DEVELOPMENT AND VALIDATION OF NEW RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF AMLODIPINE BESYLATE AND VALSARTAN IN TABLET FORMULATIONS

Dissertation

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MADURAI – 625107

APRIL-2012

CERTIFICATE

This is to certify that the dissertation entitled "**DEVELOPMENT AND VALIDATION OF NEW RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF AMLODIPINE BESYLATE AND VALSARTAN IN TABLET FORMULATIONS"** submitted by **Mr. K. KIRAN KUMAR** (**Reg. No: 26101724**) in partial fulfillment of the degree of Master of Pharmacy in Pharmaceutical Analysis, ***K. M. COLLEGE OF PHARMACY,*** MADURAI-62510, under **The Tamilnadu Dr. M. G. R. Medical University,** Chennai, carried out in **HETERO PHARMACEUITICAL LTD., UNIT –III, Hyderabad.**

It is a bonafide work carried out by him under my guidance and supervision during the academic year **2011 - 2012**. This dissertation partially or fully has not been submitted for any other degree or diploma of this university or any other universities.

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AN EXPRESION OF GRATITUDE

"With God All Things Are Possible"

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K. KIRAN KUMAR

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1. INTRODUCTION

Analysis is the study of the separation, identification and quantification of chemical components of natural and artificial materials. Analytical chemistry has been important since the early days of chemistry, providing methods for determining which elements and chemicals are present in the world around us. "Analytical chemistry is the chemistry discipline concerned with the chemical composition of materials. Analytical chemistry also is concerned with developing the tools used to examine chemical compositions" [1a, 27]. Pharmaceutical analysis plays an important role in quality assurance and quality control.

The discipline of analytical chemistry consists of [2a],

I. Qualitative analysis.

II. Quantitative analysis.

I Qualitative analysis:

- It gives an indication of the identity of the chemical species in the sample.
- Qualitative analysis seeks to establish the ways and means to detect the presence of a given element or inorganic compound in a sample [30].
- Qualitative organic analysis researches to establish the method presence of a given functional group or organic compound in a sample.

II Quantitative analysis:

- Quantitative analysis deals with the development to establish the amount of a given element or compound in a sample.
- Methods to determine of an absolute or relative abundance (often expressed as a concentration) of one, several or all particular substances present in a sample.

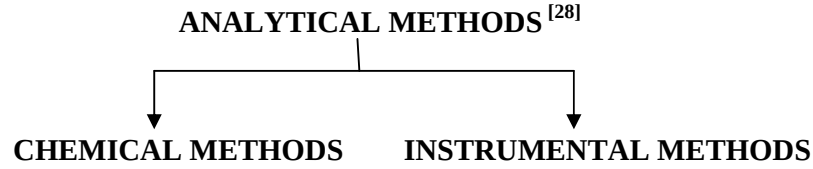
Steps involved in quantitative analysis:

- Chemical nature of the sample.
- Selection of method.
- Elimination of possible interferences.
- Measurement of an analyte.
- Calculation of results.
- Estimation of reliability of the results (validation).

Factors affecting the choice of analytical methods

- ❖ Type of analysis.
- ❖ Nature of the material.
- ❖ Interference from other components.
- ❖ Concentration range to be investigated.
- ❖ Accuracy.
- ❖ Facilities available.
- ❖ Time required for complete analysis.

The analytical methods may be categorized into,



1.1) Chemical methods / Traditional methods:

The chemical methods for separating and determining analytes still find use in many laboratories. For qualitative analysis the separated components were then treated with reagents that yield products that could be recognized by colours, boiling or melting point, the solubility in a series of solvents, odour, optical activities and their refractive indexes. For quantitative analysis, the amount of analyte was determined by gravimetric or titrimetric measurements. In gravimetric measurements, the mass of the analyte or some compounds produced from the analyte was determined [1b].

In titrimetric procedures, the volume or mass of a standard reagent required to react completely with analyte was measured.

a) Volumetric or Titrimetric

i. Acid-base or Neutralization titrations.
ii. Oxidation-Redution or Redox titrations.
iii. Precipitation titrations.
iv. Complexometric titrations.
v. Non-aqueous titrations.

b) Gravimetric

1.2) Instrumental methods:

In the instrumental methods, the measurement of physical properties of analysis such as conductivity, electrode potential, light absorption and emission. Mass to charge ratio and fluorescence is also being used for quantitative analysis of a variety of inorganic, organic and biochemical analytes [1c]. Different physical properties are exploited in different analytical techniques are listed in table no.1.

The first instrumental analysis was flame emissive spectrometry developed by Robert Bun sun and Gustav Kirchhoff who discovered rubidium and caesium in1860.

Table no. 1 Classification of analytical methods

PHYSICAL PROPERTY MEASURED	INSTRUMENTAL METHODS BASED ON MEASUREMENT OF PROPERTY
Absorption radiation	Spectrophotometry (X-ray, UV-Vis, IR, NMR)
Emission of radiation	Fluorimetry, Flame photometry
Rotation of radiation	Polarimetry, ORD, CD.
Electrical Potential	Potentiometry, Chrono-potentiometry
Electrical conductance	Conductometry.
Electrical current	Polarography, Amperometry.
Thermal properties	DTA, DSC etc.

Chromatographic and electrophoretic techniques for the separation of components complex mixtures prior to their qualitative or quantitative determination**.**

A) Microbiological methods:

Biological assays in which the compounds being quantitated either depress or stimulate the growth of a sensitive test micro organism and those assays that employs enzymes [31].

i) Diffusion assays
ii) End point determination assays
iii) Enzymatic assays

B) Chromatography

i) History of Chromatography:

The term chromatography (Greek: - Khromatos – colour and graphos –written) and its principles were first discovered by Mikhail Tswett in 1906[2b]. He primarily for the separation of plant pigments such as chlorophyll, carotenes and xanthophylls. Analytical chemistry became easier and more accurate through the development of paper chromatography.

During 1970s, most chemical separations were carried out using a variety of techniques including open-column chromatography, paper chromatography and thin-layer chromatography. High pressure liquid chromatography was developed in the mid-1970s and quickly improved with the development of column packing materials and the additional convenience of on-line detectors. In the late 1970s, new methods including reverse phase liquid chromatography was employed for improved separation between very similar compounds [29]. By the 1980s, HPLC was commonly used for the separation of chemical compounds. New techniques improved separation, identification, purification and quantification far above the previous techniques.

ii) Theory:

Chromatography may be preparative or analytical [3]. The purpose of preparative chromatography is to separate the components of a mixture for further use. Analytical chromatography is done normally with smaller amounts of material and is for measuring the relative proportions of analytes in a mixture. The two are not mutually exclusive [4a].

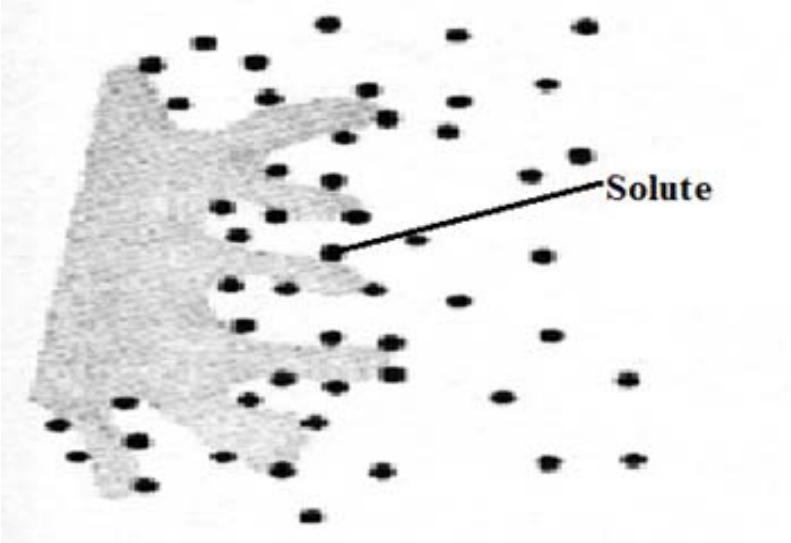
In this two mutually immiscible phases are brought into contact where one phase is stationary and other is mobile. Species in the sample undergo repeated physical interactions (partitions) between the mobile phase and stationary phase. The components are gradually separated into bands in mobile phase. It is an analytical chromatographic technique that is useful for separating ions or molecule that are dissolved in a solvent [4a].

iii) Types of Chromatography – Based on principle

❖ **Adsorption Chromatography**

Adsorption chromatography is probably one of the oldest types of chromatography around. It utilizes a mobile liquid or gaseous phase that is adsorbed onto the surface of a stationary solid phase. As shown in the fig. no.1 the equilibrates between the mobile and stationary phase accounts for the separation of different solutes [5a].

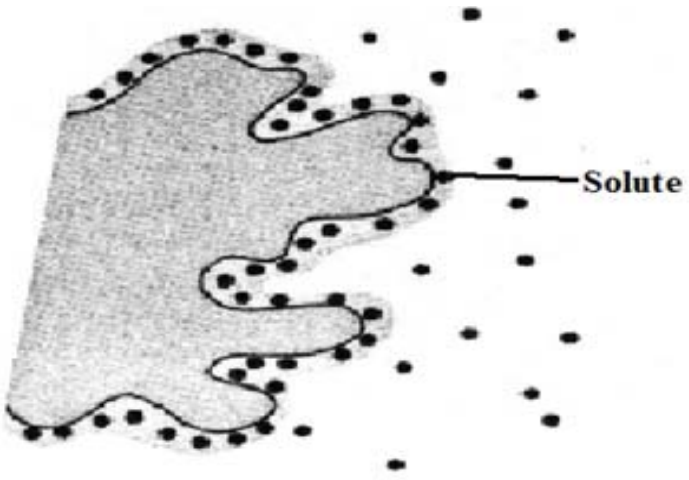
Fig. no. 1 Solute adsorbed on stationary phase in adsorption chromatography



❖ **Partition Chromatography**

This form of chromatography is based on a thin film formed on the surface of a solid support by a liquid stationary phase. As shown in the fig. no. 2 solute equilibrates between the mobile phase and the stationary liquid [4b,14].

Fig. no. 2 Solute in liquid phase distributed on surface of solid support in partition chromatography

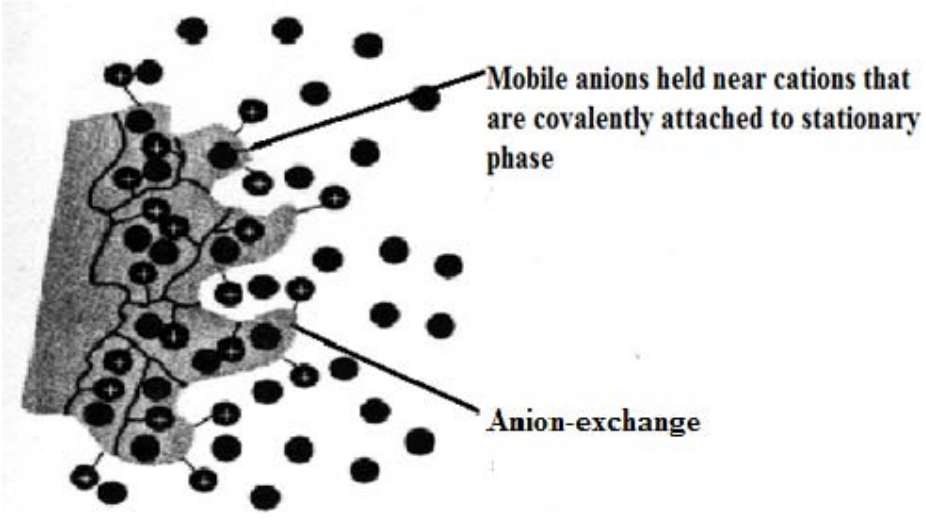


❖ **Ion Exchange Chromatography**

The chromatographic medium is an ion exchange resin which is polymer containing fixed charged groups and replaceable counter ions of the opposite charge. When a sample containing organic or inorganic ions is passed down the column the ions of the same charge as the counter ions displace the counter ions into the mobile phase and are retained on column. Cationic and anionic exchange resins have positively and negatively charged counter ions respectively, and retard the migration of the sample cations and anions respectively. The mobile phase in ion exchange chromatography is usually an aqueous solution containing one or more electrolytes.

In this type of chromatography, the use of a resin (the stationary solid phase) is used to covalently attach anions or cations onto it. Fig no.3 shows that the solute ions of opposite charge in the mobile liquid phase are attracted to the resin by electrostatic forces [4c].

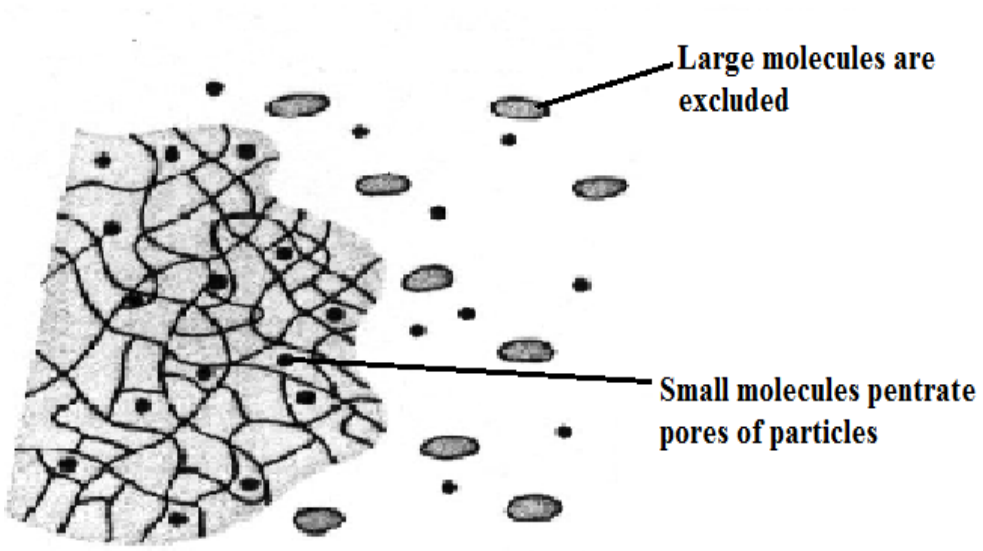
Fig. no. 3 Ions exchanged with resins in ion exchange chromatography



❖ **Molecular Exclusion Chromatography**

It is also known as gel permeation or gel filtration, this type of chromatography lacks an attractive interaction between the stationary phase and solute. The liquid or gaseous phase passes through a porous gel which separates the molecules according to its size. As shown in the fig. no. 4 the pores are normally small and exclude the larger solute molecules, but allow smaller molecules to enter the gel, causing them to flow through a larger volume. This causes the larger molecules to pass through the column at a faster rate than the smaller ones.

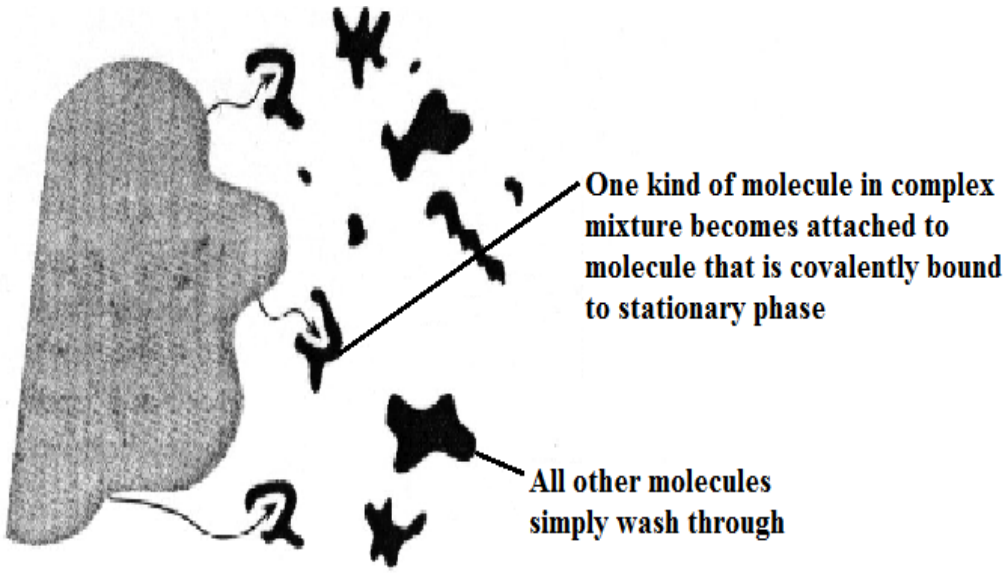
Fig. no. 4 Movement of solute molecules in molecular exclusion chromatography



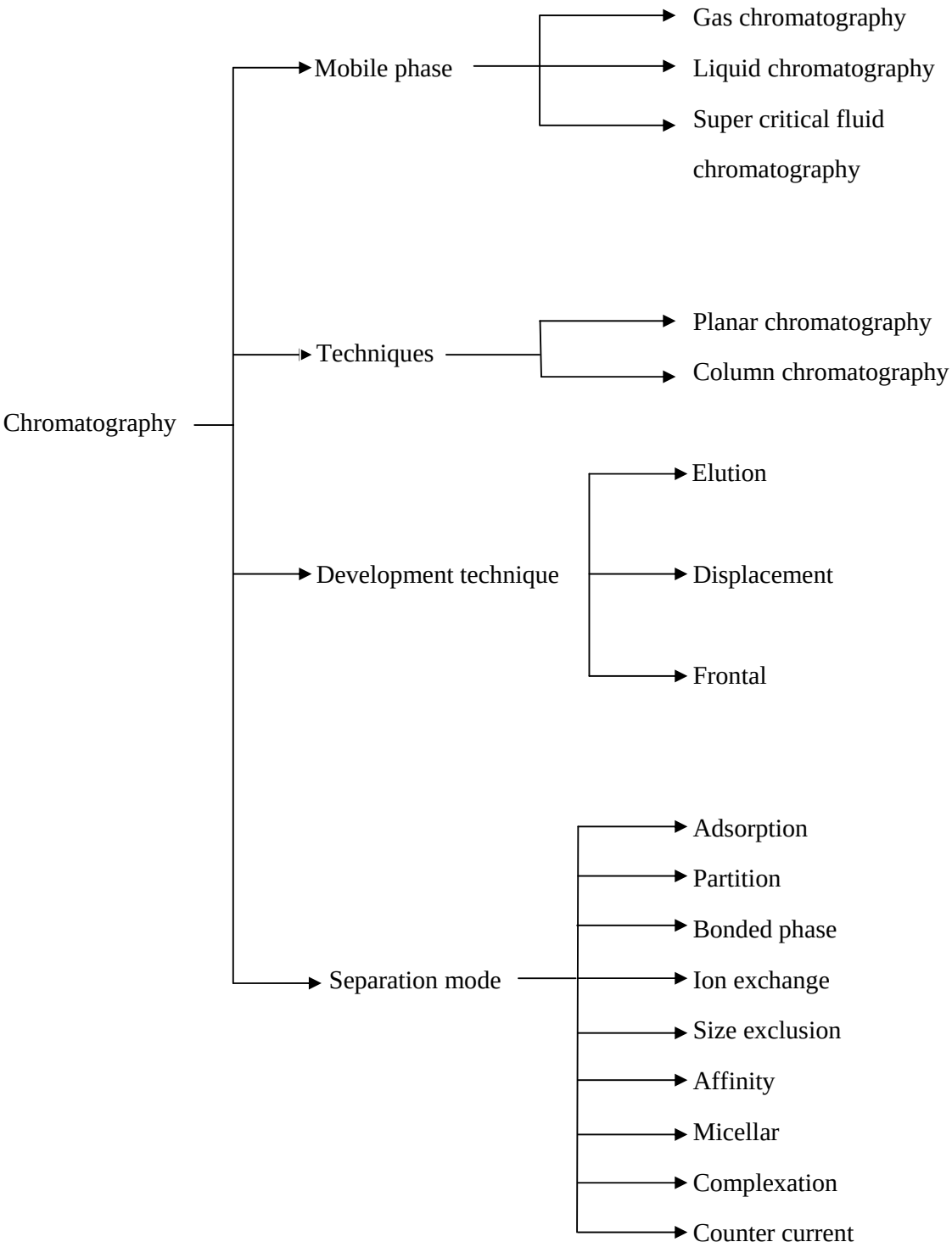
❖ **Affinity Chromatography**

This is the most selective type of chromatography employed. It utilizes the specific interaction between one kind of solute molecule and a second molecule that is immobilized on a stationary phase. For example, the immobilized molecule may be an antibody to some specific protein. When solute containing a mixture of protein passes through molecule, only the specific protein is reacted to this antibody, binding it to the stationary phase is shown in the fig. no. 5. This protein is later extracted by changing the ionic strength or pH [5b].

Fig. no. 5 Movement of molecules affinity chromatography



iv) CLASSIFICATION



v) Some special types of chromatographic techniques

a. Chiral chromatography:

Chiral chromatography involves the separation of stereoisomers. In the case of enantiomers, these have no chemical or physical differences apart from being three-dimensional mirror images. Conventional chromatography or other separation processes are incapable of separating them. To enable chiral separations to take place, either the mobile phase or the stationary phase must themselves be made chiral, giving differing affinities between the analytes. Chiral chromatography HPLC columns (with a chiral stationary phase) in both normal and reversed phase are commercially available [6].

b. Hydrophilic Interaction Liquid Chromatography (HILIC):

This is somewhat similar to normal phase chromatography using a polar stationary phase such as silica or ion exchange materials but eluted with polar mobile phases or organic solvents and aqueous buffers. It is most commonly used to separate the polar analytes and hydrophilic peptides[7].

c. Hydrophobic Interaction Chromatography (HIC):

Analogues to RPC except that mobile phases of low organic solvents content and high salt concentrations are used for the separation of proteins that can be easily denatured by mobile phases with high concentrations of organic solvents used in RPC.

d. Electro Chromatography:

Uses capillary electrophoresis (CE) equipment with packed capillary HPLC column. The mobile is driven by the electromotive force from a high voltage source as opposed to a mechanical pump. It is capable of very high efficiency [25].

e. Super Critical Fluid Chromatography (SFC):

Uses HPLC packed columns and a mobile phase of pressurized supercritical fluids (i.e. carbon dioxide with a polar organic solvent). Useful for non polar analytes and preparative applications where purified materials can be recovered easily by evaporating the carbon dioxide. HPLC pumps normal and GC type detectors are often used [26].

f. Ultra Performance Liquid Chromatography:

In this technique, columns with smaller particles (1.7 micron) and instrumentation with specialized capabilities deliver mobile phase at 15000 Psi (1000 bar). Research is going on much smaller than one micron diameter particles capable of performing at 100,000 Psi (6800 bar) [11].

g. Fast protein liquid chromatography:

Fast protein liquid chromatography (FPLC) is a term applied to several chromatography techniques which are used to purify proteins. Many of these techniques are identical to those carried out under high performance liquid chromatography, however use of FPLC techniques are typically for preparing large scale batches of a purified product[8].

h. Countercurrent chromatography:

Countercurrent chromatography (CCC) is a type of liquid-liquid chromatography, where both the stationary and mobile phases are liquids [9]. The operating principle of CCC equipment requires a column consisting of an open tube coiled around a bobbin. The bobbin is rotated in a double-axis gyratory motion (a cardioid), which causes a variable gravity (G) field to act on the column during each rotation. This motion causes the column to see one partitioning step per revolution and components of the sample separate in the column due to their partitioning coefficient between the two immiscible liquid phases used. There are many types of CCC available today. These include HSCCC (High Speed CCC) and HPCCC (High Performance CCC). HPCCC is the latest and best performing version of the instrumentation available currently [10].

i. Other forms of low pressure liquid chromatography:

Flash chromatography is a technique for sample purification using disposable glass NPC columns and mobile phase driven by gas-pressure or low-pressure pumps.

C. Reversed phase chromatography:

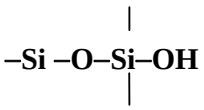
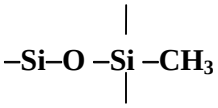
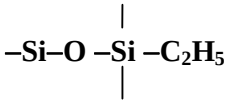
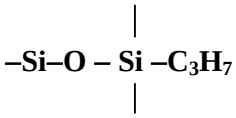
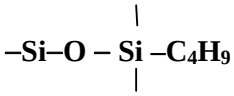
In 1960s, chromatographers started modifying the polar nature of silanol group by chemically reacting silica with organic silanes. The objective was to make less polar or non polar so that polar solvents can be used to separate water soluble polar compounds. Since the ionic nature of chemically modified silica is now reversed i.e. it is non polar and the nature of the phase is reversed. The chromatographic separation carried out with such silica is referred

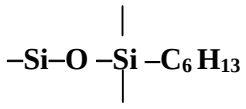
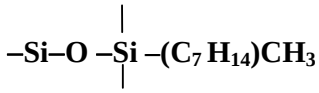
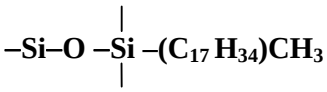
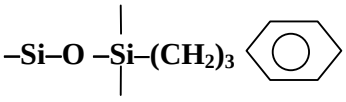
to as reversed–phase chromatography. A large number of chemically bonded stationary phases based on silica are available commercially. Table no.2 lists some of the functional groups bonded in chemically modified silica. Silica based stationary phases are still most popular in reversed phase chromatography [17]. However, other adsorbents based on polymer (styrene-divinylbenzene copolymer) are slowly gaining ground. Simple compounds are better retained by the reversed surface, the less water-soluble (i.e. the more non polar). The retention decreases in the following order: aliphatic greater than induced dipoles (i.e. CCl_4) > permanent dipoles (e.g. $CHCl_3$) > weak lewis bases (ethers, aldehydes and ketones) > strong lewis bases (amines) > weak lewis acids (alcohols and phenols) > strong lewis acids (carboxylic acids). The retention increases as the number of the carbon atoms increases [4a].

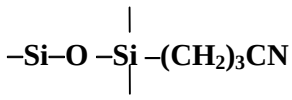
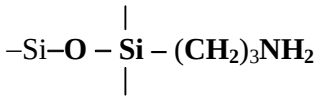
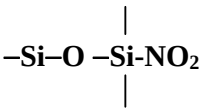
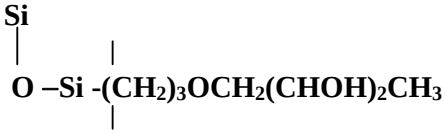
As a general rule, the retention increases with increasing in contact area between sample molecule and stationary phase i.e. with increasing in number of water molecules which are released during the adsorption of a compound. Branched chain compounds are eluted more rapidly than their normal isomers.

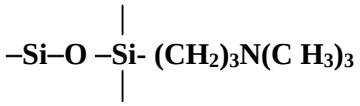
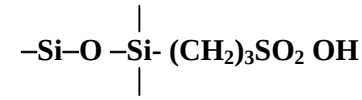
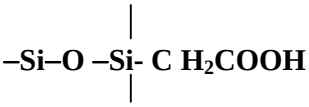
In reversed phase systems chemically bonded octadecyl silane (ODS) is alkaline with 18 carbon atoms, it is the most popular stationary phase used in pharmaceutical industry. Since most of the pharmaceutical compounds are polar and water soluble, the majority HPLC methods for quality assurance decomposition studies, quantitative analysis of both bulk drugs and their formulations use ODS columns. The solvent strength in reversed phase chromatography is reversed from that of adsorption chromatography (silica gel) as stated earlier water interacts strongly with silanol groups, so that, adsorption of sample molecules become highly restricted and they are rapidly eluted as a result. Exactly opposite applies in reversed phase systems. Water cannot wet the non polar (hydrophobic) alkyl groups such as C-18 of ODS phase and therefore does not interact with the bonded moiety; hence water is the weakest solvent of all and gives slowest elution rate. The elution time (retention time) in reversed phase chromatography increases with increasing amount of water in the mobile phase.

Table no. 2 Various bonded phases used in HPLC [12c]

Phase	Structure	Description
Si		Silica Classic normal phase material. Suitable for separating polar non-ionic organic compounds.
C_1		Reversed phase material. Unique selectively for polar and multifunctional compounds. Least retentive of all alkyl group bonded phase for non polar solvents.
C_2		Reversed phase material, less retentive than C_4, C_8, or C_{18} more retentive than C_1.
C_3		Reversed phase material, used in hydrophobic interaction chromatography (HIC) of proteins and peptides.
C_4		Reversed phase material, useful for ion-pairing chromatography offers less retention than C_8 and C_{18} phases for non polar solutes. When bonded to 300 A^o silica, it is an ideal phase for analyzing large proteins and hydrophobic peptides.

C_6		Reversed phase material, useful for ion-pairing chromatography. Less retentive than C_8 and C_{18}.
C_8		Reversed phase material, similar selectively to C_{18} but less retentive. Wide applicability (e.g. pharmaceuticals, nucleotides, steroids). When bonded to 300 A^o silica, it is an ideal phase for peptides, peptide mapping and small hydrophilic proteins.
C_{18}		Classic reversed phase material is most retentive for non-polar solutes and is excellent for ion-pairing chromatography. It is having wide applicability for the assay of nucleosides, nucleotides, steroids, pharmaceuticals, vitamins, fatty acids, and environmental compounds when bonded to 300 A^o silica this phase perfect for separating small hydrophilic compounds.
C_6H_5		It is a reversed phase material and exhibits unique selectivity. It is useful for analyzing aromatic compounds. When bonded to 300 A^o silica this phase is used for HIC.

CN		It can be employed as either a reversed phase or normal phase material. It is slightly polar and exhibits unique selectivity for polar copounds in both RP and NP modes. It equillibrates very rapidly and suitable for gradient separations. It has many pharmaceutical applications (e.g., tricyclic anti depressants).
NH_2		Amino, Amino propyl silyl can be employed as reversed phase, normal phase or weak anion exchange material. Reversed phase: useful for separating carbohydrates.
NO_2		Normal pahse material. Separates aromatic compounds and compounds with double bonds.
OH		Diol, Glycerol can be employed either a reversed phase or normal phase material. Normal phase: Similar selectivity to silica not deactivated by small amounts of water.

		Quaternary amines
SAX		Strong base. Ion exchange material, strong anion exchangers (basic) are useful for separating nucleotides, nucleocides and organic acids.
SCX		Sulphonic acid, Strong acid ion exchange material. Strong cation exchangers (acidic) are useful for separating organic bases.
WCX		Carboxy methyl weak acid ion exchange material. Weak cation exchangers (acidic) are most useful for analyzing basic proteins and peptides.

a) INSTRUMENTATION

The HPLC system consist of

I Solvent degassing system.

II Sample introduction systems.

III A column and column packing material.

IV Detector and recording unit.

The components of HPLC system and their arrangements are shown in fig.no.6.

I. Solvent delivery system

The mobile phase is pumped under pressure from one or several reservoirs and flows through the column at a constant rate, the direction of the solvent as shown in the fig.no.7 with microparticulate packing; there is a high pressure drop across a chromatographic column. Eluting power of the mobile phase is determined by its overall polarity, the polarity of the stationary phase and the nature of the sample components. For normal phase

separations, eluting power decreases with increasing solvent polarity. Optimum separating conditions can be achieved by making use of mixture of two solvents. Some other properties of the solvents, which need to be considered for successful separations, are boiling point, viscosity, detector compatibility, flammability and toxicity.

The most important component of HPLC in solvent delivery system is the pump, because its performance directly effects the retention time and reproducibility. Among the several solvent delivery systems (direct gas pressure, pneumatic intensifier, reciprocating etc.). Reciprocating pump with twin or triple pistons widely used as this system gives less base line noise good flow rate reproducibility etc. The pump must be capable of generating pressure up to 5000 psi at pulse less flow rates of upto 3 ml/min for analytical operations.

II. Sample introduction systems

Analyte introduction into the column are of two types namely a flowing stream and a stop flow injection. These techniques can be used with a syringe or an injection valve. Automatic injector is micro processor–controlled version of the manual universal injector. Usually, upto 100 samples can be loaded into the auto injector tray [4d]. The system parameters such as flow rates, gradient, run time volume to be injected etc. are chosen, stored in memory and sequentially executed on consecutive injections.

III. Column and column packing materials

The heart of the system is the column. In order to achieve high efficiency of separation the column material (micro-particles 5-10 µm size) packed in such way that highest numbers of theoretical plates are possible. Silica ($SiO_2 \times H_2O$) is the most widely used substance for the manufacturing of packing materials. It consists of a network of siloxane in cases (Si-O-Si) in a rigid three dimensional structure containing interconnecting pores [4e].

Fig. no. 6 Arrangement of components in HPLC instrumentation

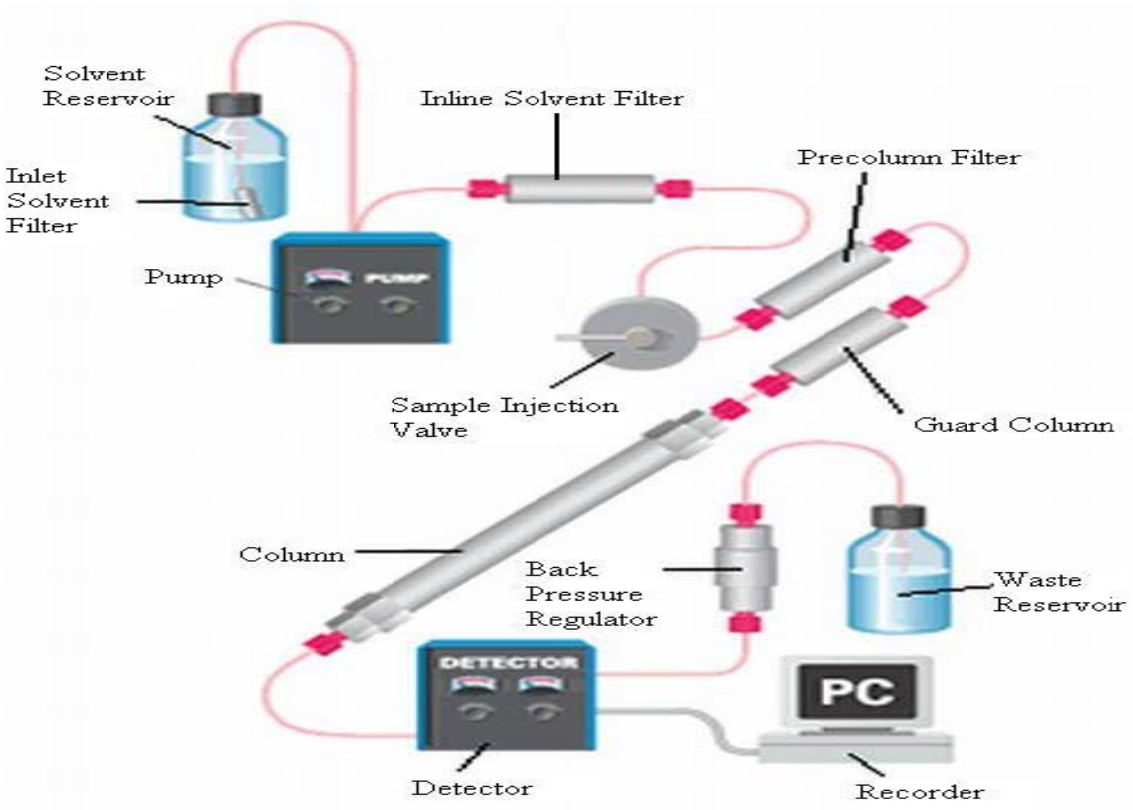
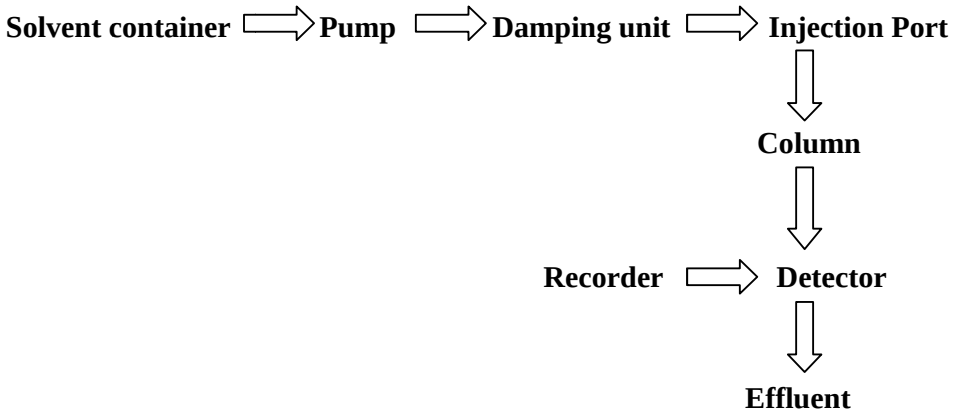


Fig no. 7 Flow chart for mobility of the solvents in HPLC systems



The silanol groups are surface of silica gives it a polar character, which is exploited adsorption chromatography using non–polar organic eluent. Silica can be drastically altered by reaction with organochlorosilanes or organoalkoxysilanes giving Si-O-Si-R linkages with the surface. The attachment of hydrocarbon chain to silica produces a non polar surface suitable for reversed phase chromatography where mixtures of water and organic solvents are used as eluents. The most popular material is octadecyl – silica (ODS-silica), which contains C_{18} chains, but materials with, C_2, C_4 and C_6 chains are also available as shown in table no.2.

During manufacture, such materials may be reacted with small mono functional silanes (e.g. trimethylchlorosilane) to reduce further the number of silanol groups remaining on the surface (end-capping). There is a vast range of materials which have intermediate surface polarities arising from the bonding to silica of other organic compounds which contains phenyl, nitro, amino, and hydroxyl groups. Strong ion exchangers are also available in which sulfuric acid or quaternary ammonium groups are bonded to silica. The useful pH range for columns is between 2 and 8, since siloxane linkages are cleaved below pH 2 while at pH values above 8, silica may dissolve [4e].

In normal phase the separation is carried out using a non polar mobile phase over polar stationary phase; but in the reverse phase separation is achieved using a polar mobile phase. The pH of the mobile phase is adjusted to suppress the ionization of the drug and thereby increase the retention on the column. For highly ionized drugs ion pair chromatography is used.

IV) Detectors:

The main function of the detector in HPLC is to monitor the mobile phase coming out of the column, which in turn emits electrical signals that are directly proportional to the characteristics either of the solute or the mobile phase [12a]. The various detectors often used in HPLC may be categorized into three major heads, namely:

i) Bulk-property detectors:

They specifically measure the difference in some physical property of the solute present in the mobile phase in comparison to the individual mobile phase, for instance:

(a) Refractive-index detectors

(b) Conductivity detectors.

(ii) Solute-property detectors:

This type of detectors critically responds to a particular physical or chemical characteristic of the solute (in question), which should be ideally and absolutely independent of the mobile phase being used. But complete independence of the mobile phase is hardly to be seen, however, signal discrimination is good enough to enable distinctly measurable experimental procedures with solvent changes, such as: gradient elution.

Ex. UV-detectors and fluorescence detectors.

(iii) Multipurpose detectors:

Besides, providing a high degree of sensitivity together with a broad linear-response-attainable range, invariably a particular situation critically demands detectors of more selective nature in the domain of 'analytical chemistry.' 'Pharmaceutical Analysis' could be accomplished by using 'multipurpose detectors', such as: Perkin-Elmer '3D' System that combines UV absorption, fluorescence and conductometric detection.

(iv) Electrochemical detectors:

Electrochemical detector in HPLC usually refers to either amperometric or coulometric detectors that specifically measures the current associated with the reduction or oxidation of solutes. As only a narrow spectrum of compounds undergoes electrochemical oxidation, such detectors are quite selective; which may be further enhanced by monitoring the potential applied to the detector so as to differentiate between various electroactive species. Naturally, electrochemical detection essentially makes use of conducting mobile phases, for instance: inorganic salts or mixtures of water with water-miscible organic solvents.

b) Derivatization:

The main purpose of derivatization in HPLC is to improve detection specifically when determining traces of solutes in complex matrices, for example :

i. Pharmaceutical substances lacking an UV-chromophore in the 254 nm region but possessing a reactive functional group [12b].
ii. Biological fluids e.g. blood, serum, urine; cerebrospinal fluid (CSF).
iii. Environmental samples.

Derivatization may be accomplished by two means, namely :

a. Pre-column off-line derivatization.
b. Post-column on-line derivatization.

These two methods shall be discussed briefly at this juncture:

1. Pre-Column Off-Line Derivatization

Merits:

a. Requires no modification to the instrument i.e. a plus point when compared to the post-column methods.
b. Imposes fewer limitations with regard to reaction time and conditions.

Demerits:

a. Formation of a stable and well defined product is an absolute necessity.
b. Presence of excess reagent or by products may invariably interfere with separation.
c. Very often derivatization may altogether change the chromatographic properties of the sample which facilitated separation [12b].

2. Post-Column On-Line Derivatization

The following experimental parameters should be maintained namely:

a. Derivatization performed in a special reactor strategically positioned between the column and the detector.
b. Reaction must be completed rapidly at moderate temperatures.
c. Derivatization reaction need not even go to completion provided it can be made reproducible.
d. No detector response should exist due to any excess reagent present.
e. Reaction must be carried out in a medium other than the mobile-phase.

Merit:

a. Post-column-on-line derivatization is ideal for the separation.
b. Detection processes can be optimized individually [12b].

D. Various Methods of quantitative analysis in HPLC

The sample or solute is analysed quantitatively in HPLC by either peak height or peak area measurements. Peak areas are proportional to the amount of constant rate. Peak heights are proportional to the amount of material only when peak width are constant and are strongly affected by the sample injection techniques [13]. Once the peak height or the peak areas are measured, there are five principle evaluation methods for quantifying the solute.

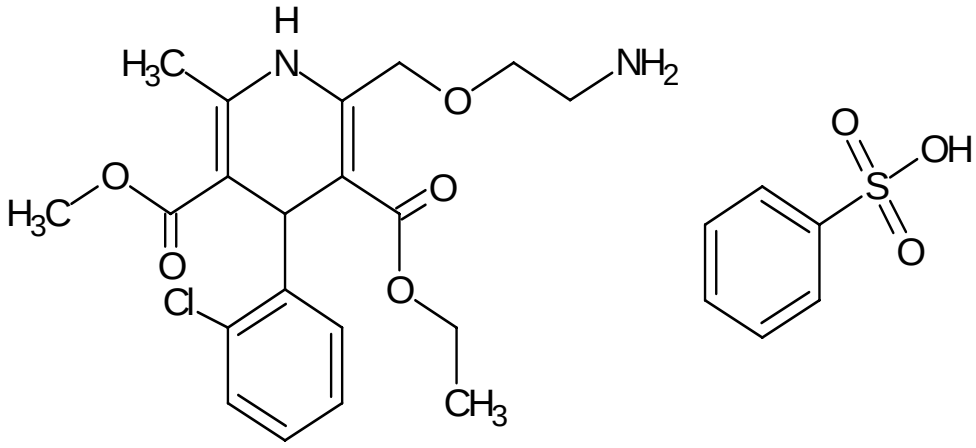
a. Calibration by Standards
b. Internal Standard Method
c. Area Normalization
d. Standard Addition Method
e. External Standard Method

2. DRUG PROFILE

2.1. AMLODIPINE:

Amlodipine is long acting calcium channel blocker. It is dihydropyridine derivative used in treatment of hypertension and coronary artery disease [21a]. Because the parent compound had very poor oral bioavailability, amlodipine is available as the amlodipine besylate pro drug, which has improved oral absorption. The molecular structure of the amlodipine was shown in the fig no. 8.

Fig no. 8 Chemical structure of amlodipine besylate



CAS number	**:**	88150-42-9
Chemical Name	**:**	2-[(2-Aminoethoxy)-4-(2-chlorophenyl)-1, 4-dihydro-6-methyl-3, 5- pyridine di-carboxylic acid-3-ethyl 5-methyl esters [24a].
Empirical Formula	**:**	$C_{20}H_{25}ClN_2O_5$ $C_6H_6O_3S$
Molecular weight	**:**	567.1 gm/mol
Description	**:**	Amlodipine is a white crystalline powder. The partition coefficient for amlodipine is 1.90 and the pKa is 8.6.
Solubility	**:**	Slightly soluble in water and sparingly soluble in ethanol.

Mechanism of action :

Amlodipine is a dihydropyridine[23] calcium antagonist (calcium ion antagonist or slow-channel blocker) that inhibits the transmembrane influx of calcium ions into vascular smooth muscle and cardiac muscle. Amlodipine binds to both dihydropyridine and non dihydropyridine binding sites. The contractile processes of cardiac muscle and vascular smooth muscle are dependent upon the movement of extracellular calcium ions into these cells through specific ion channels [22b]. Amlodipine inhibits calcium ion influx across cell membranes selectively, with a greater effect on vascular smooth muscle cells than on cardiac muscle cells. Negative inotropic effects can be detected *in-vitro* but such effects have not been seen in intact animals at therapeutic doses. Serum calcium concentration is not affected by amlodipine. Within the physiologic pH range, amlodipine is an ionized compound (pKa=8.6), and its kinetic interaction with the calcium channel receptor is characterized by a gradual rate of association and dissociation with the receptor binding site, resulting in a gradual onset of effect. Amlodipine is a peripheral arterial vasodilator that acts directly on vascular smooth muscle to cause a reduction in peripheral vascular resistance and reduction in blood pressure. Amlodipine does also act as FIASMA (functional inhibitor of acid sphingomyelinase) [22a].

Pharmacokinetics [22c]:

Absorption:

Amlodipine is slowly and almost completely absorbed from the gastrointestinal tract. Peak plasma concentrations are reached 6-12 hour following oral administration. Its estimated bioavailability is 64-90 %. Absorption is not affected by food.

Metabolism:

Hepatic metabolized extensively (90 %) to inactive metabolites via the cytochrome P_{450} $3A_4$ isozyme.

Elimination:

Amlodipine is extensively (about 90 %) converted to inactive metabolites via hepatic metabolism with 10% of the parent compound and 60 % of the metabolites excreted in the urine.

Protein binding	**:**	97. 5 %
Bioavailability	**:**	64 to 90 %
Half-life	**:**	30 to 50 hours
Excretion	**:**	Renal

Adverse reaction:

Following events are reported in < 0.1 % of patients: cardiac failure, pulse weakness, twitching, ataxia, hypertonia, migraine, cold and clammy skin, apathy, agitation, amnesia, gastritis, increased appetite, loose stools, coughing, rhinitis, dysuria, polyuria, parosmia, taste perversion, abnormal visual accommodation, and xerophthalmia [22c].

Drug interaction:

No drug interactions have been reported for amlodipine.

Dosage and administration:

Adults:

The usual initial antihypertensive oral dose of amlodipine besylate tablets is 5 mg once daily with a maximum dose of 10 mg once daily. Small, fragile or elderly individuals or patients with hepatic insufficiency may be started on 2.5 mg once daily and this dose may be used when adding amlodipine besylate tablets to other antihypertensive therapy.

Children:

The effective antihypertensive oral dose in paediatric patients ages 6-17 years in 2.5 mg to 5 mg once daily.

Brand Names:

Amlovasc 10 mg in the United Kingdom by Dr. Reddy's Laboratories.

Norvasc 5 mg in North America, China, Japan and Pakistan by Pfizer.

ATECARD-AM 5 mg in India by Alembic Ltd.

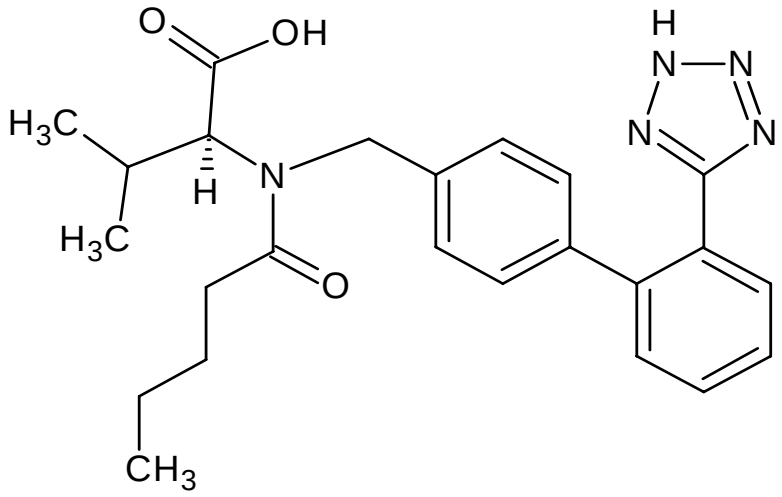
Amcard 5 mg in Bangladesh by Apex Pharma Ltd.

Amlosun 5 mg in Bangladesh by Sun Pharmaceutical (Bangladesh) Ltd.

2.2. VALSARTAN:

Valsartan (trade name Diovans) is an angiotensin-II receptor antagonist, acting on the AT-1 subtype. In the U.S, valsartan is indicated for treatment of high blood pressure, of congestive heart failure, and post-myocardial infarction [21b]. In 2005, Diovan was prescribed more than 12 million times in the United States. It is tetrazole derivative, used in treatment of hypertension. The molecular structure of the valsartan was shown in the fig no. 9.

Fig no. 9 Chemical structure of valsartan



CAS number	**:**	137862-53-4
Chemical Name	**:**	N-(1-Oxophenyl)-N-[[2'-(1H-tet-razol-5yl)[1,1'-biphenyl]-4-yl]methyl]-l-valine [24b].
Empirical formula	**:**	$C_{24}H_{29}O_3N_5$
Molecular Weight	**:**	432.51 gm/mol
Description	**:**	It is a white to practically white fine powder. The partition coefficient for valsartan is 5.8 and the pKa is 8.15.
Solubility	**:**	It is soluble in ethanol and methanol and slightly soluble in water.

Mechanism of action :

Valsartan competes with angiotensin-II for binding at the AT_1 receptor subtype [22d]. As angiotensin-II is a vasoconstrictor which also stimulates the synthesis of aldosterone, blockage of its effects results in a decrease in systemic vascular resistance.

Pharmacokinetics [22e] :

Bioavailability : 25%

Protein binding : 95%

Half-life : 6hours

Absorption : Absolute bioavailability 23% with high variability.

Distribution : Volume of distribution is 17L

Metabolism and Excretion:

Metabolism and excretion of valsartan is largely as unchanged drug (80%) and is minimally metabolized in humans [21c]. The primary circulating metabolite 4-OH – valsartan is pharmacologically inactive and produced CYP_2C_9. 4-OH – valsartan accounts for approximately 9 % of the circulating dose of valsartan. Although valsartan is metabolized by CYP_2C_9, CYP-mediated drug-drug interactions between valsartan and other drugs are unlikely.

Excretion : Renal 30 % and biliary 70 %

Therapeutic use: In patients with impaired glucose tolerance, valsartan may decrease the incidence of developing type 2 diabetes mellitus [22].

Trade names:

Diovan 160 mg in US, UK and Australia by Novartis.

Angiotan 160 mg in Pakistan Efroze.

Valtan 320 mg in India by Cipla.

Valzaar 160 mg in India by Torrent Pharmaceuticals.

Combination of both amlodipine and valsartan:

Amlodipine is a effective of hypertension in once daily doses of 2.5 mg - 10 mg while valsartan is effective in doses of 80 mg – 3320 mg. In clinical trials with amlodiopine and valsartan tablets using amlodipine doses 5 mg -10 mg and valsartan doses of 160 mg – 320 mg, the anti hypertensive effects increased with increasing doses.

Trade names: **EXFORGE** 10-320 mg in India by Pfizer.

Calcitriol 10-320 mg in Switzerland by Cerbosis-pharma.

3. LITERATURE REVIEW

Sedef Atmaca et al.,[32] developed a assay, which involves derivatization of amlodipine with 4-chloro-7-nitrobenzofurazan (NBD-Cl), solid-phase extraction on a silica column and isocratic reversed-phase chromatography with fluorescence detection. The assay was linear over the concentration range of 0.25 – 18.00 ng/ml. Both interday and intraday reproducibility and accuracy were less than 11.80 % and 12.00 % respectively.

Y. Fenag et al.,[33] developed a method after extraction by ethyl acetate using nicardipine as the internal standard, solutes are separated on a C-18 column with a mobile phase of methanol : 1% aceticacid (65:35). Detection is performed on an air pressure ionization single quadruple mass spectrometer equipped with an ESI interface and operated in positive-ionization mode.

Kanakapura Basavaiah et al.,[34] reported a new HPLC determination was carried out on a reversed phase C-18 column using 0.1% ortho-phosphoric acid (pH 3) : acetonitrile (20 : 80) at a flow rate of 1.0 ml/min with UV-detection at 238 nm. In the HPLC method, a rectilinear relationship was observed between 7.55 – 241.6 µg/ml AMBL with a detection limit of 1.51 µg/ml and quantification limit of 3.02 µg /ml.

K. R. Naidu et al.,[35] described a stability indicating HPLC method and subsequently validated for simultaneous estimation of amlodipine (AMD) present as AMBL and BH from their combination product. The proposed RP-HPLC method utilizes a Zorbax SB C-18 column, mobile phase consisting of phosphate buffer and acetonitrile in the proportion of 65 : 35 (v/v) with apparent pH adjusted to 7.0, and UV detection at 240 nm using a photodiode array detector.

Fang Shungan et al.,[36] reported a HPLC method for the contents of amlodipine besylate by detection on UV light at 236 nm. The precisions (RSD) and the recovery (RSD) were detected. They have reported a good linear correlation within the range of 1.6 - 9.6 µg/ml, r = 0.9995. This was precise with good recovery and suitable for quality control of amlodipine besylate tablet.

K. Raja Rajeswari et al., [37] reported a simple, precise, accurate and rapid HPLC method for estimation of atorvastatin and amlodipine simultaneously, in combined tablet dossage form. The mobile phase used was a mixture of acetonitrile and 0.03 M phosphate buffer pH 2.9 (55 : 45% v/v). The detection of atorvastatin and amlodipine was carried out on dual absorbance detector at 240 nm and 362 nm respectively.

M. D. Malesuik et al., [38] reported a LC analyses on a RP-18 column using a mobile phase composed of 0.1 (v/v) orthophosphoric acid (pH 3.0) : acetonitrile (60 : 40, v/v) at a flow rate of 1.0 ml/min. The detection wavelength was at 238 nm.

Afshin zarghi et al., [39] developed a chromatographic method for determination of valsartan in human plasma using a monolithic column. The assay is based on protein precipitation using acetonitrile and fluorescence detection. Reversed phase conditions using a Chromolith Performance (RP-18) column with an isocratic mobile phase consisting of 0.01 M disodium hydrogen phosphate buffer : acetonitrile (60 : 40 v/v) adjusted to pH 3.5 with diluted phosphoric acid.

D. F. Tian et al., [40] reported a simple, reproducible and efficient reverse phase high performance liquid chromatographic method for simultaneous determination of valsartan and hydrochlorothiazide in tablets. Isocratic mode of separation with mobile phase containing methanol : acetonitrile : water : isopropyl alcohol (22 : 18 : 68 : 2; adjusted to pH 8.0 using triethylamine; v/v) was used. The flow rate was 1.0 ml/min and effluent was monitored at 270 nm. The retention time for valsartan and hydrochlorothiazide were 3.42 min and 8.43 min respectively.

D. N. Vora et al., [41] developed a fast, robust and stability indicating RP-HPLC method for simultaneous determination of bisoprolol fumarate and amlodipine besylate in tablets. The mobile phase was mixture of 25 mM ammonium acetate adjusted to pH 5.0 and methanol (65 : 35) at 0.8 ml/min. The stationary phase was Luna C-18 column. UV detection was performed at 230 nm. Retention time was 1.45 and 3.91 min for bisoprolol and amlodipine respectively.

Hanaa M. Abdel Wadood et al.,[42] reported two simple and sensitive spectrofluorometric methods for determination of AMBL in tablets. The first method is based on the condensation reaction of AMBL with ninhydrin and phenylacetaldehyde in buffered medium (pH 7.0), resulting in formation of a green fluorescent product. This exhibits excitation and emission maxima at 375 and 480 nm respectively. The second method was based on the reaction of AMBL with 7-chloro-4-nitro-2, 1, 3-benzoxadiazole (NBD-Cl) in a buffered medium (pH 8.6) resulting in formation of a highly fluorescent product, which was measured fluorometrically at 535 nm (λ_{ex}, 480 nm).

R. Sunil Dhaneshwar et al.,[43] developed a TLC method for simultaneous quantitation of AMBL and VAL as the bulk drug and in tablet dossage forms. Chromatographic separation of the drugs was performed on aluminum plates pre-coated with silica gel 60 F_{254} as the stationary phase and the solvent system consisted of toluene : methanol : acetic acid 7 : 3 : 0.1 *(v/v/v).* Densitometric evaluation of the separated zones was performed at 244 nm.

S. S. Chitlange et al.,[44] described a precise, accurate and reproducible reverse phase high performance liquid chromatographic (RP-HPLC) method for simultaneous estimation of AMBL and VAL on RP C-18 column using acetonitrile : phosphate buffer (0.02 M, pH 3.0), (56 : 44 v/v) as mobile phase at a flow rate of 1.0 ml/min and the detection wavelength was 234 nm. The retention time for AMBL and VAL was found to be 3.07 min and 6.20 min respectively. This method was also used for the determination of AMBL and VAL in the presence of their degradation products formed under variety of stress conditions.

WANG Zheng-yu et al.,[45] developed a simple and specific chromatographic method by using column of phenomenex ODS C-18 was used. The detection wavelength was at 239 nm. The relationship between response and the concentration on the range of 9.952 ～99.520 mg/l was linear with the correlation coefficient of 0.9999. The average recovery were 99.09 %, 100.4 % and 101.2 %, RSD = 0.12 %.

Zong-Zhu Piao et al., [46] reported the valsartan concentration in human plasma samples using high performance liquid chromatography (HPLC) combined with ultraviolet (UV) detection. After a simple protein precipitation using methanol, the analytes were separated on a phenomenex Luna C-18 column using 42 % acetonitrile with 15 mg potassium dihydrogenphosphate in water (pH 2.0, adjusted with phosphoric acid) as the mobile phase at a flow rate of 1.2 ml/min was employed.

A. V. Ramani et al., [47] developed a high-throughput, simple, highly sensitive and specific LC-MS/MS method, for simultaneous estimation of simvastatin acid (SA), AMBL and VAL with 500 µL of human plasma using deuterated simvastatin acid as an internal standard (IS). The chromatographic method was standardized with an X-Terra C-18 column. Mobile phase consisting of 0.02 M ammonium formate (pH 4.5) : acetonitrile (20:80, v/v) at a flow rate of 0.50 ml/min.

G. Chaudhari et al., [48] described a RP-HPLC method for simultaneous estimation of VAL and AMBL from their combination dossage form. Column used was X-Terra RP-18 by gradient elution. Mobile phase consisted mixture of solution A (0.02 % trifluoro acetic acid) and solution B (water : acetonitrile : trifluoro acetic acid, 400 : 600 : 1, v/v/v) with flow rate of 1.5 ml/min and UV detection was carried out at 237 nm and 265 nm for AMBL and VAL respectively. The described method was linear over the range of 1.6-240 µg/ml and 1-30 µg/ml for VAL and AMBL, respectively. The mean recoveries were 100.12 % and 99.72 % for VAL and AMBL respectively.

S. U. Kokil et al., [49] developed a sensitive ion-paired reverse phase liquid chromatographic method for the simultaneous estimation of nebivolol hydrochloride and valsartan in their capsule formulation. The chromatographic method was standardized using a HIQ silica C-18 column with UV detection at 289 nm and flow rate of 1 ml/min. The mobile phase consisting of methanol : water (80 : 20 v/v) with addition of 0.1 % 1-hexanesulfonic acid monohydrate sodium salt as an ion-pairing reagent was selected.

Vamsikrishna Reddy et al., [50] invented a new HPLC method and validated for the determination of valsartan in rabbit plasma. Separation was carried out on a reverse phase C-18 column, using a mixture of acetonitrile and 10 mM phosphate buffer (pH 3.0) in the ratio of 40 : 60 (% v/v) at a flow rate of 1.0 ml/min with UV detection at 250 nm with run

time of 20 min. Telmisartan was used as internal standard (IS). The extraction efficiency of valsartan from rabbit plasma samples was ranged from 96.07 % to 98.1 %.

A. Azza Moustafa et al.,[51] have developed two simple, sensitive, and specific high-performance liquid chromatography and thin-layer chromatography methods for the simultaneous estimation of AMBL and VAL. Separation by HPLC was achieved using a X-Terra C-18 column and methanol : acetonitrile : water : 0.05 % triethylamine in a ratio 40:20:30:10 by volume as mobile phase, pH was adjusted to 3 ± 0.1 with O-phosphoric acid. The flow rate was 1.2 ml/min. The TLC method used silica gel 60 F_{254} plates. The optimized mobile phase was ethyl acetate : methanol : ammonium hydroxide (55:45:5 by volume). Compounds were quantified densitometrically at 237 nm.

CH. M. M. Prasada Rao et al.,[52] developed a chromatographic method for quantization of amlodipine besylate and metoprolol succinate from bulk drug and pharmaceutical formulations using a mobile phase consisting mixture of 0.02 M phosphate buffer solution and acetonitrile as 80 : 20 at the flow rate of 1 ml/min. An Inertsil ODS-CV column was used as stationary phase. The retention times of amlodipine besylate and metoprolol succinate were 3.92 min and 10.43 min.

Gaurav Patel et al.,[53] described a RP-HPLC method for the simultaneous estimation of amlodipine besylate and hydrochlorothiazide in combine dossage form. The mobile phase used was a combination of water : methanol (70 : 30). The detection of the combined dossage form was carried out at 245 nm and a flow rate employed was 0.5 mL/min. The retention time for amlodipine besylate and hydrochlorothiazide was found to be 6.95 min and 2.65 min respectively.

K. R. Gupta et al.,[54] reported a validated method for the simultaneous estimation of valsartan and amlodipine and in their combined dossage form by UV spectrophotometric methods. The method A employs estimation of drugs by simultaneous equation method (SEM) using 250 nm and 238 nm i.e. λ_{max} values for VAL and AMBL respectively. Estimation of drugs by absorption correction method (ACM) at 360 nm i.e. λ_{max} values of one drug and 236 nm an isobestic wavelength. VAL and AMBL individually and in mixture follow Beer's law over the concentration range 5-30 µg/ml at all the selected wavelengths.

D. Kul et al.,[55] developed for two-component mixtures of AMBL and VAL were analyzed by HPLC and the ratio spectra of the first derivative spectrophotometric technique. Spectrophotometric method depends on the first derivative of the ratio-spectra by measurements of the amplitudes at 234 nm for VAL and 351 nm for AMBL. In the HPLC method, C-18 RP column with the mobile phase methanol - acetonitrile - $NaH_2PO_4.H_2O$ buffer, 5 ml/l triethylamine and adjusted to pH 3.0 at 2.0 ml/min flow rate was used to separate both compounds with detection at 254 nm.

Moses Prince Francis et al.,[56] developed a LC-MS method on a C-18 analytical column, the mobile phase used was acetonitrile and 10 mM ammonium acetate in the ratio of 90 : 10 v/v and the retention times were 0.829 and 1.281 min for azithromycin (Internal standard) and amlodipine respectively.

N. K. Ramadan et al.,[57] reported three simple and sensitive methods for the determination of a mixture of AMBL and VAL without prior separation. In one method includes first derivative of ratio spectra technique was adopted by measuring its amplitude at 255.5 nm and 290 nm. In another technique 242 nm was considered as isobestic point and 360 nm for measuring the concentration of AMBL. The third method was precise ratio subtraction technique for the determination of VAL at its λ_{max} 250 nm in presence of AMBL which was determined as mentioned at 360 nm.

R. A. Shaalan et al.,[58] described a simple, sensitive and reliable spectrofluorimetric method for the simultaneous determination of the two antihypertensive drugs, AMBL and VAL in their combined tablets. In this method the native fluorescence was measured at λ_{em} 455 nm and λ_{em} 378 nm by exciting at 360 nm and 245 nm for AMBL and VAL respectively. Regression analysis showed good correlation between fluorescence intensity and concentration over the concentration ranges 0.2-3.6 and 0.008-0.080 µg/ml for AMBL and VAL respectively.

S. K. Patro et al.,[59] developed a new RP-HPLC method for the determination of valsartan in pure and tablet forms. The method reports a linear response for concentrations in the range of 50-175 µg/ml using 0.01 M $NH_4H_2PO_4$ (pH 3.5) buffer : methanol [50 : 50] as the mobile phase with detection at 210 nm and a flow rate of 1 ml/min and retention time 11.0 min.

G. Thanusha et al.,[60] described a reverse phase high performance liquid chromatographic method for the quantitation of valsartan in both pure and pharmaceutical dossage forms. A venusil XBP C-18 column in isocratic mode with mobile phase containing 0.1 M phosphate buffer : acetonitrile (20 : 80) at the flow rate of 1.0 ml/min and the effluents were monitored at 273 nm. The retention time of valsartan was found to be 4.95 min.

D. U. Vinzuda et al.,[61] urbanized a method by using thermo-hypersil ODS column. The mobile phase comprised of water : acetonitrile : glacial acetic acid (5 : 5 : 0.01). The flow rate was set as 1.0 ml/min and effluent was detected at 273 nm. The retention time of valsartan was found to be 4.6 min.

Alnajjar et al.,[62] developed a capillary electrophoresis method and validated for the simultaneous determination of AMBL and VAL in pharmaceuticals and human plasma using a UV photodiode array detector. Optimal conditions were 25 mM phosphate buffer at pH 8.0, injection time 10.0 s, voltage 25 kV, and column temperature 25 ^{o}C, with detection at 214 nm. The method was found to be linear in the range of 1.035 mg/l and 1.0350 mg/l, with weighted regression 0.9999 and 0.9994, for AMBL and VAL respectively. Validation of the method showed acceptable intraday and interday accuracy (85.595.3) and precision (RSD-1.644.2) in pharmaceutical formulation and human plasma analysis.

Arabinda Patnaik et al.,[63] have quantified AMBL by HPLC method using Microbondapak, C-18 column in the study. Mobile phase of methanol : phosphate buffer of pH-3 (65 : 35) at a flow rate of 1 ml/min. Detected at 210 nm.

A. Ibrahim Darwish et al.,[64] described highly sensitive HPLC method with fluorescence detection and on-line emission wavelength switching for the simultaneous determination of VAL and AMBL in human plasma. Irbesartan (IRB) was used as internal standard. Separations were performed in low pressure gradient mode on Hypersil phenyl analytical column using a mobile phase consisting of phosphate buffer (pH 4.0 ± 0.1) : acetonitrile : methanol (60 : 30 : 10, v/v/v) at a flow rate of 0.8 ml/min. The detection of VAL and IRB (IS) was carried out at 253 nm (for excitation) and 374 nm (for emission). After elution of VAL and IRB, the detection wavelengths were switched on-line to 393 nm (excitation) and 446 nm (emission) for detection of AMBL.

V. Bhaskara Raju et al.,[65] have estimated VAL by using a chromatographic method which was standardized using a X-terra C-18 column. The mobile phase consisting of a mixture of phosphate buffer pH 3 and acetonitrile in the ratio of 50 : 50 v/v was selected. UV detection was carried at 210 nm and flow rate of 1 ml/min. The method was validated for its sensitivity, linearity, accuracy and precision.

Bobbarala Varaprasad et al.,[66] developed a simple and accurate RP-HPLC method for the simultaneous estimation of hydrochlorothiazide, amlodipine besylate and valsartan by using C-18 column with a simple gradient elution using mobile phase comprising of 0.01 M potassium dihydrogen phosphate and acetonitrile. Flow rate was 1.0 ml/min and the detection was monitored out by UV detector at 237 nm.

Della Grace Thomas Parambi et al.,[67] developed a method for the quantitation of valsartan in tablet dossage form on a C-18 column using a mobile phase consisting of ammonium dihydrogen phosphate buffer : methanol (33.5 : 66.5) adjusted to pH 3 with formic acid at a flow rate of 1.0 ml/min and detection at 265 nm. The retention time of valsartan was found to be at 11.9 min.

K. Anandakumar et al.,[68] developed a new simple, accurate, and sensitive UV-spectrophotometric absorption correction method for simultaneous determination of AMBL, VAL and HCTZ in bulk and in combined dossage form. Methanol and distilled water were used as solvents. The wavelengths selected for the analysis were 365 nm, 250 nm and 315 nm for AMBL, VAL and HCTZ respectively. Beer's law obeyed the concentration range of 1-32 µg/ml, 4-40 µg/ml and 2-20 µg/ml for AMBL, VAL and HCTZ respectively.

Lakshmanarao et al.,[69] described a RP-HPLC method for simultaneous determination of valsartan and hydrochlorothiazide in tablets. Isocratic mode of separation with mobile phase containing acetonitrile : phosphate buffer pH 3.0 in 1:1 ratio was used. The flow rate was 0.8 ml/min and effluent was monitored at 225 nm. The retention time and linearity range for valsartan and hydrochlorothiazide were (5.59 min, 2.36 min) and (10-50 µg/ml, 10-50 µg/ml) respectively.

Nashwah gadallah Mohamed et al., [70] developed a method for simultaneous determination of AMBL and VAL without previous separation. In this method amlodipine in methanolic solution was determined using zero order UV spectrophotometry by measuring its absorbence at 360.5 nm without any interference from valsartan. Valsartan spectrum in zero order is totally overlapped with that of amlodipine. First, second and third derivative could not resolve the overlapped peaks. The first derivative of the ratio spectra technique was applied for the measurement of valsartan. The ratio spectrum was obtained by dividing the absorption spectrum of the mixture by that of amlodipine, so that the concentration of valsartan could be determined from the first derivative of the ratio spectrum at 290 nm.

Ola Moustafa Abdallah et al., [71] developed three methods for simultaneous determination of amlodipine and valsartan without previous separation. The first method depends on first derivative of the ratios spectra (DD1) by measurements of the amplitudes at 234.5 nm and 247 nm for amlodipine using 30 µg/ml of valsartan as a divisor and at 282 nm and 292 nm for valsartan using 80 µg/ml of amlodipine as a divisor. The second method describes the use of multivariate spectophotometric calibration for the simultaneous determination of the analyzed binary mixture, where the resolution is accomplished by using partial least squares (PLS) regression analysis. In the third method, HPLC separation was performed by using reversed phase column and a mobile phase composed of acetonitrile: KH_2PO_4 (50 : 50 v/v) adjusted to pH 3.5 by phosphoric acid.

P. V. Santosh Kumar et al., [72] reported a chromatographic method for the estimation of valsartan in bulk drugs and tablet formulation. The chromatographic method was standardized using a Kromasil C-18 column. The mobile phase consisting of a mixture of phosphate buffer and acetonitrile in the ratio of 55 : 45 v/v was selected. Detection was carried at 233 nm and flow rate of 1 ml/min.

Susheel John Varghese et al., [73] reported HPLC and HPTLC-densitometric methods for simultaneous determination of AMBL, VAL and HCTZ in combined tablet dossage form. RP-HPLC analysis was performed on a Phenomenex Luna C-18 column, using a mobile phase consisting of 10 mM ammonium acetate buffer (pH 6.7) and methanol in solvent gradient elution for 20 min at a flow rate of 1 ml/min. Quantification was carried out using a photodiode array UV detector at 238 nm. HPTLC analysis was carried out on an aluminum-backed sheet of silica gel $60F_{254}$ layers using chloroform : glacial acetic acid : n-butyl acetate (8 : 4 : 2, v/v/v) as the mobile phase. Quantification was achieved with UV densitometry at 320 nm.

4. AIM AND PLAN OF WORK

Combination therapy in the treatment of hypertension as an appropriate treatment option is receiving boarder acceptance amongst the clinical community. Mono therapy is often not sufficient to normalize blood pressure since the goal of treatment is to normalize both systolic and diastolic blood pressure. A hypertension treatment guideline recently issued by the European Society of Hypertension and European Society of Cardiology or, in the Guide to Management of Hypertension 2008 issued by the Australian Heart Foundation recommends the combination therapy.

It is proposed that these double combination tablets will be indicated as a substitution therapy in patients (i.e. patients are not to be started on this combination therapy) for the treatment of hypertension. As a replacement therapy in patients whose blood pressure is adequately controlled on amlodipine and valsartan used as individual or combination therapies.

Amlodipine is used in treatment of hypertension and coronary artery disease [21a,] , and valsartan used for treatment of high blood pressure, of congestive heart failure, and post-myocardial infarction[21b].

Literature survey reveals the availability of some methods for estimation of amlodipine besylate (AMBL) and valsartan (VAL) includes UV spectrometry, RP-HPLC and HPTLC alone are in combination with other drugs. Only very few HPLC estimations have been reported in the literature for the determinations of amlodipine and valsartan present in bulk, formulations and biological fluids.

The existing methods are inadequate to meet the requirements, hence it is proposed to improve the existing methods and to develop new methods for the simultaneous estimation of amlodipine and valsartan in pharmaceutical dosage forms.

The main objective for that is to improve the conditions and parameters, which could be easily adopted in the validation process.

5. EQUIPMENT AND CHEMICALS

5.1 Drug sample

Amlodipine and valsartan sample obtained from HETERO Drug Laboratories Pvt., Ltd., Hyderabad.

5.2 Instruments used for the study:

1. Waters separation module with Waters 2487 dual wavelength detector equipped with EMPOWER software.
2. Sonica ultra sonic cleaner- model 2200 MH
3. ELICO – pH meter model L1610
4. Shimadzu AUX- 220 digital balance

5.3 Chemical Reagents required:

REAGENT NAME	GRADE	MANUFATURE
Triethylamine	AR Grade	Merck, New Delhi.
Sodium Hydroxide	AR Grade	Central drug house LTD., Mumbai.
Potassium hydrogen phosphate	AR Grade	Spectrum, Mumbai.
Orthophosphoric acid	AR Grade	Merck, New Delhi.
Hydrochloric acid	AR Grade	SD fine Chem. LTD., Hyderabad.
Acetonitrile	HPLC Grade	Merck, New Delhi.
Methanol	HPLC Grade	Merck, New Delhi.
Water	Milli-Q	Milli pore, In house.

All the solutions were degassed and filtered through 0.22 µ PVD filter.

6. METHOD DEVELOPMENT

In case of analytical method development and for drugs analysts should decided whether the given analytical method is suitable for the assay of the drug. The method development of new improved method usually trailers existing approaches and instrumentation to the current analyte, as well as to the final needs or requirements of the method.

In the development stage, decision regarding choice of column, mobile phase, detectors and method of quantitation must be addressed. In this way, development considers all the parameters pertaining to any methods [15].

6.1. Different steps in method development time line:

It includes percentage of time spent on the each stage. In this approach, three critical components of HPLC method (sample preparation, HPLC analysis and standardization) are investigated [16].

Step 1: Method objective should be to determine the goals for method development (Intended use of the method) and to understand the chemistry of the analytes and the drug product.

Step 2: Development of preliminary HPLC conditions to achieve minimally acceptable separations. These HPLC conditions will be used for all subsequent method development experiments.

Step 3: Development of suitable sample preparation scheme for the drug product.

Step 4: Determination of appropriate standardization method and the use of relative response factors in calculations.

Step 5: To identify the weakness of the method and to optimize the method through experimental design. Understand the method performance with different conditions, different instrument set up and different samples.

Step 6: Complete method validation according to ICH guidelines [16].

The objective of this experiment was to optimize the assay method for simultaneous estimation of amlodipine and valsartan based on the literature survey made and the methods given in official pharmacopoeias. Trials done for optimization are as follows:

6.2. Trials

i. Trial 1:

Buffer preparation:

1.0 ml of orthophosphoric acid was mixed with 1000 ml of Milli-Q water, and shake for 15 min. Then filtered through 0.22 µ nylon membrane filter and degassed.

Mobile phase A : Buffer

Mobile phase B : Acetonitrile: methanol (1 : 1).

Chromatographic conditions

Column : Symmetry C-18, (150 × 3.4 mm, 5 µ)

Detector wavelength : 225 and 237 nm

Column temperature : 40 ^{o}C

Injection Volume : 10 µL

Flow rate : 1.2 ml/min

Runtime : 25 min

Gradient Programme: As shown in the table no. 3

Table no. 3 Gradient programme (Trial 1)

Time(min)	%A	%B
0.00	90	10
18.00	20	80
19.00	90	10
25.00	90	10

Observation:

Peak shapes were not satisfactory for both amlodipine and valsartan and the retention time of amlodipine and valsartan were found to be 10.05 and 15.216 min respectively.

ii. Trial 2:

In this trial almost similar conditions were employed except the mobile phase was exclusively acetonitrile and gradient system was slightly modified as shown in the table no. 4

Table no: 4 Gradient programme (Trial 2)

Time(min)	%A	%B
0.00	95	5
18.00	20	80
19.00	95	5
25.00	95	5

Observation:

Peaks shapes were not good fronting of the amlodipine peak was observed. Retention time of amlodipine and valsartan were found to be 8.1 and 12.5 min respectively.

iii. Trial 3:

Buffer Preparation:

2 ml triethylamine (TEA) was mixed with 1000 ml water and adjust the pH 3.0 with ortho phosphoric acid. Then filtered through 0.22 µ nylon membrane filter and degassed.

Mobile Phase Preparation:

Mobile Phase A : Buffer

Mobile Phase B : ACN

Chromatographic Condition:

Column & ID NO : Symmetry C18, (4.6 × 150, 5 µ)

Column temperature : 35 ^{o}C

Inj. Volume : 10 µl

Flow rate : 1.0 ml/min

λ_{max} : 237 nm

Gradient Programme: As shown in the table no. 5

Table no. 5 Gradient programme (trial 3)

Time (min)	%A	% B
0.01.	75	25
12.00	10	90
15.00	75	25
20.00	75	25

Fig. no. 10 Blank chromatogram for trial 3

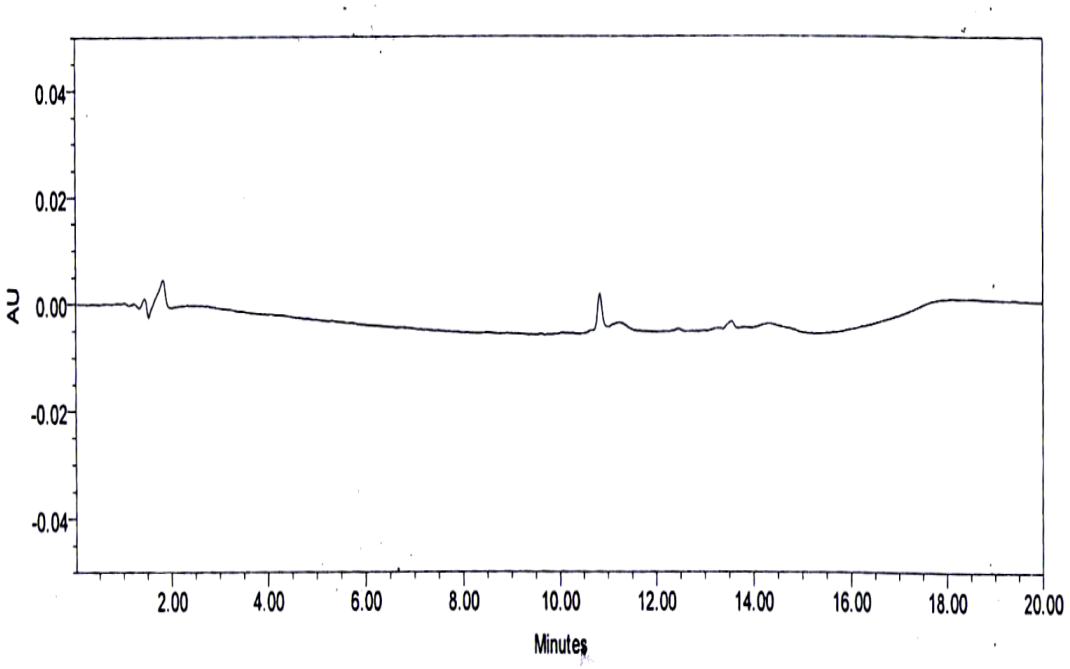
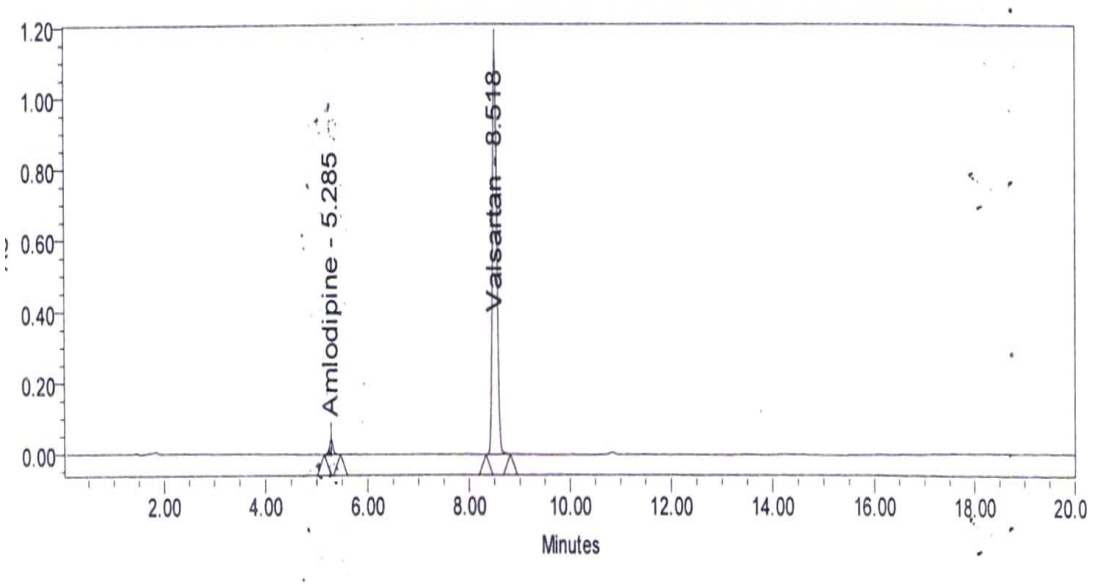


Fig. no. 11 Standard chromatogram for trial 3



Observation:

Peak shapes were not satisfactory. Retention time of amlodipine and valsartan were found to be 5.285 and 8.518 min respectively. Blank and standard chromatograms are shown in fig no. 10 and 11.

iv. Trail No.4:

Buffer Preparation:

Weighed and transferred 1.36 gm KH_2PO_4 in to 1000 ml H_2O and adjust the pH 3.0 with ortho phosphoric acid.

Mobile Phase Preparation:

Mobile Phase A	: Buffer
Mobile Phase B	: ACN
Diluent	: Buffer : Methanol (1: 1)

Chromatographic Condition:

Column Name & ID NO	: Symmetry C18, (4.6 × 150, 5 µ)
Column temperature	: 35 ^{o}C
Flow rate	: 1.0 ml/min
Inj. Volume	: 10 µl
λ_{max}	: 237 nm

Gradient Programme: As shown in the table no. 6

Table no. 6 Gradient programme (Trial 4)

Time (min)	%A	% B
0.01.	75	25
12.00	10	90
15.00	75	25
20.00	75	25

Fig. no. 12 Blank chromatogram for trial 4

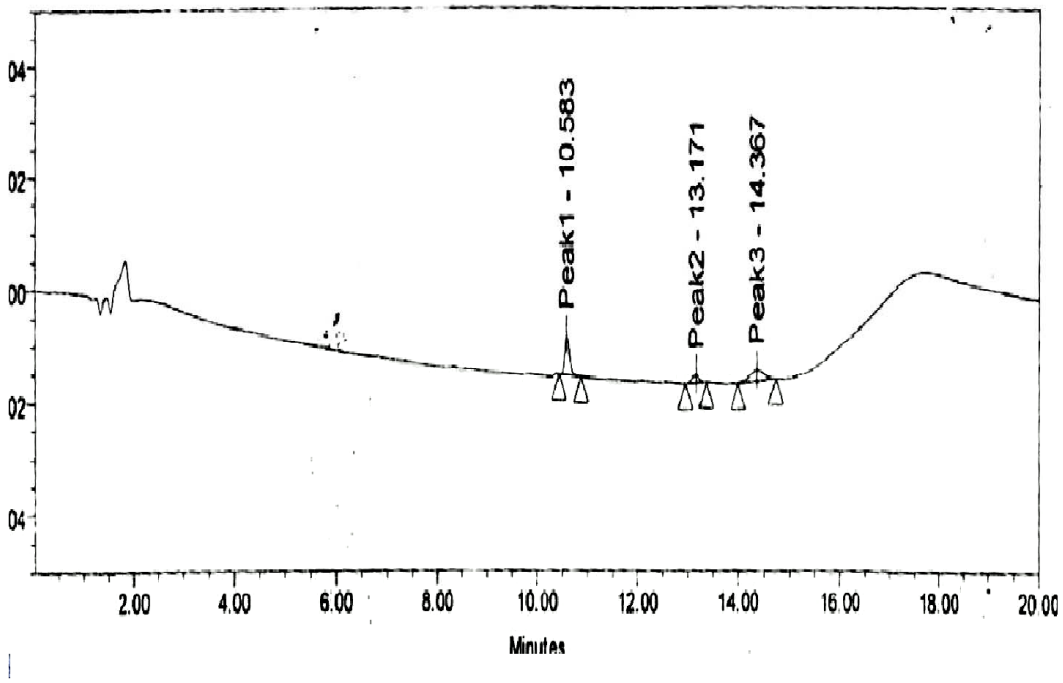
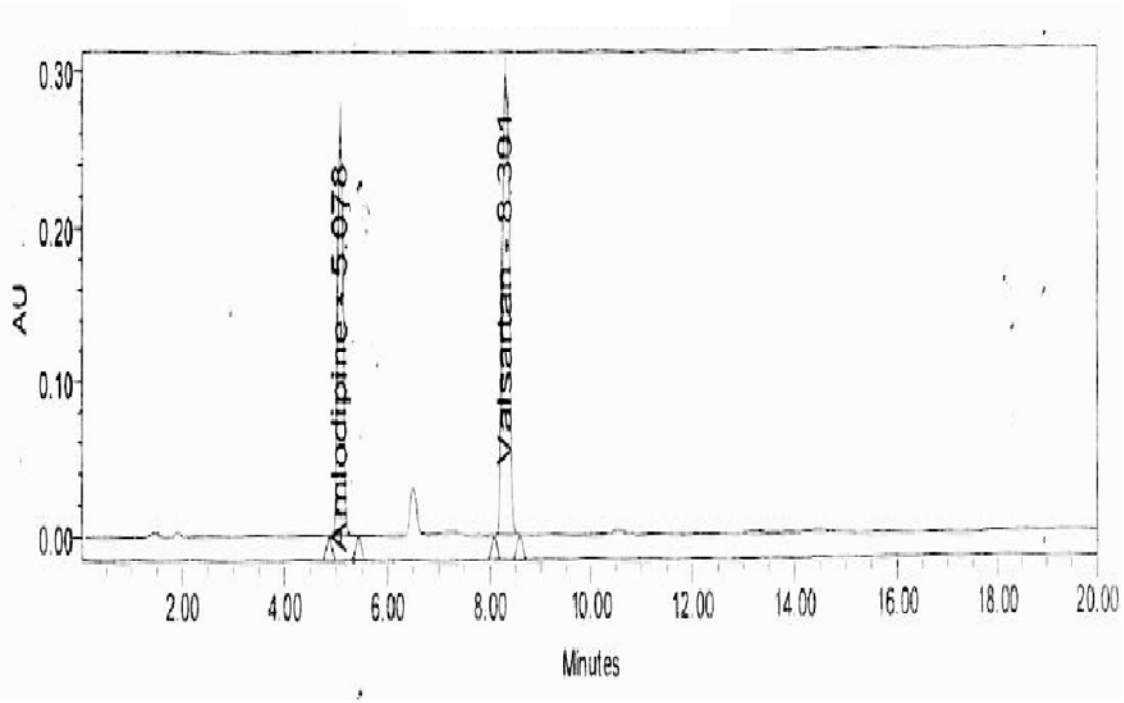


Fig. no. 13 Standard chromatogram for trial 4



Observation:

Peak shape was not good in both standard and sample preparations. Retention time is acceptable. Theoretical plates were too less and asymmetry was more than the permitted limit. Blank and standard chromatograms are shown in fig no. 12 and 13.

6.3. Optimization method:

Preparation of Buffer:

2.0 ml of triethylamine was transferred in to a beaker containing 1000 ml of water and mixed. The solution pH was adjusted to 3.0 ± 0.05 with ortho phosphoric acid, filtered through 0.22 µ membrane filter.

Mobile phase A : Buffer

Mobile Phase B : Acetonitrile

Preparation of Diluent:

Prepare a degassed mixture of buffer and methanol in the ratio of 50 : 50 v/v

Chromatographic conditions:

Column	: Symmetry C18, (150 x 4.6 mm, 3.5 µ)
Detector wavelength	: 237 nm
Injection volume	: 10 µl
Flow rate	: 1.0 ml/min
Column oven temperature	: 35 ^{o}C
Run time	: 20 min

Gradient Programme: As shown in the table no. 7

Table no.7 Gradient programme (optimized method)

Time	A%	B%
0	75	25
12	10	90
15	75	25
20	75	25

Preparation of standard stock solution:

Amlodipine besylate 70 mg and 64 mg of valsartan are weighed and transferred into 100 ml volumetric flask, sonicating for 10 min and diluted to volume with diluents and mixed.

For 10 mg / 320 mg and 5 mg / 320 mg

From the above standard solution 5 ml was pipetted and transferred into a 25 ml volumetric flask, diluted to volume with diluents and mixed.

Preparation of sample solution:

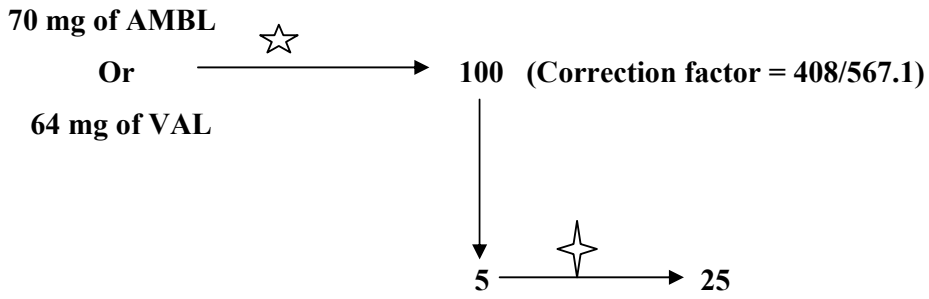
Assay tablets

The combination of AMBL and VAL are available in the ratio of 1:16, 1:32. The tablet taken for the assay contains 10 mg of AMBL and 320 mg of VAL.

Since the ratio of the combination exist in large difference. The dilution of the sample was carried out in two different stages A and B to get chromatographed appropriate peak area as shown in the table no.8 and chromatograms were shown in the fig. no. 15, 16 and 17.

Amlodipine and valsartan 10 mg/ 320 mg tablets dilution chart

Standard preparation:

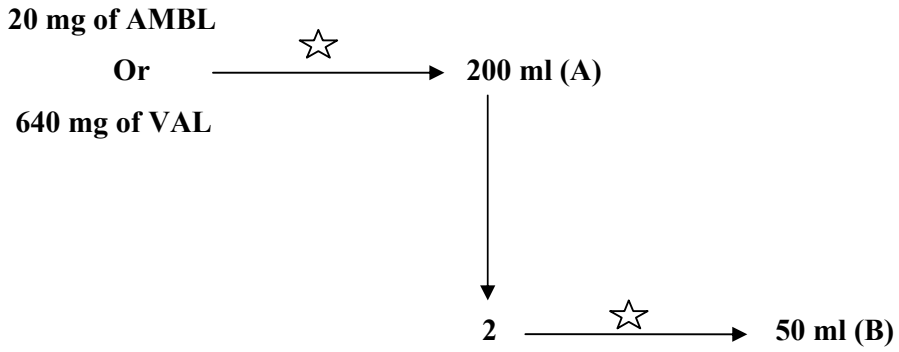


The final concentration of AMBL and VAL were 100 ppm and 128 ppm respectively.

☆ 20 ml methanol

✦ Degassed mixture of buffer: methanol in the ratio of 50:50 v/v.

Sample preparation:



The final concentration of AMBL and VAL were 100 ppm and 128 ppm respectively.

☆ Degassed mixture of buffer: methanol in the ratio of 50:50v/v.

Fig. no. 14 Blank chromatogram for optimized method

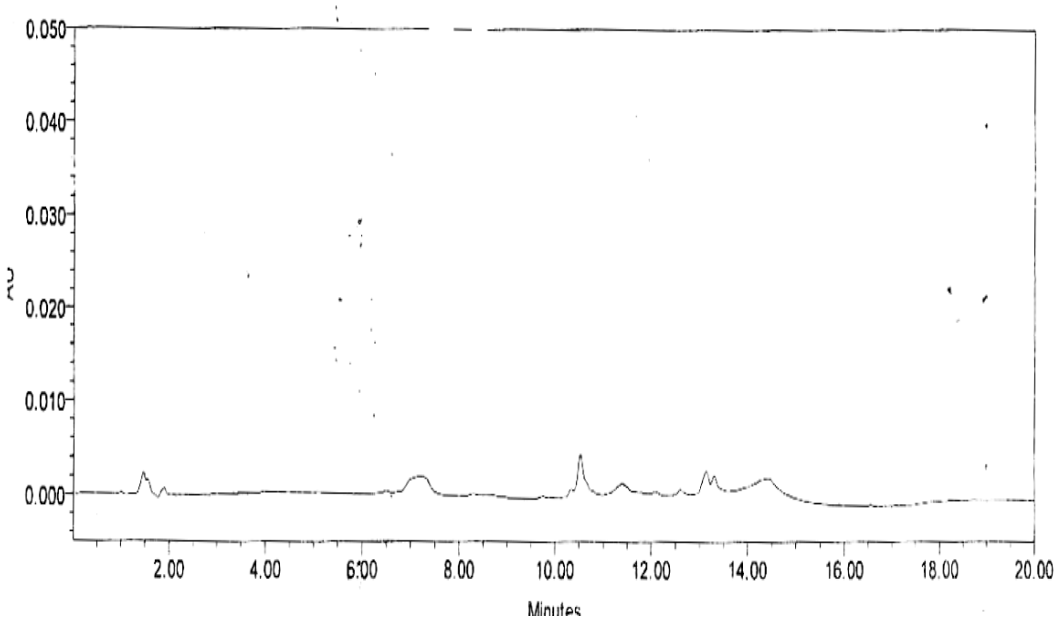


Fig. no. 15 Standard chromatogram for optimized method

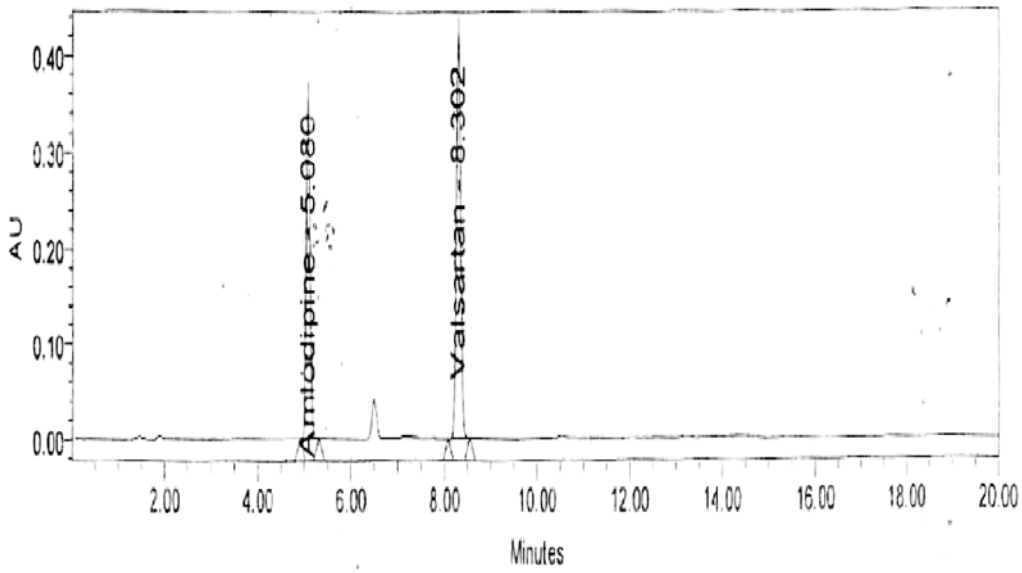


Fig. no. 16 Amlodipine sample chromatogram for optimized method

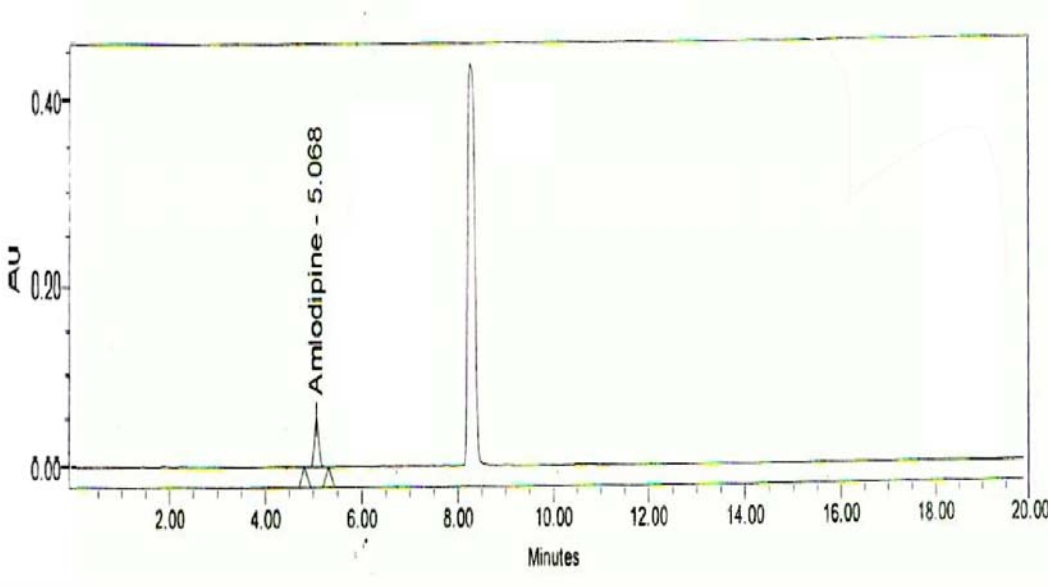
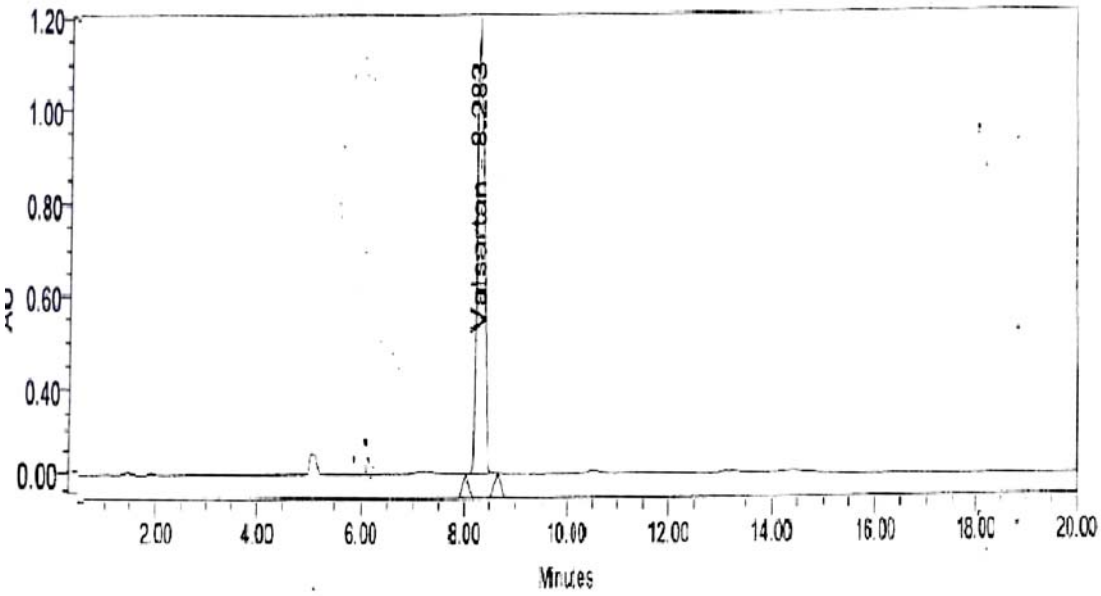


Fig. no. 17 Valsartan sample chromatogram for optimized method



Observation:

Peak shape was satisfactory in both standard and sample preparations. Retention time is acceptable. Blank, standard and sample chromatograms are shown in fig no. 14, 15, 16 and 17. Peak area, % RSD and mean were shown in the table no. 8.

Calculations:

FOR AMLODIPINE (% Labeled amount)

For Tablets assay / Blend assay

For 10 mg / 320 mg and 5 mg / 320 mg

$$= \frac{AT1}{As1} \times \frac{Ws1}{100} \times \frac{10}{25} \times \frac{200}{WT1} \times \frac{T1}{L1} \times \frac{P1}{100} \times \frac{408.0}{567.10} \times 100$$

For 10 mg /160 mg and 5 mg / 160 mg

$$= \frac{AT1}{As1} \times \frac{Ws1}{100} \times \frac{10}{25} \times \frac{200}{WT1} \times \frac{T1}{L1} \times \frac{P1}{100} \times \frac{408.0}{567.10} \times 100$$

AT1 - Area count of amlodipine peak in sample solution.

AS1 - Average area count of amlodipine peak obtained from five replicate injections of standard solution.

Ws1 - Weight of amlodipine besylate working standard solution taken, in mg

WT1 - Weight of sample taken, in mg.

P1 - % purity of amlodipine besylate working standard used.

FOR VALSARTAN (% Labeled amount)

For Tablet assay / Blend assay:

For 10 mg / 320 mg and 10 mg/ 160 mg.

$$= \frac{AT2}{As2} \times \frac{Ws2}{100} \times \frac{5}{25} \times \frac{200}{WT2} \times \frac{50}{2} \times \frac{T2}{L2} \times \frac{P2}{100} \times 100$$

AT2 - Area count of valsartan peak in sample solution.

AS2 - Average area count of valsartan peak obtained from five replicate injections of standard solution.

Ws2 - Weight of valsartan working standard solution taken, in mg

WT2 - Weight of sample taken, in mg.

P2 - % purity of valsartan working standard used.

Calculate the amount of each drug by using the following formula.

$$\text{Amlodipine} = \frac{A_A}{A_{SA}} X \frac{D_S}{D_T} X \frac{P}{100}$$

$$\text{Valsartan} = \frac{A_A}{A_{SA}} X \frac{D_S}{D_T} X \frac{P}{100}$$

Where

A_A - Average area counts of injections for amlodipine peak in the chromatograms of sample solution.

A_{SA} - Average area count of five replicate injections for amlodipine peak in the chromatogram of Standard solution.

A_{SVAL} - Average area counts of injections for valsartan peak in the chromatogram of sample solution.

A_{SVAL} - Average area counter of five replicate injections for valsartan peak in the chromatogram of standard solution.

D_S - Dilution factor of standard solution (weight ÷ dilution)

D_T - Dilution factor of sample solution

P - Percentage purity of working standard used.

$$\% \text{ Labeled Amount} = \frac{\text{Content of each drug (mg/tablet)}}{\text{Label claim (mg)}} \text{ X } 100$$

Table no. 8 Data for assay in tablet formulations

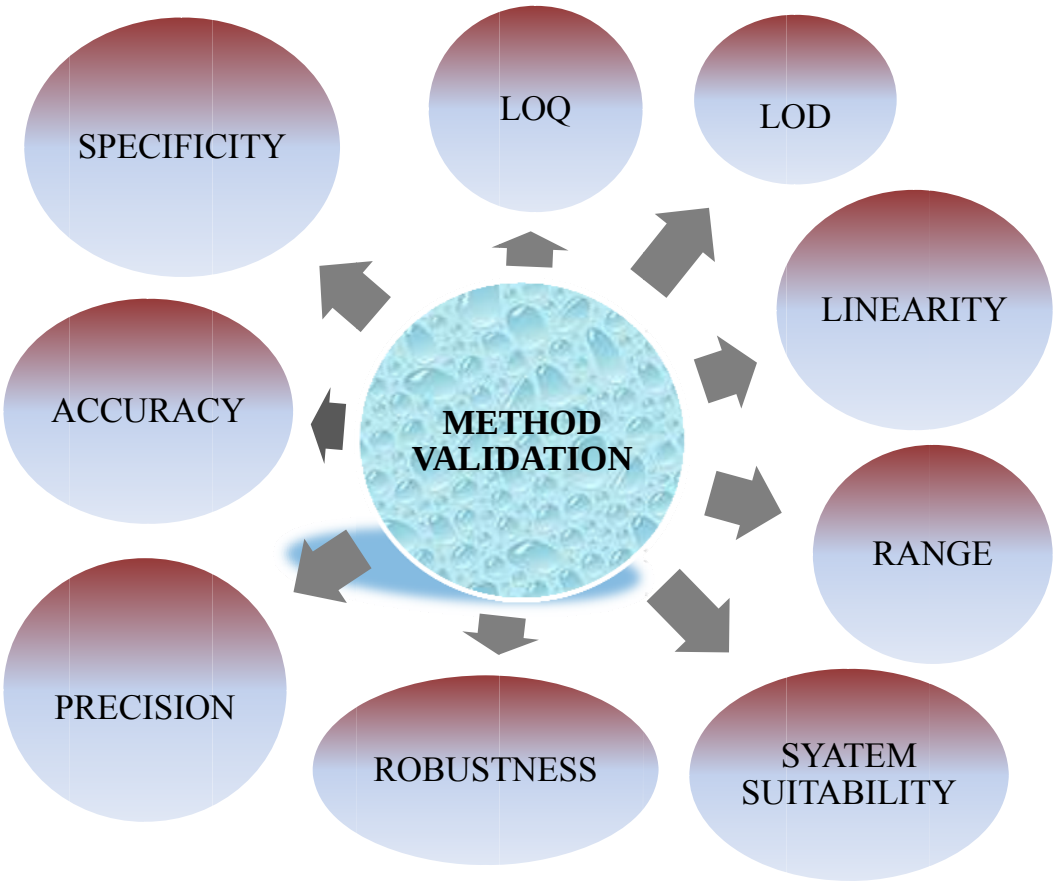
S. no.	Sample name	AMBL		VAL	
		RT	Peak area	RT	Peak area
1	Assay stock spl-1	5.068	2585376	8.288	2660937
2	Assay stock spl-2	5.068	2584325	8.283	2656499
3	Assay stock spl-3	5.063	2578138	8.273	2678468
4	Assay stock spl-4	5.080	2586304	8.269	2681350
Mean		5.070	2583535.89	8.278	2669313.61
%RSD		0.1	0.1	0.1	0.5
SD		0.007		0.009	

7. METHOD VALIDATION

According to ICH guidelines method validation can be defined as "Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics" [18]. Such validated analytical method for qualitative and quantitative testing of the drug molecule assume greater importance when they are employed to generate quality and safety compliance data during development, pre-formulation studies and post approval of drug products.

The ICH of Technical Requirements for the Registration of Pharmaceutical for human use has developed a consensus text on the validation of analytical procedures [19]. The document includes definitions for eight validation characteristics. The method validation parameters are shown in the fig no. 18.

Fig. no. 18 Method Validation Parameters



7.1. System suitability

System suitability is the checking of a system to ensure system performance before or during the analysis of unknowns. Before performing any validation experiment, HPLC method and the procedure should be capable of providing data of acceptable quality. These tests are to verify that the resolution and repeatability of the system are adequate for the analysis to be performed. It is based on the concept that equipment, electronics, analytical operations and sample constitute an integral system that can be evaluated as a whole. System suitability parameters and recommendations are shown in the table no.9

Table no. 9 System suitability parameters and recommendations

S. No	Parameters	Recommendations
1	Theoretical plates (N)	>2000
2	Tailing factor (T)	≤ 2
3	Resolution (Rs)	> 2 between peak of interest and the closest eluting potential interference
4	Repeatability	RSD ≤ 1% for N ≥ 5 is desirable
5	Capacity factor (k^1)	> 2.0
6	Relative retention	Not essential as long as the resolution is stated

Procedure:

A standard solution was prepared by using amlodipine besylate and valsartan working standards as per test method and was injected ten times into the HPLC system.

The system suitability parameters were evaluated from standard chromatograms by calculating the % RSD from ten replicate injections for amlodipine besylate and valsartan retention times and peak areas. Resulted chromatogram was shown in the chromatogram fig. no. 19.

Fig no. 19 Standard chromatogram system suitability

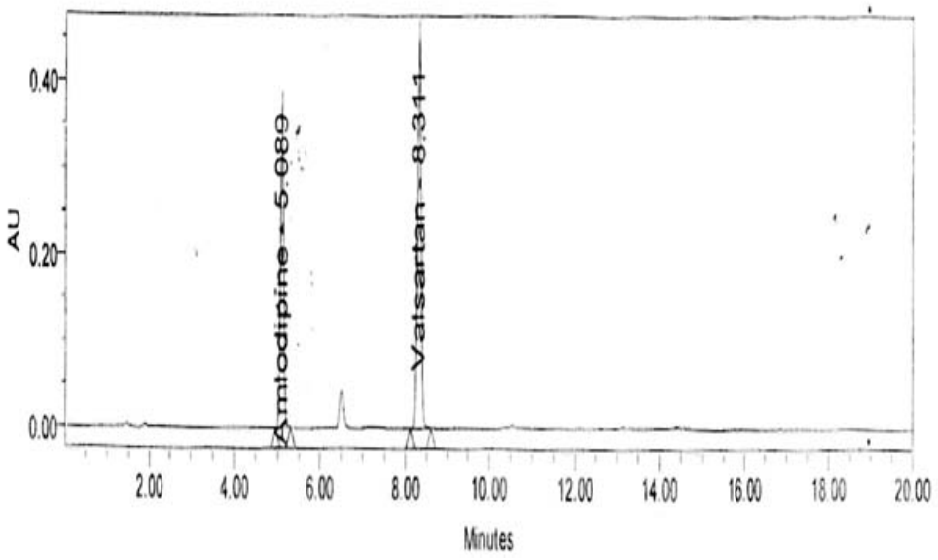


Table no. 10 Data for system suitability parameters for AMBL and VAL

Parameters & Acceptance value	AMBL			VAL		
	Mean	SD	%RSD	Mean	SD	%RSD
RT (%RSD NMT 2)	5.0735	0.006737	0.132791	8.3024	0.009107	0.109688
Peak area (%RSD NMT 2)	2585903	495.6531	0.019168	2767763	887.4316	0.032063
USP plate count (NLT 3000)	7172.233	11.4703	0.159924	11750.61	20.08686	0.170943
Tailing factor (%RSD NMT 2)	1.0891	0.011005	1.01	1.11	0.01959	1.764867

Observation: The system suitability parameters were satisfactory and exhibited in table no.10.

7.2. Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s).

This definition has the following implications:

Identification: To ensure the identity for an analyte.

Purity Tests: To ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte, i.e. related substances test, heavy metals, residual solvents content, etc.

a. Placebo Interference:

A study to establish the interference of placebo was conducted. Samples were prepared in triplicate by taking the placebo equivalent to about the weight in portion of test preparation as per the test method. Chromatogram of placebo did not show any additional peaks. This indicates that the excipients used in the formulation do not interfere in the assay of amlodipine besylate and valsartan tablets. Resulted chromatograms are shown in the fig.no. 20.

Acceptance criteria:

No interference at the retention times of amlodipine besylate and valsartan.

Fig no. 20 Amlodipine and valsartan sample chromatogram for interference study

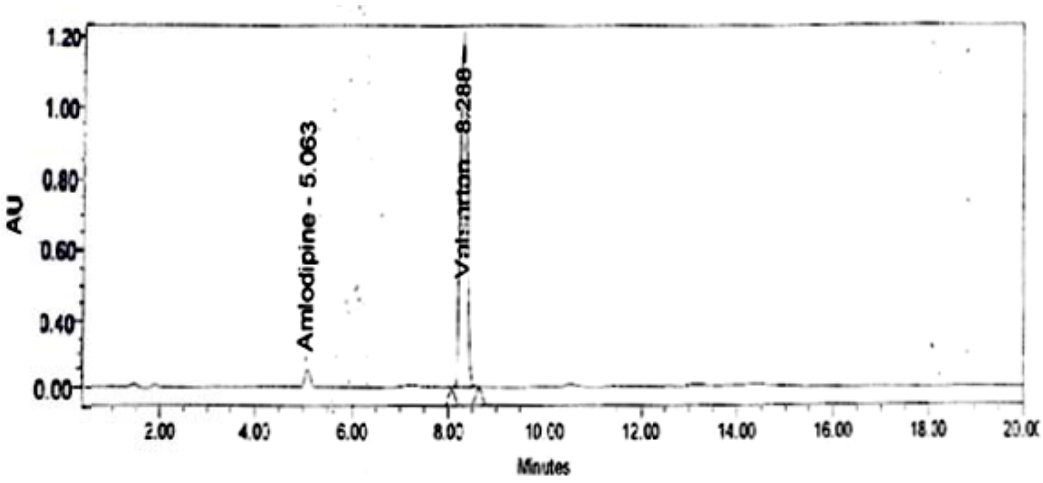


Table no.11 Data for specificity

Injections	Interference	RT
Blank	Nil	No interference at RT of Analyte Peak
Placebo	Nil	No interference at RT of Analyte Peak
Sample	Nil	AMBL - 5.0 min VAL - 8.2 min

Observation:

From the above chromatogram, it was concluded that there was no interference with placebo as no peaks were observed at the retention times of amlodipine besylate and valsartan peaks as shown in the table no. 11.

7.3. Precision:

Precision is the measure of the degree of repeatability of analytical method under normal operation and is normally expressed as % RSD for the statistically significant number of samples [5c].

Method Precision:

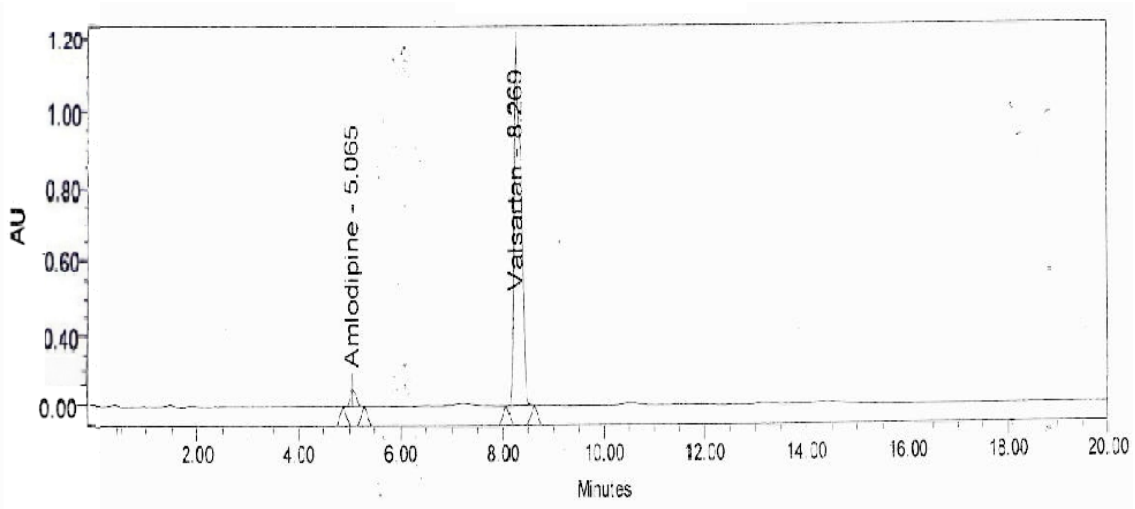
Six sample preparations were prepared individually using single batch of amlodipine besylate and valsartan tablets (1/32 mg) as per test method and injected each solution. Resulted chromatogram was shown in the fig. no. 21.

Acceptance criteria:

The % RSD of individual amlodipine besylate and valsartan tablet from the six units should be not more than 2.0 %.

The assay of amlodipine besylate and valsartan tablets is showing that the test method is precise shown in table no.12 for method precision.

Fig no. 21 Amlodipine and valsartan sample chromatogram



Observation:

% RSD of the amlodipine and valsartan tablet from six units was found to be 0.2, as shown in the table no. 12.

Table no.12 Data for method precision

S.NO	AMBL		VAL	
	RT	Peak area	RT	Peak area
1	5.089	1932075	8.311	2768666
2	5.077	1937803	8.294	2774111
3	5.078	1941938	8.298	2780808
4	5.08	1945530	8.302	2777299
5	5.082	1945255	8.304	2782394
Mean	5.081	1940519.91	8.302	2776655.4
SD	5661.8	5661.84	0.006	5497.59
%RSD	0.3	0.3	0.1	0.2

7.4. Accuracy:

The accuracy of a method is the closeness of the measured value to the true value for the sample.

Accuracy criteria for an assay method is that the mean recovery will be 100 ± 2 % over the range of 95 % - 105 % at the target concentration. For impurity method, the mean recovery will be within 0.1 % absolute of the theoretical concentration or 10 % relative, whichever is greater for impurities in the range of 0.1-2.5 % v/w.

Accuracy of test method was carried out by spiking known amount of drug substance to get concentration of amlodipine besylate and valsartan 50 %, 100 % and 150 % of target concentration in triplicate for each level. Each solution was injected in triplicate. The average % recovery of amlodipine besylate and valsartan was calculated.

Separately injected the blank and placebo of amlodipine besylate and valsartan into the system and chromatographed.

Acceptance criteria:

The mean % recovery of the amlodipine besylate and valsartan at each level should be in the range of 95 %-105 %.

Observation:

The recovery results indicating that the test method has an acceptable level of accuracy shown in table no: 13 and 14

Table no. 13 Accuracy data for amlodipine besylate

Concentration of spiked level	Amount added (ppm)	Amount found (ppm)	% Recovery	Statistical analysis of % recovery	
	50.08	49.63	99.1	Mean	99.78
50% sample	50.56	50.4	99.68	SD	0.534
	50.03	49.53	100.1	%RSD	0.535
	100.01	98.46	98.36	Mean	99.28
100% sample	100.13	99.391	99.26	SD	0.462
	100.23	100.07	100.15	%RSD	0.468
	150.12	150.05	99.95	Mean	99.28
150% sample	150.23	150.15	99.96	SD	0.447
	150.3	149.94	99.76	%RSD	0.4471

Table no. 14 Accuracy data for valsartan

Concentration of spiked level	Amount added(ppm)	Amount found (ppm)	% Recovery	Statistical analysis of % Recovery	
	64.58	64.32	99.59	Mean	9.67
50% sample	64.98	64.17	98.75	SD	0.505
	64.63	64.01	99.04	%RSD	0.595
	128.86	127.23	99.07	Mean	99.39
100% sample	129.03	128.76	99.79	SD	0.701
	129.45	128.69	99.41	%RSD	0.698
	192.56	192.36	99.89	Mean	100.22
150% sample	193.98	191.64	98.79	SD	0.35
	192.66	192.24	100.23	%RSD	0.249

7.5. Linearity:

Linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods. For example, calculation of a regression line by the method of least square. Therefore data from regression line itself may be helpful to provide mathematical estimates of the degree of linearity. Linearity was performed by preparing AMBL and VAL standard solutions in the range of 50-150% of target concentration (50%, 60% 80%, 100% 120% and 150%). Measure the peak area response of solution at Level 1 and Level 6 six times and Level 2 to Level 5 two times.

Acceptance criteria: Correlation coefficient should be not less than 0.9990.

% of RSD for Level 1 and Level 6 should be not more than 2.0 %.

Observation:

The correlation coefficient was found to be 0.999 and 1 for AMBL and VAL respectively. Refer tables 15 and16. From the above study it was established that the linearity of test method is from 50 % to 150 % of target concentration shown in linearity plots figure no. 22.

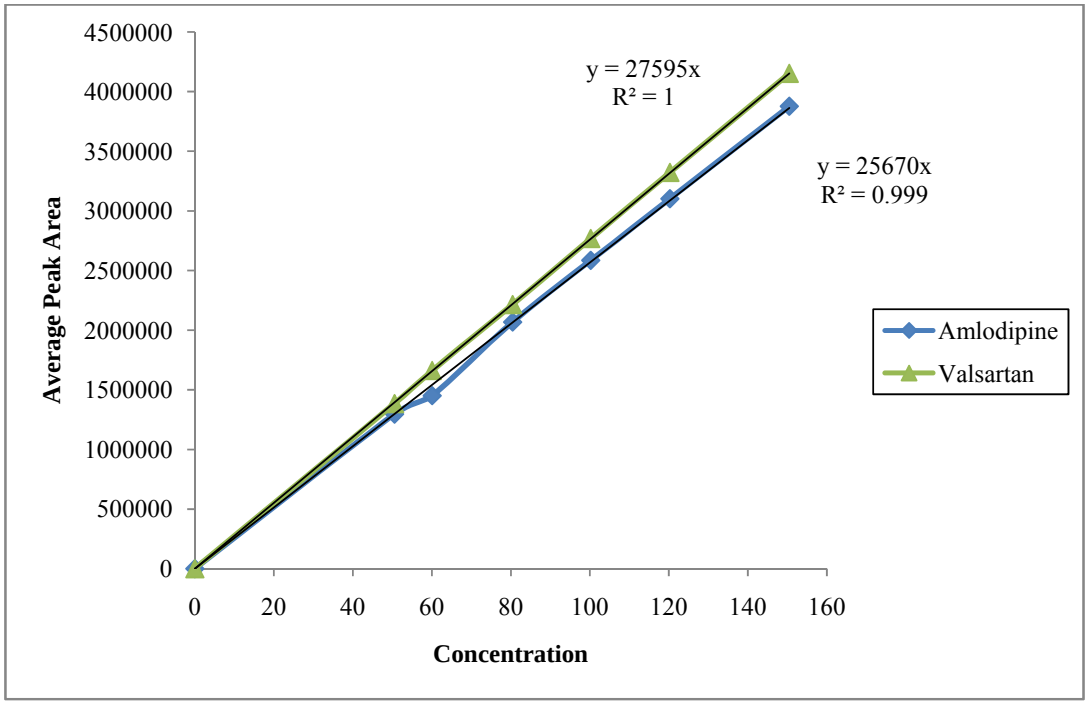
Table no.15 Linearity data for amlodipine

Linearity Level	Concentration(ppm)	Average area	%RSD	Statistical Analysis	
L1-50%	50.56	1292693.3	0.58	Slope	16028
L2-60%	60.08	1551226.8	0.47	Y-intercept	25670
L3-80%	80.45	2068302.4	0.36	% of Y-intercept	0.132
L4-100%	100.23	2585378.2	0.57	Correlation Coefficient R2	0.999
L5-120%	120.26	3102453.6	0.49		
L6-150%	150.5	3878068.2	0.52		

Table no. 16 Linearity data for valsartan

Linearity Level	Concentration (ppm)	Average area	%RSD	Statistical Analysis	
L1-50%	64.3	1384334.6	0.45	Slope	16379
L2-60%	76.8	1661201.52	0.35	Y-intercept	27595
L3-80%	102.4	2214935.36	0.56	% of Y-intercept	0.149
L4-100%	128	2768669.2	0.58		
L5-120%	153.6	3322403.04	0.39	Correlation Coefficient R2	1.00
L6-150%	193.2	4153003.8	0.48		

Fig no. 22 Linearity graph amlodipine and valsartan



7.6. Ruggedness of test method:

The United States pharmacopoeia (USP) define ruggedness as the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions such as different labs, different analysis, different lots of reagents etc. Ruggedness is a measure of reproducibility of test results under normal expected operational conditions from laboratory to laboratory and from analyst to analyst.

a) System to system variability:

System to system variability study was conducted on different HPLC systems under similar conditions at different times. Six samples were prepared and each was analyzed as per test method. The relative standard deviation for amlodipine besylate and valsartan were found to be below 2 % on the systems. Comparison of both the results obtained on two different HPLC systems variability.

Acceptance Criteria:

The % RSD of amlodipine besylate and valsartan from the six sample preparations should be not more than 2.0 %.

The % recovery of amlodipine besylate and valsartan should be between 95.0 % to 105 %.

Observation:

The %RSD was found within the limits were shown in table no: 17 and 18.

Table no. 17 Data for the ruggedness study in system-1

Tablet ID	% Assay		Statistical Analysis		
	AMBL	VAL		AMBL	VAL
1	99.24	98.28	Mean	98.878	99.29
2	98.19	99.36			
3	98.34	99.78	SD	1.01	0.68
4	98.12	99.04			
5	100.5	100.03	%RSD	1.02	0.68

Table no. 18 Data for the ruggedness study in system-2

Tablet ID	% Assay		Statistical Analysis		
	AMBL	VAL		AMBL	VAL
1	99.19	99.28	Mean	98.834	98.838
2	98.14	99.06			
3	99.32	98.78	SD	0.698	0.48
4	98.02	98.04			
5	99.5	99.03	%RSD	0.7	0.48

b) Bench top stability of standard and Test preparation:

A study to establish stability of amlodipine besylate and valsartan standards and test preparations on bench top was conducted over period of two days. Amlodipine besylate and valsartan test preparation spiked to target concentration are injected initial, 6.0 hr, 12 hr and18 hr. The difference in % of amlodipine besylate and valsartan from initial to 18hrs is within the limits. In the similar way standard preparations were injected initial, 6.0 hr and 18 hr.

Acceptance Criteria:

The difference between initial and bench top stability sample for % RSD of amlodipine besylate and valsartan should be not more than 2.0.

Observation:

The % assay was found with the limits. Table no: 19 and 20

Table no. 19 Data for interday variation

Tablet ID	% Assay		Statistical Analysis		
	AMBL	VAL		AMBL	VAL
1	99.54	99.34			
2	98.02	99.13	Mean	99.39	99.4
3	99.87	101			
4	101.02	99.76	SD	1.05	0.592
5	98.5	98.76			
6	99.43	101.16	% RSD	1.06	0.598

Table no. 20 Data for intraday variation

Tablet ID	% Assay		Statistical Analysis		
	AMBL	VAL		AMBL	VAL
1	99.14	99.38			
2	98.24	98.46	Mean	98.954	99.185
3	98.77	99.78			
4	100.12	100.04	SD	0.732107	0.684
5	98.5	99.9	% RSD	0.739845	0.69

7.7. Robustness:

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

i) Effect of variation of flow rate:

Mobile phase flow rate as per proposed analytical method is 1.0 ml/min. Change in flow rate was made at a level of 10 percentages i.e. at 0.9 and 1.1 ml/min. The effect due to change in flow rate on the system suitability parameters are compared. Results are tabulated in table no. 21.

Acceptance Criteria:

The tailing factor of amlodipine and valsartan standards should be not more than 2.0 for variation in flow.

Table no.21 Data for variation in flow rate

Parameters		AMBL		VAL	
		RT	**Peak area**	**RT**	**Peak area**
Normal (1.0 ml/min, %RSD-0.04, 0.5)		5.1	2583537	8.2	2669313
Flow rate	0.9 ml/min	7.3	2623612	10.7	2854365
Flow rate	1.1 ml/min	4.5	2183133	6.3	2296323
%RSD			0.205		0.27

Observation:

The % RSD for amlodipine and valsartan are found to be within the limits as shown in the table no. 21.

ii) Effect of Variation of temperature:

The column oven temperature as per proposed analytical method is 35 ^{o}C. Change in column oven temperature by 5 ^{o}C both by increasing and decreasing fashion. The effects due

to change column oven temperature on the system suitability parameters are compared. Results are tabulated in table no. 22 and 24.

***Acceptance criteria*:**

The % RSD of amlodipine and valsartan standard and sample solutions should be not more than 2.0 for variation in temperature.

Table no.22 Data for variation in column temperature

Parameters		AMBL		VAL	
		RT	**Peak area**	**RT**	**Peak area**
Normal (35 °C, % RSD-0.04, 0.5)		5.1	2583537	8.2	2669313
Column temperature	30 °C	5.2	2482427	8.3	2774128
Column temperature	40 °C	5.1	2468953	8.1	2796459
%RSD			0.095		0.055

Observation:

Increase in temperature resulted in early elution of all two active ingredients. Decrease in temperature resulted in late elution of all two actives but still within the proposed run time as shown in the table no. 22 and 24.

iii) Effect of variation in pH

The pH employed for the analytical method is 3.0. To study the effect of pH the buffer pH was changed to 2.8 and 3.2. The effects due to change pH on the system suitability parameters are compared. Results are tabulated in table no. 23.

Acceptance criteria:

The % RSD Factor amlodipine and valsartan were found to be within the limits as shown in the table no. 23 and 24.

Table no.23 Data for variation in pH

Parameters		AMBL		VAL	
		RT	Peak area	RT	Peak area
Normal (pH 3.0, %RSD-0.04, 0.5)		5.1	2583537	8.2	2669313
Buffer pH	2.8	5.9	2543198	9.6	2687432
Buffer pH	3.2	4.9	2574944	8.0	2753421
%RSD			0.245		0.255

Observation:

Increase in pH resulted in early elution of all two active ingredients. Decrease in pH resulted in late elution of all two actives but still within the proposed run time as shown in table no. 23 and 24.

Table no. 24 Remarks in robustness parameters

Parameters	Optimum range	Conditions in procedure	Remarks
Flow rate ml/min	0.9-1.1	1	Increase in flow rate resulted in early elution of all two active ingredients. Decrease in flow rate in late elution of all three actives but still within the proposed run time.
Temperature	30-40 ^{o}C	35 ^{o}C	Increase in temperature resulted in early elution of all two active ingredients. Decrease in temperature resulted in late elution of all two actives but still within the proposed run time.

pH	2.8-3.2	3.0	Increase in pH resulted in early elution of all two active ingredients. Decrease in pH resulted in late elution of all two actives but still within the proposed run time

CONCLUSION: From the above results it is concluded that the method was robust.

8. RESULTS AND DISCUSSION

Combination therapy in the treatment of hypertension as an appropriate treatment option is receiving boarder acceptance amongst the clinical community. Amlodipine is an effective of hypertension in once daily doses of 2.5 mg - 10 mg while valsartan is effective in doses of 80 mg – 320 mg.

Only very few HPLC estimations have been reported in the literature for the determinations of amlodipine and valsartan present in bulk, formulations and biological fluids.

Method development:

The developed method has an advantage of determination of AMBL and Val by RP-HPLC. HPLC determination was carried out on a reversed phase C18 column. Following tables give the results of the method development, quantitation and validation parameters.

Table no. 25 Validation parameters and acceptance criteria for AMBL and VAL

S. No	Parameters	AMBL	VAL	Acceptance Criteria
1.	SPECIFICITY	99.12%	99.18%	No interference of excipients.
2.	LINEARITY	1	0.999	0.99
3.	ACCURACY	98.4%	100.4%	95 – 105%
4.	PRECISION			
	System Precision	0.86%	0.58%	2% (RSD)
	Method Precision	0.1%	0.2%	2% (RSD)

5.	RUGGEDNESS	99.17% 99.29% 0.895% 0.644%	95-105% 2% (RSD)
6.	ROBUSTNESS a) Change in flow rate • 0.9 ml/min • 1.0 ml/min	0.34% 0.42% 0.07% 0.12%	2% (RSD)
	b) change in the buffer pH • 2.8 • 3.2	0.10% 0.14% 0.39% 0.37%	2%(RSD)
	c) Change in column temperature • 30 °C • 40 °C	0.06% 0.04% 0.13% 0.07%	2% (RSD)

7.	SYSTEM SUITABILITY a) Theoretical Plates	7172.23	11750.61	NLT 2000
	b) Tailing factor	1.08	1.11	NMT 2
	c) RSD	0.13	0.10	NMT 2.0%

Table no.26 Assay method

S. No.	Content	Label claim (mg)	Peak area	RT	Percentage Content
1.	AMBL	10 mg	2583535.89	5.07	99.28 %
2.	VAL	320 mg	2669313.61	8.2	99.39 %

9. CONCLUSION

More rapid, precise, specific, sensitive, economic and reproducible gradient reverse phase HPLC method was developed and validated for simultaneous determination of amlodipine besylate and valsartan tablets. The method was validated for specificity, linearity, and precision as per ICH guidelines.

Chromatographic conditions:

Column	: Symmetry C-18, (150 x 4.6 mm, 3.5 µ)
Flow rate	: 1.0 ml/min
Detector wavelength	: 237 nm
Injection volume	: 10 µL
Column oven temperature	: 35 ^{o}C
Run time	: 20 min

The RP-HPLC method for AMBL and VAL detector wave length is 237 nm, the beer's law was obeyed in the concentration of 100 ppm of AMBL and 128 ppm of VAL and retention time found to be 5.06 and 8.28 min respectively.

Conclusion and recommendations:

A new method was developed for the quantification of AMBL and VAL in combined tablet formulation. This method seems to obey the validation parameters and cost effective. This method can be routinely employed for the analysis of this combination. The reason behind selection of two different categories of antihypertensive drugs was also justified properly because of synergistic effect on lowering of blood pressure and reduction of side effects of one drug by another. Side effects of the individual drugs can be mitigated by using a complementary agent rather than increasing the dose of a single agent.

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GLOSSARY

1. **Degassing:** The process of removing dissolved gas from the liquid mobile phase before or during use. Degassing is done by heating, by vacuum, or by helium purging.
2. **Drift:** The change in the baseline value over time, expressed line mathematically as the slope of the least line squares line fitted to the base line in a specified region chromatogram.
3. **Eluent:** Mobile phase used to perform a chromatographic separation. The liquid that exists through a chromatographic column during a separation.
4. **Equilibration:** The process of bringing a chromatographic solvent (solvent, column and interactive surfaces) to a thermally and chemically stable, usually indicated by a drift_ free base line.
5. **External standard:** A separate sample that contains known quantities of the same compounds that are in unknown sample. External standards are used for quantitation by matching the detector response of a component peak to a point on a calibration curve for that component. The calibration curve is generated from a separately processed standard (or a set of standards).
6. **Fronting peak:** An asymmetrically shaped chromatographic peak in which the front part of the peak (before the apex) contains more area than the rear of the peak. The asymmetry factor for fronting peak has value less than one. The opposite of fronting peak is a tailing peak.
7. **Fused peak:** Two or more no baseline – resolved peaks in a chromatogram that share the same base line, the same base line start and end points, and the same slope and offset.
8. **Gradient elution:** Also called as solvent programming, a technique for decreasing the separation time by increasing the mobile phase strength over time during a chromatographic separation. Gradients can be continuous or stepwise. Binary (2-solvent), ternary (3-solvent) and quaternary (4- solvent) gradients are used routinely in HPLC.
9. **Integration:** The mathematically process of calculating an area such as a chromatographic peak that is bounded in a part or in a whole by a curved line.

10. **Isocratic:** The condition in which the solvent composition, flow rate and the temperature are constant during a chromatographic run, the condition in which the solvent composition is constant during a chromatographic run.
11. **Linearity:** The condition in which detector response is directly proportional to the concentration or amount of a component over a specified range of component concentration or amounts. A calibration curve is a straight line when the standard concentration is within the linear response range of the detector. The chromatography, accurate quantitation requires linearity of the detector response over the range of actual sample concentration or amounts.
12. **Mobile phase:** The fluid (gas or liquid) that carries solutes through a chromatographic column. In lc the liquid that is pumped through the fluid path of the chromatographic system and into which the samples are injected.
13. **Plate count:** A measure of the observed chromatographic resolution based on its equivalency to the number of the theoretical plates that would provide the same resolution.
14. **Resolution:** The extent to which a chromatographic column separates components from each other. Mathematically defined, resolution is the difference between the peak retention time of a selected peak and the peak preceding it's multiplied by a constant of 1.178, and then divided by the sum of the peak widths at 50% of peak height. It is used to monitor the separation of eluting components and to establish system efficiency.
15. **Retention time:** The time that elapses between the injection of a sample and the appearance of the peak maximum of a component in a sample.
16. **Rt ratio:** The retention time of a component divided by the retention time of its reference peak.
17. **System suitability**: An application that applies a set of standard criteria to test if an entire chromatographic system and the methodology are working within acceptable limits. Empower software bases the system suitability testes on standard laboratory calculations, including United States pharmacopoeia (USP) guidelines and calculations. Empower software procedures reports showing statistical accuracy and reproducibility of the chromatographic system data.

18. **Tailing factor:** A measure of peak symmetry where a symmetrical peak has a tailing factor of 1. As tailing increases peak symmetry increases for system suitability the tailing factor is the width of the peak at 5% height divided by two times the distance from the peak maximum to the leading edge of the peak (where the distance is measured at point 5% of the peak height from the baseline).
19. **Capacity factor:** A chromatographic parameter that measures retention time of a sample molecule relative to the column dead volume.
20. **Acceptance criteria:** Numerical limits ranges or other suitable for acceptance of the results of analytical procedures.
21. **Detection limit:** The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value.
22. **Drug product:** A finished dosage form, for example a capable tablet or solution that contains a drug substance, but not necessarily in association with one or more other ingredients.
23. **Drug substance:** Active ingredient: an active ingredient that is intended to furnish pharmacological activity or other direct effect the structure or any function of the human body. The active ingredient does not include intermediates used in the synthesis of such ingredient. The term includes those components that may undergo chemical change in the manufacture of the drug product and be present in the drug product in a modified form intended to furnish the specified activity or effect.
24. **Reagent:** For analytical procedures any substance used in a reaction for the purpose of detecting measuring examining or analyzing other substance.
25. **Specification:** The quality standards (i.e.., tests, analytical procedures, and acceptance criteria) provided in an approved application to confirm the quality of the drug substances, drug products, intermediates, raw materials, reagents, and other components including container closure systems, and in-process materials.
26. **Spiking:** The addition of small known amount of a known compound to a standard, sample, or placebo, typically for the purpose of confirming the performance of an analytical procedure of an analytical procedure or the calibration of an instrument.
27. **Working standards:** A standard that is qualified against and used instead of the reference standard (also known as in-house or secondary standards).