_2SO_4. Beer's law obeyed in the concentration range of 5-40 μg/ ml for Ramipril and 2-20 μg/ ml for Telmisartan. Method B was graphical absorbance method which is based on measurement of absorbance of Ramipril and Telmisartan at 222.0 nm (iso-absorptive point of Ramipril and Telmisartan) and 291.0 nm (λmax of Telmisartan).

15. Priyanka R. Patil *et al*., (2009), reported **"Simultaneous Estimation of Ramipril and Amlodipine by UV-Spectrophotometric Method".** The method involved the estimation of Ramipril and Ambroxol by simultaneous equation method. The

wavelengths selected for the analysis are 210 nm λmax of Ramipril and 238 nm λmax of Ambroxol. The solvent used was methanol.

16. Gupta K.R. *et al*., (2008), reported **"New Spectrophotometric Method for Simultaneous Determination of Metoprolol Tartrate and Hydrochlorthiazide in Tablets".** The two-wavelength method for simultaneous determination of Metoprolol and Hydrochlorothiazide in fixed dose combination tablet. The wavelengths selected for method were 257.8 nm, 282.9 nm and 315.0 nm. The absorbance difference at first two wavelengths was used for determination of Metoprolol and the latter was used for determination of Hydrochlorthiazide**.**

17. Lincy Joseph *et al*., (2008), reported **"Simultaneous Estimation of Atorvastatin and Ramipril by RP-HPLC and Spectroscopy".** Separation was done by Intersil ODS column (250 × 4.6 mm) 5µ at 40ºC using 50% acetonitrile and 50% Sodium perchlorate buffer pH 2.5 with dilute phosphoric acid as mobile phase. With the flow rate was 1.2ml/ min at 215 nm. The simultaneous equation method has selected two wavelengths 247 nm and 208 nm the λmax of Atorvastatin Calcium and Ramipril, respectively.

18. Chandra Bose R.J. *et al*., (2011), reported **"Validated RP-HPLC Method for the Simultaneous Estimation of Ramipril and Metoprolol Tartrate in Bulk and Tablet Dosage Form".** The separation was achieved on a Hypersil C_{18}, 150 mm x 4.6 mm, 5 µm particle size in isocratic mode with mobile phase acetonitrile: methanol: 10mM Acetate buffer (30: 50: 20 v/v/v) and pH adjusted to 5 ± 0.1 with triethanolamine was used. The flow rate was 1.0 ml/ min and absorbance of individual component was measured at 210 nm. The retention times of Metoprolol tartrate and Ramipril were found to be 2.85 and 3.88 min, respectively.

19. Shah D.A. *et al.*, (2004), reported **"Development and Validation of a RP-HPLC Method for Determination Atorvastatin Calcium and Aspirin in a Capsule Dosage Form"**. The separation was achieved on a A Phenomenex Gemini C_{18}, 5 mm column having 250 x 4.6 mm i.d. in isocratic mode, with mobile phase containing 0.02 M potassium di hydrogen phosphate: methanol (20:80) adjusted to pH 4 using

ortho phosphoric acid was used. The flow rate was 1.0 ml/ min and effluents were monitored at 240 nm. The retention times of Atorvastatin Calcium and Aspirin were 5.4 min and 3.4 min, respectively.

20. Shah D.A. *et al*., (2008), reported **"Stability Indicating RP-HPLC Estimation of Atorvastatin Calcium and Amlodipine Besylate in Pharmaceutical Formulations".** The separation was carried out on a Phenomenex Gemini C_{18}, 5 µm column having 250×4.6 mm i.d. in isocratic mode, with mobile phase containing 0.02 M potassium dihydrogen phosphate:acetonitrile:methanol (30:10:60, v/v/v) adjusted to pH 4 using ortho phosphoric acid was used. The flow rate was 1.0 ml/ min and effluents were monitored at 240 nm. The retention times of Atorvastatin Calcium and Amlodipine besylate were 11.6 min and 4.5 min, respectively.

21. Jain N. *et al*., (2008), reported **"Development and Validation of RP-HPLC Method for Simultaneous Estimation of Atorvastatin Calcium and Fenofibrate in Tablet Dosage Forms".** The separation was achieved by Luna C_{18} column and methanol: acetate buffer pH 3.7 (82:18 v/v) as mobile phase, at a flow rate of 1.5 ml/ min. Detection was carried out at 248 nm. Retention times of Atorvastatin Calcium and Fenofibrate was found to be 3.02+0.1 and 9.05+0.2 min, respectively.

22. Srinivasa Rao K. *et al*., (2010), reported **"RP-HPLC Method for the Determination of Losartan Potassium and Ramipril in Combined Dosage Form".** The separation was achieved by Hypersil ODS C_{18}, 4.6×250 mm, 5 µm column in isocratic mode, with mobile phase acetonitrile:methanol:10 mM tetra butyl ammonium hydrogen sulphate in water in the ratio of 30:30:40% v/v/v was used. The flow rate was 1.0 ml/ min and effluent was monitored at 210 nm. The retention times of Losartan Potassium and Ramipril were 4.7 and 3.3 min, respectively.

23. Kurade V.P. *et al.,* (2009), reported **"RP-HPLC Estimation of Ramipril and Telmisartan in Tablets".** The separation was achieved by Genesis C_{18} column having dimensions of 4.6×250 mm and particle size of 5 µm in isocratic mode, with mobile phase containing a mixture of 0.01 M potassium dihydrogen phosphate buffer (adjusted

to pH 3.4 using ortho phosphoric acid): methanol : acetonitrile (15:15:70 v/v/v) was used. The mobile phase was pumped at a flow rate of 1.0 ml/ min and the eluents were monitored at 210 nm. The selected chromatographic conditions were found to effectively separate Ramipril (R_t: 3.68 min) and Telmisartan (R_t: 4.98 min) having a resolution of 3.84.

24. Rawool N.D. *et al*., (2011), reported **"Analytical Method for the Simultaneous Estimation of Metoprolol Tartrate and Hydrochlorthiazide using RP-HPLC".** The separation is done on a C_{18} column using phosphate buffer along with methanol as mobile phase, in the proportion of 60:40 and it is estimated at a λ_{max} of 226 nm with a flow rate of 1 ml/ min.

25. Sohan S. Chitlange *et al*., (2008), reported **"RP-HPLC Method for Simultaneous Estimation of Metoprolol Tartrate And Amlodipine Besylate in Tablet Formulation".** The determination was carried out on a Kromasil C_{18} (250 x 4.6 mm, 5 µm) column using a mobile phase of 0.02 M phosphate buffer solution: acetonitrile (70:30v/v, pH 3.0). The flow rate was 1.0ml/ min with detection at 221 nm. The retention time for Amlodipine was 2.57 min and for Metoprolol 4.49 min.

26. Sagar S. Panda *et al*., (2010), reported **"Ion-Pairing RP-HPLC Method for Simultaneous Determination of Aspirin and Clopidogrel Bisulphate in Tablet and Capsule Dosage Form".** The proposed RP-HPLC method utilized LiChroCART-LiChrospher 100;C_{18} column (250mm × 4mm i.d.,5µm) and mobile phase consisting of acetonitrile:0.01M TBAHS (50:50% v/v) at a flow rate of 1.0ml/ min. Quantification was achieved with UV detection at 240 nm. The retention times were 3.167min and 5.758min for Aspirin and Clopidogrel, respectively.

27. Swapnil D. Jadhav *et al*., (2010), reported **"Spectrophotometric Methods for Estimation of Atorvastatin Calcium from Tablet Dosage Forms".** In the present incestigation, hydrotropic solubilization is employed to enhance the aqueous solubilities of poorly water- soluble drugs like Atorvastatin Calcium in tablet dosage forms. This method utilizes 2.0 M urea solution as, hydrotropic solubilizing agent. In the urea

solution Atorvastatin Calcium shows maximum absorbance at 240 nm. The 2.0 M urea solution does not show any interference with the sampling wavelength. Another method is formation of green color complex between the drug Atorvastatin Calcium and 0.3 % w/v ferric chloride and 0.02 % w/v potassium ferricyanide. The green colored complex shows the maximum absorbance at 787 nm.

28. Anil kumar Sharma *et al*., (2010), reported **"Simultaneous Estimation of Atorvastatin Calcium, Ramipril and Aspirin in Capsule Dosage Form using HPTLC"**. The method was developed using precoated silica gel 60F254 as stationary phase. The mobile phase used was a mixture of benzene: ethyl acetate: toluene: methanol: glacial acetic acid (4.0:4.5:1.0:0.5:0.1 v/v/v/v/v). Detection was carried out with ultra-violet detection at 220 nm. The Rf values were about 0.45±0.02, 0.28±0.01 and 0.72±0.02 for Atorvastatin Calcium, Ramipril and Aspirin, respectively.

29. Nagavalli D. *et al*., (2010), reported **"Simultaneous Estimation of Atorvastatin Calcium, Ezetimibe and Fenofibrate in Pure and in Combined Dosage Form by RP-HPLC and HPTLC Methods".** In method A, Atorvastatin Calcium, Ezetimibe and Fenofibrate were chromatographed on Phenomenax Luna ODS analytical column (150 mm x 4.6 mm i.d, 5µ) using Methanol: Acetonitrile: Water (50:30:20 v/v/v) as the mobile phase and scanned at 254 nm. Rt values of Atorvastatin Calcium, Ezetimibe and Fenofibrate were found to be 1.77 ± 0.02, 2.62 ± 0.01 and 9.26 ± 0.03 respectively. In method B, Atorvastatin Calcium, Ezetimibe and Fenofibrate were chromatographed on silica gel 60 F_{254} TLC plate using chloroform: Toluene: Methanol: Glacial acetic acid (6:3:1:0.5 v/v/v/v) as the mobile phase and scanned at 254 nm. The Rf value Atorvastatin Calcium, Ezetimibe and Fenofibrate were found to be 0.48 ± 0.02, 0.56 ± 0.03 and 0.87 ± 0.02 respectively.

30. Stephan Rathinaraj B. *et al*., (2010), reported **"Determination of Atorvastatin Calcium and Ezetimibe by using HPTLC Method".** The stationary phase used was precoated silica gel 60F 254. The mobile phase used was a mixture of benzene, methanol, acetone and triethylamine (7:2:1:0.2 v/v/v/v). The detection spots were carried out at 266 nm.

31. Gaikwad A.V. *et al.*, (2011), reported **"Simultaneous Estimation of Ramipril and Valsartan in Tablets by HPTLC".** Identification and determination were performed on 10 cm × 10 cm aluminum-backed TLC plates, coated with 0.2 mm layers of silica gel 60 F254, previously washed with methanol using ethyl acetate : chloroform: glacial acetic acid, (8:2:0.2, *v/v*) as mobile phase. Detection was carried out densitometrically using UV detector at 220 nm. The Rf values were 0.15 for Ramipril and 0.49 for Valsartan.

32. Jitendra A. Wayadande *et al.*, (2011), reported **"Validated HPTLC Method for Simultaneous Estimation of Ramipril and Metazolone in Bulk Drugs and Formulation".** Chromatographic separation of the drugs was performed on aluminum plates precoated with silica gel 60 F254 as the stationary phase and the solvent system consisted of toluene : ethyl acetate : methanol : glacial acetic acid (4 : 4 : 1 : 0.2 v/v/v/v). Densitometric evaluation of the separated zones was performed at 223 nm. The two drugs were satisfactorily resolved with RF values 0.33 ± 0.02 and 0.59 ±0.02 for Ramipril and Metolazone respectively.

33. Prajakta S. Nawale *et al.*, (2012), reported **"Normal and RP-HPTLC Methods for Simultaneous Estimation of Telmisartan and Metoprolol Succinate in Pharmaceutical Formulation".** Method I was developed with aluminium plates precoated with silica gel 60F254S, and toluene : propanol : methanol : triethylamine (8 : 1 : 1 : 0.5 v/v) was used as as mobile phase. Method II was carried out using aluminium coated with RP-18 silica gel 60 F254S HPTLC plates using methanol : water : triethylamine (6 : 4 : 0.5 v/v) as mobile phase. Both analyses were scanned with a densitometer at 242 nm. In Method I, good separation and resolution of drugs were achieved with Rf values 0.45 ± 0.02 (Telmisartan) and 0.70 ± 0.02 (Metoprolol), while in Method II, Telmisartan and Metoprolol showed Rf values 0.55 ± 0.02 and 0.41 ± 0.02, respectively.

34. Savitha S. Yadhav *et al.*, (2005), reported **"A Simple and Sensitive HPTLC Method for the Determination of Content Uniformity of Atorvastatin Calcium Tablets".** The

stationary phase was precoated silica gel 60 F254. The mobile phase used was a mixture of benzene: methanol (7:3 v/v). Combination of benzene: methanol offered optimum migration (Rf = 0.46±0.02). Detection of the spot was carried out at 281nm.

35. Nageswara Roa Pilli *et al*., (2011), reported **"Simultaneous Determination of Atorvastatin Calcium, Amlodipine Besylate, Ramipril and Benazepril in Human Plasma by LC-MS/MS and its Application to a Human Pharmakinetic Study".** The API-4000 LC-MS/MS was operated under the multiple-reaction monitoring mode using electrospray ionization. The Analytes and IS were extracted from plasma by simple liquid–liquid extraction technique using ethyl acetate. The reconstituted samples were chromatographed on C_{18} column by pumping 0.1% formic acid–acetonitrile (15:85, v/v) at a flow rate of 1 ml/ min.

36. Gowda K.V. *et al*., (2007), reported **"Liquid Chromatography Tandem Mass Spectrometry Method for Simultaneous Determination of Metoprolol Tartrate, Ramipril in Human Plasma".** The chromatographic separation was performed on a reversed-phase C_8 column with a mobile phase of 10 mM ammonium formate-methanol (3:97, v/v). The protonated analyte was quantitated in positive ionization by multiple reactions monitoring with a mass spectrometer.

37. Altuntas T.G., *et al*., (2004), reported **"Liquid Chromatographic Determination of Atorvastatin in Bulk Drug, Tablets and Human Plasma"** A simple, specific, and accurate high performance liquid chromatographic (HPLC) method for determination of atorvastatin in bulk drug, tablets, and human plasma have been developed. Liquid chromatography was performed on a RP-Supelcosil C_{18} (5 µm, 150 × 4.6 mm) column and the mobile phase consisted of an acetonitrile:methanol:water (45:45:10 v/v/v), and a flow rate of 1.0 mL/min. The effluent was monitored on a UV detector at 240 nm.

AIM AND PLAN OF WORK

3. AIM OF WORK

3.1 AIM OF WORK

Hypertension is a chronic disorder very commonly seen among people. Current hypertension treatment guidelines recommend a goal of <140/90 mmHg for population with uncomplicated hypertension and goals are even lower (< 130/80 mmHg) for patients with diabetes or renal disease. These recommendations are supported by long-term trials suggesting that the greater the reduction in Blood Pressure, the greater the reduction in risk of cardiovascular events. Major clinical studies have shown that most patients require two or more drugs to achieve their Blood Pressure goals. Combination therapy should be used as initial treatment for patients in whom the probability of achieving BP control with monotherapy is low. Given the number of antihypertensive agents available, the number of potential combinations is large. However, rational choices should be based on some requirements.

The prevalence of cardiovascular disease will undoubtedly increase as the mean age of the populations' throughput the world increases. Heart disease is generally complex and multifactorial. Despite considerable advances in therapy, the underlying mechanisms are still rather poor. Efforts are directed towards the development of new drugs through the modification of the physicochemical properties of drug molecule, and the design and synthesis of new drugs with particular emphasis on drug delivery systems, pharmaceutical materials research, controlled drug release and targeting of drugs to the site of action to enhance the therapeutic effects. The perspective of the user enables the pharmacist and clinicians in understanding the factors leading to the suboptimal use of drugs in society. Identifying, solving and preventing drug-related problems in order to improve health and quality of life through pharmaceutical care in the community are therefore also important. Equally, the optimal utilization of pharmacist and clinician know – how will bring tremendous benefits to the patients from the viewpoint of social Pharmacy in the promotion of safe and efficient use of drugs.

The use of combination drug therapy for cardiovascular (CV) disease risk reduction is the established approach to multiple risk factor reduction. The spectrum of rational combination products in CV disease prevention and treatment alone is extremely

broad. Development of fixed-dose combination drug products requires information beyond that needed for approval of single active ingredient products. Establishing the rationale and target populations for novel combinations, as well as the contributions of the component drugs to the claimed effects, and characterizing the pharmacokinetics, the pharmacodynamics, and clinical safety and efficacy of the combination are all necessary to support approval.

Nowadays, Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate are the antihypertensive drugs in combined dosage form used for the treatment of prolonging hypertension and decrease LDL in CABG patients, and improve the quality of life. There are several methods were reported for the estimation of Atorvastatin Calcium, Aspirin Ramipril and Metoprolol Tartrate individually, two or three drugs combinations and the combination of these drugs with other drugs. But there are no methods were reported for estimation of these drugs in combined dosage form without prior separation.

The non- availability of any UV-spectroscopy and HPTLC until now for the simultaneous analysis of the combination made it a worth-while objective to pursue the present work.

Hence the present work was aimed to develop simple, precise and accurate methods for the estimation of Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate in bulk and in combined pharmaceutical dosage form and to validate the developed methods by UV-spectroscopy and HPTLC.

3.2 PLAN OF WORK

3.2.1 Survey on Literature

A complete literature survey was made on the drugs of Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate for the various physicochemical properties such as solubility, melting point and storage conditions etc. This survey gives some basic information of the drugs and the reported analytical methods of these drugs which help in the process of analytical method development.

3.2.2 Procurement of Raw Material and Formulation

The standard raw materials drugs of Atorvastatin Calcium, Aspirin, Ramipril, and Metoprolol Tartrate were obtained as the gift samples from Madras Pharmaceuticals, Chennai, India. ZYCAD-4, containing Atorvastatin Calcium- 10 mg, Aspirin- 75 mg, Ramipril- 5 mg and Metoprolol Tartrate- 50 mg was procured from Vasantha Medicals; Choolaimedu; Chennai.

3.2.3 Method Development

The solubility of individual drugs was checked and from the list of solvents the common and stable solvent for the four drugs was selected. The solvent selected for both UV spectroscopy and HPTLC must be cheap and readily available.

The various steps involved in the method development as follows,

UV Spectroscopy

- Selection of appropriate analytical wavelength and selection of suitable method
- Determination of working concentration range
- Analysis of synthetic mixture
- Simultaneous analysis of the formulation by using the developed method

HPTLC Method

- Determination of suitable detection wavelength
- Optimization of chromatographic conditions
- Analysis of formulation
- System suitability testing

3.2.4 Validation

The method to be developed should be validated as per ICH and USFDA guidelines. The various parameters of validation are Linearity, Range, Precision, Accuracy, LOD, LOQ and Ruggedness.

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 Drug Sample

The raw materials were obtained from Madras Pharmaceuticals Chennai.

4.1.2 Formulation Used

The formulations ZYCAD-4, containing Atorvastatin Calcium- 10 mg, Aspirin- 75 mg, Ramipril- 5 mg and Metoprolol Tartrate- 50 mg was purchased from Vasantha Medicals, Choolaimedu, Chennai.

4.1.3 Chemicals and Solvents Used

Methanol (AR grade), Methanol (HPLC grade), Benzene (AR grade), Toluene (AR grade), Ethyl Acetate (AR grade), Chloroform (AR grade) and Glacial Acetic Acid (AR grade) were purchased from Qualigens India Pvt. Limited, Mumbai and Loba Chemie India Limited, Mumbai. Distilled water was obtained from Double distillation unit in our laboratory.

4.1.4 Instruments Used

Different instruments used to carry out the work are,

- Shimadzu AUX- 220 Digital balance.
- Shimadzu- 1700 Double Beam UV-Visible Spectrophotometer with a pair of 10 mm matched quartz cells.
- ELICO SL-210 Double Beam UV- Visible Spectrophotometer with pair of 10 mm matched quartz cells.
- Soltec - Sonica Ultrsonic Cleaner - Model 2200 MH.
- Remi Centrifuge Apparatus
- Cyberlab Micropipette.

4.1.5 Specifications of Instruments

4.1.5.1 Shimadzu AUX- 220 digital balance (Shimadzu instruction manual)

SPECIFICATIONS	
Weighing capacity	200 gms
Minimum display	0.1 mg
Standard deviation	≤ 0.1 mg
Operating temperature range	5 to 40° C

4.1.5.2 Double beam UV-Visible Spectrophotometer (Shimadzu Instruction Manual)

Model: Shimadzu, UV-1700; Cuvetts: 1 cm matched quartz cells.

SPECIFICATIONS	
Light source	20 W halogen lamp, Deuterium lamp. Light source position automatic adjustment mechanism
Monochromators	Aberration- correcting concave blazed holographic grating
Detector	Silicon photodiode
Stray Light	0.04% or less (220 nm: NaI 10 g/lt) 0.04% or less (340 nm: $NaNO_2$ 50 g/lt)
Measurement wavelength range	190 ~ 1100 nm
Spectral band width	1nm or less (190 to 900 nm)
Wavelength accuracy	±0.5 nm on board automatic wavelength calibration mechanism
Recording range	Absorbance: -3.99~3.99 Abs Transmittance: -3.99~3.99 Abs
Photometric accuracy	±0.004 Abs (at 1.0 Abs), ±0.002 Abs (at 0.5 Abs)
Operating temperature/ humidity	Temperature range: 15 to 35° C Humidity range: 35 to 80% (15 to below 30° C) 35 to 70% (30 to below 35° C)

4.1.5.3 Elico - SL 210 Double Beams UV- Visible Spectrophotometer

(Elico Instruction Manual)

SPECIFICATIONS	
Light source	Tungsten halogen lamp (W), Deuterium lamp (D_2). Light source positions automatic adjustment mechanism
Monochromators	Concave holographic grating with 1200 lines/mm
Detector	Photodiode
Stray light	<0,05%T at 220 nm with NaI 10 g/lt
Measurement Wavelength range	190 to 1100 nm
Spectral Band Width	1.8 nm
Wavelength Accuracy	±0.5 nm
Spectral repeatability	±0.2 nm
Spectral readability	0.1 nm
Recording range	±3.0000 Abs
Photometric accuracy	±0.005 Abs (at 1.0 Abs), ± 0.010 Abs (at 1.5 abs)
Operating temperature/ Humidity	Temperature range: 15 to 35º C Humidity range: 35 to 80% (15 to below 30º C) 35 to 70% (30 to below 35º C)

4.1.5.4 CAMAG Automatic TLC Sampler 4 (ATS4)

APPLICATOR SPECIFICATIONS	
Spray gas	Nitrogen
Sample solvent type	Methanol
Spray gas temperature	Unheated
Syringe size	25 µl
Number of tracks	15
Application type	Band
Band length	8 mm

4.1.5.5 CAMAG TLC Scanner 3

SPECIFICATIONS	
Lamp	D_2 and W lamp
Scanning Speed	20 mm/ sec
Data Resolution	100 µm/ step
Measurement Type	Remission
Measurement Mode	Absorption
Optical Filter	Second Order
Detector Mode	Automatic

4.2 METHODS

An effort was made to develop and validate simple, accurate and precise methods for the simultaneous analysis of Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate in bulk and in pharmaceutical dosage form by

1. UV Spectroscopy

   First order derivative spectrophotometry

2. HPTLC method.

4.2.1 UV Spectroscopy

4.2.1.1 First Order Derivative Spectrophotometry

Derivative spectrophotometry involves the conversion of a normal spectrum to its first, second or higher derivative spectrum. The transformations that occur in the derivative spectra are understood by reference to a Gaussian band which represents an ideal absorption band. In the context of derivative spectrophotometry, the normal absorption spectrum is referred to as the fundamental, zero order or D° spectrum. The first derivative (D_1) spectrum is a plot of the ratio of change of absorbance with wavelength against wavelength.

4.2.1.1.2 Selection of Solvent

The solubility of drugs was determined in a variety of polar and non-polar solvents as per specification. The common and stable solvent was found to be Methanol and further dilutions were made with distilled water for the analysis of Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate for the proposed method.

4.2.1.1.3 Preparation of Standard Stock Solutions

15 mg of Atorvastatin Calcium was weighed accurately and transferred in to 10 ml volumetric flask. Dissolved with methanol and made up to the volume with methanol. The stock solution contains 1.5 mg/ ml of Atorvastatin Calcium

50 mg of Aspirin, Ramipril and Metoprolol Tartrate were accurately weighed and transferred in to 50 ml volumetric flask separately. Dissolved in methanol and made up to the volume to 50 ml. The solution contains 1 mg/ ml of Aspirin, Ramipril and Metoprolol Tartrate.

4.2.1.1.4 Selection of Wavelength

10 µg/ ml concentration solutions of Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate were prepared from the stock solution and the spectra were recorded between 200 and 400 nm by using distilled water as blank. The zero order spectra were derivitized to first order and the spectra were overlained. From the overlain spectra, the wavelength selected for the analysis were 291.5 nm, 247 nm 242.5 nm and 229.5 nm for Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate, respectively.

4.2.1.1.5 Stability Studies

The stability of Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate were checked using 10 µg/ ml solutions at the selected wavelengths in different time intervals. It was found that Atorvastatin Calcium was stable for up to 3 hours, Aspirin up to 3 hours and 30 minutes, Ramipril up to 1 hour and 30 minutes, and Metoprolol Tartrate up to 1 hour.

4.2.1.1.6 Linearity and Calibration

5 ml of Atorvastatin Calcium stock solution was pipetted out in to 50 ml volumetric flask and made up to 50 ml with distilled water. From that 1 – 7 ml were transferred in to a series of 50 ml volumetric flasks and made up to the volume with distilled water to get concentrations of 3 to 21 µg/ ml. 1 – 7 ml of the stock solutions of Aspirin, Ramipril and Metoprolol Tartrate were transferred in to series of 100 ml volumetric flask individually and made up to the volume with distilled water to get the concentrations of 10 – 70 µg/ ml of Aspirin, Ramipril and Metoprolol Tartrate.

4.2.1.1.7 Synthetic Mixture

4 µg/ ml of Atorvastatin Calcium and 30 µg/ ml of Aspirin, Ramipril and Metoprolol Tartrate were prepared individually from their corresponding stock solutions. 1 to 5 ml were pipetted out from each stock solution into a series of six100 ml volumetric flasks and made up to 100 ml with distilled water to get a mixture of Atorvastatin Calcium, Aspirin, Ramipril, and Metoprolol Tartrate in the concentration range of 4 to 20 µg/ ml for Atorvastatin Calcium and 30 to 70 µg/ ml for Aspirin, Ramipril, and Metoprolol Tartrate. The absorbances of the prepared synthetic mixtures were measured at the selected wavelengths. The amount of drugs in the prepared synthetic mixture was calculated.

4.2.1.1.8 Quantification of Formulation

Twenty capsules (ZYCAD-4, containing Atorvastatin Calcium – 10 mg, Aspirin – 75 mg, Ramipril – 5 mg, Metoprolol Tartrate – 50 mg) were accurately weighed and the average weight was calculated. The capsules containing powder were crushed and made in to a fine powder. The mixed contents of capsule powder equivalent to 25 mg of Aspirin was accurately weighed and transferred in to a series of 25 ml volumetric flasks and added 15 mg/ ml solution of Ramipril. Dissolved in methanol and sonicated for15 minutes. The volume was made up to 25 ml with methanol and was centrifuged for 15 minutes at 2000 rpm. The solution was filtered through whatmann filter paper No.41. 3 ml of the stock solution was further diluted to 100 ml volumetric flask and made up to with distilled water to get the theoretical concentrations of 4 µg/ ml, 30 µg/ ml, 20µg/ ml and 20 µg/ ml of Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate, respectively. The absorbances of solutions were measured at 291.5 nm, 247 nm, 242.5 nm and 229.5 nm. The procedure was repeated for six times. The amounts of these drugs were calculated.

4.2.1.1.9 Recovery Studies

4.2.1.1.9.1 Preparation of Raw Material Standard Stock Solutions

10 mg of Atorvastatin Calcium raw material was weighed accurately in to a 10 ml volumetric flask, dissolved with methanol and made up to 10 ml with methanol to get a concentration of 1 mg/ ml of Atorvastatin Calcium. Similarly 100 mg of Aspirin, Ramipril and Metoprolol Tartrate raw material were weighed accurately and transferred in to 10 ml volumetric flasks separately, dissolved with methanol and made up to 10 ml with methanol to get concentrations of 10 mg/ ml of Aspirin, Ramipril and Metoprolol Tartrate.

4.2.1.1.9.2 Recovery Procedure

The recovery study was done by adding a specified quantity of the drug to the pre-analyzed formulation. The tablet powder equivalent to 25 mg of Aspirin was weighed accurately and added 15 mg/ ml solution of Ramipril in to three separate 25 ml volumetric flasks. To this 2 ml, 3 ml, 4 ml of Atorvastatin Calcium, 2.0 ml, 2.5 ml, 3.0 ml of Aspirin, 1.0 ml, 1.5 ml, 2.0 ml of Ramipril and Metoprolol Tartrate stock solutions were added. Dissolved in methanol, sonicated for 15 minutes and made up to 25 ml with

methanol. The solution was centrifuged for 15 minutes at 2000 rpm and was filtered through whatmann filter paper No.41. From each solution, 3 ml was transferred to six 100 ml volumetric flasks and made up to the volume with distilled water. The absorbances of the solutions were measured at 291.5 nm, 247 nm, 242.5 nm and 229.5 nm. The amount of drug recovered was calculated. The procedure was repeated three times for each concentration.

4.2.1.1.10 Validation of Developed Methods

Linearity

A calibration curve was plotted with concentration versus the absorbance value. The linearity was checked for Atorvastatin Calcium in the concentration range of 3 to 21 µg/ ml, Aspirin, Ramipril and Metoprolol Tartrate in the concentration range of 10 to 70 µg/ ml. The drugs were found to be linear in the specified concentration ranges.

Precision

The repeatability of the method was confirmed by repeated analysis of the formulation for six times with the same concentration. The amount of drug present in the formulation was calculated. The percentage RSD value was calculated. Confidence Interval (95%) was also calculated.

The intermediate precision was confirmed by the intraday and inter day analysis i.e. the analysis was performed three times on the same day and on three successive days. The amount of drug present in the formulation was calculated. The percentage RSD values were calculated. Confidence Interval (95%) was also calculated.

Ruggedness

Ruggedness of the method was confirmed by the analysis of formulation in different analyst and by different instrument. The amount of drug was calculated and the percentage RSD values were also calculated. Confidence Interval (95%) was also calculated.

Accuracy

Accuracy of the method was confirmed by recovery studies. To the pre-analyzed formulation a known quantity of the standard drug solutions were added and the amount

of drug recovered was calculated. The percentages RSD values were calculated. Confidence Interval (95%) was also calculated.

LOD and LOQ

The linearity study was carried out for six times. The LOD and LOQ were calculated based up on the calibration curve method. The LOD and LOQ were calculated using the average of slope and standard deviation of intercept.

4.2.2 HPTLC

In HPTLC, the separation of the components of a mixture is based on the principle of adsorption. The HPTLC differ from the TLC in the size of silica gel used as the stationary phase and automated sampling application and detection. In the present study a twin through chamber and silica 60 F 254 were used.

Selection of Stationary Phase

The resolution of Atorvastatin Calcium, Ramipril, Aspirin and Metoprolol Tartrate was achieved using TLC plate made up of silica gel G60 F 254 coated on an Aluminium support (E.Merck). The size of the silica gel particle was 2 μ and thickness of sorbent layer was 0.2 mm. The plates were supplied in 20 × 10 cm size which was cut in to appropriate sizes for method development.

Selection of Mobile Phase

The mobile phase system was chosen based on the solubility and polarity of four drugs. The solution of drugs was prepared in methanol and used for spotting. Methanol gets vaporized soon after application on to the plate under nitrogen stream. After trying different mobile phase system an ideal system was chosen based on the resolution between compounds. The fixed mobile phase system for the separation of four drugs consisted of (mobile phase) with an appropriate Rf values. The drugs were scanned at 224 nm after the development.

The velocity of mobile phase in HPTLC is affected by the nature of the stationary phase (porosity, packing, particle size, etc), as well as mobile phase properties (viscosity, surface tension, vapour pressure of solvents, etc). Generally the velocity of mobile phase decreases during chromatographic development due to higher resistance of stationary phase densely packed with fine particles.

Different mixtures of mobile phase were tried to choose the ideal mobile phase with good resolution for the analysis. For that purpose select the mobile phase from previous journals and books the common mobile phase was Benzene, Ethyl Acetate, Toluene, Methanol, Chloroform and Glacial Acetic Acid They include the following

Various mobile phase trials

Mobile Phase	**Ratio**
Benzene: Ethyl Acetate: Toluene: Methanol: Glacial Acetic acid	(4.5:4.0:1.0:0.5:0.1% v/v/v/v/v)
Benzene: Ethyl Acetate: Toluene: Methanol: Glacial acetic acid	(6.0:4.0:1.5:1.5:0.1% v/v/v/v/v)
Benzene: Chloroform: Toluene: Methanol: Glacial Acetic acid	(3.5:3.5:2.0:1.0:0.1% v/v/v/v/v)
Benzene: Chloroform: Toluene: Methanol: Glacial Acetic Acid	(3.5:3.5:1.5:1.5:0.1% v/v/v/v/v)
Benzene: Chloroform: Toluene: Methanol: Glacial Acetic Acid	(3.0:3.5:1.5:2.0:0.1% v/v/v/v/v)
Benzene: Chloroform: Toluene: Methanol: Glacial Acetic Acid	(3.0:3.0:1.5:2:5:0.1% v/v/v/v/v)
Benzene: Chloroform: Toluene: Methanol: Glacial Acetic Acid	(3.0:2.5:1.5:3.0:0.1% v/v/v/v/v)
Benzene: Chloroform: Toluene: Methanol: Glacial Acetic Acid	(2.5:3.0:1.5:3.0:0.1% v/v/v/v/v)
Benzene: Chloroform: Toluene: Methanol: Glacial Acetic Acid	(2.0:.3.0:1.5:3.5:0.1% v/v/v/v/v)
Benzene: Carbon Tetra Chloride: Toluene: Methanol: Glacial Acetic Acid	(4.0:4.5:0.5:1.0:0.1% v/v/v/v/v)
Benzene: Toluene: Methanol: Glacial Acetic Acid	(6.5:2.0:1.5:0.1% v/v/v/v)
Benzene: Toluene: Methanol: Glacial Acetic Acid	(7.5:1.0:1.5:0.1% v/v/v/v)

From the above list of mobile phase the mobile phase Benzene: Toluene: Methanol: Glacial Acetic Acid (7.5:1.0:1.5:0.1% v/v/v/v) was found to be an ideal mobile phase with good resolution between the spots.

Optimization of Variants in TLC

The composition of mobile phase, chamber saturation (equilibration time), plate equilibration time, the distance of solvent development and bandwidth of the spot are a few variants affect Rf values of drugs.

Chamber Saturation (Equilibration time)

Chamber saturation is done so that equilibration is established eventually between the components of developing solvents and their vapour phase and the formation of secondary solvent fronts could be avoided

Hence in the current study chamber saturation was taken in to consideration to achieve reproducible Rf values and peak area. The mobile phase was placed on one side of twin through chamber and shaken well. Different saturation times were maintained for different mobile phase. The chamber saturation time for Benzene: Toluene: Methanol: Glacial Acetic Acid (7.5:1.0:1.5:0.1% v/v/v/v) was 30 minutes.

Selection of Detection Wavelength

By comparing the spectral characters of Atorvastatin Calcium, Ramipril, Aspirin and Metoprolol Tartrate the detection wavelength was selected for the method 224 nm was selected for as the detection wavelength with reference to the spectral conformation graph.

4.2.2.1Optimized Chromatographic Conditions

After conforming with the mobile phase and detection wavelength, the optimized conditions for the method was as follows

Stationary Phase	-	Silica Gel 60 F 254 HPTLC Plates
Mobile Phase	-	Benzene: Toluene: Methanol: Glacial Acetic Acid
Mobile Phase ratio	-	7.5: 1.0: 1.5: 0.1 % v/v/v/v
Detection	-	CAMAG TLC scanner 3, at 224 nm
Temperature	-	Room Temperature
Chamber	-	Twin through Chamber
Development Mode	-	Ascending Mode

4.2.2.2 Preparation of Standard Stock Solution

100 mg of Metoprolol Tartrate, 10 mg of Ramipril, 20 mg of Atorvastatin Calcium, and 75 mg of Aspirin raw materials were accurately weighed in to 100 ml volumetric flask, dissolved in methanol and made up to the volume with methanol. This solution contains 1 mg/ ml of Metoprolol Tartrate, 100 µg/ ml of Ramipril, 200 µg/ ml of Atorvastatin Calcium and 750 µg/ ml of Aspirin.

4.2.2.3 Linearity and Calibration Curve

1 ml of the stock solutions was pipetted out in to 10 ml volumetric flasks and made up to the volume with methanol. 1 ng/ µl – 6 ng/ µl were spotted on TLC plates and to get the concentration range of 100 – 600 ng/ µl, 10 – 60 ng/ µl, 20 – 120 ng/ µl and 75 – 450 ng/ µl of Metoprolol Tartrate, Ramipril, Atorvastatin Calcium and Aspirin, respectively.

4.2.2.4 Quantification of Formulation

Twenty capsules were weighed accurately and the average weight of each capsule was determined. Powdered the mixed contents of capsule powder and weighed accurately a quantity of the capsule powder equivalent to 75 mg of Aspirin in to a series of six 100 ml volumetric flasks. Dissolved in methanol and sonicated for 15 minutes. The volume was made up to 50 ml with methanol and was centrifuged for 15 minutes at 2000 rpm. The solution was filtered through whatmann filter paper No.41. 1 ml of the filtrate was further diluted to 10 ml with methanol. The solution contains 100 ng/ µl, 10 ng/ µl, 20 ng/ µl and 150 ng/ µl of Metoprolol Tartrate, Ramipril, Atorvastatin Calcium and Aspirin theoretically. 1 µl spots were placed on the plates and the chromatogram was developed in the twin through chamber. From the peak area and amount of drugs were calculated. The procedure was repeated for six times.

4.2.2.5 Recovery Studies

4.2.2.5.1 Preparation of Raw Material Standard Stock Solutions

200 mg of Metoprolol Tartrate, 20 mg of Ramipril, 40 mg of Atorvastatin Calcium and 300 mg of Aspirin raw materials were weighed accurately in to 10 ml volumetric flasks individually, dissolved with methanol and made up to the volume with methanol to get a concentration of 20 mg/ ml, 2 mg/ ml, 4 mg/ ml and 30 mg/ ml of Metoprolol Tartrate, Ramipril, Atorvastatin Calcium and Aspirin.

4.2.2.5.2 Recovery Procedure

The recovery study was done by adding a specified quantity of the drug to the pre-analyzed formulation. The tablet powder equivalent to 75 mg of Aspirin was weighed accurately in to three separate 50 ml volumetric flasks. To this 2 ml, 2.5 ml and 3 ml of Metoprolol Tartrate, Ramipril, Atorvastatin Calcium and Aspirin stock solutions were added. Dissolved in methanol, sonicated for 15 minutes and made up to 50 ml with methanol. The solutions were centrifuged for 15 minutes at 2000 rpm and were filtered through Whatmann filter paper No.41. From each solution, 1 ml was transferred to six 10 ml volumetric flasks and made up to the volume with methanol. From this solution, 1 µl quantity of sample was spotted and the chromatogram was recorded. From the peak area, the amount of drug recovered was calculated. The procedure was repeated three times for each concentration.

4.2.2.6 Validation of Developed Method

Linearity

A calibration curve was plotted with concentration versus the peak area. The linearity range was checked for in the concentration range of 100 – 600 ng/ µl, 10 – 60 ng/ µl, 20 – 120 ng/ µl and 75 – 450 ng/ µl of Metoprolol Tartrate, Ramipril, Atorvastatin Calcium and Aspirin, respectively. The drugs were found to be linear in the specified concentration ranges.

Precision

The repeatability of the method was checked by repeated analysis of the formulation for six times with the same concentration. The amount of drug present in the formulation was calculated. The percentage RSD value was calculated. Confidence Interval (95%) was also calculated.

The intermediate precisions were confirmed by the intraday and inter day analysis i.e. the analysis was performed three times on the same day and on three successive days. The amount of drug present in the formulation was calculated. The percentage RSD values were calculated. Confidence Interval (95%) was also calculated.

Accuracy

Accuracy of the method was confirmed by recovery studies. To the pre-analyzed formulation a known quantity of the standard drug solutions were added and the amount of drug recovered was calculated. The percentages RSD values were calculated. Confidence Interval (95%) was also calculated.

LOD and LOQ

The linearity study was carried out for three times. The LOD and LOQ were calculated based up on the calibration curve method. The LOD and LOQ were calculated using the average of slope and standard deviation of intercept.

RESULTS AND DISCUSSION

5. RESULTS AND DISSCUSION

Simultaneous estimation of four drugs in a formulation has more advantages such as accurate, less use of reagent and less time requirement for the estimation rather than individual estimation of four drugs. The simple, precise and accurate analytical techniques were developed for the simultaneous estimation of Atorvastatin Calcium, Ramipril, Metoprolol Tartrate and Aspirin in bulk and in pharmaceutical dosage form. The methods include

1. UV Spectroscopy

- First Order Derivative Spectrophotometry

2. HPTLC Method

5.1 UV Spectroscopy

5.1.1 First Order Derivative Spectrophotometry

A simple, precise and accurate first order derivative spectrophotometric method was developed and validated for the simultaneous estimation of Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate in bulk and in combined dosage form. The drugs were identified by the melting point and IR spectrum, the IR spectrum of Atorvastatin Calcium, Ramipril, Metoprolol Tartrate and Aspirin were given in figures 1, 2, 3 and 4, respectively. The solubility of drugs in various polar and non polar solvents checked as per I.P guidelines. All the drugs were exhibited different solubility characters. From the solubility data, the common solvents were found to be methanol, acetonitrile, acetone, dichloromethane and alkaline borate buffer (pH 9). The solvents such as acetonitrile, acetone, dichloromethane and alkaline borate buffer (pH 9) were not selected because the cut off wavelength of these solutions were above 240 nm and the solvent interference should be expected during the analysis. Hence, methanol was selected as the common solvent and it was used for the preparation of stock solution. Further dilutions were made with distilled water. The solubility profile of Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate were given in tables 1, 2, 3, and 4, respectively.

10 µg/ ml concentration solutions of Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate were prepared and the spectra were recorded. The overlain zero order

spectra are shown in figure 5. By observing the spectral characters of Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate, the methods used for the multi component analysis viz. simultaneous equation method, absorption correction method and absorption ratio method were not applied, because the interference were more. Hence the normal curve was derivitized to first order, and overlained and shown in figure 6. From the overlain spectra 291.5 nm, 247 nm, 242.5 nm and 229.5 nm were selected for the simultaneous estimation of Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate, respectively. At 291.5 nm, Atorvastatin Calcium has the absorbance where as Aspirin, Ramipril and Metoprolol Tartrate has no absorbance. Hence this wavelength selected for the analysis of Atorvastatin Calcium without the interference of other three drugs. At 247 nm Atorvastatin Calcium and Aspirin were showed marked absorbance whereas Ramipril and Metoprolol Tartrate have zero crossing points. The absorbance of Atorvastatin Calcium was interfered in the analysis of Aspirin. Hence, the absorbance of Atorvastatin Calcium was corrected for interference from the total absorbance value. With the help of corrected absorbance the amount of Aspirin was calculated at 247 nm. At 242.5 nm Atorvastatin Calcium, Aspirin and Ramipril were showed marked absorbance and Metoprolol Tartrate have zero crossing point. The absorbance of Atorvastatin Calcium and Aspirin were interfered in the analysis of Ramipril. Hence, the absorbance of Atorvastatin Calcium and Aspirin were corrected for interference from the total absorbance value. With the help of the corrected absorbance, the amount of Ramipril was calculated at 242.5 nm. At 229.5 nm Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate were showed the absorbance of Atorvastatin Calcium, Aspirin and Ramipril were interfered in the analysis of Metoprolol Tartrate. Hence, the absorbance of Atorvastatin Calcium, Aspirin and Ramipril were corrected for interference from the total absorbance value. The corrected absorbance value was used for the analysis of Metoprolol Tartrate at 229.5 nm.

The stability of Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate were checked at the selected wavelengths using methanol and distilled water as solvent. It was found that Atorvastatin Calcium stable for about 3 hours, Aspirin for about 3 hours and 30 minutes, Ramipril for about 1 hour and 30 minutes and Metoprolol Tartrate for about 1 hour.

Various aliquots of Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate were prepared in the concentration range of 3 - 21 µg/ ml, 10 – 70 µg/ ml, 10 - 70 µg/ ml and 10 - 70 µg/ ml, respectively. The absorbances of these solutions were measured at the selected wavelengths. The calibration curve was constructed using concentration versus absorbance. The preparation of calibration curve was repeated for six times for each drug at their selected wavelengths. The optical parameters like Molar absorptivity, Sandell's sensitivity, Correlation coefficient, Slope, Intercept, LOD and LOQ were calculated. The correlation coefficient for all the four drugs was found to be above 0.999. This indicates that all the drugs obey Beer's law in the selected concentration ranges. Hence the concentrations were found to be linear. The calibration graph for Atorvastatin Calcium at 291.5 nm is shown in figure 7. The calibration curves for Atorvastatin Calcium and Aspirin at 247 nm are shown in figure 8 and 9, respectively. The calibration curves for Atorvastatin Calcium, Aspirin and Ramipril at 242.5 nm are shown in figure 10, 11 and 12, respectively. At 229.5 nm, the calibration curves for Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate are shown in figure 13, 14, 15 and 16, respectively. The optical characteristics of the drugs at their selected wavelengths are shown in tables 5, 6, 7, and 8, respectively.

The developed method was applied for the analysis of synthetic mixture to find out the developed method was correct or not. The amount of Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate were found to be in the range of 98.00 - 100.87%, 98.16 - 100.36%, 99.22 - 100.84% and 100.23 – 101.21%, respectively. The results are listed in table 9. The amount found was good agreement with the expected concentration. Hence it was planned to apply for the analysis of formulation.

The capsule formulation ZYCAD-4 (containing Atorvastatin Calcium- 10 mg, Aspirin – 75 mg, Ramipril- 5 mg and Metoprolol Tartrate- 50 mg) was selected for the analysis. The drugs Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate in the ratio of 2: 15: 1: 10 in the formulation. The percentage purity of the drugs in the formulation was found to be 102.76 ± 1.4487, 99.00 ± 1.5795, 99.95 ± 1.7250 and 98.66 ± 0.8496 for Atorvastatin Calcium, Aspirin, Ramipril, and Metoprolol Tartrate, respectively. The results are listed in table 10.The confidence interval (95%) for drugs

was found to be in the range of 101.23 – 104.28, 97.34 – 100.65, 98.13 – 101.76 and 97.76 – 99.55 for Atorvastatin Calcium, Aspirin, Ramipril, and Metoprolol Tartrate, respectively. The precision of the method was confirmed by the repeated analysis of formulation for six times. The percentage RSD values were found to be 1.4097, 1.5954, 1.7257 and 0.8612 for Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate, respectively.

Further the precision of the method was confirmed by intraday and inter day analysis. Intraday and inter day analysis of formulation was done on three times on same day and one time on three consecutive days. The percentage RSD for the intraday and inter day precision was found to be 0.1528 and 0.0680 for Atorvastatin Calcium, 0.8116 and 0.3145 for Aspirin, 1.9063 and 1.1612 for Ramipril and 1.7204 and 1.0513 for Metoprolol Tartrate, respectively. The results are listed in table 11. The low percentage RSD values indicated that the precision of the method was confirmed. The confidence interval (95%) of intraday analysis were found to be in the range of 101.48 – 102.25, 97.15 – 101.14, 96.06 – 105.61 and 95.25 – 103.76 for Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate, respectively. The confidence interval (95%) of inter day analysis were found to be in the range of 101.72 – 102.07, 98.57 – 100.12, 98.25 – 104.08 and 96.24 – 101.41 for Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate, respectively.

The ruggedness of the method was validated by using different analysts and different instruments. The percentage RSD for analyst 1 and analyst 2 were found to be 1.3842 and 0.2887 for Atorvastatin Calcium, 1.5954 and 1.8396 for Aspirin, 1.7256 and 1.8602 for Ramipril and 0.8611 and 0.8367 for Metoprolol Tartrate, respectively. The percentage RSD for instrument 1 and instrument 2 were found to be 1.1602 and 1.5153 for Atorvastatin Calcium, 0.6684 and 0.4462 for Aspirin, 1.9820 and 1.0782 for Ramipril and 1.2887 and 0.8223 for Metoprolol Tartrate, respectively. The results are listed in table 12. The Confidence interval (95%) of different analyst 1 was found to be in the range of 101.23 -104.22, 97.34 – 100.65, 98.13 – 101.76 and 97.76 – 99.55 for Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate, respectively. The Confidence interval (95%) of different analyst 2 was found to be in the range of

101.63 -102.24, 97.60 – 101.45, 98.03 – 101.94 and 98.20 – 99.93 for Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate, respectively. The Confidence interval (95%) of different instrument 1 was found to be in the range of 101.87 -102.22, 98.70 – 100.09, 99.00 – 103.21 and 97.63 – 100.30 for Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate, respectively. The Confidence interval (95%) of different instrument 2 was found to be in the range of 98.37 - 101.54, 98.54 – 99.47, 98.67 – 100.92 and 98.17 – 99.88 for Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate, respectively.

The accuracy of the method was confirmed by the recovery studies. To the pre-analyzed formulation, a known quantity of raw material was added and the percentage recovery was calculated. The percentage of raw material added was 60%, 90%, and 120% for Atorvastatin Calcium, Ramipril and Metoprolol Tartrate and 80%, 100% and 120% for Aspirin. The percentage recovery was found to be in the range of 99.76 - 100.45% for Atorvastatin Calcium, 100.38 - 101.08% for Aspirin, 98.58 - 101.64% for Ramipril and 98.33 - 101.69% for Metoprolol Tartrate. The percentage RSD value was found to be 0.3508 for Atorvastatin Calcium, 0.3506 for Aspirin, 1.5580 for Ramipril and 1.8860 for Metoprolol Tartrate. The low percentage RSD indicated there was no interference due to excipients used in formulation. Hence, the accuracy method was conformed. The results are listed in table 13. The confidence interval (95%) for drugs was found to be in the range of 99.52 – 101.27, 99.82 – 101.57, 96.40 – 104.17 and 94.85 – 104.18 for Atorvastatin Calcium, Aspirin, Ramipril, and Metoprolol Tartrate, respectively.

5.2 HPTLC Method

An effort was made to develop a simple, precise and accurate method for the simultaneous estimation of Metoprolol Tartrate, Ramipril, Atorvastatin Calcium and Aspirin in bulk and in pharmaceutical dosage form by HPTLC method.

The initial separation was based up on the solubility of drugs, the different mobile phase were tried to get the better resolution. The different mixtures of the mobile phase tried were Benzene : Ethyl Acetate : Toluene : Methanol : Glacial Acetic Acid, Benzene : Chloroform : Toluene : Methanol : Glacial Acetic Acid and Benzene : Toluene :

Methanol : Glacial Acetic Acid with different ratios. In Benzene : Toluene : Methanol : Glacial Acetic Acid, all the drugs were well separated with good resolution when compared to other mobile phases. By altering the composition of Benzene the separation was affected more. Hence the chromatograms were recorded by changing the concentration of Benzene. In the mobile phase, Benzene : Toluene : Methanol : Glacial Acetic Acid in the ratio of 7.5: 1.0: 1.5: 0.1, all the drugs were eluted with better resolution. Hence this was selected as the mobile phase for the analysis of Metoprolol Tartrate, Ramipril, Atorvastatin Calcium and Aspirin. With the above selected mobile phase the UV spectra of all the drugs were recorded and overlained. From the overlain spectra, at 224 nm all the drugs showed marked absorbance. Hence this was selected as the detection wavelength and shown is figure 17.

With the optimized chromatographic conditions, the chromatograms were recorded and shown in figures 18, 19, 20 and 21 for Metoprolol Tartrate, Ramipril, Atorvastatin Calcium and Aspirin, respectively. The Rf value for Metoprolol Tartrate, Ramipril, Atorvastatin Calcium and Aspirin were found to be 0.14 ± 0.01, 0.26 ± 0.01, 0.40 ± 0.01 and 0.99 ± 0.01, respectively.

The linearity range was fixed as 100 – 600 ng/ µl for Metoprolol Tartrate, 10 – 60 ng/ µl for Ramipril, 20 – 120 ng/ µl for Atorvastatin Calcium and 75 – 450 ng/ µl for Aspirin in methanol and shown in figures 22 – 27. The calibration graph was recorded using peak area and concentration and these are shown in figures 28 – 31. The correlation coefficients were found to be 0.9999, 0.9998, 0.9998 and 0.9998 for Metoprolol Tartrate, Ramipril, Atorvastatin Calcium and Aspirin, respectively. The optical characteristics such as the Correlation coefficient, Slope, Intercept, LOD and LOQ and were calculated and shown in tables 14 – 17. The correlation coefficient values indicated that the selected concentration was linear.

The capsule dosage form ZYCAD-4 was selected for the analysis. The chromatogram for the analysis of formulation was shown in figures 32 – 37. The percentage purity of Metoprolol Tartrate, Ramipril, Atorvastatin Calcium and Aspirin were found to be 100.24 ± 0.8348, 99.93 ± 0.5910, 99.00 ± 0 .6526 and 99.97 ± 0.0829,

respectively. The results of analysis are shown in table 18. The confidence interval (95%) for drugs was found to be in the range of 99.36 – 101.11, 99.30 – 100.55, 98.31 – 99.68 and 99.88 – 100.05 for Metoprolol Tartrate, Ramipril, Atorvastatin Calcium and Aspirin, respectively. Precision of the method was confirmed by repeated analysis of formulation for six times. The percentage RSD values were found to be 0.8328, 0.5914, 0.6593 and 0.0830 for Metoprolol Tartrate, Ramipril, Atorvastatin Calcium and Aspirin, respectively.

Further the precision of the method was confirmed by intraday and inter day analysis. Intraday and inter day analysis of formulation was done on three times on same day and one time on three consecutive days. The percentage RSD values for the intraday and inter day precision was found to be 0.7639 and 1.2832 for Metoprolol Tartrate, 0.7197 and 1.1052 for Ramipril, 0.1547 and 2.0167 for Atorvastatin Calcium and 0.3158 and 0.1399 for Aspirin. The results of analysis are shown in table 19. The confidence interval (95%) of intraday analysis were found to be in the range of 98.14 – 101.93, 98.40 – 101.99, 98.35 – 99.10 and 98.97 - 100.54 for Metoprolol Tartrate, Ramipril, Atorvastatin Calcium and Aspirin, respectively. The confidence interval (95%) of inter day analysis were found to be in the range of 96.88 – 101.27, 96.92 – 102.39, 95.20 – 105.19 and 99.69 – 100.38 for Metoprolol Tartrate, Ramipril, Atorvastatin Calcium and Aspirin, respectively.

The accuracy of the method was confirmed by the recovery studies. To the pre-analyzed formulation, a known quantity of raw material was added and the percentage recovery was calculated. The percentage of raw material added was 80%, 100% and 120% for all four drugs. The chromatograms for the recovery analysis are shown in figures 38 – 40. The percentage recovery was found to be in the range of 99.00 - 99.99%, for Metoprolol Tartrate 99.86 - 101.26% for Ramipril, 98.03 - 101.98 for Atorvastatin Calcium and 98.40 - 98.71 for Aspirin. The percentage RSD values were found to be 0.5087, 0.6978, 1.6383 and 0.1787 for Metoprolol Tartrate, Ramipril, Atorvastatin Calcium and Aspirin, respectively. The low percentage RSD value indicates that there was no interference due to the excipients used in formulation during the analysis. The data of recovery analysis are listed in table 20. The confidence interval (95%) for drugs was found to be in the range of 98.29 – 100.80, 98.84 – 102.33,

96.49 – 104.70 and 98.16 – 99.03 for Metoprolol Tartrate, Ramipril, Atorvastatin Calcium and Aspirin, respectively.

SUMMARY AND CONCLUSION

6. SUMMARY AND CONCLUSION

Two simple, precise and accurate methods were developed for the simultaneous estimation of Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate in bulk and in pharmaceutical dosage form.

The methods developed were

1. UV Spectroscopy

- First Order Derivative Spectrophotometry

2. HPTLC Method

6.1 UV Spectroscopy

6.1.1 First Order Derivative Spectrophotometry

From the solubility profile, methanol was chosen as the common solvent for the simultaneous analysis of Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate. The spectra of all the drugs recorded and are derivitized to first order. From the overlain first order derivative spectra 291.5 nm, 247 nm, 242.5 nm and 229.5 nm were selected for the analysis of Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate, respectively.

Beer's law obeyed the concentration range of 3 to 21 µg/ ml, 10 to 70 µg / ml, 10 to 70 µg/ ml and 10 to 70 µg / ml for Atorvastatin Calcium, Aspirin, Ramipril, and Metoprolol Tartrate, respectively. The calibration graphs were plotted. The correlation coefficient values for all the drugs were more than 0.999. The optical parameters like the Molar absorptivity, Sandell's sensitivity, Correlation coefficient, Slope, Intercept, LOD and LOQ, and were calculated.

The developed method was applied for the analysis of synthetic mixture to find out the developed method was correct or not. The amount of Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate were found to be in the range of 98.00 - 100.87%, 98.16 - 100.36%, 99.22 - 100.84% and 100.23 – 101.21%, respectively.

The amount found was good agreement with the expected concentration. Hence it was planned to apply for the analysis of formulation.

ZYCAD-4 capsule containing Atorvastatin Calcium 10 mg, Aspirin 75 mg, Ramipril 5 mg and Metoprolol Tartrate 50 mg and was selected for the analysis. The percentage of drugs in the formulation was found to be 102.76 ± 1.4487, 99.00 ± 1.5795, 99.95 ± 1.7250 and 98.66 ± 0.8496 for Atorvastatin Calcium, Aspirin, Ramipril, and Metoprolol Tartrate, respectively. The confidence interval (95%) for drugs was found to be in the range of 101.23 – 104.28, 97.34 – 100.65, 98.13 – 101.76 and 97.76 – 99.55 for Atorvastatin Calcium, Aspirin, Ramipril, and Metoprolol Tartrate, respectively.

The precision of the method was confirmed by the repeatability studies. The percentages RSD were found to be 1.4097, 1.5954, 1.7257 and 0.8612 for Atorvastatin Calcium, Aspirin, Ramipril, and Metoprolol Tartrate, respectively.

Further the precision of the method was confirmed by intraday and inter day analysis. The percentages RSD values for intraday and inter day were found to be 0.1528 and 0.0680 for Atorvastatin Calcium, 0.8116 and 0.3145 for Aspirin, 1.9063 and 1.1612 for Ramipril and 1.7204 and 1.0513 for Metoprolol Tartrate, respectively. The confidence interval (95%) of intraday analysis were found to be in the range of 101.48 – 102.25, 97.15 – 101.14, 96.06 – 105.61 and 95.25 – 103.76 for Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate, respectively. The confidence interval (95%) of inter day analysis were found to be in the range of 101.72 – 102.07, 98.57 – 100.12, 98.25 – 104.08 and 96.24 – 101.41 for Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate, respectively.

The ruggedness of the method was confirmed by performing the analysis with the different analysts and different instruments. The percentage RSD values for different analysts were found to be 1.3842 and 0.2887 for Atorvastatin Calcium, 1.5954 and 1.8396 for Aspirin, 1.7256 and 1.8602 for Ramipril and 0.8611 and 0.8367 for Metoprolol Tartrate, for analyst 1 and analyst 2, respectively. The percentage RSD values for different instruments were found to be 1.1602 and 1.5153 for Atorvastatin Calcium, 0.6684 and 0.4463 for Aspirin, 1.9820 and 1.0782 for Ramipril and 1.2887 and

0.8223 for Metoprolol Tartrate, for instrument 1 and instrument 2, respectively. The Confidence interval (95%) of different analyst 1 was found to be in the range of 101.23 - 104.22, 97.34 – 100.65, 98.13 – 101.76 and 97.76 – 99.55 for Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate, respectively. The Confidence interval (95%) of different analyst 2 was found to be in the range of 101.63 -102.24, 97.60 – 101.45, 98.03 – 101.94 and 98.20 – 99.93 for Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate, respectively. The Confidence interval (95%) of different instrument 1 was found to be in the range of 101.87 -102.22, 98.70 – 100.09, 99.00 – 103.21 and 97.63 – 100.30 for Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate, respectively. The Confidence interval (95%) of different instrument 2 was found to be in the range of 98.37 - 101.54, 98.54 – 99.47, 98.67 – 100.92 and 98.17 – 99.88 for Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate, respectively.

The accuracy of the method was confirmed by recovery studies. To the preanalysed formulation the different amount of raw material were added and the amount of drugs recovered were calculated. The percentage recovery was found to be in the range of 99.76 - 100.45% for Atorvastatin Calcium, 100.38 - 101.08% for Aspirin, 98.58 -101.64% for Ramipril and 98.33 - 101.69% for Metoprolol Tartrate. The percentage RSD value was found to be 0.3508 for Atorvastatin Calcium, 0.3506 for Aspirin, 1.5580 for Ramipril and 1.8860 for Metoprolol Tartrate. The confidence interval (95%) for drugs was found to be in the range of 99.52 – 101.27, 99.82 – 101.57, 96.40 – 104.17 and 94.85 – 104.18 for Atorvastatin Calcium, Aspirin, Ramipril, and Metoprolol Tartrate, respectively.

6.2 HPTLC Method

The simple, precise and accurate HPTLC method was developed for the simultaneous estimation of Metoprolol Tartrate, Ramipril, Atorvastatin Calcium and Aspirin in bulk and in pharmaceutical dosage form.

The mobile phase consists of Benzene: Toluene: Methanol: Glacial Acetic Acid (7.5: 1.0: 1.5: 0.1% v/v/v/v) was selected for the analysis. From the spectral characteristics, 224 nm was selected as detection wavelength for the analysis.

Beer's law obeyed the concentration range of 100 – 600 ng/ µl, 10 – 60 ng/ µl, 20 – 120 ng/ µl and 75 – 450 ng/ µl for Metoprolol Tartrate, Ramipril, Atorvastatin Calcium and Aspirin, respectively. The calibration graphs were plotted. The correlation coefficient values for all the drugs were more than 0.999. The optical parameters like the Correlation coefficient, Slope, Intercept, LOD and LOQ were calculated.

ZYCAD-4 capsule containing Atorvastatin Calcium 10 mg, Aspirin 75 mg Ramipril 5 mg and Metoprolol Tartrate 50 mg was selected for the analysis. The percentage of drugs in the formulation was found to be 100.24 ± 0.8348, 99.93 ± 0.5910, 99.00 ± 0.6526 and 99.97 ± 0.0829 for Metoprolol Tartrate, Ramipril, Atorvastatin Calcium and Aspirin, respectively**.** The confidence interval (95%) for drugs was found to be in the range of 99.36 – 101.11, 99.30 – 100.55, 98.31 – 99.68 and 99.88 – 100.05 for Metoprolol Tartrate, Ramipril, Atorvastatin Calcium and Aspirin, respectively.

Precision of the method was confirmed by repeated analysis of formulation for six times. The percentage RSD values were found to be 0.8328, 0.5914, 0.6593 and 0.0830 for Metoprolol Tartrate, Ramipril, Atorvastatin Calcium and Aspirin, respectively**.**

Further the precision of the method was confirmed by intraday and inter day analysis. The percentage RSD values for the intraday and inter day precision was found to be 0.7639 and 1.2832 for Metoprolol Tartrate, 0.7197 and 1.1052 for Ramipril, 0.1547 and 2.0167 for Atorvastatin Calcium and 0.3158 and 0.1399 for Aspirin. The confidence interval (95%) of intraday analysis were found to be in the range of 98.14 – 101.93, 98.40 – 101.99, 98.35 – 99.10 and 98.97 100.54 for Metoprolol Tartrate, Ramipril, Atorvastatin Calcium and Aspirin, respectively. The confidence interval (95%) of inter day analysis were found to be in the range of 96.88 – 101.27, 96.92 – 102.39, 95.20 – 105.19 and 99.69 – 100.38 for Metoprolol Tartrate, Ramipril, Atorvastatin Calcium and Aspirin, respectively.

The accuracy of the method was confirmed by the recovery studies. To the pre-analyzed formulation, a known quantity of raw material was added and the percentage recovery was calculated. The percentage recovery was found to be in the

range of 99.00 - 99.99%, for Metoprolol Tartrate 99.86 - 101.26% for Ramipril, 98.03 - 101.98 for Atorvastatin Calcium and 98.40 - 98.71 for Aspirin. The percentage RSD values were found to be 0.5087, 0.6978, 1.6383 and 0.1787 for Metoprolol Tartrate, Ramipril, Atorvastatin Calcium and Aspirin, respectively. The confidence interval (95%) for drugs was found to be in the range of 98.29 – 100.80, 98.84 – 102.33, 96.49 – 104.70 and 98.16 – 99.03 for Metoprolol Tartrate, Ramipril, Atorvastatin Calcium and Aspirin, respectively.

Two instrumental analytical methods were successfully developed for the simultaneous estimation of Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate and in bulk and in pharmaceutical dosage form.

The two methods were found to be accurate, precise and rapid for the simultaneous estimation of these drugs. This was confirmed by low percentage RSD values. The spectrophotometric method is found to be economical when compared to the HPTLC method. But HPTLC is more sensitive than UV spectrophotometric method. The low percentage RSD value in the recovery studies suggests that the excipients do not interfere in the analysis of formulation and hence all the methods are accurate. HPTLC is found to be more sensitive than other method. Because the linearity range, LOD, LOQ were less in HPTLC method than UV spectroscopic method. Hence it is suggested that these two methods can be applied successfully for the routine quality control analysis for the simultaneous estimation of Atorvastatin Calcium, Aspirin, Ramipril and Metoprolol Tartrate in bulk and in pharmaceutical dosage form and the obtained results will be presented elsewhere.

FIGURES

Figure 1

IR SPECTRUM OF ATORVASTATIN CALCIUM

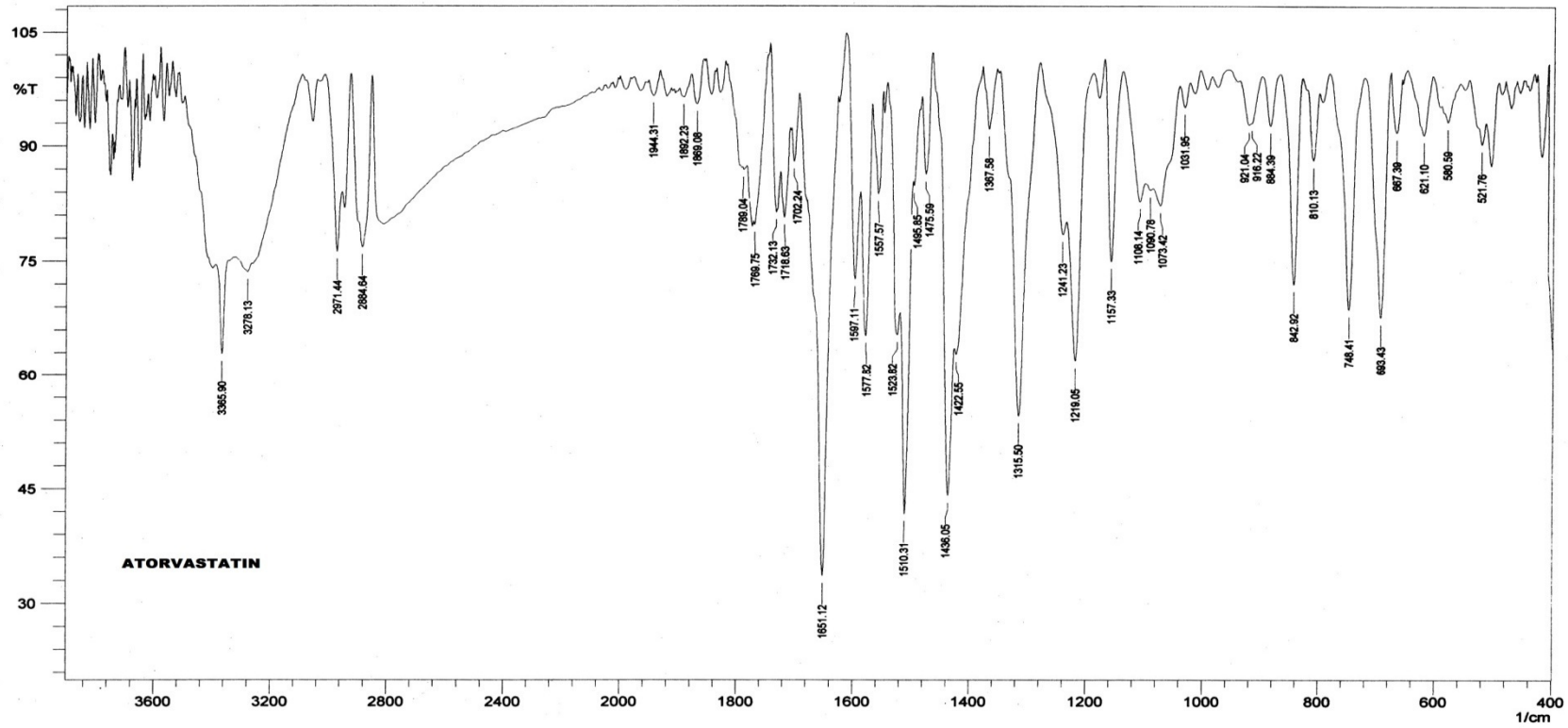


Figure 2

IR SPECTRUM OF ASPIRIN

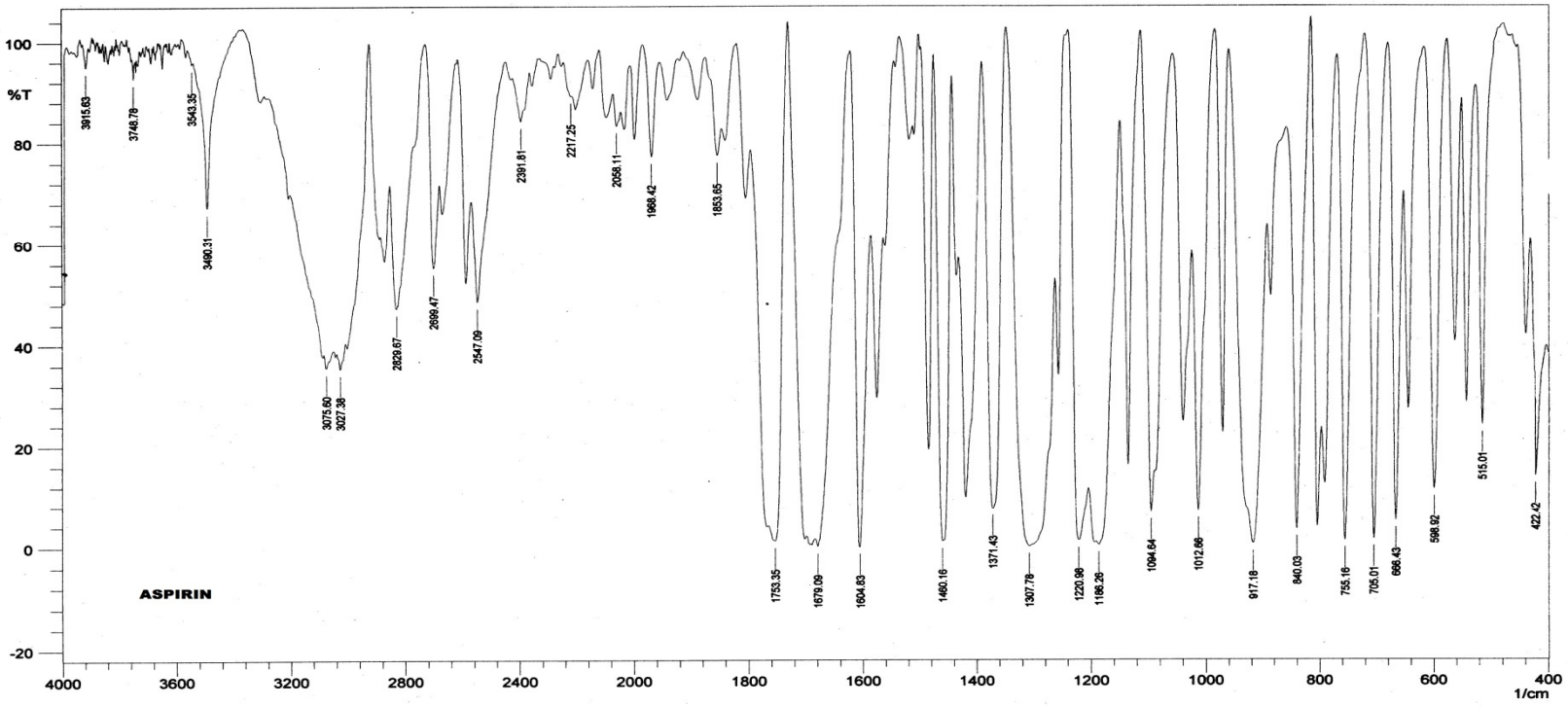


Figure 3

IR SPECTRUM OF RAMIPRIL

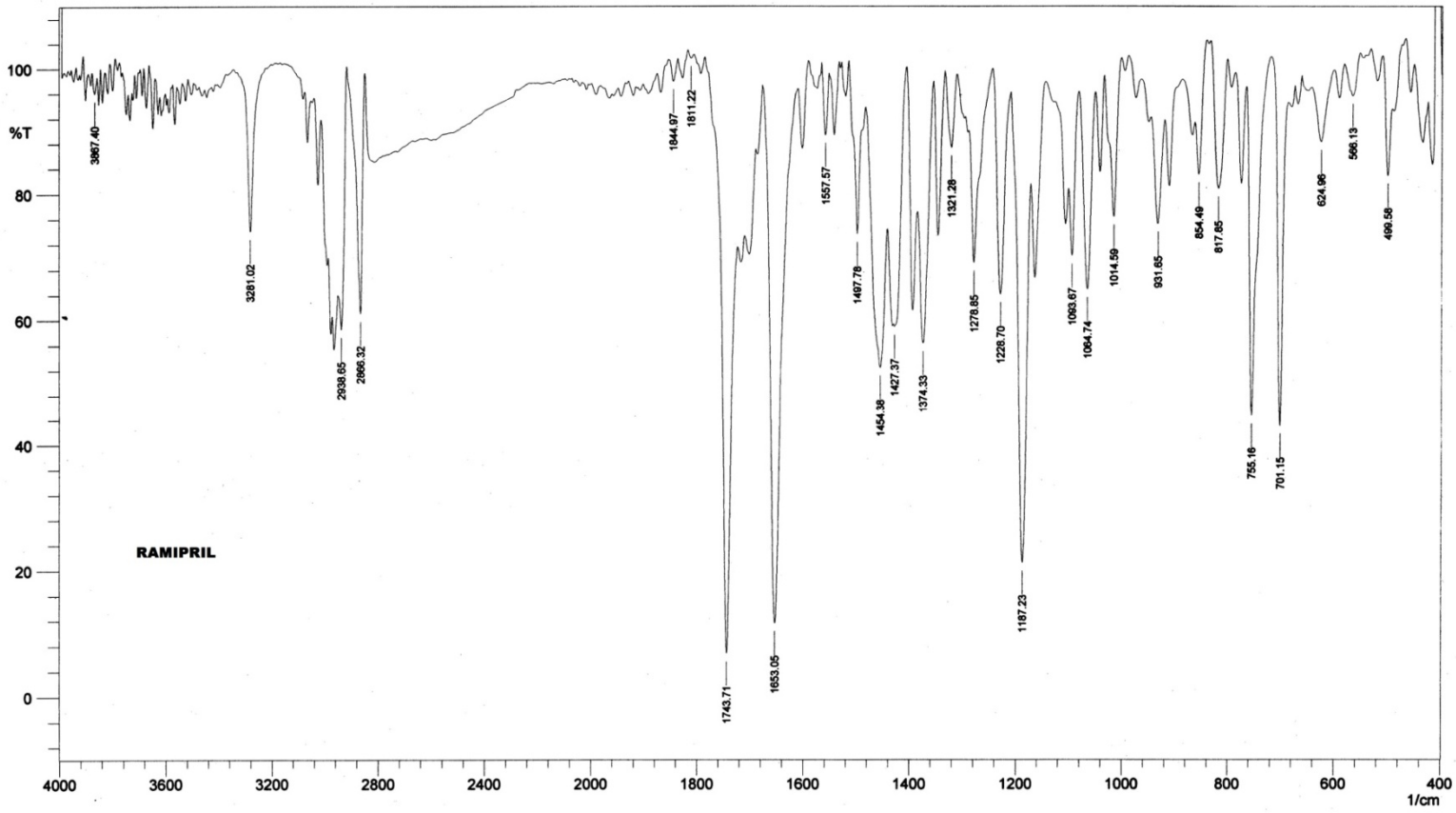


Figure 4

IR SPECTRUM OF METOPROLOL TARTRATE

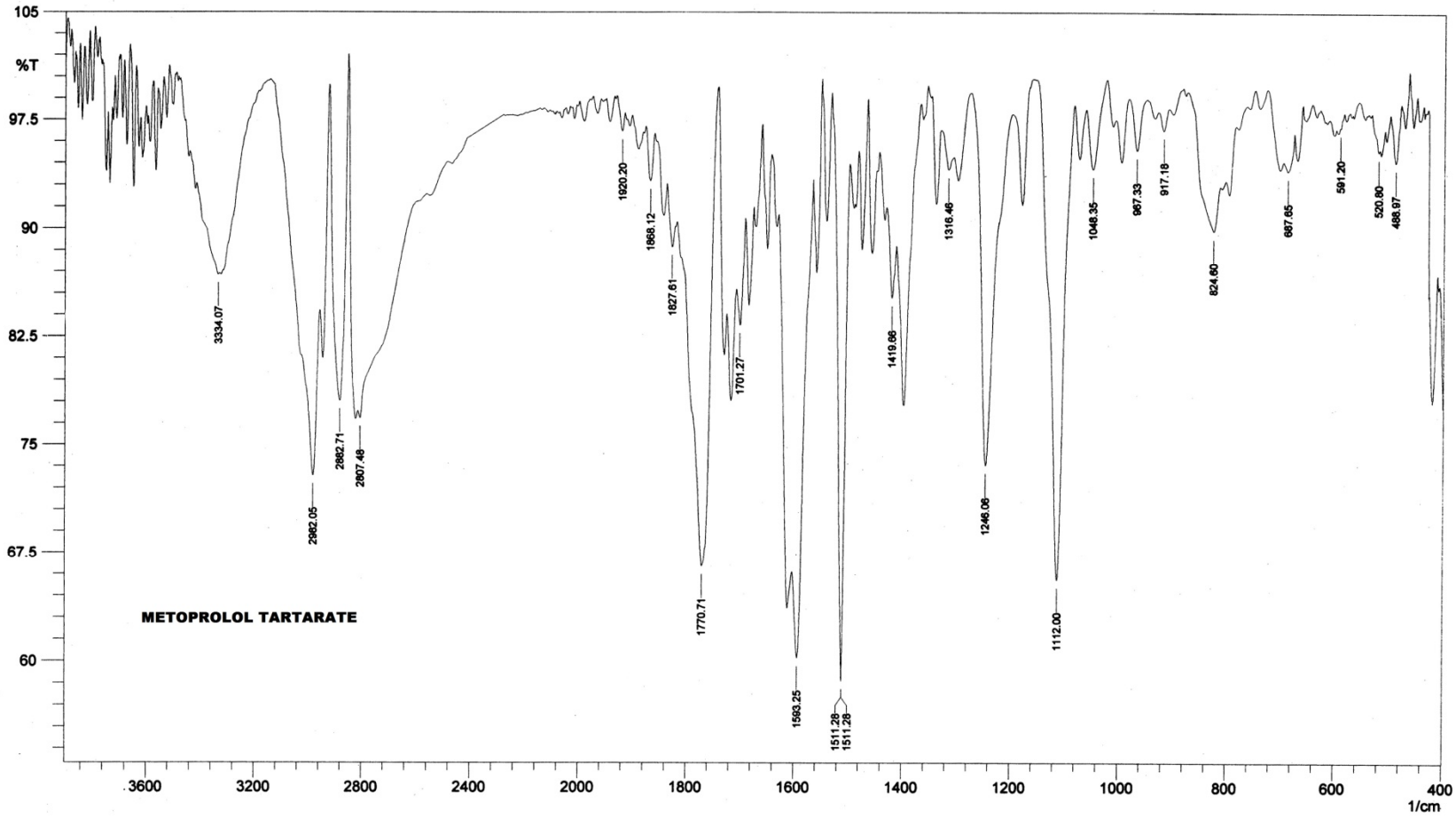


Figure 5

OVERLAIN ZERO ORDER SPECTRA OF ATORVASTATIN CALCIUM, ASPIRIN, RAMIPRIL AND METOPROLOL TARTRATE

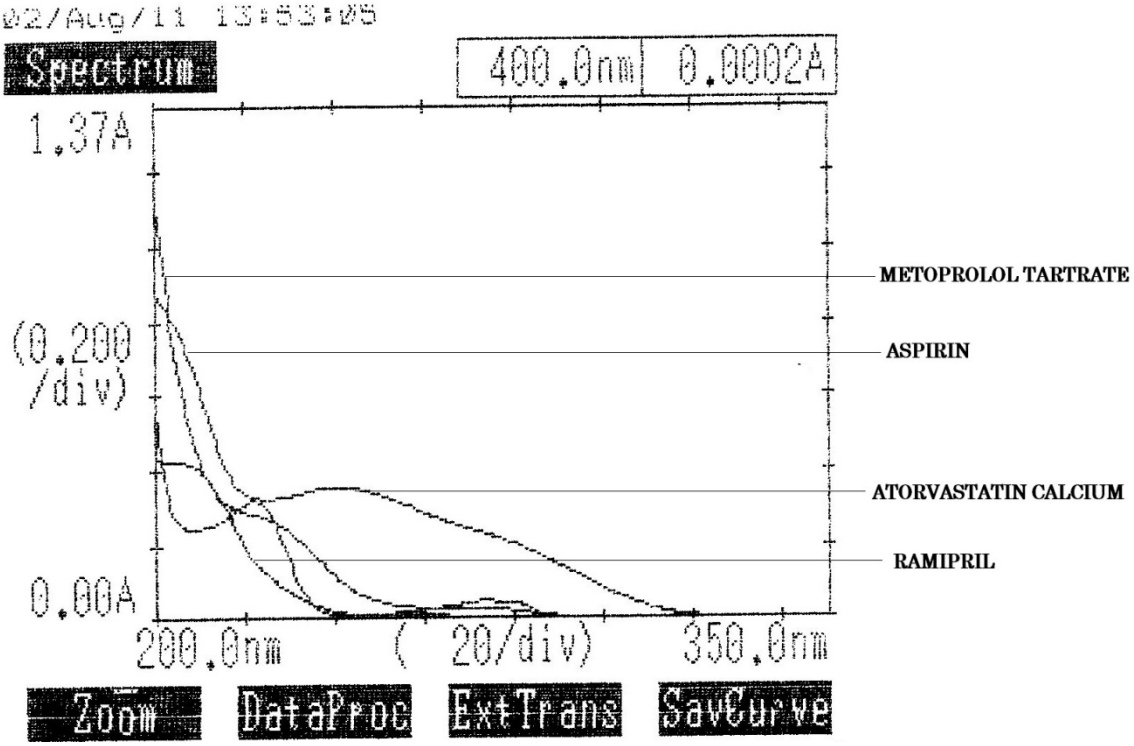


Figure 6

OVERLAIN FIRST ORDER DERIVATIVE SPECTRUM OF ATORVASTATIN CALCIUM, ASPIRIN, RAMIPRIL AND METOPROLOL TARTRATE

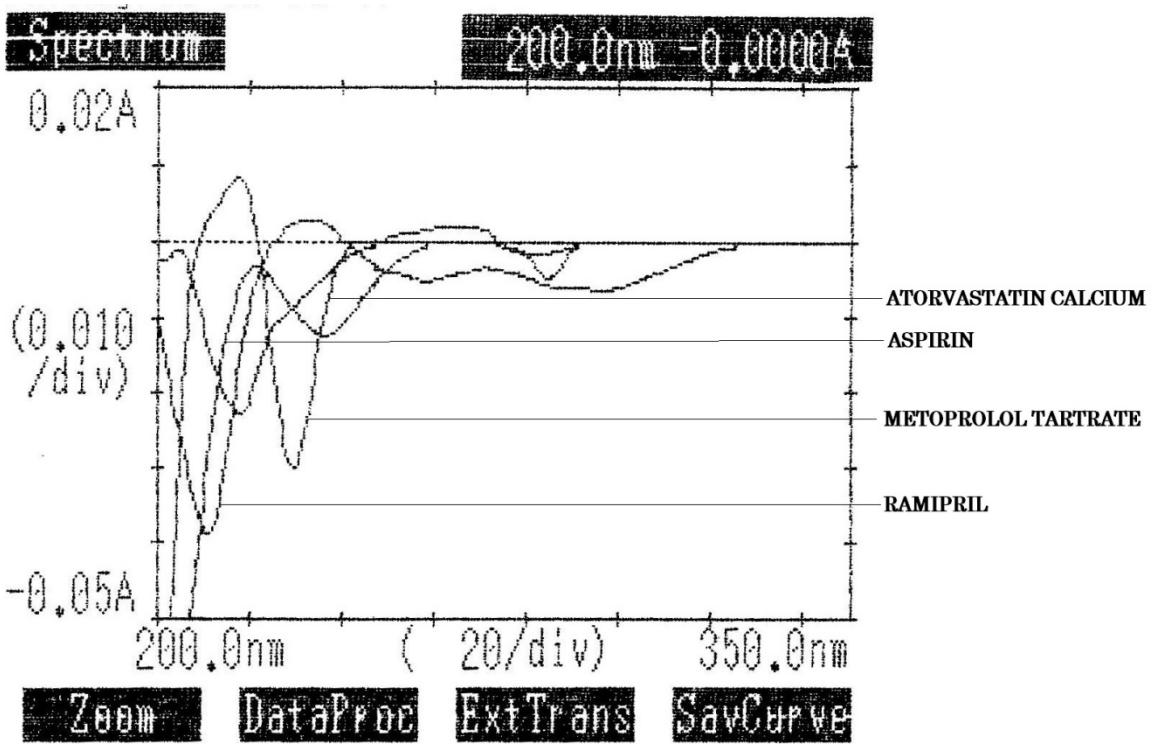


Figure 7

CALIBRATION GRAPH OF ATORVASTATIN CALCIUM AT 291.5 nm

(FIRST ORDER DERIVATIVE METHOD)

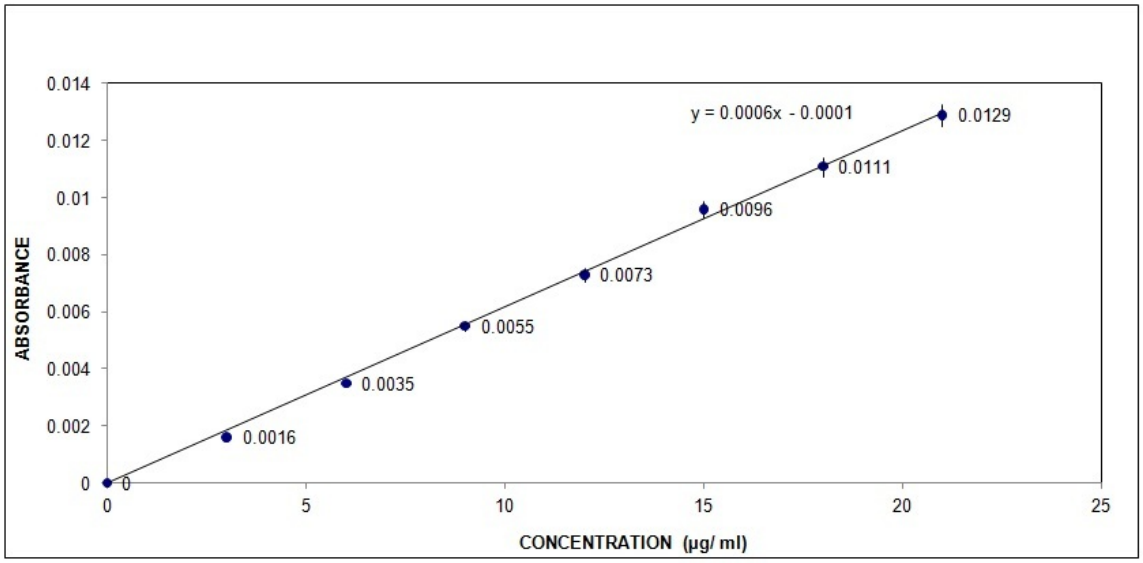


Figure 8

CALIBRATION GRAPH OF ATORVASTATIN CALCIUM AT 247 nm

(FIRST ORDER DERIVATIVE METHOD)

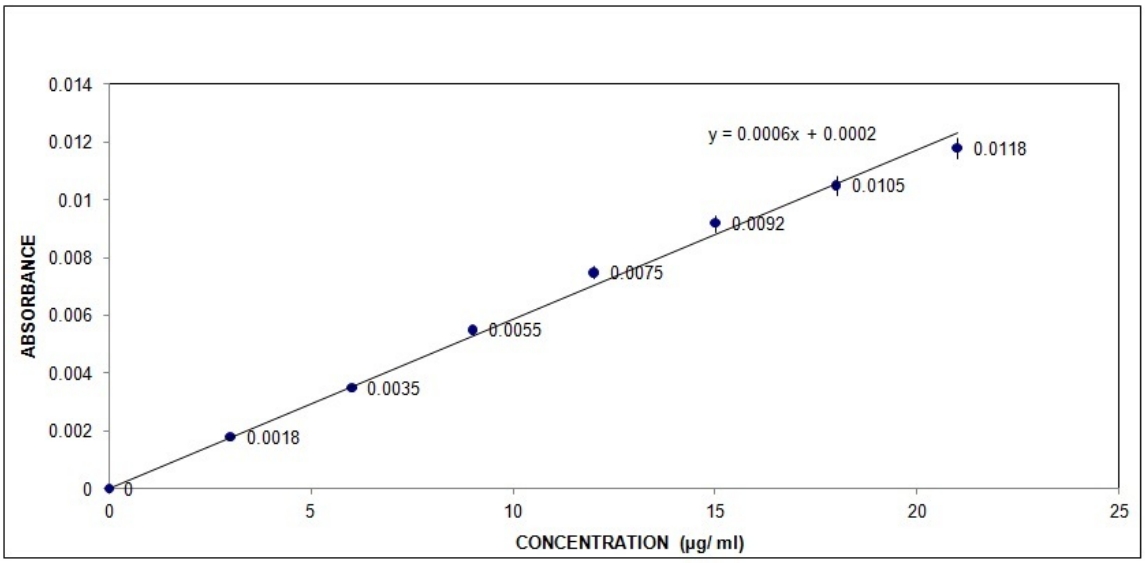


Figure 9

CALIBRATION GRAPH OF ASPIRIN AT 247 nm

(FIRST ORDER DERIVATIVE METHOD)

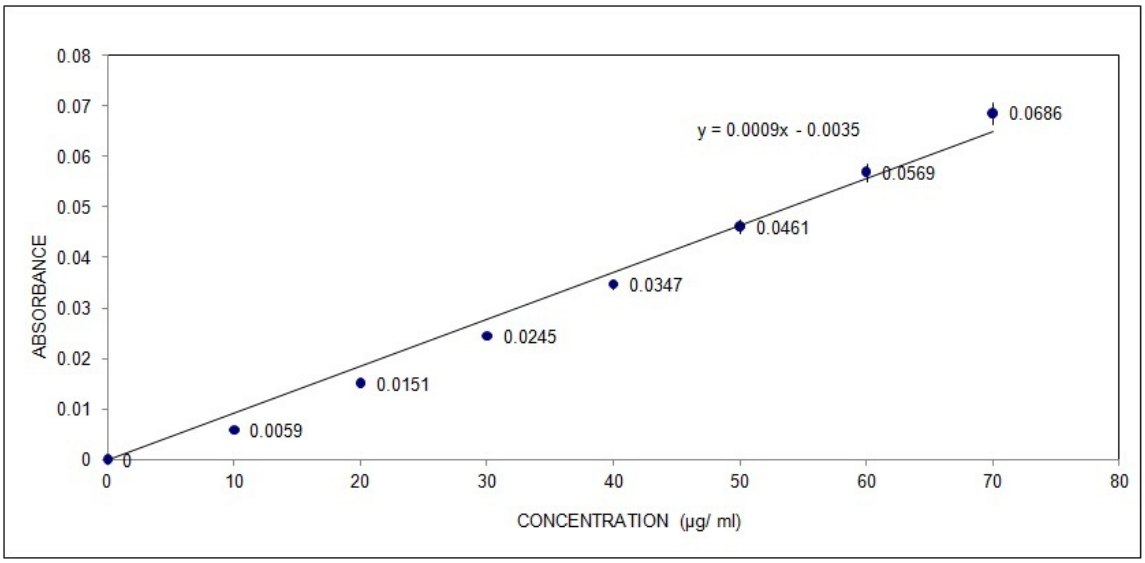


Figure 10

CALIBRATION GRAPH OF ATORVASTATIN CALCIUM AT 242.5 nm

(FIRST ORDER DERIVATIVE METHOD)

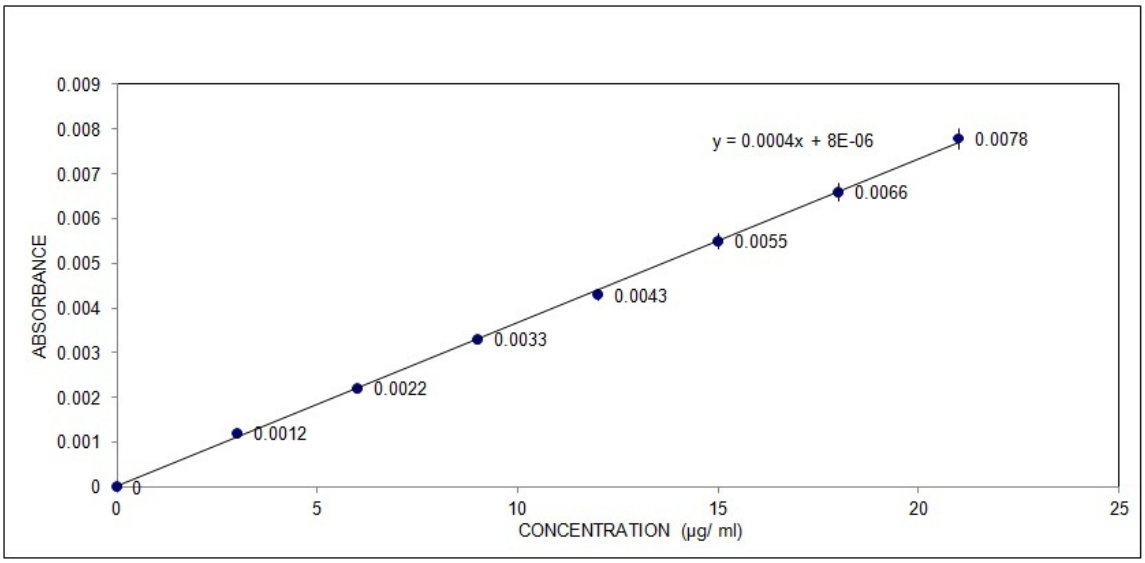


Figure 11

CALIBRATION GRAPH OF ASPIRIN AT 242.5 nm

(FIRST ORDER DERIVATIVE METHOD)

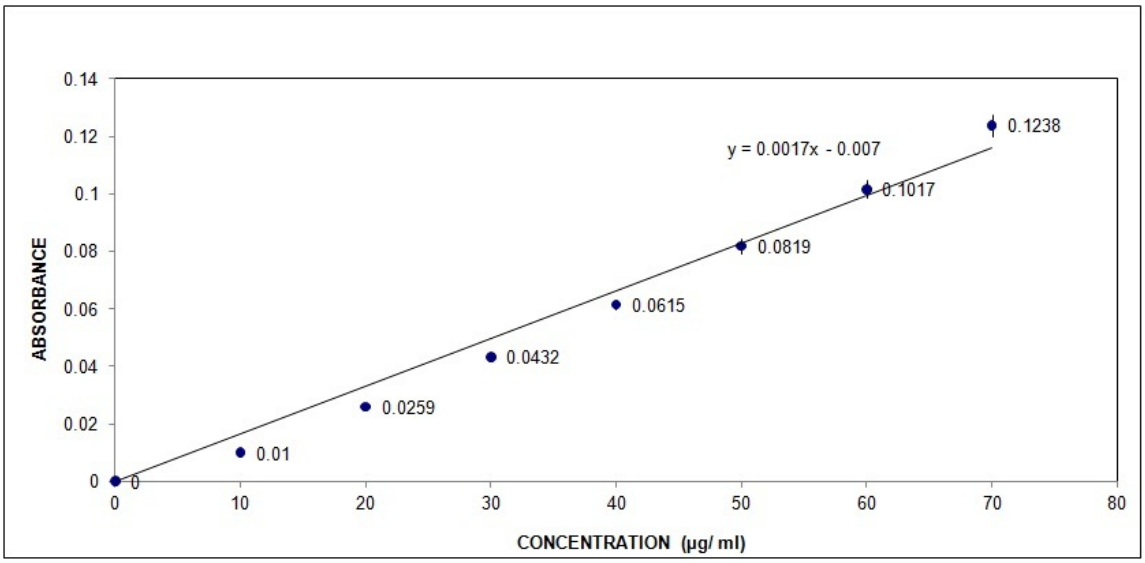


Figure 12

CALIBRATION GRAPH OF RAMIPRIL AT 242.5 nm

(FIRST ORDER DERIVATIVE METHOD)

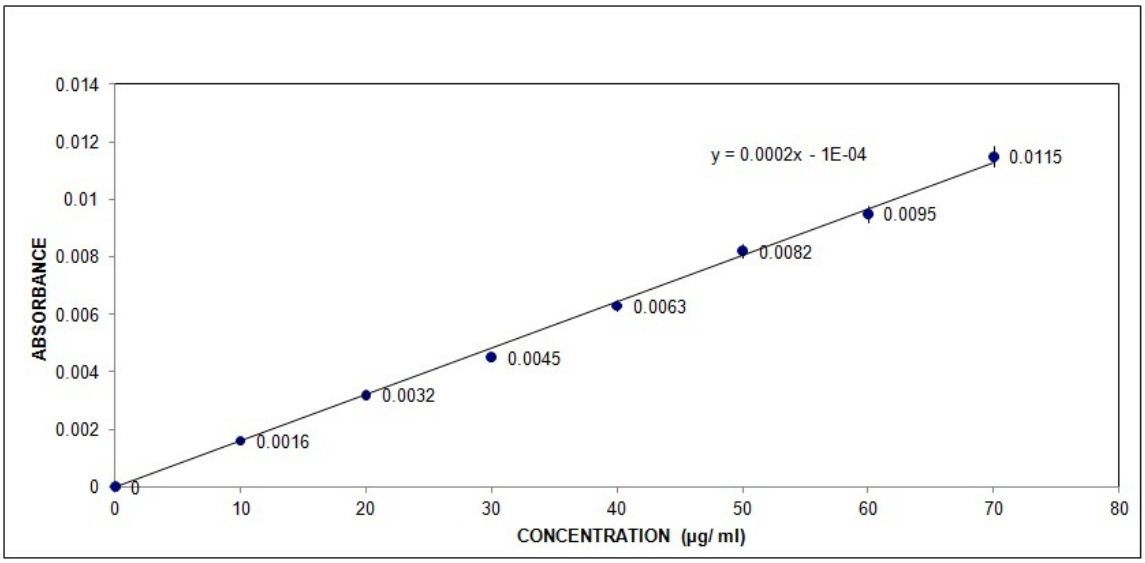


Figure 13

CALIBRATION GRAPH OF ATORVASTATIN CALCIUM AT 229.5 nm

(FIRST ORDER DERIVATIVE METHOD)

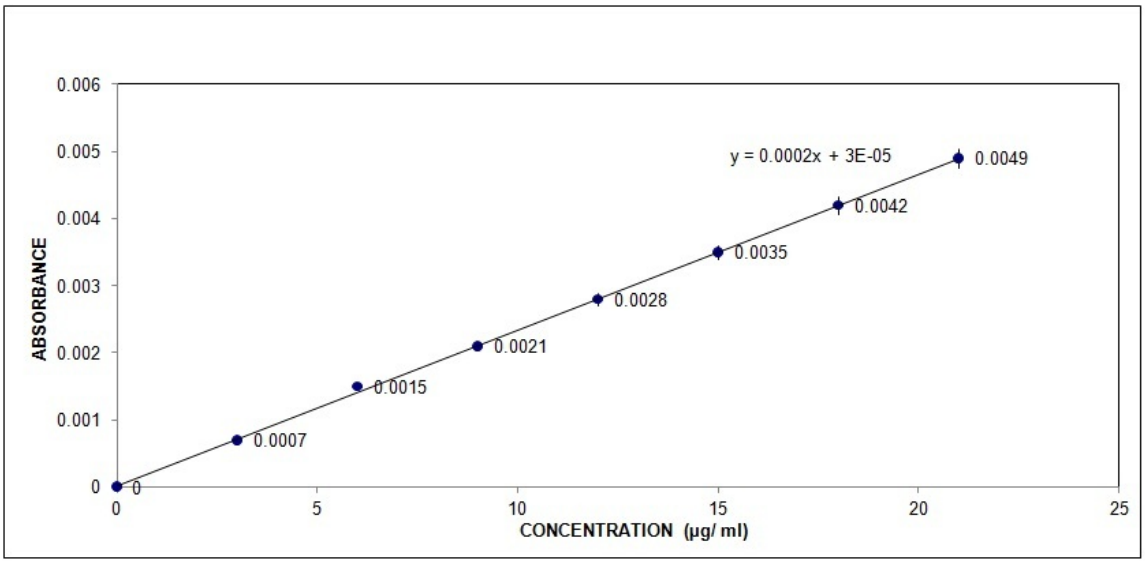


Figure 14

CALIBRATION GRAPH OF ASPIRIN AT 229.5 nm

(FIRST ORDER DERIVATIVE METHOD)

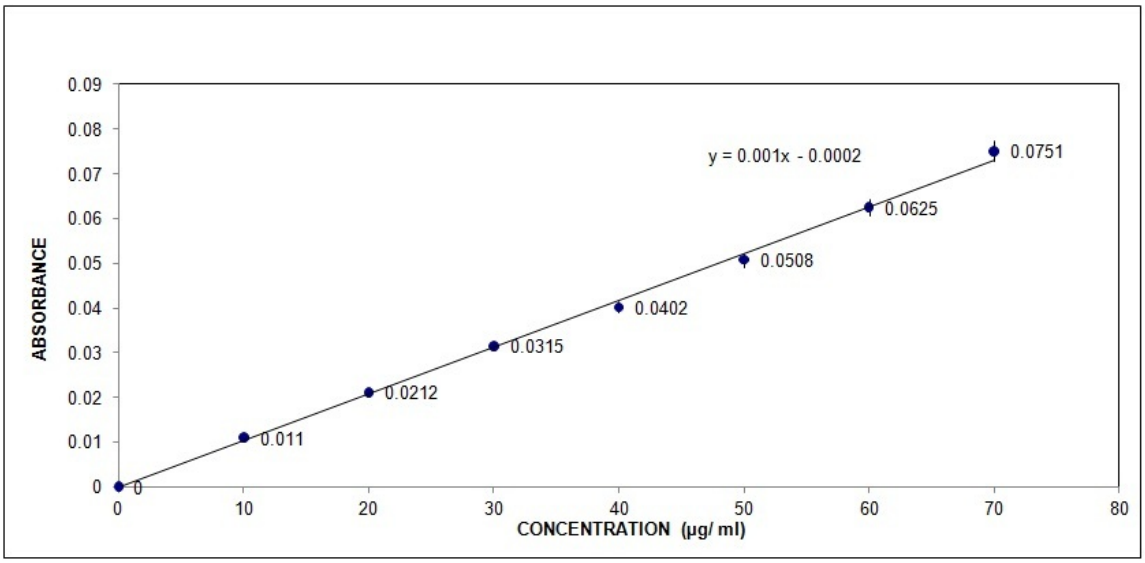


Figure 15

CALIBRATION GRAPH OF RAMIPRIL AT 229.5 nm

(FIRST ORDER DERIVATIVE METHOD)

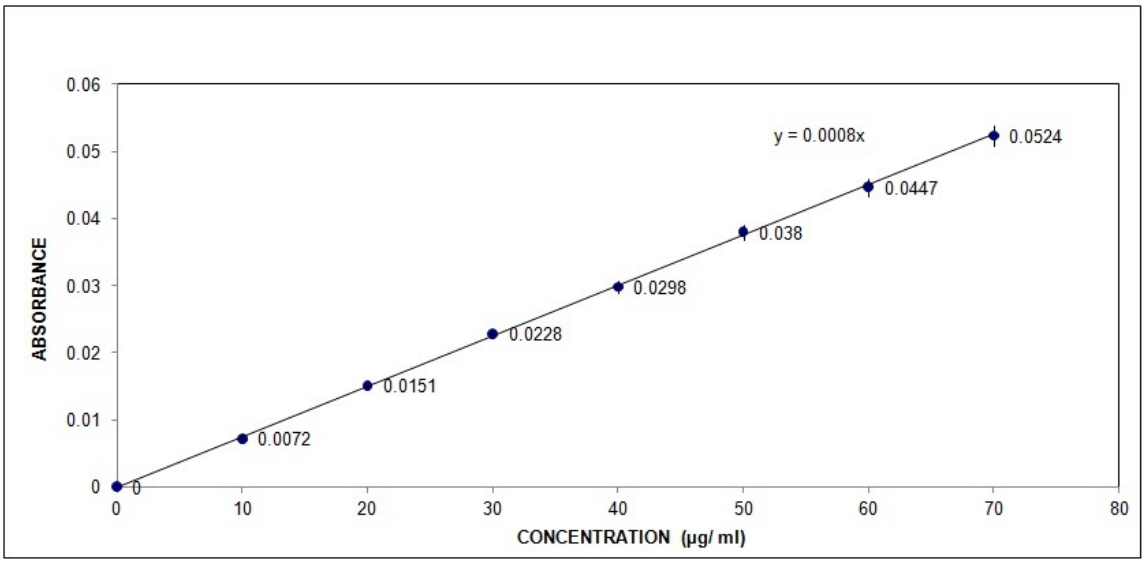


Figure 16

CALIBRATION GRAPH OF METOPROLOL TARTRATE AT 229.5 nm

(FIRST ORDER DERIVATIVE METHOD)

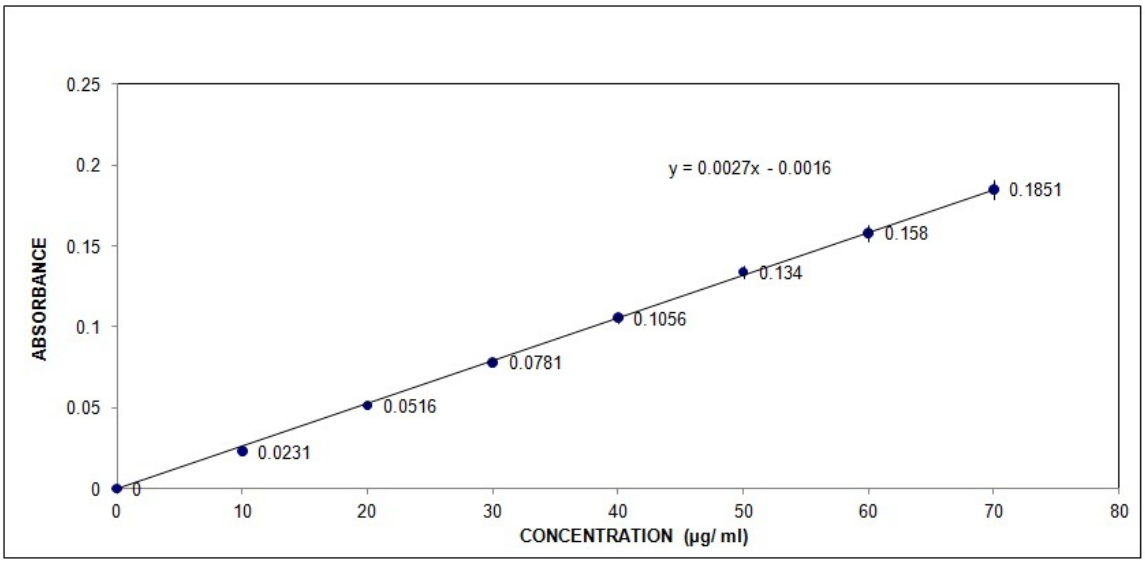


Figure 17

UV SPECTRUM OF METOPROLOL TARTRATE, RAMIPRIL, ATORVASTATIN CALCIUM AND ASPIRIN IN BENZENE: TOLUENE: METHANOL: GLACIAL ACETIC ACID (7.5:1.0:1.5:0.1 % V/V/V/V)

(DETECTION WAVELENGTH)

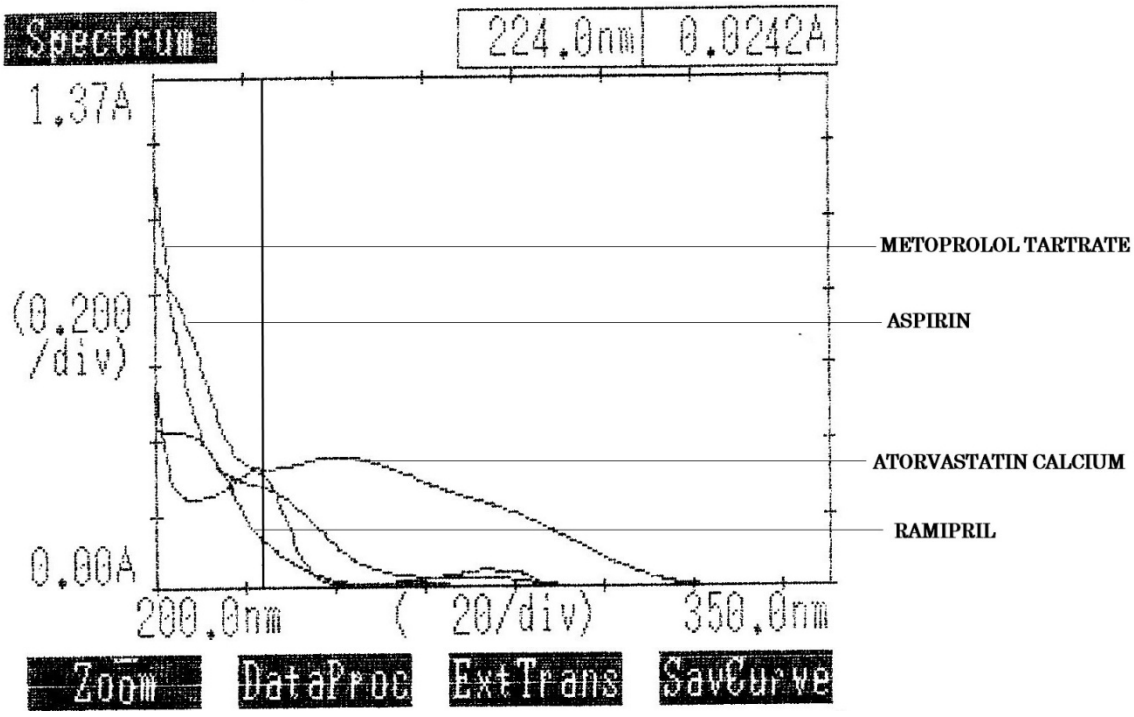
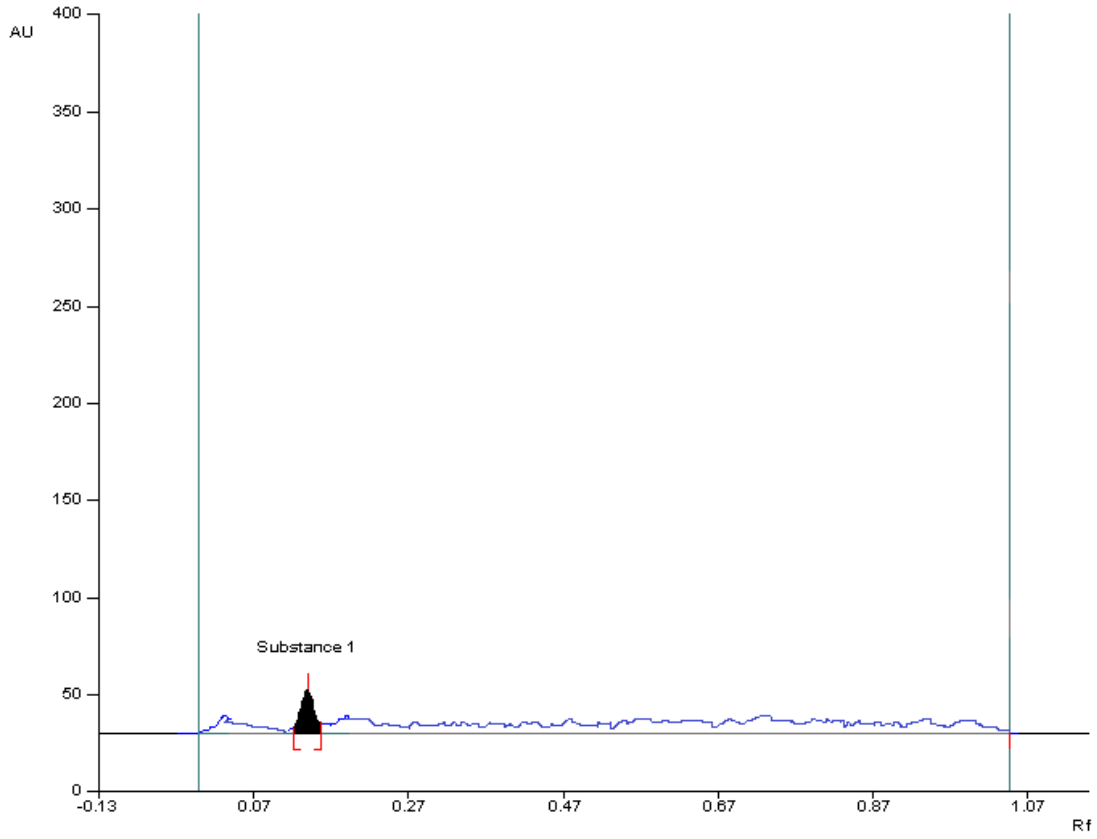


Figure 18

CHROMATOGRAM FOR METOPROLOL TARTRATE BY HPTLC

(100 ng/ µl)

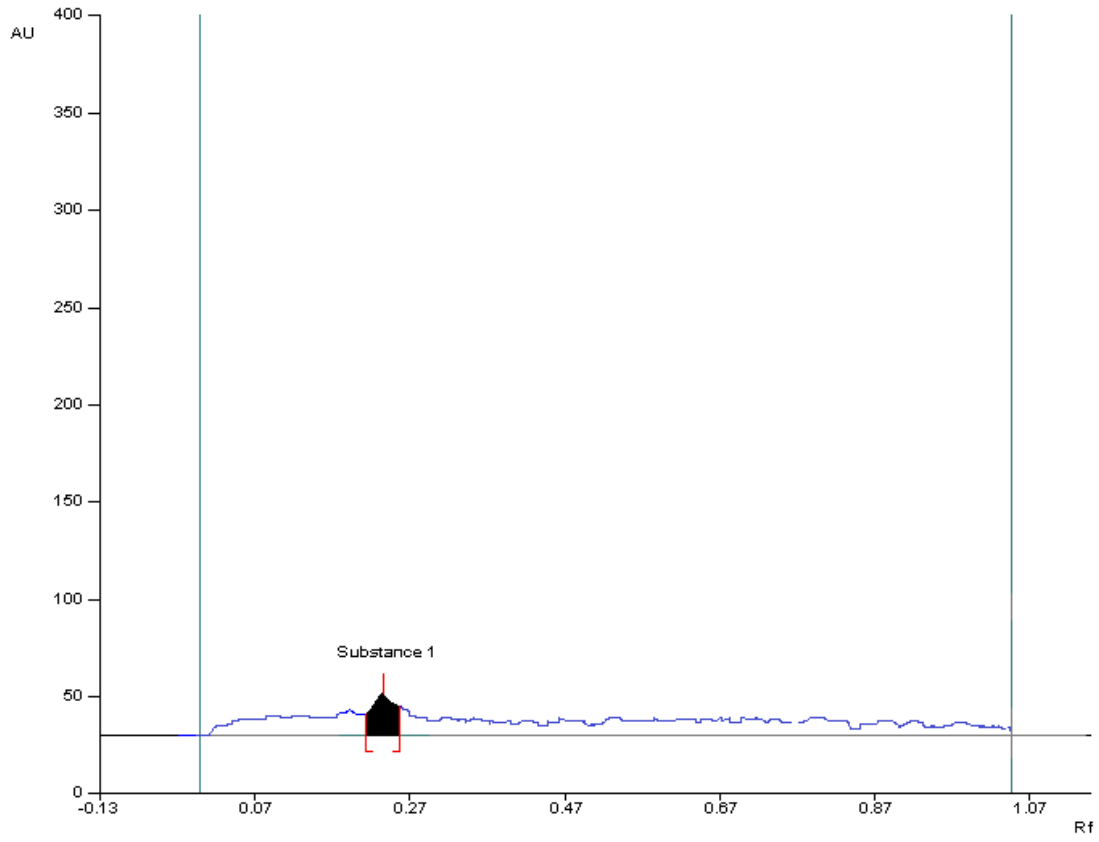


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.12	3.4	0.14	46.3	10.71	0.18	4.9	237.1	100

Figure 19

CHROMATOGRAM FOR RAMIPRIL BY HPTLC

(10 ng/ µl)

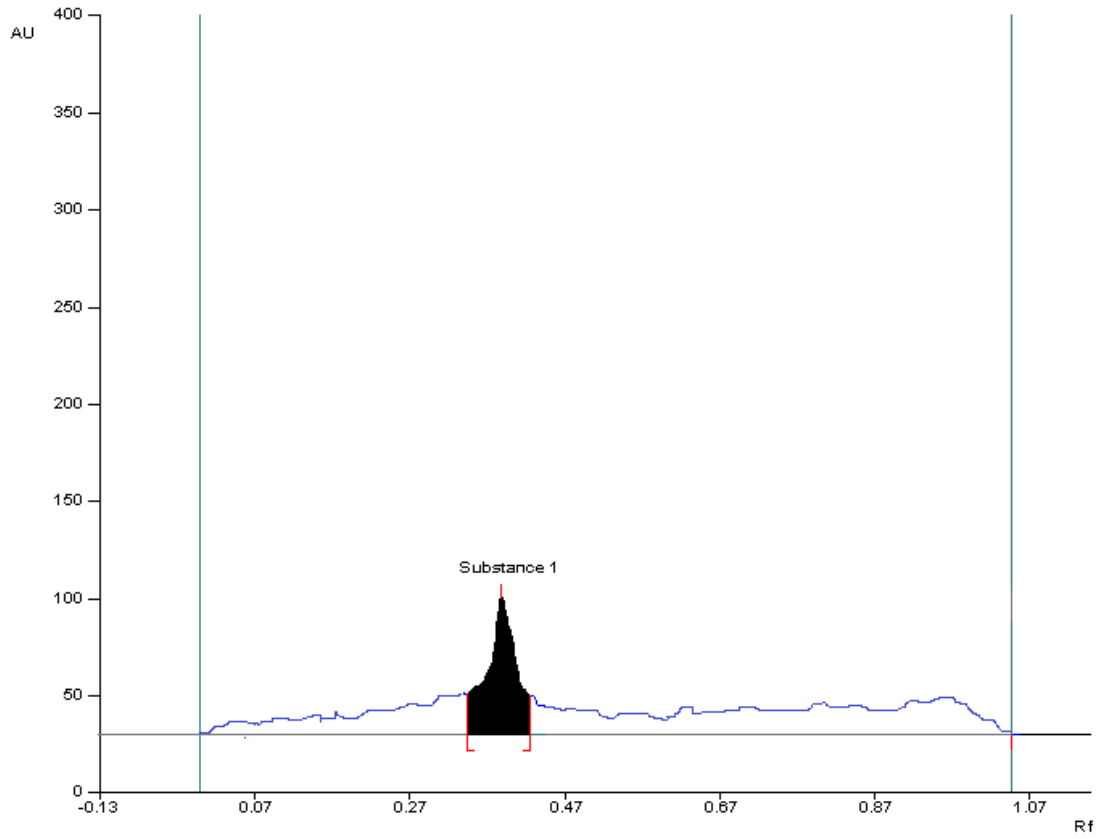


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.22	12.3	0.26	18.3	4.64	0.28	10.5	206.2	100

Figure 20

CHROMATOGRAM FOR ATORVASTATIN CALCIUM BY HPTLC

(20 ng/ µl)

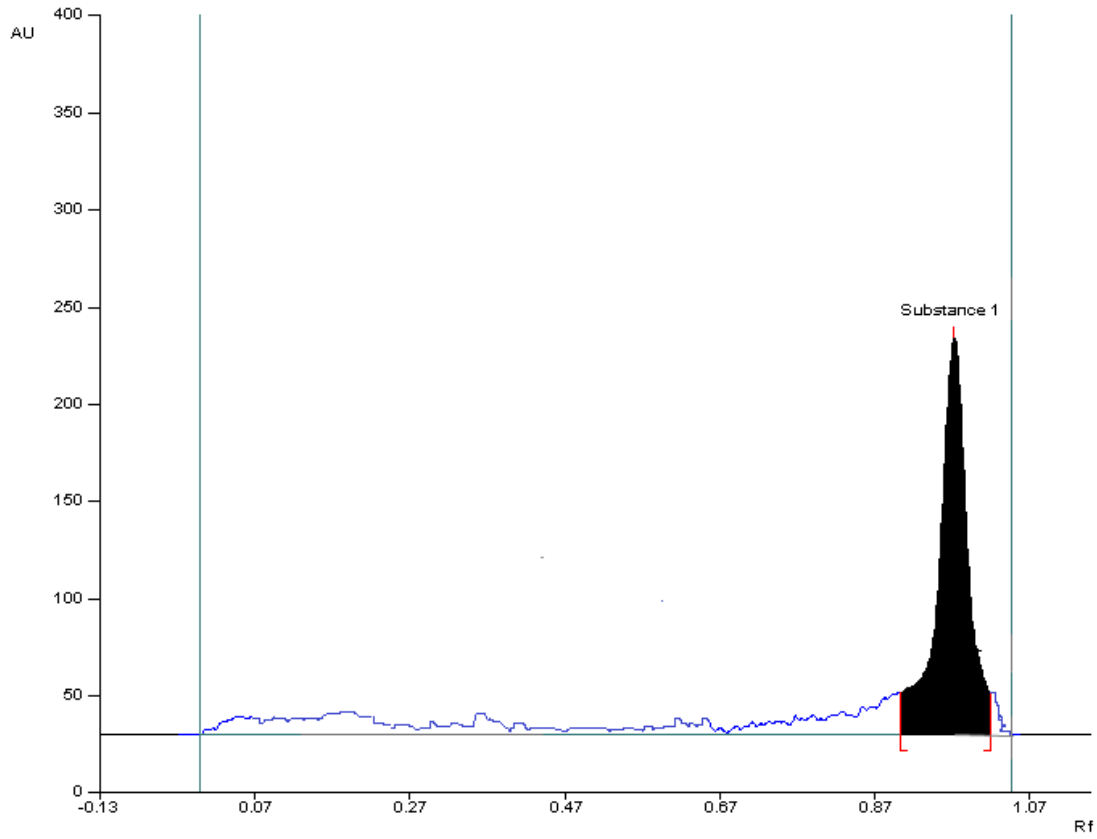


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.36	27.3	0.40	78.9	18.21	0.43	16.9	618.9	100

Figure 21

CHROMATOGRAM FOR ASPIRIN BY HPTLC

(75 ng/ µl)

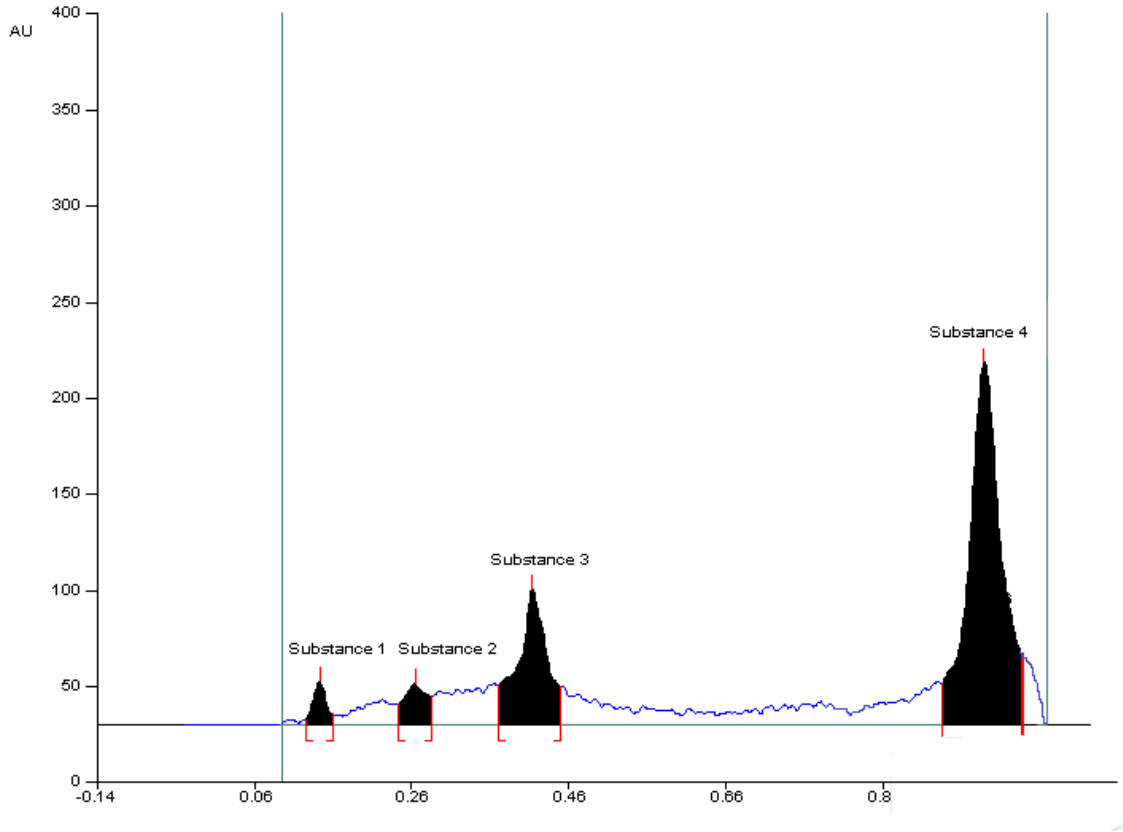


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.96	23.4	0.99	206.01	45.6	1.05	35.3	1304	100

Figure 22

LINEARITY CHROMATOGRAM FOR METOPROLOL TARTRATE, RAMIPRIL, ATORVASTATIN CALCIUM AND ASPIRIN BY HPTLC

(100+10+20+75 ng/ µl)

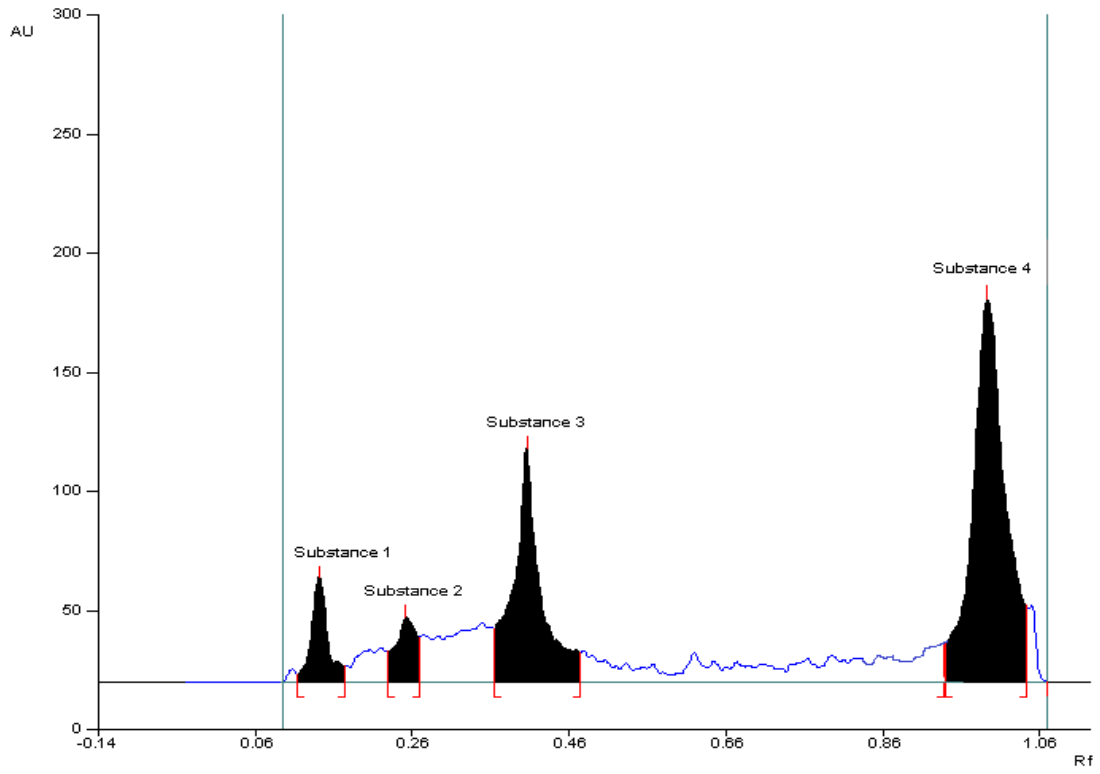


Peak	Start Rf	Start Height	Max Rf	Max height	Max %	End Rf	End Height	Area	Area %
1	0.12	2.4	0.14	43.3	9.93	0.17	4.4	222.8	9.18
2	0.23	10.6	0.25	16.2	3.72	0.28	11.5	193.8	7.97
3	0.37	24.1	0.40	76.4	16.06	0.45	18.1	636.3	26.24
4	0.94	22.3	0.99	19.71	42.28	1.03	37.5	1372.2	50.59

Figure 23

LINEARITY CHROMATOGRAM FOR METOPROLOL TARTRATE, RAMIPRIL, ATORVASTATIN CALCIUM AND ASPIRIN BY HPTLC

(200+20+40+150 ng/ µl)

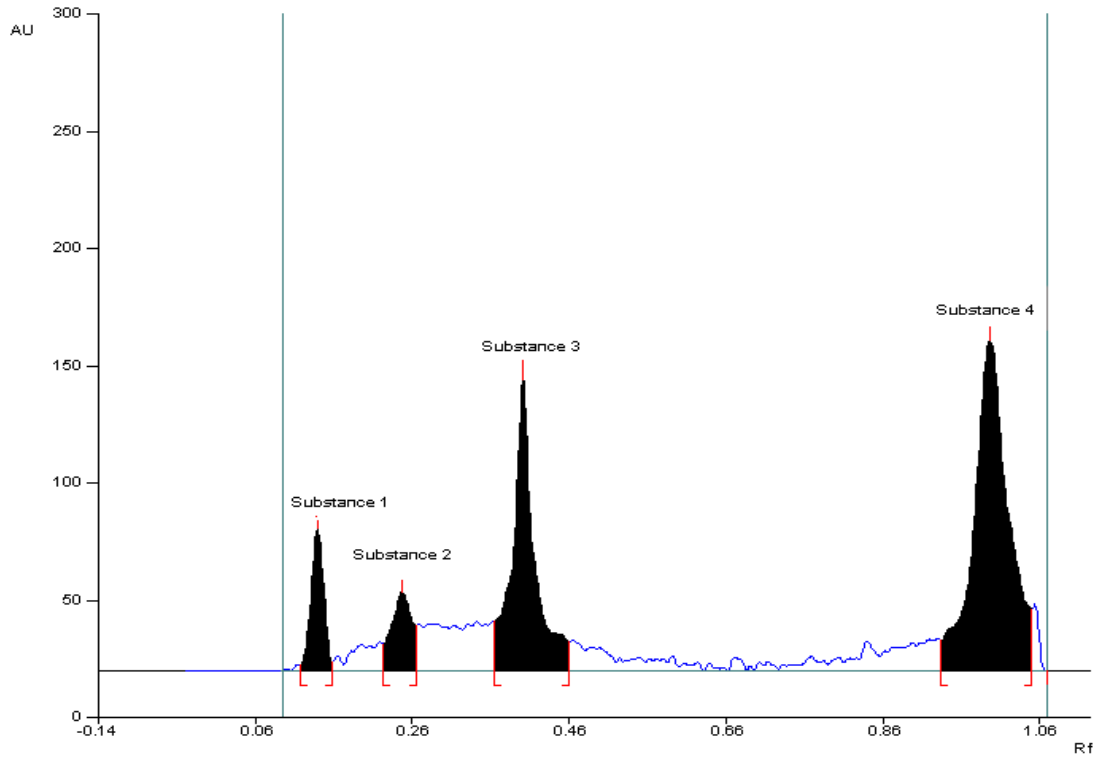


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Rf	Area	Area %
1	0.13	2.7	0.14	43.0	6.02	0.16	5.5	456.2	9.41
2	0.24	10.7	0.25	21.3	5.58	0.29	15.0	387.3	8.03
3	0.37	20.4	0.40	70.3	18.43	0.45	19.6	1263.4	25.69
4	0.94	21.6	0.99	188.8	49.49	1.04	37.8	2756.6	56.80

Figure 24

LINEARITY CHROMATOGRAM FOR METOPROLOL TARTRATE, RAMIPRIL, ATORVASTATIN CALCIUM AND ASPIRIN BY HPTLC

(300+30+60+225 ng/ µl)

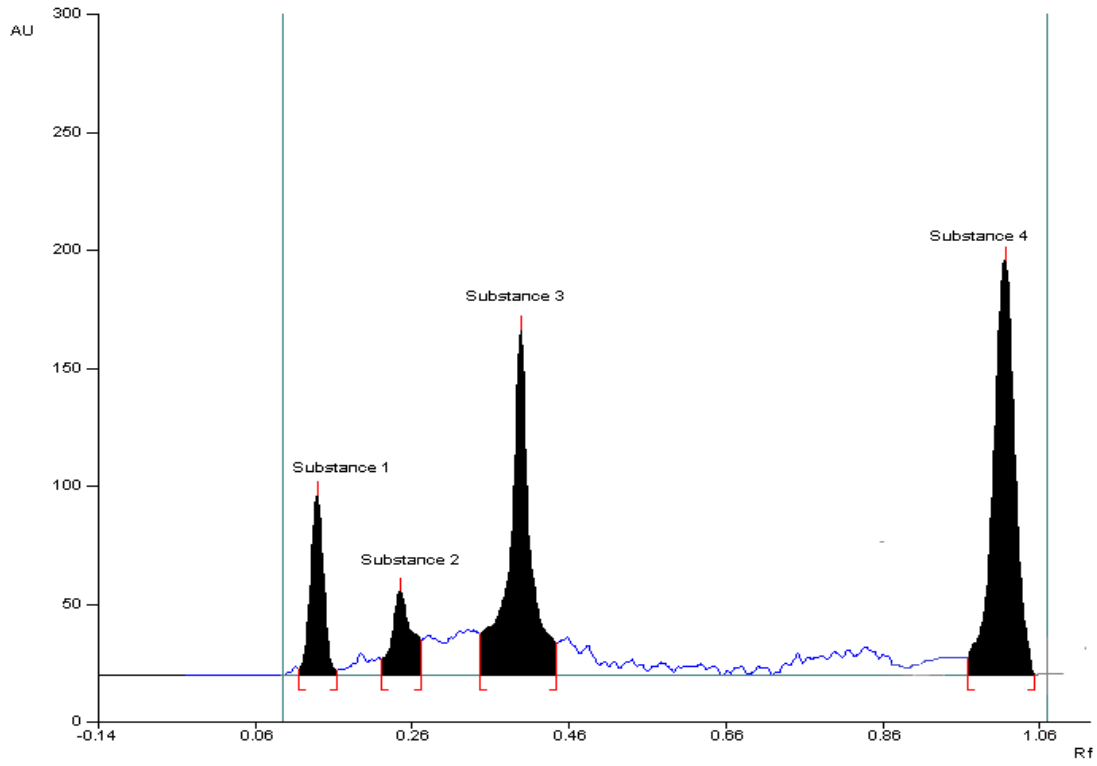


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.11	3.3	0.14	44.3	12.36	0.17	6.8	664.2	9.10
2	0.23	13.1	0.25	27.4	7.63	0.27	18.9	589.2	8.07
3	0.36	22.6	0.40	98.1	27.37	0.47	12.2	1927.5	25.42
4	0.94	24.3	0.99	208.9	54.88	1.04	39.4	4112.4	56.38

Figure 25

LINEARITY CHROMATOGRAM FOR METOPROLOL TARTRATE, RAMIPRIL, ATORVASTATIN CALCIUM AND ASPIRIN BY HPTLC

(400+40+80+300 ng/ µl)

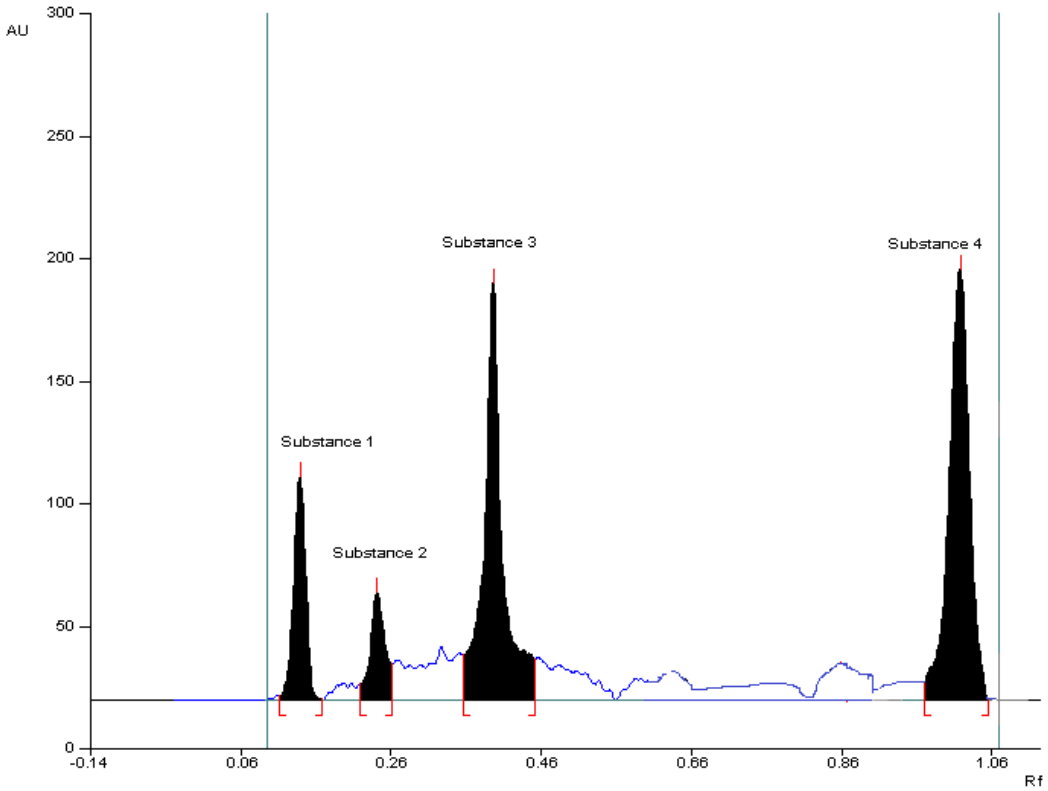


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.12	2.3	0.14	60.6	16.84	0.16	3.9	889.2	9.18
2	0.22	11.5	0.25	33.6	9.35	0.27	19.5	773.8	8.05
3	0.36	21.4	0.40	124.5	34.62	0.46	12.1	2598.5	26.18
4	0.94	26.2	0.99	219.2	59.19	1.05	41.3	5483.7	56.57

Figure 26

LINEARITY CHROMATOGRAM FOR METOPROLOL TARTRATE, RAMIPRIL, ATORVASTATIN CALCIUM AND ASPIRIN BY HPTLC

(500+50+100+375 ng/ µl)

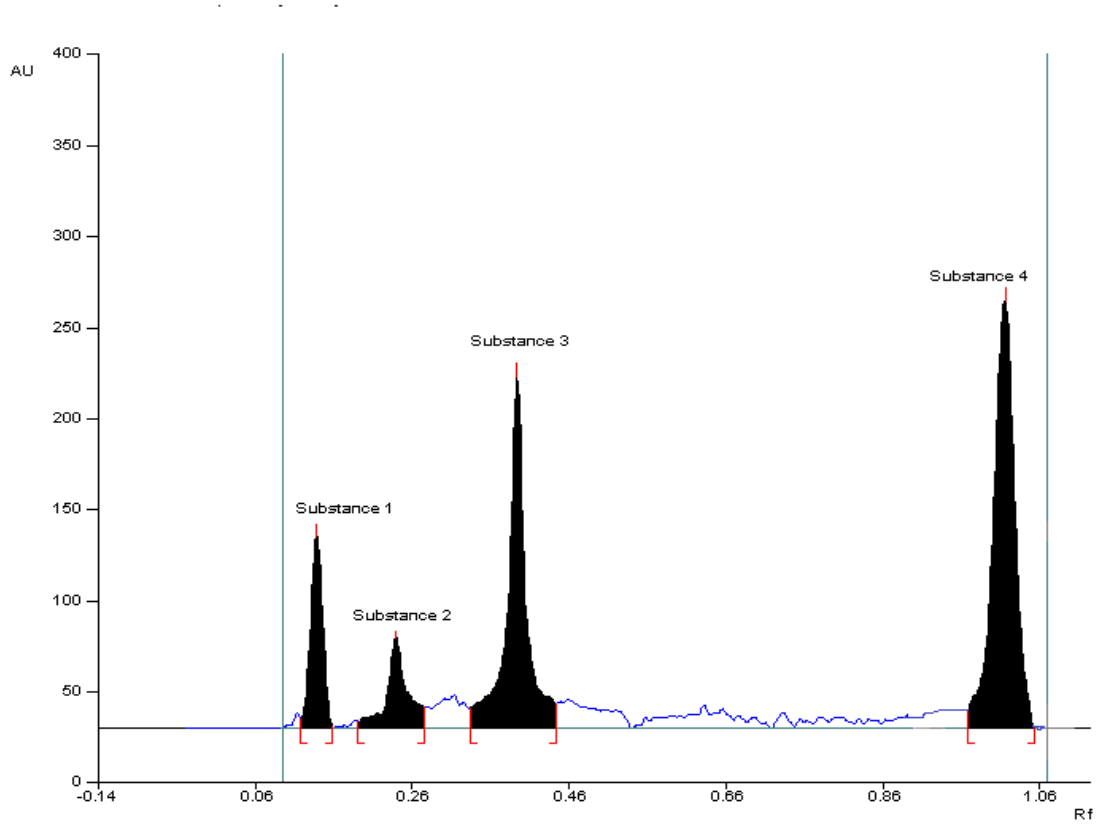


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.12	2.7	0.14	76.1	29.41	0.16	2.1	1121.4	9.05
2	0.22	6.8	0.25	36.0	13.91	0.27	15.1	960.4	7.90
3	0.35	17.6	0.40	146.6	56.68	0.45	13.4	3151.4	26.76
4	0.94	27.9	0.99	232.0	62.12	1.05	41.9	6859.2	56.27

Figure 27

LINEARITY CHROMATOGRAM FOR METOPROLOL TARTRATE, RAMIPRIL, ATORVASTATIN CALCIUM AND ASPIRIN BY HPTLC

(600+60+120+450 ng/ µl)



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.11	1.6	0.14	91.6	25.61	0.17	0.5	1338.2	9.02
2	0.22	6.6	0.25	44.3	12.40	0.26	15.1	1134.3	7.82
3	0.36	18.8	0.40	170.1	47.57	0.45	17.0	3816.3	26.92
4	0.94	28.2	0.99	241.2	63.13	1.06	42.5	8430.2	56.23

Figure 28

CALIBRATION GRAPH FOR METOPROLOL TARTRATE BY HPTLC

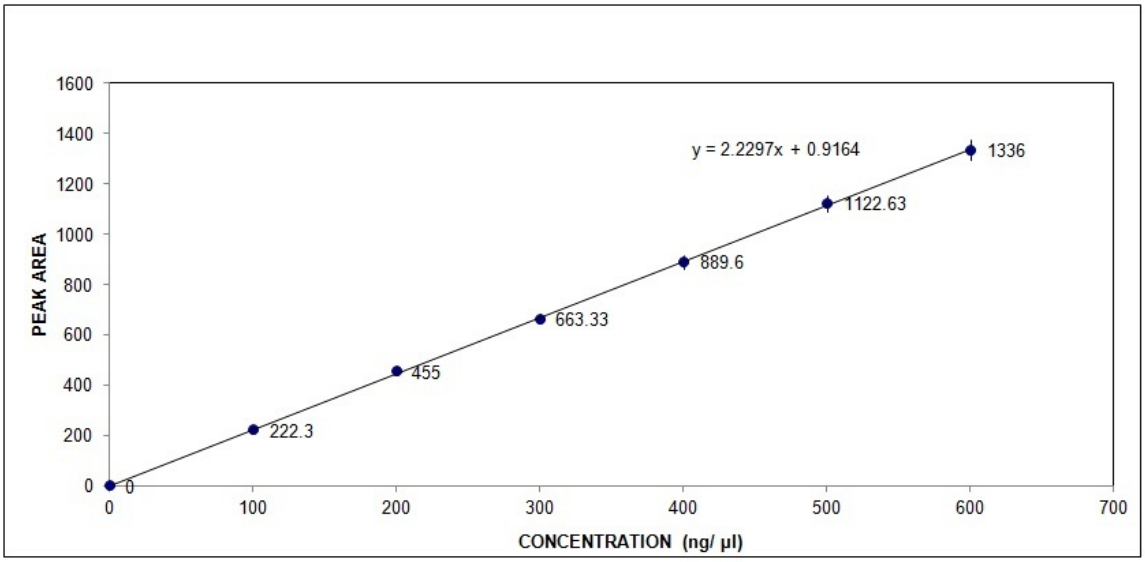


Figure 29

CALIBRATION GRAPH FOR RAMIPRIL BY HPTLC

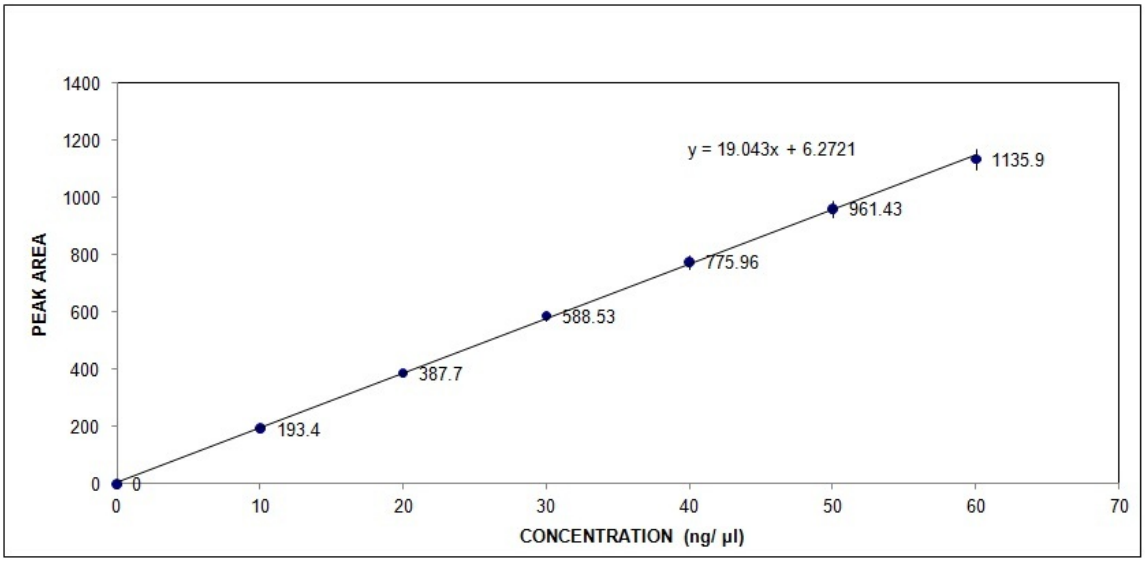


Figure 30

CALIBRATION GRAPH FOR ATORVASTATIN CALCIUM BY HPTLC

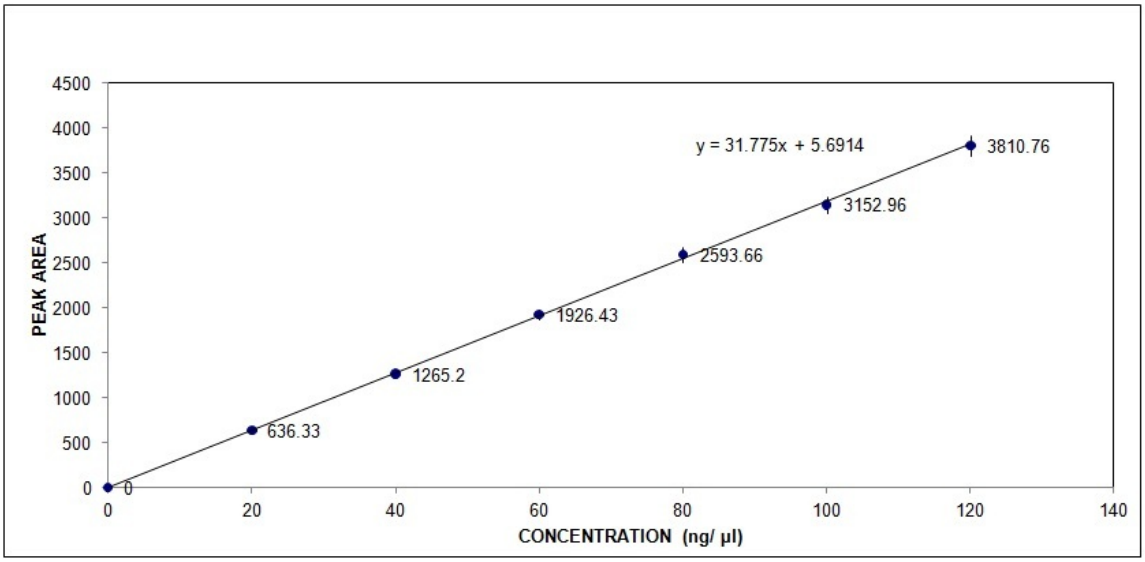


Figure 31

CALIBRATION GRAPH FOR ASPIRIN BY HPTLC METHOD

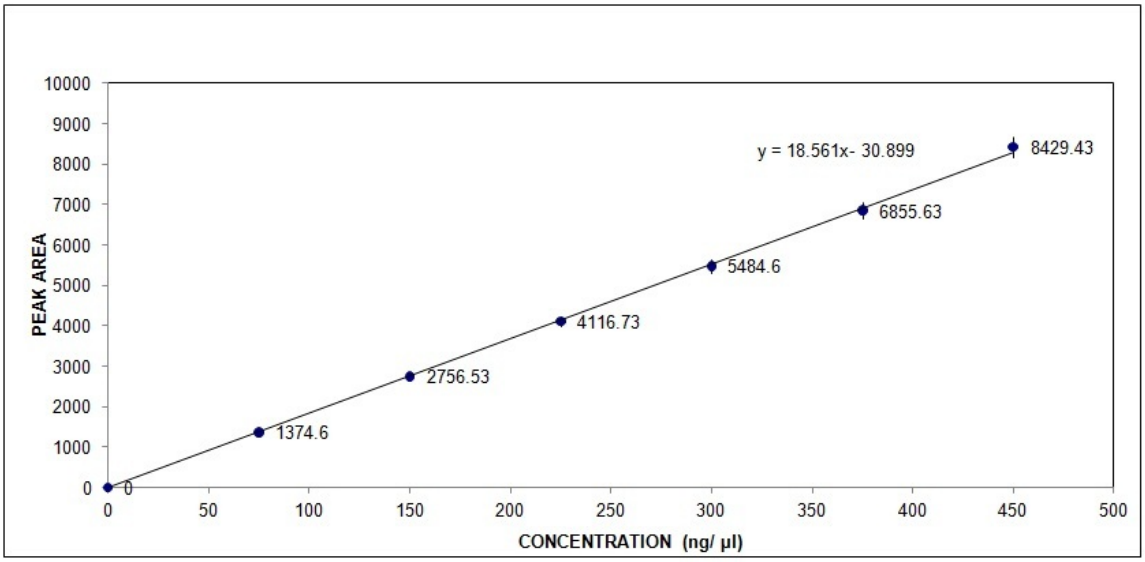
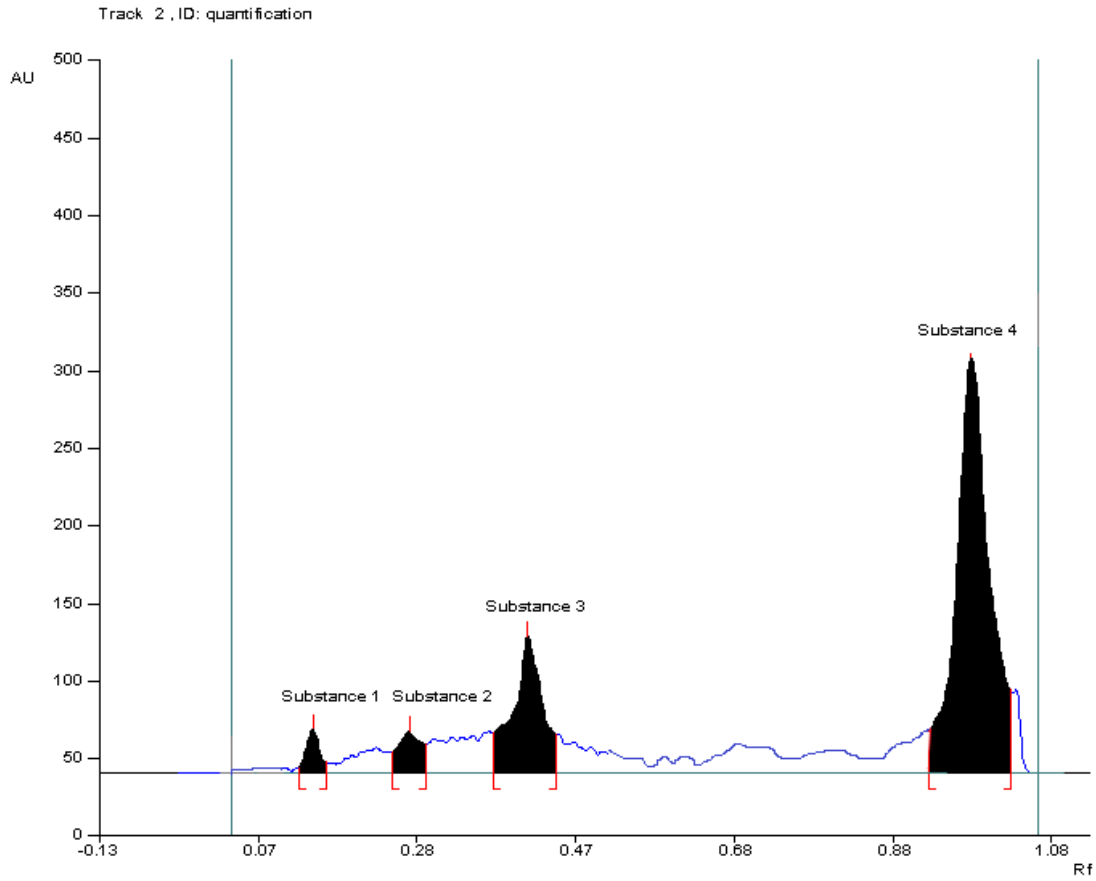


Figure 32

CHROMATAGRAM FOR FORMULATION ANALYSIS BY HPTLC

REPEATABILITY 1

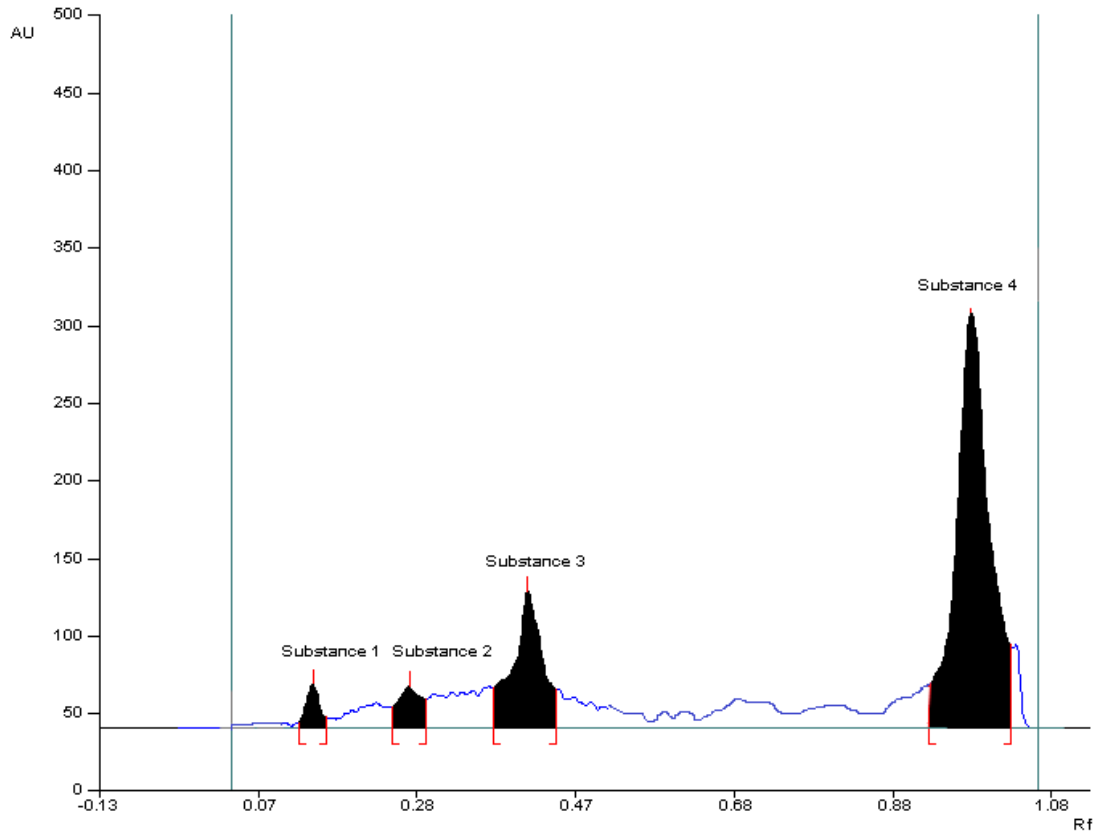


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.12	7.8	0.15	16.9	3.23	0.17	12.3	221.9	9.40
2	0.23	15.7	0.25	27.4	5.25	0.27	13.6	195.3	7.88
3	0.37	27.8	0.40	88.7	16.54	0.43	21.6	632.6	26.02
4	0.94	24.6	0.99	205.2	54.82	1.04	39.4	2752.6	56.68

Figure 33

CHROMATOGRAM FOR FORMULATION ANALYSIS BY HPTLC

REPEATABLITY 2

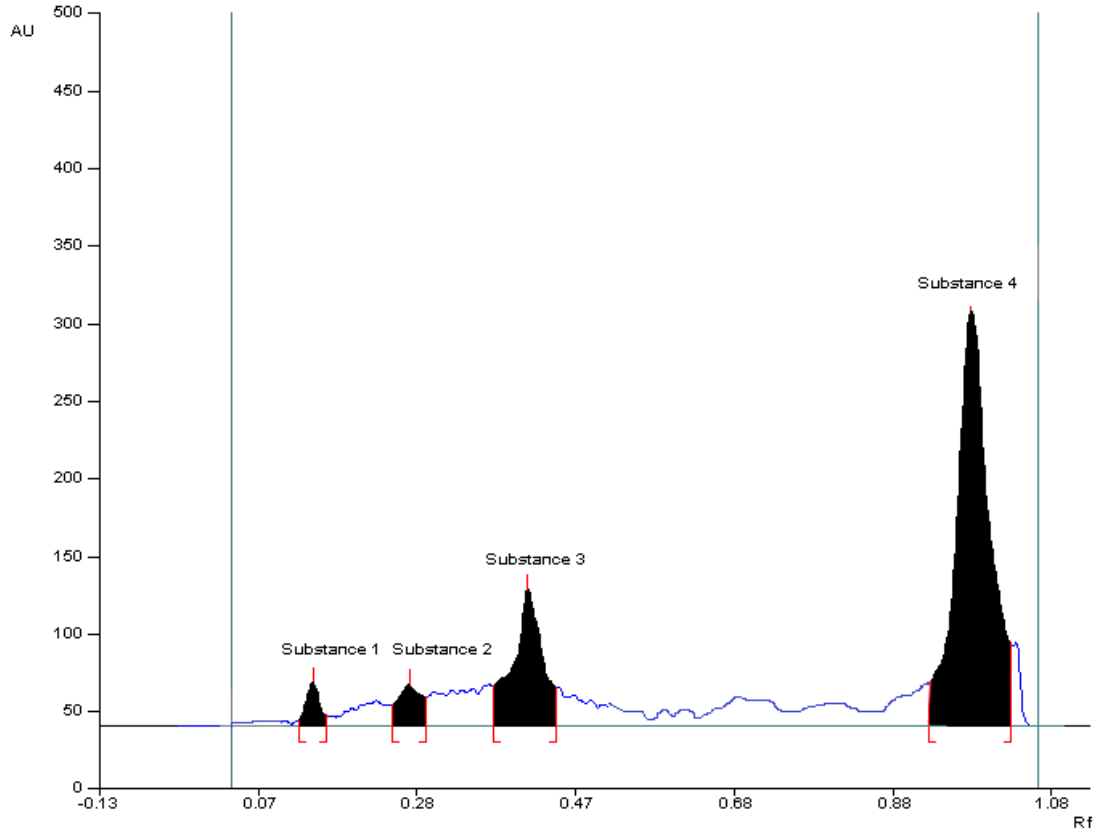


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.12	7.6	0.15	16.7	3.29	0.17	12.3	223.5	9.34
2	0.23	15.6	0.25	27.5	5.25	0.27	13.4	197.3	7.96
3	0.37	27.4	0.40	88.5	16.40	0.43	21.2	631.4	26.09
4	0.95	23.9	0.99	204.6	55.85	1.05	39.8	2756.7	56.59

Figure 34

CHROMATOGRAM FOR FORMULATION ANALYSIS BY HPTLC

REPEATABILITY 3

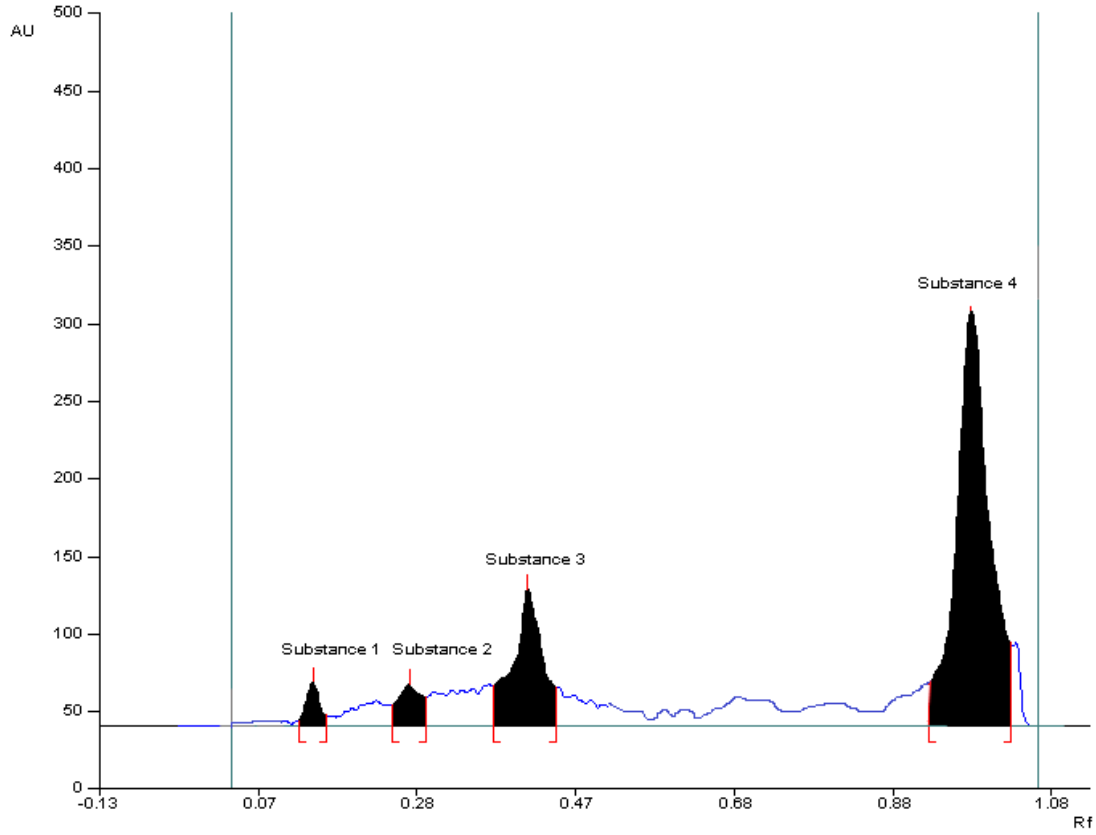


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.13	8.8	0.15	21.3	4.57	0.18	12.8	225.2	9.22
2	0.23	14.3	0.25	25.1	5.39	0.27	19.3	198.1	7.92
3	0.38	24.9	0.40	95.2	20.43	0.43	18.2	634.6	25.70
4	0.95	25.8	0.99	209.7	52.73	1.04	39.7	2755.3	57.19

Figure 35

CHROMATOGRAM FOR FORMULATION ANALYSIS BY HPTLC

REPEATABILITY 4

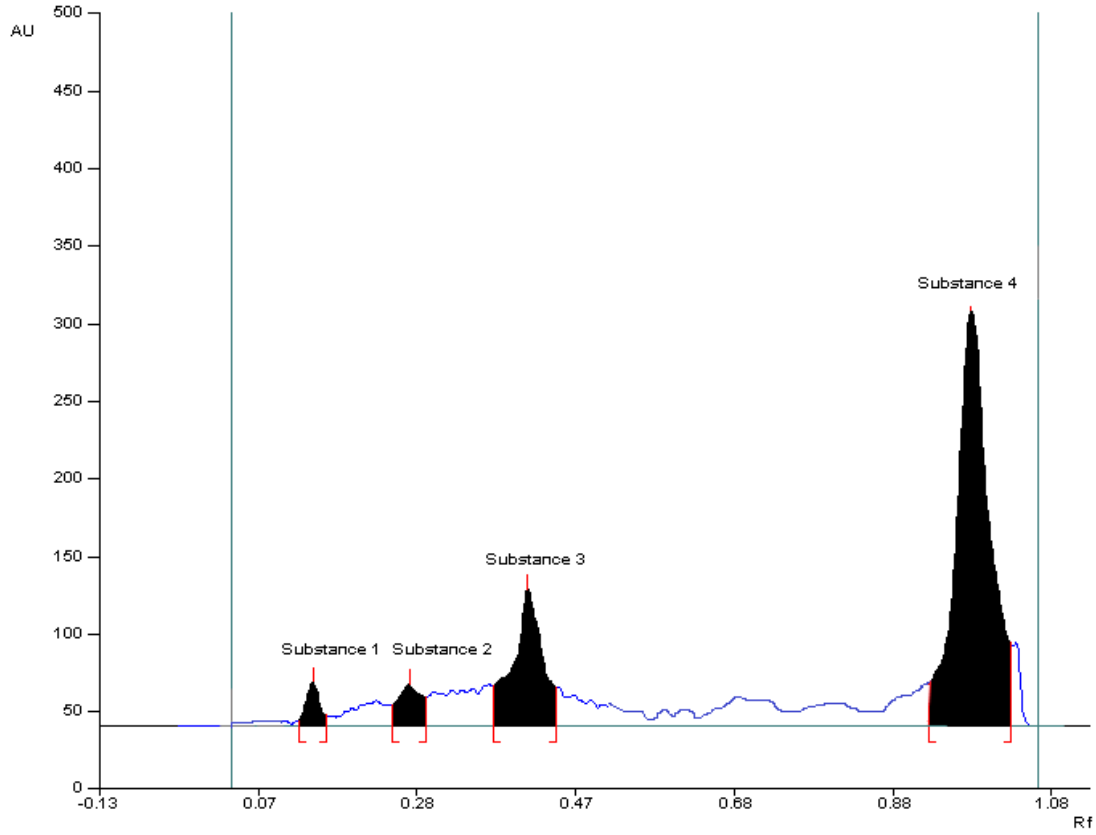


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.14	8.1	0.15	18.6	4.18	0.19	9.6	224.6	9.26
2	0.22	14.4	0.25	24.6	5.36	0.27	20.9	196.4	7.96
3	0.37	23.9	0.40	95.2	23.57	0.44	18.5	640.2	25.64
4	0.95	25.1	0.99	211.3	53.89	1.06	39.9	2759.7	57.11

Figure 36

CHROMATOGRAM FOR FORMULATION ANALYSIS BY HPTLC

REPEATABILITY 5

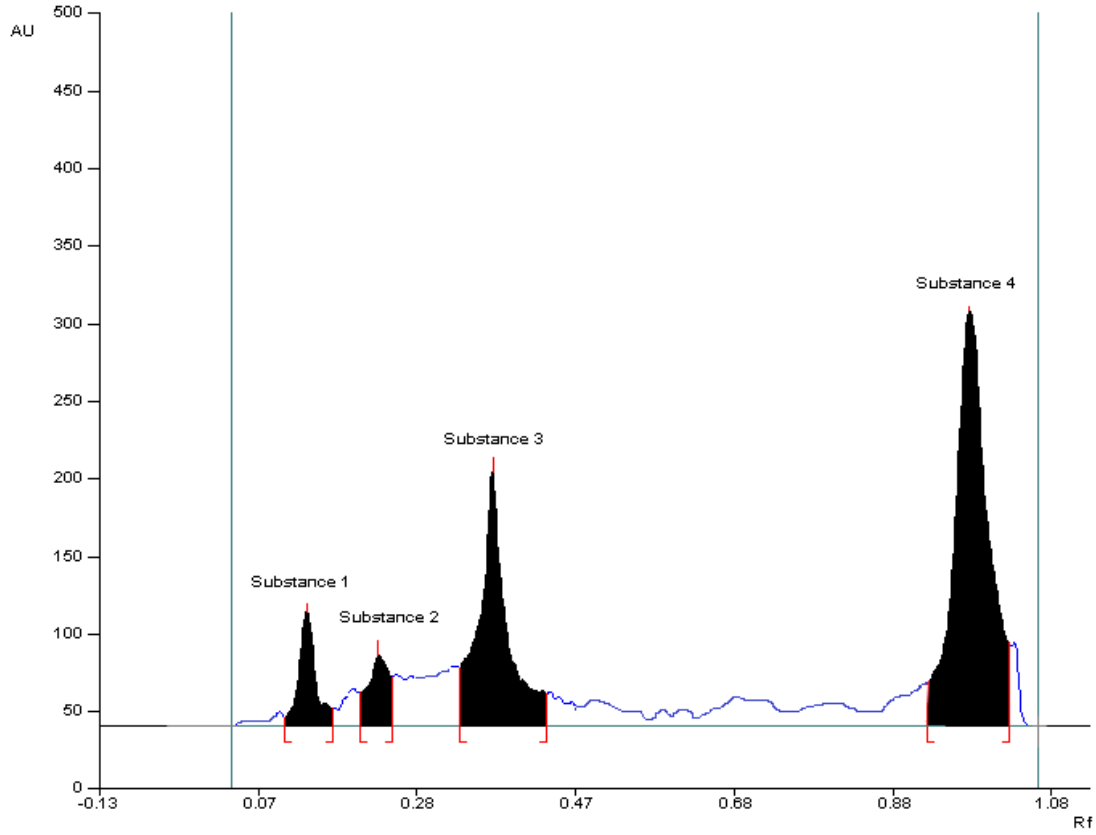


Peak	Start Rf	Start Height	Max Rf	Max height	Max %	End Rf	End Height	Area	Area %
1	0.14	7.8	0.14	16.1	3.75	0.16	10.5	226.2	9.49
2	0.22	14.5	0.25	26.4	5.81	0.27	20.5	196.2	8.06
3	0.39	23.1	0.40	85.0	24.28	0.43	19.3	633.9	25.77
4	0.95	25.6	0.99	215.0	53.15	1.06	39.8	2754.8	56.66

Figure 37

CHROMATOGRAM FOR FORMULATION ANALYSIS BY HPTLC

REPEATABILITY 6

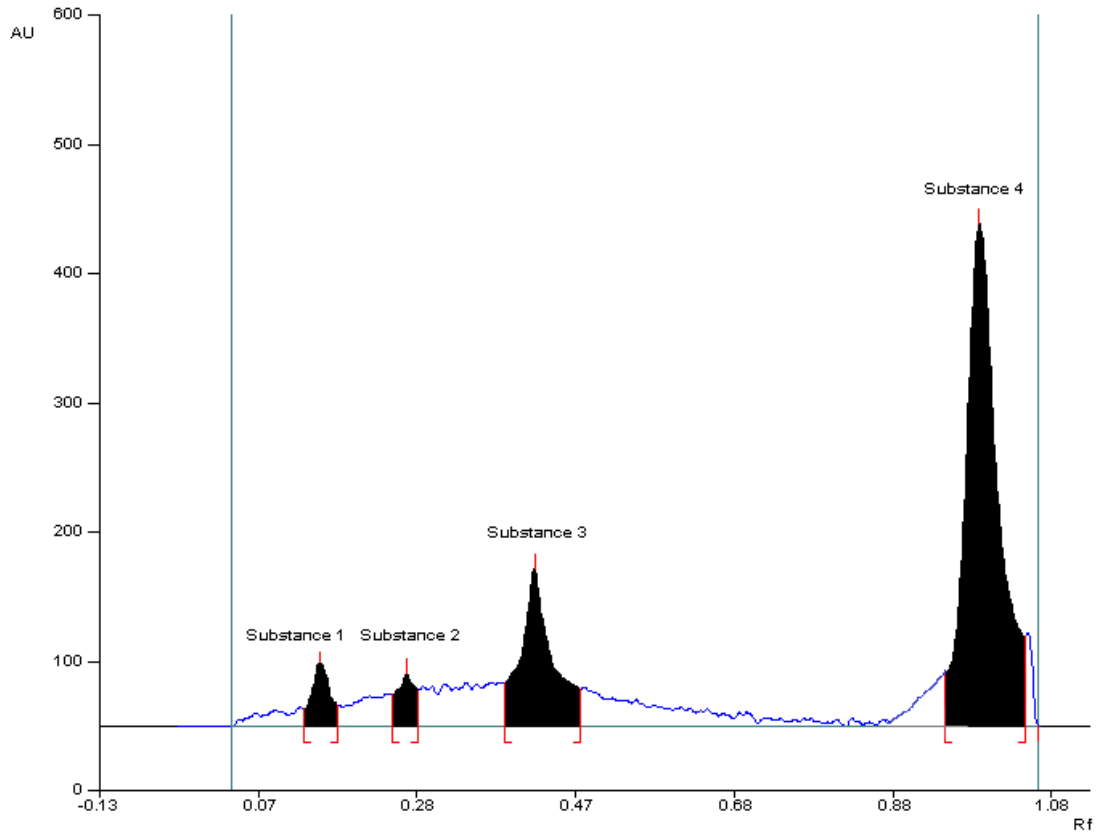


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.14	8.5	0.14	18.9	4.24	0.16	12.3	227.1	9.54
2	0.23	14.9	0.25	24.7	6.63	0.26	20.9	198.2	7.95
3	0.37	23.9	0.40	92.8	25.82	0.42	19.9	641.5	25.98
4	0.96	24.8	0.99	219.3	53.64	1.05	37.6	2758.6	56.03

Figure 38

CHROMATOGRAM FOR THE RECOVERY ANALYSIS OF FORMULATION

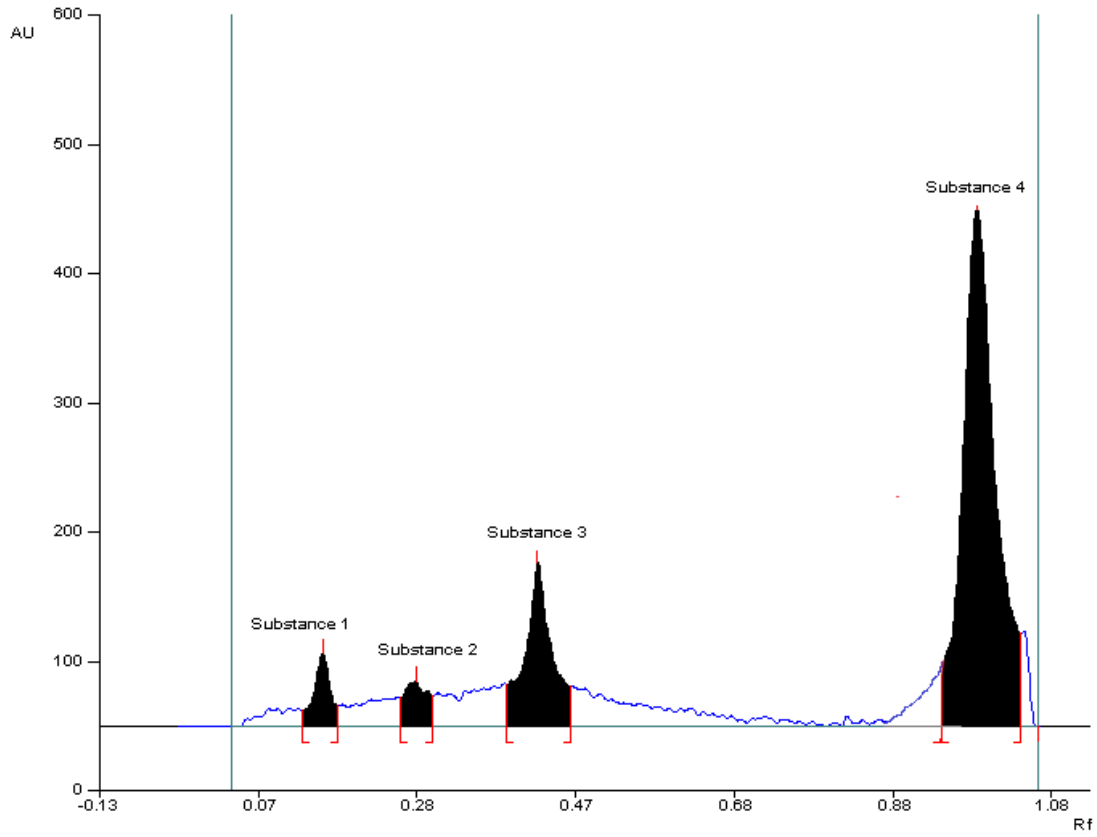
RECOVERY 1



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.12	13.7	0.15	53.3	8.66	0.18	15.5	402.8	9.47
2	0.24	22.7	0.26	36.6	7.11	0.28	25.5	353.2	8.02
3	0.39	32.7	0.41	122.6	40.44	0.43	31.3	1152.1	25.71
4	0.95	42.9	0.99	393.1	61.44	1.04	68.9	4956.2	56.78

Figure 39

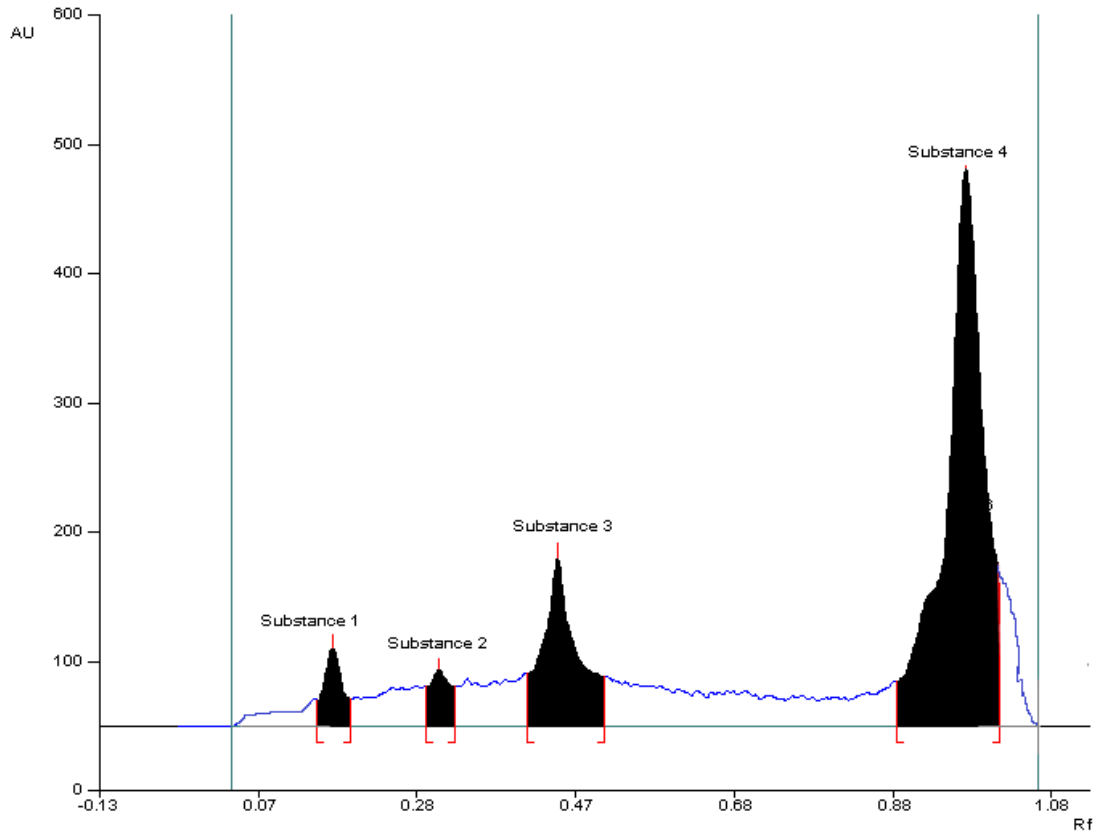
CHROMATOGRAM FOR THE RECOVERY ANALYSIS OF FORMULATION RECOVERY 2



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.13	11.9	0.15	57.0	8.52	0.18	15.8	446.4	9.43
2	0.23	22.1	0.25	34.3	5.13	0.28	23.7	393.2	7.96
3	0.38	32.4	0.40	126.2	18.87	0.45	30.8	1281.9	25.74
4	0.94	49.9	0.99	400.1	59.65	1.02	72.8	5507.3	56.86

Figure 40

CHROMATOGRAM FOR THE RECOVERY ANALYSIS OF FORMULATION RECOVERY 3



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.13	18.8	0.15	15.3	2.01	0.17	10.7	492.5	9.45
2	0.23	25.8	0.25	39.1	5.15	0.28	28.7	432.6	8.07
3	0.37	34.8	0.40	130.2	17.15	0.44	33.8	1411.3	25.58
4	0.97	17.4	0.99	418.7	54.92	1.03	94.2	6059.4	56.89

TABLES

Table 1

SOLUBILITY PROFILE OF ATORVASTATIN CALCIUM

S.NO	SOLVENT	EXTENT OF SOLUBILTY	CATEGORY
1.	Distilled water	More than 100 ml	Practically insoluble
2.	Methanol	10 mg in 0.1 ml	Freely soluble
3.	Ethanol	10 mg in 0.3 ml	soluble
4.	Acetonitrile	10 mg in 0.3 ml	Soluble
5.	Dimethylformamide	10 mg in 3 ml	Slightly soluble
6.	Acetone	10 mg in 0.5 ml	Sparingly soluble
7.	Acetic Acid	More than 100 ml	Practically insoluble
8.	Ethyl Acetate	10 mg in 20 ml	Very slightly soluble
9.	Chloroform	10 mg in 20 ml	Very slightly soluble
10.	n-Butanol	More than 100 ml	Practically insoluble
11.	Benzene	More than 100 ml	Practically insoluble
12.	Toluene	More than 100 ml	Practically insoluble
13.	Isopropyl alcohol	More than 100 ml	Practically insoluble
14.	Hexane	10 mg in 6 ml	Slightly soluble
15.	0.1 M Hydrochloric acid	More than 100 ml	Practically insoluble
16.	0.1 M Sodium hydroxide	More than 100 ml	Practically insoluble
17.	Dichloromethane	10 mg in 1 ml	Sparingly soluble
18.	Diethyl ether	More than 100 ml	Practically insoluble
19.	Acid phthalate buffer(pH3)	More than 100 ml	Practically insoluble
20.	Neutralized buffer (pH5)	More than 100 ml	Practically insoluble
21.	Phosphate buffer (pH7)	More than 100 ml	Practically insoluble
22.	Alkaline buffer (pH8)	10 mg in 10 ml	Slightly soluble

Table 2

SOLUBILITY PROFILE OF ASPIRIN

S.NO	SOLVENT	EXTENT OF SOLUBILTY	CATEGORY
1.	Distilled water	10 mg in 11 ml	Very slightly soluble
2.	Methanol	10 mg in 50 µl	Freely soluble
3.	Ethanol	10 mg in 70 µl	Freely soluble
4.	Acetonitrile	10 mg in 70 µl	Freely soluble
5.	Dimethylformamide	10 mg in 50 µl	Freely soluble
6.	Acetone	10 mg in 0.5 ml	Sparingly soluble
7.	Acetic Acid	10 mg in 1.5 ml	Slightly soluble
8.	Ethyl Acetate	10 mg in 0.2 ml	Soluble
9.	Chloroform	10 mg in 0.3 ml	Soluble
10.	n-Butanol	10 mg in 0.5 ml	Sparingly soluble
11.	Benzene	10 mg in 1.1 ml	Slightly soluble
12.	Toluene	10 mg in 5 ml	Slightly soluble
13.	Isopropyl alcohol	10 mg in 50 µl	Freely soluble
14.	Hexane	10 mg in 2 ml	Slightly soluble
15.	0.1 M Hydrochloric acid	10 mg in 5 ml	Slightly soluble
16.	0.1 M Sodium hydroxide	10 mg in 0.5 ml	Sparingly soluble
17.	Dichloromethane	10 mg in 0.2 ml	Soluble
18.	Diethyl ether	10 mg in 0.5 ml	Sparingly soluble
19.	Acid phthalate buffer pH3	10 mg in 5.1 ml	Slightly soluble
20.	Neutralized buffer (pH5)	10 mg in 4.1 ml	Slightly soluble
21.	Phosphate buffer (pH7)	10 mg in 1 ml	Sparingly soluble
22.	Alkaline buffer (pH9)	10 mg in 0.5 ml	Sparingly soluble

Table 3

SOLUBILITY PROFILE OF RAMIPRIL

S.NO	SOLVENT	EXTENT OF SOLUBILTY	CATEGORY
1.	Distilled water	10 mg in 10 ml	Very slightly soluble
2.	Methanol	10mg in 70 µl	Freely soluble
3.	Ethanol	10 mg in 80 µl	Freely soluble
4.	Acetonitrile	10 mg in 70 µl	Freely soluble
5.	Dimethylformamide	10 mg in 1.5 ml	Slightly soluble
6.	Acetone	10 mg in 0.1 ml	Soluble
7.	Acetic Acid	10 mg in 50 µl	Freely soluble
8.	Ethyl Acetate	10 mg in 9 ml	Slightly soluble
9.	Chloroform	10 mg in 30 µl	Freely soluble
10.	n-Butanol	More than 100 ml	Practically insoluble
11.	Benzene	10 mg in 1.5 ml	Slightly soluble
12.	Toluene	10 mg in 0.1 ml	Soluble
13.	Isopropyl alcohol	10 mg in 0.6 ml	Sparingly soluble
14.	Hexane	More than 100 ml	Practically inSoluble
15.	0.1 M Hydrochloric acid	10 mg in 0.5 ml	Sparingly soluble
16.	0.1 M Sodium hydroxide	10 mg in 0.9 ml	Practically insoluble
17.	Dichloromethane	10 mg in 30 µl	Freely soluble
18.	Diethyl ether	10 mg in 5 ml	Slightly soluble
19.	Acid phthalate buffer pH3	10 mg in 1.5 ml	Slightly soluble
20.	Neutralized buffer (pH5)	More than 100 ml	Practically insoluble
21.	Phosphate buffer (pH7)	10 mg in 10 ml	Slightly soluble
22.	Alkaline buffer (pH9)	10 mg in 5 ml	Slightly soluble

Table 4

SOLUBILITY PROFILE OF METOPROLOL TARTRATE

S.NO	SOLVENT	EXTENT OF SOLUBILTY	CATEGORY
1.	Distilled water	10 mg in 10 µl	Very soluble
2.	Methanol	10 mg in 10 µl	Very soluble
3.	Ethanol	10 mg in 40 µl	Freely soluble
4.	Acetonitrile	10 mg in 0.4 ml	Sparingly soluble
5.	Dimethylformamide	10 mg in 0.6 ml	Sparingly soluble
6.	Acetone	10 mg in 1.3 ml	Slightly soluble
7.	Acetic Acid	10 mg in 1 ml	Sparingly soluble
8.	Ethyl Acetate	10 mg in 0.3ml	Soluble
9.	Chloroform	10 mg in 0.6ml	Sparingly soluble
10.	n-Butanol	10 mg in 0.5 ml	Sparingly soluble
11.	Benzene	10 mg in 0.2 ml	Soluble
12.	Toluene	10 mg in 2 ml	Slightly soluble
13.	Isopropyl alcohol	10 mg in 70 µl	Freely soluble
14.	Hexane	10 mg in 0.2 ml	Soluble
15.	0.1 M Hydrochloric acid	10 mg in 0.5 ml	Sparingly soluble
16.	0.1 M Sodium hydroxide	10 mg in1.5 ml	Slightly soluble
17.	Dichloromethane	10 mg in 2 ml	Slightly soluble
18.	Diethyl ether	10 mg in 0.6 ml	Sparingly soluble
19.	Acid phthalate buffer pH3	10 mg in 0.2 ml	Soluble
20.	Neutralized buffer (pH5)	10 mg in 50 µl	Freely soluble
21.	Phosphate buffer (pH7)	10 mg in 100 µl	Freely soluble
22.	Alkaline buffer (pH9)	10 mg in 100 µl	Freely soluble

Table 5

OPTICAL CHARACTERISTICS OF ATORVASTATIN CALCIUM

(FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY) µg/ml

PARAMETERS	**AT 291.5 nm***
Beers law limit (µg/ ml)	3 - 21
Molar absorptivity (L $mol^{-1}cm^{-1}$)	740.67
Sandell's sensitivity (µg/cm^2/0.001 A.U)	1.5960
Correlation coefficient (r)	0.9999
Regression equation (Y=mx+c)	Y = 0.0006x +(-) 0.0001
Slope (m)	0.0006
Intercept (c)	(-) 0.0001
LOD (µg/ ml)	0.6176
LOQ (µg/ ml)	1.8714
Standard Error	0.00003

(*Mean of six observations)

Table 6

OPTICAL CHARACTERISTICS OF ASPIRIN

(FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY)

PARAMETERS	**AT 247 nm***
Beers law limit (µg/ ml)	10 - 70
Molar absorptivity (L $mol^{-1}cm^{-1}$)	117.52
Sandell's sensitivity (µg/cm^2/0.001 A.U)	1.0017
Correlation coefficient (r)	0.9968
Regression equation (Y=mx+c)	Y = 0.0009x+ (-) 0.0035
Slope (m)	0.0009
Intercept (c)	(-) 0.0035
LOD (µg/ ml)	0.1432
LOQ (µg/ ml)	0.4343
Standard Error	0.00002

(*Mean of six observations)

Table 7

OPTICAL CHARACTERISTICS OF RAMIPRIL

(FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY)

PARAMETERS	**AT 242.5 nm***
Beers law limit (µg/ ml)	10 - 70
Molar absorptivity (L $mol^{-1}cm^{-1}$)	66.66
Sandell's sensitivity (µg/cm^2/0.001 A.U)	6.1907
Correlation coefficient (r)	0.9996
Regression equation (Y=mx+c)	Y = 0.0002x+ (-) 0.0001
Slope (m)	0.0002
Intercept (c)	(-) 0.0001
LOD (µg/ ml)	1.6587
LOQ (µg/ ml)	5.0263
Standard Error	0.00002

(*Mean of six observations)

Table 8

OPTICAL CHARACTERISTICS OF METOPROLOL TARTRATE

(FIRST ORDER DERIVATIVE SPECTROPHOTOMETRY)

PARAMETERS	**AT 229.5 nm***
Beers law limit (µg/ ml)	10 - 70
Molar absorptivity (L $mol^{-1}cm^{-1}$)	672.118
Sandell's sensitivity (µg/cm^2/0.001 A.U)	0.3742
Correlation coefficient (r)	0.9998
Regression equation (Y=mx+c)	Y = 0.0027x+ (-) 0.0016
Slope (m)	0.0027
Intercept (c)	(-) 0.0016
LOD (µg/ ml)	0.1310
LOQ (µg/ ml)	0.3969
Standard Error	0.00003

(*Mean of six observations)

Table 9

ANALYSIS OF SYNTHETIC MIXTURE

Drug	Concentration prepared (µg/ ml)	Amount Found (µg/ ml)*	Percentage Purity*	Average (%)	SD	RSD	SE
ATR	4 8 12 16 20	3.92 8.07 12.06 15.89 20.04	98.00 100.87 100.50 99.31 100.20	99.78	1.1481	1.1506	0.0459
ASP	30 40 50 60 70	30.11 39.60 49.30 58.90 69.74	100.36 99.00 98.60 98.16 99.62	99.15	0.8646	0.8720	0.0346
RAM	30 40 50 60 70	30.16 39.77 50.42 60.20 69.46	100.53 99. 42 100.84 100.33 99.22	100.07	0.7101	0.7096	0.0284
MET	30 40 50 60 70	30.07 40.37 50.43 60.73 70.24	100.23 100.92 100.86 101.21 100.34	100.71	0.4135	0.4106	0.0165

(* Mean of six observations)

Table 10
QUANTIFICATION OF FORMULATION (ZYCAD-4)

Drug	Label Claim (mg/tab)	Amount Found (mg/tab)*	Percentage Obtained*	Average (%)	SD	RSD	SE
ATR	10	10.1849	101.84	102.76	1.4487	1.4097	0.0402
	10	10.1747	101.74				
	10	10.4575	104.57				
	10	10.4692	104.69				
	10	10.1911	101.91				
	10	10.1826	101.82				
ASP	75	73.9410	98.58	99.00	1.5795	1.5954	0.0438
	75	72.3707	96.49				
	75	74.2635	99.01				
	75	75.8524	101.11				
	75	73.9855	98.64				
	75	75.1467	100.19				
RAM	5	5.0974	101.94	99.95	1.7250	1.7257	0.0479
	5	4.9926	99.85				
	5	4.9648	99.29				
	5	4.8815	97.63				
	5	5.1005	102.01				
	5	4.9515	99.03				
MET	50	49.2274	98.45	98.66	0.8496	0.8612	0.0236
	50	49.0784	98.15				
	50	49.0568	98.11				
	50	49.0374	98.07				
	50	49.4569	98.91				
	50	50.1394	100.27				

(*Mean of six observations)

Table 11

INTRADAY AND INTER DAY ANALYSIS OF FORMULATION (ZYCAD-4)

Drug	Amount labeled (mg/tab)	Percentage Obtained*		SD		%RSD	
		Intra day	Inter day	Intra day	Inter day	Intra day	Inter day
ATR	10	102.02	101.82	0.1557	0.0693	0.1528	0.0680
	10	101.71	101.94				
	10	101.89	101.94				
Mean		101.87	101.90				
ASP	75	98.75	98.99	0.8048	0.3124	0.8116	0.3145
	75	100.08	99.55				
	75	98.63	99.51				
Mean		99.15	99.35				
RAM	5	101.61	102.52	1.9222	1.1748	1.9063	1.1612
	5	102.26	100.38				
	5	98.65	100.61				
Mean		100.84	101.17				
MET	50	98.62	98.58	1.7119	1.0391	1.7204	1.0513
	50	98.42	99.98				
	50	101.48	97.95				
Mean		99.51	98.83				

(*Mean of six observations)

Table 12

RUGGEDNESS STUDY

Drug	Condition	Percentage Obtained	SD	%RSD	SE
ATR	Analyst 1	102.73	1.4218	1.3842	0.0394
	Analyst 2	101.94	0.2943	0.2887	0.0081
	Instrument 1	102.05	0.1635	0.1602	0.0045
	Instrument 2	99.96	1.5147	1.5153	0.0420
ASP	Analyst 1	99.00	1.5795	1.5954	0.0438
	Analyst 2	99.53	1.8311	1.8396	0.0509
	Instrument 1	99.40	0.6644	0.6684	0.0184
	Instrument 2	99.01	0.4419	0.4463	0.0128
RAM	Analyst 1	99.95	1.7249	1.7256	0.4791
	Analyst 2	99.99	1.8625	1.8602	0.0517
	Instrument 1	101.11	2.0040	1.9820	0.0557
	Instrument 2	99.80	1.0762	1.0783	0.0299
MET	Analyst 1	98.66	0.8496	0.8611	0.0236
	Analyst 2	99.07	0.8289	0.8367	0.0230
	Instrument 1	98.97	1.2756	1.2887	0.0354
	Instrument 2	99.03	0.8143	0.8223	0.0226

Table 13

RECOVERY ANALYSIS OF FORMULATION (ZYCAD-4)

Drug	Amount Present (µg/tab)	Amount Added (µg/tab)*	Amount Estimated (µg/tab)*	Amount Recovered (µg/tab)*	Percentage Recovered*	SD	%RSD	SE
ATR	4.1333 4.1333 4.1333	2.4799 3.7199 4.9599	6.6244 7.8615 9.0816	2.4911 3.7282 4.9483	100.45 100.22 99.76	0.3513	0.3508	0.0390
				Mean	100.40			
ASP	29.8667 29.8667 29.8667	23.8933 29.8667 35.8400	53.8515 60.0584 65.9412	23.9848 30.1917 36.0745	100.38 101.08 100.65	0.3530	0.3506	0.0392
				Mean	100.70			
RAM	20.1010 20.1010 20.1010	12.0606 18.0909 24.1212	31.9905 38.4895 44.3816	11.8895 18.3885 24.2806	98.58 101.64 100.66	1.5626	1.5580	0.1736
				Mean	100.29			
MET	19.8492 19.8492 19.8492	11.9095 17.8642 23.8190	31.5875 37.4166 44.0712	11.7383 17.5674 24.2220	98.56 98.33 101.69	1.8770	1.8860	0.2086
				Mean	99.52			

(* Mean of three observations)

Table 14

OPTICAL CHARACTERISTICS OF METOPROLOL TARTRATE

(HPTLC METHOD)

PARAMETERS	**METOPROLOL TARTRATE***
Detection Wavelength	224 nm
Beers law limit (ng/ µl)	100 - 600
Correlation coefficient (r)	0.9999
Regression equation (Y=mx+c)	Y = 2.2297x+ 0.9164
Slope (m)	2.2297
Intercept (c)	0.9167
LOD (ng/ µl)	0.7632
LOQ (ng/ µl)	2.3128
Standard Error	0.4449

(*Mean of three observations)

Table 15

OPTICAL CHARACTERISTICS OF RAMIPRIL

(HPTLC METHOD)

PARAMETERS	RAMIPRIL*
Detection Wavelength	224 nm
Beers law limit (ng/ µl)	10 - 60
Correlation coefficient (r)	0.9998
Regression equation (Y=mx+c)	Y = 19.043x+ 6.272
Slope (m)	19.043
Intercept (c)	6.272
LOD (ng/ µl)	0.1025
LOQ (ng/ µl)	0.3105
Standard Error	0.1427

(*Mean of three observations)

Table 16

OPTICAL CHARACTERISTICS OF ATORVASTATIN CALCIUM

(HPTLC METHOD)

PARAMETERS	ATORVASTATIN CALCIUM*
Detection Wavelength	224 nm
Beers law limit (ng/ µl)	20 to 120
Correlation coefficient (r)	0.9998
Regression equation (Y=mx+c)	Y = 31.775x+ 5.6914
Slope (m)	31.7751
Intercept (c)	5.6917
LOD (ng/ µl)	0.1272
LOQ (ng/ µl)	0.3855
Standard Error	0.1471

(*Mean of three observations)

Table 17

OPTICAL CHARACTERISTICS OF ASPIRIN

(HPTLC METHOD)

PARAMETERS	ASPIRIN*
Detection Wavelength	224 nm
Beers law limit (ng/ µl)	75 to 450
Correlation coefficient (r)	0.9998
Regression equation (Y=mx+c)	Y = 18.561x+ (-)30.8631
Slope (m)	18.5616
Intercept (c)	(-) 30.8631
LOD (ng/ µl)	0.2955
LOQ (ng/ µl)	0.8954
Standard Error	0.0821

(*Mean of three observations)

Table 18
QUANTIFICATION OF FORMULATION (ZYCAD-4) BY HPTLC

Drug	Label Claim (mg/tab)	Amount Found (mg/tab)*	Percentage Obtained	Average (%)*	SD	RSD	SE
MET	50	49.4905	98.98	100.24	0.8348	0.8328	0.0232
	50	49.8501	99.70				
	50	50.2296	100.45				
	50	50.0948	100.90				
	50	50.4543	100.90				
	50	50.6541	101.30				
RAM	5	4.9540	99.08	99.93	0.5910	0.5914	0.0164
	5	5.0089	100.07				
	5	5.0289	100.57				
	5	4.9840	99.68				
	5	4.9790	99.58				
	5	5.0289	100.57				
ATR	10	9.8481	98.48	99.00	0.6526	0.6593	0.0181
	10	9.8331	98.33				
	10	9.8831	98.83				
	10	9.9680	99.68				
	10	9.8731	98.73				
	10	9.9929	99.92				
ASP	75	74.8950	99.86	99.97	0.0829	0.0830	0.0023
	75	74.9948	99.99				
	75	74.9599	99.94				
	75	75.0797	100.10				
	75	74.9449	99.92				
	75	75.0498	100.01				

(*Mean of six observations)

Table 19

INTRADAY AND INTER DAY ANALYSIS OF FORMULATION (ZYCAD-4)

(HPTLC)

Drug	Amount Labeled (mg/ tab)	Percentage Obtained*		SD		%RSD	
		Intra day	Inter day	Intra day	Inter day	Intra day	Inter day
MET	50	99.24	101.16	0.7643	1.2842	0.7639	1.2832
	50	100.14	98.66				
	50	100.76	100.42				
Mean		100.04	100.08				
RAM	5	100.80	100.80	0.7211	1.1015	0.7197	1.1052
	5	100.40	98.60				
	5	99.40	99.60				
Mean		100.20	99.66				
ATR	10	98.90	102.70	0.1527	2.0113	0.1547	2.0167
	10	98.70	98.50				
	10	98.60	99.40				
Mean		98.73	100.20				
ASP	75	99.96	100.18	0.3150	0.1400	0.3158	0.1399
	75	99.93	99.90				
	75	100.04	100.04				
Mean		99.76	100.04				

(*Mean of six observations)

Table 20

RECOVERY ANALYSIS OF FORMULATION (ZYCAD-4) BY HPTLC

Drug	Amount Present (µg/ ml)	Amount Added (µg/ ml)*	Amount Estimated (µg/ ml)*	Amount Recovered (µg/ ml)*	Percentage Recovered*	SD	%RSD	SE
MET	100.3783 100.3783 100.3783	80.3026 100.3786 120.4539	180.6733 199.7600 220.4533	80.2950 99.3817 120.0750	99.99 99.00 99.68	0.5064	0.5087	0.0563
				Mean	99.55			
RAM	10.0066 10.0066 10.0066	8.0052 10.0066 12.0079	18.1100 20.0000 22.0933	8.1034 9.9934 12.0867	101.26 99.86 100.65	0.7019	0.6978	0.0780
				Mean	100.59			
ATR	19.8233 19.8233 19.8233	15.8586 19.8233 23.7879	35.5285 40.0052 44.2833	15.7052 20.1819 24.2600	98.03 101.80 101.98	1.6536	1.6383	0.1837
				Mean	100.60			
ASP	150.1550 150.1550 150.1550	120.4400 150.1550 180.1860	268.8700 298.3766 328.0066	118.5150 148.2216 177.8516	98.40 98.71 98.70	0.1762	0.1787	0.0196
				Mean	98.60			

(* Mean of three observations)

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