DEVELOPMENT OF ANALYTICAL METHODS FOR SOME CARDIAC DRUGS

THESIS SUBMITTED FOR THE FULFILLMENT OF
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IN THE FACULTY OF PHARMACEUTICAL SCIENCES

BY

SAVITA SHIVRAM YADAV

M. Pharm.

BHARATI VIDYAPEETH DEEMED UNIVERSITY
POONA COLLEGE OF PHARMACY
PUNE- 411038

2014

Dr. K. R. Mahadik
Principal and Professor,
Bharati Vidyapeeth Deemed University
Poona College of Pharmacy,
Pune – 411038.

CERTIFICATE

This is to certify that the work presented in the thesis entitled **'DEVELOPMENT OF ANALYTICAL METHODS FOR SOME CARDIAC DRUGS'** for the degree of **Doctor of Philosophy** in the subject of Pharmacy under the Faculty of Pharmaceutical Sciences has been carried out by **Ms. Savita Shivram Yadav**, in the laboratories of Bharati Vidyapeeth Deemed University, Poona College of Pharmacy, Pune; under the guidance of **Dr. (Mrs.) Janhavi R. Rao, Professor, Dept. of Pharmaceutical Chemistry, Poona College of Pharmacy, Pune** during the period from December 2008 to November 2014.

Date:
Place: Pune

Dr. K. R. Mahadik

Dr. (Mrs.) Janhavi R. Rao
Professor, Dept. of Pharmaceutical Chemistry,
Bharati Vidyapeeth Deemed University
Poona College of Pharmacy,
Pune – 411038.

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This is to certify that the work incorporated in the thesis entitled **'DEVELOPMENT OF ANALYTICAL METHODS FOR SOME CARDIAC DRUGS'** submitted by **Ms. Savita Shivram Yadav** for the degree of **Doctor of Philosophy** in the subject of Pharmacy under the Faculty of Pharmaceutical Sciences has been carried out in the Department of Pharmaceutical Chemistry, Bharati Vidyapeeth Deemed University, Poona College of Pharmacy, Pune during the period from December 2008 to November 2014 under my guidance and to my satisfaction.

Date:
Place: Pune

Dr. (Mrs.) Janhavi R. Rao
(Research Guide)

DECLARATION BY THE CANDIDATE

I declare that the thesis entitled **'DEVELOPMENT OF ANALYTICAL METHODS FOR SOME CARDIAC DRUGS'** submitted by me for the degree of **Doctor of Philosophy** is the record of work carried out by me during the period from December 2008 to November 2014 under the guidance of **Dr. (Mrs.) Janhavi R. Rao** and has not formed the basis for the award of any degree, diploma, associateship, fellowship, titles in this or any other University or other institutions of Higher learning.

I further declare that the material obtained from other sources has been duly acknowledged in the thesis.

Date: Signature of the candidate

Place: Pune **Savita Shivram Yadav**

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1.1. General Introduction:

Generally methods for chemical analysis are at best selective, few if any, are truly specific. Consequently the separation of analyte from potential interferences is more often than not, a vital step in analytical procedures. Until the middle of twentieth century, analytical separations were largely carried out by such classical methods as precipitation, distillation and extraction. By now however, analytical separations are most commonly carried out by chromatography and electrophoresis, particularly with samples that are complex and multicomponent.

Chromatography[1-7] is a powerful separation method that finds application in all branches of science. It encompasses a diverse and important group of methods that permit the scientist to separate closely related components of complex mixtures, many of these separations are impossible by other means. Chromatography was first invented by Russian botanist named Tswett[8-9] in 1872. In 1903, Tswett allowed plant extract to percolate through a bed of powdered calcium carbonate and found that coloured bands of plant pigment were produced on the adsorbent. This introduced the term chromatography to describe the separation process, combining the Greek Word 'chromos' meaning colour, with "grafe" meaning writing. Although colour has little to do with modern chromatography, the name has persisted and still used to describe all separation techniques that employ a mobile phase and stationary phase.

In all chromatographic separations, the sample is transported in a mobile phase, which may be a gas, a liquid or a supercritical fluid. This mobile phase is then forced through an immiscible, stationary phase, which is fixed in place in a column or a solid surface. The phases are chosen so that the components of the sample distribute themselves between the mobile and stationary phase to varying degrees. Those components that are strongly retained by the stationary phase move only slowly with the flow of mobile phase. In contrast components that are weakly held by stationary phase travel rapidly. As a consequence of these differences in mobility of sample components, separate into discrete bands or zones, that can be analyzed qualitatively and quantitatively.

Hence chromatography is probably the most powerful analytical technique available to modern chemists. Its power arises from its capacity to determine quantitatively many individual components present in a mixture during one single analytical procedure. Its versatility comes from its capacity to handle a very wide variety of samples that may be gaseous, liquid or solid in nature. In addition, the samples can range in complexity from a single substance to a multicomponent mixture containing widely differing chemical species.

Another aspect of versatility is that the analysis can be carried out, at once, on a very costly and complex instrument, and at other, on a simple, inexpensive thin plate[10].

1.1.1. Classification of chromatographic methods:

Chromatographic methods can be categorised in two ways:

(I) Based upon the physical means by which stationary and mobile phases are brought into contact. For example,

(a) Column Chromatography - stationary phase is held in a narrow tube through which the mobile phase is forced under pressure.

(b) Planar Chromatography.

Stationary phase supported on a flat plate or in the interstices of a paper. Here mobile phase moves through the stationary phase by capillary action or under influence of gravity.

(II) Based upon the types of mobile and stationary phases and the kind of equilibria involved in the transfer of solutes between phases. For example,

(a) Liquid chromatography (b) gas chromatography and super critical fluid chromatography.

Liquid chromatography can be performed either in columns or on plane surfaces.

Gas chromatography and super critical fluid chromatography on the other hand is restricted to column procedures so that the column walls contain the mobile phase.

In 1941, Martin and Synge[11] introduced liquid - liquid chromatography by supporting stationary phase, in this case water on silica in the form of packed column, which was used to separate some acetyl amino acids. In the same year, Martin and Synge suggested that it would be advantageous to replace the liquid mobile phase by gas to improve the rate transfer between the phases and thus enhance the separation. This was termed as gas chromatography. In the next decade, gas chromatography became the well-established analytical technique. During that time, the foundations of chromatography, column theory were laid down which were to guide the development of liquid chromatography in the sixties and seventies. Consequently, both Gas chromatography (GC) and Liquid chromatography (LC) were succeeded. In contrast to GC, the progress of LC had been slow. Despite this, today, LC is a well-established separation technique and has applications more than GC.

The technique, as practised until the mid 1960's, generally involved using fairly large column containing a packed bed of adsorbent, most commonly silica gel or alumina, coated with a stationary liquid for partition application. The separation was

carried out by percolating liquid through the bed under the force of gravity. The progress of separation as most often monitored by collecting fractions of the column eluent and subsequently performing some independent method of quantification. This usually involved evaporating the fractions to dryness so that the residue could be weighed. It should be apparent that a method involving so many steps is wasteful of reagents, operator time and sample material. This technique, therefore, tends to be unpopular where rapid and precise method was required for minute quantities of sample mixture to be separated and detected. Because of the limitations of existing technique, a number of closely related methods have been developed for separating mixtures of chemical substances in the liquid phase. The most widely practiced of these chromatographic methods include paper chromatography (PC) and thin layer chromatography (TLC).

The most recent developments in column chromatography have been concerned with the transition of the technique from these methods to a refined instrumental method. This transition was chiefly brought about by the increased separating power achieved by use of pellicular or solid core packing in the late 1960's. These materials were only to be superseded a few years later by the microparticulate, totally porous packings that yield even higher efficiencies. At the same time, the use of chemically bonded stationary phases eliminated much of the time consuming operations. Terms used to describe this latest approach to column chromatography include **high-pressure liquid chromatography, (HPLC) or high-performance liquid chromatography.** The technique is called as high pressure because mobile phase is pumped to the column with high pressure.

1.1.2. Applications of chromatography:

Chromatography has grown to be the premiere method for separating closely related chemical species. In addition it can be employed for qualitative identification and quantitative determination, of separated species[12].

i) Qualitative analysis:

A chromatogram provides only a single piece of qualitative, information about each species in a sample, namely retention time, or its position on the stationary phase after a certain elution period. Still the amount of information obtainable by chromatography is small compared with the amount provided by a single IR, NMR or Mass Spectrum. It is important to note that while chromatograms may not lead to positive identification of species present in a sample; they often provide some evidence of the absence of certain compounds. Thus if the sample does not produce peak at the sample retention time as a standard run under identical condition, it can be assumed that

the compound in question is absent (or is present at a concentration level below the detection limit of the procedure).

ii) Quantitative analysis[13-14]:

Quantitative chromatography is based upon a comparison of either the height or the area of the analyte peak with that of one or more standards. We normally choose conditions under which the response is linear, which means that the area or height of a peak is proportional to the amount of that component.

a) Analysis based on peak height :

The height of a chromatographic peak is obtained by connecting the baselines on the two sides of the peak by a straight line and measuring the perpendicular distance from this line to the peak. This measurement can ordinarily be made with reasonably high precision and yields accurate results, provided variations in column conditions do not alter peak width during the period required to obtain chromatograms for sample and standards. Relative errors of 5 to 10% due to this cause are not unusual with syringe injection.

b) Analysis based on peak area:

Peak area is a more satisfactory analytical parameter than peak height. Many modern chromatographic instruments are equipped with electronic integrators that provide precise measurements of relative peak areas. A simple method that works well for symmetric peaks with reasonable widths is to multiply peak height by the width at one-half peak height

c) Calibration with standards:

The most straightforward method for quantitative chromatographic analyses involves the preparation of a series of standard solutions that approximate the composition of the unknown. Chromatograms for the standards are then obtained, and peak heights or areas are plotted as a function of concentration. A plot of the data should yield a straight line that passes through the origin; analyses are based upon this plot. Frequent restandardization is necessary for highest accuracy.

1.1.3 Quantification methods:

Various methods for obtaining quantitative information from chromatograms are:

a) Normalizing peak area method:

The area of each peak is obtained from a series of replicate injections of a mixture containing equal (on known) amounts of all the components. One component is chosen as reference and the relative responses of the other components are determined by dividing the peak areas by that of the reference component. Detector response may then

be used to calculate corrected peak areas for other analysis involving these components and hence there percentage ratios in mixture may be determined.

For component x:

$$D_{RF} = \frac{A_x}{A_{REF}}$$

Where D_{RF} = detector response factor

A_x = area for component x

A_{REF} = area for reference.

Corrected area for a component in a sample is

$A_{correct} = D_{RF} X A_{chrom}$

A_{chrom} = area of the chromatogram

% of component x in the sample is calculated as

$$\text{Area \%} = \frac{A_{correct\ x}}{\Sigma A_{correct}} X\ 100$$

Where $A_{chrom.}$ Is the area from the chromatogram and $\Sigma A_{correct}$ is the sum of the corrected peak areas for all peaks in the chromatogram.

b) Internal standard method:

It is recommended for accurate quantitation. It eliminates the needs for accurate injections since a reference standard is included in each sample analyzed. An internal standard is selected which has retention time such that it is eluted in a suitable gap in the chromatogram. The procedure involves analyzing a test sample containing known amounts of each component plus a predetermined amount of the internal standard. Since peak area is proportional to the amount of an eluted component and the detector response factor,

For an individual component x:

$$\mathbf{A_x = D_{RFX}\ X\ C_x}$$

For the internal standard:

$$\mathbf{A_{IS} = D_{RF,\ IS}\ X\ C_{IS}}$$

Where C is the amount of component x or internal standard IS and D_{RF} is detector response factor.

The relative response of a component $D_{RF"x}$ to the internal standard is therefore

$$\mathbf{D_{RF"x} = (A_X / A_{IS})\ X\ (C_{IS} / C_x)}$$

Response factors for all components are calculated in the same way. Analysis of unknown mixture is achieved by adding an accurately known amount of internal

standard and then carrying out the chromatography. The concentration of each component can be calculated by following equation:

$$C_x = (A_X / A_{IS}) \text{ X } (C_{IS} / D_{RFx})$$

The precision of analysis is not dependent on injection of an accurately known amount of sample, but does depend on accurate measurement of peak areas. The highest precision for quantitative chromatography is obtained by using internal standards because the uncertainties introduced by sample injection are avoided. In this procedure, a carefully measured quantity of an internal-standard substance (I.S.) is introduced into each standard and sample, and the ratio of analyte peak area (or height) to internal-standard peak area (or height) is the analytical parameter. For this method to be successful, it is necessary that the internal-standard peak be well separated from the peaks of all other components in the sample ($R_s > 1.25$), but it must appear close to the analyte peak. With a suitable internal standard, precisions of 0.5 to 1 % relative are reported.

c) External standard method:

A set of standard mixtures containing known concentration of the analyte is analyzed and their peak area recorded. A calibration graph of area versus concentration can be drawn for each analyte to confirm a linear detector response and from which the amount of analysis in a mixture can be determined. Alternatively for an established method a replicate series of one standard mixture is injected and the area / unit amount of analyte calculated.

$$A_{STANDARD} = x \text{ mg / litre}$$

The mixture is then analyzed and the amount of components in the sample calculated using the peak area data for the standard mixture. Therefore, if the recorded peak area for the component in a sample mixture is A_{MIX} then the amount of component x is

$$\text{Amount}_x = (xA_{MIX} / A_{STANDARD}) \text{ mg/litre}$$

d) Standard addition method:

It is used in many techniques in analytical chemistry. It is of limited use in chromatography because of difficulty of injecting accurately known amounts of sample. A sample mixture is analyzed for the analyte of interest by adding a specified amount this analyte to the sample, thus increasing its concentration. The analysis is then repeated and the resulting increase in peak area due to addition of standard amount is noted. From the increase in the area of the peak concentration of analyte in the original sample may be calculated. If the peak area for first analyte is A_1 and with the standard addition of is

A_2, then the peak area corresponding to x mg is (A_2 - A_1). Thus the original amount of the analyte x in the sample corresponding to A_1 is given by:

$$\mathbf{Amount_x = (x\ A_1) / (A_2 - A_1)\ mg/litre}$$

1.2 High Performance Liquid Chromatography [15, 16]:

HPLC consists of two main components, stationary phase (adsorbent) and mobile phase (eluent). Depending upon the type of mobile phase and stationary phase used, HPLC can be classified as **Normal Phase and Reversed Phase HPLC**. In normal phase HPLC, stationary phase is polar and mobile phase is non-polar, whereas, in reversed phase HPLC, mobile phase is polar and stationary phase is non-polar.

1.2.1 Mobile phases used in HPLC[17, 18]:

The power of HPLC in terms of being able to resolve many compounds is mainly due to the diversity of mobile phases. A mobile phase in HPLC has great influence on the retention of the solutes and the separation of component mixtures.

a) Characteristics of mobile phases:

Various properties such as viscosity, compressibility, refractive index, UV cut-off, polarity, vapour pressure, and flash point of the solvents needs due consideration while selecting a mobile phase.

In case of the solvents, the viscosity generally increases with the number of carbons. Also, as viscosity of the mobile phases increases, the efficiency of the system, as measured by the number of theoretical plates, decreases.

Compressibility is the tendency of a solvent to be compressed in volume during pumping cycle. Solvents with a high degree of compressibility may show greater pulsation with reciprocating piston pumps.

When a differential refractometer is used as detector, the refractive index of a solvent plays a crucial role. Since this type of detector measures differences between the refractive index of the mobile phase and the solute, the sensitivity of the detection is related to the difference between the respective refractive indices.

The UV-cut-off is defined as the wavelength below which the solvent will absorb more than 1.0 absorbance unit in a 1-cm cell. This value is of prime importance while using UV or fluorescence detector.

The polarity of the solvent is a measure of the solvent strength or ability to elute a particular type of compound.

b) Reversed phase mobile phases:

The primary constituent of mobile phases in reversed phase chromatography is

water. Water miscible solvents such as methanol, ethanol, acetonitrile, dioxane, tetrahydrofuran and dimethyl formamide are added to adjust the polarity of the mobile phase. Additionally, acids, bases, buffers, and/or ionic surfactants are sometimes added. Water to be used, as mobile phase needs to be of high quality, either distilled or demineralised. The most widely used polarity modifiers are methanol, acetonitrile, and tetrahydrofuran. Methanol and acetonitrile have comparable polarities, but latter is an aprotic solvent. Aprotic nature is very important if hydrogen bonding plays a significant role in the separation. When inorganic salts and ionic surfactants are used, the mobile phase should be filtered before use since these additives frequently contain a significant amount of water-insoluble contaminants that may harm the column. Reversed phase mobile phases are generally non-flammable due to high water content. In order to remove the dissolved gases in the solvents it is very important to degas the mobile phases.

c) Normal phase mobile phases:

These phases are generally non-polar. Linear hydrocarbons such as pentane, hexane, heptane and isooctane are frequently used. Aliphatic halides such as dichloromethane, dichloroethane, butyl chloride and chloroform are also used. Modifiers such as alcohols, tetrahydrofuran and acetic acid are common components of these mobile phases. These mobile phases may also be saturated with respect to water. Generally, dissolved gases are not a problem with normal phase solvents. Since these solvents are less viscous, lower pressures are necessary to maintain an adequate flow rate. A major drawback of some normal phase solvents is their flammability and the hazards arising due to the same.

1.2.2 Stationary phases (column packings) in HPLC[19]:

Column packing materials can be irregular or spherical particles. In general, irregular packing assumes a more closely packed configuration, thus causing a greater resistance to solvent flow. In addition, the structural strength of a bed of irregular particles is less when compared with a well ordered spherical particle. Thus, columns containing spherical particles tend to be slightly more stable to mechanical shocks and solvent pressures.

Most column packings are based on silica matrix. These packings are rigid and can withstand pressures more than 10,000 psi. Silica particles are also available in wide range of porosity. Since the surface of the silica contains silanol groups, organic moieties may be chemically bound to the surface for bonded-phase chromatography. Other rigid solid that can be used as support material is, particles of polystyrene cross-linked with

divinylbenzene. These polymers are more resistance to chemical attack than silica particles and may find more use with mobile phases of high as well as low pH values.

The size of the packing material has a major effect on the resolving power of the system. As particle size decreases, the height equivalent to theoretical plate decreases. However, as the diameter of the particle decreases, the resistance to the solvent flow increases. With smaller diameter particles, a greater number of plates are generated per unit length of column.

Depending upon these stationary phases there are different types of HPLC techniques. These include,

a) Adsorption chromatography (liquid-solid chromatography):

In this mode, the stationary phase is polar material with less polar mobile phases. Non-polar solutes elute first with more-polar solutes eluting later. Silica and alumina are most widely used stationary materials, since both contain surface hydroxyl groups, which undergo interactions with the solutes. If mobile phase is relatively more non-polar, water is added as modifier.

b) Partition chromatography:

In this case, stationary phase consists of an organic moiety chemically bonded to the surface of silica through the surface silanol groups. Since organic moieties are generally long chain hydrocarbons, the mobile phases are generally polar in nature. Here more polar solute elutes first while the nonpolar compounds are retained.

c) Ion-exchange HPLC:

This utilizes stationary phase that can exchange cations or anions with the mobile phase. In this mode, a reversible exchange of ions between two phases accounts for separation. Components that are preferentially attracted through electrostatic interactions to the stationary phase are retained.

d) Ion pair HPLC:

It is based on the formation of ion pairs from charged analytes and oppositely charged pairing agents. It is practised as reversed phase HPLC with water-methanol and water-acetonitrile mobile phases bonded long chain alkyl packings.

e) Size exclusion HPLC:

It is also known as gel permeation, gel filtration and steric exclusion chromatography. It separates analytes based on molecular size. Its principle use has come in the separation of large molecules such as proteins and other macromolecules. It is often the method of choice for solutes of molecular weight greater than 2,000.

Advantages of HPLC [20, 21, 22]:

- Separation fast and efficient (high resolving power).
- Can be applied to the separation and analysis of very complex mixtures.
- Accurate quantitative measurement.
- Both aqueous and non-aqueous samples can be analyses with little or no sample pretreatment.
- Separated components can be easily collected and isolated from the mobile phase for further analysis or characterization.

1.2.3 Applications of HPLC[22]:

- Qualitative and quantitative analysis of the compounds.
- Analysis of drugs metabolites, stability studies and compound identification.
- Helps to evaluate the metabolic profile, plasma concentration and bio-availability of the formulations or chemical moieties under development.
- Helpful for extraction of analytes in food matrices, dyes and synthetic colours, artificial sweetners, antioxidants, aflatoxins and additives.
- Analysis of fat soluble vitamins and water soluble vitamins.
- Determination of sugars like glucose, fructose, maltose and other saccharides
- Separation of organic, inorganic, biological compounds, and thermally labile compounds.

1.2.4 System suitability test[23-25]:

a) Chromatogram:

If a detector that responds to solute concentration is placed at the end of the column and its signal is plotted as function of time (or of volume of the added mobile phase) a series of peak is obtained. Such plot is called a chromatogram.

b) Retention time:

The time analyte takes after sample injection for the analyte peak to reach the detector is called retention time and is given the symbol t_R.

c) Distribution constant:

The distribution equilibrium involved in chromatography is described by relatively straightforward equations that involve the transfer of an analyte between mobile and stationary phase.

$$\mathbf{A_{mobile} = A_{stationary}}$$

Where A is analyte species.

The equilibrium constant K for this reaction is called the distribution constant.

$$K = C_s/C_m$$

Where C_s is the molar concentration of the solute in the stationary phase and

C_m is the molar concentration of the solute in the mobile phase.

d) Column resolution:

The resolution R_s of a column provides a quantitative measure of its ability to separate two analytes. Column resolution is defined as

$$R_s = \frac{2[(t_R)_B - (t_R)_A]}{W_A + W_B}$$

Column resolution (R_S) should be either greater than or equal to 2 for a good separation between adjacent peaks.

Where $(t_R)_B - (t_R)_A$ = retention time of compound B & A respectively.

W_A, W_B = width at the base of the peak in time units of peak A and B.

Resolution of 1.5 gives essentially complete separation of the two components, whereas a resolution of 0.75 does not. At a resolution of 1.0, the overlap between two peaks is 4% and at a resolution of 1.5, the overlap is about 0.3 %. The Resolution for a given stationary phase can be improved by lengthening the column, thus increasing the number of plates. An adverse consequence of added plates is an increase in the time required for the separation.

e) Selectivity factor:

The selectivity factor,$\propto$, of a column for the two species A and B is defined as

$$\propto = \frac{K_B}{K_A}$$

Where K_B is distribution constant for more strongly retained species B.

K_A is distribution constant for less strongly or more rapidly eluted species A. $\propto$ is always greater than unity for a good separation.

f) Column efficiency:

Plate height, H, and plate count, N, are widely used as quantitative measures of chromatographic column efficiency

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

L is the length of column packing.

N can be calculated from two time measurements t_R and W to obtain H. The length of column packing L must also be known.

$$N = 16 \frac{(t_R)^2}{(W)^2}$$

The following parameters must be checked, preferably during method development.

g) Capacity factor (K'):

$$K' = \frac{t_R - t_o}{t_o}$$

Where t_R is retention time of analyte and t_o is elution time of the void volume or non-retained components. The capacity factor is an important parameter that describes the migration rates of solutes on column.

Generally the value of K' is > 2.

h) Precision:

Precision is expressed as RSD (Relative Standard Deviation). It indicates the performance of HPLC, which includes the pumping, column and environmental conditions at the time of analysis of the samples.

RSD ≤ 1-2 % in 10 injections of the standard for good precision.

i) Relative retention:

It is defined by the equation,

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

and is a measure of the relative location of two peaks.

j) Tailing factor:

Tailing factor is defined as

$$T = \frac{W_x}{2f}$$

Where W_x is width of the peak determined at either 5% or 10% from the base line of the peak height and f is distance between peak maximum and peak front. The accuracy of quantitation decreases with increase in peak tailing

$$\mathbf{0.5 \leq T < 2}$$

k) Theoretical plate number:

It is a measure of column efficiency and determines the number of peaks that can be located per unit run time of the chromatogram.

$$\mathbf{N = 16 (t_R/t_W)^2 = L/H}$$

N is fairly constant for each peak on a chromatogram with fixed set of operating conditions. H or HETP, the height equivalent of theoretical plate measures the column efficiency per unit length (L) of the column. Parameters, which can affect N or H, include peak position, particle size in column, flow rate of mobile phase, column temperature, viscosity of mobile phase and molecular weight of analyte.

1.3 High Performance Thin Layer Chromatography:

Thin layer chromatography (TLC) [26] is a type of planar liquid chromatographic (LC) technique[27]. A sample mixture (solutes) is introduced into chromatography system by depositing it at a certain place on the layer. A solvent (mobile phase) passes through the system and the separation occurs based on relative solubility and adsorption. This is the basic principle of TLC.

There are some advantages of TLC[28]

- Sample preparation is very simple.
- Unlike HPLC, the stationary phase in TLC is disposable. Therefore, contamination of stationary phase by sample matrix can be accepted.
- Large sample volumes can be applied using a special spray-on technique.
- A large number of samples are chromatographed side by side, making TLC even quicker than fully automated HPLC.
- Low detection limits can be achieved by using sample spray-on technique.
- Spraying on large sample volumes can influence detection limits.
- Any solvent may be used irrespective of its 'UV cut-off'.
- In TLC, solvent is evaporated prior to its detection.
- All fractions are permanently stored on the plate. This makes it possible to repeat detection of one, several / all fractions of the chromatogram with the same or with different parameters.

These are some of the major aspects, which make quantitative TLC a choice for many analytical tasks.

TLC as practiced today is in two forms, some are using it as qualitative tool for separation of simple mixture, where speed, low cost, simplicity are required, others use it as powerful separation tool for quantitative analysis with high sample throughput. The later now referred to as high performance thin layer chromatography (HPTLC).

Table 1.01: Characteristics of TLC and HPTLC[29, 30]:

Parameters	**TLC**	**HPTLC**
Size of the chromatographic plate	20 X 20 cm	20 X 10 cm / 10 X 10 cm
Particle size of stationary phase	250 μm	100 μm
Injection Volume	1 - 5 μl	0.1 - 0.3 μl
Distance of migration	10 - 15 cm	3 - 6 cm
Separation time	30 - 200 min	3 - 20 min

It is evident from the above table that, by reducing the mean particle size of the stationary phase, better separation can be achieved in shorter migration distance. This

reduces the analysis time greatly[31]. Solvent consumption for HPTLC is also substantially lower than that for conventional TLC.

However, a major drawback of HPTLC is small sample capacity. Consequently, it is necessary to concentrate the sample as much as possible, before spotting. To obtain optimum resolution and sensitivity in HPTLC, the spot should be no greater than 1-3 mm in diameter. This translates to sample sizes of 0.1 µl - 0.3 µl. A problem arises when a solution to be spotted has a high viscosity. Incomplete transfer may occur during this case.

1.3.1 Stationary phases in HPTLC[31, 32]:

In HPTLC/TLC, stationary phase is in the form of laminated sheet. Majorities of TLC separations are carried out employing silica/silica base material as the stationary phase. However, there are a significant number of alternative materials that have been employed for special separations, but few of them have achieved common use.

The TLC coating can serve two purposes. The surface of the coated material can contain chemical groups that actually interact directly with the solutes themselves, thus determine the extent of their retention and separation, and consequently behave as stationary phase. Depending on these properties several stationary phases are available, which include silica gel, alumina, kieselghur, cellulose, magnesia etc. These stationary phases can be coated on certain supports (glass / plastic / aluminium sheet). In the initial stages all the stationary phase material were coated manually. However, with the availability of pre-coated plates, the use of hand made plates is on decline.

Commercially available pre-coated plates with their applications:

- Silica gel 60F (unmodified): Largely used.
- Silica gel with chemically modified silanol groups includes -NH_2 (amino), -CN (cyano) and reversed phases.
- Alumina: It is similar to silica but is not used extensively. These plates do not provide the efficiencies that can be expected from those coated with silica. These plates can be used for separation of basic substances, alkaloids and steroids.
- Cellulose: It is used in TLC as a support for holding liquid stationary phases and not as adsorbent. Employing a solvent mixture that has a strongly polar component (e.g. acetonitrile / water), the more polar component (water) absorbs on the cellulose and acts as a liquid stationary phase. It has been used satisfactory in the separation of a number of amino acid mixtures and in the form of ion exchange cellulose, for the separation of inorganic ions.

1.3.2 Mobile phases in HPTLC[33]:

The choice of the mobile phase for a given separation constitutes a very important stage in realizing a good separation in HPTLC. Solvents used in HPTLC should follow the criteria such as, ease of purification, cost, low viscosity, and compatible with thin layer material, support plate and binder used. Sample mixtures to be separated should be soluble in these solvents.

Optimization of mobile phase[34]:

1. Neat solvents such as ethanol, methanol, methylene chloride, ethyl acetate should be tested.
2. If required solvent strength should be adjusted with solvent modifier, e.g. hexane, water.
3. Mixtures of solvents (binary, ternary) should be tried. If needed small amounts of acidic / basic modifiers are, added.
4. Small variations in the proportions are carried out to achieve best results.

1.3.3 Sample preparation in HPTLC:

The solid samples of test material are dissolved in a suitable solvent and applied to thin layer in the form of solution. Organic solvents with low boiling points such as ethanol, methanol acetone and chloroform are usually used. The quantity of the sample is usually 1-100 μg depending on the sensitivity of detection method and purpose of the analysis.

HPTLC is 'off-line' technique. In this technique sample application, chromatographic development and quantitative assessment are carried out independently and various devices are separated from each other in time and in space.

1.3.4 Advantages of 'OFF-LINE' technique:

1. The quantitative evaluation of any fraction can be repeated without repeating any fraction.
2. The selectivity of the determination can be significantly improved by changing the scanning mode / detection wavelength from one fraction to another of the same run.
3. The sensitivity of detection is improved in the absence of mobile phase.

1.3.5 Sample application in HPTLC[35-39]:

Successful quantitative HPTLC is strongly dependent on the quality of sample application. Reproducibility of sample amount and spot size are obviously quite

important but to achieve good chromatographic resolution, and sensitivity of detection, the shape of the applied sample is also of great importance. The methods used for sample application are summarised.

A) Micro-capillary pipette:

It is one of the simplest and most useful methods. Fixed volume capillary pipettes consist of Pt-Ir tube of calibrated length, sealed into a glass holder. This device has the advantage of closely controlled tolerances and inertness and the tip of the capillary will not damage the surface of the layer. It is suitable for pre-calibrated application at either 100nL–200nL. The area of the tip that contacts the adsorbent surface is extremely small. A slight deformation of adsorbent is necessary for the liquid in the pipette to be exposed to the adsorbent and be drawn into the layer by capillary action. The volume precision of this type of disposable sample applicator is about 1%.

B) Micro-syringes:

An alternative to the capillary pipette is the micro syringe, which offers greater flexibility in the choice of sample volumes. It delivers the sample solution by displacement rather than by capillary action. The needle of the syringe needs only to be brought close to the sorbent surface. Direct contact during application with the adsorbent can thus be avoided.

C) Contact spotting:

This method combines sample concentration and application, removing the solvent before introduction of the sample on the chromatographic plate. A relatively large volume of sample solution is evaporated on a specially prepared polymer film. Due to large contact angle between the solution and the polymer surface, the sample forms a symmetrical droplet / bead and retains its symmetry until evaporation is complete. The dried sample is then transferred to the sorbent layer by simple contact with the film.

D) Sample application with continuous evaporation of solvent:

Sample solution is pumped on to the layer from syringe that normally feeds the eluent. Simultaneously, the plate is heated from behind. With this method, extreme enrichment of the sample can be achieved, but sample application procedure is time consuming and difficult, therefore it has not been widely used in practice.

E) Spray-on technique:

An atomizer operating with a controlled stream of nitrogen sprays the sample from the syringe forming narrow, homogeneous bands on the layer. The length of the band can be pre-selected. This band wise application of sample offers additional advantages compared to spot wise sample application, the chromatographic resolution is improved, and the accuracy can be increased.

When sample bands are sprayed on, the dimensions of the resulting sample origin zone are practically independent of the elution properties of the sample solvent. Any type of solvent can be selected to dissolve the sample. The spray-on technique permits the application of different volumes of sample and calibration standards without creating additional errors.

1.3.6 Development of chromatoplate in HPTLC [40, 41]:

In HPTLC, the development of the plate can take several forms. Chromatographic separation can be influenced by interaction between layer material and atmosphere inside the developing chamber. Interaction between the dry layer and gas phase can be suppressed by placing a counter plate opposite the chromatographic layer. Such an arrangement is called 'sandwich configuration'. Good separation can be achieved by either preconditioning of the plate or by saturation of the chambers. Pre-conditioning / pre-equilibrium of the plates depends on the solvents to be used for the development. Saturation of chambers is done by thick filter paper. These filter papers are used to line the inner walls of a developing chamber. There are two types of chambers.

1) **Flat bottom chamber:** In this, saturation of chamber is done by adding solvent in chamber and filter papers are lined to walls of the chamber before development of chromatographic plate.
2) **Twin trough chamber:** In this, 10-20 ml of the solvent is sufficient for the development. This not only saves the solvent, but also reduces the waste disposal problem, as there is almost no solvent left when the run is completed. For pre-equilibrium, the plate is placed in the empty trough, which contains the solvent. The user decides the pre-conditioning time.

1.3.7 Detection in HPTLC[42, 43]:

In situ measurement of the absorbance or fluorescence characteristics of a solute represents the most widely used approach in pharmaceutical analysis due to the sensitivity and reproducibility of the method. Modern instrumentation makes it possible to make rapid measurements under variety of conditions such as absorbance / fluorescence and either by transmission or reflectance modes. The instrument is called as densitometer. All scanning densitometers have certain features in common such as

1. Light source
2. Wavelength selection device and
3. Photosensing detector

In addition, a mechanism is necessary to move the plate under the focused light beam in order to scan the plate.

1. Light source:

Lamps to be employed in photometry should, produce radiation that is as constant as possible both in origin and intensity. Be a point source in order to facilitate the production of parallel beams. There are two types of sources.

a. **Continuous sources:** These sources have to be employed to record absorption spectra. For example, hydrogen / deuterium and incandescent tungsten lamps. High-pressure xenon lamps are primarily employed in the visible region.
b. **Spectral line sources:** These deliver spectrally purer light in the region of their emission line.

   A mercury lamp is one of the examples of spectral line sources. Fluorescence is usually excited with mercury vapour lamps, in the region of their major bands they radiate more powerful than do xenon lamps.

2. Wavelength selectors:

Wavelength selectors used are monochromators instead of filters as it is advantageous since it allows for facile wavelength changes and produce beam of light that contains fewer wavelengths. Quartz prism monochromators are usually employed.

3. Photosensing detector:

The commercial instruments employ detectors of various types. Their utility depends fundamentally on

- The constancy of time of the photocurrent at constant radiation levels and constant external conditions.
- The proportionality of the photocurrent to the intensity of illumination.
- The signal to noise ratio of the photodetector.

Different detectors used are photocells, photomultipliers, photoelements and photodiodes. Out of these, photomultipliers are commonly used detectors. When the photomultiplier tube is placed below the plate, the instrument operates in the transmission mode (T). Placing the photomultipliers tube above the plate allows for the reflectance mode. Depending on this configuration i.e. reflectance mode, different types of instruments are available.

a) Single beam instrument:

The incident beam of monochromatic radiation is projected onto the plate at 0^0. The reflected light is measured by photomultiplier at an angle of 45^0. For transmission measurements, a second photomultiplier is operated on the opposite side of the layer. For simultaneous measurement, photomultiplier signals from the reflectance and

transmission modes are electronically added. For fluorescence measurement, a cut-off filter is inserted between the sample and the photomultiplier.

b) Double beam instrument:

In this instrument, beam splitter is employed to focus two beams of the same wavelength to the surface of the plate. One beam scans the portion of the plate in order to correct for interferences inherent in the plate. This configuration requires a pair of matched photomultiplier tubes in order to obtain maximum system stability. The photomultiplier tubes are placed at 45^0 to the plate for operation in the reflectance mode. The ratio between the signals of two photomultipliers is recorded.

c) Zigzag scanning instrument:

Even though the double beam configuration reduces interferences inherent in the plate, small irregularities in the surface and undesirable components in the sample may still pose some problems. To further reduce these problems a dual wavelength instrument may be employed. In this configuration, two monochromators alternately supply light to the plate by means of chopping motor (CM). One wavelength is shown to have minimal sample absorbance while other is chosen to have maximum sample absorbance. An advantage of this type is that both the sample and reference scan the same part of the plate surface thereby eliminating effects caused by variations in the plate. For two-dimensional scanning, the light beam is fixed and the stage holding the thin layer plate is moved in zigzag manner by two independent stepping motors on the X and Y-axis. Once the samples on the plate have been detected, results obtained are quantified.

1.3.8 Quantitation in HPTLC[44- 46]:

Quantitative TLC never enjoyed as much popularity as attained by GC and HPLC due to problems in sample application, development and evaluation that can contribute to poor reproducibility of conventional TLC. However, recent developments in the instrumentation for TLC have lead to improvement in precision and accuracy. Quantitation of analyte by TLC involves two types of methods. The first involves the layer such as area measurement directly, visible comparison, or densitometry, while the other involves removal of the analyte from the plate followed by quantitation step.

Although the importance of direct method has increased considerably, the spot elution technique is a very popular method, owing to its simplicity. After elution from the chromatoplate colourimetric, fluorimetric, spectrophotometric and other methods can quantify the separated compounds.

a) Quantitation using direct, *in situ* densitometry:

Direct densitometry can be defined as, resolving the compounds to be separated on the

chromatoplate and measuring the optical density of the separated spots directly on the plate. The amount of compounds is determined by comparing them to standard curve from reference material chromatographed simultaneously under the same conditions. In this case, each plate is scanned in different modes such as absorption measurements either in reflectance or transmission mode. This mode can be applied to UV or visible range. Fluorescence radiation can be measured in both the reflectance and transmission mode and the results obtained are similar. For a low concentration a linear response is obtained between the signal and the amount of sample, hence the fluorescence measurements are practically independent of the shape of the spot.

b) Calculations of the results:

As in any quantitative method based on the interpretation of detector responses several methods exist to evaluate the results obtained. In quantitative TLC three different evaluations are normally used,

1. Peak height measurement
2. Peak area measurement
3. Calibration
    - Single level calibration
    - Multilevel calibration
        a. Linear regression
        b. Nonlinear regression

c) Quantitation using spot elution technique:

The spot elution technique is widely used and popular method to quantitate compounds after T.L.C. separation. The spot elution technique consists of the following steps.

1. Drying of the chromatoplate after the run.
2. Location of the separated spots.
3. Removal of compounds from active sites on the sorbent surface.
4. Quantitation by colourimetric, fluorimetric, spectrophotometric and other methods.

1.3.9 Applications of high performance chromatography (HPTLC) in the pharmaceutical testing [47-53]

- **Manufacturing:** For process monitoring of bulk drugs, fermentation broth analysis, residue analysis and in process testing.
- **Quality control:** For raw material assays, analysis of multicomponent formulations, uniformity of content testing, impurity profiling and method validations.
- **Research and development:** analysis of formulations, stability testing, sustained release and bioavailability studies. HPTLC is also used for preparative work and process optimization in synthetic R & D work.
- **Herbal product analysis:** For fingerprinting of crude drugs and their formulations.

1.4 Statistical parameters[54, 55]:

i) Mean:

It is a measure of center of distribution if the data are symmetrically distributed below and above the average. It simply involves the summing of the individual results and division by the number of results. It is denoted by $\mathbf{X_m}$.

$$\mathbf{X_m = \Sigma X_1/N}$$

Where ΣX_1 = sum of all observations and

N is number of observations.

ii) Standard deviation:

It is a measure of the spread of data about the mean and is given by

$$\mathbf{S = \frac{\Sigma X^2 - (\Sigma X)^2/N}{N - 1}}$$

Where ΣX^2 is sum of each value squared,

(ΣX^2) is square of sum of all values and

$(\Sigma X)^2/N$ is correction term

iii) Standard deviation of mean:

It is a measure of variability of mean.

$$S_{Xm} = S/(N)^{1/2}$$

Where S_{Xm} is the standard deviation of mean

S is the standard deviation and

N is the number of observations.

iv) Variance of mean:

It can be calculated as

$$VOM = S^2 / N$$

v) Linear regression and calibration:

Calibration involves establishing the relationship between an instrument response and one or more reference values. The term regression is used to describe group of methods that summarize the degree of association between one variable and another variable.

It uses the method of least squares to determine the best linear equation to describe a set of X & Y data points. The method of least square minimizes the sum of square of the residuals - the difference between a measured data point and a hypothetical point on a line. A common application of linear regression in analytical chemistry is to determine best linear equation for calibration data to generate a calibration curve. The conc. of analyte in a sample can then be determined by comparing a measurement of the unknown to the calibration curve.

a) Linear regression equation:

For the linear equation: $\mathbf{Y = mX + b}$

Where **Y** = estimated response / dependent variable

m is slope of the regression line and

b is intercept (Y value when X = 0)

This straight-line model is only appropriate if the data approximately fit the assumption of linearity.

Slope of the equation is defined as,

$$\mathbf{m = \frac{S_{XY}}{S_{XX}}}$$

Where, $\mathbf{S_{XY} = \Sigma(X_i - X_m)\,(Y_1 - Y_m)}$ and $\mathbf{S_{XX} = \Sigma(X_i - X_m)^2}$

b) Correlations:

Correlation is a measure of the relation between two or more variables. Correlation coefficients can range from -1.00 to -1.00. The value of -1.00 represents a perfect negative correlation while a value of +1.00 represents a perfect positive correlation. A value of 0.00 represents a lack of correlation.

The most widely used type of correlation coefficient is Pearson r, also called linear or product - moment correlation.

Simple linear correlation (Pearson r):

Pearson correlation assumes that the two variables are measured on at least interval scales and it determines the extent to which values of the two variables are "proportional" to each other.

The correlation coefficient (r) represents the linear relationship between two variables. If the correlation coefficient is squared, then the resulting value (r^2, the coefficient of determination) will represent the proportion of common variation in the two variables (i.e., the "strength" or "magnitude" of the relationship).

Significance of correlation:

The significance level calculated for each correlation is a primary source of information about the reliability of the correlation. The significance of a correlation coefficient of a particular magnitude will change depending on the size of the sample from which it was computed. The test of significance is based on the assumption that the distribution of the residual values (i.e., the deviations from the regression line) for the dependent variable γ follows the normal distribution, and that the variability of the residual values is the same for all values of the independent variable χ.

Pearson Chi-square test:

The Pearson Chi-square is the most common test for significance of the relationship between categorical variables. The measure is based on the computation of the expected frequencies in a two-way table. The value of the Chi-square and its significance level depends on the overall number of observations and the number of cells in the table. Relatively small deviations of the relative frequencies across cells from the expected pattern will prove significant if the number of observations is large. The only assumption underlying the use of the Chi-square is that the expected frequencies are not very small.

Yates correction:

The approximation of the Chi-square statistic in small 2 x 2 tables can be improved by reducing the absolute value of differences between expected and observed frequencies by 0.5 before squaring (Yates' correction). This correction, which makes the estimation more conservative, is usually applied when the table contains only small observed frequencies, so that some expected frequencies become less than 10.

Fisher exact test:

This test is only available for 2x2 tables. It assumes that in the population the two factors in the table are not related. For small n, that probability can be computed exactly by counting all possible tables that can be constructed based on the marginal frequencies.

Thus, the Fisher exact test computes the exact probability under the null hypothesis of obtaining the current distribution of frequencies across cells.

Coefficient of variation:

It is a measure of relative variability. It can be calculated as

$$CV = S/X_m$$

CV of 0.1 or 10% means that standard deviation is one tenth of the mean.

Confidence limit:

The confidence limits are another statistical measure of the precision for a series of repetitive measurements. They are calculated from the standard deviation using:

$$\mu = X \pm t*S/N^{1/2}$$

We would say that with some confidence, for example 95%, the true value is between the confidence limits.

The t term is taken from table for the number of degrees of freedom and the degree of confidence desired.

Confidence interval:

It is the span between the confidence limits.

$$C.I. = 2*t*S/N^{1/2}$$

vi) Student's T-Test:

a) T-Test for independent samples:

The *t*-test is the most commonly used method to evaluate the differences in means between two groups. The t-test can be used even if the sample sizes are very small. The *p*-level reported with a *t*-test represents the probability of error involved in accepting the hypothesis about the existence of a difference. If the difference is in the predicted direction, consider only one half (one "tail") of the probability distribution and thus divide the standard *p*-level reported with a *t*-test (a "two-tailed" probability) by two.

b) T-test for dependent samples: Within-group variation:

The t-test for dependent samples has an advantage in which an important source of within-group variation can be easily identified and excluded from the analysis. If two groups of observations are based on the same sample of subjects who were tested twice, then a considerable part of the within-group variation in both groups of scores can be attributed to the initial individual differences between subjects. The assumption of the t-test for independent samples is that the paired differences should be normally distributed. The long term average (population mean, μ) with a sample mean

$$t = \frac{X_m - \mu}{S/(N)^{1/2}}$$

The difference between two means (e.g. two analytical methods)

- For a Two tailed test :

$$t = \frac{|d_m| \; X \; (N)^{1/2}}{S_d}$$

- For a One tailed test

$$t = \frac{d_m \; X \; (N)^{1/2}}{S_d}$$

The difference between independent sample means with equal variances.

$$t = \frac{X_{m1} - Xm2}{S_d \; [1/n_1 + 1/n_2]^{1/2}}$$

The difference between independent sample means with unequal variances.

$$t = \frac{X_{m1} - Xm2}{[S_1^2/n_1 + S_2^2/n_2]^{1/2}}$$

Where,

X_m = sample mean

μ = Population mean

S = standard deviation for the sample

n = number items in the sample

$|d_m|$ = absolute mean difference between pairs

d_m = mean difference between pairs

S_d = sample standard deviation for the pairs

X_{ml} & X_{m2} = two independent sample means

vii) F – test:

An F - test compares the spread of results in two data sets to determine if they could reasonably be considered to come from the same parent distribution. The measure of spread used in F - test is variance, which is simply the square of the standard deviation. The variances are rationed (i.e., divide the variance of one set of data by the variance of the other) to get the test value.

$$F = \frac{S_1^2}{S_2^2}$$

The F_{crit} value is found from tables using $(N_1 - 1)$ & $(N_2 - 1)$ degrees of freedom at the appropriate level of confidence. If $F_{crit} > F_{calculated}$ we can conclude that the spread of results in the two data sets are not significantly different.

viii) P-value:

The p-value represents an inverse index of the reliability of the statistic (i.e., the probability of error in accepting the observed results as valid). If we are comparing two means to see if they are different a p - value of 0.10 is equivalent to saying we are 90% certain that the means are different, 0.05 is equivalent to saying we are 95% certain that the means are different, and 0.01 we are 99% certain that the means are different.

i.e. [(1-p) X 100%]

P-levels ≤ 0.05 are statistically significant. If the p value is less than 0.05 (5%), correlation must be greater than the threshold value, so that result is statistically significant.

Bigger correlations would have been smaller p values and would be statistically significant.

P values and confidence intervals:

If the observed effect is positive, then half of the p value is the probability that the true effect is negative. "Each p value represents twice the probability that the true value of the effect has any value with sign opposite to that of the observed value. The p value is calculated for both tails of the distribution of the statistic. P values for one-tailed tests are half those for two-tailed tests. If the level of significance is 5% (also called an alpha level), then any result with a p value of less than 0.05 significant.

Results that are significant at the p 0.01 level are commonly considered statistically significant, and p 0.005 or p 0.001 levels are often called "highly" significant.

ix) Analysis of variance (ANOVA):

ANOVA is a technique of separating the total variability in a set of data into component parts, represented by statistical model. It is particularly useful in analyzing data from designed experiments whose objective is to compare two or more group means.

Statistical analysis and interpretation of ANOVA is based on following assumptions:

1. The errors are normal with constant variance.
2. The errors are independent.

a) One-way ANOVA (completely randomized design):

It should be used when we are comparing several sets of observations. One-way ANOVA should be used when there is only one factor being considered and replicate data from changing the level of that factor are available. If $F_{calculated} > F_{tabular}$ for a stated

level of confidence means that the difference being tested is statistically significant at that level.

b) Two-way ANOVA (randomized blocks):

It is the method used when there are two separate factors that may be influencing a result. Statistical analysis and interpretation of Two-Way ANOVA is based on following assumptions:

1. Subjects must be chosen at random.
2. Variable under study must have normality characteristic i.e.,

   Coefficient of skewness = 0

   Coefficient of kurtosis = 3

1 Between comparable groups variance are mostly same.

2 There is no interaction between the two factors.

If $F_{calculated} > F_{tabular}$ for a stated level of confidence means that the difference being tested is statistically significant at that level.

1.5 Validation of Analytical Method [56- 64]:

Validation:

Validation of a method is the process by which a method is tested by the developer or user for reliability, accuracy and preciseness of its intended purpose.

Parameters for method validation:

The parameters for method validation as defined by the ICH (International conference on harmonization) guidelines and by other organizations are summarized below:

A. Specificity
B. Selectivity
C. Precision
   a. Repeatability
   b. Intermediate precision
   c. Reproducibility
D. Accuracy
E. Trueness
F. Bias
G. Linearity
H. Range
I. Limit of detection
J. Limit of quantitation

K. Robustness

L. Ruggedness

Terminologies included in ICH guidelines but are not part of required parameters.

Specificity:

It is the ability to measure accurately and specifically the analyte of interest in the presence of other components that may be expected to be present in sample matrix. It is measure of degree of interference from such things as other active ingredients, excipients, impurities and degradation product ensuring that peak response is due to single component only. Specificity is measured by resolution, plate count and tailing factor.

Selectivity:

It refers to a method, which provides responses for a number of chemical entities that may or may not be distinguished from each other. If the response is distinguished from all other responses the method is safe to be selected.

Precision:

It is measure of degree of repeatability of an analytical method under normal operation and it is normally expressed as % Relative Standard Deviation.

$$\% \text{ RSD} = \frac{s}{x} \times 100$$

Where s = standard deviation

x = mean

Repeatability:

It is obtained when the analysis is carried out in one laboratory by one operator using one piece of equipment over relatively short time span at least 5 or 6 determinations of three different matrices at 2 or 3 different concentrations. The acceptance criteria for compound analysis are 1% RSD.

Intermediate precision:

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

Reproducibility:

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

Accuracy:

It is the measure of how close the experimental value is to the true value. Accuracy studies for drug substance and drug product are recommended to be performed at 80%, 100% and 120% levels of label claim. Three replicates of each concentration should be there and the mean is an estimate of accuracy.

Linearity:

It is ability of method to elicit test results that are directly proportional to analyte concentration within a given range. It is generally reported as variance of slope of regression line. It is determined by series of three to six injections of five or more standards whose concentration spans 80% to 120% of expected concentration range.

Range:

It is interval between the upper and lower levels of analyte studied. The range is normally expressed in the same units as the test results (e.g. %, ppm) obtained by the analytical method. The ICH guidelines specify a minimum of five concentration levels, along with certain minimum specified ranges.

For assay, minimum specified range is from 80-120% of the target concentration.

Limit of detection:

It is defined as the lowest concentration of an analyte in a sample that can be detected not quantitated. LOD is expressed as a concentration at a specified signal to noise ratio. In chromatography detection limit is the injected amount that results in a peak with a height at least twice or three times as high as base line noise level.

S/N = 2: 1 or 3: 1

It may also be calculated based on standard deviation (SD) of the response and slope of the curve. (S)

$$LOD = \frac{3.3\ (SD)}{S}$$

The signal-to-noise ratio (S/N) is calculated from the equation:

$$S/N = \frac{2H}{h_n}$$

H = height of the peak corresponding to the component concerned in the chromatogram obtained with the reference solution.

h_n=absolute value of the largest noise fluctuation from the baseline in a chromatogram obtained after injection of a blank and observed over a distance equal to twenty times the width at half-height of the peak in the chromatogram obtained with the prescribed reference solution, and situated equally around the place where this peak would be found.

Limit of quantitation:

It is defined as lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under stated operational conditions of the method. LOQ is expressed as a concentration at a specified signal to noise ratio.

In chromatography detection limit is the injected amount that results in a peak with a height ten times as high as base line noise level.

$$S/N = 10/1$$

It may also be calculated based on standard deviation (SD) of the response and slope of the curve. (S)

$$LOQ = \frac{10\ (SD)}{S}$$

A number of sample with decreasing amount of analyte are injected six times. The calculated % RSD of the precision is plotted against the analyte amount. The amount that corresponds to the previously defined required precision is equal to limit of quantitation.

Robustness:

It is the capacity of method to remain unaffected by small deliberate variations in method parameters. It is evaluated by varying chromatographic method parameters such as flow rate, column temperature, injection volume, detection wavelength, mobile phase composition etc. If the influence of parameter is within specified tolerance, the parameter is said to be within the method robustness range. According to ICH guidelines robustness should be considered early in the development of a method.

Ruggedness:

According to USP it is degree of reproducibility of the results obtained under variety of conditions expressed as %RSD. These conditions include different laboratories, analysts, instruments, reagents, days etc. It is not included in ICH guidelines.

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Scope of this work:

The numbers of drugs introduced into the market are increasing every year. These formulations may be either new entities or partial structural modification of the existing one. Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors. Under these conditions, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. There is a scope, therefore to develop newer analytical methods for such drugs. Analytical methods development and validation play important role in the discovery, development, and manufacture of pharmaceuticals. Pharmaceutical products formulated with more than one drug, typically referred to as combination products, are intended to meet previously unmet patients need by combining the therapeutic effects of two or more drugs in one product. These combination products can present daunting challenges to the analytical chemist responsible for the development and validation of analytical methods. Analytical techniques like HPLC and HPTLC have increased in the past decades and theses methods are highly suitable for qualitative and quantitative analysis in broad areas like pharmaceutical, environmental, toxicological and forensic research. Such techniques have been proven to be efficient techniques for the separation and analysis of combination products. The multicomponent products are existing for the treatment of various ailments. Among those life threatening diseases, cardiovascular disease (also called heart disease) is a class of diseases that involve the heart, the blood vessels (arteries, capillaries, and veins) or both. There are many risk factors associated with heart diseases.

Hypertension is the single biggest risk factor nowadays. Hence, antihypertensive therapy considerably reduces the risk of developing cardiovascular complications that cause a high mortality rate. Use of evidence-based multidrug regimens for patients at high risk for cardiovascular disease would be cost-effective in low-income and middle-income countries. For prevention of high risk for cardiovascular disease various combinations of antihypertensive drugs along with aspirin, a calcium-channel, an angiotensin-converting-enzyme inhibitor and a statin are available. Such combinations of drugs are used in the treatment of hypertension to improve the tolerability and decrease toxicity profile of the therapy. It becomes essential to develop highly specific, precise, accurate and robust analytical methods for the analysis of such multicomponent formulations and validate as per the regulatory guidelines. The official test methods that

result from these processes will be of greater importance for the quality control laboratories to ensure the identity, purity, potency, and performance of drug products.

2.1 Hypothesis:

Nowadays, most of the official monographs state chromatographic techniques for identification tests, related impurities and assays. An optimized analytical method designed by exploiting advantages of chromatographic techniques enables to achieve selectivity, sensitivity, accuracy and economy.

2.2 Objective:

- To select some suitable anti-hypertensive drug candidates and drug formulations.
- To develop suitable chromatographic methods for the determination of selected drug candidates using HPLC and HPTLC
- To validate the methods as per ICH guidelines using HPLC and HPTLC.
- To apply the developed methods for the analysis of selected drug candidates in formulations.
- To carry out comparative evaluation with the established methods

2.3 Plan of work:

1. Selection of combinations of drugs

Literature and market survey was carried out and suitable drug combinations were selected on the basis of physicochemical parameters and potential of drug. The selected combinations of drugs are as follows.

- Nebivolol hydrochloride and Indapamide
- Amlodipine besylate and Indapamide
- Ramipril and Amlodipine besylate
- Amlodipine besylate and Hydrochlorothiazide
- Amlodipine besylate, Losartan potassium and Hydrochlorothiazide
- Ramipril, Losartan potassium and Hydrochlorothiazide
- Atenolol, Hydrochlorothiazide and Losartan potassium
- Atorvastatin calcium, Losartan potassium, Atenolol and Aspirin
- Aspirin, Atenolol, Hydrochlorothiazide, Ramipril and Simvastatin
- Atenolol and Indapamide

- Propranolol hydrochloride and Hydrochlorothiazide
- Bisoprolol fumarate and Hydrochlorothiazide
- Nebivolol hydrochloride and Hydrochlorothiazide

2. Selection of analytical techniques

Chromatographic methods such as Reverse Phase High Performance Liquid Chromatography (RP-HPLC) and High Performance Thin Layer Chromatography (HPTLC) were selected.

3. Method development

Optimization of method was carried out through well designed experiments and statistical analysis of data.

- **HPLC method development**
  - Selection of suitable detection wavelength
  - Optimization of mobile phase
  - System suitability tests
  - Validation and statistical evaluation
  - Analysis of marketed pharmaceutical formulation
- **HPTLC method development**
  - Selection of suitable detection wavelength
  - Optimization of mobile phase
  - Validation and statistical evaluation
  - Analysis of marketed pharmaceutical formulation

2.4 Analytical method validation:

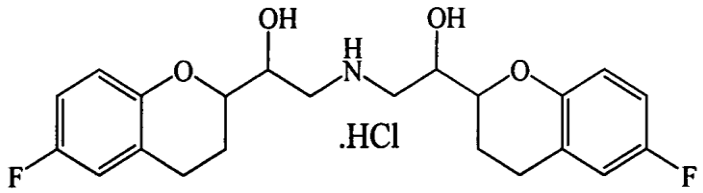
The methods were validated as per ICH guidelines for linearity, accuracy, precision, limit of detection, limit of quantification and sensitivity.

2.5. Comparative evaluation:

The methods developed were subjected to comparative analysis with existing methods.

Drug Profile:

3.1 Nebivolol Hydrochloride[1, 2, 3]:



Molecular formula : $C_{22}H_{25}F_2NO_4$•HCl

Molecular weight : 441.9

Chemical name: (1RS,1′RS)-1,1′-[(2RS,2′SR)-bis(6-fluoro-3,4-dihydro-2H-1-benzopyran-2-yl)]- 2,2′-iminodiethanol hydrochloride

Description : White to off-white powder

Solubility : Soluble in methanol, dimethyl sulfoxide, sparingly soluble in ethanol, insoluble in water

Melting point : 220-222 °C

Category : Beta-Adrenergic Blocking Agents

Storage : Store at room temperature

Pharmacology:

Nebivolol is a selective β1-receptor antagonist. Activation of β1-receptors by epinephrine increases the heart rate and the blood pressure, and the heart consumes more oxygen. Nebivolol blocks these receptors which reverses the effects of epinephrine, lowering the heart rate and blood pressure. In addition, beta blockers prevent the release of renin, which is a hormone produced by the kidneys which leads to constriction of blood vessels. At high enough concentrations, this drug may also bind beta 2 receptors.

Pharmacokinetics:

Absorption:

Absorbed rapidly from the Gastro Intestinal tract (oral). Peak plasma concentrations after 0.5-4 hours.

Distribution:

In human plasma, approximately 98% of nebivolol is bound to protein (mostly to albumin), regardless of nebivolol concentration, and the drug is widely distributed into tissues, including the brain.

Metabolism:

Nebivolol is extensively metabolised, partly to active hydroxy-metabolites. Nebivolol is metabolised via alicyclic and aromatic hydroxylation, N-dealkylation and

glucuronidation. In addition, glucuronides of the hydroxy-metabolites are formed.

Excretion:

Via urine and faeces (as metabolites). Elimination half-life: 10 hours (nebivolol), 24 hours (hydroxy metabolites).

Literature survey [4-16]:

- **IP method of assay**

  Column: stainless steel C18 column (5µ particles size) (250 mm X 4.6 mm)

  Mobile phase: Acetonitrile: buffer: dethylamine in ratio 28: 72: 0.3

  Flow rate: 1.0ml/min, λ max = 220nm, Detector: UV

- **A validated UV spectrophotometric method for estimation of nebivolol hydrochloride in bulk and pharmaceutical formulation.**

  Method: UV spectrophotometric

  Solvent: Methanol, λ max = 282 nm

- **RP-HPLC Method for Estimation of Nebivolol in Pharmaceutical Dosage Form.**

  Column: Hypersil BDS C18 column (5µ particles size) (250 mm X 4.6 mm)

  Mobile phase: Acetonitrile: 0.3M potassium dihydrogen phospate in ratio 50:50 (pH 3.2 adjusted with orthophosphoric acid)

  Flow rate: 1.2ml/min, λ max = 278 nm, Detector: UV

- **Development & validation of stability indicating method for quantification of nebivolol & their related substances by HPLC-UV-PDA detection in its pharmaceutical drug product.**

  Column: Hypersil BDS Phenyl (250mmx 4.6mm) 5µm

  Mobile phase: buffer and acetonitrile (80:20 v/v)

  Flow rate: 1.2ml/min, λ max = 220 nm, Detector: UV

- **RP-HPLC and HPTLC methods for the estimation of nebivolol hydrochloride in tablet dosage form.**

  For HPLC:

  Column: Lichrospher 100 C-18, 5 µm

  Mobile phase: 50mM KH_2PO_4 buffer (pH 3.0±0.1): acetonitrile :(45:55 v/v)

  Flow rate: 1.0 ml/min, λ max: 282 nm, Detector: UV

  For HPTLC:

  Stationary phase: Precoated silica gel 60F 254

  Mobile phase: ethyl acetate: toluene: methanol: ammonium hydroxide (1:6:2:0.1)

  λ max: 282 nm, Detector: UV

- **Stability-Indicating TLC-densitometric determination of nebivolol hydrochloride in bulk and pharmaceutical dosage form.**

  Stationary phase: TLC aluminium plates precoated with silica gel 60F254

  Mobile phase: toluene-methanol-triethylamine (3.8:1.2:0.2 v/v/v)

  λ max: 281 nm, Detector: UV

- **Simultaneous Estimation of Nebivolol hydrochloride and Amlodipine besylate by UV Spectrophotometric Method.**

  Method: simultaneous equation method

  Solvent: Methanol, λ max = 280 nm for NEB and 239 nm for AMLO

- **Simultaneous Estimation of Nebivolol Hydrochloride and Valsartan in Bulk and Capsule Dosage Form by Simultaneous Equation Method.**

  Method: Simultaneous equation method

  Solvent: Methanol, λ max = 218 nm for NEB and 251 nm for VAL

- **Validated HPTLC Method for Simultaneous Estimation of Nebivolol and Indapamide in Solid Dosage Form.**

  Stationary Phase: silica gel 60 F254 TLC plates

  Mobile phase: ethyl acetate: methanol: dil. ammonia, (8.5: 0.8: 1.0 v/v/v)

  λ max: 274 nm, Detector: UV

- **Simultaneous estimation of nebivolol hydrochloride and valsartan using RP HPLC.**

  Column: HIQ sil C_{18} column (250×4.6 mm i.d., 5 µm particle size)

  Mobile phase: methanol: water (80:20 v/v) with addition of 0.1 percent 1-hexanesulfonic acid monohydrate sodium salt as an ion-pairing reagent

  Flow rate: 1.0 ml/min, λ max: 289 nm, Detector: UV

- **New RP-HPLC Method for Simultaneous Estimation of Nebivolol Hydrochloride and Hydrochlorothiazide in Dosage Forms.**

  Mobile phase: acetonitrile and potassium dihydrogen phosphate buffer (pH 3.2 ± 0.1) in the ratio of 50:50 v/v

  Flow rate: 1.2 ml/min, λ max: 282 nm, Detector: UV

- **Method Development and Validation of Indapamide and Nebivolol Hydrochloride by RP-HPLC Method.**

  Column: Kromosil C 18 column (250×4.6 mm i.d., 3.µm particle size)

  Mobile phase: Ammonium acetate buffer pH 4.5: Methanol (45:55 v/v)

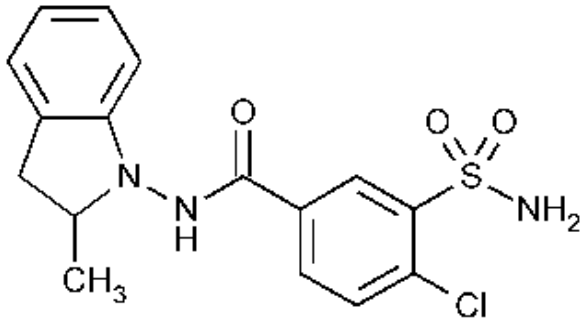
  Flow rate: 1.0 mL/min, Detector wavelength: 226 nm

- **Development and Validation of High Performance Liquid Chromatography Method for Simultaneous Estimation of Nebivolol and Indapamide in Their Combined Tablet Dosage Form.**

  Column: BDS Hypersil C_{18} column (250×4.6 mm i.d., 3.µm particle size)

  Mobile phase: (0.05M KH_2PO_4) buffer-pH 3.5: triethylamine: ACN (40:0.5:60 v/v), Flow rate: 1.0 mL/min, Detector wavelength: 286 nm

3.2 Indapamide [17, 18]:



Molecular formula : $C_{16}H_{16}ClN_3O_3S$

Molecular weight : 365.84

Chemical name : Benzamide,3-(aminosulfonyl)-4-chloro-*N*-(2,3-dihydro-2-methyl-1*H*-indol-1-yl)-4-Chloro-*N*-(2-methyl-1-indolinyl)-3-sulfamoylbenzamide

Description : White to off-white powder

Solubility: Soluble in methanol, ethanol, acetic acid, ethyl acetate, very slightly soluble in ether, chloroform, benzene insoluble in water

Melting point : 160-162 °C

Category : Diuretics

Storage : Store at room temperature

Pharmacology:

Indapamide is classed as a diuretic; its main mechanism of lowering blood pressure is due to a direct action on the blood vessels. Indapamide causes the blood vessels to widen, which reduces the pressure inside the blood vessels. This helps to lower blood pressure.

Pharmacokinetics:

Absorption:

Rapidly and completely absorbed from GI tract (oral). Peak plasma levels are achieved within 2-2.5 hours.

Distribution:

Widely distributed, preferentially and reversibly bound to erythrocytes.

Metabolism:

Extensively metabolized in the liver.

Excretion:

Via urine (60-70% as metabolites, 5-7% as unchanged), via faeces (16-23% remaining dose); 14 hours (elimination half-life).

Literature survey [19-30]:

- **IP method of assay**

  Column: stainless steel C18 column (5µ particles size) (30 cm X 4 mm)

  Mobile phase: Water: ACN: methanol: glacial acetic acid (65: 17.5: 17.5: 0.1)

  Flow rate: 2.0ml/min, λ max = 254 nm

- **USP method of assay**

  Column: stainless steel C18 column (5µ particles size) (30 cm X 4 mm)

  Mobile phase: Water: ACN: methanol: glacial acetic acid (650: 175: 175: 1)

  Flow rate: 2.0ml/min, λ max = 254 nm

- **Development of New Spectrophotometric methods for the determination of Indapamide in Bulk and Pharmaceutical formulations**

  Method: spectrophotometric methods

  Reagent: p-dimethylamino cinnamaldehyde (PDAC) and p-dimethylamino benzaldehyde (PDAB) in the acidic medium

  λ max = 682.0nm and 602.0nm

- **Validated RP-HPLC Method for the Determination of Indapamide in Bulk and Tablet Dosage Form**

  Column: C18 column (250 X 4.6mm i.d., 5µm)

  Mobile phase: Acetonitrile: Methanol: Water (40:50:10)

  Flow rate: 1.0ml/min, λ max = 242 nm, Detector: UV

- **Development and Validation of RP-HPLC Method for Quantitative estimation of Indapamide in Bulk and Pharmaceutical dosage forms**

  Column: supelco RP C-18 Column (25cm x 4.6 mm i.d., particle size 5 µm)

  Mobile phase: OPA (0.05%) buffer of pH 3.0 and Acetonitrile (60: 40)

  Flow rate: 1.0ml/min, λ max = 240 nm, Detector: UV

- **Quantitative Determination of Indapamide in Pharmaceuticals and Urine by High-Performance Liquid Chromatography with Amperometric Detection**

  Column: muBondapak C18 column

  Mobile phase: ACN-H_2O mixture (45:55, 5 mM) in KH_2PO_4-K_2HPO_4 (pH 4.0)

  Flow rate: 1.0ml/min

  Electrode = glassy carbon working electrode

Detector: amperometric detector (+1200 mV)

- **A selective HPLC method for the determination of Indapamide in human whole blood: Application to a bioequivalence study in Chinese volunteers**

  Column: Inertsil ODS-3 analytical column (25 cm x 4.6mm i.d. 5µm)

  Mobile phase: phosphate buffer: ACN: Methanol (55: 40: 5)

  Flow rate: 1.0ml/min, λ max = 240 nm, Detector: UV

- **Visible spectrophotometric estimation of aceclofenac and indapamide from tablets using folin–ciocalteu reagent**

  Method: spectrophotometric methods

  Reagent: Folin-Ciocalteu reagent.

  λ max = 642.6 nm and 783.2 nm

- **Method Development and Validation for Simultaneous Estimation of Perindopril Erbumine and Indapamide by RP-HPLC In Pharmaceutical Dosage Forms**

  Column: Inertsil ODS-3V (250mm x 4.6mm i.d. 5µm)

  Mobile phase: potassium dihydrogen phosphate buffer adjusted to pH 3.0 using ortho phosphoric acid and acetonitrile (60:40 v/v)

  Flow rate: 1.0ml/min, λ max = 215nm, Detector: UV

- **Method Development, Validation and Stability Study for Simultaneous Estimation of Telmisartan and Indapamide by Reverse Phase-High Performance Liquid Chromatography in Pure and Marketed Formulation**

  Column: Amazone C18, 5 microm, 150 x 4.6 mm

  Mobile phase: (Buffer: acetonitrile: methanol) (45+25+30) KH2PO4 & Triethaylamine pH 3.0 with ortho phosphoric acid buffer

  Flow rate: 1.0ml/min, λ max = 285nm, Detector: UV

- **A Validated RP-HPLC Method for Simultaneous Estimation of Atenolol and Indapamide in Pharmaceutical Formulations**

  Column: Waters C18 column (250×4.6 mm, 5 µ particle size)

  Mobile phase: methanol and water (adjusted to pH 2.7 with 1% ortho phosphoric acid) in the ratio of 80:20

  Flow rate: 1.0ml/min, λ max = 230nm, Detector: UV

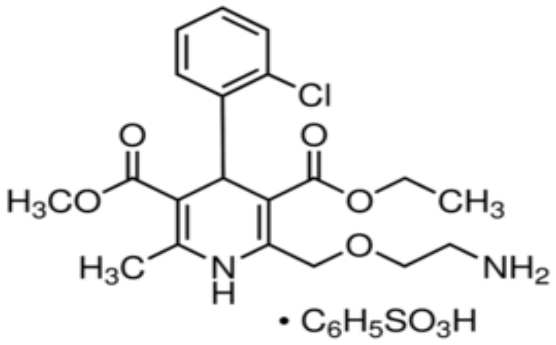
- **Simultaneous HPTLC Analysis of Atenolol and Indapamide in Tablet Formulation**

  Stationary phase: aluminum foil plates precoated with silica gel 60F254

  Mobile phase: Toluene: ethyl acetate: methanol: ammonia 5:3:3:0.1 (v/v)

  λ max = 229 nm, Detector: UV

3.3 Amlodipine besylate [31-33]:



Molecular formula : $C_{26}H_{31}ClN_2O_8S$

Molecular weight : 567.1

Chemical name : 3,5-Pyridinecarboxylic acid, 2-((aminoethoxy) methyl)-4-(2-chlorophenyl)-1,4-dihydro-6-methyl,3-ethyl-5-methylester

Description : White powder

Solubility : Slightly soluble in water; soluble in ethanol and methanol

Melting point : 195-204 ^{o}C

Category : Calcium channel blockers used as an anti-hypertensive, antianginal

Storage : Store at room temperature

Pharmacology:

Amlodipine relaxes peripheral and coronary vascular smooth muscle. It produces coronary vasodilation by inhibiting the entry of Ca ions into the voltage-sensitive channels of the vascular smooth muscle and myocardium during depolarisation. It also increases myocardial O2 delivery in patients with vasospastic angina.

Pharmacokinetics:

Absorption:

Well absorbed from the Gastro intestinal tract (oral); peak plasma concentrations after 6-12 hr.

Distribution:

Protein-binding: 97.5%.

Metabolism:

Amlodipine is extensively metabolised by the liver. Amlodipine is metabolized through the cytochrome P450 system, mainly via CYP 3A4 isoenzyme. Amlodipine is extensively (about 90%) converted to inactive with 10% of the parent compound and 60% of the metabolites excreted in the urine. Ex vivo studies have shown that

approximately 93% of the circulating drug is bound to plasma proteins in hypertensive patients.

Excretion:

Via urine (mainly as metabolites, 10% as unchanged); 35-50 hr (elimination half-life).

Literature survey [34-52]:

- **IP method of assy**

  Column - ODS (15 cm, 3.9 mm internal diameter, 5 µ).

  Mobile phase - acetonitrile: methanol: water containing 0.7 % triethylamine (pH adjusted to 3.0 ± 0.1 with phosphoric acid).

  Flow rate - 1.0 ml/min, λ max=237 nm.

- **BP method of assay**

  Column - ODS (15 cm, 3.9 mm internal diameter, 5 µ).

  Mobile phase - acetonitrile: methanol: water containing 0.7 % triethylamine (pH adjusted to 3.0 ± 0.1 with phosphoric acid).

  Flow rate - 1.0 ml/min,

  λ max=237 nm.

- **USP method of assay**

  Column - ODS (15 cm, 3.9 mm internal diameter, 5 µ).

  Mobile phase - buffer: methanol: acetonitrile (50:35:15)

  Flow rate - 1.0 ml/min.,

  λ max=237 nm.

- **Validated spectrophotometric methods for the determination of amlodipine besylate in drug formulations using 2,3-dichloro 5,6-dicyano 1,4-benzoquinone and ascorbic acid**

  Method: spectrophotometric methods

  Reagent: 2,3-dichloro 5,6-dicyano 1,4-benzoquinone and ascorbic acid

  λ max = 580 nm and 530nm

- **Sensitive spectrophotometric determination of amlodipine and felodipine using iron(III) and ferricyanide**

  Method: spectrophotometric method

  Reagent: Iron (II) with ferricyanide

  λ max = 760 nm

- **Development and validation of a HPLC analytical assay method for amlodipine besylate tablets: A Potent Ca+2 channel blocker**

  Column: WATERS C18 column 250 mm × 4.6 mm (5µm)

Mobile phase: acetonitrile: 70mM potassium dihydrogen orthophosphate buffer: methanol (15:30:55) and pH adjusted to 3.0 using OPA

Flow rate: 1.0 ml/min, λ max = 240 nm, Detector: UV

- **Validated HPLC method for determination of amlodipine in human plasma and its application to pharmacokinetic studies**

  Column: analytical 125 × 4.6 mm i.d. Nucleosil C8 column

  Mobile phase: 0.01 M sodium dihydrogen phosphate buffer and acetonitrile (63:37, v/v) adjusted to pH 3.5

  Flow rate: 1.5 ml/min, λ max = 239 nm, Detector: UV

- **Determination of amlodipine in human plasma by liquid chromatography-tandem mass spectrometry**

  Column: Zorbax C8 column

  Mobile phase: acetonitrile-water-formic acid (75:35:1), Flow rate: 0.4 ml/min

  Transitions monitored = 409 to 238 nm

  Detector: Electrospray ionization detector in positive ionization mode

- **Enantiomeric determination of amlodipine in human plasma by liquid chromatography coupled to tandem mass spectrometry**

  Column: Chiral AGP column.

  Mobile phase: 10 - mM acetate buffer (pH 4.5) : 1 - propanol (99:1, v/v).

  Detection: MS/MS transitions monitered at 409 to 238 nm.

- **Micellar electrokinetic chromatography as a fast screening method for the determination of 1, 4-dihydropyridine calcium antagonists.**

  The effect of buffer (concentration and pH): 50 mM of borate buffer, pH 8.2.

  Concentration of organic modifier (sodium dodecyl sulfate): 20 mM.

  Acetonitrile 15 % v/v.

- **Quantitative determination of amlodipine in serum by liquid chromatography with atmospheric pressure chemical ionization tandem mass spectrometry**

  Ion source: atmospheric pressure chemical ionization interface.

  Detection: selected reactive monitoring mode.

- **Enantioselective gas chromatographic assay with electron-capture detection for amlodipine in biological samples.**

  Gas chromatography involves derivatization with chiral reagent (±) (S) - alpha-methoxy - alpha- triflouromethyl phenyl acetyl chloride and subsequent separation.

- **Development and Validation of Spectrophotometric Methods for Simultaneous Estimation of Amlodipine Besylate and Indapamide in Combined Dosage Form**

  Method: (A) Absorbance ratio and (B) Area under curve

  Solvent: Methanol

  λ max = 237 nm and 240 nm

- **Simultaneous estimation of Atorvastatin Calcium & Amlodipine Besylate from tablets**

  Method: UV-spectrophotometric method

  Solvent: Methanol

  λ max = 246 nm and 360 nm

- **Spectrophotometric Methods for Simultaneous Estimation of Nebivolol Hydrochloride and Amlodipine Besylate in Tablets**

  Method: simultaneous equation method

  Solvent: Methanol

  λ max = 238nm and 360 nm

- **RP- HPLC Method for Simultaneous Estimation of Losartan potassium and Amlodipine besylate in Tablet Formulation**

  Column: Microsorb-MV 100-5, 250 x 4.6 mm

  Mobile phase: 0.02% triethylamine in water: acetonitrile (60:40), pH adjusted to 2.5 with O- phosphoric acid

  Flow rate: 1.0ml/min, λ max = 226 nm, Detector: UV

- **Stability indicating RP-HPLC method for simultaneous estimation of Valsartan and Amlodipine in capsule formulation**

  Column: C-18 Column (Kromasil, 250 x 4.6 mm)

  Mobile phase: acetonitrile: phosphate buffer (0.02M, pH 3.0), (56:44 v/v)

  Flow rate: 1.0ml/min, λ max = 234 nm, Detector: UV

- **Method development and validation of indapamide and nebivolol hydrochloride by RP-HPLC method**

  Column: Kromosil C_{18} column (250 × 4.6 mm i.d., 3 µm particle size)

  Mobile phase: Ammonium acetate buffer pH 4.5: Methanol (45:55 v/v)

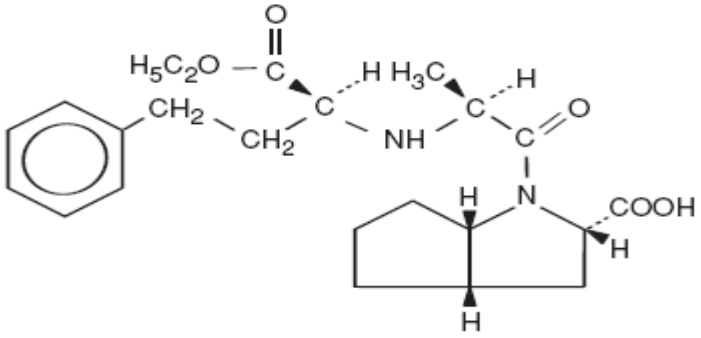
  Flow rate: 1.0 mL/min, Detector wavelength: 226 nm

- **Development and validation of high performance liquid chromatography method for simultaneous estimation of nebivolol and indapamide in their combined tablet dosage form**

  Column: BDS Hypersil C_{18} column (250 × 4.6 mm i.d., 3 µm particle size)

Mobile phase: (0.05M KH_2PO_4) buffer-pH 3.5: triethylamine: ACN (40: 0.5: 60 v/v/v), Flow rate: 1.0 mL/min, Detector wavelength: 286 nm

3.4 Ramipril [53-54]:



Molecular formula : $C_{23}H_{32}N_2O_5$

Molecular weight : 416.5

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

Description : White, crystalline substance

Solubility : Soluble in polar organic solvents and buffered aqueous solutions

Melting point : 105-112 °C

Category : Angiotensin-converting-enzyme inhibitor

Storage : Store below 40 °C.

Pharmacology:

Ramipril is an ACE inhibitor which is metabolised into the active metabolite ramiprilat. It competitively inhibits angiotensin-converting enzyme (ACE) from converting angiotensin I to angiotensin II resulting in increased plasma renin activity and reduced aldosterone secretion. It also increases bradykinin levels. By these mechanisms, ramipril produces a hypotensive effect and a beneficial effect in CHF.

Pharmacokinetics:

Absorption: 50-60% is absorbed from the GI tract (oral); peak plasma concentrations after 2-4 hr (ramiprilat).

Distribution:

The serum protein binding of ramipril is about 73% and that of ramiprilat about 56%.

Metabolism:

Ramipril is almost completely metabolised to ramiprilat, and to the diketopiperazine ester, the diketopiperazine acid, and the glucuronides of ramipril and ramiprilat.

Excretion:

Via urine (60% of the dose), via faeces (remaining dose); 13-17 hr (elimination half-life),

may be prolonged in renal impairment.

Literature survey [55-68]:

- **IP method of assay**

  Column: ODS stainless steel C18 column (5 µm, 250×4.6 mm i.d.)

  Mobile phase: Mobile phase A: sodium perchlorate+ triethylamine pH 3.6 with OPA acetonitrile (80:20) Mobile phase B: sodium perchlorate+ triethylamine pH 2.6 with OPA acetonitrile (30:70)

  Flow rate: 1.0ml/min, λ max = 210 nm Detector: UV

- **USP method of assay**

  Column: ODS stainless steel C18 column (5 µm, 250×4.6 mm i.d.)

  Mobile phase: Mobile phase A: sodium perchlorate: triethylamine pH 3.6 with OPA acetonitrile (80:20) Mobile phase B: sodium perchlorate: triethylamine pH 2.6 with OPA acetonitrile (30:70)

  Flow rate: 1.0ml/min, λ max = 210 nm, Detector: UV

- **Development and Validation of Ramipril Estimation from Capsules Using Visible Spectrophotometric Method**

  Method: visible spectrophotometric

  Reagent: Folin reagent, λ max = 456.5 nm

- **Spectrophotometric and AAS determination of ramipril and enalepril through ternary complex formation.**

  Method: Spectrophotometric and atomic absorption spectrometric method

  Reagent: (iron III, thiocyanate and ramipril) was extracted in methylene chloride, λ max= 436.6 nm

- **Determination of Ramipril in Pharmaceutical Preparations by High-Performance Liquid Chromatography**

  Column: Ace C18 column (5 µm, 250×4.6 mm i.d.)

  Mobile phase: 20 mM phosphate buffer (pH 2.5) containing 0.1% trifluoroacetic acid (TFA)-acetonitrile (50:50, v/v)

  Flow rate: 1.0ml/min, λ max = 208 nm, Detector: UV

- **Development of a method for the detection of ramipril using electron capture gas chromatography.**

  Electron capture gas chromatography-gas column coated with methyl silicone at 295^{O}C with carrier gas (25 psi) and 63_{Ni} ECD.

- **The voltametric study and determination of ramipril in dosage forms and biological fluids.**

  Method: Cyclic voltammetry, direct current polarography (DCt), differential pulse polarography (DPP) and alternating current polarography (ACt)

  Solvent: Britton–Robinson buffers over the pH range 6–12

- **RP-HPLC Estimation of Ramipril and Telmisartan in Tablets**

  Column: Genesis C18 column 4.6×250 mm, 5 µm

  Mobile phase: 0.01 M potassium dihydrogen phosphate buffer (adjusted to pH 3.4 using orthophosphoric acid): methanol:acetonitrile (15:15:70 v/v/v)

  Flow rate: 1.0ml/min, λ max = 210 nm, Detector: UV

- **Simultaneous Determination of Ramipril, Hydrochlorothiazide and Telmisartan By RP-HPLC**

  Column: 250 x 4.6 mm, 5µ Inertsil C18 column

  Mobile phase: methanol: water (pH adjusted to 4.5 using dilute orthophosphoric acid) in the ratio of 72:28 (v/v)

  Flow rate: 1.0ml/min, λ max = 270 nm, Detector: UV

- **Validated HPTLC Method for Simultaneous Estimation of Ramipril and Metolazone in Bulk Drug and Formulation**

  Stationary phase: aluminum plates precoated with silica gel 60 F254

  Mobile phase: toluene: ethyl acetate: methanol: glacial acetic acid (4: 4: 1: 0.2 v/v/v/v), λ max = 223 nm, Detector: UV

- **Development and Validation of RP-HPLC Method for Simultaneous Estimation of Ramipril, Aspirin and Atorvastatin in Pharmaceutical Preparations**

  Column: 25cm×4.6 mm i.d, 5µm particle, C18 column

  Mobile phase: Mixture of (A) acetonitrile methanol (65:35) and (B) 10 mM sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$) buffer and mixture of A: B (60:40 v/v) adjusted to pH 3.0 with o-phosphoric acid (5%v/v)

  Flow rate: 1.5 ml/min , λ max = 230 nm, Detector: UV

- **Development and validation of an RP-HPLC method for simultaneous determination of Ramipril and Amlodipine in tablets**

  Column: Inertsil ODS-3 column (250 mm×4.0 mm, 3 µm) (10 mm×4.0 mm (i.d.) 5 µm)

  Mobile phase: A consisted of 60 mM sodium perchlorate buffer (containing 7.2 mM triethylamine)-acetonitrile (60:40, v/v) and mobile phase B was 60 mM

sodium perchlorate buffer (containing 7.2 mM triethylamine)-acetonitrile (20:80, v/v) pH 2.6 with phosphoric acid

Flow rate: 1.0 ml/min, λ max = 210 nm, Detector: PDA

- **Isocratic reverse phase high performance liquid chromatographic estimation of ramipril and amlodipine in pharmaceutical dosage form**

  Column: Phenomenex C_{18} column (150×4.6 mm i.d., 5µm particle size)

  Mobile phase: Phosphate buffer (0.02 M potassium di-hydrogen orthophosphate and 0.002 M di-potassium hydrogen phosphate anhydrous, pH 6.8) and acetonitrile (60:40 v/v), Flow rate: 1.0 ml/min, Detector wavelength: 237 nm

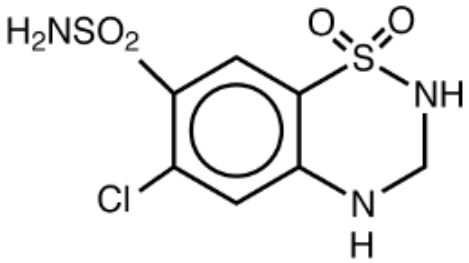
- **Simultaneous Estimation of Losartan Potassium, Ramipril and Hydrochlorothiazide in Bulk as well as in Pharmaceutical Formulation by RP-HPLC**

  Column: Symmetry C18 column (5µ particles size, 150 cm X 4.6 mm) Hypersil

  Mobile phase: Potassium dihydrogen phosphate: acetonitrile (68:32% v/v)

  Flow rate: 0.9 ml/min, λ max = 210 nm

3.5 Hydrochlorothiazide [69-71]:



Molecular formula : $C_7H_8ClN_3O_4S_2$

Molecular weight : 297.73

Chemical name : 6-Chloro-3,4-dihydro-2H-l,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide

Description : White powder

Solubility : Slightly soluble in water, sparingly soluble in dilute sodium hydroxide solution, freely soluble in n-butylamine and dimethylformamide, sparingly soluble in methanol, and insoluble in ether, chloroform, and dilute mineral acids

Melting point : 273-275°C

Category : Thiazide diuretic

Storage : Store below 40 °C. Protect from moisture.

Pharmacology:

Hydrochlorothiazide, a thiazide diuretic, by blocking the sodium-chloride symporter, hydrochlorothiazide effectively reduces the osmotic gradient and water reabsorption throughout the nephron.

Pharmacokinetics:

Absorption:

Bioavailability is 65% to 75%, C_{max} is 70 to 490 ng/mL (dose dependent), and T_{max} is 1 to 5 h. Food reduces the bioavailability 10% and the C_{max} 20% and increases the T_{max} from 1.6 to 2.9 h. Plasma concentrations are linearly related to administered dose.

Distribution:

Protein binding is 40% to 68% and crosses the placenta but not the blood brain barrier. It is also excreted in breast milk.

Metabolism:

Hydrochlorothiazide is not metabolized.

Excretion:

Hydrochlorothiazide is eliminated primarily by renal pathways (as unchanged by the kidneys; 55% to 77% of the administered dose appears in urine with more than 95% of the absorbed dose excreted in urine unchanged). Plasma half-life is 5.6 to 14.8 h.

Literature survey [72-85]:

- **IP method of assay**

  Column: ODS stainless steel C18 column (5 µm, 10 cm ×4.6 mm i.d.)

  Mobile phase: Mobile phase A: 940 ml phosphate buffers pH 3.2: 60 ml methanol: 10 ml tretrahydrofuran, Mobile phase B: 500 mlphosphate buffers pH 3.2+ 500 ml methanol: 500 ml tretrahydrofuran,

  Flow rate: 0.8ml/min, λ max = 224 nm, Detector: UV

- **Spectrophotometric estimation and validation of hydrochlorothiazide in tablet dosage forms by using different solvents**

  Method: UV Spectrophotometric method

  Solvent: Distilled water and 0.01N NaOH

  λ max = 272 nm

- **Stability Indicating HPLC Method for the Determination of Hydrochlorothiazide in Pharmaceutical Dosage form**

  Column: C-18 column

  Mobile phase: Methanol: Buffer pH- 3.2 (60:40 v/v)

  Flow rate: 1.0 ml/min, λ max = 270 nm, Detector: UV

- **Simple method for determination of hydrochlorothiazide in human urine by high performance liquid chromatography utilizing narrow bore chromatography**

  Column: C18 analytical column

  Mobile phase: 0.1 % aqueous acetic acid: acetonitrile (93:3, v/v) pH 3

  Detector: UV

- **New sensitive method for the determination of hydrochlorothiazide in human serum by high-performance liquid chromatography with electrochemical detection**

  Column: Reverse phase C18 column.

  Mobile phase - Phosphate buffer: acetonitrile (90:10) v/v.

  Detector: Coulometric cell for hydrochlorothiazide and ultraviolet detector for para amino benzoic acid

- **Simultaneous spectrophotometric estimation of Hydrochlorothiazide and bisoprolol fumarate in combined dosage forms**

  Method: Ratio spectra derivative spectrophotometry and simultaneous equation

  Solvent: methanol, λ max = 212.6 & 230 nm and 223 and 271.6 nm

- **Application of derivative Spectrophotometry for Determination of Enalapril, Hydrochlorothiazide and Valsartan in Complex Pharmaceutical Preparations**

  Method: Derivative spectrophotometry method

  Solvent: methanol, λ max = 225 nm and 278 nm

- **Derivative and Q-analysis Spectrophotometric Methods for Estimation of Hydrochlorothiazide and Olmesartan Medoxomil in Tablets**

  Method: Q-analysis method and derivative spectrophotometric method

  Solvent: methanol, λ max = 264 nm and 271 nm

- **Simultaneous Determination of Valsartan and Hydrochlorothiazide in Tablets by RP-HPLC**

  Column: 200 x 4.6 mm, 5µ Inertsil C18 column

  Mobile phase: methanol: acetonitrile: water: isopropyl alcohol (22:18: 68: 2; adjusted to pH 8.0 using triethylamine; v/v)

  Flow rate: 1.0ml/min, λ max = 270 nm, Detector: UV

- **A liquid chromatography/ tandem mass spectrometry method for the simultaneous quantification of valsartan and hydrochlorothiazide in human plasma**

  Column: Zorbax SB-Aq C18 column

Mobile phase: acetonitrile: 10 mM ammonium acetate (60:40, v/v, pH 4.5)

Flow rate: 1.2 ml/min, Detector: multiple reaction monitoring

- **Validated RP-HPLC Method for Simultaneous Determination of Telmisartan and Hydrochlorothiazide in Pharmaceutical Formulation**

  Column: C18 column

  Mobile phase: methanol and acetonitrile (70:30 v/v)

  Flow rate: 1.0 ml/min, λ max = 270 nm, Detector: UV

- **High-pressure liquid chromatographic determination of chlorothiazide and Hydrochlorothiazide in plasma and urine: preliminary results of clinical studies**

  Column: ODS reverse phase.

  Mobile phase: 15 % methanol in 0.01M acetic acid for plasma and 4% acetonitrile in 0.01 M sodium perchlorate adjusted to pH 4.6 for urine.

  Flow rate: 2.5 ml/min.

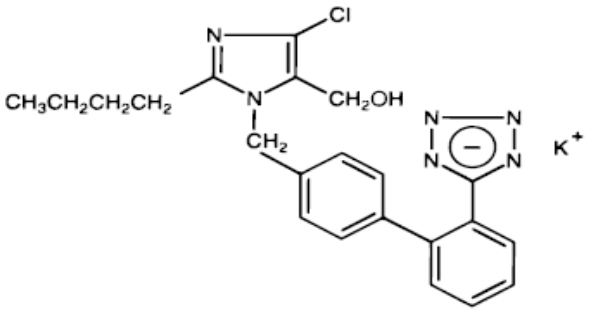
- **Analytical method development and validation of Amlodipine and Hydrochlorothiazide in combined dosage form by RP-HPLC**

  Column: Phenomenex C_{18} column (250×4.6 mm i.d., 5 µm particle size)

  Mobile phase: Triethylamine: Acetonitrile: Methanol (50:25:25 v/v/v) (pH adjusted to 3.0 with Ortho-phosphoric acid)

  Flow rate: 2 mL/min, Detector wavelength: 235 nm

3.6 Losartan potassium [86-88]:



Molecular formula : $C_{22}H_{22}ClKN_6O$

Molecular weight : 461.01

Chemical name : 2-butyl-4-chloro-1-[p-(o-1H-tetrazol-5ylphenyl)benzyl] imidazole -5- methanol monopotassium salt

Description : White to off-white free-flowing crystalline powder

Solubility : Freely soluble in water, soluble in alcohols, and slightly soluble in common organic solvents, such as acetonitrile and methyl ethyl

ketone

Melting point **:** 263-265 °C

Category **:** Antihypertensive -angiotensin II receptor antagonist

Storage : Store at room temperature, away from light and moisture

Pharmacology:

Angiotensin II [formed from angiotensin I in a reaction catalyzed by angiotensin converting enzyme (ACE, kininase II)], is a potent vasoconstrictor, the primary vasoactive hormone of the renin-angiotensin system and an important component in the pathophysiology of hypertension. It also stimulates aldosterone secretion by the adrenal cortex. Losartan and its principal active metabolite block the vasoconstrictor and aldosterone-secreting effects of angiotensin II by selectively blocking the binding of angiotensin II to the AT1 receptor found in many tissues, (e.g., vascular smooth muscle, adrenal gland).

Pharmacokinetics:

Absorption:

Well absorbed. Food decreases absorption but has only minor effects on losartan AUC or AUC of active metabolite. Systemic bioavailability is about 33%. T_{max} is 1 h (losartan) and 3 to 4 h (metabolite). While C_{max} of drug and active metabolite are equal, metabolite AUC is 4 times greater than that of losartan.

Distribution:

Losartan and active metabolite are highly bound to plasma proteins, primarily albumin. Neither losartan nor metabolite accumulates in plasma upon repeated daily dosing.

Metabolism:

Undergoes substantial first-pass metabolism by CYP-450 2C9 and 3A4 enzymes. Fourteen percent of an oral dose is converted to an active carboxylic acid metabolite that is responsible for most of the angiotensin II receptor antagonist activity.

Excretion:

The $t_{½}$ is 2 h (losartan) and 6 to 9 h (metabolite). Renal Cl is 75 mL/min (losartan) and 25 mL/min (metabolite). Total plasma Cl is 600 mL/min (losartan) and 50 mL/min (metabolite). Biliary excretion contributes to the elimination of losartan and metabolite. About 4% is excreted unchanged in the urine and 6% excreted as active metabolite in urine.

Literature survey [89-102]:

- **IP method of assay**

  Column: ODS stainless steel C18 column (5 µm, 25 cm ×4.0 mm i.d.)

  Mobile phase: Mobile phase A: o.1 % OPA in water, Mobile phase B: Acetonitrile

  Flow rate: 1ml/min, λ max = 220 nm, Detector: UV

- **Quantitative Estimation of Losartan Potassium in Pharmaceutical Dosage Forms by UV Spectrophotometry**

  Method: Second derivative UV- spectrophotometric method

  Solvent: Methanol, λ max = 234 nm

- **Sensitive and accurate estimation of losartan potassium formulation by high performance thin-layer chromatography**

  Stationary phase: aluminum-backed silica gel 60F254 HPTLC plates

  Mobile phase: acetonitrile-methanol-0.1% acetic acid (3.5:2.6:3.9, v/v)

  λ max = 235 nm, Detector: UV

- **Simultaneous UV Spectrophotometric Method for Estimation of Losartan Potassium and Amlodipine Besylate in Tablet Dosage Form**

  Method: simultaneous equation and Q value analysis

  Solvent: Methanol

  λ max = 208 nm & 237.5 nm and 242.5 nm & 237.5 nm

- **Simultaneous Estimation of Losartan Potassium and Hydrochlorothiazide in Combination**

  Method: simultaneous equation

  Solvent: Methanol, λ max = 236 and 270 nm

- **Determination of Losartan, Telmisartan, and Valsartan by Direct Injection of Human Urine into a Column-Switching Liquid Chromatographic System with Fluorescence Detection**

  Column: 25 microm C(18) alkyl-diol support (ADS)

  Mobile phase: 5mM phosphate buffer (pH 3.8)-acetonitrile-methanol (65:20:15)

  Flow rate: 3 mL/min

  λ max = 259 and 399 nm as excitation and emission , Detector: fluorescence

- **Simultaneous Analysis of Losartan Potassium, Atenolol and Hydrochlorothiazide in Bulk and in Tablets by High-Performance Thin-layer Chromatography with UV Absorption Densitometry**

  Stationary phase: prewashed silica gel plates

  Mobile phase: toluene–methanol–triethylamine 6.5:4:0.5 (v/v)

λ max = 274 nm, Detector: UV

- **Simultaneous Determination of Losartan Potassium, Atenolol and Hydrochlorothiazide in Pharmaceutical Preparations by Stability-Indicating UPLC**

  Column: Zorbax C18 50 mm × 4.6 mm 1.5 µm

  Mobile phase: water: acetonitrile: triethyl amine: ortho phosphoric acid (60:40:0.1:0.1), Flow rate: 0.7 mL/min, λ max = 225 nm, Detector: UV

  Mobile phase: [0.1% triethylamine-0.1% acetic acid (pH 7.1)]-acetonitorile (65:35, v/v)

- **Simple high-performance liquid chromatographic method for determination of Losartan and E-3174 metabolite in human plasma, urine and dialysate**

  Column: phenyl analytical column C18, 4.6×250 mm, 5 µm column

  Mobile phase: a gradient mobile phase consisting of 25 mM potassium phosphate and acetonitrile pH 2.2 and isocratic 25 m*M*potassium phosphate and acetonitrile (60:40, v/v) pH 2.2 detection, Detector: fluorescence.

- **Determination of losartan and its degradates in COZAAR® tablets by reversed - phase high - performance thin - layer chromatography.**

  A reversed - phase high - performance thin - layer chromatography method has been developed for the determination of losartan and its low level dimeric degradates (E and F). The method has been validated.

  C18 HPTLC plate (silica on glass).

  Mobile phase – Acetonitrile: methanol: 0.1 % acetic acid (7:5:8) v/v/v.

  Detection wavelength – 254 nm.

- **Optimization and validation of a capillary zone electrophoretic method for the analysis of several angiotensin – II - receptor antagonists.**

  Capillary zone electrophoretic method was used for the separation of six angiotensin - II - receptor antagonists (ARA-IIs): candesartan, eprosartan, irbesartan, losartan potassium, telmisartan, and valsartan. A three - level, full - factorial design using 60 mM sodium phosphate buffer (pH 2.5).

- **Identification of losartan degradates in stressed tablets by LC - MS and LC - MS - MS.**

  Column – 5µm spherisorb C-8 column (25 cm × 4.6 mm internal diameter) at 40^{O}C, Gradient elution: Flow rate - 1.5 ml/min.

  Mobile phase – Acetonitrile: 1 mM - phosphate buffer 2.3 pH [40 - 75%] in 25 min., Detection – 230 nm.

  LC - MS mobile phase - aqueous 0.1 %TFA: acetonitrile [35 – 75 %] in 25min.

- **Spectrophotometric and HPLC Methods for Simultaneous Estimation of Amlodipine Besilate, Losartan Potassium and Hydrochlorothiazide in Tablets**

  Column: Kromasil C18 column (5µ particles size, 25 cm X 4.6 mm)

  Mobile phase: 0.025 M phosphate buffer (pH 3.7): acetonitrile (57:43% v/v)

  Flow rate: 1.0 to 1.3 ml/min, λ max = 232nm

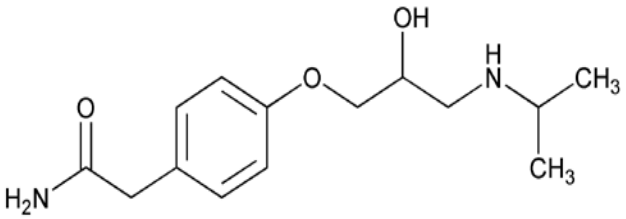
- **Simultaneous estimation of hydrochlorothiazide, amlodipine, and losartan in tablet dosage form by RP-HPLC**

  Column: Phenomenex luna CN100R, (5µ particles size, 25 cm X 4.6 mm)

  Mobile phase: acetonitrile, water and 0.4% of potassium dihydrogen phosphate buffer pH 2.7 adjusted with ortho-phosphoric acid (45:35:20)

  Flow rate: 1.0 ml/min, λ max = 230 nm

3.7 Atenolol [103-106]:



Molecular formula : $C_{14}H_{22}N_2O_3$

Molecular weight : 266.33

Chemical name : 4-(2-hydroxy-3-[(1-methylethyl) amino] propoxy] benzene acetamide

Description : White or almost white powder

Solubility : sparingly soluble in water, soluble in alcohol and practically insoluble in ether

Melting point : 152-155 ^oC

Category : β 1 selective (cardioselective) β adrenergic receptor blocking agent

Storage : Store in a well closed container, Below 40°C

Pharmacology:

Atenolol belongs to a class of medications called beta-blockers. Atenolol is a beta-adrenergic (beta-1 selective) agent that blocks beta receptors on the heart. This action primarily affects the heart by slowing it down and decreasing blood pressure. The beta-adrenerigic receptor blocking activity is demonstrated by the ability of atenolol to reduce the resting and exercising heart rate, reduction of blood pressure at resting and after

exercising, inhibition of tachycardia (fast heart rate) and reduction of orthostatic tachycardia (fast heart rate after standing up quickly).

Pharmacokinetics:

Absorption:

Atenolol is incompletely absorbed (about 50%), but most of the absorbed dose reaches the systemic circulation.

Distribution:

Only a small amount (6-16%) is protein-bound resulting in relatively consistent plasma drug levels with about a four-fold inter-patient variation.

Metabolism:

Peak blood levels are reached between two and four hours after ingestion. Unlike propranolol or metoprolol, atenolol undergoes little or no metabolism by the liver.

Excretion:

Over 85% of intravenous dose is excreted in urine within 24 hours compared with 50% for an oral dose. The elimination half-life of atenolol is 6 to 7 hours and there is no alteration of kinetic profile of drug by chronic administration.

Literature survey [107-116]:

- **IP method of assay**

  Column: ODS stainless steel column 5 µm, 30 cm × 3.9 mm i.d.

  Mobile phase: sodium heptane suphonate+ dibasic sodium phosphate in water+ dibultylamine, pH 3 wth 0.8 M OPA + methanol

  Flow rate: 0.6 mL/min, λ max = 226 nm, Detector: UV

- **High-Performance Liquid Chromatographic Determination of Atenolol in Tablets**

  Column: Pinkerton column ISRP (GFF-S5–80) 5 µm, 150 × 4.6 mm i.d.

  Mobile phase: 0.0612 M potassium hydrogen phosphate-isopropanol-tetrahydrofuran (84:10:6) v/v). The pH was adjusted to 6.7 with phosphate buffer

  Flow rate: 1.0 mL/min, λ max = 272 nm, Detector: UV

- **Estimation of Atenolol by Reverse Phase High Performance Liquid Chromatography**

  Column: ODS C18 (250 x 4.6 mm, 5µ)

  Mobile phase: mixture of phosphate buffer and acetonitrile (53:47 v/v)

  Flow rate: 1.2 mL/min, λ max = 230 nm, Detector: UV

- **Stability-indicating HPLC Method for the Determination of Atenolol in Pharmaceutical Preparations**

  Column: Hibar® pre-packed column RT 250-4 LiChrospher® 100-RP8 (250

mm×4.0 mm i.d., 5 µm particle size

Mobile phase: acetonitrile: methanol: 0.02 M phosphate buffer, pH 5 [20:20:60]

Flow rate: 1.0 mL/min, λ max = 226 nm, Detector: UV

- **Validation of UV Spectrophotometric and HPLC Methods for Quantitative Determination of Atenolol in Pharmaceutical Preparations**

  For UV Spectrophotometry

  Method: UV- spectrophotometric method

  Solvent: sodium acetate solution, λ max = 226 nm

  For HPLC

  Column: Purospher RP-18 (250 mm x 4.6 mm, 5 µm)

  Mobile phase: 10 mM ammonium acetate buffer (pH 7.0) and acetonitrile (80:20)

  Flow rate: 0.8 mL/min, λ max = 275 nm, Detector: UV-DAD

- **A simple spectrophotometric method for the determination of β-blockers in dosage forms**

  Method: UV- spectrophotometric method

  Solvent: chloroform with 0.1% chloroformic solutions of acidic sulphophthalein dyes, λ max = 415 nm

- **Simultaneous high-performance liquid chromatographic determination of Atenolol and Amlodipine in pharmaceutical-dosage form**

  Column: shim-pack CLC ODS (C18), 4.6 mm × 25 cm & 0.5 µm

  Mobile phase: ammonium acetate buffer (the pH was adjusted to 4.5 ± 0.05 with glacial acetic acid), acetonitrile and methanol (35:30:35 v/v)

  Flow rate: 1.5 mL/min, λ max = 237 nm, Detector: UV

- **Simultaneous analysis of losartan potassium, atenolol, and hydrochlorothiazide in bulk and in tablets by high-performance thin-layer chromatography with UV absorption densitometry**

  Stationary phase: silica gel 60F254 HPTLC plates

  Mobile phase: toluene–methanol–triethylamine 6.5:4:0.5 (v/v)

  λ max = 274 nm, Detector: UV

- **RP-HPLC Method for Simultaneous Estimation of Atenolol, Hydrochlorothiazide and Losartan in Tablet Dosage Form**

  Column: Luna C18 column (5µ particles size, 25 cm X 4.6 mm) Phenomenex

  Mobile phase: mixture of (A) acetonitrile, methanol (65:35) and (B) 10 mM NaH_2PO_4,H_2O buffer with 0.4% v/v TEA and mixture of A: B (60:40 v/v) pH 3.0 with OPA (5% v/v)

  Flow rate: 1.5 ml/min, Detection wavelength = 230nm.

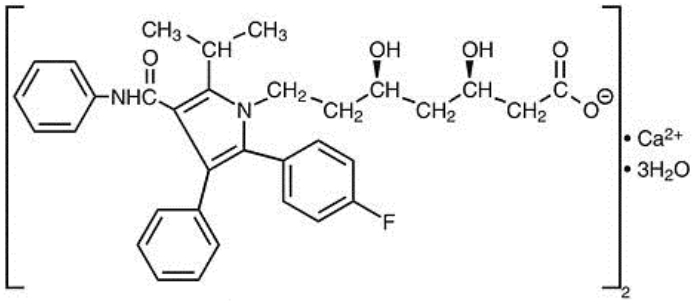
- **Simultaneous Estimation and validation of Atenolol, Hydrochlorothiazide and Losartan K in Tablet Dosage Form by RP-HPLC method**

  Column: Thermo scientific C18 column (5µ particles size, 20 cm X 4.6 mm)

  Mobile phase: Acetonitrile: Phosphate buffer pH 3.6 adjusted with Na_2HPO_4

  Flow rate: 1.2 ml/min, Detection wavelength = 229 nm.

3.8 Atorvastatin Calcium[117-118]:



Molecular formula: $(C_{33}H_{34}\ FN_2O_5)2Ca•3H_2O$

Molecular weight : 1209.42

Chemical name : [R-(R*, R*)]-2-(4-fluorophenyl)-β, δ-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino)carbonyl]-1Hpyrrole- 1-heptanoic acid, calcium salt (2:1) trihydrate

Description : White to off-white crystalline powder

Solubility : Very slightly soluble in distilled water, pH 7.4 phosphate buffer, and acetonitrile; slightly soluble in ethanol; and freely soluble in methanol.

Melting point : 159-161 °C

Category : lipid-lowering agent (HMG CoA reductase inhibitors or "statins")

Storage : Store at room temperature. Protect from light.

Pharmacology:

Atorvastatin selectively and competitively inhibits the hepatic enzyme HMG-CoA reductase. As HMG-CoA reductase is responsible for converting HMG-CoA to mevalonate in the cholesterol biosynthesis pathway, this result in a subsequent decrease in hepatic cholesterol levels. Decreased hepatic cholesterol levels stimulate upregulation of hepatic LDL-C receptors which increases hepatic uptake of LDL-C and reduces serum LDL-C concentrations.

Pharmacokinetics:

Absorption:

Atorvastatin is rapidly absorbed after oral administration with maximum plasma concentrations achieved in 1 to 2 hours. The absolute bioavailability of atorvastatin

(parent drug) is approximately 14% and the systemic availability of HMG-CoA reductase inhibitory activity is approximately 30%. The low systemic bioavailability is due to presystemic clearance by gastrointestinal mucosa and first-pass metabolism in the liver.

Distribution:

Mean volume of distribution is approximately 381 liters and is ≥ 98% bound to plasma proteins. A blood/plasma ratio of approximately 0.25 indicates poor drug penetration into red blood cells.

Metabolism:

Atorvastatin is extensively metabolized to ortho and parahydroxylated derivatives and various beta-oxidation products. In vitro inhibition of HMG-CoA reductase by ortho- and parahydroxylated metabolites is equivalent to that of atorvastatin. Approximately 70% of circulating inhibitory activity for HMG-CoA reductase is attributed to active metabolites. CYP3A4 is also involved in the metabolism of atorvastatin.

Excretion:

Eliminated primarily in bile after hepatic and/or extrahepatic metabolism. Does not appear to undergo significant enterohepatic recirculation. Less than 2% of the orally administered dose is recovered in urine.

Literature survey [119-128]:

- **IP method of assay**

  Column: ODS C8 column

  Mobile phase: A solution: 92.5: 7.5 (ACN: tetrahydrofran) ,B solution 58: 42 buffer: water C solution 20: 20: 60 (buffer: A solution: methanol)

  λ max = 246 nm, Detector: UV

- **A simple and rapid HPLC method for the determination of atorvastatin in human plasma with UV detection and its application to pharmacokinetic studies**

  Column: 125 x 4 mm (i.d.) Nucleosil C8 column (5 microm particle size)

  Mobile phase: NaH_2PO_4 buffer-acetonitrile (60:40, v/v) pH 5.5

  Flow rate: 1.5 ml/min, λ max = 245 nm, Detector: UV

- **HPTLC Determination of Atorvastatin in Plasma**

  Stationary phase: silica gel 60F254 HPTLC plates

  Mobile phase: toluene–methanol 70:30 (v/v)

  λ max = 280 nm, Detector: UV

- **Determination of Atorvastatin in Human Serum by Reversed-Phase High-Performance Liquid Chromatography with UV Detection**

  Column: C18 analytical column

  Mobile phase: sodium phosphate buffer (0.05 M, pH 4.0) and methanol (33:67, v/v), Flow rate: 1.0 ml/min, λ max = 247 nm, Detector: UV

- **Spectrophotometric Method for Simultaneous Estimation of Atorvastatin and Amlodipine in Tablet Dosage Form**

  Method: Simultaneous equations method

  Solvent: Methanol, λ max = 242nm and 364nm

- **Simultaneous Estimation and Validation of Atorvastatin Calcium and Ubidecarenone (Coenzyme Q10) in Combined Tablet Dosage Form by RP-HPLC Method**

  Column: PEERLESS C8 reverse phase column (250 x 4.6mm, 5 µ, L 7 pack)

  Mobile phase: methanol and acetonitrile 80: 20%v/v

  Flow rate: 1.5 ml/min, λ max = 290 nm, Detector: UV

- **Simultaneous Estimation of Atorvastatin and Ramipril by RP-HPLC and Spectroscopy**

  For Spectroscopy

  Method: simultaneous equation (Vierodt's equation)

  Solvent: Methanol, λ max = 247 nm & 208 nm

- **For HPLC**

  Column: Intersil ODS (250x46 m.m) 5µ. I

  Mobile Phase: 50% acetonitrile and 50% buffer.

  Flow rate: 1.2 ml/min, λ max =215 nm, Detector: UV

- **RP-HPLC method for the simultaneous determination of Atorvastatin and Amlodipine in tablet dosage form**

  Column: RP-C_{18} column (150x4.6 mm I.D.; particle size 5 mm)

  Mobile Phase: mixture of acetonitrile and 0.03M phosphate buffer pH 2.9 (55:45% v/v)

  Flow rate: 1.0 ml/min, λ max = 240 nm and 362 nm, Detector: UV

- **A stability-indicating High Performance Liquid Chromatographic (HPLC) Assay for the Simultaneous Determination of Atorvastatin and Amlodipine in Commercial Tablets**

  Column: Perfectsil® Target ODS-3, 5 µm, 250 mm × 4.6 mm i.d.

  Mobile Phase: acetonitrile–0.025 M NaH2PO4 buffer (pH 4.5) (55:45, v/v)

  Flow rate: 1.0 ml/min

λ max =237 nm, Detector: UV

- **RP-HPLC Method for the Simultaneous Determination of Aspirin, Atorvastatin and Pioglitazone in Capsule Dosage Form**

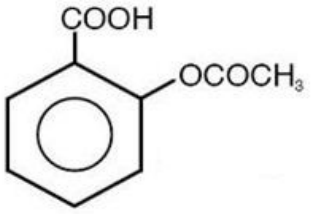
  Column: Zorbax SBCN, 5µm, 4.6 x 250mm, AGILENT column

  Mobile Phase: Acetonitrile and phosphate buffer with pH 3.5 (40 %: 60 % v/v)

  Flow rate: 1.0 ml/min

  λ max =261 nm, Detector: PDA

3.9 Aspirin[129-132]:



Molecular formula : $C_9H_8O_4$

Molecular weight : 180.15

Chemical name : 2-(acetyloxy) benzoic acid

Description : White crystalline powder

Solubility : Slightly soluble in water, soluble in ethanol, methanol and DMSO

Melting point : 138 - 140 °C

Category : Analgesic, antipyretic

Storage : Stored at room temperature

Pharmacology:

The mechanism by which aspirin exerts its anti-inflammatory, analgesic and antipyretic actions. Aspirin and other non-steroid anti-inflammatory drugs (NSAIDs) inhibit the activity of the enzyme called cyclooxygenase (COX) which leads to the formation of prostaglandins (PGs) that cause inflammation, swelling, pain and fever. However, by inhibiting this key enzyme in PG synthesis, the aspirin-like drugs also prevented the production of physiologically important PGs which protect the stomach mucosa from damage by hydrochloric acid, maintain kidney function and aggregate platelets when required.

Pharmacokinetics:

Absorption:

Aspirin is absorbed rapidly from the stomach and intestine by passive diffusion. Aspirin is a prodrug, which is transformed into salicylate in the stomach, in the intestinal mucosa, in the blood and mainly in the liver. Salicylate is the active metabolite responsible for

most anti-inflammatory and analgesic effects (but acetylsalicylate is the active moiety for the antiplatelet-aggregating effect).

Distribution:

Salicylate distributes rapidly into the body fluid compartments. It binds to albumin in the plasma. With increasing total plasma salicylate concentrations, the unbound fraction increases. Salicylate may cross the placental barrier and distributes into breast milk.

Metabolism:

Aspirin is rapidly biotransformed into the active metabolite, salicylate. Therefore, aspirin has a very short half-life. Salicylate, in turn, is mainly metabolized by the liver. This metabolism occurs primarily by hepatic conjugation with glycin or glucuronic acid, each involving different metabolic pathways. The predominant pathway is the conjugation with glycin, which is saturable.

Excretion:

Urinary excretion of unchanged salicylate accounts for 10% of the total elimination of salicylate. Excretion of salicylate results of glomerular filtration, active proximal tubular secretion through the organic acid transporters and passive tubular reabsorption. Salicylate metabolites are also excreted in the urine.

Literature survey [133-141]:

- **IP method of Assay**

  Column: ODS C18 (25cm x 4.6 mm 5µ) column

  Mobile Phase: 0.2: 60:40 (Phosphoric acid+acetonitrile+water)

  Flow rate: 1.0ml/min, λ max =237 nm, Detector: UV

- **Development and validation of HPLC method for the simultaneous determination of aspirin**

  Column: Hypersil BDSC18 (100 x 4.6 mm 5µ) column

  Mobile Phase: mixing sodium chlorate buffer (pH 2.5), acetonitrile and isopropyl alcohol (85:1:14 %)

  Flow rate: 1.5 ml/min, λ max =275 nm, Detector: UV

- **Kinetic spectrophotometric determination of acetylsalicylic acid in dosage form "ACELYSIN-KMP"**

  Method: kinetic spectrophotometric method

  Reagents: indicator reaction of catalytic p-phenetidine oxidation by hydrogen peroxide in a weak alkaline medium

  λ max =358 nm

- **A Validated Stability Indicating HPTLC Method for Determination of Aspirin and Clopidogrel Bisulphate in Combined Dosage Form**

  Stationary Phase: TLC aluminum plates precoated with silica gel 60 F254

  Mobile phase: carbon tetrachloride-acetone (6: 2.4 v/v)

  λ max =220 nm, Detector: UV

- **Stability-indicating HPLC method for simultaneous determination of aspirin and prasugrel**

  Column: Kromasil 100 C_{18} (150×4.6 mm, 5 µ)

  Mobile Phase: acetonitrile: methanol: water (30:10:60, v/v), pH 3.0 with OPA

  Flow rate: 1.0 ml/min, Detector: UV and PDA

- **Development and validation of RP-HPLC method for simultaneous determination of metoprolol and aspirin in fixed dose combinations**

  Column: Phenomenex-Luna, C18 (250 mm x 4.6 mm i.d., 5 µm)

  Mobile Phase: phosphate buffer (pH adjudted to 4.6 with ortho-phosphoric acid): methanol (20:80 v/v)

  Flow rate: 0.8 ml/min, λ max =230 nm, Detector: UV

- **Development and validation of a RP-HPLC method for determination of atorvastatin calcium and aspirin in a capsule dosage form**

  Column: phenomenex Gemini C-18, 5 mm column having 250 x 4.6 mm i.d

  Mobile Phase: 0.02 M potassiumdihydrogen phosphate: methanol (20:80) adjusted to pH 4 using ortho phosphoric acid

  Flow rate: 1.0 ml/min, λ max =240 nm, Detector: UV

- **RP-HPLC Method for Simultaneous Estimation of Atorvastatin Calcium, Losartan Potassium, Atenolol, and Aspirin From Tablet Dosage Form and Plasma**

  Column: KYA TECH HiQSil C18HS (5µ particles size, 25 cm X 4.6 mm)

  Mobile phase: acetonitrile: 0.02 M potassium dihydrogen phosphate buffer (pH 3.4) (70:30% v/v), Flow rate: 1 ml/min, λ max = 236nm.

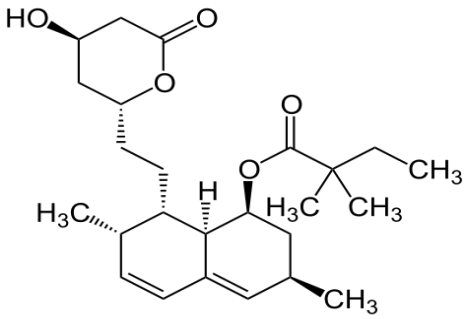
- **Quantitative Application to a Polypill by the Development of Stability Indicating LC Method for the Simultaneous Estimation of Aspirin, Atorvastatin, Atenolol and Losartan Potassium**

  Column: Intersil ODS c18 (5µ particles size, 150mm X 4.6 mm)

  Mobile phase: Mobile phase A: 0.1 % Orthophosphoric acid pH 2.9 adjusted with TEA, Mobile phase B- Acetonitrile:

  Flow rate: 1 ml/min, λ max = 230 nm.

3.10 Simvastatin[142-144]:



Molecular formula : $C_{25}H_{38}O_5$

Molecular weight : 418.57

Chemical name : butanoic acid, 2,2-dimethyl-,1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-(tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl)-ethyl]-1-naphthalenyl ester, [1S-[1α,3α,7β,8β(2S*,4S*),-8aβ]].

Description : White to off-white, non-hygroscopic, crystalline powder

Solubility : Freely soluble in chloroform, methanol and ethanol.

Melting point : 135-138 °C

Category : lipid-lowering agent (HMG CoA reductase inhibitors or "statins")

Storage : Store at room temperature. Protect from light.

Pharmacology:

Simvastatin is a prodrug and is hydrolyzed to its active β-hydroxyacid form, simvastatin acid, after administration. Simvastatin is a specific inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the enzyme that catalyzes the conversion of HMG-CoA to mevalonate, an early and rate limiting step in the biosynthetic pathway for cholesterol. In addition, simvastatin reduces VLDL and TG and increases HDL-C.

Pharmacokinetics:

Absorption:

T_{max} is 4 h. Bioavailability is less than 5%.

Distribution:

Approximately 95% bound to plasma proteins.

Metabolism:

Simvastatin undergoes extensive first pass metabolism in the liver. Rapidly hydrolyzed in the beta-hydroxyacid of simvastatin and its 6′-hydroxy, 6′-hydroxymethyl, and 6′-exomethylene derivatives.

Excretion:

13% is excreted in urine; 60% in feces.

Literature survey [145-157]:

- **IP method of Assay**

  Column: ODS C18 (3.3cm x 4.6 mm 3µ) column

  Mobile Phase: Mobile phase: A solution: 50.50 (ACN : 0.1 % OPA) ,B solution o.1 % OPA in acetonitrile Gradient

  Flow rate: 3ml/min, λ max =238 nm

- **Development and Validation of Reversed Phase HPLC Method for Estimation of Simvastatin in Pharmaceutical Dosage Form**

  Column: Promosil C-18, (250 mm, 4.6 mm, 5µm)

  Mobile phase: buffer: methanol pH 6.8 (96: 4 v/v)

  Flow rate: 1.0 mL/min

  λ max = 254 nm, Detector: UV

- **Spectrophotometric Methods for Estimation of Simvastatin in Bulk Drug and Its Dosage Form**

  Method: Spectrophotometric Method

  Solvent: Methanol, 2-propanol and conc.H2SO4

  λ max =230, 236 and 415 nm

- **Development and Validation of HPLC Method for the Determination of Simvastatin in Bulk and Pharmaceutical Formulation**

  Column: Poroshell SB C18 column (150x4.6 mm, 2.7 µm)

  Mobile Phase: methanol and 0.1% ortho phosphoric acid in water (10:90)

  Flow rate: 1.0 ml/min, λ max =238nm, Detector: UV.

- **Analysis of Simvastatin using a Simple and Fast High Performance Liquid Chromatography-Ultra Violet Method: Development, Validation and Application in Solubility Studies**

  Column: 4.6 mm x 150 mm, 5µ particle size

  Mobile Phase: methanol and 0.01M KH_2PO_4 phosphate buffer (80:20) and pH 5.5 with 2M phosphoric acid

  Flow rate: 1.0 ml/min, λ max =238nm, Detector: UV

- **Determination of Simvastatin in Pharmaceutical Dosage Forms by Optimized and Validated Method Using HPLC/UV**

  Column: Phenomenex ODS Luna column (250 mm × 4.6 mm I.D. particle size 5 µm) and Hypersil ODC column (250 mm × 4.6 mm I.D. particle size 5 µm)

  Mobile Phase: acetonitrile/water (100:0 v/v) and methanol/water (97:3 v/v)

  Flow rate: 1.5 ml/min

  λ max =238nm, Detector: UV

- **RP-HPLC Method for Simultaneous Estimation of Simvastatin and Ezetimibe in Bulk Drug and its Combined Dosage Form**

  Column: Luna C18 column (250×4.6 mm i.d.)

  Mobile Phase: methanol: water: acetonitrile (75: 18.75: 6.25 % v/v/v)

  Flow rate: 1.8 ml/min, λ max =231 nm, Detector: UV-PDA

- **Simultaneous HPTLC Estimation of Simvastatin and Ezetimibe in Tablet Dosage Form**

  Stationary phase: silica gel 60 F-254

  Mobile phase: mixture of chloroform and methanol (9.5: 0.5 %v/v)

  λ max = 254 nm, Detector: UV

- **Second-Derivative UV Spectrometric Determination of Simvastatin in its Tablet Dosage Form**

  Method: Second derivative UV spectroscopic method

  Solvent: Methanol, λ max = 243 nm

- **High-Performance Liquid Chromatographic Determination of Simvastatin in Medical Drugs**

  Column: C18-Hypersil column (250 × 4.6 mm, i.d. 5 µm)

  Mobile phase: Acetonitril–phosphate buffer–methanol (50 : 30 : 10, v/v/v)

  Flow rate: 2.5 mL/min, λ max = 230 nm, Detector: UV

- **Stability Indicating RP-HPLC Method for Simultaneous Determination of Simvastatin and Ezetimibe from Tablet Dosage Form**

  Column: C18 ODS Hypersil column

  Mobile phase: acetonitrile: phosphate buffer (pH 4.5, 0.01M) (65:35 v/v)

  Flow rate: 1.0 mL/min, λ max = 232 nm and at 238 nm, Detector: UV

- **A Simple RP-HPLC Method for Simultaneous Analysis of Aspirin, Atenolol, Hydrochlorothiazide, Ramipril and Simvastatin in Pharmaceutical Solid Dosage Forms**

  Column: X-Terra C18 column (5µ particles size, 150 cm X 4.6 mm)

  Mobile phase: Solution A: Sodium Per chlorate, Solution B: acetonitrile

  Flow rate: 0.8 ml/min, λ max = 220 nm

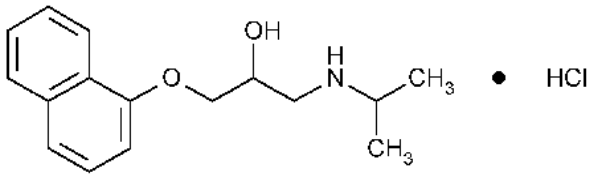
- **Simultaneous Determination of Ramipril, Atenolol, Hydrochlorothiazide, Simvastatin and Aspirin in Ramipril, Atenolol, Hydrochlorothiazide, Simvastatin as Tablets and Aspirin as Enteric Coated Tablets in Capsules by RP-HPLC**

  Column: X-Terra C18 column (5µ particles size, 150 cm X 4.6 mm)

Mobile phase: Solution A: Sodium Per chlorate pH 2.2 adjust with OPA, Solution B: acetonitrile

Flow rate: 1.5 ml/min, λ max = 210 nm

3.11 Propranolol Hydrochloride [158-160]:



Molecular formula : $C_{16}H_{21}NO_2 \cdot HCl$

Molecular weight : 295.80

Chemical name : (+)-1-(isopropylamino)-3-(1-naphthyloxy)-2-propanol hydrochloride

Description : White, crystalline solid

Solubility : Soluble in water and ethanol

Melting point : 163-165 °C

Category : Beta-adrenergic receptor blocking agent

Storage : Store in a well closed container, Below 40°C. Protect from Sunlight and Moisture

Pharmacology:

Propranolol competes with sympathomimetic neurotransmitters such as catecholamines for binding at beta (1)-adrenergic receptors in the heart, inhibiting sympathetic stimulation. This results in a reduction in resting heart rate, cardiac output, systolic and diastolic blood pressure, and reflex orthostatic hypotension.

Pharmacokinetics:

Absorption:

Propranolol is almost completely absorbed from the GI tract; however, plasma concentrations attained are quite variable among individuals.

Distribution:

Approximately 90% of circulating propranolol is bound to plasma proteins (albumin and alpha-1-acid glycoprotein). The binding is enantiomer-selective. The S(-)-enantiomer is preferentially bound to alpha-1-glycoprotein and the R(+)-enantiomer is preferentially bound to albumin. The volume of distribution of propranolol is approximately 4 liters/kg.

Metabolism:

Propranolol is metabolized through three primary routes: aromatic hydroxylation (mainly

4-hydroxylation), N-dealkylation followed by further side-chain oxidation, and direct glucuronidation.

Excretion:

Propranolol is extensively metabolized with most metabolites appearing in the urine.

Literature survey [161-168]:

- **IP method of Assay**

  Column: ODS C18 (25cm x 4.6 mm 5µ) column

  Mobile Phase: SDS, tetrabutyl ammonium dihydrogen phosphate+sulphuric acid: ACN (45:55 v/v)

  Flow rate: 1.8ml/min, λ max =292 nm

- **Quantitative Analysis of Propranolol Hydrochloride by High Performance Thin Layer Chromatography**

  Stationary phase: aluminium backed silica gel 60 $F_{254\ TLC}$ plates

  Mobile phase: Isopropanol:ethyl acetate:ammonia (1:8.5:0.5 v/v/v)

  λ max = 290 nm, Detector: UV

- **An HPLC-ESI-MS method for the determination of propranolol in human plasma and its application to pharmacokinetic studies**

  Column: Agilent ZORBAX Extend-C18 column (5 µm, 150 mm × 2.1 mm i.d)

  Mobile phase: 10 mmol/l ammonium acetate buffer containing 0.1% formic acid-methanol (55: 45, v/v)

  Flow rate: 0.25 ml/min, Detector: Ion monitoring (SIM) mode

- **Development and validation of HPLC method for estimation of propranolol HCl in human plasma**

  Column: C18 column (300 mm x 3.9 mm i.d., 5 µm)

  Mobile phase: acetonitrile: pH 4.5 phosphate buffer (35:65)

  Flow rate: 1.0 ml/min, λ max = 214 nm, Detector: PDA

- **UV Spectrophotometric Method Development and Validation for Simultaneous Determination of Flunarizine Dihydrochloride and Propranolol Hydrochloride in Combined Capsule Dosage Form**

  Method: UV - spectrophotometric isobestic point

  Solvent: methanol

  λ max = 262.5 nm and 288.8 nm, Detector:UV

- **Simultaneous Determination of Propranolol Hydrochloride and Flunarizine Dihydrochloride in Bulk and Capsule Using Reversed - Phase High Performance Thin Layer Chromatography / Densitometry**

  Stationary phase: silica gel 60 RP-18 F254S HPTLC plates

Mobile phase: methanol: toluene: ammonia (7:3:0.5 v/v)

λ max = 267 nm, Detector: UV

- **Simultaneous high-performance liquid chromatographic assay of furosemide and propranolol HCL and its application in a pharmacokinetic study**

  Column: Nucleosil C18 column

  Mobile phase: 0.02 M potassium dihydrogen phosphate and acetonitrile (80:20, v/v) adjusted to pH 4.5

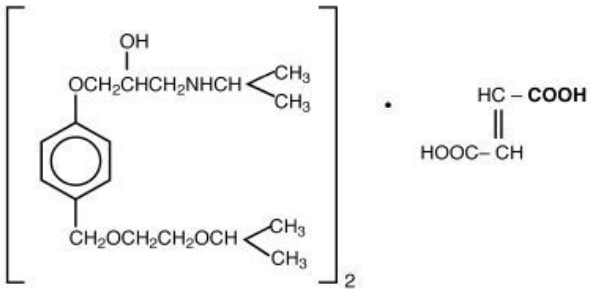
  Flow rate: 1.0 ml/min, λ max =235 nm, Detector: UV

- **Validated RP HPLC Method for Simultaneous Determination of Propranolol hydrochloride and Alprazolam in Bulk and in Pharmaceutical formulations** Column: Waters C18 column (250 x 4.6 mm, i.d. 5 Î¼)

  Mobile phase: acetonitrile: water (adjusted to pH 2.3 with ortho phosphoric acid) in the ratio of 60:40 v/v

  Flow rate: 1.0 ml/min, λ max = 214 nm, Detector: UV

3.12 Bisoprolol Fumarate [169-170]:



Molecular formula : $(C_{18}H_{31}NO_4)_2 \bullet C_4H_4O_4$

Molecular weight : 766.97

Chemical name : (±)-l-[4-[[2-(l-methylethoxy)ethoxy]methyl]phenoxy]-3-[(l-methylethyl) amino]-2-propanol(E)-2-butenedioate (2:1) (salt).

Description : White crystalline powder

Solubility : soluble in water, methanol, ethanol, and chloroform

Melting point : 100 ^oC

Category : Beta1-adrenergic blocker used as an Antihypertensive

Storage : Store at room temperature. Protect from light

Pharmacology:

Bisoprolol selectively blocks catecholamine stimulation of β1-adrenergic receptors in the heart and vascular smooth muscle. This result in a reduction of heart rate, cardiac output, systolic and diastolic blood pressure, and possibly reflexes orthostatic hypotension. At higher doses (e.g. 20 mg and greater) bisoprolol may competitively block β2-adrenergic

receptors in bronchial and vascular smooth muscle causing bronchospasm and vasodilation.

Pharmacokinetics:

Absorption:

Bisoprolol is almost completely (>90%) absorbed from the gastrointestinal tract and, because of its small first pass metabolism of about 10%-15%, has an absolute bioavailability of about 85-90% after oral administration. The bioavailability is not affected by food. Peak plasma concentrations occur within 2-3 hours.

Distribution:

Bisoprolol is extensively distributed. The volume of distribution is 3.5 L/kg. Binding to plasma proteins is approximately 35%; uptake into human blood cells was not observed.

Metabolism:

Approximately 50% of the dose is metabolized primarily metabolized by CYP3A4 to inactive metabolites. In vitro studies, bisoprolol is also metabolized by CYP2D6 though this does not appear to be clinically significant. Approximately half the administered dose is excreted in unchanged in urine.

Excretion:

The total clearance of the drug is 15.6 ± 3.2 L/h with renal clearance being 9.6 ± 1.6 L/h. In a study with 14C-labelled bisoprolol the total urinary and fecal excretion was 90 ± 2.7% and 1.4 ± 0.1% of the dose, respectively (mean ± SEM recoveries of the total dose within 168 hours). Bisoprolol has an elimination half-life of 10-12 hours.

Literature survey [171-177]:

- **USP method of assay**

  Column: stainless steel C18 column (5µ particles size) (12.5 cm X 4.6 mm)

  Mobile phase: Water: ACN (65:35) heptafluorobutyric acid: diethylamine:formic acid

  Flow rate: 1.0ml/min, λ max = 273 nm

- **Development and Validation of RP-HPLC Method for the Determination of Bisoprolol Fumarate Tablets**

  Column: Promosil C-18, (250 mm, 4.6 mm, 5µm)

  Mobile phase: buffer (pH 5.6) and acetonitrile (750: 250)

  Flow rate: 1.0 mL/min, λ max = 226 nm, Detector: PDA

- **Spectrophotometric Determination of Bisoprolol Using Methyl Orange as Reagent**

  Method: Spectrophotometric method

  Solvent: Methyl orange, in acid medium, λ max = 427 nm

- **Estimation of Bisoprolol Fumarate in Pharmaceutical Preparations by HPTLC** Stationary Phase: precoated 60 F254 silica gel on aluminum sheet
  Mobile phase: Methanol: Toluene: Ammonia (2:4:0.1, v/v/v)
  λ max = 229 nm, Detector: UV
- **RP-HPLC method for simultaneous estimation of bisoprolol fumarate and hydrochlorothiazide in tablet formulation**
  Column: Inertsil ODS 3V (25 cm × 4.6 mm) 5 μm
  Mobile phase: 0.1 M KH_2PO_4 buffer and acetonitrile (70:30, v/v)
  Flow rate: 1.0 ml/min, λ max = 228 nm, Detector: UV
- **Development and Validation of a Simultaneous HPLC Method for Estimation of Bisoprolol Fumarate and Amlodipine Besylate from Tablets**
  Column: Luna C18-2 column (3 μ, 50×4.6 mm ID)
  Mobile phase: 25 mM ammonium acetate adjusted to pH 5.0 and methanol (65: 35), Flow rate: 0.8 ml/min,λ max = 230 nm, Detector: UV
- **High Performance Thin Layer Chromatographic Method for Simultaneous Estimation of Amlodipine Besilate and Bisoprolol Fumarate in Pharmaceutical Preparations**
  Stationary phase: HPTLC plate precoated 60 F254 silica gel on aluminum sheet
  Mobile phase: ethyl acetate: methanol: ammonia (6:0.5:0.5 v/v/v)
  λ max = 229 nm, Detector: UV

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Development of analytical methods for the simultaneous analysis of anti-hypertensive formulations containing two components by HPLC

Introduction [1-7]:

Combination therapy rises as an alternative for the patients who fail to lower their blood pressure by monotherapy. Obtaining the target blood pressure level by monotherapy can be challenging, especially for the patients who are suffering from other diseases. It is well established that a majority of hypertensive patients need two or more antihypertensive drugs to lower blood pressure effectively. Fixed-dose formulation can be defined as a single pharmaceutical formulation containing several active agents.

Fixed dose combinations as a promising choice to hypertensive patients may have some potential superiorities like synergistic effect in a perfect combination, convenience and compliance, less side effects and low cost. Since drugs in formulations from different classes exert their effect based on individual mechanism with different action sites and action time, fixed dose combinations in hypertension have a potential for modest and a long term action.

There are several common drug classes which can be used in tackling hypertension: such as beta (β)–blockers, calcium channel blockers (CCB's), angiotensin-converting enzyme (ACE) inhibitors and diuretics. Since the use of fixed-dose combinations in hypertension is being new trend and method in clinical practice, more and more combination products are developed in treating hypertension especially with combination of drugs comprising of diuretics and a beta (β)–blockers /calcium channel blockers (CCB's) or a calcium channel blockers (CCB's) and an angiotensin-converting enzyme (ACE) inhibitor.

The combination of a beta blocker and a diuretic produces additive effects compared with monotherapy using either agent alone. It is known that beta blockers can reduce cardiac output, inhibit the secretion of renin and also cause retention of sodium and water. Diuretics can cause mild volume reduction that leads to an increase in renin secretion by the kidney. The rationale for combining beta blockers with diuretics is twofold: beta blockers blunt the increase in the plasma renin level that is induced by diuretics, and diuretics decrease the sodium and water retention that is caused by beta blockers. e.g Nebivolol hydrochloride and Indapamide, Atenolol and Indapamide, Bisoprolol and hydrochlorothiazide, Propranolol and hydrochlorothiazide, Metoprolol and hydrochlorothiazide etc.

Calcium channel blockers work by interrupting the movement of calcium into the heart and blood vessel cells, which in turn decreases the force of contraction of the myocardium (muscle of the heart) and relaxes and widens blood vessels. Calcium channel blockers

increases renal sodium excretion, although not to the same extent as diuretics. Moreover, long –term treatment with both classes is associated with vasodilation, given that volume depletion does not occur with diuretics. e.g Amlodipine and Indapamide and Amlodipine and Hydrochlorothiazide etc.

ACE inhibitors and calcium channel blockers work effectively in combination to lower blood pressure. Angiotensin-converting enzyme (ACE) inhibitors widen blood vessels and decrease the workload of the heart. They treat high blood pressure and can also help to protect the heart and kidneys. Calcium-channel blockers (CCBs), or calcium antagonists, exert their antihypertensive effect through a vasodilatory action. This is useful in the treatment of angina (chest pain) and some arrhythmias (abnormal heart rhythms). e.g Amlodipine and Ramipril, Amlodipine and Benazepril, Amlodipine and Enalapril, Amlodipine and Lisinopril etc.

In view of the above concepts and considering the need to develop new analytical methods, the following combinations were selected for the present study.

Sr. No.	Combination	Brand name	Manufacturer	Label Claim
1	Nebivolol HCl and Indapamide	Nebula-D Tablets	Zydus Cadila Healthcare Ltd.	Nebivolol HCl IP 5.0 mg Indapamide USP 1.5 mg
2	Amlodipine besylate and Indapamide	Amlodac -D Tablet	Zydus Cadila Healthcare Ltd.	Amlodipine IP 5.0 mg Indapamide USP 2.5 mg
3	Amlodipine besylate and Hydrochlorothiazide	Amlokin d-H Tablet	Mankind Pharmaceuticals Pvt. Ltd.	Amlodipine 5.0 mg Hydrochlorothiazide 12.5 mg
4	Amlodipine besylate and Ramipril	Stamace Capsule	Dr. Reddy's Laboratory Ltd.	Amlodipine 5.0 mg Ramipril 2.5 mg

Literature survey revealed that a numerous methods have been reported for estimation of 1. Nebivolol hydrochloride and Indapamide, 2. Amlodipine besylate and Indapamide, 3. Amlodipine besylate and Hydrochlorothiazide and 4. Amlodipine besylate and Ramipril individually or in combination with each other and also with other drugs.

However, reported methods for the above combinations suffer from certain drawbacks like buffered mobile phases which reduce the column life and time consuming preparation methods, pH adjustment, linearity at higher concentration ranges and reproducibility of the methods. The purpose of the present study was to develop methods for the above combinations which eliminate the drawbacks mentioned above. Hence reliable and rugged method for the simultaneous analysis of drugs from above combinations was taken up.

4.1 Simultaneous analysis of Nebivolol hydrochloride and Indapamide by HPLC

Nebivolol hydrochloride, (1RS, 1′RS)-1, 1′-[(2RS, 2′SR)-bis (6-fluoro-3, 4-dihydro-2H-1-benzopyran-2-yl)] - 2, 2′-iminodiethanol hydrochloride, is a highly cardioselective vasodilatory beta-1 receptor blocker used in treatment of hypertension. Indapamide, benzamide, 3-(aminosulfonyl)-4-chloro-*N*-(2, 3-dihydro-2-methyl-1*H*-indol-1-yl) -4-chloro-*N*-(2-methyl-1-indolinyl)-3-sulfamoylbenzamide, is classed as a diuretic and antihypertensive agent.

The drug profiles and literature survey of Nebivolol HCl (NEB) and Indapamide (IND) are given in 3.1 and 3.2.

4.1.1 Experimental:

- **Instrumentation:**

  The HPLC system consisted of Intelligent HPLC pump model (Jasco PU 1580). The solutions were injected into the chromatograph through a Rheodyne valve, with a 20 µL loop with auto sampler (AS 1555). The column used was Thermo Hypersil Gold C_{18} (250 mm × 4.6 mm, 5.0 µ), operating at room temperature. The elution was carried out isocratically at flow rate of 1mL/min. using methanol: water (80:20 v/v) as mobile phase. The detector consisted of a UV/ VIS (Jasco UV 1575) model operated at a wavelength of 265 nm. Data was integrated using Jasco Borwin version 1.5, LC-Net II/ADC system.

- **Chemicals and reagents:**

  Reference standards of Nebivolol hydrochloride (99.3%) and Indapamide (99.4%) were procured from Torrent Pharmaceuticals Ltd, and Emcure Pharmaceuticals Ltd., respectively. The tablets Nebula-D (Nebivolol hydrochloride-5 mg, Indapamide-1.5 mg) of Zydus Cadila Healthcare Ltd. was procured from the market. For HPLC work double distilled water was prepared in the laboratory. Methanol used was of HPLC grade and was purchased from Merck Chemicals, Mumbai, India.

- **Stationary Phase:**

  Thermo Hypersil Gold C_{18} (250 mm × 4.6 mm, 5.0 µ) was used.

- **Preparation of mobile phases:**

  HPLC grade methanol (800 mL) was mixed with (200 mL) of double distilled water. This solution was filtered through 0.45µ membrane filter paper and degassed in an ultrasonic bath before use.

- **Preparation of standard stock solution-I:**

  50 mg of Nebivolol HCl (NEB) and 15 mg of Indapamide (IND) were accurately weighed and transferred to a 50 mL volumetric flask and dissolved in methanol. Volume was made up to the mark with mobile phase to get a solution containing NEB 1 mg/mL and IND 0.3 mg/mL respectively. 1 mL of this solution was further diluted to 10 mL with mobile phase to get **Standard stock solution-I** containing NEB 0.1 mg/mL and IND 0.03 mg/mL, respectively.

- **Working standard solution-II:**

  1 mL of the standard stock solution-I was diluted to 10 mL with mobile phase to get a concentration of 10 µg/mL of NEB and 3 µg/mL of IND. This solution was used as working standard.

- **Sample solution:**

  Twenty tablets were weighed accurately and powered. Tablet powder equivalent to 5 mg of NEB and 1.5 mg of IND was weighed accurately and transferred into a clean and dry 50 mL volumetric flask. The powder was first dissolved in sufficient volume of mobile phase and kept in an ultrasonic bath for 15 min. and the volume was made to 50 mL with mobile phase, filtered through 0.45µ membrane filter paper and solution was further diluted with mobile phase to obtain a solution of NEB (10 µg/mL) and IND (3 µg/mL), respectively. The sample solution (20 µL) was injected into the chromatograph and the chromatogram was recorded.

- **System suitability:**

  20 µL of standard solution containing NEB 10 µg/mL and IND 3 µg/mL was injected into the chromatograph to test the system suitability to determine resolution (Rs), retention time (t_R), theoretical number of plates (N) and tailing factor (T).

- **Analytical Method Validation:**

  Validation was done as per ICH guideline. The developed method was validated with respect to parameters such as linearity, LOD and LOQ, precision, accuracy and specificity.

- **Linearity:**

  Several aliquots of standard stock solution-I of NEB and IND were taken in different 10 mL volumetric flask and diluted up to the mark with mobile phase such that the final concentration of NEB and IND were in the range of 10–60 µg/mL and 3-18 µg/mL respectively. Evaluations of two drugs were performed with UV detector set at 265 nm. The peak area versus concentration data was collected and subjected to regression analysis. The correlation coefficient, slope and y-intercept values were calculated from the calibration plot obtained.

- **Limit of detection and limit of quantitation:**

  The limit of detection (LOD) and limit of quantitation (LOQ) for NEB and IND estimated at a signal-to-noise ratio of 3:1 and 10:1 respectively, by injecting a series of dilute solutions with known concentration.

- **Precision:**

  The precision of the method was verified by intra-day and inter-day precision studies. Intra-day precision studies were performed by analysis of three different concentrations 20, 40, 60 µg/mL for NEB and 6, 12, 18 µg/mL for IND three times on the same day. The inter-day precision of the method was checked by repeating studies on three different days.

- **Robustness:**

  To determine the robustness of the developed method, experimental conditions were deliberately changed by changing the composition and flow rate of the mobile phase. The relative standard deviation for replicate injections of NEB and IND were evaluated. Flow rate was changed by ± 0.1 units to 0.9 and 1.1 mL/min. The effect of % of methanol in the mobile phase was studied at (± 5%).

- **Recovery studies:**

  To check the accuracy of the proposed method recovery experiment was carried out. Twenty tablets from the same lot of the commercial formulation were weighed accurately and finely powered. Powder equivalent to 5 mg of NEB and 1.5 mg of IND was weighed and transferred to different 50 mL volumetric flask. Standard drug was added at 80%, 100% and 120% level. The contents were dissolved by sonicating in 20 mL of mobile phase for 15 minutes and filtered through 0.45µ membrane filter paper and further diluted up to the mark using mobile phase. 1 mL of the filtrate was taken in 10 mL volumetric flask and diluted up to the mark with mobile phase. 20µL of sample solution was injected into the chromatograph and chromatograms were

recorded. Percentage recovery and relative standard deviation were calculated. Each level was repeated three times.

- **Analysis of marketed formulation:**

  20 µL of working standard solution-II (NEB 10 µg/mL and IND 3 µg/mL) and sample solutions were injected into the liquid chromatograph and the chromatograms were recorded. From the peak area of NEB and peak area of IND the amount of the drugs in the sample were calculated.

- **Specificity:**

  The specificity of the method was ascertained by analysing drug standards and samples. The chromatogram was overlaid to study the specificity. The identities of the peak for NEB and IND from the sample were confirmed by comparing the t_R with those of standards.

- **Comparison of the proposed method with the reported methods:**

  The proposed method was compared with the following reported methods from the literature:

1 Method development and validation of indapamide and nebivolol hydrochloride by RP-HPLC method[8]

  Column: Kromosil C_{18} column (250 × 4.6 mm i.d., 3 µm particle size)

  Mobile phase: Ammonium acetate buffer pH 4.5: Methanol (45:55 v/v)

  Flow rate: 1.0 mL/min, Detector wavelength: 226 nm

2 Development and validation of high performance liquid chromatography method for simultaneous estimation of nebivolol and indapamide in their combined tablet dosage form[9]

  Column: BDS Hypersil C_{18} column (250 × 4.6 mm i.d., 3 µm particle size)

  Mobile phase: (0.05M KH_2PO_4) buffer-pH 3.5: triethylamine: ACN (40: 0.5: 60 v/v/v), Flow rate: 1.0 mL/min, Detector wavelength: 286 nm

4.1.2. Results and Discussion:

- **Selection of column:**

  As the drugs were soluble in polar solvents such as methanol and acetonitrile, C_{18} reverse phase column was selected with particle size of 5.0 µ, length 250 mm and internal diameter 4.6 mm.

- **Selection of suitable wavelength:**

  Stock solutions of individual drugs were prepared in methanol and the solutions were scanned individually in a UV spectrophotometer in the range of 200-400 nm and their spectras were recorded. The spectras were overlaid and the isobestic point, 265 nm was selected as the suitable wavelength as both the drugs showed good absorption (Fig.4.01) at this wavelength. The wavelength was checked and confirmed in the optimised mobile phase also.

- **Selection of mobile phase:**

  Initially different mobile phase compositions were tried using methanol and water such as 50:50 v/v, 60:40 v/v and 70:30 v/v. But these trails did not result in good peak shape and showed tailing and fronting. After several trails satisfactory peak shape was obtained with methanol: water in the ration of 80:20 v/v and was selected for further study. The selected mobile phase was found to resolve peaks of NEB (t_R-7.767min.) and IND (t_R -3.383 min.), respectively.

- **System suitability:**

  System suitability tests were carried out using freshly prepared standard stock solution of NEB and IND to determine efficiency, resolution factor, symmetry factor and precision of the instrument and found to comply with USP requirement. Parameters obtained with 20 µL injection volume are shown in (Table 4.01). Column efficiency in term of theoretical plates number (N) was 4632.70 for IND and 3643.01 for NEB, respectively.

- **Validation of the method:**

  Using optimised chromatographic conditions the RP-HPLC method developed was validated in terms of linearity, LOD and LOQ, precision, robustness and accuracy.

  - **Linearity:**

    Linear regression data for the calibration plots revealed good linear relationships between response and concentration over the ranges 10–60 µg/mL for NEB and 3-18 µg/mL for IND, respectively. The linear regression equations were Y=2408X - 5688

(r^2= 0.9992) for NEB and Y= 4040X +380.0 (r^2= 0.9991) for IND. The residuals plot shows a random pattern, indicating a good fit for a linear model. The calibration curves and residual plots are given in Fig.4.03 and Fig.4.04 for NEB and IND, respectively and Table 4.02 (A), and Table 4.02 (B).

- **Limits of Detection and Quantitation:**

  The signal to noise ratios of 3: 1 and 10: 1 were considered as LOD and LOQ respectively. The results of the LOD and LOQ were found to be 1.0 µg/mL, 2.2 µg/mL and 0.1 µg/mL, 0.5 µg/mL for NEB and IND, respectively.

- **Precision:**

  The precision of the developed method was demonstrated by intra-day and inter-day precision studies. This was done by three replicate analysis of the sample. The precision of the method was expressed as relative standard deviation (RSD, %). The % RSD of intra-day and inter-day precision values for NEB and IND were found to be 1.16 and 1.13 and 1.11 and 1.16 %, respectively (Table 4.03). The RSD values were found to be <2 %, which indicates that the proposed method is precise.

- **Robustness:**

  There were no significant changes in the retention times of NEB and IND when the composition of the mobile phase (± 5%) and flow rate (± 0.1 mL/min.) were changed. The low values of the % RSD indicate the robustness of the method, as shown in Table 4.04.

- **Accuracy:**

  To study the accuracy of the proposed method recovery experiment were carried out by standard addition method. The proposed method when used for extraction and subsequent estimation of NEB and IND from pharmaceutical dosage form after spiking with 80%, 100% and 120% of the additional drug afforded mean recovery of 99.43% for NEB and 99.26 % for IND. The mean recoveries indicate non-interference from excipients (Table 4.05).

- **Analysis of marketed formulation:**

  The chromatogram of the sample extracted from conventional tablets showed peaks of IND (t_R 3.383min.) and NEB (t_R -7.767 min) well resolved from other tablet excipients, shown in Fig. 4.02. The mean contents of NEB and IND per tablet by proposed method was found to be 4.980 mg and 1.488 mg, respectively. The results are summarized in Table 4.06.

- **Comparison of the proposed method with the reported methods:**

  The proposed method was compared with the reported methods. The proposed method uses simple mobile phase of methanol: water (80:20 v/v) as against buffered mobile phases used in the reported methods. The proposed method gave better resolution and low LOD and LOQ for NEB and IND than the reported methods. Accuracy and precision of the proposed method are comparable to the reported methods with low % RSD. (Table 4.07)

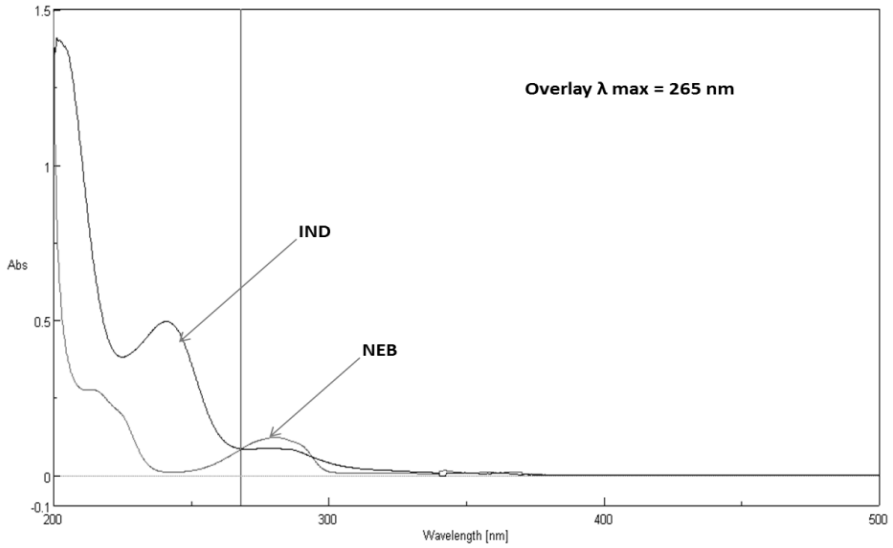

Fig.4.01. Overlay absorption spectra of NEB and IND

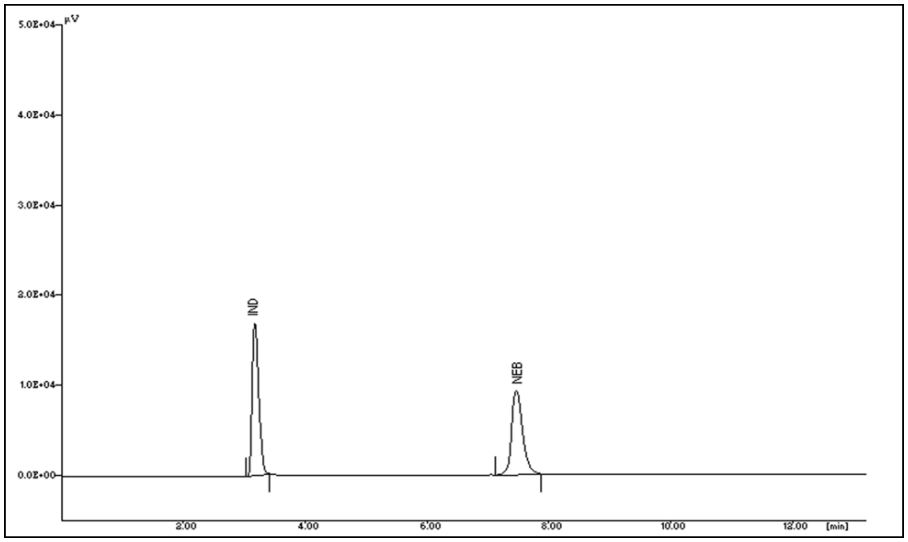

Fig.4.02. Typical chromatogram of NEB and IND

Table 4.01: System suitability parameters (n=6)

Parameters	**IND**	**NEB**
Retention Time (t_R) in min	3.383	7.767
Resolution (Rs)	--	9.921
Theoretical plates number (N)	4632.70	3643.01
Tailing Factor (T)	1.131	1.124

Table 4.02 (A): Data for NEB from linearity study

Concentration (µg/mL)	HPLC (n= 3)	
	Mean peak area ± SD	**RSD (%)**
10	20150.5 ± 209.57	1.04
20	41265.25 ± 45804.43	1.11
30	64934.4 ± 779.21	1.20
40	90602 ± 987.56	1.09
50	115752.5 ± 1308	1.13
60	138903 ± 1652.95	1.19
Regression coefficient r^2		**0.9992**
Slope		**2408**
Intercept		**- 5688**

Table 4.02 (B): Data for IND from linearity study

Concentration (µg/mL)	HPLC (n= 3)	
	Mean peak area ± SD	**RSD (%)**
3	12351.9 ± 145.75	1.18
6	24088.9 ± 255.34	1.06
9	37056.8 ± 407.62	1.10
12	49407.6 ± 597.83	1.21
15	61759.5 ± 722.59	1.17
18	72111.4 ± 814.86	1.13
Regression coefficient r^2		**0.9991**
Slope		**4040**
Intercept		**380.0**

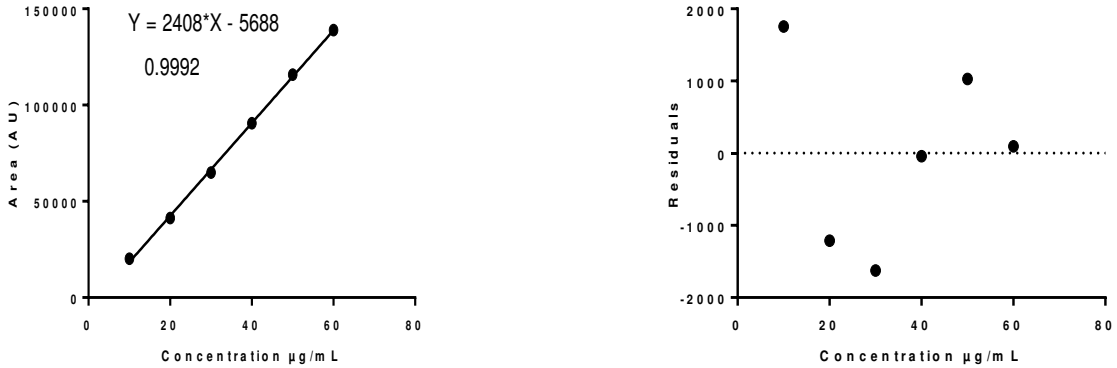

Fig.4.03. Calibration curve and residual plot for NEB

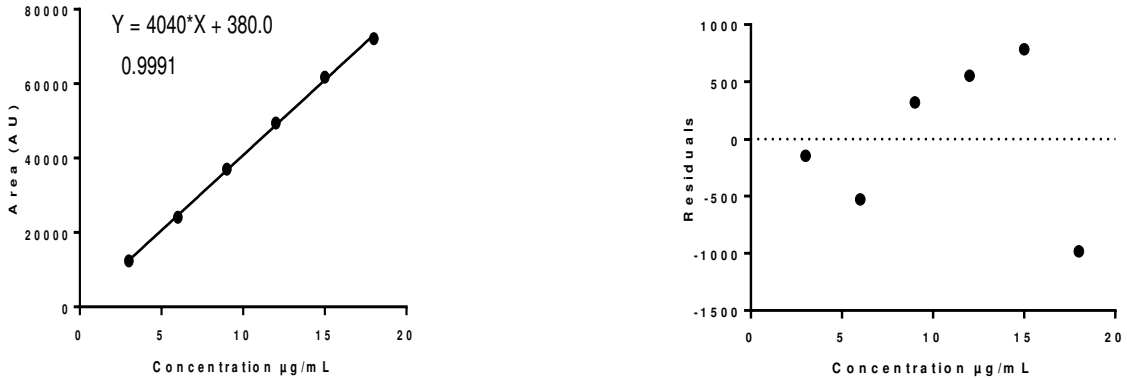

Fig.4.04. Calibration curve and residual plot for IND

Table 4.03: Precision studies of IND and NEB

Conc. (µg/mL)	Intra-day precision (n=3)			Inter-day precision (n=3)		
	Measured Conc. ± SD	(%) RSD	Recovery (%)	Measured Conc. ±SD	(%) RSD	Recovery (%)
Indapamide						
6	5.975 ± 0.062	1.04	99.58	5.964 ± 0.069	1.16	99.40
12	11.92 ± 0.140	1.17	99.33	11.980 ± 0.13	1.09	99.17
18	17.93 ± 0.201	1.12	99.61	17.90 ± 0.22	1.23	99.44
Nebivolol HCl						
20	19.85 ± 0.220	1.11	99.25	19.83 ± 0.24	1.21	99.15
40	39.69 ± 0.48	1.20	99.23	39.65 ± 0.40	1.01	99.13
60	59.70 ± 0.69	1.16	99.50	59.65 ± 0.70	1.17	99.42

Table 4.04: Robustness evaluation of IND and NEB (n=3)

Conditions	Level	IND		NEB	
		t_R (min.)	% RSD of peak area	t_R (min.)	% RSD of peak area
A: Flow rate (± 0.1 mL/min.)					
0.9	-0.1	3.400	1.01	7.800	1.14
1	0.0	3.383	1.13	7.767	1.10
1.1	+0.1	3.364	1.16	7.753	1.02
Mean ± SD		3.386 ± 0.013	1.10 ± 0.079	7.776 ± 0.021	1.087 ± 0.061
B: % of methanol in the mobile phase (± 5%)					
75	-5.0	3.412	1.11	7.801	1.08
80	0.0	3.383	1.07	7.767	1.03
85	+5.0	3.367	1.14	7.750	1.10
Mean ± SD		3.388 ± 0.022	1.11 ± 0.035	7.773 ± 0.026	1.07 ± 0.036

Table 4.05: Recovery study for IND and NEB (n=3)

Label claim (mg/tablet)	Amount Added (%)	Total amount (mg)	Amount recovered (mg)	(%) Recovery	Mean (%) Recovery (± SD)
IND 1.5	80	2.7	2.678	99.19	99.43 ± 0.022
	100	3	2.984	99.45	
	120	3.3	3.288	99.64	
NEB 5.0	80	9	8.93	99.22	99.26 ± 0.040
	100	10	9.93	99.30	
	120	11	10.92	99.27	

Table 4.06: Assay results of IND and NEB in tablets (n=3)

Nebula-D tablet contains	Label claim (mg/tablet)	Amount found (mg ± SD)	RSD (%)	Recovery (%)
IND	1.5 mg	1.488 ± 0.017	1.15	99.20
NEB	5 mg	4.980 ± 0.054	1.08	99.60

Table 4.07: Comparison of the proposed method with the reported methods:

Parameter	Reported method 1		Reported method 2		Proposed method	
	IND	**NEB**	**IND**	**NEB**	**IND**	**NEB**
Mobile Phase	Ammonium acetate buffer pH4.5: Methanol (45:55 v/v)		(0.05M KH_2PO_4) buffer-pH 3.5: triethylamine: ACN (40:0.5:60 v/v)		**Methanol: Water (80:20 v/v)**	
Mode	Isocratic		Isocratic		**Isocratic**	
Detection wavelength (nm) & Flow Rate	226 1.0 mL/min.		286 1.0 mL/min.		**265 1.0 mL/min.**	
Retention time (t_R) in min.	2.35	3.49	5.730	3.587	**3.383**	**7.767**
Linearity range (µg/mL)	9.0-21	30-70	7.5-22.5	25-75	**3.0-18**	**10-60**
LOD (µg/mL) and LOQ (µg/mL)	2.12 6.42	2.57 7.78	0.101 0.306	0.855 2.590	**0.1 0.5**	**1.0 2.2**
% Recovery (n=3)	100.25	100.01	99.47%	99.85	**99.43**	**99.26**
Precision (% RSD) Intra-day (n=3) Inter-day (n=3)	0.70-0.89 ----	0.49-0.78 ----	1.188 1.01	0.904 1.14	**1.11 1.16**	**1.16 1.13**

4.1.3. Conclusion:

In the present work, RP-HPLC method has been developed for simultaneous estimation of Nebivolol HCl and Indapamide from pharmaceutical dosage form. The method has been validated statistically for linearity, specificity, accuracy and precision. The common excipients and other additives are usually present in the tablet mixture do not interfere in the analysis of NEB and IND. The analytical conditions and solvent system developed provided a good separation for NEB and IND.

The proposed method uses simple mobile phase of methanol: water (80: 20 v/v) as against buffered mobile phases used in the reported methods which increases the life of column employed. The proposed method gives better resolution and low LOD and LOQ for NEB and IND than the reported methods. Accuracy and precision of the proposed method are comparable to the reported methods with low % RSD. The method has several advantages, including a simple mobile phase, easy sample preparation and lack of complicated procedures, in contrast with previous methods. Thus, the method can be proposed for routine analysis and for dissolution studies.

4.2 Simultaneous analysis of Amlodipine besylate and Indapamide by HPLC

Amlodipine besylate, 3, 5-Pyridinecarboxylic acid, 2-((aminoethoxy) methyl)-4-(2-chlorophenyl) -1, 4-dihydro-6-methyl, 3-ethyl-5-methyl ester, a calcium channel blocker used as an anti-hypertensive. Indapamide, Benzamide, 3-(aminosulfonyl)-4-chloro-N-(2,3-dihydro-2-methyl-1H-indol-1-yl)-4-chloro-N-(2-methyl-1-indolinyl)-3-sulfamoylbenzamide, is classed as a diuretic and antihypertensive agent.

The drug profiles and literature survey of Indapamide (IND) and Amlodipine besylate (AMLO) are given in 3.2 and 3.3.

4.2.1 Experimental:

- **Instrumentation:**

  The HPLC system consisted of Intelligent HPLC pump model (Jasco PU 1580). The solutions were injected into the chromatograph through a Rheodyne valve, with a 20 µL loop with auto sampler (AS 1555). The column used was Thermo Hypersil Gold C_{18} (250 mm × 4.6 mm, 5.0 µ), operating at room temperature. The elution was carried out isocratically at flow rate of 1mL/min. using methanol: water (80:20 v/v) as mobile phase. The detector consisted of a UV/ VIS (Jasco UV 1575) model operated at a wavelength of 238 nm. Data was integrated using Jasco Borwin version 1.5, LC-Net II/ADC system.

- **Chemicals and reagents:**

  Reference standards of Amlodipine besylate (99.92 %) and Indapamide (99.4%) were procured from Lupin Research Park and Emcure Pharmaceuticals Ltd., respectively. The tablets Amlodac-D (Amlodipine 5 mg, Indapamide 2.5 mg) of Zydus Cadila Healthcare Ltd. was procured from the market. For HPLC work double distilled water was prepared in the laboratory. Methanol used was of HPLC grade and was purchased from Merck Chemicals, Mumbai, India.

- **Stationary Phase:**

  Thermo Hypersil Gold C_{18} (250 mm × 4.6 mm, 5.0 µ) was used.

- **Preparation of mobile phases:**

  HPLC grade methanol (800 mL) was mixed with (200 mL) of double distilled water.

This solution was filtered through 0.45µ membrane filter paper and degassed in an ultrasonic bath before use.

- **Preparation of standard stock solution-I:**

  50 mg of Amlodipine besylate (AMLO) and 25 mg of Indapamide (IND) were accurately weighed and transferred to a 50 mL volumetric flask and dissolved in methanol. Volume was made up to the mark with mobile phase to get a solution containing AMLO 1 mg/mL and IND 0.5 mg/mL respectively. 1 mL of this solution was further diluted to 10 mL with mobile phase to get **Standard stock solution-I** containing AMLO 0.1 mg/mL and IND 0.05 mg/mL, respectively.

- **Working standard solution-II:**

  0.1 mL of the standard stock solution-I was diluted to 10 mL with mobile phase to get a concentration of 1 µg/mL of AMLO and 0.5 µg/mL of IND. This solution was used as working standard.

- **Sample solution:**

  Twenty tablets were weighed accurately and powered. Tablet powder equivalent to 5 mg of AMLO and 2.5 mg of IND was weighed accurately and transferred into a clean and dry 50 mL volumetric flask. The powder was first dissolved in sufficient volume of mobile phase and kept in an ultrasonic bath for 15 min. and the volume was made to 50 mL with mobile phase, filtered through 0.45µ membrane filter paper and solution was further diluted with mobile phase to obtain a solution of AMLO (1 µg/mL) and IND (0.5µg/mL), respectively. The sample solution (20 µL) was injected into the chromatograph and the chromatogram was recorded.

- **System suitability:**

  20 µL of standard solution containing AMLO 1 µg/mL and IND 0.5µg/mL was injected into the chromatograph to test the system suitability to determine resolution (Rs), retention time (t_R), theoretical number of plates (N) and tailing factor (T).

- **Analytical Method Validation:**

  Validation was done as per ICH guidelines. The developed method was validated with respect to parameters such as linearity, LOD and LOQ, precision, accuracy and specificity.

- **Linearity:**

  Several aliquots of standard stock solution-I of AMLO and IND were taken in different 10 mL volumetric flask and diluted up to the mark with mobile phase such

that the final concentration of AMLO and IND were in the range of 1-6 µg/mL and 0.5-3µg/mL respectively. Evaluations of two drugs were performed with UV detector set at 238 nm. The peak area versus concentration data was collected and subjected to regression analysis. The correlation coefficient, slope and y-intercept values were calculated from the calibration plot obtained.

- **Limit of detection and limit of quantitation:**

  The Limit of detection (LOD) and Limit of quantitation (LOQ) for AMLO and IND estimated at a signal-to-noise ratio of 3:1 and 10:1 respectively, by injecting a series of dilute solutions with known concentration.

- **Precision:**

  The precision of the method was verified by intra-day and inter-day precision studies. Intra-day precision studies were performed by analysis of three different concentrations 2, 4, 6 µg/mL for AMLO and 1, 2, 3 µg/mL for IND three times on the same day. The inter-day precision of the method was checked by repeating studies on three different days.

- **Robustness:**

  To determine the robustness of the developed method, experimental conditions were deliberately changed by changing the composition and flow rate of the mobile phase. The relative standard deviation for replicate injections of AMLO and IND were evaluated. Flow rate was changed by ± 0.1 units to 0.9 and 1.1 mL/min. The effect of % of methanol in the mobile phase was studied at (± 5%).

- **Recovery studies:**

  To check the accuracy of the proposed method recovery experiment was carried out. Twenty tablets from the same lot of the commercial formulation were weighed accurately and finely powered. Powder equivalent to 5 mg of AMLO and 2.5 mg of IND was weighed and transferred to different 50 mL volumetric flask. Standard drug was added at 80%, 100% and 120% level. The contents were dissolved by sonicating in 20 mL of mobile phase for 15 minutes and filtered through 0.45µ membrane filter paper and further diluted up to the mark using mobile phase. 0.1 mL of the filtrate was taken in 10 mL volumetric flask and diluted up to the mark with mobile phase. 20 µL of sample solution was injected into the chromatograph and chromatograms were recorded. Percentage recovery and relative standard deviation were calculated. Each level was repeated three times.

- **Analysis of marketed formulation:**

  20 µL of working standard solution-II (AMLO 1 µg/mL and IND 0.5µg/mL) and sample solutions were injected into the liquid chromatograph and the chromatogram was recorded. From the peak area of AMLO and peak area of IND the amount of the drugs in the sample were calculated.

- **Specificity:**

  The specificity of the method was ascertained by analysing drug standards and samples. The chromatogram was overlaid to study the specificity. The identities of the peak for AMLO and IND from the sample were confirmed by comparing the t_R with those of standards.

- **Comparison of the proposed method with the reported methods.**

  The proposed method was compared with the reported methods from the literature:

1. Simultaneous estimation of amlodipine besylate and indapamide in a pharmaceutical formulation by a high performance liquid chromatographic (RP-HPLC) method[10]

   Column: Brownlee C_{18} column (250 × 4.6 mm i.d., 3µm particle size)

   Mobile phase: 0.02 M KH_2PO_4–methanol (30:70 v/v) pH-adjusted to 3 with OPA

   Flow rate: 1.0 ml/min, Detector wavelength: 242 nm

2. Development and validation of reverse phase high performance liquid chromatographic method for simultaneous estimation of amlodipine besylate and indapamide in combined dosage form[11]

   Column: Intersil C_{18} column (250×4.6 mm i.d., 5 µm particle size)

   Mobile phase: ACN: Acid Phthalate Buffer (pH 3.0 ± 0.05) (50:50, v/v)

   Flow rate: 1.0 ml/min, Detector wavelength: 240 nm

4.2.2. Results and Discussion:

- **Selection of column:**

  As the drugs were soluble in polar solvents such as methanol and acetonitrile, C_{18} reverse phase column was selected with particle size of 5.0 µ, length 250 mm and internal diameter 4.6 mm.

- **Selection of suitable wavelength:**

  Stock solutions of individual drugs were prepared in methanol and the solutions were scanned individually in a UV spectrophotometer in the range of 200-400 nm and their spectras were recorded. The spectras were overlaid and the isobestic point, 238 nm was selected as the suitable wavelength as both the drugs showed good absorption (Fig.4.05) at this wavelength. The wavelength was checked and confirmed in the optimised mobile phase also.

- **Selection of mobile phase:**

  Initially different mobile phase compositions were tried using methanol and water such as 50:50 v/v, 60:40 v/v and 70:30 v/v. But these trails did not result in good peak shape and showed tailing and fronting. After several trails satisfactory peak shape was obtained with methanol: water in the ratio of 80:20 v/v and was selected for further study. The selected mobile phase was found to resolve peaks of AMLO (t_R-7.550 min.) and IND (t_R-3.392 min.), respectively.

- **System suitability**

  System suitability tests were carried out on freshly prepared standard stock solution of AMLO and IND to determine efficiency, resolution factor, symmetry factor and precision of the instrument and found to comply with USP requirement. Parameters obtained with 20 µL injection volume are shown in (Table 4.08). Column efficiency in term of theoretical plates number (N) was 4589.17 for IND and 3318.15 for AMLO, respectively.

- **Validation of the method**

  Using optimised chromatographic conditions the RP-HPLC method developed was validated in terms of linearity, LOD and LOQ, precision, robustness and accuracy.

  - **Linearity:**

    Linear regression data for the calibration plots revealed good linear relationships between response and concentration over the ranges 1-6 µg/mL for AMLO and 0.5-3µg/mL for IND, respectively. The linear regression equations were Y=11513X +

1333 (r^2= 0.9992) for AMLO and Y= 4004X +268.3 (r^2= 0.9995) for IND.The residuals plot shows a random pattern, indicating a good fit for a linear model. The calibration curves and residual plots are given in Fig. 4.07 and Fig.4.08 for AMLO and IND, respectively and Table 4.09 (A), and Table 4.09 (B).

- **Limits of Detection and Quantitation:**

  The signal to noise ratios of 3: 1 and 10: 1 were considered as LOD and LOQ respectively. The results of the LOD and LOQ were found to be 0.4 µg/mL, 0.8 µg/mL and 0.1 µg/mL, 0.5 µg/mL for AMLO and IND, respectively.

- **Precision:**

  The intra-day precision was determined by analysing AMLO and IND solutions in the concentration range of 2, 4 and 6 µg/mL and 1, 2 and 3 µg/mL for three times on the same day while inter-day precision was determined by analysing corresponding standards daily for three days over a period of one week. The intra-day and inter day precision % RSD were found to be less than 2 reveal the high precision of the method. The results listed in Table 4.10.

- **Robustness:**

  There were no significant changes in the retention times of AMLO and IND when the composition of the mobile phase (± 5 %) and flow rate (± 0.1 mL/min.) were changed. The low values of the % RSD indicate the robustness of the method, as shown in Table 4.11.

- **Accuracy:**

  To study the accuracy of the proposed method recovery experiment were carried out by standard addition method. The proposed method when used for extraction and subsequent estimation of AMLO and IND from pharmaceutical dosage form after spiking with 80%, 100% and 120% of the additional drug afforded mean recovery of 99.42% for AMLO and 99.28 % for IND. The mean recoveries indicate non-interference from excipients (Table 4.12).

- **Analysis of marketed formulation:**

  The chromatogram of the sample extracted from conventional tablets showed peaks of IND (t_R 3.392min.) and AMLO (t_R-7.550 min.) well resolved from other tablet excipients, shown in Fig. 4.06. The mean contents of AMLO and IND per tablet by proposed method was found to be 4.979 mg and 2.478 mg, respectively. The results are summarized in Table 4.13.

- **Comparison of the proposed method with the reported methods:**

  The proposed method was compared with the reported methods. The proposed method uses simple mobile phase of methanol: water (80:20 v/v) as against buffered mobile phases used in the reported methods. The proposed method gives better resolution for AMLO and IND than the reported methods. Accuracy and precision of the proposed method are comparable to the reported methods with low % RSD (Table 4.14).

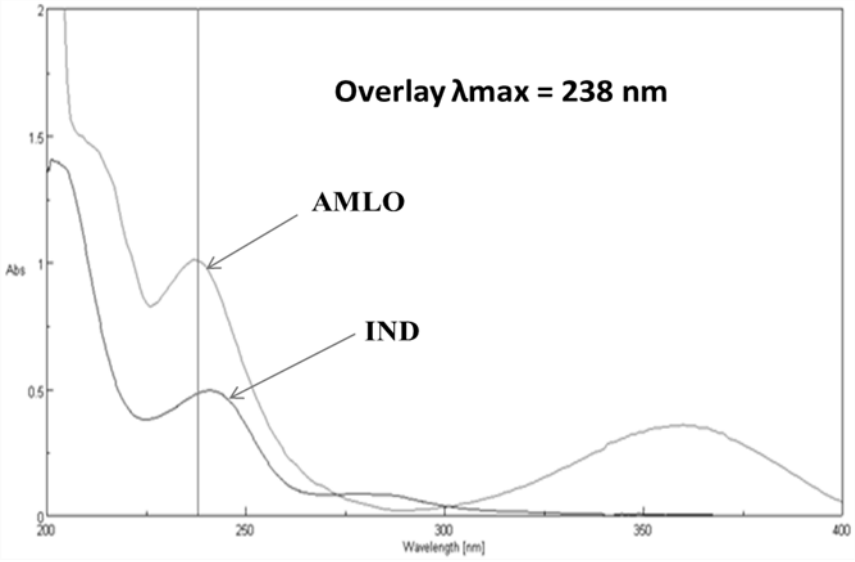

Fig. 4.05. Overlay absorption spectra of AMLO and IND

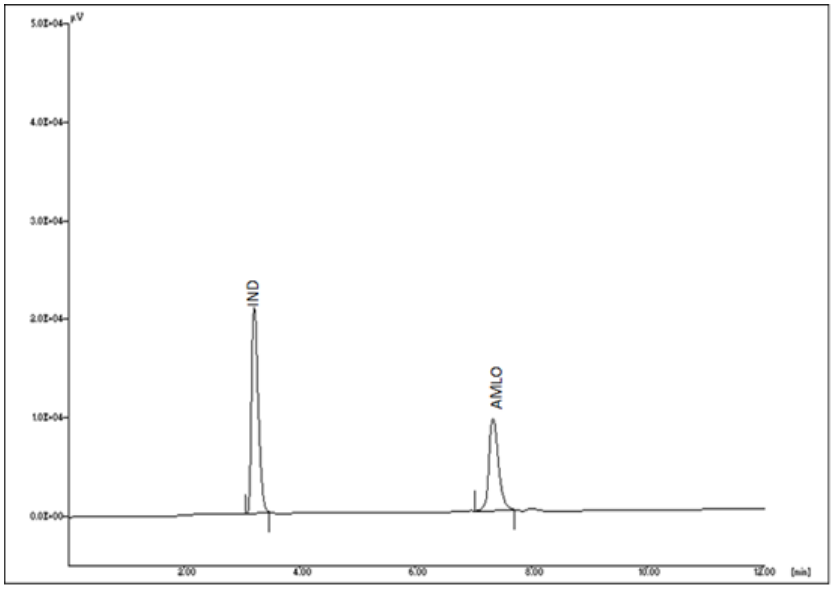

Fig.4.06. Typical chromatogram of IND and AMLO

Table 4.08: System suitability parameters (n=6)

Parameters	IND	AMLO
Retention Time (t_R) in min	3.392	7.550
Resolution (Rs)	--	9.741
Theoretical plates number (N)	4589.17	3318.15
Tailing Factor (T)	1.126	1.257

Table 4.09 (A): Data for AMLO from linearity study

Concentration (µg/mL)	HPLC (n= 3)	
	Mean peak area ± SD	**RSD (%)**
1	13798.23 ±155.92	1.13
2	23596.45 ± 259.56	1.10
3	35394.68 ± 361.02	1.02
4	47192.91 ± 538.0	1.14
5	58991.13 ± 701.99	1.19
6	70789.36 ± 934.42	1.32
Regression coefficient r^2		**0.9992**
Slope		**11513**
Intercept		**1333**

Table 4.09 (B): Data for IND from linearity study

Concentration (µg/mL)	HPLC (n= 3)	
	Mean peak area ± SD	**RSD (%)**
0.5	2378.77 ± 27.12	1.14
1	4257.53 ± 43.85	1.03
1.5	6136.3 ± 66.89	1.09
2	8234.6 ± 94.70	1.15
2.5	10293.25 ± 125.58	1.22
3	12351.9 ± 143.28	1.16
Regression coefficient r^2		**0.9995**
Slope		**4004**
Intercept		**268.3**

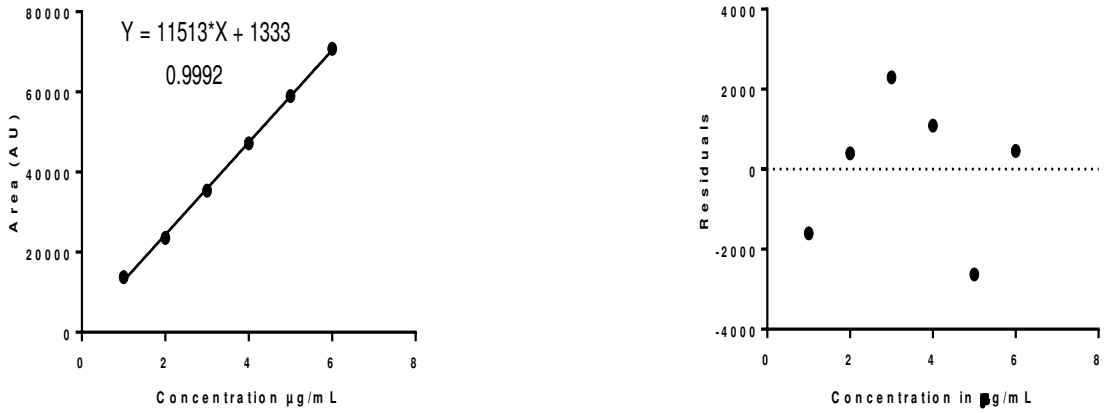

Fig. 4.07. Calibration curve and residual plot for AMLO

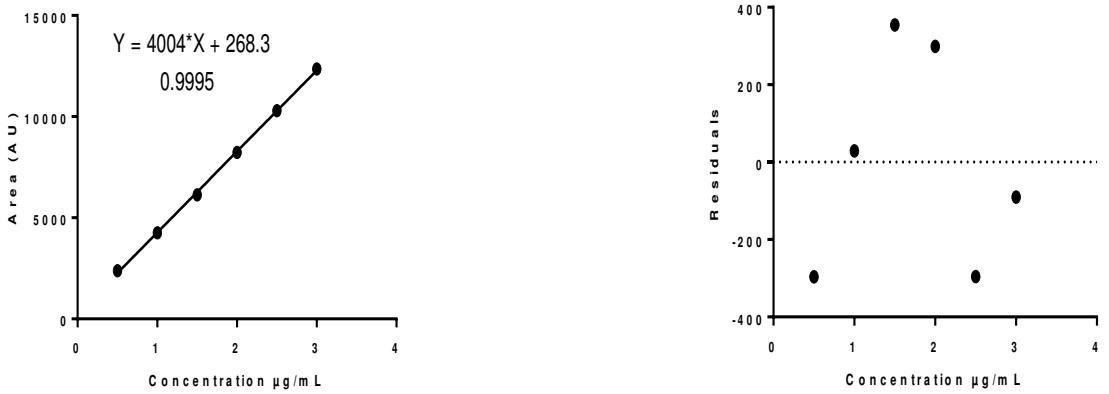

Fig. 4.08. Calibration curve and residual plot for IND

Table 4.10: Precision studies of IND and AMLO

Conc. (µg/mL)	Intra-day precision (n=3)			Inter-day precision (n=3)		
	Measured Conc. ± SD	(%) RSD	Recovery (%)	Measured Conc. ±SD	(%) RSD	Recovery (%)
Indapamide						
1	0.989 ± 0.011	1.08	98.90	0.987 ± 0.010	1.06	98.70
2	1.982 ± 0.022	1.11	99.10	1.980 ± 0.024	1.20	99.00
3	2.979 ± 0.031	1.04	99.30	2.972 ± 0.034	1.16	99.07
Amlodipine						
2	1.989 ± 0.020	1.01	99.45	1.980 ± 0.022	1.11	99.00
4	3.967 ± 0.047	1.18	99.18	3.962 ± 0.048	1.21	99.05
6	5.975 ± 0.074	1.24	99.58	5.968 ± 0.069	1.15	99.47

Table 4.11: Robustness evaluation of IND and AMLO (n=3)

Conditions	Level	IND		AMLO	
		t_R (min.)	% RSD of peak area	t_R (min.)	% RSD of peak area
A: Flow rate (0.1 mL/min.)					
0.9	-0.1	3.433	1.12	7.650	1.19
1.0	0.0	3.392	1.09	7.550	1.13
1.1	+0.1	3.287	1.21	7.412	1.10
Mean ± SD		3.371 ± 0.075	1.14 ± 0.062	7.558 ± 0.15	1.14 ± 0.046
B: % of methanol in the mobile phase (± 5%)					
75	-5.0	3.441	1.13	7.656	1.17
80	0.0	3.392	1.02	7.550	1.22
85	+5.0	3.270	1.10	7.410	1.15
Mean ± SD		3.368 ± 0.088	1.083± 0.0568	7.547 ± 0.12	1.18 ± 0.0360

Table 4.12: Recovery study for IND and AMLO (n=3)

Label claim (mg/tablet)	Amount Added (%)	Total amount (mg)	Amount recovered (mg)	Recovery (%)	Mean (%) Recovery (± SD)
IND 2.5	80 100 120	4.5 5 5.5	4.475 4.979 5.458	99.44 99.58 99.24	99.42 ± 0.17
AMLO 5.0	80 100 120	9 10 11	8.953 9.93 10.895	99.48 99.30 99.05	99.28 ± 0.215

Table 4.13: Assay results of IND and AMLO in tablets

Amlodac D tablet contains	Label claim (mg/tablet)	Amount found (mg ± SD)	RSD (%)	Recovery (%)
IND	2.5 mg	2.478 ± 0.026	1.07	99.12
AMLO	5 mg	4.979 ± 0.061	1.22	99.58

Table 4.14: Comparison of the proposed method with the reported methods

Parameter	Reported method 1		Reported method 2		Proposed method	
	IND	**AMLO**	**IND**	**AMLO**	**IND**	**AMLO**
Mobile Phase	0.02 M KH_2PO_4– methanol pH-adjusted to 3 with OPA (30:70 v/v)		ACN : Acid Phthalate Buffer (pH 3.0 ± 0.05) (50:50, v/v)		**Methanol: Water (80:20 v/v)**	
Mode	Isocratic		Isocratic		**Isocratic**	
Detection wavelength (nm) &Flow Rate	242 1 mL/min.		240 1 mL/min.		**238 1 mL/min.**	
Retention time (t_R) in min.	3.6	5.9	5.11	2.31	**3.392**	**7.550**
Linearity range (µg/mL)	0.075–10.5	0.25–35	3-15	10-50	**0.5-3**	**1-6**
LOD (µg/mL) and LOQ (µg/mL)	0.99 (ng) 3 (ng)	2.9 (ng) 8.8 (ng)	0.19 0.58	0.69 2.08	**0.1 0.5**	**0.4 0.8**
% Recovery (n=3)	99.69	99.55	100.13	100.09	**99.42**	**99.28**
Precision (% RSD) Intra-day (n=3) Inter-day (n=3)	1.06-1.86 0.96-1.26	1.23-1.078 1.02-1.53	0.79-1.59 0.08-1.21	1.06-1.51 0.40-1.39	**1.077 1.14**	**1.14 1.16**

4.2.3. Conclusion:

RP-HPLC method has been developed for simultaneous estimation of Amlodipine besylate and Indapamide in bulk drug and pharmaceutical dosage form. The developed method was validated according to the ICH guidelines. Based on the results, it can be concluded that there is no other co-eluting peak with the main peaks and that the method is specific for estimation of AMLO and IND. The proposed method has linear response in the stated range and is accurate and precise.

The method has several advantages, including rapid analysis, ease of sample preparation and its simplicity. The proposed method can be used for simultaneous analysis of AMLO and IND in bulk and formulation. The developed method can also be conveniently adopted for dissolution testing of tablets containing AMLO and IND.

4.3 Simultaneous analysis of Amlodipine besylate and Hydrochlorothiazide by HPLC

Amlodipine besylate, 3, 5-Pyridinecarboxylic acid, 2-((aminoethoxy) methyl)-4-(2-chlorophenyl) -1, 4-dihydro-6-methyl, 3-ethyl-5-methyl ester, a calcium channel blocker is used as an anti-hypertensive agent. Hydrochlorothiazide chemically, 6-Chloro-3, 4-dihydro-2H-l, 2, 4-benzothiadiazine-7-sulfonamide 1, 1-dioxide is a thiazide diuretic.

The drug profiles and literature survey of Amlodipine besylate (AMLO) and Hydrochlorothiazide (HCTZ) are given in 3.3 and 3.5.

4.3.1 Experimental:

- **Instrumentation:**

  The HPLC system consisted of Intelligent HPLC pump model (Jasco PU 1580). The solutions were injected into the chromatograph through a Rheodyne valve, with a 20 µL loop with auto sampler (AS 1555). The column used was Thermo Hypersil Gold C_{18} (250 mm × 4.6 mm, 5.0 µ), operating at room temperature. The elution was carried out isocratically at flow rate of 0.8 mL/min. using methanol: water (95:5 v/v) as mobile phase. The detector consisted of a UV/ VIS (Jasco UV 1575) model operated at a wavelength of 230nm. Data was integrated using Jasco Borwin version 1.5, LC-Net II/ADC system.

- **Chemicals and reagents:**

  Reference standards of Amlodipine besylate (99.92 %) and Hydrochlorothiazide (99.97%) were procured from Lupin Research Park and Emcure Pharmaceuticals Ltd., respectively. The tablets Amlokind-H (Amlodipine 5 mg, Hydrochlorothiazide 12.5 mg) of Mankind Pharmaceuticals Pvt. Ltd. was procured from the market. For HPLC work double distilled water was prepared in the laboratory. Methanol used was of HPLC grade and were purchased from Merck Chemicals, Mumbai, India

- **Stationary Phase:**

  Thermo Hypersil Gold C_{18} (250 mm × 4.6 mm, 5.0 µ) was used.

- **Preparation of mobile phases:**

  HPLC grade methanol (950 mL) was mixed with (50 mL) of double distilled water. This solution was filtered through 0.45µ membrane filter paper and degassed in an ultrasonic bath before use.

- **Preparation of standard stock solution-I:**

  50 mg of Amlodipine besylate (AMLO) and 125 mg of Hydrochlorothiazide (HCTZ) were accurately weighed and transferred to a 100 mL volumetric flask and dissolved in methanol. Volume was made up to the mark with mobile phase to get a solution containing AMLO 0.5 mg/mL and HCTZ 1.25 mg/mL respectively. 1 mL of this solution was further diluted to 10 mL with mobile phase to get **Standard stock solution-I** containing AMLO 0.05 mg/mL and HCTZ 0.125 mg/mL, respectively.

- **Working standard solution-II:**

  0.1 mL of the standard stock solution-I was diluted to 10 mL with mobile phase to get a concentration of 0.5 µg/mL of AMLO and 1.25 µg/mL of HCTZ. This solution was used as working standard.

- **Sample solution:**

  Twenty tablets were weighed accurately and powered. Tablet powder equivalent to 5 mg of AMLO and 1.25 mg of HCTZ was weighed accurately and transferred into a clean and dry 100 mL volumetric flask. The powder was first dissolved in sufficient volume of mobile phase and kept in an ultrasonic bath for 15 min. and the volume was made to 100 mL with mobile phase, filtered through 0.45µ membrane filter paper and solution was further diluted with mobile phase to obtain a solution of AMLO (0.5 µg/mL) and HCTZ (1.25 µg/mL), respectively. The sample solution (20µL) was injected into the chromatograph and the chromatogram was recorded.

- **System suitability:**

  20µL of standard solution containing AMLO 0.5µg/mL and HCTZ 1.25 µg/mL was injected into the chromatograph to test the system suitability to determine resolution (Rs), retention time (t_R), theoretical number of plates (N) and tailing factor (T).

- **Analytical Method Validation:**

  Validation was done as per ICH guidelines. The developed method was validated with respect to parameters such as linearity, LOD and LOQ, precision, accuracy and specificity.

  - **Linearity:**

    Several aliquots of standard stock solution-I of AMLO and HCTZ were taken in different 10 mL volumetric flask and diluted up to the mark with mobile phase such that the final concentration of AMLO and HCTZ were in the range of 0.5–3 µg/mL and 1.25-7.5 µg/mL, respectively. Evaluations of two drugs were performed with UV

detector set at 230 nm. The peak area versus concentration data was collected and subjected to regression analysis. The correlation coefficient, slope and y-intercept values were calculated from the calibration plot obtained.

- **Limit of detection and limit of quantitation:**

  The Limit of detection (LOD) and Limit of quantitation (LOQ) for AMLO and HCTZ estimated at a signal-to-noise ratio of 3:1 and 10:1 respectively, by injecting a series of dilute solutions with known concentration.

- **Precision:**

  The precision of the method was verified by intra-day precision and inter-day precision studies. Intra-day precision studies were performed by analysis of three different concentrations 1, 2, 3 µg/mL for AMLO and 2.5, 5, 7.5 µg/mL for HCTZ three times on the same day. The inter-day precision of the method was checked by repeating studies on three different days.

- **Robustness:**

  To determine the robustness of the developed method, experimental conditions were deliberately changed by changing the composition and flow rate of the mobile phase. The relative standard deviation for replicate injections of AMLO and HCTZ were evaluated. Flow rate was changed by ± 0.1 units to 0.7 and 0.9 mL/min. The effect of % of methanol in the mobile phase was studied at (± 5%).

- **Recovery studies:**

  To check the accuracy of the proposed method recovery experiment was carried out. Twenty tablets from the same lot of the commercial formulation were weighed accurately and finely powered. Powder equivalent to 5 mg of AMLO and 1.25 mg of HCTZ was weighed and transferred to different 100 mL volumetric flask. Standard drug was added at 80%, 100% and 120% level. The contents were dissolved by sonicating in 20 mL of mobile phase for 15 minutes and filtered through 0.45µ membrane filter paper and further diluted up to the mark using mobile phase. 0.1 mL of the filtrate was taken in 10 mL volumetric flask and diluted up to the mark with mobile phase. 20 µL of sample solution were injected into the chromatograph and chromatograms were recorded. Percentage recovery and relative standard deviation were calculated. Each level was repeated three times.

- **Analysis of marketed formulation:**

  20 µL of working standard solution-II (AMLO 0.5µg/mL and HCTZ 1.25 µg/mL) and sample solutions were injected into the liquid chromatograph and the

chromatogram was recorded. From the peak area of AMLO and HCTZ the amount of the drugs in the sample was calculated.

- **Specificity:**

  The specificity of the method was ascertained by analysing standards and samples. The chromatogram was overlaid to study the specificity. The identities of the peak for AMLO and HCTZ from the sample were confirmed by comparing the t_R with those of standards.

- **Comparison of the proposed method with the reported method:**

  The proposed method was compared with the reported method from the literature:

  Analytical method development and validation of Amlodipine and Hydrochlorothiazide in combined dosage form by RP-HPLC[12]

  Column: Phenomenex C_{18} column (250×4.6 mm i.d., 5 µm particle size)

  Mobile phase: Triethylamine: Acetonitrile: Methanol (50:25:25 v/v/v) (pH adjusted to 3.0 with Ortho-phosphoric acid)

  Flow rate: 2 mL/min, Detector wavelength: 235 nm

4.3.2. Results and Discussion:

- **Selection of column:**

  As the drugs were soluble in polar solvents such as methanol and acetonitrile, C_{18} reverse phase column was selected with particle size of 5 µ, length 250 mm and internal diameter 4.6 mm.

- **Selection of suitable wavelength:**

  Stock solutions of individual drugs were prepared in methanol and the solutions were scanned individually in a UV spectrophotometer in the range of 200-400 nm and their spectras were recorded. The spectras were overlaid and the isobestic point, 230 nm was selected as the suitable wavelength as both the drugs showed good absorption (Fig.4.09) at this wavelength. The wavelength was checked and confirmed in the optimised mobile phase also.

- **Selection of mobile phase:**

  Initially different mobile phase compositions were tried using methanol and water such as 50:50 v/v, 60:40 v/v, 70:30 v/v, 80:20 v/v and 90:10 v/v. But these trails did not result in good peak shape. After several trails satisfactory peak shape was obtained with methanol: water in the ratio of 95:5 v/v and was selected for further study. The selected mobile phase was found to resolve peaks of AMLO at t_R-7.325 min. and HCTZ at t_R-3.867 min. respectively.

- **System suitability**

  System suitability tests were carried out on freshly prepared standard stock solution of AMLO and HCTZ to determine efficiency, resolution factor, symmetry factor and precision of the instrument and found to comply with USP requirement. Parameters obtained with 20 µL injection volume are shown in (Table 4.15). Column efficiency in term of theoretical plates number (N) was 5135.35 for HCTZ and 4739.75 for AMLO, respectively.

- **Validation of the method**

  Using optimised chromatographic conditions the RP-HPLC method developed was validated in terms of linearity, LOD and LOQ, precision, robustness and accuracy.

  - **Linearity:**

    Linear regression data for the calibration plots revealed good linear relationships between response and concentration over the ranges 0.5-3µg/mL for AMLO and 1.25-7.5µg/mL for HCTZ, respectively. The linear regression equations were Y=

8794X -128.9 (r^2= 0.9996) for AMLO and Y= 19380X + 2795 (r^2= 0.9996) for HCTZ. The residuals plot shows a random pattern, indicating a good fit for a linear model. The calibration curves and residuals plots are given in Fig. 4.11 and Fig.4.12 for AMLO and HCTZ, respectively and Table 4.16 (A), and Table 4.16 (B).

- **Limits of Detection and Quantitation:**

  The signal to noise ratios of 3: 1 and 10: 1 were considered as LOD and LOQ respectively. The results of the LOD and LOQ were found to be 0.2µg/mL, 0.5µg/mL and 0.6µg/mL, 1.0µg/mL for AMLO and HCTZ, respectively.

- **Precision:**

  The % RSD of intraday and inter-day precision values for AMLO and HCTZ were found to be 1.077 and 1.13 and 1.15 and 1.037 %, respectively (Table 4.17). The RSD values were found to be <2 %, which indicates that the proposed method is precise.

- **Robustness:**

  There were no significant changes in the retention times of AMLO and HCTZ when the composition of the mobile phase (± 5%) and flow rate (± 0.1mL/min.) were changed. The low values of the RSD indicate the robustness of the method, as shown in Table 4.18.

- **Accuracy:**

  To study the accuracy of the proposed method recovery experiment were carried out by standard addition method. The proposed method when used for extraction and subsequent estimation of AMLO and HCTZ from pharmaceutical dosage form after spiking with 80%, 100% and 120% of the additional drug afforded mean recovery of 99.23% for AMLO and 99.47 % for HCTZ. The mean recoveries indicate non-interference from excipients (Table 4.19).

- **Analysis of marketed formulation:**

  The chromatogram of the sample extracted from conventional tablets showed peaks of HCTZ (t_R 3.867 min.) and AMLO (t_R-7.325 min.) that are well resolved from other tablet excipients as shown in Fig. 4.10. The mean contents of AMLO and HCTZ per tablet by proposed method was found to be 4.96 mg and 12.42 mg, respectively. The results are summarized in Table 4.20.

- **Comparison of the proposed method with the reported method:**

  The proposed method was compared with the reported method. The proposed method uses simple mobile phase of methanol: water (95:5 v/v) at flow rate of 0.8 mL/min. as against buffered mobile phases used in the reported method. The proposed method gives better column efficiency and resolution than the reported method. Linearity concentration range of the proposed method is 1.25 - 7.5 µg/mL for AMLO and 0.5-3 µg/mL for HCTZ as against 200-300 µg/mL for AMLO and 80-120 µg/mL for HCTZ of the reported method. LOD and LOQ for AMLO and HCTZ of the proposed method are lower than the reported method. Hence proposed method can be applied for routine analysis. Accuracy and precision of the proposed method are comparable to the reported method with low % RSD (Table 4.21).

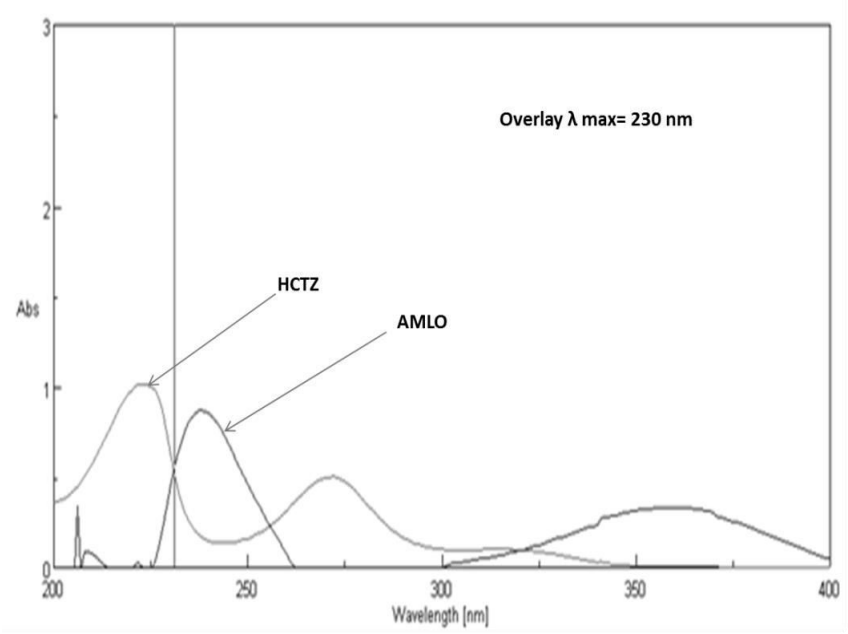

Fig. 4.09. Overlay absorption spectra of AMLO and HCTZ

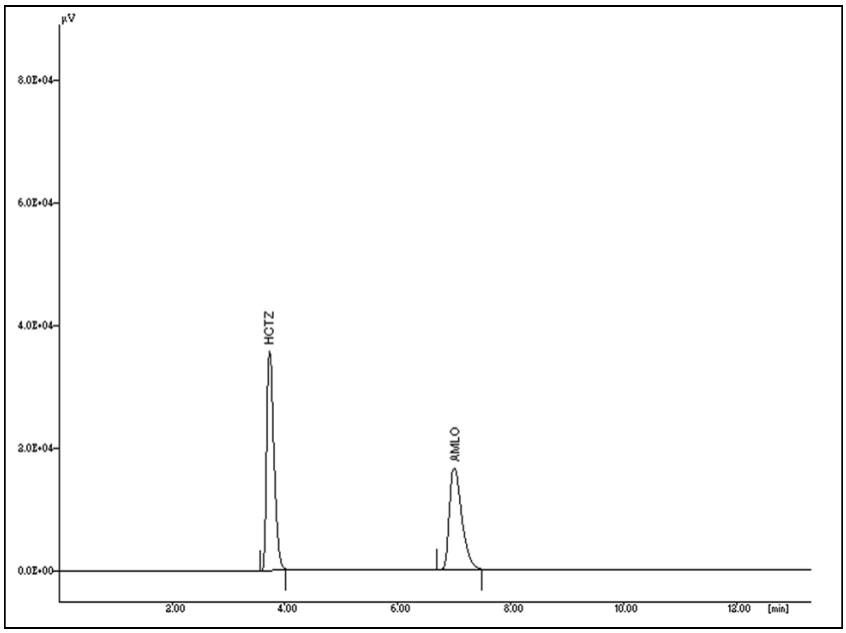

Fig.4.10. Typical chromatogram of HCTZ and AMLO

Table 4.15: System suitability parameters (n=6)

Parameters	HCTZ	AMLO
Retention Time(t_R) in min	3.867	7.325
Resolution (Rs)	--	9.056
Theoretical plates number (N)	5135.35	4739.75
Tailing Factor (T)	1.144	1.262

Table 4.16 (A): Data for AMLO from linearity study

Concentration (µg/mL)	**HPLC (n= 3)**	
	Mean peak area ± SD	**RSD (%)**
0.5	4398.5 ± 46.18	1.05
1.0	8429.0 ± 100.31	1.19
1.5	13114.0 ± 161.30	1.23
2.0	17423.0 ± 191.65	1.10
2.5	22063.0 ± 229.46	1.04
3.0	26135.0 ± 326.69	1.25
Regression coefficient r^2		**0.9996**
Slope		**8794**
Intercept		**-128.9**

Table 4.16 (B): Data for HCTZ from linearity study

Concentration (µg/mL)	**HPLC (n= 3)**	
	Mean peak area ± SD	**RSD (%)**
1.25	26078 ± 294.68	1.13
2.5	51115.5 ± 536.71	1.05
3.75	76455.0 ± 886.88	1.16
5.0	100924.5 ± 1211.09	1.20
6.25	123745.5 ± 1373.58	1.11
7.5	147183.5 ± 1501.27	1.02
Regression coefficient r^2		**0.9996**
Slope		**19380**
Intercept		**2795**

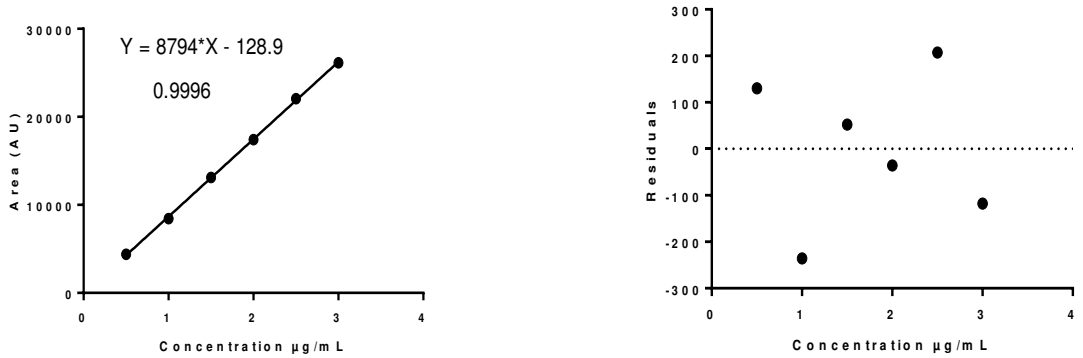

Fig. 4.11. Calibration curve and residual plot for AMLO

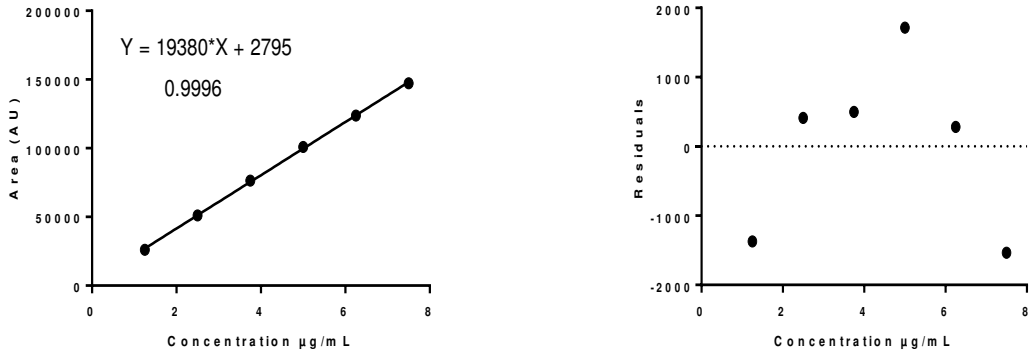

Fig. 4.12. Calibration curve and residual plot for HCTZ

Table 4.17: Precision studies of AMLO and HCTZ

Conc. (µg/mL)	Intra-day precision (n=3)			Inter-day precision (n=3)		
	Measured Conc. ± SD	(%) RSD	Recovery (%)	Measured Conc. ±SD	(%) RSD	Recovery (%)
Amlodipine						
1	0.992 ± 0.011	1.08	99.20	0.989 ± 0.010	1.05	98.90
2	1.981 ± 0.021	1.04	99.50	1.979 ± 0.022	1.13	98.95
3	2.973 ± 0.033	1.11	99.10	2.970 ± 0.036	1.21	99.00
Hydrochlorothiazide						
2.5	2.479 ± 0.028	1.12	99.16	2.475 ± 0.029	1.19	99.00
5.0	4.973 ± 0.062	1.24	99.46	4.970 ± 0.055	1.10	99.40
7.5	7.450 ± 0.081	1.09	99.33	7.430 ± 0.061	0.82	99.07

Table 4.18: Robustness evaluation of HCTZ and AMLO (n=3)

Conditions	Level	HCTZ		AMLO	
		t_R (min.)	% RSD of peak area	t_R (min.)	% RSD of peak area
A: Flow rate (± 0.1 mL/min.)					
0.7	-0.1	3.890	1.05	7.354	1.13
0.8	0.0	3.867	1.11	7.325	1.04
0.9	+0.1	3.807	1.01	7.305	1.17
Mean ± SD		3.855 ± 0.043	1.057±0.050	7.328 ± 0.025	1.113 ±0.066
B: % of methanol in the mobile phase (± 5%)					
90	-5.0	3.883	1.20	7.356	1.09
95	0.0	3.867	1.18	7.325	1.11
100	+5.0	3.803	1.12	7.297	1.15
Mean ± SD		3.851 ± 0.042	1.167±0.042	7.326 ± 0.030	1.117± 0.031

Table 4.19: Recovery study for AMLO and HCTZ (n=3)

Label claim (mg/tablet)	Amount Added (%)	Total amount (mg)	Amount recovered (mg)	Recovery (%)	Mean (%) Recovery (± SD)
AMLO 5.0	80 100 120	9 10 11	8.94 9.91 10.92	99.33 99.10 99.27	99.23 ± 0.12
HCTZ 12.5	80 100 120	22.5 25 27.5	22.42 24.90 27.27	99.64 99.60 99.16	99.47 ± 0.27

Table 4.20: Assay results of AMLO and HCTZ in tablets

Amlokind-H tablet contains	Label claim (mg/tablet)	Amount found (mg ± SD)	RSD (%)	Recovery (%)
AMLO	5 mg	4.96 ± 0.058	1.16	99.20
HCTZ	12.5 mg	12.42 ± 0.14	1.11	99.36

Table 4.21: Comparison of the proposed method with the reported method

Parameter	Reported method		Proposed method	
	HCTZ	**AMLO**	**HCTZ**	**AMLO**
Mobile Phase	TEA: ACN: MeOH (50:25:25 v/v/v) pH adjusted to 3.0 with OPA		**Methanol: Water (95:5 v/v)**	
Mode	Isocratic		**Isocratic**	
Detection wavelength (nm) & Flow Rate	235 2 mL/min.		**230** **0.8 mL/min.**	
Retention time (t_R) in min.	2.183	6.631	**3.867**	**7.325**
Linearity range (µg/mL)	200-300	80-120	**1.25-7.5**	**0.5-3**
LOD (µg/mL) and LOQ (µg/mL)	3.40 10.31	0.80 2.43	**0.6** **1.0**	**0.2** **0.5**
% Recovery (n=3)	99.04	99.51	**99.47**	**99.23**
Precision (% RSD) Intra-day (n=3) Inter-day (n=3)	----	----	**1.15** **1.037**	**1.077** **1.13**

4.3.3. Conclusion:

RP-HPLC method has been developed for simultaneous estimation of Amlodipine besylate and Hydrochlorothiazide from pharmaceutical dosage form. The proposed method is simple and do not involve laborious time-consuming sample preparation and is performed at lower linear concentration range. The method uses simple mobile phase of methanol: water (95:5 v/v) at flow rate of 0.8 ml/min. as against buffered mobile phases used in the reported methods. The proposed method gives better column efficiency and resolution than the reported method. Linearity concentration range of the proposed method is 1.25 - 7.5 µg/mL for AMLO and 0.5 - 3µg/mL for HCTZ as against 200-300 µg/mL for AMLO and 80-120 µg/mL for HCTZ of the reported method. LOD and LOQ for AMLO and HCTZ of the proposed method are lower than the reported method. The validation study shows that the developed method is accurate, rapid, and precise, with acceptable correlation co-efficient, RSD (%) and standard deviations which make it valuable for simultaneous determination of AMLO and HCTZ in pharmaceutical dosage forms. Hence proposed method can be applied for routine analysis.

4.4 Simultaneous analysis of Amlodipine besylate and Ramipril by HPLC

Amlodipine besylate, 3, 5-Pyridinecarboxylic acid, 2-((aminoethoxy) methyl)-4-(2-chlorophenyl) -1, 4-dihydro-6-methyl, 3-ethyl-5-methyl ester, is a calcium channel blockers used as an anti-hypertensive. Ramipril, (2S,3aS,6aS)-1[(S)-N-[(S)-1-Carboxy-3-phenylpropyl] alanyl] octa hydro cyclopenta [b]pyrrole-2-carboxylic acid, 1-ethyl ester, is an angiotensin converting enzyme, used to treat high blood pressure (hypertension).

The drug profiles and literature survey of Amlodipine besylate (AMLO) and Ramipril (RAM) are given in 3.3 and 3.4.

4.4.1 Experimental:

- **Instrumentation:**

  The HPLC system consisted of Intelligent HPLC pump model (Jasco PU 1580). The solutions were injected into the chromatograph through a Rheodyne valve, with a 20 µL loop with auto sampler (AS 1555). The column used was Thermo Hypersil Gold C_{18} (250 mm × 4.6 mm, 5.0 µ), operating at room temperature. The elution was carried out isocratically at flow rate of 1.0 mL/min. using methanol: water (95: 5 v/v) as mobile phase. The detector consisted of a UV/ VIS (Jasco UV 1575) model operated at a wavelength of 227 nm. Data was integrated using Jasco Borwin version 1.5, LC-Net II/ADC system.

- **Chemicals and reagents:**

  Reference standards of Amlodipine besylate (99.92 %) and Ramipril (99.4%) were procured from Lupin Research Park and Emcure Pharmaceuticals Ltd., respectively. The capsules Stamace (Amlodipine 5 mg, Ramipril 2.5 mg) of Dr. Reddy's Laboratories Ltd. was procured from the market. For HPLC work double distilled water was prepared in the laboratory. Methanol used was of HPLC grade and were purchased from Merck Chemicals, Mumbai, India.

- **Stationary Phase:**

  Thermo Hypersil Gold C_{18} (250 mm × 4.6 mm, 5.0 µ) was used.

- **Preparation of mobile phases:**

  HPLC grade methanol (950 mL) was mixed with (50 mL) of double distilled water. This solution was filtered through 0.45µ membrane filter paper and degassed in an ultrasonic bath before use.

- **Preparation of standard stock solution-I:**

  50 mg of Amlodipine besylate (AMLO) and 25 mg of Ramipril (RAM) were accurately weighed and transferred to a 50 mL volumetric flask and dissolved in methanol. Volume was made up to the mark with mobile phase to get a solution containing AMLO 1 mg/mL and RAM 0.5 mg/mL respectively. 1mL of this solution was further diluted (AMLO 1 mg/mL and RAM 0.5 mg/mL) to 10 mL with mobile phase to get **Standard stock solution-I** containing AMLO 0.1 mg/mL and RAM 0.05 mg/mL, respectively.

- **Working standard solution-II:**

  0.1 mL of the standard stock solution-I was diluted to 10 mL with mobile phase to get a concentration of 1 µg/mL of AMLO and 0.5 µg/mL of RAM. This solution was used as working standard.

- **Sample solution:**

  Twenty capsules were weighed accurately and powered. Capsule powder equivalent to 5 mg of AMLO and 2.5 mg of RAM was weighed accurately and transferred into a clean and dry 50 mL volumetric flask. The powder was first dissolved in sufficient volume of mobile phase and kept in an ultrasonic bath for 15 min. and the volume was made to 50 mL with mobile phase, filtered through 0.45µ membrane filter paper and solution was further diluted with mobile phase to obtain a solution of AMLO (1µg/mL) and RAM (0.5µg/mL), respectively. The sample solution (20µL) was injected into the chromatograph and the chromatogram was recorded.

- **System suitability:**

  20µl of standard solution containing AMLO 1 µg/mL and RAM 0.5µg/mL was injected into the chromatograph to test the system suitability to determine resolution (Rs), retention time (t_R), theoretical number of plates (N) and tailing factor (T).

- **Analytical Method Validation:**

  Validation was done as per ICH guidelines. The developed method was validated with respect to parameters such as linearity, LOD and LOQ, precision, accuracy and specificity.

  - **Linearity:**

    Several aliquots of standard stock solution-I of AMLO and RAM were taken in different 10 mL volumetric flask and diluted up to the mark with mobile phase such that the final concentration of AMLO and RAM were in the range of 1–6 µg/mL and

0.5-3 µg/mL respectively. Evaluations of two drugs were performed with UV detector set at 227 nm. The peak area versus concentration data was collected and subjected to regression analysis. The correlation coefficient, slope and y-intercept values were calculated from the calibration plot obtained.

- **Limit of detection and limit of quantitation:**

  The Limit of detection (LOD) and Limit of quantitation (LOQ) for AMLO and RAM estimated at a signal-to-noise ratio of 3:1 and 10:1 respectively, by injecting a series of dilute solutions with known concentration.

- **Precision:**

  The precision of the method was verified by intra-day precision and inter-day precision studies. Intra-day precision studies were performed by analysis of three different concentrations of 2, 4, 6 µg/mL for AMLO and 1, 2, 3 µg/mL for RAM three times on the same day. The inter-day precision of the method was checked by repeating studies on three different days.

- **Robustness:**

  To determine the robustness of the developed method, experimental conditions were deliberately changed by changing the composition and flow rate of the mobile phase. The relative standard deviation for replicate injections of AMLO and RAM were evaluated. Flow rate was changed by ± 0.1 units to 0.9 and 1.1 mL/min. The effect of % of methanol in the mobile phase was studied at (± 5%).

- **Recovery studies:**

  To check the accuracy of the proposed method recovery experiment was carried out. Twenty capsules from the same lot of the commercial formulation were weighed accurately and finely powered. Powder equivalent to 5 mg of AMLO and 2.5 mg of RAM was weighed and transferred to different 50 mL volumetric flask. Standard drug was added at 80%, 100% and 120% level. The contents were dissolved by sonicating in 20 mL of mobile phase for 15 minutes and filtered through 0.45µ membrane filter paper and further diluted up to the mark using mobile phase. 0.1 mL of the filtrate was taken in 10 mL volumetric flask and diluted up to the mark with mobile phase. 20 µL of sample solution were injected into the chromatograph and chromatograms were recorded. Percentage recovery and relative standard deviation were calculated. Each level was repeated three times.

- **Analysis of marketed formulation:**

  20 µL of working standard solution-II (AMLO 1 µg/mL and RAM 0.5µg/mL) and sample solutions were injected into the liquid chromatograph and the chromatogram was recorded. From the peak area of AMLO and peak area of RAM the amount of the drugs in the sample were calculated.

- **Specificity:**

  The specificity of the method was ascertained by analysing drug standards and samples. The chromatogram was overlaid to study the specificity. The identities of the peak for AMLO and RAM from the sample were confirmed by comparing the t_R with those of standards.

- **Comparison of the proposed method with the reported methods.**

  The proposed method was compared with the reported methods from the literature:

1. Development and validation of an RP-HPLC method for simultaneous determination of ramipril and amlodipine in tablets[13]

   Column: Intersil ODS-3 C_{18} column (250×4.6 mm i.d., 3 µm particle size)

   Mobile phase: Mobile phase A: 60 mM sodium perchlorate buffer (containing 7.2 mM TEA)-ACN (60:40 v/v), Mobile phase B: 60mM sodium perchlorate buffer (containing 7.2 mM TEA)-ACN (20:80, v/v) pH 2.6 with phosphoric acid (Gradient) Flow rate: 1.0 ml/min, Detector wavelength: 210 nm.

2. Isocratic reverse phase high performance liquid chromatographic estimation of ramipril and amlodipine in pharmaceutical dosage form[14]

   Column: Phenomenex C_{18} column (150×4.6 mm i.d., 5µm particle size)

   Mobile phase: Phosphate buffer (0.02 M potassium di-hydrogen orthophosphate and 0.002 M di-potassium hydrogen phosphate anhydrous, pH 6.8) and acetonitrile (60:40 v/v), Flow rate: 1.0 ml/min, Detector wavelength: 237 nm.

4.4.2. Results and Discussion:

- **Selection of column:**

  As the drugs were soluble in methanol and acetonitrile, C_{18} reverse phase column was selected with particle size of 5.0 µ, length 250 mm and internal diameter 4.6 mm.

- **Selection of suitable wavelength:**

  Stock solutions of individual drugs were prepared in methanol and the solutions were scanned individually in a UV spectrophotometer in the range of 200-400 nm and their spectras were recorded. The spectras were overlaid and the isobestic point, 227 nm was selected as the suitable wavelength as both the drugs showed good absorption (Fig.4.13) at this wavelength. The wavelength was checked and confirmed in the optimised mobile phase also.

- **Selection of mobile phase:**

  Different mobile phase were selected and tried. The preliminary trials using different composition of mobile phases such as methanol with water in a ratios 50:50 v/v, 60:40 v/v, 70:30 v/v, 80:20 v/v and 90:10 v/v did not gave good peak shape or desired t_R. Finally the mobile phase composition of methanol: water (95:5, v/v) resulted in satisfactory peak shape and desired t_R. The selected mobile phase was found to resolve peaks of AMLO (t_R-7.192min.) and RAM (t_R-2.630 min.), respectively.

- **System suitability**

  System suitability tests were carried out on freshly prepared standard stock solution of AMLO and RAM to determine efficiency, resolution factor, symmetry factor and precision of the instrument and found to comply with USP requirement. Parameters obtained with 20 µL injection volume are shown in (Table 4.22). Column efficiency in term of theoretical plates number (N) was 4623.45 for RAM and 4247.15 for AMLO, respectively.

- **Validation of the method**

  Using optimised chromatographic conditions the RP-HPLC method developed was validated in terms of linearity, LOD and LOQ, precision, robustness and accuracy.

  - **Linearity:**

    Linear regression data for the calibration plots revealed good linear relationships

between response and concentration over the ranges 1–6 µg/mL for AMLO and 0.5-3µg/mL for RAM, respectively. The linear regression equations were Y= 130214X + 3266 (r^2= 0.9998) for RAM and Y= 8895X – 521.8 (r^2= 0.9997) for AMLO. The residuals plot shows a random pattern, indicating a good fit for a linear model. The calibration curves and residuals plots are given in Fig. 4.15 and Fig.4.16 for RAM and AMLO, respectively and Table 4.23(A), and Table 4.23(B).

- **Limits of Detection and Quantitation:**

  The signal to noise ratios of 3: 1 and 10: 1 were considered as LOD and LOQ respectively. The results of the LOD and LOQ were found to be 0.2 µg/mL, 0.5 µg/mL and 0.2 µg/mL, 0.5 µg/mL for AMLO and RAM, respectively.

- **Precision:**

  For intra-day precision studies three concentrations were injected in triplicate in a day and for inter-day precision studies three concentrations were injected in triplicate for three days. % RSD of intra-day and inter-day precision were found to be 1.14, 1.18 for RAM and 1.11 and 1.15 for AMLO, respectively that proves the method is precise. The results are listed in Table 4.24.

- **Robustness:**

  There were no significant changes in the retention times of AMLO and RAM when the composition (± 5%) and flow rate (± 0.1 mL/min.) of the mobile phase were changed. The low values of the RSD indicate the robustness of the method, as shown in Table 4.25.

- **Accuracy:**

  To study the accuracy of the proposed method recovery experiment were carried out by standard addition method. The proposed method when used for extraction and subsequent estimation of AMLO and RAM from pharmaceutical dosage form after spiking with 80%, 100% and 120% of the additional drug afforded mean recovery of 99.30 % for AMLO and 99.37 % for RAM. The mean recoveries indicate non-interference from excipients (Table 4.26).

- **Analysis of marketed formulation:**

  The chromatogram of the sample extracted from conventional capsules showed well resolved peaks of RAM (t_R, 2.630 min.) and AMLO (t_R, 7.192 min.) from other capsule excipients, shown in Fig.4.14. The mean contents of RAM and AMLO per

capsule by proposed method was found to be 4.978 mg and 2.486 mg, respectively. The results are summarized in Table 4.27.

- **Comparison of the proposed method with the reported methods:**

  The proposed method was compared with the reported methods. The proposed method uses simple mobile phase of methanol: water (95:5 v/v) as against buffered mobile phases used in the reported methods. The proposed method gives better column efficiency and resolution than the reported method. Linearity concentration range of the proposed method is 1.0- 6.0 (µg/mL) for AMLO and 0.5 – 3 (µg/mL) for RAM which is quiet lower as compared to the reported methods. Accuracy and precision of the proposed method are comparable to the reported methods with low % RSD (Table 4.28).

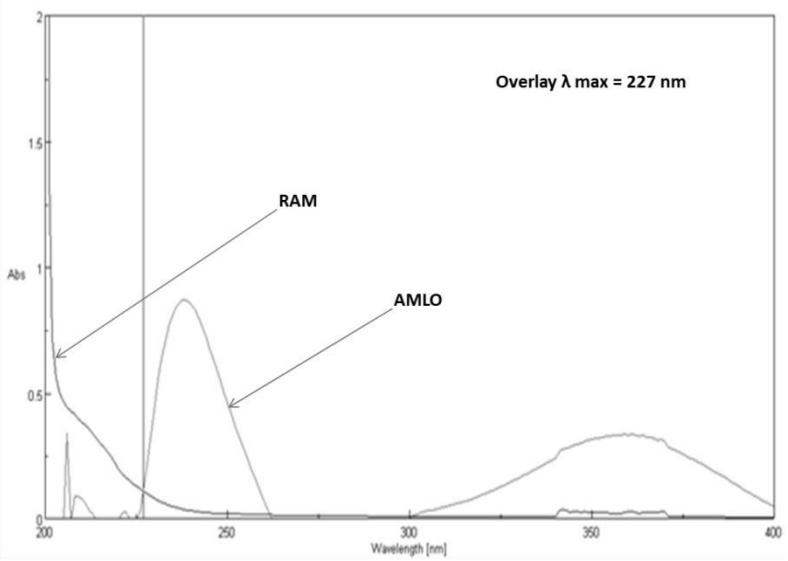

Fig.4.13. Overlay absorption spectra of RAM and AMLO

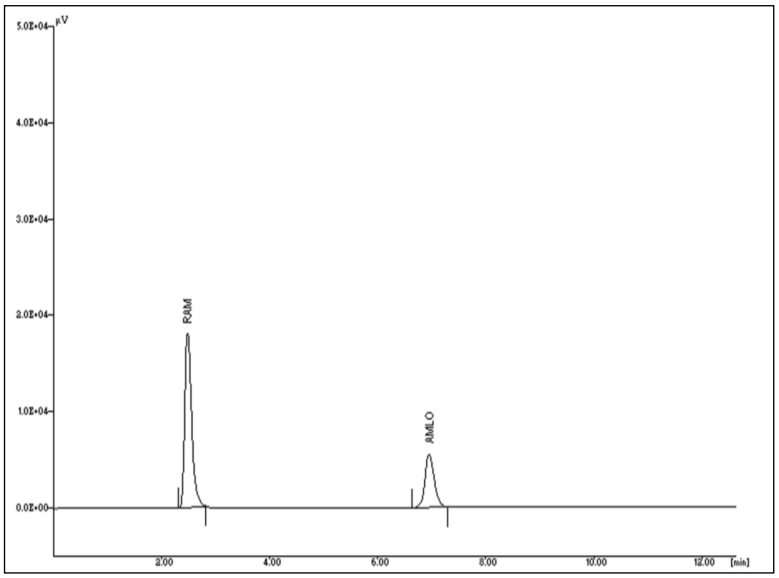

Fig.4.14. Typical chromatogram of RAM and AMLO

Table 4.22: System suitability parameters (n=6)

Parameter	RAM	AMLO
Retention Time (t_R) in min	2.630	7.192
Resolution (Rs)	--	9.576
Theoretical plates number (N)	4623.45	4247.15
Tailing Factor (T)	1.143	1.210

Table 4.23 (A): Data for RAM from linearity study

Concentration (µg/mL)	HPLC (n= 3)	
	Mean peak area ± SD	**RSD (%)**
0.5	66993 ± 703.43	1.05
1	132985.5 ± 1489.44	1.12
1.5	200977.5 ± 2472.02	1.23
2	263971 ± 3114.86	1.18
2.5	329968.8 ± 3365.68	1.02
3	391955 ± 4507.48	1.15
Regression coefficient r^2		**0.9998**
Slope		**130214**
Intercept		**3266**

Table 4.23 (B): Data for AMLO from linearity study

Concentration (µg/mL)	HPLC (n= 3)	
	Mean peak area ± SD	**RSD (%)**
1	8429 ± 96.09	1.14
2	17423 ± 179.46	1.03
3	26135 ± 282.26	1.08
4	34846.67 ± 400.74	1.15
5	43557.5 ± 527.05	1.21
6	53270 ± 591.30	1.11
Regression coefficient r^2		**0.9997**
Slope		**8895**
Intercept		**– 521.8**

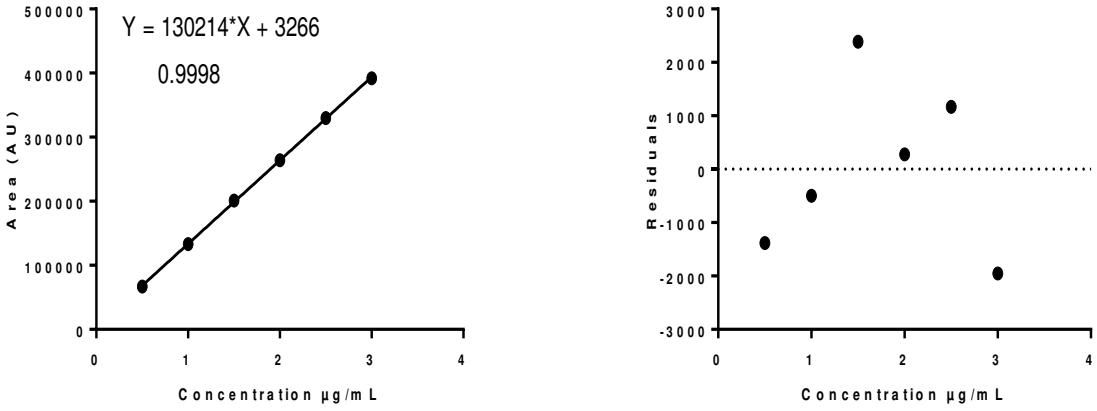

Fig.4.15. Calibration curve and residual plot for RAM

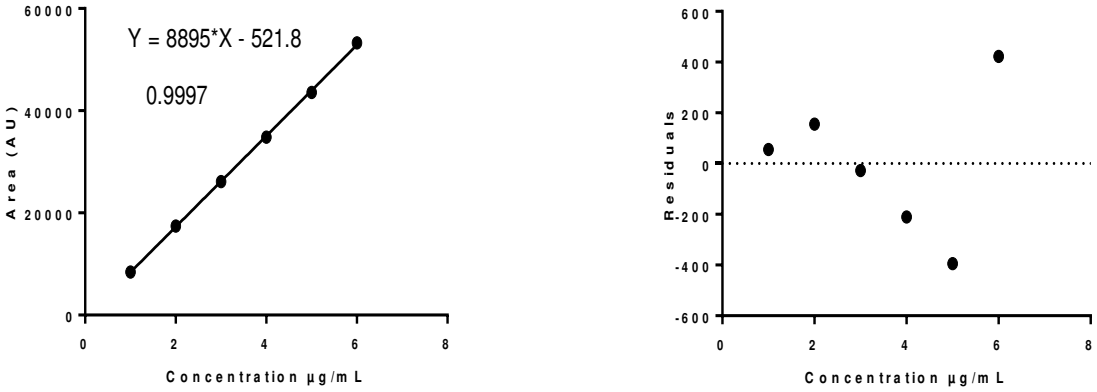

Fig. 4.16. Calibration curve and residual plot for AMLO

Table 4.24: Precision studies of RAM and AMLO

Conc. (µg/mL)	Intra-day precision (n=3)			Inter-day precision (n=3)		
	Measured Conc. ± SD	(%) RSD	Recovery (%)	Measured Conc. ±SD	(%) RSD	Recovery (%)
Ramipril						
1	0.992 ± 0.011	1.15	99.20	0.99 ± 0.012	1.24	99.00
2	1.985 ± 0.024	1.21	99.25	1.983 ± 0.023	1.17	99.15
3	2.974 ± 0.032	1.07	99.13	2.972 ± 0.034	1.13	99.07
Amlodipine						
2	1.988± 0.021	1.03	99.40	1.981 ± 0.023	1.14	99.05
4	3.982 ± 0.045	1.12	99.55	3.979 ± 0.042	1.05	99.48
6	5.98 ± 0.071	1.19	99.67	5.97 ± 0.076	1.27	99.50

Table 4.25: Robustness evaluation of RAM and AMLO (n=3)

Conditions	Level	RAM		AMLO	
		t_R (min.)	% RSD of peak area	t_R (min.)	% RSD of peak area
A: Flow rate (± 0.1 mL/min.)					
0.9	-0.1	2.690	1.09	7.216	1.08
1	0.0	2.630	1.13	7.192	1.12
1.1	+0.1	2.597	1.01	7.162	1.15
Mean ± SD		2.636 ± 0.052	1.077± 0.06	7.190 ± 0.027	1.12 ± 0.035
B: % of methanol in the mobile phase (± 5%)					
90	-5.0	2.686	1.05	7.212	1.14
95	0.0	2.630	1.12	7.192	1.08
100	+5.0	2.592	1.04	7.150	1.11
Mean ± SD		2.636 ± 0.047	1.07 ± 0.044	7.185 ± 0.032	1.11± 0.03

Table 4.26: Recovery study for RAM and AMLO (n=3)

Label claim (mg/capsule)	Amount Added (%)	Total amount (mg)	Amount recovered (mg)	Recovery (%)	Mean (%) Recovery (± SD)
RAM 2.5	80 100 120	4.5 5 5.5	4.471 4.974 5.460	99.36 99.48 99.27	99.37 ± 0.11
AMLO 5.0	80 100 120	9 10 11	8.930 9.932 10.93	99.22 99.32 99.36	99.30 ± 0.072

Table 4.27: Assay results of RAM and AMLO in capsules (n=3)

Stamace capsule contains	Label claim (mg/capsule)	Amount found (mg ± SD)	RSD (%)	Recovery (%)
RAM	2.5 mg	2.486 ± 0.030	1.19	99.44
AMLO	5 mg	4.978 ± 0.056	1.12	99.56

Table 4.28: Comparison of the proposed method with the reported methods:

Parameter	Reported method 1		Reported method 2		Proposed method	
	RAM	**AMLO**	**RAM**	**AMLO**	**RAM**	**AMLO**
Mobile Phase	Mobile phase A: 60 mM $NaClO_4$ buffer (containing 7.2 mM TEA)-ACN (60:40), Mobile phase B: 60 mM $NaClO_4$ buffer (containing 7.2 mM TEA)-ACN (20:80, v/v) pH 2.6 with H_3PO_4		phosphate buffer (0.02 M KH_2PO_4 and 0.002 M di-K_2HPO_4 anhydrous, pH 6.8) and ACN (60:40 % v/v)		**Methanol: Water (95:5 v/v)**	
Mode	Gradient		Isocratic		**Isocratic**	
Detection wavelength (nm) & Flow Rate	210 1 mL/min.		237 1 mL/min.		**227 1 mL/min.**	
Retention time (t_R) in min.	10	12	2.13	5.18	**2.630**	**7.192**
Linearity range (µg/mL)	10-250	14-360	1.25-7.5	2.5-15	**0.5-3**	**1-6**
LOD (µg/mL) and LOQ (µg/mL)	0.06 0.2	0.02 0.07	0.46 1.42	1.05 3.18	**0.2** **0.5**	**0.2** **0.5**
% Recovery (n=3)	99.7	100.5	99.99	100.65	**99.37**	**99.30**
Precision (% RSD) Intra-day (n=3) Inter-day (n=3)	 0.46 1.6	 0.55 1.8	 0.32 0.28	 0.31 0.32	 **1.14** **1.18**	 **1.11** **1.15**

4.4.3. Conclusion:

RP-HPLC method has been developed for simultaneous estimation of Amlodipine besylate and Ramipril in bulk drug and pharmaceutical dosage form. The method was validated for linearity, accuracy, precision and specificity. The run time is relatively short (less than 10 min), which enables rapid quantification of many samples in routine and quality control analysis of formulation. The method also uses a solvent system with the same composition as the mobile phase for dissolving, extracting drugs from the matrices and washing of the column, thus minimizing noise. Thus the proposed method is rapid, selective, requires a simple sample preparation procedure and it is suitable for analysis of AMLO and RAM in their formulation in a single isocratic run, in contrast with previous gradient method. Therefore the developed method can be used for routine analysis.

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Development of analytical methods for the simultaneous analysis of antihypertensive formulations containing three components by HPLC

Introduction[1-6]:

Antihypertensive regimens that combine agents from different antihypertensive drug classes can facilitate attainment of blood pressure goals and improve cardiovascular outcomes at lower drug doses. When hypertensive patients can't obtain target blood pressure with monotherapy or with formulations containing two drugs, triple fixed-dose combinations are preferred for lowering blood pressure effectively.

For patients whose antihypertensive therapy includes multiple medications, the use of a single-pill, triple fixed-dose combination therapy can significantly improve compliance and thereby help patients to achieve BP goals.

Several classes of antihypertensive agents with different mechanisms of action including: angiotensin- converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs), beta (β)-blockers, calcium channel blockers (CCBs), and thiazide-type diuretics have demonstrated efficacy for lowering BP.

Triple fixed dose combination that are available for the management of hypertensive patients with moderate, high or very high risk include,

- A thiazide diuretic, an angiotensin II receptor blockers (ARBs) and a calcium channel blocker. e.g. Hydrochlorothiazide, Amlodipine and Losartan/Valsartan/ Olmesartan/ Telmisartan etc.

  Thiazide diuretics reduce intravascular volume and total body sodium. Calcium channel blockers prevent the entry of calcium to the cellular cytosol in arteriolar smooth muscle cells. Angiotensin II receptor blockers impede the activation of angiotensin II AT1 receptors, preventing the vasoconstriction induced by angiotensin II, while blockade of the receptor in the renal cells helps prevent the renal retention of sodium.

- A thiazide diuretic, an angiotensin II receptor blocker (ARBs) and an angiotensin-converting enzyme (ACE) inhibitor. e.g. Hydrochlorothiazide, Amlodipine and Ramipril.

  Triple fixed dose combinations consist of thiazide diuretics, angiotensin- converting enzyme (ACEI) inhibitors and angiotensin II receptor blockers (ARBs) can often

perform a favorable effect demonstrating that thiazide diuretics would induce increased sodium loss and intravascular volume depletion when exerting their effects which can lead to an activation of renin-angiotensin-aldosterone system thus boosting the antihypertensive effect of angiotensin- converting enzyme (ACEI) inhibitors. Based upon the same mechanism, angiotensin II receptor blockers (ARBs) show an additive antihypertensive effect. ARB can reduce the potassium loss and hyperuricemia caused by thiazide diuretics owing to the interdiction of aldosterone secretion.

- A thiazide diuretic, an angiotensin II receptor blocker (ARBs) and a beta (β)–blocker. e.g Atenolol, hydrochlorothiazide and Losartan, seems a reasonable and efficacious combinations.

  Thiazide diuretics blocks sodium chloride reabsorption at distal convoluted tubule. The beta blockers inhibits activation by direct suppression of renin release, inhibit beta adrenergic sympathetic stimulation decreasing myocardial contractility and heart rate. ARBs cause the antagonism of angiotensin II at the vascular and myocardial level by direct AT-1 receptor blockade.

In view of the above concepts and considering the need to develop new analytical methods, the following combinations were selected for the present study.

Sr. No.	Combination	Brand name	Manufacturer	Label Claim
1	Losartan, Amlodipine and Hydrochlorothiazide	Losagem-AH Tablet	Genetic Pharma.	Losartan 50 mg Amlodipine 5.0 mg Hydrochlorothiazide 12.5 mg
2	Losartan, Ramipril and Hydrochlorothiazide	Loram-H	Unichem Laboratories Ltd, India	Losartan 50 mg Ramipril 5.0 mg Hydrochlorothiazide 12.5 mg
3	Losartan, Atenolol and Hydrochlorothiazide	Repalol-H	Sun Pharma. Industries Ltd.	Losartan 50 mg Atenolol 50 mg Hydrochlorothiazide 12.5 mg

Literature survey revealed that few HPLC methods have been reported for estimation of 1. Losartan, Amlodipine and Hydrochlorothiazide, 2. Losartan, Ramipril and Hydrochlorothiazide, 3. Losartan, Atenolol and Hydrochlorothiazide individually or in combination with each other and also with other drugs. However, reported methods for the above combinations suffer from certain drawbacks like buffered mobile phases which required time consuming preparation methods, pH adjustment, linearity at higher concentration ranges and reproducibility of the methods. The purpose of the present study was to develop methods for the above combinations which eliminate the drawbacks mentioned above. Hence reliable and rugged method for the simultaneous analysis of drugs from above combinations was taken up.

5.1 Simultaneous analysis of Losartan potassium, Hydrochlorothiazide and Amlodipine besylate by HPLC

Losartan potassium, 2-butyl-4-chloro-1-[p-(o-1H-tetrazol-5ylphenyl)benzyl] imidazole -5- methanol monopotassium salt, is an angiotensin II receptor blocker and is used as an antihypertensive agent. Hydrochlorothiazide, chemically, 6-Chloro-3, 4-dihydro-2H-l, 2, 4-benzothiadiazine-7-sulfonamide 1, 1-dioxide, is a thiazide diuretic. Amlodipine besylate, 3, 5-Pyridinecarboxylic acid, 2-((aminoethoxy) methyl)-4-(2- chlorophenyl) -1, 4-dihydro-6-methyl, 3-ethyl-5-methyl ester, a calcium channel blockers is used as an anti-hypertensive.

The drug profiles and literature reviews of Amlodipine besylate (AMLO), Hydrochlorothiazide (HCTZ) and Losartan potassium (LOS) are given in 3.3, 3.5 and 3.6.

5.1.1 Experimental:

- **Instrumentation:**

  The HPLC system consisted of Intelligent HPLC pump model (Jasco PU 1580). The solutions were injected into the chromatograph through a Rheodyne valve, with a 20 µL loop with auto sampler (AS 1555). The column used was Thermo Hypersil Gold C_{18} (250 mm × 4.6 mm, 5.0 µ), operating at room temperature. The elution was carried out isocratically at flow rate of 0.8 mL/min. using methanol: water (95:5 v/v) as mobile phase. The detector consisted of a UV/ VIS (Jasco UV 1575) model operated at a wavelength of 230 nm. Data was integrated using Jasco Borwin version 1.5, LC-Net II/ADC system.

- **Chemicals and reagents:**

  Reference standards of, Losartan potassium (100.2%) and Hydrochlorothiazide (99.97 %) were procured from Emcure Pharmaceuticals Ltd. Pune and Amlodipine besylate (99.92%) was procured from Lupin research park Pune, India. The tablets Losagem-AH (Losartan 50 mg, Amlodipine 5 mg and Hydrochlorothiazide 12.5 mg) of Genetic Pharma. was procured from the market. For HPLC work double distilled water was prepared in the laboratory. Methanol used was of HPLC grade and was purchased from Merck Chemicals, Mumbai, India.

- **Stationary Phase:**

  Thermo Hypersil Gold C_{18} (250 mm × 4.6 mm, 5.0 µ) was used.

- **Preparation of mobile phases:**

  HPLC grade methanol (950 mL) was mixed with (50 mL) of double distilled water. This solution was filtered through 0.45µ membrane filter paper and degassed in an ultrasonic bath before use.

- **Preparation of standard stock solution-I:**

  500 mg of Losartan potassium (LOS), 125 mg of Hydrochlorothiazide (HCTZ) and 50 mg of Amlodipine besylate (AMLO) were accurately weighed and transferred to a 50 mL volumetric flask and dissolved in methanol. Volume was made up to the mark with mobile phase to get a solution containing LOS 10 mg/mL, AMLO 1 mg/mL and HCTZ 0.25 mg/mL respectively. 1mL of this solution was further diluted to 10 ml with mobile phase to get **Standard stock solution-I** containing LOS 1 mg/mL, AMLO 0.1 mg/mL and HCTZ 0.025 mg/mL, respectively.

- **Working standard solution-II:**

  0.1mL of the standard stock solution-I was diluted to 10 mL with mobile phase to get a concentration of 10 µg/mL of LOS, 2.5 µg/ml HCTZ and 1 µg/mL of AMLO. This solution was used as working standard.

- **Sample solution:**

  Twenty tablets were weighed accurately and powered. Tablet powder equivalent to 50 mg of LOS, 12.5 mg of HCTZ and 5 mg of AMLO was weighed accurately and transferred into a clean and dry 50 mL volumetric flask. The powder was first dissolved in sufficient volume of methanol and kept in an ultrasonic bath for 15 min. and the volume was made to 50 mL with mobile phase, filtered through 0.45µ membrane filter paper and solution was further diluted with mobile phase to obtain a solution of LOS (10 µg/mL), HCTZ (2.5 µg/mL) and AMLO (1 µg/mL), respectively. The sample solution (20µL) was injected into the chromatograph at the flow rate of 0.8 mL/min. and the chromatogram was recorded.

- **System suitability**

  20µL of standard solution containing LOS (10 µg/mL), HCTZ (2.5 µg/mL) and AMLO (1 µg/mL) was injected into the chromatograph to test the system suitability

to determine resolution (Rs), retention time (t_R), theoretical number of plates (N) and tailing factor (T).

- **Analytical Method Validation**

  Validation was done as per ICH guidelines. The developed method was validated with respect to parameters such as linearity, LOD and LOQ, precision, accuracy and specificity.

- **Linearity:**

  Several aliquots of standard stock solution-I of LOS, HCTZ and AMLO were taken in different 10 mL volumetric flask and diluted up to the mark with mobile phase such that the final concentration of LOS, HCTZ and AMLO were in the range of 10–60 µg/mL, 2.5-15 µg/mL and 1-6 µg/mL, respectively. Evaluations of three drugs were performed with UV detector set at 230 nm. The peak area versus concentration data was collected and subjected to regression analysis. The correlation coefficient, slope and y-intercept values were calculated from the calibration plot obtained.

- **Limit of detection and limit of quantitation:**

  The Limit of detection (LOD) and Limit of quantitation (LOQ) for LOS, HCTZ and AMLO and estimated at a signal-to-noise ratio of 3:1 and 10:1 respectively, by injecting a series of dilute solutions with known concentration.

- **Precision:**

  The precision of the method was verified by intra-day and inter-day precision studies. Intra-day precision studies were performed by analysis of three different concentrations 20, 40, 60 µg/mL for LOS, 5, 10, 15 µg/mL for HCTZ and 2, 4, 6 µg/mL for AMLO three times on the same day. The inter-day precision of the method was checked by repeating studies on three different days.

- **Robustness:**

  To determine the robustness of the developed method, experimental conditions were deliberately changed and the relative standard deviation for replicate injections of LOS, AMLO and HCTZ were evaluated. Flow rate was changed by ± 0.1 units to 0.7 and 0.9 mL/min. The effect of % of methanol in the mobile phase was studied at (± 5%).

- **Recovery studies:**

  To check the accuracy of the proposed method recovery experiment was carried out. Twenty tablets from the same lot of the commercial formulation were weighed accurately and finely powered. Powder equivalent to 50 mg of LOS, 12.5 mg of HCTZ and 5 mg of AMLO was weighed and transferred to different 50 mL volumetric flask. Standard drug was added at 80%, 100% and 120% level. The contents were dissolved by sonicating in 20 mL of mobile phase for 15 minutes and filtered through 0.45µ membrane filter paper and further diluted up to the mark using mobile phase. 0.1 mL of the filtrate was taken in 10 mL volumetric flask and diluted up to the mark with mobile phase. 20µL of sample solution were injected into the chromatograph and chromatograms were recorded. Percentage recovery and relative standard deviation were calculated. Each level was repeated three times.

- **Analysis of marketed formulation:**

  20 µL of working standard solution-II (10 µg/mL of LOS, 2.5 µg/mL HCTZ and 1 µg/mL of AMLO) and sample solutions were injected into the liquid chromatograph and the chromatogram was recorded. From the peak areas of LOS, HCTZ and AMLO the amount of the drugs in the sample were calculated.

- **Specificity:**

  The specificity of the method was ascertained by analysing drug standards and samples. The chromatogram was overlaid to study the specificity. The identities of the peak for LOS, HCTZ and AMLO from the sample were confirmed by comparing the t_R with those of standards.

- **Comparison of the proposed method with the reported methods:**

  The proposed method was compared with the following reported methods from the literature:

1. Spectrophotometric and HPLC Methods for Simultaneous Estimation of Amlodipine Besilate, Losartan Potassium and Hydrochlorothiazide in Tablets[7]

   Column: Kromasil C_{18} column (5µ particles size, 25 cm X 4.6 mm)

   Mobile phase: 0.025 M phosphate buffer (pH 3.7): acetonitrile (57:43% v/v)

   Flow rate: 1.0 to 1.3 ml/min, λ max = 232nm.

2. Simultaneous estimation of hydrochlorothiazide, amlodipine, and losartan in tablet dosage form by RP-HPLC[8]

   Column: Phenomenex luna CN100R, (5µ particles size, 25 cm X 4.6 mm)

   Mobile phase: acetonitrile, water and 0.4% of potassium dihydrogen phosphate buffer pH 2.7 adjusted with ortho-phosphoric acid (45:35:20)

   Flow rate: 1.0 ml/min, λ max = 230 nm.

5.1.2. Results and Discussion:

- **Selection of column:**

  As the drugs were soluble in polar solvents such as methanol and acetonitrile, C_{18} reverse phase column was selected with particle size of 5.0 µ, length 250 mm and internal diameter 4.6 mm.

- **Selection of suitable wavelength:**

  Stock solutions of individual drugs were prepared in methanol and the solutions were scanned individually in a UV spectrophotometer in the range of 200-400 nm and their spectras were recorded. The spectras were overlaid and the isobestic point, 230 nm was selected as the suitable wavelength as all three drugs showed good absorption at this wavelength (Fig.5.01).The wavelength was checked and confirmed in the optimised mobile phase also.

- **Selection of mobile phase:**

  Different mobile phase were selected and tried. In the preliminary trials using different composition of methanol with water in a ratios of 50:50 v/v, 60:40 v/v and 75:25 v/v, two peaks was resolved but did not give well defined peak in a short time. With 80:20%, 90:10% methanol and water ratios, three peaks were resolved however asymmetry was not good for AMLO. Keeping mobile phase composition as methanol: water (95:5, v/v), satisfactory resolution with good peak shape was obtained. Finally the mobile phase 95:5 v/v methanol: water was selected. With this optimized mobile phase retention time were found to be for LOS (t_R-2.850 min.), HCTZ (t_R-3.875) and AMLO (t_R-7.333 min.), respectively.

- **System suitability**

  System suitability tests were carried out on freshly prepared standard stock solution of LOS, HCTZ and AMLO to determine efficiency, resolution factor, symmetry

factor and precision of the instrument and found to comply with USP requirement. Parameters obtained with 20 µL injection volume are shown in Table 5.01. Column efficiency in terms of theoretical plates number (N) was for 3272.51 for LOS, 6185.40 for HCTZ and 10318.15 for AMLO, respectively.

- **Validation of the method**

  Using optimised chromatographic conditions the RP-HPLC method developed was validated in terms of linearity, LOD and LOQ, precision, robustness and accuracy.

- **Linearity:**

  Linear regression data for the calibration plots revealed good linear relationships between response and concentration over the ranges 10–60 µg/mL for LOS, 2.5-15 for µg/mL HCTZ and 1-6 µg/mL for AMLO, respectively. The linear regression equations were Y=15938X + 25057 (r^2= 0.9994) for LOS, Y= 19655X + 1857 (r^2= 0.9994) for HCTZ and Y= 8784X -201.9 (r^2= 0.9997) for AMLO. The residuals plot shows a random pattern, indicating a good fit for a linear model. The calibration curves and residuals plots are given in Fig. 5.03, Fig. 5.04 and Fig. 5.05 and Table 5.02 (A), Table 5.02 (B) and Table 5.02 (C) for LOS, HCTZ and AMLO, respectively.

- **Limits of Detection and Quantitation:**

  The signal to noise ratios of 3: 1 and 10: 1 were considered as LOD and LOQ respectively. The results of the LOD and LOQ were found to be 0.6 µg/mL and 1.8 µg/mL for LOS, 0.2 µg/mL and 0.5 µg/mL for HCTZ and 0.4 µg/mL and 0.8 µg/mL for AMLO, respectively,

- **Precision:**

  The precision of the developed method was demonstrated by intra-day and inter-day precision studies. This was done by three replicate analysis of the composite sample. The precision of the method was expressed as relative standard deviation (RSD, %). The % RSD of intra-day and inter-day precision values was found to be 1.00 % and 1.01%, for LOS, 1.11% and 1.03 % for HCTZ and 1.017% and 1.02% for AMLO respectively (Table 5.03). The RSD values were found to be <2 %, which indicates that the proposed method is precise.

- **Robustness:**

  There were no significant changes in the retention times of LOS, HCTZ and AMLO when the flow rate (± 0.1mL/min.) and composition of the mobile phase (± 5%) were changed. The low values of the RSD indicate the robustness of the method, as shown in Table 5.04

- **Accuracy:**

  Accuracy of the proposed method was evaluated by carrying out a recovery study. The known concentration of standard drugs were added to a preanalysed tablet sample at three different levels namely 80%, 100% and 120%. Total amount of the drugs were determined by the proposed method. The average recoveries obtained as 99.52 % for LOS and 99.52 for HCTZ and 99.17 % for AMLO indicate good accuracy of the method (Table 5.05).

- **Analysis of marketed formulation:**

  The chromatogram of the sample extracted from conventional tablets showed principal peaks of LOS (t_R 2.850 min.), HCTZ (t_R 3.875 min.) and AMLO (t_R 7.333 min) that are well resolved from other tablet excipients, as shown in Fig. 5.02. The mean content of LOS, HCTZ and AMLO per tablet by proposed method was found to be 50.35 mg, 12.71 mg and 5.01mg, respectively. The results are summarized in Table 5.06.

- **Comparison of the proposed method with the reported methods:**

  The proposed method was compared with the reported methods. The proposed method uses simple mobile phase of methanol: water (95:5 v/v) at flow rate of 0.8 mL/min. as against buffered mobile phases at the flow rate of 1 to 1.3 mL/min which was change during the analysis. Linearity concentration range of the proposed method is 10-60 µg/mL for LOS, 2.5-15 µg/mL for HCTZ and 1-6 µg/mL for AMLO which is quiet lower as compared to the reported methods. The proposed method gives better column efficiency and resolution than the reported methods. (Table 5.07)

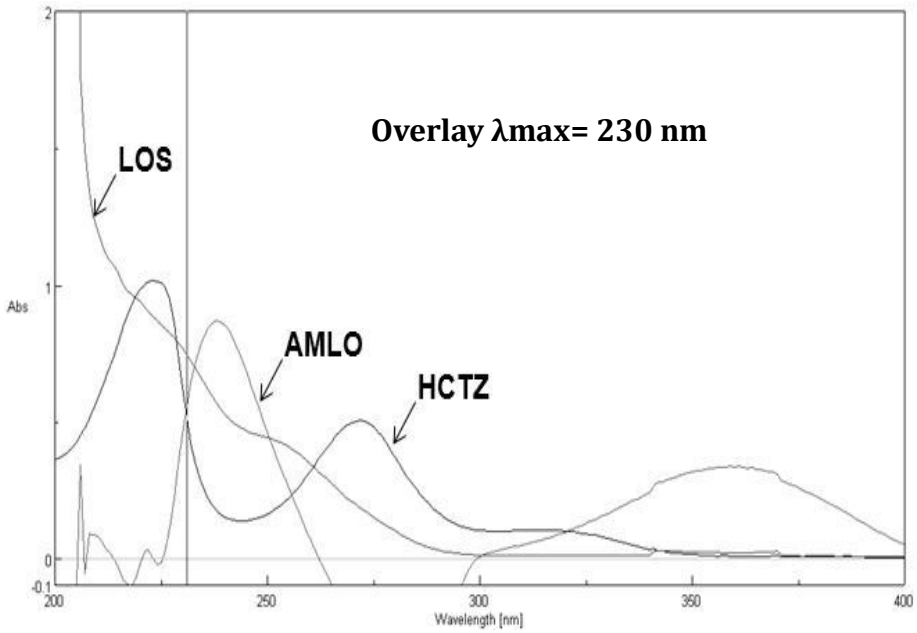

Fig. 5.01. Overlay absorption spectra of LOS, AMLO and HCTZ

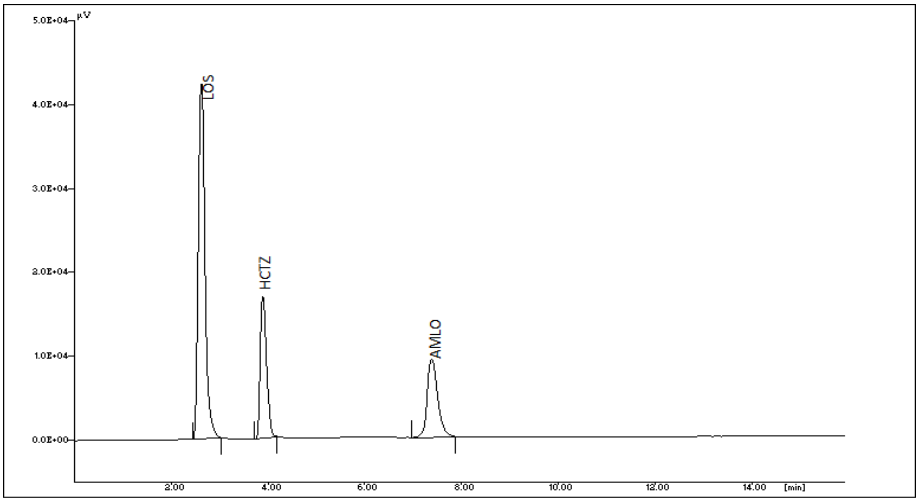

Fig.5.02. Typical chromatogram of LOS, HCTZ and AMLO

Table 5.01: System suitability parameters (n=6)

Parameters	**LOS**	**HCTZ**	**AMLO**
Retention Time in min	2.850	3.875	7.333
Resolution (Rs)	--	4.359	14.339
Theoretical plates number (N)	3272.51	6185.40	10318.15
Tailing Factor	1.071	1.255	1.320

Table 5.02 (A): Data for LOS from linearity study

Concentration (µg/mL)	HPLC (n= 3)	
	Mean peak area ± SD	**RSD (%)**
10	187042 ± 2244.50	1.20
20	339157 ± 3934.22	1.16
30	496247 ± 6550.46	1.32
40	673334 ± 7272.0	1.08
50	827070 ± 9428.60	1.14
60	974570 ± 10233.0	1.05
Regression coefficient r^2		**0.9994**
Slope		**15938**
Intercept		**25057**

Table 5.02 (B): Data for HCTZ from linearity study

Concentration (µg/mL)	HPLC (n= 3)	
	Mean peak area ± SD	**RSD (%)**
2.5	50156 ± 486.51	0.97
5	98231 ± 1021.60	1.04
7.5	150910 ± 1720.37	1.14
10	201849 ± 2482.74	1.23
12.5	247491 ± 2920.40	1.18
15	294367 ± 3562.0	1.21
Regression coefficient r^2		**0.9994**
Slope		**19655**
Intercept		**1857**

Table 5.02 (C): Data for AMLO from linearity study

Concentration (µg/mL)	HPLC (n= 3)	
	Mean peak area ± SD	**RSD (%)**
1	8797 ± 93.25	1.06
2	16978 ± 214.0	1.26
3	26228 ± 346.21	1.32
4	34846 ± 390.28	1.12
5	44126 ± 560.40	1.27
6	52270 ± 564.12	1.08
Regression coefficient r^2		**0.9997**
Slope		**8784**
Intercept		**-201.9**

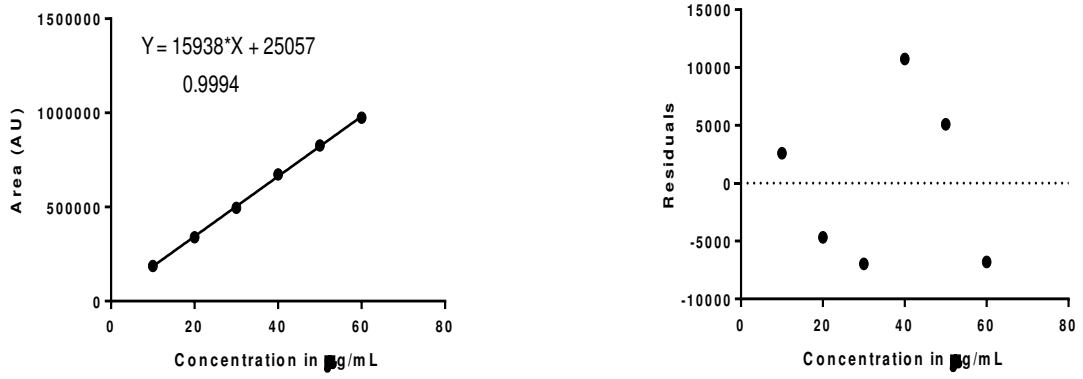

Fig. 5.03. Calibration curve and residual plot for LOS

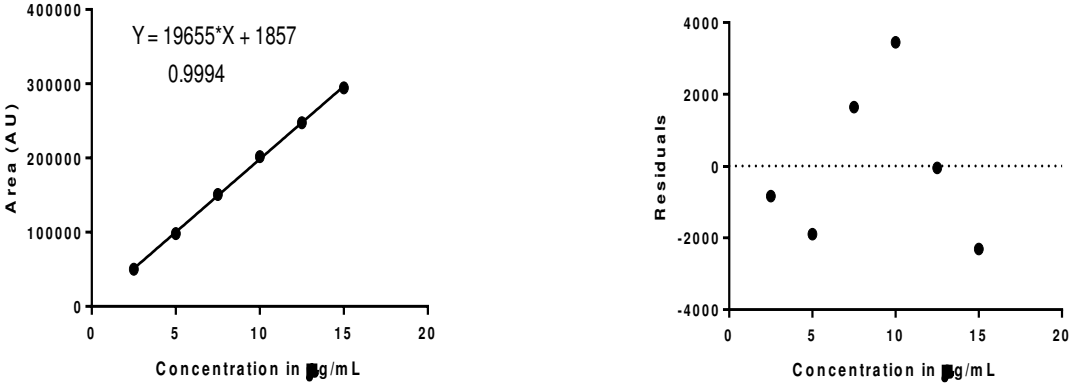

Fig. 5.04. Calibration curve and residual plot for HCTZ

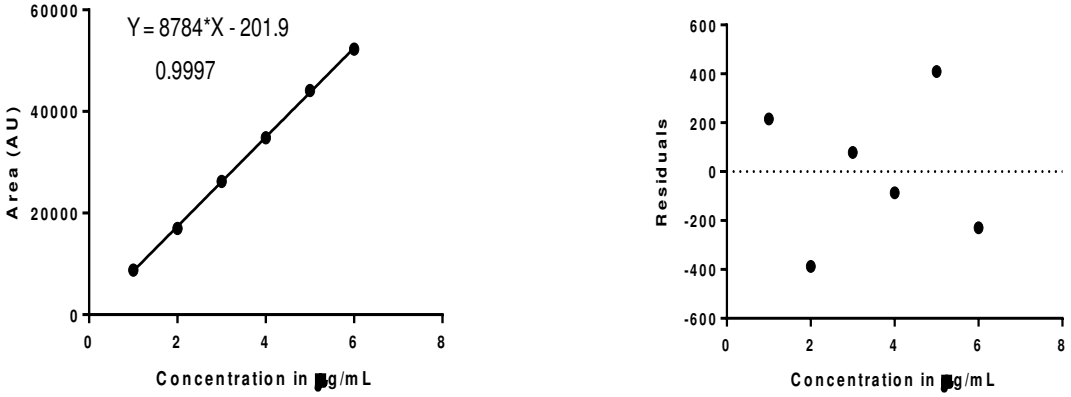

Fig. 5.05. Calibration curve and residual plot for AMLO

Table 5.03: Precision studies of LOS, HCTZ and AMLO

Conc. (µg/mL)	Intra-day precision (n=3)			Inter-day precision (n=3)		
	Measured Conc. ± SD	(%) RSD	Recovery (%)	Measured Conc. ±SD	(%) RSD	Recovery (%)
Losartan						
20	19.90 ± 0.183	0.92	99.50	19.87 ± 0.20	1.03	99.35
40	39.92 ± 0.41	1.03	99.80	39.80 ± 0.39	0.98	99.50
60	59.96 ± 0.64	1.07	99.93	59.82 ± 0.61	1.02	99.70
Hydrochlorothiazide						
5	4.94 ± 0.052	1.05	98.80	4.92 ± 0.054	1.10	98.40
10	9.94 ± 0.11	1.08	99.40	9.90 ± 0.10	1.04	99.00
15	14.85 ± 0.18	1.21	99.00	14.83 ± 0.14	0.97	98.86
Amlodipine						
2	1.98 ± 0.020	1.02	99.00	1.97 ± 0.020	1.03	98.50
4	3.97 ± 0.042	1.05	99.25	3.96 ± 0.040	1.02	99.00
6	5.95 ± 0.058	0.98	99.16	5.93 ± 0.060	1.01	98.83

Table 5.04: Robustness evaluation of LOS, HCTZ and AMLO (n=3)

Conditions	Level	LOS		HCTZ		AMLO	
		t_R (min.)	% RSD of peak area	t_R (min.)	% RSD of peak area	t_R (min.)	% RSD of peak area
A: Flow rate (± 0.1mL/min.)							
0.7	-0.1	2.892	1.12	3.883	1.09	7.350	1.01
0.8	0.0	2.850	0.97	3.875	1.04	7.333	1.08
0.9	+0.1	2.817	1.08	3.867	1.11	7.308	1.16
Mean ± SD		2.853 ± 0.038	1.057 ± 0.078	3.875 ± 0.008	1.08 ± 0.036	7.330 ± 0.0211	1.083 ± 0.075
B: % of methanol in the mobile phase (± 5%)							
90	-5.0	2.898	0.99	3.885	1.15	7.356	1.09
95	0.0	2.850	1.07	3.875	1.18	7.333	1.14
100	+5.0	2.810	1.14	3.850	1.01	7.292	1.19
Mean ± SD		2.853 ± 0.044	1.067 ± 0.075	3.87 ± 0.018	1.113 ± 0.091	7.327 ± 0.032	1.14 ± 0.050

Table 5.05: Recovery study of LOS, HCTZ and AMLO (n=3)

Label claim (mg/tablet)	Amount Added (%)	Total amount (mg)	Amount recovered (mg)	Recovery (%)	Mean (%) Recovery (± SD)
LOS 50	80	90	89.45	99.38	99.52
	100	100	99.89	99.89	
	120	110	109.21	99.28	
HCTZ 12.5	80	22.5	22.40	99.55	99.52
	100	25	24.92	99.68	
	120	27.5	27.32	99.34	
AMLO 5	80	9	8.92	99.11	99.17
	100	10	9.94	99.40	
	120	11	10.89	99.00	

Table 5.06: Assay results of LOS, AMLO and HCTZ in tablets

Losagem-AH tablet contains	Label claim (mg/tablet)	Amount found (mg ± SD)	RSD (%)	Recovery (%)
LOS	50 mg	50.35 ± 0.564	1.12	100.7
HCTZ	12.5 mg	12.71 ± 0.16	1.25	101.68
AMLO	5 mg	5.01 ± 0.076	1.52	100.23

Table 5.07: Comparison of the proposed method with the reported methods

Parameter	**Reported Method 1**	**Reported Method 2**	**Propose Method**
Mobile Phase	0.025 M phosphate buffer (pH 3.7): ACN (57:43%v/v)	ACN, water and 0.4% of KH_2PO_4 buffer pH 2.7 adjusted with OPA (45:35:20 v/v/v)	**Methanol: Water (95:5 v/v)**
Mode	Isocratic	Isocratic	**Isocratic**
Detection wavelength (nm) & Flow Rate	232nm 1 to 1.3mL/min	230nm 1 mL/min	**230nm** **0.8 mL/min**
Retention time (t_R) in min.	LOS- 8.02 HCTZ- 3.42 AMLO- 5.12	LOS- 5.8 HCTZ- 3.9 AMLO- 4.9	**LOS-2.850** **HCTZ- 3.875** **AMLO- 7.333**
Linearity range (µg/mL)	LOS- 20-140 HCTZ- 5-40 AMLO- 2-14	LOS-50-250 HCTZ- 12.5-62.5 AMLO- 2.5 – 12.5	**LOS-10-60** **HCTZ-2.5-15** **AMLO-1-6**
LOD (µg/mL) and LOQ (µg/mL)	-----	LOS-0.108 and 0.228 HCTZ- 0.03 and 0.1 AMLO-0.03 and 0.1	**LOS-0.6 and 1.8** **HCTZ-0.2 and 0.5** **AMLO-0.4 and 0.8**
% Recovery (n=3)	LOS-99.96 HCTZ- 100.33 AMLO-99.97	LOS-99.92 HCTZ- 99.31 AMLO-99.58	**LOS-99.52** **HCTZ- 99.52** **AMLO-99.17**
Precision (% RSD) Intra-day (n=3) Inter-day (n=3)	------	LOS-0.373 & 0.567 HCTZ-0.495 &0.57 AMLO-0.927 &0.962	**LOS-1.00 &1.01** **HCTZ-1.11 &1.03** **AMLO-1.017 &1.2**

5.1.3. Conclusion:

A simple, rapid and reliable RP-HPLC method has been developed for simultaneous determination of Losartan potassium, Hydrochlorothiazide and Amlodipine besylate in bulk and in pharmaceutical formulation. The method has been validated statistically for linearity, specificity, accuracy and precision. The linear regression data for the calibration plot are indicative of a good linear relationship between peak area and concentration over a wide range. The correlation coefficient was indicative of high significance. The low values of the standard deviation, slope and the intercept of the ordinate showed the calibration plot did not deviate from linearity.

The developed method has several advantages including a simple mobile phase, simple sample preparation and no requirement of change in flow rate during analysis as compared to previous methods. This makes the method suitable for routine analysis in quality control laboratories.

5.2 Simultaneous analysis of Losartan potassium, Ramipril and Hydrochlorothiazide by HPLC

Losartan potassium, 2-butyl-4-chloro-1-[p-(o-1H-tetrazol-5ylphenyl)benzyl] imidazole -5-methanol monopotassium salt is an angiotensin II receptor blocker and is used as an antihypertensive agent. Hydrochlorothiazide chemically, 6-Chloro-3, 4-dihydro-2H-l, 2, 4-benzothiadiazine-7-sulfonamide 1, 1-dioxide is a thiazide diuretic. Ramipril is (2*S*, 3aS, 6aS)-1[(*S*)-N-[(*S*)-1-Carboxy-3-phenylpropyl]alanyl] octahydrocyclopenta [*b*]pyrrole-2-carboxylic acid, 1-ethyl ester, is an angiotensin converting enzyme.

The drug profiles and literature reviews of Losartan potassium (LOS), Ramipril (RAM) and Hydrochlorothiazide (HCTZ) by HPLC are given in 3.4, 3.5 and 3.6.

5.2.1 Experimental:

- **Instrumentation:**

  The HPLC system consisted of Intelligent HPLC pump model (Jasco PU 1580). The solutions were injected into the chromatograph through a Rheodyne valve, with a 20 µL loop with auto sampler (AS 1555). The column used was Thermo Hypersil Gold C_{18} (250 mm × 4.6 mm, 5.0 µ), operating at room temperature. The elution was carried out isocratically at flow rate of 1 mL/min. using methanol: water (95:5 v/v) as mobile phase. The detector consisted of a UV/ VIS (Jasco UV 1575) model operated at a wavelength of 218nm. Data was integrated using Jasco Borwin version 1.5, LC-Net II/ADC system.

- **Chemicals and reagents:**

  Reference standards of, Losartan potassium (100.2%), Hydrochlorothiazide (99.97%) and Ramipril (99.4%) were procured from Emcure Pharmaceuticals Ltd. The tablets LORAM-H (Losartan-50 mg, Ramipril-5 mg and Hydrochlorothiazide 12.5 mg) of Unichem Laboratories Ltd, India. was procured from the market. For HPLC work double distilled water was prepared in the laboratory. Methanol used was of HPLC grade and was purchased from Merck Chemicals, Mumbai, India.

- **Stationary Phase:**

  Thermo Hypersil Gold C_{18} (250 mm × 4.6 mm, 5.0 µ) was used.

- **Preparation of mobile phases:**

  HPLC grade methanol (950 mL) was mixed with (50 mL) of double distilled water. This solution was filtered through 0.45µ membrane filter paper and degassed in an ultrasonic bath before use.

- **Preparation of standard stock solution-I:**

  500 mg of Losartan potassium (LOS), 50 mg of Ramipril (RAM) and 125 mg of Hydrochlorothiazide (HCTZ) were accurately weighed and transferred to a 50 mL volumetric flask and dissolved in mobile phase. Volume was made up to the mark with mobile phase to get a solution containing LOS 10 mg/mL, RAM 1mg/mL and HCTZ 0.25 mg/mL respectively. 0.1mL of this solution was further diluted (LOS 10 mg/mL, RAM 1 mg/mL and HCTZ 0.25 mg/mL) to 10 mL with mobile phase to get **Standard stock solution I** containing LOS 0.1 mg/mL, RAM 0.01 mg/mL and HCTZ 0.0025 mg/mL, respectively.

- **Working standard solution-II:**

  0.2 ml of the standard stock solution-I was diluted to 10 mL with mobile phase to get a concentration of 2 µg/mL of LOS, 0.2 µg/mL of RAM and 0.5 µg/mL HCTZ. This solution was used as working standard.

- **Sample solution:**

  Twenty tablets were weighed accurately and powered. Tablet powder equivalent to 50 mg of LOS, 5 mg of RAM and 12.5 mg of HCTZ was weighed accurately and transferred into a clean and dry 50 mL volumetric flask. The powder was first dissolved in sufficient volume of mobile phase and kept in an ultrasonic bath for 15 min. and the volume was made to 50 mL with mobile phase, filtered through 0.45µ membrane filter paper and solution was further diluted with mobile phase to obtain a solution of LOS (2µg/mL), RAM (0.2µg/mL) and HCTZ (0.5 µg/mL), respectively. The sample solution (20µL) was injected into the chromatograph at the flow rate of 1mL/min and the chromatogram was recorded.

- **System suitability**

  20µL of standard solution containing LOS (2µg/mL), RAM (0.2µg/mL) HCTZ (0.5 µg/mL) and was injected into the chromatograph to test the system suitability to determine resolution (Rs), retention time (t_R), theoretical number of plates (N) and tailing factor (T).

- **Analytical Method Validation**

  Validation was done as per ICH guidelines. The developed method was validated with respect to parameters such as linearity, LOD and LOQ, precision, accuracy and specificity.

- **Linearity:**

  Several aliquots of standard stock solution-I of LOS, RAM and HCTZ were taken in different 10 mL volumetric flask and diluted up to the mark with mobile phase such that the final concentration of LOS, RAM and HCTZ were in the range of 2–12µg/mL, 0.2-1.2µg/mL and 0.5-3µg/mL, respectively. Evaluations of three drugs were performed with UV detector set at 218 nm. The peak area versus concentration data was collected and subjected to regression analysis. The correlation coefficient, slope and y-intercept values were calculated from the calibration plot obtained.

- **Limit of detection and limit of quantitation:**

  The limit of detection (LOD) and limit of quantitation (LOQ) for LOS, RAM and HCTZ estimated at a signal-to-noise ratio of 3:1 and 10:1 respectively, by injecting a series of dilute solutions with known concentration.

- **Precision:**

  The precision of the method was verified by intra-day precision and inter-day precision studies. Intra-day precision studies were performed by analysis of three different concentrations of 4, 8, 12 µg/mL for LOS, 0.4, 0.8, 1.2 µg/mL for RAM and 1, 2, 3 µg/mL for HCTZ three times on the same day. The inter-day precision of the method was checked by repeating studies on three different days.

- **Robustness:**

  To determine the robustness of the developed method, experimental conditions were deliberately changed and the relative standard deviation for replicate injections of LOS, RAM and HCTZ were evaluated. Flow rate was changed by ± 0.1 units to 0.9 and 1.1 mL/min. The effect of % of methanol in the mobile phase was studied at (± 5%).

- **Recovery studies:**

  To check the accuracy of the proposed method recovery experiment was carried out. Twenty tablets from the same lot of the commercial formulation were weighed accurately and finely powered. Powder equivalent to 50 mg of LOS, 5 mg of RAM

and 12.5 mg of HCTZ was weighed and transferred to different 50 mL volumetric flask. Standard drug was added at 80%, 100% and 120% level. The contents were dissolved by sonicating in 20 mL of mobile phase for 15 minutes and filtered through 0.45µ membrane filter paper and further diluted up to the mark using mobile phase. 0.2 mL of the filtrate was taken in 10 mL volumetric flask and diluted up to the mark with mobile phase. 20µL of sample solution was injected into the chromatograph and chromatograms were recorded. Percentage recovery and relative standard deviation were calculated. Each level was repeated three times.

- **Analysis of marketed formulation:**

  20 µL of working standard solution-II (2 µg/mL of LOS, 0.2 µg/mL of RAM and 0.5 µg/ml of HCTZ) and sample solutions were injected into the liquid chromatograph and the chromatogram was recorded. From the peak areas of LOS, RAM and HCTZ the amount of the drugs in the sample were calculated.

- **Specificity:**

  The specificity of the method was ascertained by analysing drug standards and samples. The chromatogram was overlaid to study the specificity. The identities of the peak for LOS, RAM and HCTZ from the sample were confirmed by comparing the t_R with those of standards.

- **Comparison of the proposed method with the reported method:**

  The proposed method was compared with the reported method from the literature:

  Simultaneous Estimation of Losartan Potassium, Ramipril and Hydrochlorothiazide in Bulk as well as in Pharmaceutical Formulation by RP-HPLC[9]

  Column: Symmetry C_{18} column (5µ particles size, 150 cm X 4.6 mm) Hypersil

  Mobile phase: Potassium dihydrogen phosphate: acetonitrile (68:32% v/v)

  Flow rate: 0.9 ml/min, λ max = 210 nm.

5.2.2. Results and Discussion:

- **Selection of column:**

  As the drugs were soluble in polar solvents such as methanol and acetonitrile, C_{18} reverse phase column was selected with particle size of 5.0 µ, length 250 mm and internal diameter 4.6 mm.

- **Selection of suitable wavelength:**

  Stock solutions of individual drugs were prepared in methanol and individually the solutions were scanned in the UV range 200-400 nm in a UV spectrophotometer and their spectras were recorded. The spectras were overlaid and the isobestic point, 218 nm was selected as the suitable wavelength as all three drugs showed good absorption at that wavelength (Fig.5.06).The wavelength was checked and confirmed in the optimised mobile phase also.

- **Selection of mobile phase:**

  Different mobile phases were tried. The preliminary trials using different composition of mobile phases consisting of methanol with water in ratios of 50:50 v/v, 60:40 v/v and 75:25 v/v did not result in desired separation and peak shapes. With 80:20 v/v, 90:10 v/v methanol and water ratios, three peaks were resolved however asymmetry was not good for LOS and RAM. The mobile phase composition of methanol: water (95:5, v/v) resulted in well resolved peaks with good shape. Thus the mobile phase methanol: water 95:5 v/v was selected. With this optimized mobile phase retention times were found to be for LOS (t_R 2.383min.), RAM (t_R 2.658 min.) and HCTZ (t_R 3.108 min.), respectively.

- **System suitability**

  System suitability test is commonly used to verify resolution, column efficiency, and repeatability of a chromatographic system to ensure its adequacy for a particular analysis. System suitability tests were carried out on freshly prepared standard stock solutions LOS, HCTZ and RAM. The parameters obtained are shown in Table 5.08. Column efficiency in term of theoretical plates number (N) was 3423 for LOS, 5528 for RAM and 6782 for HCTZ, respectively.

- **Validation of the method**

  Using optimised chromatographic conditions the RP-HPLC method developed was validated in terms of linearity, LOD and LOQ, precision, robustness and accuracy.

- **Linearity:**

  Linear regression data for the calibration plots revealed good linear relationships between response and concentration over the ranges 2–12µg/mL for LOS, 0.2-1.2 for µg/mL RAM and 0.5-3µg/mL for HCTZ, respectively.The linear regression equations were Y=127014X + 20134 (r^2= 0.9995) for LOS, Y= 133128X - 399.9 (r^2= 0.9996) for RAM and Y= 853148X + 19333 (r^2= 0.9995) for HCTZ. The residuals plot shows a random pattern, indicating a good fit for a linear model. The calibration curves and residuals plots are given in Fig. 5.08, Fig. 5.09and Fig. 5.10 and Table 5.09 (A), Table 5.09 (B) and Table 5.09 (C) for LOS, RAM and HCTZ, respectively.

- **Limits of Detection and Quantitation:**

  The signal to noise ratios of 3: 1 and 10: 1 were considered as LOD and LOQ respectively. The results of the LOD and LOQ were found to be 0.6 µg/mL and 1.8 for LOS, 0.08 µg/mL and 0.2 µg/mL for RAM and 0.2µg/mL and 0.5 µg/mL for HCTZ, respectively

- **Precision:**

  The precision of the developed method was demonstrated by intra-day precision and inter-day precision studies. This was done by three replicate analysis of the sample. The precision of the method was expressed as relative standard deviation (RSD, %). The % RSD of intraday and inter-day precision values were found to be 1.11 and 1.09 % for LOS, 1.11 and 1.037 % for RAM and 1.16 and 1.157% for HCTZ respectively (Table 5.10). The RSD values were found to be <2 %, which indicates that the proposed method is precise.

- **Robustness:**

  There were no significant changes in the retention times of LOS, RAM and HCTZ when the flow rate (± 0.1mL/min) and composition of the mobile phase (± 5%) were changed. The low values of the RSD indicate the robustness of the method, as shown in Table 5.11.

- **Accuracy:**

  To study the accuracy of the proposed method recovery experiment were carried out by standard addition method. The proposed method when used for extraction and subsequent estimation of LOS, RAM and HCTZ from pharmaceutical dosage form

after spiking with 80%, 100% and 120% of the additional drug afforded mean recovery of 99.51% for LOS and 99.35 for RAM and 99.29 % for HCTZ. The mean recoveries indicate non-interference from excipients (Table 5.12).

- **Analysis of marketed formulation:**

  The chromatogram of the sample extracted from conventional tablets showed peaks of LOS (Rt-2.383 min.), RAM (Rt-2.658 min) and HCTZ (Rt- 3.108 min.) that are well resolved from other tablet excipients as shown in Fig. 5.7. The mean content of LOS, RAM and HCTZ per tablet by proposed method was found to be 49.92 mg, 4.97 mg and 12.46 mg, respectively. The results are summarized in Table 5.13.

- **Comparison of the proposed method with the reported method:**

  The proposed method was compared with the reported method. The proposed method uses simple mobile phase of methanol: water (95:5 v/v) as compared to buffered mobile phases used in the reported method. Linearity concentration range of the proposed method is 2- 12 µg/mL for LOS, 0.2- 1.2µg/mL for RAM and 0.5–3.0 µg/mL for HCTZ as against 50-110 µg/mL for LOS, 1.255-2.75 for RAM and 12.5-27.5 for µg/mL for HCTZ of the reported method. The proposed method gives better column efficiency and intraday & inter-day precision than the reported method. The peaks are well resolved and the run time for the simultaneous analysis for LOS, RAM and HCTZ is less than 7 mins as compared to the reported method which requires more than 12 mins (Table no 5.14).

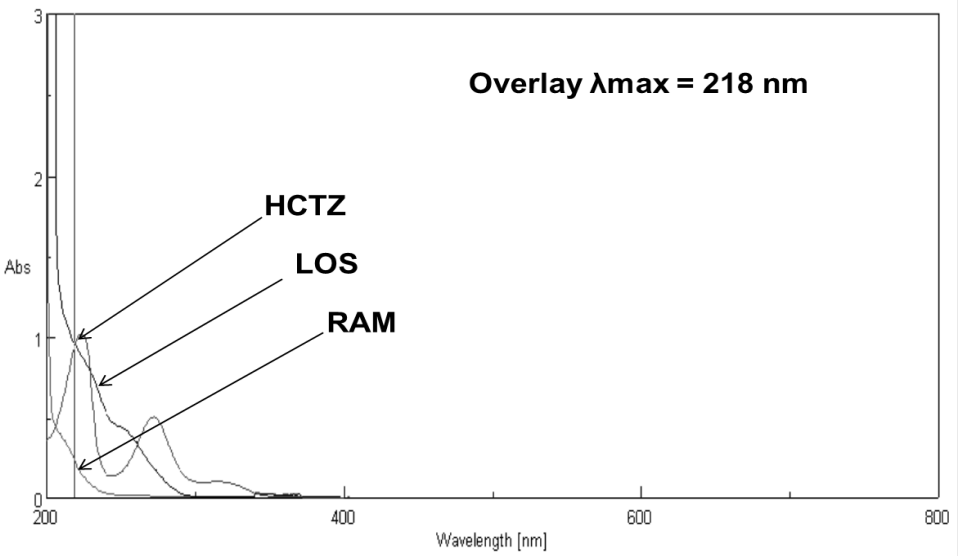

Fig. 5.06. Overlay absorption spectra of LOS, RAM and HCTZ

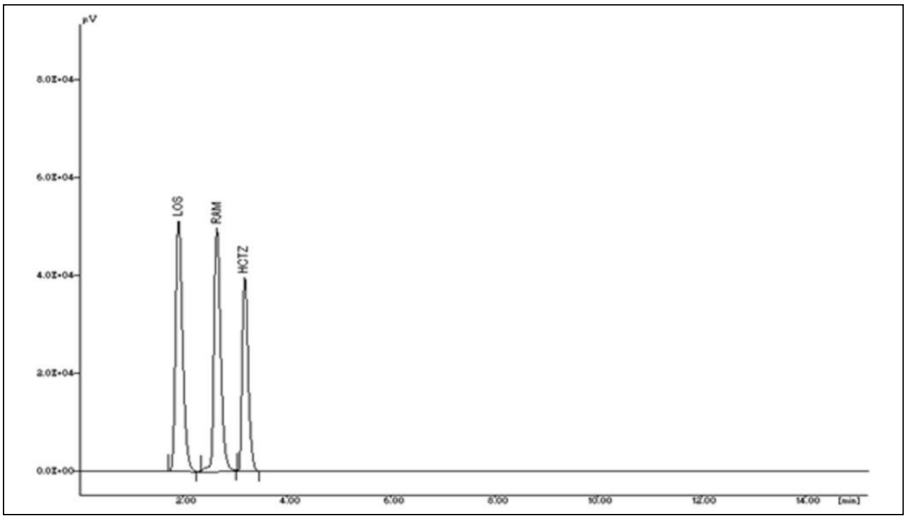

Fig.5.07. Typical chromatogram of LOS, RAM and HCTZ

Table 5.08: System suitability parameters (n=6)

Parameter	LOS	RAM	HCTZ
Retention Time in min (t_R)	2.383	2.658	3.108
Resolution (Rs)	--	2.581	4.346
Theoretical plates number (N)	3423	5528	6782
Tailing Factor	1.08	1.14	1.10

Table 5.09 (A): Data for LOS from linearity study

Concentration (µg/mL)	HPLC (n= 3)	
	Mean peak area ± SD	**RSD (%)**
2	267970.9 ± 3028.07	1.13
4	525941.8 ± 6206.11	1.18
6	801912.7 ± 8339.89	1.04
8	1031884.0 ± 13517.68	1.31
10	1279855.0 ± 15230.27	1.19
12	1547825.0 ± 19193.03	1.24
Regression coefficient r^2		**0.9995**
Slope		**127014**
Intercept		**20134**

Table 5.09 (B): Data for RAM from linearity

Concentration (µg/mL)	HPLC (n= 3)	
	Mean peak area ± SD	**RSD (%)**
0.2	26797.2 ± 340.32	1.27
0.4	53594 ± 589.53	1.10
0.6	78391.3 ± 838.79	1.07
0.8	105188 ± 1314.85	1.25
1.0	131985.5 ± 1715.81	1.30
1.2	160782 ± 1929.384	1.20
Regression coefficient r^2		**0.9996**
Slope		**133128**
Intercept		**- 399.9**

Table 5.09 (C): Data for HCTZ from linearity study by HPLC

Concentration (µg/mL)	HPLC (n= 3)	
	Mean peak area ± SD	**RSD (%)**
0.5	449717 ± 4901.92	1.09
1	889434 ± 10228.49	1.15
1.5	1289151 ± 14567.41	1.13
2	1698868 ± 18177.89	1.07
2.5	2148584 ± 26427.58	1.23
3	2598302 ± 29880.47	1.15
Regression coefficient r^2		**0.9995**
Slope		**853148**
Intercept		**19333**

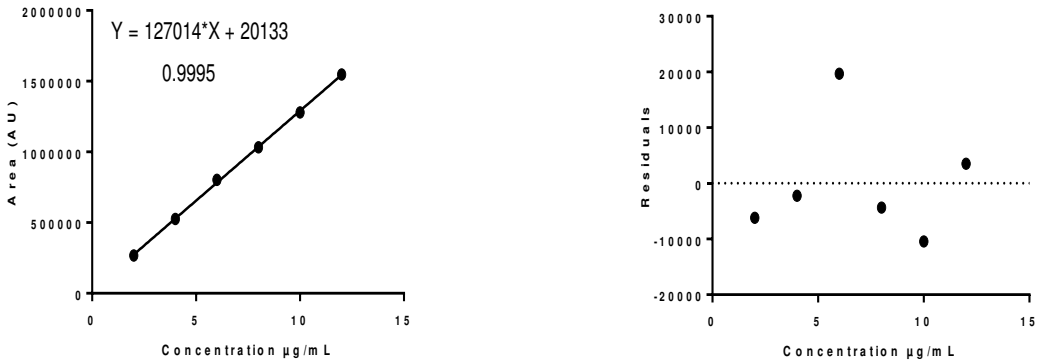

Fig. 5.08. Calibration curve and residual plot for LOS

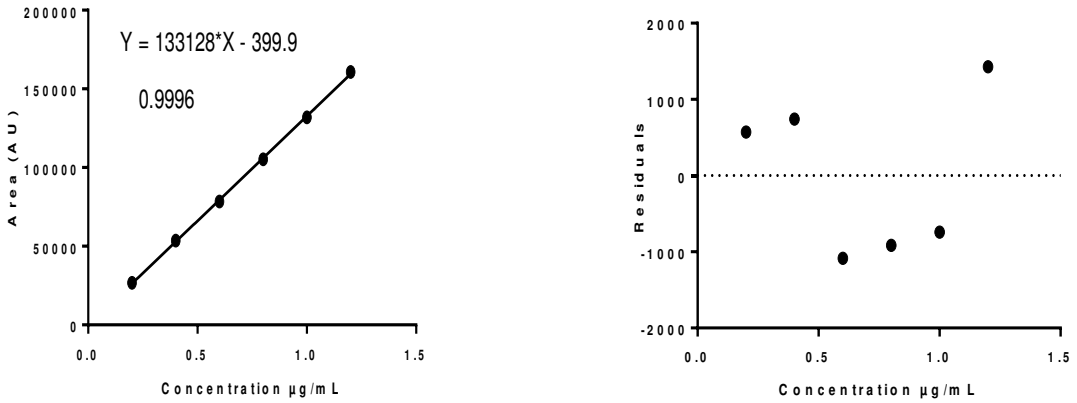

Fig.5.09. Calibration curve and residual plot for RAM

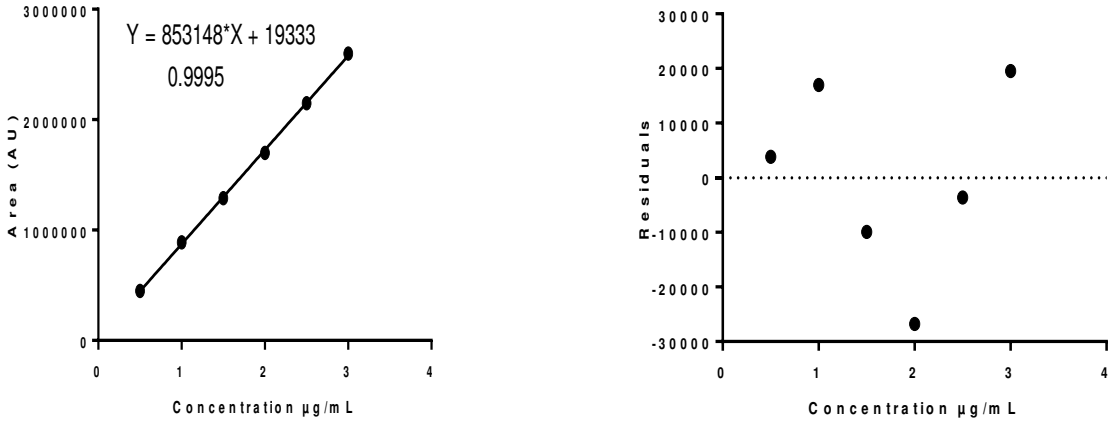

Fig. 5.10. Calibration curve and residual plot for HCTZ

Table 5.10: Precision studies of LOS, RAM and HCTZ

Conc. (µg/mL)	Intra-day precision (n= 3)			Inter-day precision (n=3)		
	Measured Conc. ± SD	(%) RSD	Recovery (%)	Measured Conc. ± SD	(%) RSD	Recovery (%)
Losartan						
4	3.982 ± 0.042	1.05	99.55	3.980 ± 0.045	1.13	99.50
8	7.97 ± 0.089	1.12	99.63	7.95 ± 0.083	1.05	99.38
12	11.96 ± 0.14	1.17	99.67	11.93 ± 0.13	1.09	99.42
Ramipril						
0.4	0.397 ± 0.005	1.16	99.25	0.396± 0.0048	1.21	99.00
0.8	0.792 ± 0.008	1.07	99.00	0.791 ± 0.0070	0.88	98.88
1.2	1.190 ± 0.013	1.10	99.17	1.189 ± 0.012	1.02	99.08
Hydrochlorothiazide						
1	0.993 ± 0.011	1.15	99.30	0.991 ± 0.012	1.21	99.10
2	1.979 ± 0.024	1.21	98.95	1.967 ± 0.025	1.17	98.35
3	2.972 ± 0.034	1.13	99.07	2.969 ± 0.032	1.09	98.97

Table 5.11: Robustness evaluation of LOS, RAM and HCTZ (n=3)

Conditions	Level	LOS		RAM		HCTZ	
		t_R (min.)	% RSD of peak area	t_R (min.)	% RSD of peak area	t_R (min.)	% RSD of peak area
A: Flow rate (± 0.1 mL/min.)							
0.9	-0.1	2.412	1.14	2.716	1.20	3.155	1.15
1.0	0.0	2.383	1.10	2.658	1.13	3.108	1.19
1.1	+0.1	2.333	1.09	2.572	1.18	3.005	1.15
Mean ± SD		2.376 ± 0.040	1.11 ±0.026	2.649 ± 0.072	1.17 ±0.036	3.0893 ± 0.077	1.163 ± 0.023
B: % of methanol in the mobile phase (± 5%)							
90	-5.0	2.432	1.17	2.712	1.13	3.167	1.11
95	0.0	2.383	1.11	2.658	1.09	3.108	1.19
100	+5.0	2.303	1.10	2.553	1.16	3.002	1.08
Mean ± SD		2.373 ± 0.065	1.13 ± 0.038	2.6377 ± 0.077	1.127 ± 0.035	3.0923 ± 0.084	1.127 ± 0.057

Table 5.12: Recovery study of LOS, RAM and HCTZ (n=3)

Label claim (mg/tablet)	Amount Added (%)	Total amount (mg)	Amount recovered (mg)	Recovery (%)	Mean (%) Recovery (± SD)
LOS 50	80	90	89.40	99.33	99.51 ± 0.29
	100	100	99.84	99.84	
	120	110	109.30	99.36	
RAM 5	80	9	8.927	99.19	99.35 ±0.28
	100	10	9.967	99.67	
	120	11	10.91	99.19	
HCTZ 12.5	80	22.5	22.35	99.33	99.29 ±0.032
	100	25	24.82	99.28	
	120	27.5	27.30	99.27	

Table 5.13: Assay results of LOS, RAM and HCTZ in tablets

Loram-H tablet contains	Label claim (mg/tablet)	Amount found (mg ± SD)	RSD (%)	Recovery (%)
LOS	50 mg	49.92 ± 0.574	1.15	99.84
RAM	5 mg	4.97 ± 0.055	1.11	99.40
HCTZ	12.5 mg	12.46 ± 0.144	1.16	99.68

Table 5.14: Comparison of the proposed method with the reported method:

Parameter	Reported Method	Propose Method
Mobile Phase	KH_2PO_4 and ACN (68:32 v/v)	**Methanol: Water (95:5 v/v)**
Mode	Isocratic	Isocratic
Detection wavelength (nm) & Flow Rate	210nm 0.9 mL/min	**218nm** **1mL/min**
Retention time (t_R) in min.	LOS- 11.603 RAM-8.702 HCTZ- 2.571	**LOS-2.383** **RAM- 2.658** **HCTZ- 3.108**
Linearity range (µg/mL)	LOS- 50-110 RAM- 1.255- 2.75 HCTZ- 12.5 – 27.5	**LOS- 2-12** **RAM- 0.2 –1.2** **HCTZ- 0.4 -3**
LOD (µg/mL) and LOQ (µg/mL)	LOS- 0.07 and 0.24 RAM-0.078 and 0.27 HCTZ-0.01 and 0.05	**LOS-0.6 and 1.8** **RAM-0.08 and 0.2** **HCTZ-0.2 and 0.5**
% Recovery (n=3)	LOS-99.5 RAM-99.8 HCTZ-99.8	**LOS-99.84** **RAM-99.40** **HCTZ-99.68**
Precision (% RSD) Intra-day (n=3) Inter-day (n=3)	Intra-day LOS-1.86 RAM-1.42 HCTZ-0.44	**LOS-1.11 & 1.09** **RAM-1.11 & 1.037** **HCTZ-1.11 & 1.157**

5.2.3. Conclusion:

RP-HPLC method has been developed for simultaneous estimation of LOS, RAM and HCTZ from fixed dosage form. The method has been validated statistically for linearity, specificity, accuracy and precision. The run time was relatively short i.e. less than 5 min. as compared to the method reported previously, which enables rapid quantitation of many samples in routine and quality control analysis of formulations. The method was validated and found to be simple, sensitive, accurate, precise and economical. Percentage of recovery shows that the method is free from interference of the excipients used in the formulation. Thus, the proposed HPLC method can be successfully applied for the routine quality control analysis of LOS, RAM and HCTZ from their fixed dosage form and analysis of raw materials.

5.3 Simultaneous analysis of Losartan potassium, Hydrochlorothiazide and Atenolol by HPLC

Losartan potassium, 2-butyl-4-chloro-1-[p-(o-1H-tetrazol-5ylphenyl)benzyl] imidazole -5- methanol monopotassium salt, is an angiotensin II receptor blocker and is used as an antihypertensive agent. Hydrochlorothiazide, chemically, 6-Chloro-3, 4-dihydro-2H-l, 2, 4-benzothiadiazine-7- sulfonamide 1, 1-dioxide, is a thiazide diuretic. Atenolol, 4-(2-hydroxy-3-[(1-methylethyl) amino] propoxy] benzene acetamide, is a β 1 selective (cardioselective) β adrenergicreceptor blocking agent.

The drug profiles and literature reviews of Losartan potassium (LOS), Hydrochlorothiazide (HCTZ) and Atenolol (ATN) are given in 3.5, 3.6 and 3.7.

5.3.1 Experimental:

- **Instrumentation:**

  The HPLC system consisted of Intelligent HPLC pump model (Jasco PU 1580). The solutions were injected into the chromatograph through a Rheodyne valve, with a 20 µL loop with auto sampler (AS 1555). The column used was Thermo Hypersil Gold C_{18} (250 mm × 4.6 mm, 5.0 µ), operating at room temperature. The elution was carried out isocratically at flow rate of 0.8 mL/min. using methanol: water (95:5 v/v) as mobile phase. The detector consisted of a UV/ VIS (Jasco UV 1575) model operated at a wavelength of 225nm. Data was integrated using Jasco Borwin version 1.5, LC-Net II/ADC system.

- **Chemicals and reagents:**

  Reference standards of, Losartan potassium (100.2 %), Hydrochlorothiazide (99.97%) and Atenolol (99.7%) were procured from Emcure Pharmaceuticals Ltd. The tablets Repalol-H (Losartan-50 mg, Atenolol-50 mg and Hydrochlorothiazide 12.5 mg) of Sun Pharmaceutical Industries Ltd. was procured from the market. For HPLC work double distilled water was prepared in the laboratory. Methanol used was of HPLC grade and was purchased from Merck Chemicals, Mumbai, India.

- **Stationary Phase:**

  Thermo Hypersil Gold C_{18} (250 mm × 4.6 mm, 5.0 µ) was used.

- **Preparation of mobile phases:**

  HPLC grade methanol (950 mL) was mixed with (50 mL) of double distilled water. This solution was filtered through 0.45µ membrane filter paper and degassed in an ultrasonic bath before use.

- **Preparation of standard stock solution-I:**

  500 mg of Losartan potassium (LOS), 125 mg of Hydrochlorothiazide (HCTZ) and 500 mg of Atenolol (ATN) were accurately weighed and transferred to a 50 mL volumetric flask and dissolved in mobile phase. Volume was made up to the mark with mobile phase to get a solution containing LOS 10 mg/mL, ATN 10 mg/mL and HCTZ 0.25 mg/mL respectively. 0.1ml of this solution was further diluted (LOS 10 mg/mL, ATN 10mg/mL and HCTZ 0.25 mg/mL) to 10 ml with mobile phase to get **Standard stock solution-I** containing LOS 0.1 mg/mL, ATN 0.1 mg/mL and HCTZ 0.0025 mg/mL, respectively.

- **Working standard solution-II:**

  0.2 ml of the standard stock solution-I was diluted to 10 mL with mobile phase to get a concentration of 2 µg/mL of LOS, 0.5 µg/ml HCTZ and 2 µg/mL of ATN. This solution was used as working standard.

- **Sample solution:**

  Twenty tablets were weighed accurately and powered. Tablet powder equivalent to 50 mg of LOS, 12.5 mg of HCTZ and 50 mg of ATN was weighed accurately and transferred into a clean and dry 50 mL volumetric flask. The powder was first dissolved in sufficient volume of mobile phase and kept in an ultrasonic bath for 15 min. and the volume was made to 50 mL with mobile phase, filtered through 0.45µ membrane filter paper and solution was further diluted with mobile phase to obtain a solution of LOS (2µg/mL), HCTZ (0.5 µg/mL) and ATN (2 µg/mL), respectively. The sample solution (20µl) was injected into the chromatograph at the flow rate of 0.8 mL/min and the chromatogram was recorded.

- **System suitability**

  20µL of standard solution containing LOS (2 µg/mL), HCTZ (0.5 µg/mL) and ATN (2µg/mL) was injected into the chromatograph to test the system suitability to determine resolution (Rs), retention time (t_R), theoretical number of plates (N) and tailing factor (T).

- **Analytical Method Validation**

  Validation was done as per ICH guidelines. The developed method was validated with respect to parameters such as linearity, LOD and LOQ, precision, accuracy and specificity.

- **Linearity:**

  Several aliquots of standard stock solution-I of LOS, HCTZ and ATN were taken in different 10 mL volumetric flask and diluted up to the mark with mobile phase such that the final concentration of LOS, HCTZ and ATN were in the range of 2–12µg/mL, 0.5-3µg/mL and 2-12µg/mL, respectively. Evaluations of three drugs were performed with UV detector set at 225 nm. The peak area versus concentration data was collected and subjected to regression analysis. The correlation coefficient, slope and y-intercept values were calculated from the calibration plot obtained.

- **Limit of detection and limit of quantitation:**

  The Limit of detection (LOD) and Limit of quantitation (LOQ) for LOS, HCTZ and ATN and estimated at a signal-to-noise ratio of 3:1 and 10:1 respectively, by injecting a series of dilute solutions with known concentration.

- **Precision:**

  The precision of the method was verified by intra-day and inter-day precision studies. Intra-day precision studies were performed by analysis of three different concentrations 4, 8, 12 µg/mL for both LOS and ATN and 1, 2, 3 µg/mL for HCTZ three times on the same day. The inter-day precision of the method was checked by repeating studies on three different days.

- **Robustness:**

  To determine the robustness of the developed method, experimental conditions were deliberately changed and the relative standard deviation for replicate injections of LOS, HCTZ and ATN were evaluated. Flow rate was changed by ± 0.1 units to 0.7 and 0.9 mL/min. The effect of % of methanol in the mobile phase was studied at (± 5%).

- **Recovery studies:**

  To check the accuracy of the proposed method recovery experiment was carried out. Twenty tablets from the same lot of the commercial formulation were weighed accurately and finely powered. Powder equivalent to 50 mg of LOS, 12.5 mg of

HCTZ and 50 mg of ATN was weighed and transferred to different 50 mL volumetric flask. Standard drug was added at 80%, 100% and 120% level. The contents were dissolved by sonicating in 20 mL of mobile phase for 15 minutes and filtered through 0.45µ membrane filter paper and further diluted up to the mark using mobile phase. 0.2 mL of the filtrate was taken in 10 mL volumetric flask and diluted up to the mark with mobile phase. 20µL of sample solution were injected into the chromatograph and chromatograms were recorded. Percentage recovery and relative standard deviation were calculated. Each level was repeated three times.

- **Analysis of marketed formulation:**

  20µL of working standard solution-II (2 µg/mL of LOS, 0.5 µg/mL HCTZ and 2 µg/mL of ATN) and sample solutions were injected into the liquid chromatograph and the chromatogram was recorded. From the peak areas of LOS, HCTZ and ATN the amount of the drugs in the sample were calculated.

- **Specificity:**

  The specificity of the method was ascertained by analysing drug standards and samples. The chromatogram was overlaid to study the specificity. The identities of the peak for LOS, HCTZ and ATN from the sample were confirmed by comparing the t_R with those of standards.

- **Comparison of the proposed method with the reported methods:**

  The proposed method was compared with the following reported methods from the literature:

1. RP-HPLC Method for Simultaneous Estimation of Atenolol, Hydrochlorothiazide and Losartan in Tablet Dosage Form[10]

   Column: Luna C_{18} column (5µ particles size, 25 cm X 4.6 mm) Phenomenex

   Mobile phase: mixture of (A) acetonitrile, methanol (65:35) and (B) 10 mM NaH_2PO_4,H_2O buffer with 0.4% v/v TEA and mixture of A: B (60:40 v/v) pH 3.0 with OPA (5% v/v)

   Flow rate: 1.5 ml/min, Detection wavelength = 230nm.

2. Simultaneous Estimation and validation of Atenolol, Hydrochlorothiazide and Losartan K in Tablet Dosage Form by RP-HPLC method [11]

   Column: Thermo scientific C_{18} column (5µ particles size, 20 cm X 4.6 mm)

Mobile phase: Acetonitrile: Phosphate buffer pH 3.6 adjusted with Na_2HPO_4

Flow rate: 1.2 ml/min, Detection wavelength = 229 nm.

5.3.2. Results and Discussion:

- **Selection of column:**

  As the drugs were soluble in polar solvents such as methanol and acetonitrile, C_{18} reverse phase column was selected with particle size of 5.0 µ, length 250 mm and internal diameter 4.6 mm.

- **Selection of suitable wavelength:**

  Stock solutions of individual drugs were prepared in methanol and the solutions were scanned individually in a UV spectrophotometer in the range of 200-400 nm and their spectras were recorded. The spectras were overlaid and the isobestic point, 225 nm was selected as the suitable wavelength as all three drugs showed good absorption at this wavelength (Fig.5.11). The wavelength was checked and confirmed in the optimised mobile phase also.

- **Selection of mobile phase:**

  Initial trails were carried out on trial and error basis and with the knowledge of the previously published reports. The preliminary trials using different composition of mobile phases consisting of water with methanol in a ratios 50:50 v/v, 60:40%, 75:25% and 80:20%, resulted in poor resolution and did not give well defined peak in a short time. With 90:10% methanol and water ratio, three peaks were resolved but sharp peaks were not found for ATN. Keeping mobile phase composition as methanol: water (95:5, v/v), satisfactory peak shape was obtained. Finally the mobile phase 95:5 v/v methanol: water was selected. The optimized mobile phase was found to resolve peaks of LOS (t_R-2.633min.), HCTZ (t_R-3.883) and ATN (t_R-7.783 min.), respectively.

- **System suitability**

  System suitability tests were carried out on freshly prepared standard stock solution of LOS, HCTZ and ATN to determine efficiency, resolution factor, symmetry factor and precision of the instrument and found to comply with USP requirement. Parameters obtained with 20 µL injection volume are shown in (Table 5.15). Column

efficiency in terms of theoretical plates number (N) was 5989.38 for LOS, 6236.65 for HCTZ and 8684.15 for ATN, respectively.

- **Validation of the method**

  Using optimised chromatographic conditions the RP-HPLC method developed was validated in terms of linearity, LOD and LOQ, precision, robustness and accuracy.

➢ **Linearity:**

The calibration curves were prepared by plotting the areas of the drugs which were in linear in the concentration range of 2–12µg/mL for both LOS and ATN and 0.5-3µg/mL for HCTZ, respectively. Peak areas ratios and concentrations subjected to least square linear regression analysis to calculate the calibration equations and correlation coefficients. The mean regression equations were found as Y= 52287X + 120009 (r^2= 0.9993) for LOS, Y= 126353X + 6931 (r^2= 0.9992) for HCTZ and Y= 28808X + 18283 (r^2= 0.9996) for ATN. The residuals plot shows a random pattern, indicating a good fit for a linear model. The calibration curves and residuals plots are given in Fig. 5.13, Fig. 5.14 and Fig. 5.15 and Table 5.16 (A), Table 5.16(B) and Table 5.16 (C) for LOS, HCTZ and ATN, respectively.

➢ **Limits of Detection and Quantitation:**

The results of the LOD and LOQ were found to be 0.6 µg/mL and 1.8 for LOS, 0.2µg/mL and 0.5 µg/mL for HCTZ and 0.8µg/mL, 2 µg/mL for ATN at a signal to noise ratios of 3:1 and 10:1. The signal to noise ratios of 3: 1 and 10: 1.

➢ **Precision:**

The precision of the developed method was demonstrated by intra-day and inter-day precision studies. This was done by three replicate analysis of the sample. The precision of the method was expressed as relative standard deviation (RSD, %). The % RSD of intraday and inter-day precision values was found to be 1.059 and 1.10% for LOS, 1.103 and 1.17% for HCTZ and 1.12 and 1.18 % for ATN, respectively, indicating good precision (Table 5.17).

➢ **Robustness:**

The robustness of the method was found out by testing the effect of small deliberate changes in the chromatographic conditions. The method was found to be robust enough that the peak area was not apparently affected by small variation in the chromatographic conditions and there were no significant changes in the retention

times of LOS, HCTZ and ATN. The low values of the RSD indicate the robustness of the method, as shown in Table 5.18

- **Accuracy:**

  To study the accuracy of the proposed method recovery experiment were carried out by standard addition method. The proposed method when used for extraction and subsequent estimation of LOS, HCTZ and ATN from pharmaceutical dosage form after spiking with 80%, 100% and 120% of the additional drug afforded mean recovery of 99.56% for LOS, 99.50% for HCTZ and 99.40 % for ATN. The mean recoveries indicate non-interference from excipients (Table 5.19).

- **Analysis of marketed formulation:**

  The chromatogram of the sample extracted from conventional tablets showed principal peaks of LOS (t_R-2.633 min.), HCTZ (t_R - 3.883 min.) and ATN (t_R -7.783 min) well resolved from other tablet excipients, shown in Fig. 5.12. The mean content of LOS, HCTZ and ATN per tablet by proposed method was found to be 50.175 mg, 12.475 mg and 49.58 mg, respectively. The results are summarized in Table 5.20.

- **Comparison of the proposed method with the reported methods:**

  The proposed method was compared with the reported methods. The proposed method uses simple mobile phase of methanol: water (95:5) at the flow rate of 0.8 ml/min. as compared to buffered mobile phases. The flow rate of the proposed method is 0.8 ml/min as against 1.5 mL/min and 1.2 mL/min used in the reported methods. LOD and LOQ for LOS, HCTZ and ATN of the proposed method is lower than the reported method. Resolution for peaks of the proposed method is better than the reported methods. Accuracy and precision of the proposed method are comparable to the reported methods with low % RSD. (Table 5.21)

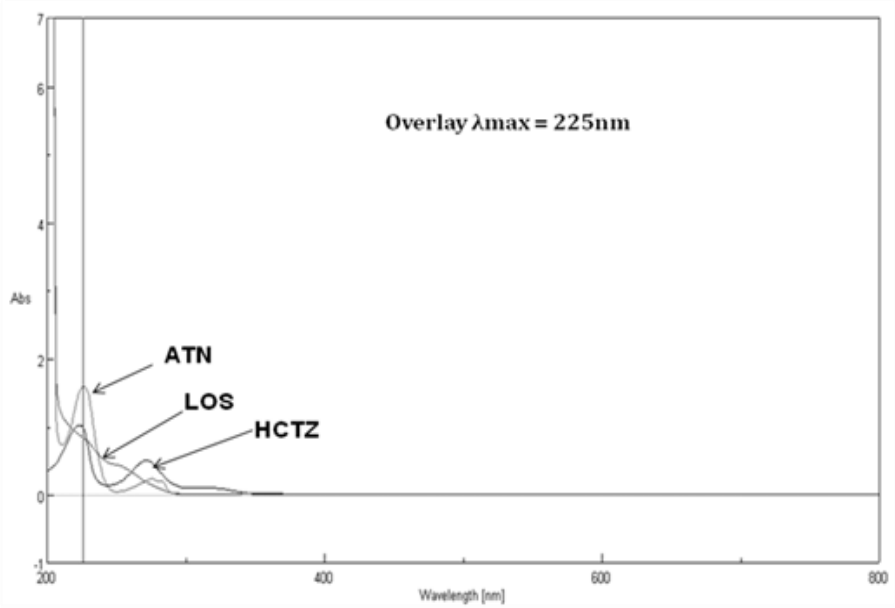

Fig. 5.11. Overlay absorption spectrum of LOS, HCTZ and ATN

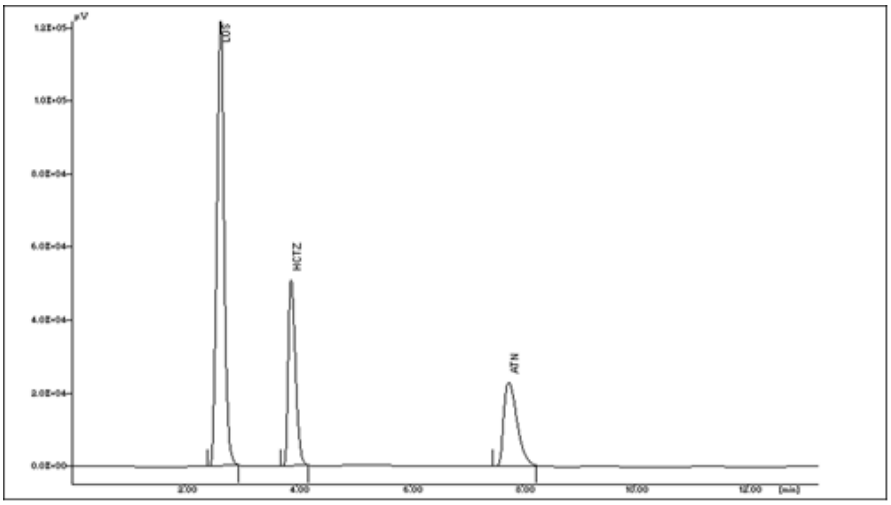

Fig.5.12. Typical chromatogram of LOS, HCTZ and ATN

Table 5.15: System suitability parameters (n=6)

Parameters	**LOS**	**HCTZ**	**ATN**
Retention Time in min	2.633	3.883	7.783
Resolution (Rs)	0.00	6.436	14.159
Theoretical plates number (N)	5989.38	6236.65	8684.15
Tailing Factor	1.175	1.160	1.247

Table 5.16 (A): Data for LOS from linearity study

Concentration (µg/mL)	HPLC (n= 3)	
	Mean peak area ± SD	**RSD (%)**
2	223184 ± 2388.07	1.07
4	336175 ± 4135.0	1.23
6	431913 ± 6133.16	1.42
8	532028 ± 5958.71	1.12
10	639779 ± 7613.37	1.19
12	753010 ± 7981.91	1.06
Regression coefficient r^2		**0.9993**
Slope		**52287**
Intercept		**120009**

Table 5.16 (B): Data for HCTZ from linearity study

Concentration (µg/mL)	HPLC (n= 3)	
	Mean peak area ± SD	**RSD (%)**
0.5	74830 ± 695.92	0.93
1	129821 ± 1557.85	1.20
1.5	193425 ± 2147.02	1.11
2	257892 ± 3507.33	1.36
2.5	325657 ± 4168.41	1.28
3	386672 ± 4833.4	1.25
Regression coefficient r^2		**0.9992**
Slope		**126353**
Intercept		**6931**

Table 5.16 (C): Data for ATN from linearity study

Concentration (µg/mL)	HPLC (n= 3)	
	Mean peak area ± SD	**RSD (%)**
2	75807 ± 940.0	1.24
4	134659 ± 1454.32	1.08
6	192573 ± 2541.96	1.32
8	245273 ± 3114.97	1.27
10	304875 ± 3140.21	1.03
12	366452 ± 4250.84	1.16
Regression coefficient r^2		**0.9996**
Slope		**28808**
Intercept		**18283**

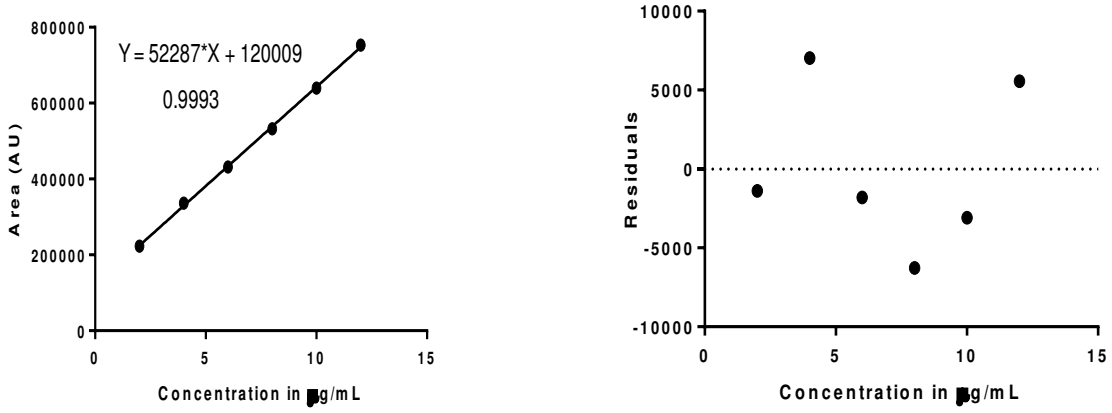

Fig. 5.13. Calibration curve and residual plot for LOS

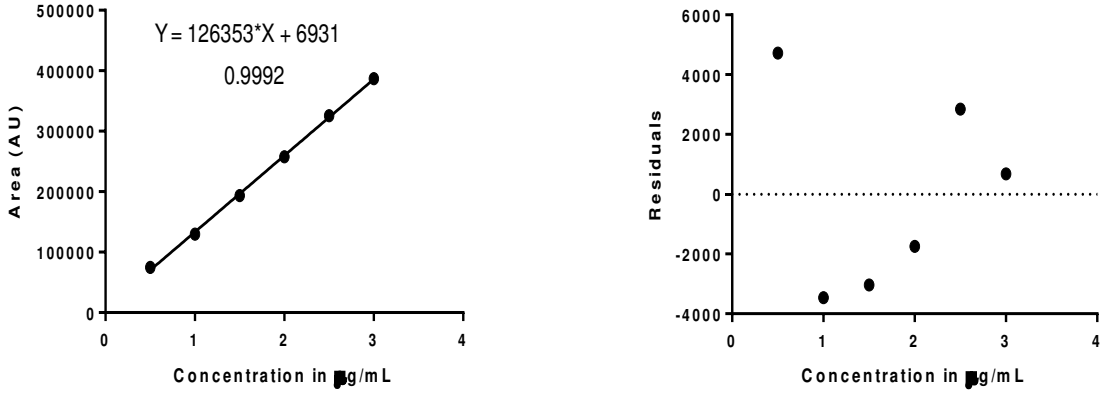

Fig. 5.14. Calibration curve and residual plot for HCTZ

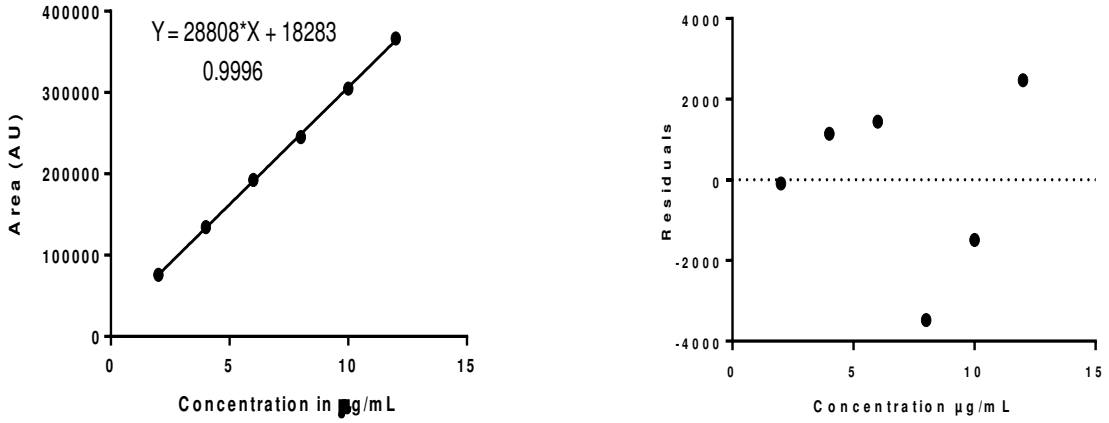

Fig. 5.15. Calibration curve and residual plot for ATN

Table 5.17: Precision studies of LOS, HCTZ and ATN

Conc. (µg/mL)	Intra-day precision (n=3)			Inter-day precision (n=3)		
	Measured Conc. ± SD	(%) RSD	Recovery (%)	Measured Conc. ±SD	(%) RSD	Recovery (%)
Losartan						
4	3.96 ± 0.042	1.06	99.00	3.94 ± 0.043	1.09	98.50
8	7.94 ± 0.087	1.096	99.25	7.92 ± 0.089	1.12	99.00
12	11.96 ± 0.122	1.02	99.66	11.93 ± 0.13	1.09	99.42
Hydrochlorothiazide						
1	0.99 ± 0.011	1.11	99.00	0.98 ± 0.011	1.12	98.00
2	1.97 ± 0.022	1.12	98.50	1.96 ± 0.023	1.17	98.00
3	2.97 ± 0.032	1.08	99.00	2.96 ± 0.036	1.21	98.66
Atenolol						
4	3.98 ± 0.043	1.08	99.50	3.96 ± 0.047	1.19	99.00
8	7.97 ± 0.089	1.12	99.63	7.95 ± 0.096	1.21	99.38
12	11.95 ± 0.14	1.17	99.58	11.92 ± 0.137	1.15	99.33

Table 5.18: Robustness evaluation of LOS, HCTZ and ATN (n=3)

Conditions	Level	LOS		HCTZ		ATN	
		t_R (min.)	% RSD of peak area	t_R (min.)	% RSD of peak area	t_R (min.)	% RSD of peak area
A: Flow rate (± 0.1 mL/min.)							
0.7	-0.1	2.662	1.10	3.892	1.15	7.800	1.13
0.8	0.0	2.633	1.03	3.883	1.10	7.783	1.11
0.9	+0.1	2.601	1.08	3.867	1.17	7.750	1.16
Mean ± SD		2.632 ± 0.030	1.07 ± 0.036	3.883 ± 0.015	1.14 ± 0.036	7.778 ± 0.025	1.13 ± 0.025
B: % of methanol in the mobile phase (± 5%)							
90	-5.0	2.665	1.04	3.897	1.12	7.808	1.18
95	0.0	2.633	1.07	3.883	1.09	7.783	1.12
100	+5.0	2.600	1.11	3.850	1.18	7.715	1.15
Mean ± SD		2.633 ± 0.033	1.073 ± 0.035	3.877 ± 0.024	1.13 ± 0.046	7.769 ± 0.048	1.15 ± 0.03

Table 5.19: Recovery study of LOS, HCTZ and ATN (n=3)

Label claim (mg/tablet)	Amount Added (%)	Total amount (mg)	Amount recovered (mg)	Recovery (%)	Mean (%) Recovery (± SD)
LOS 50	80	90	89.55	99.50	99.56 ± 0.21
	100	100	99.79	99.79	
	120	110	109.32	99.38	
HCTZ 12.5	80	22.5	22.35	99.33	99.50 ±0.20
	100	25	24.93	99.72	
	120	27.5	27.35	99.45	
ATN 50	80	90	89.42	99.36	99.40 ±0.15
	100	100	99.56	99.56	
	120	110	109.20	99.27	

Table 5.20: Assay results of LOS, HCTZ and ATN in tablets

Repalol-H tablet contains	Label claim (mg/tablet)	Amount found (mg ± SD)	RSD (%)	Recovery (%)
LOS	50 mg	50.175 ± 0.37	0.74	100.35
HCTZ	12.5 mg	12.475 ± 0.15	1.20	99.80
ATN	50 mg	49.58 ± 0.60	1.21	99.16

Table 5.21: Comparison of the proposed method with the reported methods:

Parameter	Reported Method 1	Reported Method 2	Propose Method
Mobile Phase	Mixture of (A) ACN, MeOH (65:35) and (B) 10 mM NaH_2PO_4, H_2O buffer with 0.4% v/v TEA and mixture of A: B (60:40 v/v) pH 3.0 with OPA (5%)	Acetonitrile: Phosphate buffer pH 3.6 adjusted with Na_2HPO_4 (70:30 v/v)	**Methanol: Water (95: 5% v/v)**
Mode	Isocratic	Isocratic	**Isocratic**
Detection wavelength (nm) & Flow Rate	230 nm 1.5mL/min	229 nm 1.2mL/min	**225 nm** **0.8 mL/min**
Retention time (t_R) in min.	LOS- 9.010 HCTZ- 4.211 ATN- 3.152	LOS- 9.2 HCTZ- 5.6 ATN- 2.6	**LOS-2.633** **HCTZ- 3.883** **ATN-7.783**
Linearity range (µg/mL)	LOS- 1-25 HCTZ- 1-25 ATN- 1-25	LOS- 10-90 HCTZ- 5-25 ATN- 10-90	**LOS- 2-12** **HCTZ-0.5- 3** **ATN-2-12**
LOD and LOQ (µg/mL)	LOS- 0.129-0.129 HCTZ- 0.118-0.343 ATN-0.014 – 0.933	LOS- 0.54 – 5.60 HCTZ- 0.20-3.20 ATN-0.50-4.50	**LOS-0.6 and 1.8** **HCTZ-0.2 and 0.5** **ATN-0.8 and 2**
% Recovery (n=3)	LOS-99.64 HCTZ- 100.20 ATN-100.17	LOS-100.21 HCTZ- 99.58 ATN-99.69	**LOS-99.64** **HCTZ- 99.50** **ATN-99.39**
Precision (% RSD) Intra-day (n=3) Inter-day (n=3)	 ATN- 1.652 &1.500 HCTZ- 0.769 & 0.751 LOS-1.013 &1.124	 ATN- 0.7148 & 0.837 HCTZ- 0.259 & 0.4543 LOS-0.62 & 0.446	 **ATN-1.123 & 1.100** **HCTZ- 1.103 & 1.660** **LOS-1.058 & 1.833**

5.3.3. Conclusion:

The proposed RP-HPLC method developed for simultaneous estimation of LOS, HCTZ and ATN in bulk and its formulation is simple, precise, accurate and specific. The proposed method is sufficiently sensitive and reproducible for the analysis of the LOS, HCTZ and ATN tablet dosage form within a short time of analysis. The method was proved to be superior to most of the reported methods. The mobile phase is simple to prepare and economical. It is suitable for analysis of antihypertensive agents in their triple fixed dose formulation in a single isocratic run using a simple mobile phase of methanol: water (95: 5% v/v). The method has been validated according to ICH guidelines. Low % RSD values indicate that the method is precise, accurate and robust. The proposed method can be used for the routine analysis of LOS, HCTZ and ATN from its dosage forms.

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Development of analytical methods for the simultaneous analysis of antihypertensive formulations containing more than three drugs by HPLC

Introduction[1-5]:

One of the treatment approach 'polypill' concept is used as an alternative for the patients who fail to lower their blood pressure and associated multiple risk factors with formulations containing two drugs or triple fixed-dose combinations.

The polypill concept aims to reduce multiple risk factors simultaneously (such as blood pressure, low density lipoprotein cholesterol and platelet function) through the administration of a single formulation that combines various effective medications at lower doses. The ploypill has two or three BP-lowering agents and has been shown to be superior to the combination of these antihypertensive with statin and aspirin.

Polypill- a single pill that would include a number of key drugs. These include aspirin, statins, an angiotensin II receptor blockers, beta-blockers, angiotensin-converting–enzyme (ACE) inhibitors and diuretics.

Fixed-dose combinations (FDC) containing four and five drugs that are available for reduction of multiple risk factors associated with hypertension includes:

1. HMG CoA reductase inhibitors or statins, an Angiotensin II receptor blockers (ARBs), beta-blockers and analgesic. e.g. Atorvastatin calcium, Losartan potassium, Atenolol and Aspirin.
2. HMG CoA reductase inhibitors or statins, angiotensin-converting–enzyme (ACE) inhibitors, beta-blockers, diuretics and analgesic. e.g. Simvastatin, Ramipril, Atenolol, Hydrochlorothiazide and Aspirin.

Considering the potential of these combinations and the need to develop new analytical methods, the following combinations were selected for the present study.

Sr. No.	Combination	Brand name	Manufacturer	Label Claim
1	Losartan Aspirin Atorvastatin Atenolol	Starpill Tablet	Gena Pharmaceutical Ltd	Losartan 50 mg Aspirin 75 mg Atorvastatin 10 mg Atenolol 50 mg
2	Ramipril Aspirin Hydrochlorothiazide Simvastatin Atenolol	Polycap Capsule	Cadila Pharmaceuticals Ltd. India	Ramipril 5.0 mg Aspirin 100 mg Hydrochlorothiazide 12.5 mg Simvastatin 20 mg Atenolol 50 mg

Literature survey revealed that few methods have been reported for simultaneous estimation of 1. Atorvastatin calcium, Losartan potassium, Atenolol and Aspirin; 2. Simvastatin, Ramipril, Atenolol, Hydrochlorothiazide and Aspirin. In addition there are many methods are available to estimate the above drugs individually or in combination with each other and also with other drugs.

However, reported methods for the above combinations suffer from certain drawbacks like buffered mobile phases, gradient mode of separation, time consuming preparation methods, pH adjustment, linearity at higher concentration ranges and lack of reproducibility of the methods. The purpose of the present study was to develop methods for the above combinations which eliminate the drawbacks mentioned above. Hence reliable and rugged method for the simultaneous analysis of drugs from above combinations was taken up.

6.1 Simultaneous analysis of Losartan potassium, Aspirin, Atorvastatin and Atenolol by HPLC

Losartan potassium, 2-butyl-4-chloro-1-[p-(o-1H-tetrazol-5ylphenyl)benzyl] imidazole -5- methanol monopotassium salt is an angiotensin II receptor blocker and is used as an antihypertensive agent. Aspirin, 2-(acetyloxy) benzoic acid, exerts its anti-inflammatory, analgesic and antipyretic actions and maintain kidney function and aggregate platelets when required. Atorvastatin chemically is [R-(R*, R*)]-2-(4-fluorophenyl)-β, δ-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino) carbonyl]-1Hpyrrole- 1-heptanoic acid, calcium salt (2:1) trihydrate. It is a lipid-lowering agent (HMG CoA reductase inhibitors or statins). Atenolol is 4-(2-hydroxy-3-[(1-methylethyl) amino] propoxy] benzene acetamide, β1 selective (cardioselective) β adrenergic receptor blocking agent.

The drug profiles and literature reviews of Losartan potassium (LOS), Atenolol (ATN), Atorvastatin (ATV) and Aspirin (ASP) are given in 3.6, 3.7, 3.8 and 3.9.

6.1.1 Experimental:

- **Instrumentation:**

  The HPLC system consisted of Intelligent HPLC pump model (Jasco PU 1580). The solutions were injected into the chromatograph through a Rheodyne valve, with a 20 µL loop with auto sampler (AS 1555). The column used was Thermo Hypersil Gold C_{18} (250 mm × 4.6 mm, 5.0 µ), operating at room temperature. The elution was carried out isocratically at flow rate of 1 mL/min. using methanol: water (60:40 v/v) as mobile phase. The detector consisted of a UV/ VIS (Jasco UV 1575) model operated at a wavelength of 230nm. Data was integrated using Jasco Borwin version 1.5, LC-Net II/ADC system.

- **Chemicals and reagents:**

  Reference standard of Atorvastatin calcium (99.91%) was kindly supplied by Cipla Ltd, Atenolol (99.7%) and Losartan potassium (100.2 %) were kindly supplied by Emcure Pharmaceuticals Ltd. and Aspirin (99.90 %) was kindly supplied by Wockhardt Ltd., respectively. The tablets Starpill (Losartan-50 mg, Aspirin-75 mg, Atorvastatin-10 mg and Atenolol-50 mg) of Gena Pharmaceutical Ltd., India, was procured from the market. For HPLC work double distilled water was prepared in

the laboratory. Methanol used was of HPLC grade and were purchased from Merck Chemicals, Mumbai, India.

- **Stationary Phase:**

  Thermo Hypersil Gold C_{18} (250 mm × 4.6 mm, 5.0 µ) was used.

- **Preparation of mobile phases:**

  HPLC grade methanol (600 mL) was mixed with (400 mL) of double distilled water. This solution was filtered through 0.45µ membrane filter paper and degassed in an ultrasonic bath before use.

- **Preparation of standard stock solution-I:**

  50 mg of Losartan potassium (LOS), 75 mg of Aspirin (ASP), 10 mg of Atorvastatin (ATV) and 50 mg of Atenolol (ATN) were accurately weighed and transferred to a 25 mL volumetric flask and dissolved in diluent (95:5 v/v). Volume was made up to the mark with diluent to get a solution containing LOS (2 mg/mL), ASP (3 mg/mL), ATV (0.4 mg/mL) and ATN (2 mg/mL), respectively. 1mL of this solution was further diluted to 10 mL with diluent to get **Standard stock solution-I** containing LOS 0.2 mg/mL, ASP 0.3 mg/mL, ATV 0.04 mg/mL and ATN 0.2 mg/mL, respectively.

- **Working standard solution-II:**

  0.1mL of the standard stock solution-I was diluted to 10 mL with diluent to get a concentration of 2 µg/mL of LOS, 3 µg/mL of ASP, 0.4 µg/mL of ATV and 2µg/mL of ATN. This solution was used as working standard.

- **Sample solution:**

  From the triturate of 20 tablets, an amount equivalent to 50 mg of ATN and LOS, 10 mg of ATV, and 75 mg of ASP was weighed transferred into a clean and dry 50 mL volumetric flask and dissolved in sufficient volume of methanol and kept in an ultrasonic bath for 15 min. and the volume was made to 50 mL with diluent, filtered through 0.45µ membrane filter paper and solution was further diluted with diluent to obtain a solution of LOS (2 µg /mL), ASP (3 µg /mL), ATV (0.4 µg /mL) and ATN (2 µg /mL), respectively. The sample solution (20µL) was injected into the chromatograph and the chromatogram was recorded.

- **System suitability:**

  20 µL of standard solution containing LOS (2 µg /mL), ASP (3 µg /mL), ATV (0.4 µg /mL) and ATN (2 µg /mL) were injected into the chromatograph to test the

system suitability to determine retention time (t_R), resolution (Rs), theoretical number of plates (N) and tailing factor (T).

- **Analytical Method Validation**

  Validation was done as per ICH guidelines. The developed method was validated with respect to parameters such as linearity, LOD and LOQ, precision, accuracy and specificity.

- **Linearity:**

  Several aliquots of standard stock solution-I of LOS, ASP, ATV and ATN were taken in different 10 mL volumetric flask and diluted up to the mark with diluent such that the final concentration of LOS, ASP, ATV and ATN were in the range of 2–12 µg/mL, 3–18 µg/mL, 0.4–2.4 µg/mL and 2–12 µg/mL, respectively. Evaluations of four drugs were performed with UV detector set at 230 nm. The peak area versus concentration data was collected and subjected to regression analysis. The correlation coefficient, slope and y-intercept values were calculated from the calibration plot obtained.

- **Limit of detection and limit of quantitation:**

  The Limit of detection (LOD) and Limit of quantitation (LOQ) for LOS, ASP, ATV and ATN and estimated at a signal-to-noise ratio of 3:1 and 10:1 respectively, by injecting a series of dilute solutions with known concentration.

- **Precision:**

  The precision of the method was verified by intra-day precision and inter-day precision studies. Intra-day precision studies were performed by analysis of three different concentrations 4, 8, 12 µg/mL for LOS and ATN, 6, 12, 18 µg/mL for ASP and 0.8, 1.6, 2.4 µg/mL for ATV three times on the same day. The inter-day precision of the method was checked by repeating studies on three different days.

- **Robustness:**

  To determine the robustness of the developed method, experimental conditions were deliberately changed and the relative standard deviation for replicate injections of LOS, ASP, ATV and ATN were evaluated. Flow rate was changed by ± 0.1 units to 0.9 and 1.1 mL/min. The effect of % of methanol in the mobile phase was studied at (± 5%).

- **Recovery studies:**

  To check the accuracy of the proposed method recovery experiment was carried out. Twenty tablets from the same lot of the commercial formulation were weighed accurately and finely powered. Powder equivalent to 50 mg of LOS, 75 mg of ASP, 10 mg of ATV and 50 mg of ATN were weighed and transferred to different 25 mL volumetric flask. Standard drug was added at 80%, 100% and 120% level. The contents were dissolved by sonicating in 20 mL of diluent for 15 minutes and filtered through 0.45µ membrane filter paper and further diluted up to the mark using diluent. 0.1 mL of the filtrate was taken in 10 mL volumetric flask and diluted up to the mark with mobile phase. 20µL of sample solution were injected and chromatograms were recorded. Percentage recovery and relative standard deviation were calculated. Each level was repeated three times.

- **Analysis of marketed formulation:**

  20µL of working standard solution-II (2 µg/mL of LOS, 3 µg/mL ASP, 0.4 µg/mL ATV and 2µg/mL ATN) and sample solutions were injected into the liquid chromatograph and the chromatogram was recorded. From the peak areas of LOS, ASP, ATV and ATN the amount of the drugs in the sample were calculated.

- **Specificity:**

  The specificity of the method was ascertained by analysing drug standards and samples. The chromatogram was overlaid to study the specificity. The identities of the peak for LOS, ASP, ATV and ATN from the sample were confirmed by comparing the t_R with those of standards

- **Comparison of the proposed method with the reported methods:**

  The proposed method was compared with the following reported methods from the literature:

1. RP-HPLC Method for Simultaneous Estimation of Atorvastatin Calcium, Losartan Potassium, Atenolol, and Aspirin From Tablet Dosage Form and Plasma[6]

   Column: KYA TECH HiQSil C_{18}HS (5µ particles size, 25 cm X 4.6 mm)

   Mobile phase: acetonitrile: 0.02 M potassium dihydrogen phosphate buffer (pH 3.4) (70:30% v/v), Flow rate: 1 ml/min, λ max = 236nm.

2. Quantitative Application to a Polypill by the Development of Stability Indicating LC Method for the Simultaneous Estimation of Aspirin, Atorvastatin, Atenolol and Losartan Potassium[7]

Column: Intersil ODS C_{18} (5µ particles size, 150mm X 4.6 mm)

Mobile phase: Mobile phase A: 0.1 % Orthophosphoric acid pH 2.9 adjusted with TEA, Mobile phase B- Acetonitrile (Gradient)

Flow rate: 1 ml/min, λ max = 230 nm.

6.1.2. Results and Discussion:

- **Selection of column:**

  As the drugs were soluble in polar solvents such as methanol and acetonitrile, C_{18} reverse phase column was selected with particle size of 5.0 µ, length 250 mm and internal diameter 4.6 mm.

- **Selection of suitable wavelength:**

  Stock solutions of individual drugs were prepared in methanol and the solutions were scanned individually in a UV spectrophotometer in the range of 200-400 nm and their spectras were recorded. The spectras were overlaid and the isobestic point, 230 nm was selected as the suitable wavelength as all four drugs showed good absorption at this wavelength (Fig.6.01). The wavelength was checked and confirmed in the optimised mobile phase also.

- **Selection of mobile phase:**

  Different mobile phase were selected and tried. Our preliminary trials using different composition of mobile phases consisting of water with methanol in a ratios 50:50 % and 55:45%, peaks were resolved but did not give well defined peak in a short time. Keeping mobile phase composition as methanol: water (60:40, v/v), satisfactory peak shape was obtained. Finally the mobile phase 60:40 methanol: water was selected. With this optimized mobile phase retention time were found to be for ATN (t_R-2.475min.), ASP (t_R-2.942 min.), LOS (t_R-3.550) and ATV (t_R-5.267 min.), respectively.

- **System suitability:**

  System suitability tests were carried out on freshly prepared standard stock solution of LOS, ASP, ATV and ATN to check various parameters such as efficiency, retention time and peak tailing which was found to comply with the USP requirements. Parameters obtained with 20 µL injection volume are shown in Table 6.01. Column efficiency in term of theoretical plates number (N) were 5342 for ATN, 5478 for ASP, 6002 for LOS and 4989 for ATV, respectively.

- **Validation of the method:**

  Using optimised chromatographic conditions the RP-HPLC method developed was validated in terms of linearity, LOD and LOQ, precision, robustness and accuracy.

- **Linearity:**

  Linear regression data for the calibration plots revealed good linear relationships between response and concentration over the ranges 2-12 µg/mL, 3-18 µg/mL, 0.4-2.4 µg/mL and 2-12 µg/mL for LOS, ASP, ATV and ATN, respectively. The linear regression equations were Y= 102786X + 6311 (r^2= 0.9998) for LOS, Y= 25991X + 786.7 (r^2= 0.9996) for ASP, Y=27806X + 459.0 (r^2= 0.9991) for ATV, and Y= 44575X + 419.7 (r^2= 0.9998) for ATN. The residuals plot shows a random pattern, indicating a good fit for a linear model. The calibration curves and residuals plots are given in Figures 6.03, 6.04, 6.05 and 6.06 for LOS, ASP, ATV and ATN, respectively and Table 6.02 (A), Table 6.02 (B), Table 6.02 (C) and Table 6.02 (D).

- **Limits of Detection and Quantitation:**

  The results of the LOD and LOQ were found to be 0.6 µg/mL and 1.8 µg/mL for LOS, 0.3 µg/mL and 1.2 µg/mL for ASP, 0.1µg/mL, 0.3 µg/mL for ATV and 0.8µg/mL, 2 µg/mL for ATN, respectively.

- **Precision:**

  Precision study was performed for three consecutive days at three times to find out intra-day and inter-day variations. The % relative standard deviation (RSD) for intra-day precision was 1.15%, 1.15%, 1.146%, and 1.073% for LOS, ASP, ATV and ATN, respectively; and for inter-day, precision was 1.033%, 1.12%, 0.91 %, and 1.147% for LOS, ASP, ATV and ATN, respectively, which is less than 2%, which indicates that the proposed method is precise (Table 6.03).

- **Robustness:**

  The robustness study was carried out by making small changes in the optimized method parameters like flow rate (± 0.1mL/min) and composition of methanol in the mobile phase (± 5%). There was no significant change in the retention times of drugs. The results of the robustness study indicated that the method is robust and is unaffected by small variations in the chromatographic conditions. The results of robustness study are reported in Table 6.04.

- **Accuracy:**

  Accuracy of the proposed method was evaluated by carrying out a recovery study. The known concentration of standard drugs were added to a preanalysed tablet sample at three different levels namely 80%, 100% and 120%. Total amount of the drugs were determined by the proposed method. The average recoveries obtained as 99.32 % for LOS, 99.15% for ASP, 99.18% for ATV and 99.36% for ATN indicate good accuracy of the method. (Table 6.05).

- **Analysis of marketed formulation:**

  The chromatogram of the sample extracted from conventional tablets showed peaks of ATN (t_R-2.475 min.), ASP (t_R- 2.942 min.), LOS (t_R-3.550 min.) and ATV (t_R-5.267min.) well resolved from other tablet excipients, shown in Fig. 6.2. The mean content of LOS, ASP, ATV and ATN per tablet by proposed method was found to be 49.92 mg, 74.80 mg, 9.93 mg and 49.90 mg, respectively. The results are summarized in Table 6.06.

- **Comparison of the proposed method with the reported methods:**

  The proposed method was compared with the reported methods. The proposed method uses simple mobile phase of methanol: water (95:5 v/v) as compared to buffered mobile phases. Linearity concentration range of the proposed method is 2–12 µg/mL for ATN, 3–18µg/mL for ASP, 0.4–2.4µg/mL for ATV and 2–12 µg/mL for LOS which is quiet lower as compared to the reported methods. The peaks obtained are well resolved. Accuracy and precision of the proposed method are comparable to the reported methods with low % RSD (Table 6.07).

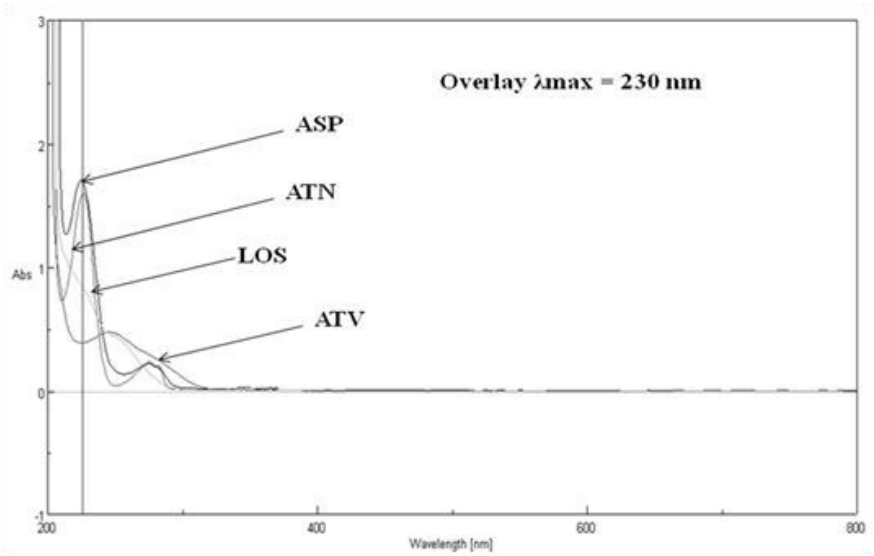

Fig.6.01. Overlay absorption spectra of LOS, ASP, ATV, and ATN

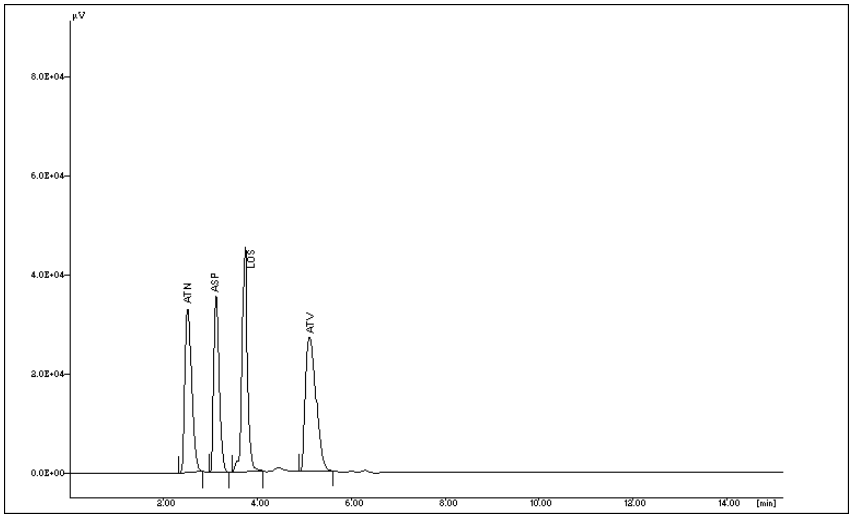

Fig.6.02. Typical chromatogram of LOS, ASP, ATV and ATN

Table 6.01: System suitability parameters (n=6)

Parameter	**ATN**	**ASP**	**LOS**	**ATV**
Retention Time in min	2.475	2.942	3.550	5.267
Resolution (Rs)	--	2.446	4.166	6.508
Theoretical plates number (N)	5342	5478	6002	4989
Tailing Factor	1.125	1.172	1.191	1.241

Table 6.02 (A): Data for LOS from linearity study

Concentration (µg/mL)	HPLC (n= 3)	
	Mean peak area ± SD	**RSD (%)**
2	207106 ± 1303.84	0.63
4	424452 ± 7293.833	1.72
6	625915 ± 8941.76	1.43
8	825024 ± 11322.54	1.37
10	1028530 ± 13038.4	1.27
12	1243836 ± 18708.29	1.50
Regression coefficient r^2		**0.9998**
Slope		**102786**
Intercept		**6311**

Table 6.02 (B): Data for ASP from linearity study

Concentration (µg/mL)	HPLC (n= 3)	
	Mean peak area ± SD	**RSD (%)**
3	77607 ± 1140.175	1.47
6	155814 ± 1978.84	1.27
9	234221 ± 447.2136	0.20
12	318228 ± 5674.504	1.78
15	390435 ± 547.7226	0.14
18	465842 ± 4381.78	0.95
Regression coefficient r^2		**0.9996**
Slope		**25991**
Intercept		**786.7**

Table 6.02 (C): Data for ATV from linearity study by HPLC

Concentration (µg/mL)	HPLC (n= 3)	
	Mean peak area ± SD	**RSD (%)**
0.4	11257.36 ± 130.384	1.158212
0.8	22475.0 ± 130.384	0.580129
1.2	34773.0 ± 433.5897	1.246915
1.6	47069.4 ± 642.6508	1.365326
2.0	56867.0 ± 83.666	0.147126
2.4	66464.16 ± 894.4272	1.345729
Regression coefficient r^2		**0.9991**
Slope		**27806**
Intercept		**459.0**

Table 6.02 (D): Data for ATN from linearity study by HPLC

Concentration (µg/mL)	HPLC (n= 3)	
	Mean peak area ± SD	**RSD (%)**
2	88929.4 ± 583.8193	0.66
4	177433 ± 1140.175	0.64
6	268150 ± 4037.326	1.51
8	361266 ± 3563.706	0.99
10	445202 ± 4892.034	1.10
12	533698.4 ± 4174.326	0.78
Regression coefficient r^2		**0.9998**
Slope		**44575**
Intercept		**419.7**

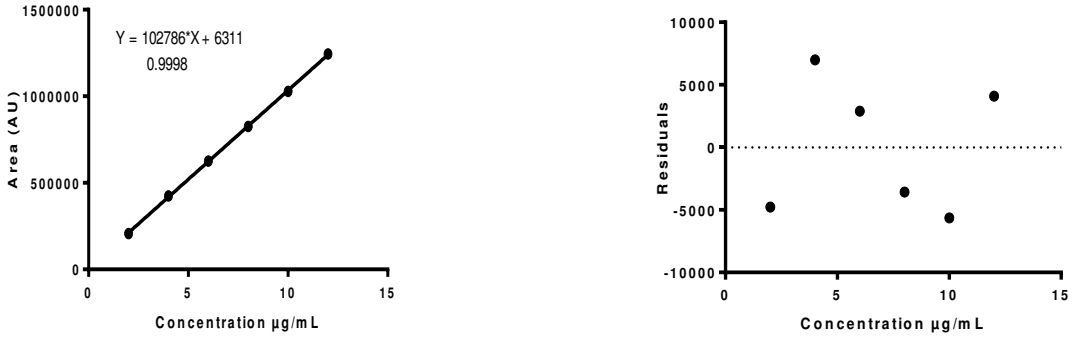

Fig.6.03. Calibration curve and residual plot for LOS

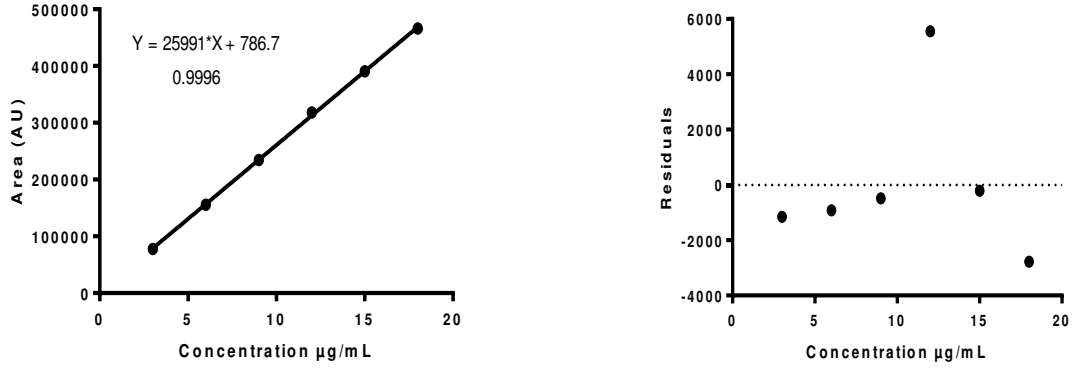

Fig.6.04. Calibration curve and residual plot for ASP

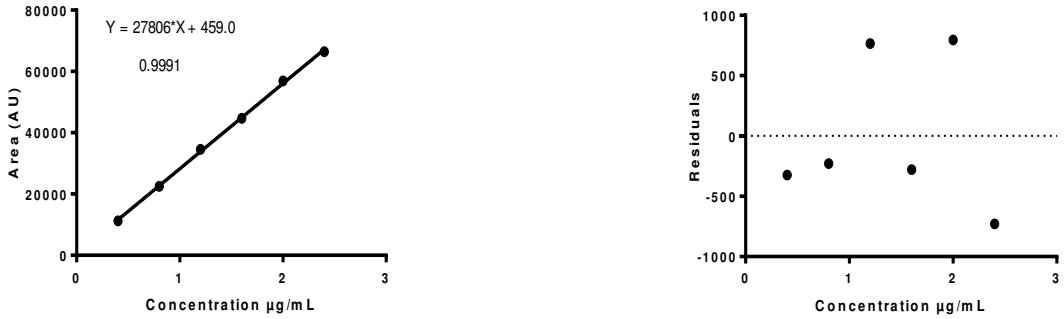

Fig. 6.05. Calibration curve and residual plot for ATV

Fig. 6.06. Calibration curve and residual plot for ATN

Table 6.03: Precision studies of ATN, ASP, LOS and ATV

Conc. (µg/mL)	Intra-day precision (n= 3)			Inter-day precision (n=3)		
	Measured Conc. ± SD	(%) RSD	Recovery (%)	Measured Conc. ± SD	(%) RSD	Recovery (%)
Atenolol						
4	3.95 ± 0.044	1.12	98.75	3.929 ± 0.039	1.16	99.23
8	7.97 ± 0.081	1.02	99.63	7.94 ± 0.084	1.05	99.25
12	11.92 ± 0.13	1.08	99.33	11.92 ± 0.15	1.23	99.33
Losartan potassium						
4	4.037 ± 0.055	1.37	100.92	3.97 ± 0.039	0.98	99.25
8	7.98 ± 0.054	0.67	99.75	7.96 ± 0.062	0.78	99.50
12	11.94 ± 0.167	1.40	99.50	11.92 ± 0.16	1.34	99.33
Aspirin						
6	5.95 ± 0.067	1.12	99.16	5.93 ± 0.056	0.95	98.83
12	11.95 ± 0.13	1.09	99.58	11.92 ± 0.14	1.15	99.33
18	17.85 ± 0.22	1.23	99.16	17.83 ± 0.23	1.27	99.05
Atorvastatin						
0.8	0.795 ± 0.01	1.26	99.38	0.793 ± 0.008	1.05	99.13
1.6	1.594 ± 0.018	1.14	99.63	1.593 ± 0.015	0.92	99.56
2.4	2.395 ± 0.025	1.04	99.79	2.392 ± 0.018	0.73	99.67

Table 6.04: Robustness evaluation of ATN, ASP, LOS and ATV (n=3)

Parameter	Level	ATN		ASP		LOS		ATV	
		t_R	% RSD	t_R	% RSD	t_R	% RSD	t_R	% RSD
Flow rate mL/min.	0.9	2.531	1.12	2.975	1.08	3.584	1.11	5.310	1.18
	1.0	2.475	1.15	2.942	1.11	3.550	1.15	5.267	1.13
	1.1	2.381	1.06	2.890	1.10	3.406	1.12	5.015	1.19
Mean ± SD		**2.462 ± 0.076**	**1.11 ± 0.046**	**2.936 ± 0.043**	**1.097 ± 0.015**	**3.513 ± 0.095**	**1.127 ± 0.021**	**5.197 ± 0.16**	**1.167 ± 0.032**
Methanol (± 5%)	65	2.528	1.10	2.982	1.09	3.586	1.21	5.316	1.04
	60	2.475	1.02	2.942	1.13	3.550	1.17	5.267	1.12
	55	2.354	1.08	2.881	1.16	3.403	1.19	5.011	1.15
Mean ± SD		**2.452 ± 0.089**	**1.067± 0.0416**	**2.935 ± 0.051**	**1.127 ±0.035**	**3.513 ± 0.097**	**1.19 ±0.02**	**5.198 ± 0.16**	**1.103 ± 0.057**

Table 6.05: Recovery study of ATN, ASP, LOS and ATV (n= 3)

Label claim (mg/tablet)	Amount Added (%)	Total amount (mg)	Amount recovered (mg)	Recovery (%)	Mean (%) Recovery (± SD)
ATN 50	80	90	89.25	99.17	99.36 ± 0.39
	100	100	99.80	99.80	
	120	110	109.0	99.10	
ASP 75	80	135	133.8	99.11	99.15 ± 0.106
	100	150	148.6	99.07	
	120	165	163.8	99.27	
LOS 50	80	90	89.54	99.49	99.32 ± 0.275
	100	100	99.00	99.00	
	120	110	109.4	99.46	
ATV 10	80	18	17.89	99.39	99.18 ± 0.184
	100	20	19.82	99.10	
	120	22	21.79	99.05	

Table 6.06: Assay results of ATN, ASP, LOS and ATV in tablets (n=3)

Starpill tablet contains	Label claim (mg/tablet)	Amount found (mg ± SD)	RSD (%)	Recovery (%)
ATN	50 mg	49.90 ± 0.633	1.27	99.80
ASP	75 mg	74.80 ± 0.98	1.31	99.73
LOS	50 mg	49.92 ± 0.614	1.23	99.84
ATV	10 mg	9.93 ± 0.108	1.09	99.3

Table 6.07: Comparison of the proposed method with the reported methods

Parameter	Reported Method 1	Reported Method 2	Propose Method
Mobile Phase	acetonitrile: 0.02 M KH_2PO_4 buffer (pH 3.4) (70:30% V /V)	Solution:(A) Buffer (0.1% OPA) and (B) ACN (20:80)	**Methanol: Water (60:40 v/v)**
Mode	Isocratic	Gradient	**Isocratic**
Detection wavelength (nm) & Flow Rate	236nm 1 mL/min	230nm 1mL/min	**230nm** **1mL/min**
Retention time (t_R) in min.	ATN- 2.18 ASP-3.2 LOS- 3.63 ATV- 4.83	ATN- 3.3 ASP-7.6 LOS- 10.7 ATV- 12.9	**ATN- 2.475** **ASP-2.942** **LOS- 3.550** **ATV- 5.267**
Linearity range (µg/mL)	ATN-20-120 ASP- 30-180 LOS-20-120 ATV-4.0-24	ATN-25-100 ASP- 37.5-150 LOS-25-100 ATV-5.0-20	**ATN-2.0-12** **ASP- 3.0-18** **LOS-2.0–12** **ATV-0.4-2.4**
LOD (µg/mL) and LOQ (µg/mL)	ATN-0.15 and 0.4 ASP- 0.1 and 0.3 LOS-0.02 and 0.06 ATV-0.2 and 0.7	ATN-0.15 and 0.4 ASP- 0.1 and 0.3 LOS-0.02 and 0.06 ATV-0.2 and 0.7	ATN-0.2 and 0.7 ASP- 0.3 and 1.2 LOS-0.3 and 0.8 ATV-0.1 and 0.4
% Recovery (n=3)	ATN-99.97 ASP- 100.10 LOS-99.34 ATV-99.01	ATN-99.41 ASP- 99.60 LOS-99.62 ATV-99.30	ATN-99.36 ASP- 99.15 LOS-99.32 ATV-99.18
Precision (% RSD) Intra-day (n=3) Inter-day (n=3)	For Intra-day & Intra-day ATN-3.5 & 2.023 ASP-1.8 & 2.19 LOS-2.7 & 2.6 ATV-1.75 & 2.9	For Intra-day & Intra-day ATN-0.4 & 0.6 ASP-0.4 & 0.5 LOS-0.5 & 0.7 ATV-0.8 & 0.8	**For Intra-day & Intra-day** **ATN-1.073 &1.147** **ASP- 1.15 &1.12** **LOS-1.15 & 1.033** **ATV-1.146 &0.91**

6.1.3 Conclusion:

RP-HPLC method has been developed for the simultaneous determination of Losartan potassium, Aspirin, Atorvastatin and Atenolol from tablet formulations. The method has been validated statistically for linearity, precision, specificity and accuracy. The method thus developed is free from interferences due to other tablet excipients. The developed RP-HPLC method for tablet analysis is accurate, precise, and selective and can be employed successfully for the estimation of LOS, ASP, ATV and ATN in both bulk and multi-component formulations. The run time required to elute all the four active components of the formulation is less than 7 min which clearly indicates the utility of the developed method for rapid and accurate analysis of formulations containing these drugs with acceptable precision. The method can be adopted for routine quality control analysis of the above mentioned drugs in bulk and in formulations.

6.2 Simultaneous analysis of Aspirin, Ramipril, Hydrochlorothiazide, Simvastatin and Atenolol by HPLC

Aspirin, 2-(acetyloxy) benzoic acid, is known for its anti-inflammatory, analgesic and antipyretic actions. Ramipril, (2S,3aS,6aS)-1[(S)-N-[(S)-1-Carboxy-3-phenylpropyl] alanyl] octa hydro cyclopenta [b] pyrrole-2-carboxylic acid, 1-ethyl ester, is an angiotensin converting enzyme, used to treat high blood pressure (hypertension) or congestive heart failure. Hydrochlorothiazide chemically, 6-Chloro-3, 4-dihydro-2H-l, 2, 4-benzothiadiazine-7- sulfonamide 1, 1-dioxide, is a thiazide diuretic. Atenolol, 4-(2-hydroxy-3-[(1-methylethyl) amino] propoxy] benzene acetamide, is a β 1 selective (cardioselective) β adrenergic receptor blocking agent. Simvastatin is butanoic acid, 2,2-dimethyl-,1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-(tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl)-ethyl]-1-naphthalenyl ester, [1S-[1α,3α,7β,8β(2S*,4S*),-8aβ]], a lipid-lowering agent (HMG CoA reductase inhibitors or statins).

The drug profiles and literature reviews of Ramipril (RAM), Hydrochlorothiazide (HCTZ), Atenolol (ATN), Aspirin (ASP), and Simvastatin (SIM) are given in 3.4, 3.5, 3.7, 3.9 and 3.10.

6.2.1. Experimental:

- **Instrumentation:**

  The HPLC system consisted of Intelligent HPLC pump model (Jasco PU 1580). The solutions were injected into the chromatograph through a Rheodyne valve, with a 20 µL loop with auto sampler (AS 1555). The column used was Thermo Hypersil Gold C_{18} (250 mm × 4.6 mm, 5.0 µ), operating at room temperature. The elution was carried out isocratically at flow rate of 1 mL/min. using methanol: water (95:5 v/v) as mobile phase. The detector consisted of a UV/ VIS (Jasco UV 1575) model operated at a wavelength of 230 nm. Data was integrated using Jasco Borwin version 1.5, LC-Net II/ADC system.

- **Chemicals and reagents:**

  Reference standards of Aspirin (99.90 %) was kindly supplied by Wockhardt Pharmaceuticals Ltd, Hydrochlorothiazide (99.97 %), Ramipril (99.4%) and Atenolol (99.7 %) were kindly supplied by Emcure Pharmaceuticals Ltd. and Simvastatin (100 %) Smilax Laboratories Ltd, respectively. The Capsules Polycap

(Aspirin-100 mg, Ramipril-5.0 mg, Hydrochlorothiazide-12.5 mg, Simvastatin- 20 mg and Atenolol-50 mg) of Cadila Pharmaceuticals Ltd. India was procured from the market. For HPLC work double distilled water was prepared in the laboratory. Methanol used was of HPLC grade and were purchased from Merck Chemicals, Mumbai, India.

- **Stationary Phase:**

  Thermo Hypersil Gold C_{18} (250 mm × 4.6 mm, 5.0 µ) was used.

- **Preparation of mobile phases:**

  HPLC grade methanol (950 mL) was mixed with (50 mL) of double distilled water. This solution was filter through 0.45µ membrane filter paper and degassed in an ultrasonic bath before use.

- **Preparation of standard stock solution-I:**

  100 mg of Aspirin (ASP), 5 mg of Ramipril (RAM), 12.5 mg of Hydrochlorothiazide (HCTZ), 20 mg of Simvastatin (SIM) and 50 mg of Atenolol (ATN) were accurately weighed and transferred to a 50 mL volumetric flask and dissolved in mobile phase. Volume was made up to the mark with mobile phase to get a solution containing ASP 2.0 mg/mL, RAM 0.1 mg/mL, HCTZ 0.25 mg/mL, SIM 0.4 mg/mL and ATN 1.0 mg/mL, respectively. 1 mL of this solution was further diluted to 10 mL with mobile phase to get **Standard stock solution-I** containing ASP 0.2 mg/mL, RAM 0.01 mg/mL, HCTZ 0.025 mg/mL, SIM 0.04 mg/mL and ATN 0.1 mg/mL, respectively.

- **Working standard solution-II:**

  1.0 mL of the standard stock solution-I was diluted to 10 mL with mobile phase to get a concentration of ASP 20 µg/mL, RAM 1.0 µg/mL, HCTZ 2.5 µg/mL, SIM 4.0 µg/mL and ATN 10 µg/mL. This solution was used as working standard.

- **Sample solution:**

  Twenty capsules were weighed accurately and powered and powder equivalent to, 100 mg of ASP, 5.0 mg RAM, 12.5 mg of HCTZ, 20 mg of SIM and 50 mg of ATN were weighed into clean and dry 50 mL volumetric flask. The powder was first dissolved in sufficient volume of mobile phase and kept in an ultrasonic bath for 15 min. and the volume was made to 50 mL with mobile phase, filtered through 0.45µ membrane filter paper and solution was further diluted with mobile phase to obtain a solution of ASP (20 µg/mL), RAM (1.0 µg/mL), HCTZ (2.5 µg/mL), SIM (4.0

µg/mL) and ATN (10 µg/mL), respectively. The sample solution (20 µL) was injected into the chromatograph and the chromatogram was recorded.

- **System suitability:**

  20 µL of standard solution containing ASP (20 µg/mL), RAM (1.0 µg/mL), HCTZ (2.5 µg/mL), SIM (4.0 µg/mL) and ATN (10 µg/mL) was injected into the chromatograph to test the system suitability to determine resolution (Rs), retention time (t_R), theoretical number of plates (N) and tailing factor (T).

- **Analytical Method Validation:**

  Validation was done as per ICH guidelines. The developed method was validated with respect to parameters such as linearity, LOD and LOQ, precision, accuracy and specificity.

- **Linearity:**

  Several aliquots of standard stock solution-I of ASP, RAM, HCTZ, SIM and ATN were taken in different 10 mL volumetric flask and diluted up to the mark with mobile phase such that the final concentration of ASP, RAM, HCTZ, SIM and ATN were in the range of 20-120 µg/mL, 1.0-6.0 µg/mL, 2.5-15 µg/mL, 4.0-24 µg/mL and 10–60 µg/mL, respectively. Evaluations of five drugs were performed with UV detector set at 230 nm. The peak area versus concentration data was collected and subjected to regression analysis. The correlation coefficient, slope and y-intercept values were calculated from the calibration plot obtained.

- **Limit of detection and limit of quantitation:**

  The Limit of detection (LOD) and Limit of quantitation (LOQ) for ASP, RAM, HCTZ, SIM and ATN estimated at a signal-to-noise ratio of 3:1 and 10:1 respectively, by injecting a series of dilute solutions with known concentration.

- **Precision:**

  The precision of the method was verified by intra-day precision and inter-day precision studies. Intra-day precision studies were performed by analysis of three different concentrations 40, 80, 120 µg/mL for ASP, 2, 4, 6 µg/mL for RAM, 5, 10, 15 µg/mL for HCTZ, 8, 16, 24 µg/mL for SIM and 20, 40, 60 µg/mL ATN three times on the same day. The inter-day precision of the method was checked by repeating studies on three different days.

- **Robustness:**

  To determine the robustness of the developed method, experimental conditions were deliberately changed and the relative standard deviation for replicate injections of ASP, RAM, HCTZ, SIM and ATN were evaluated. Flow rate was changed by ± 0.1 units to 0.9 and 1.1 mL/min. The effect of % of methanol in the mobile phase was studied at (± 5%).

- **Recovery studies:**

  To check the accuracy of the proposed method recovery experiments were carried out. Twenty capsules from the same lot of the commercial formulation were weighed accurately and finely powered. Powder equivalent to, 100 mg of ASP, 5 mg RAM, 12.5 mg of HCTZ, 20 mg of SIM and 50 mg of ATN were weighed and transferred to different 50 mL volumetric flask. Standard drug was added at 80%, 100% and 120% level. The contents were dissolved by sonicating in 20 mL of mobile phase for 15 minutes and filtered through 0.45µ membrane filter paper and further diluted up to the mark using mobile phase. 1.0 mL of the filtrate was taken in 10 mL volumetric flask and diluted up to the mark with mobile phase. 20 µL of sample solution were injected and chromatograms were recorded. Percentage recovery and relative standard deviation were calculated. Each level was repeated three times.

- **Analysis of marketed formulation:**

  20 µL of working standard solution-II (20 µg/mL of ASP, 1.0 µg/mL of RAM, 2.5 µg/mL of HCTZ, 4.0 µg/mL of SIM and 10 µg/mL ATN) and sample solutions were injected into the liquid chromatograph and the chromatograms were recorded. From the peak areas of ASP, RAM, HCTZ, SIM and ATN the amount of the drugs in the sample were calculated.

- **Specificity:**

  The specificity of the method was ascertained by analysing drug standards and samples. The chromatogram was overlaid to study the specificity. The identities of the peak for, ASP, RAM, HCTZ, SIM and ATN from the sample were confirmed by comparing the t_R with those of standards.

- **Comparison of the proposed method with the reported methods:**

The proposed method was compared with the reported methods from the literature:

1. A Simple RP-HPLC Method for Simultaneous Analysis of Aspirin, Atenolol, Hydrochlorothiazide, Ramipril and Simvastatin in Pharmaceutical Solid Dosage Forms[8]

   Column: X-Terra C_{18} column (5µ particles size, 150 cm X 4.6 mm)

   Mobile phase: Solution A: Sodium perchlorate, Solution B: acetonitrile

   Flow rate: 0.8 ml/min, λ max = 220 nm

2. Simultaneous Determination of Ramipril, Atenolol, Hydrochlorothiazide, Simvastatin and Aspirin in Ramipril, Atenolol, Hydrochlorothiazide, Simvastatin as Tablets and Aspirin as Enteric Coated Tablets in Capsules by RP-HPLC[9]

   Column: X-Terra RP-8 column (5µ particles size, 150 cm X 4.6 mm)

   Mobile phase: Solution A: Sodium perchlorate pH 2.2 adjust with OPA, Solution B: acetonitrile

   Flow rate: 1.5 ml/min, λ max = 210 nm

6.2.2. Results and Discussion:

- **Selection of column:**

  As the drugs were soluble in polar solvents such as methanol and acetonitrile, C_{18} reverse phase column was selected with particle size of 5.0 µ, length 250 mm and internal diameter 4.6 mm.

- **Selection of suitable wavelength:**

  Stock solutions of individual drugs were prepared in methanol and individually the solutions were scanned in the UV range 200-400 nm in a UV spectrophotometer and their spectras were recorded. The spectras were overlaid and the isobestic point, 230 nm was selected as the suitable wavelength as all the drugs showed good absorption at that wavelength (Fig.6.07). The wavelength was checked and confirmed in the optimised mobile phase also.

- **Selection of mobile phase:**

  Different mobile phase compositions were tried to achieve good resolution with short run time. Initial trials using mobile phases consisting of methanol with water in a ratios 50:50 %, 60:40% and 75:25%, resulted in poor separation. With 80:20%, 90:10% methanol and water ratios, peaks were resolved however asymmetry was not

good for ATN. Keeping mobile phase composition as methanol: water (95:5, v/v), satisfactory peak shape was obtained. Finally the mobile phase consisting of methanol: water 95:5 v/v was selected. The optimised mobile phase was found to resolve peaks of ASP (t_R-1.983min.), RAM (t_R-2.525min.), HCTZ (t_R-3.108 min.), SIM (t_R-3.867 min.) and ATN (t_R-7.833min.), respectively.

- **System suitability:**

  System suitability tests were carried out on freshly prepared standard stock solutions ASP, RAM, HCTZ, SIM and ATN to determine efficiency, resolution factor, symmetry factor and precision of the instrument and found to comply with USP requirement. The parameters obtained are shown in Table 6.08. Column efficiency in term of theoretical plates number (N) were 3423 for ASP, 5528 for RAM, 6782 for HCTZ, 4152 for SIM and 4567 for ATN.

- **Validation of the method:**

  Using optimised chromatographic conditions the RP-HPLC method developed was validated in terms of linearity, LOD and LOQ, precision, robustness and accuracy.

  - **Linearity:**

    A calibration curve were plotted using peak areas obtained against respective concentrations of ASP, RAM, HCTZ, SIM and ATN, respectively. Linear regression data for the calibration plots revealed good linear relationships between response and concentration over the ranges 20–120 µg/mL for ASP, 1.0-6.0 µg/mL for RAM, 2.5-15 µg/mL for HCTZ, 4.0-24 µg/mL for SIM and 10-60 µg/mL for ATN, respectively. The linear regression equations were Y=5254X + 105362 (r^2= 0.9996) for ASP, Y= 42392X + 6327 (r^2= 0.9990) for RAM, Y= 21553X + 56927 (r^2= 0.9995) for HCTZ, Y= 14133X + 47241 (r^2= 0.9996) for SIM and Y= 9089X + 62236 (r^2= 0.9996) for ATN. The residuals plot shows a random pattern, indicating a good fit for a linear model. The calibration curves and residuals plots are given in Fig. 6.09, Fig. 6.10, 6.11, 6.12 and Fig. 6.13 and Table 6.09 (A), Table 6.09 (B), Table 6.09 (C), Table 6.09 (D) and Table 6.09 (E) for ASP, RAM, HCTZ, SIM and ATN, respectively.

  - **Limits of Detection and Quantitation:**

    The signal to noise ratios of 3: 1 and 10: 1 were considered as LOD and LOQ respectively. The results of the LOD and LOQ were found to be 0.6 µg/mL and 1.4 µg/mL for ASP, 0.2 µg/mL and 0.5 µg/mL for RAM, 0.23 µg/mL and 0.6 µg/mL for

HCTZ, 1.32 µg/mL and 3.97 µg/mL for SIM and 0.98 µg/mL, 0.5 µg/mL for ATN, respectively.

- **Precision:**

  The precision of the developed method was demonstrated by intra-day and inter-day precision studies. This was done by three replicate analysis of the sample. The precision of the method was expressed as relative standard deviation (% RSD). The % RSD of intra-day and inter-day precision values were found to be (0.51% and 0.82%) for ASP, (0.81% and 1.0%) for RAM, (0.85% and 0.55%) for HCTZ, (0.62% and 0.47%) for SIM and (0.33% and 0.54%) for ATN, respectively (Table 6.10). The % RSD values were found to be <2 %, which indicates that the proposed method is precise.

- **Robustness:**

  The robustness study was carried out by making small changes in the optimized method. Parameters like composition of the mobile phase (± 5%) and flow rate (± 0.1 mL/min). There were no significant changes in the retention times of ASP, RAM, HCTZ, SIM and ATN. The low values of the % RSD indicate the robustness of the method, as shown in Table 6.11

- **Accuracy:**

  To study the accuracy of the proposed method recovery experiment were carried out by standard addition method. The proposed method when used for extraction and subsequent estimation of ASP, RAM, HCTZ, SIM and ATN from pharmaceutical dosage form after spiking with 80%, 100% and 120% of the additional drug afforded mean recovery of 99.60 % for ASP, 99.40 % for RAM, 99.13 % for HCTZ, 99.46 % for SIM and 99.54 % for ATN. The mean recoveries indicate non-interference from excipients (Table 6.12).

- **Analysis of marketed formulation:**

  The chromatogram of the sample extracted from conventional capsules showed peaks of ASP (t_R 1.983min.), RAM (t_R 2.525min.), HCTZ (t_R 3.108 min.), SIM (t_R 3.867 min.) and ATN (t_R 7.833min.) that are well resolved from other capsule excipients as shown in Fig. 6.08. The mean content of ASP, RAM, HCTZ, SIM and ATN per capsule by proposed method was found to be 99.92 mg, 4.98 mg, 12.40 mg, 19.93 mg and 49.91 mg, respectively. The results are summarized in Table 6.13.

- **Comparison of the proposed method with the reported method:**

  The proposed method was compared with the reported methods. A simple isocratic method uses methanol: water 95:5 v/v, at a flow rate of 1 ml/min was found to be a suitable mobile phase, allowing rapid analysis of multicomponent formulation as against buffered mobile phases used in the reported gradient methods. The chromatographic run time of 8 min. allows the analysis of large number of samples in a short period of time as compared to reported method which requires more than 30 mins. of analysis run time. Linearity concentration range of the proposed method is 20–120 µg/mL for ASP, 1.0-6.0 µg/mL for RAM, 2.5-15 µg/mL for HCTZ, 4.0-24 µg/mL for SIM and 10-60 µg/mL for ATN which is quiet lower as compared to the reported methods. Accuracy and precision of the proposed method are comparable to the reported methods with low % RSD (Table 6.14).

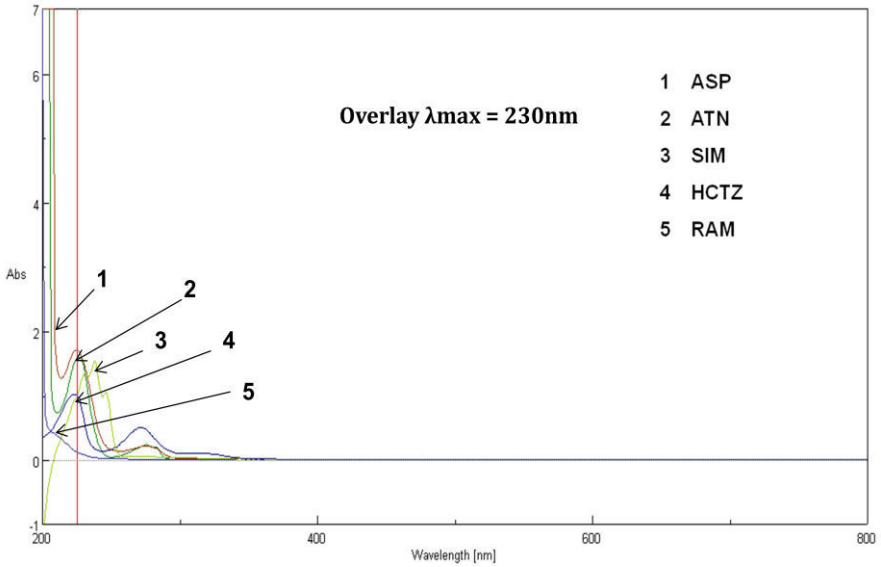

Fig.6.07. Overlay absorption spectra of ASP, RAM, HCTZ, SIM and ATN

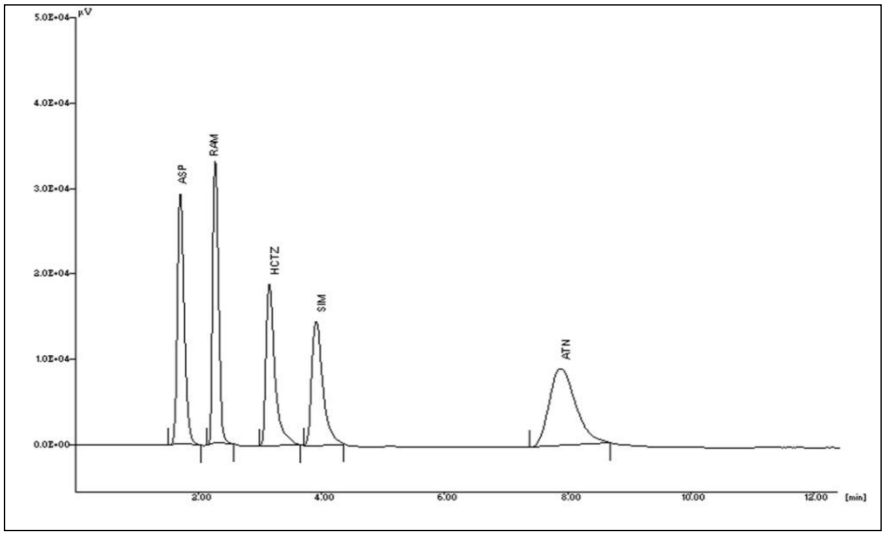

Fig.6.08. Typical chromatogram of ASP, RAM, HCTZ, SIM and ATN

Table 6.08: System suitability parameters

Parameter	ASP	RAM	HCTZ	SIM	ATN
Retention Time in min	1.983	2.525	3.108	3.867	7.833
Resolution (Rs)	--	2.446	3.146	4.460	8.235
Theoretical plates no. (N)	3423	5528	6782	4152	4567
Tailing Factor	1.02	1.20	1.16	1.12	1.32

Table 6.09 (A): Data for ASP from linearity study by HPLC

Concentration (µg/mL)	HPLC (n= 3)	
	Mean peak area ± SD	RSD (%)
20	209495.4 ± 2136.85	1.02
40	319617.4 ± 2844.6	0.89
60	418623.9 ± 4730.45	1.13
80	520291.9 ± 3902.2	0.75
100	635705.8 ±7882.75	1.24
120	735043.8 ± 8011.98	1.09
Regression coefficient r^2		0.9996
Slope		5254
Intercept		105362

Table 6.09 (B): Data for RAM from linearity study by HPLC

Concentration (µg/mL)	HPLC (n= 3)	
	Mean peak area ± SD	RSD (%)
1	46564.32 ± 530.83	1.14
2	91128.64 ± 1093.54	1.20
3	136693 ± 1339.6	0.98
4	175194.5 ± 1804.50	1.03
5	220820 ± 2362.77	1.07
6	257791.8 ± 3119.28	1.21
Regression coefficient r^2		0.9990
Slope		42392
Intercept		6327

Table 6.09(C): Data for HCTZ from linearity study by HPLC

Concentration (µg/mL)	HPLC (n= 3)	
	Mean peak area ± SD	RSD (%)
2.5	113910.8 ± 1104.93	0.97
5.0	160820 ± 1672.53	1.04
7.5	217900 ± 2440.48	1.12
10	272922.6 ± 3384.24	1.24
12.5	327421.5 ± 3601.6	1.10
15	380123.5 ± 3231.05	0.85
Regression coefficient r^2		0.9995
Slope		21553
Intercept		56927

Table 6.09 (D): Data for SIM from linearity study by HPLC

Concentration (µg/mL)	HPLC (n= 3)	
	Mean peak area ± SD	RSD (%)
4	105194.5 ± 1062.46	1.01
8	160821 ± 1881.61	1.17
12	216008.8 ± 2030.48	0.94
16	270648.5 ± 2246.38	0.83
20	328666.8 ± 3878.26	1.18
24	389284.5 ± 4904.98	1.26
Regression coefficient r^2		0.9996
Slope		14133
Intercept		47241

Table 6.09 (E): Data for ATN from linearity study by HPLC

Concentration (µg/mL)	HPLC (n= 3)	
	Mean peak area ± SD	RSD (%)
10	155755.1 ± 1401.80	0.90
20	244969.1 ± 3013.12	1.23
30	332552 ± 3924.11	1.18
40	422075.5 ± 4347.37	1.03
50	514241.6 ± 6530.87	1.27
60	612519.5 ± 7043.97	1.15
Regression coefficient r^2		0.9996
Slope		9089
Intercept		62236

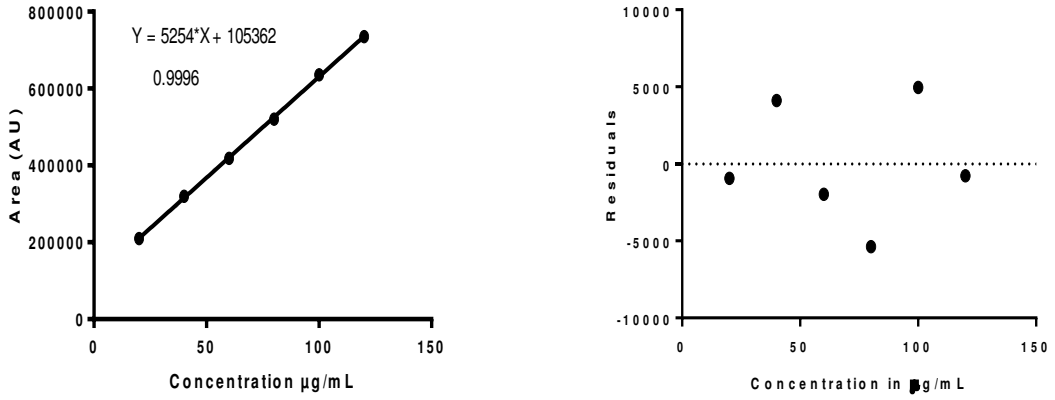

Fig. 6.09. Calibration curve and residual plot for ASP

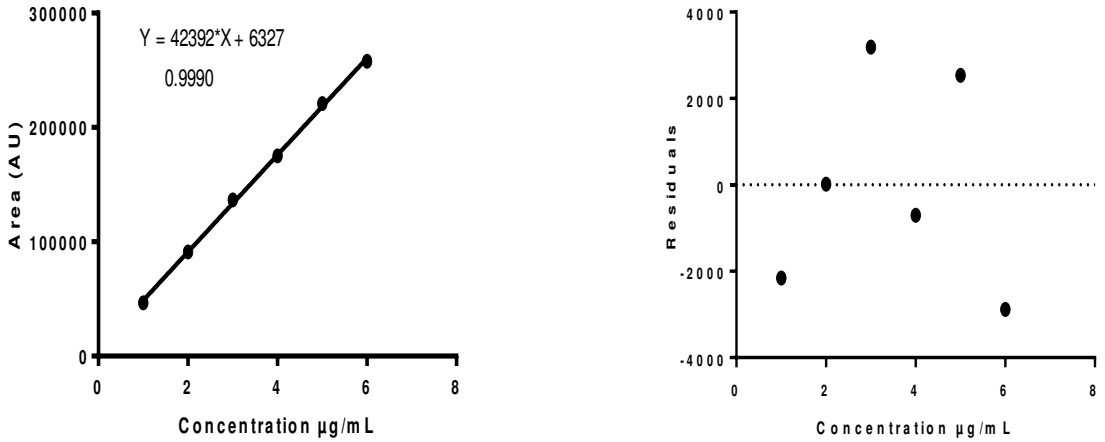

Fig. 6.10. Calibration curve and residual plot for RAM

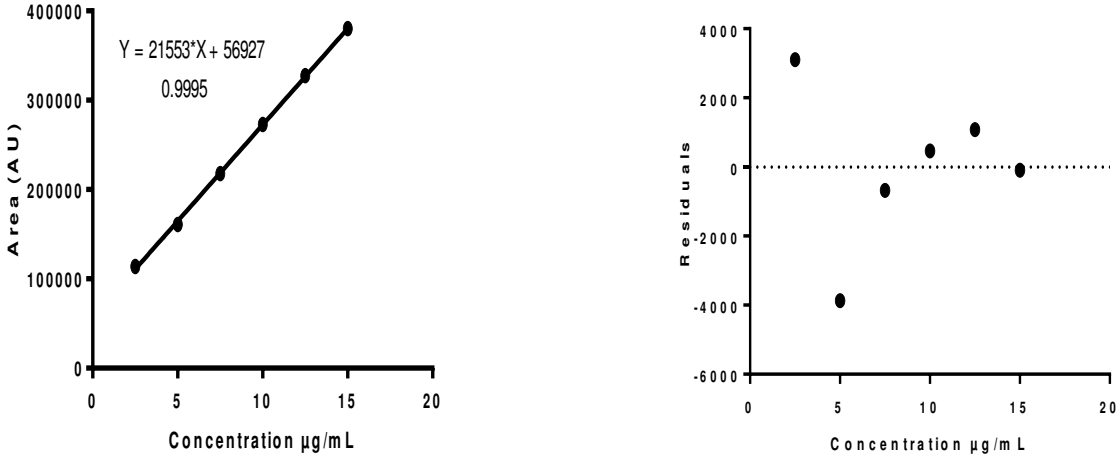

Fig. 6.11. Calibration curve and residual plot for HCTZ

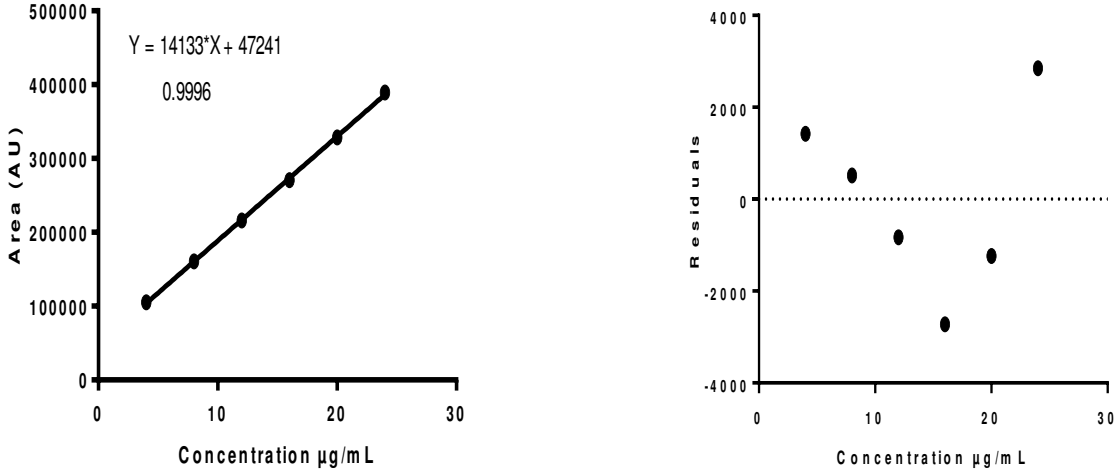

Fig.6.12. Calibration curve and residual plot for SIM

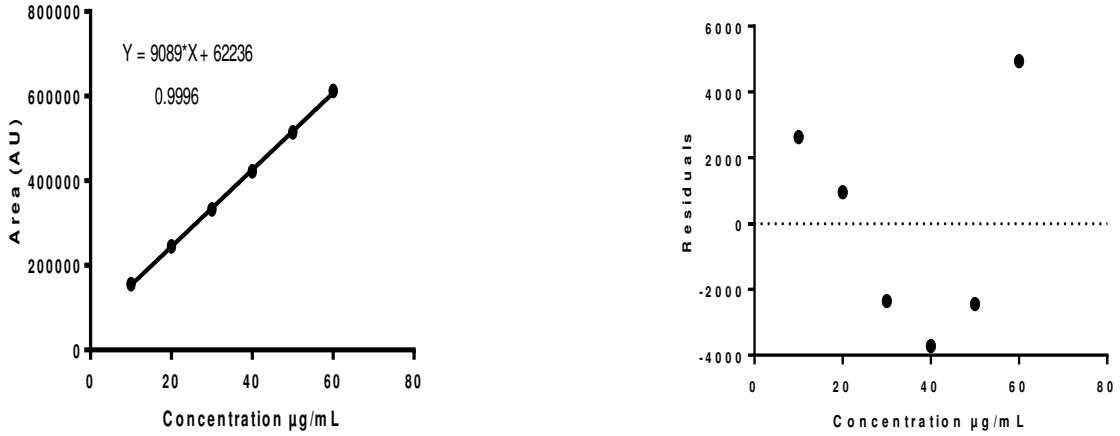

Fig.6.13. Calibration curve and residual plot for ATN

Table 6.10: Precision studies of ASP, RAM, HCTZ, SIM and ATN

Conc. (µg/mL)	Intra-day precision (n= 3)			Inter-day precision (n=3)		
	Measured Conc. ± SD	(%) RSD	Recovery (%)	Measured Conc. ± SD	(%) RSD	Recovery (%)
Aspirin						
40	39.90 ± 0.18	0.45	99.75	39.87 ± 0.35	0.88	99.67
80	79.92 ± 0.31	0.39	99.90	79.80 ± 0.64	0.80	99.75
120	119.96 ± 0.84	0.70	99.97	119.82 ± 0.95	0.79	99.85
Ramipril						
2	1.98 ± 0.012	0.60	99.00	1.97 ± 0.020	1.01	98.50
4	3.98 ± 0.051	1.28	99.50	3.95 ± 0.039	0.98	98.75
6	5.98 ± 0.034	0.56	99.67	5.95± 0.061	1.02	99.17
Hydrochlorothiazide						
5	4.96 ± 0.058	1.17	99.20	4.94 ± 0.064	1.29	98.80
10	9.96 ± 0.091	0.91	99.60	9.94 ± 0.025	0.25	99.40
15	14.89 ± 0.068	0.46	99.27	14.86 ± 0.017	0.11	99.07
Simvastatin						
8	7.98 ± 0.090	1.13	99.75	7.95 ± 0.080	1.00	99.38
16	15.97 ± 0.062	0.39	99.81	15.93 ± 0.050	0.31	99.56
24	23.95 ± 0.078	0.33	99.79	23.93 ± 0.024	0.10	99.71
Atenolol						
20	19.82 ± 0.092	0.46	99.10	19.87 ± 0.080	0.40	99.35
40	39.77 ± 0.082	0.20	99.43	39.86 ± 0.35	0.88	99.65
60	59.85 ± 0.192	0.32	99.75	59.55 ± 0.21	0.35	99.25

Table 6.11: Robustness evaluation of ASP, RAM, HCTZ, SIM and ATN (n=3)

Level	ASP		RAM		HCTZ		SIM		ATN	
	t_R	% RSD	t_R	% RSD	t_R	% RSD	t_R	% RSD	t_R	% RSD
A: Flow rate mL/min.(± 0.1)										
0.9	2.021	1.10	2.560	1.10	3.152	1.12	3.890	1.11	7.875	1.18
1.0	1.983	1.13	2.525	1.01	3.108	1.05	3.867	1.07	7.833	1.14
1.1	1.981	1.09	2.500	1.14	3.008	1.02	3.821	1.15	7.801	1.19
Mean ± SD	**1.99 ± 0.02**	**1.10 ± 0.02**	**2.53 ± 0.03**	**1.08 ± 0.07**	**3.09 ± 0.07**	**1.06 ± 0.051**	**3.86 ± 0.035**	**1.11 ± 0.04**	**7.836 ± 0.04**	**1.17 ± 0.026**
B: % of methanol in the mobile phase (± 5%)										
90	2.024	1.10	2.562	1.15	3.154	1.07	3.891	1.19	7.876	1.12
95	1.983	1.02	2.525	1.08	3.108	1.10	3.867	1.16	7.833	1.14
100	1.982	1.08	2.501	1.10	3.007	1.11	3.822	1.14	7.803	1.17
Mean ± SD	**1.99 ± 0.02**	**1.07 ± 0.04**	**2.53 ± 0.03**	**1.11 ± 0.04**	**3.09 ± 0.07**	**1.093 ± 0.021**	**3.86 ± 0.035**	**1.163 ± 0.02**	**7.84 ± 0.04**	**1.143 ± 0.025**

Table 6.12: Recovery study of ASP, RAM, HCTZ, SIM and ATN (n= 3)

Label claim (mg/capsule)	Amount Added (%)	Total amount (mg)	Amount recovered (mg)	Recovery (%)	Mean (%) Recovery (± SD)
ASP 100	80	180	178.5	99.17	99.60 ± 0.38
	100	200	199.8	99.90	
	120	220	219.4	99.72	
RAM 5.0	80	9	8.95	99.44	99.40 ± 0.12
	100	10	9.95	99.50	
	120	11	10.92	99.27	
HCTZ 12.5	80	22.5	22.30	99.11	99.13 ± 0.059
	100	25	24.80	99.20	
	120	27.5	27.25	99.09	
SIM 20	80	36	35.85	99.58	99.46 ± 0.11
	100	40	39.75	99.37	
	120	44	43.75	99.43	
ATN 50	80	90	89.52	99.47	99.54 ± 0.25
	100	100	99.82	99.82	
	120	110	109.27	99.34	

Table 6.13: Assay of ASP, RAM, HCTZ, SIM and ATN (n=3)

Polycap Capsule	Label claim (mg/capsule)	Amount found (mg ± SD)	RSD (%)	Recovery (%)
ASP	100 mg	99.92 ± 1.029	1.03	99.92
RAM	5 mg	4.98 ± 0.057	1.14	99.60
HCTZ	12.5 mg	12.40 ± 0.15	1.22	99.20
SIM	20 mg	19.93 ± 0.28	1.40	99.65
ATN	50 mg	49.91 ± 0.68	1.37	99.82

Table 6.14: Comparison of the proposed method with the reported methods

Parameter	Reported Method 1	Reported Method 2	Propose Method
Mobile Phase	Solution:(A) $NaClO_4$ and (B) ACN	Solution A: Sodium Per chlorate pH 2.2 adjust with OPA, Solution B: ACN	**Methanol: Water (95:5 v/v)**
Mode	Gradient	Gradient	**Isocratic**
Detection wavelength (nm) & Flow Rate	220 nm 0.8 mL/min	210 nm 1.5 mL/min.	**230 nm** **1mL/min**
Retention time (Rt) in min.	ASP- 2.6 RAM- 8.6 HCTZ- 7.6 SIM-13.0 ATN- 6.2	ASP- 9.322 RAM- 19.153 HCTZ- 6.743 SIM-24.799 ATN- 4.355	**ASP- 1.983** **RAM- 2.525** **HCTZ- 3.108** **SIM- 3.867** **ATN- 7.833**
Linearity range (µg/mL)	ASP- 10-60 RAM-10-60 HCTZ- 10-60 SIM- 10-60 ATN- 10-60	ASP- 200-604 RAM-19-60 HCTZ- 50-150 SIM- 80-240 ATN- 199-599	**ASP- 20-120** **RAM- 1.0-6.0** **HCTZ- 2.5-15** **SIM- 4.0-24** **ATN- 10-60**
LOD (µg/mL) and LOQ (µg/mL)	ASP- 0.27 and 0.9 RAM- 0.3 and 0.95 HCTZ- 0.12 and 0.4 SIM-0.22 and 0.71 ATN- 0.2 and 0.68	Not reported	**ASP- 0.6 and 1.4** **RAM- 0.2 and 0.5** **HCTZ- 0.23 and 0.6** **SIM-1.32 and 3.97** **ATN- 1.98 and 5.0**
% Recovery (n=3)	ASP- 99.32 RAM- 99.21 HCTZ-98.58 SIM- 98.72 ATN- 100.17	ASP- 99.5 RAM- 105.4 HCTZ-102.7 SIM- 99.1 ATN- 104.3	**ASP- 99.60** **RAM- 99.40** **HCTZ-99.13** **SIM-99.46** **ATN-99.54**
Precision (% RSD) Intra-day (n=3) Inter-day (n=3)	Intra-day & Intra-day ASP- 0.79 and 0.74 RAM- 0.72 and 0.66 HCTZ-0.66 and 0.73 SIM-1.01 and 1.00 ATN- 0.82 and 0.84	Intra-day ASP- 0.7 RAM- 1.8 HCTZ-1.7 SIM-1.7 ATN- 1.3	**Intra-day & Intra-day** **ASP- 0.51 and 0.82** **RAM- 0.81 and 1.0** **HCTZ-0.85 and 0.55** **SIM-0.62 and 0.47** **ATN-0.33 and 0.54**

6.2.3 Conclusion:

A simple isocratic RP-HPLC method has been developed and validated as per ICH guidelines for the simultaneous quantitative analysis of ASP, RAM, HCTZ, SIM and ATN from capsule dosage form. The validation results reveal that, method is precise, linear, robust and accurate, which proves the reliability of the proposed method. The excipients present in the capsule dosage form do not interfere with the selectivity of the method. The mobile phase of methanol: water (95:5 v/v), at a flow rate of 1 mL/min was found to resolve peaks of ASP, RAM, HCTZ, SIM and ATN efficiently. Each analysis requires short run time of 8 min. as compared to previously reported longer run time and gradient elution methods. Hence, the proposed method can be conveniently adopted for the routine quality control analysis in the multicomponent formulation.

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Development of analytical methods for the simultaneous analysis of antihypertensive formulations containing two components by HPTLC

Introduction[1-7]:

High Performance Thin Layer Chromatography (HPTLC) is a powerful method equally suitable for qualitative and quantitative analytical tasks. Important features of HPTLC include:

1. Offline process wherein various stages are carried out independently,
2. Simultaneous processing of large number of samples along with a series of standards so as to offer better accuracy and precision of quantification,
3. Wide variety of solvents can be explored as mobile phases since they get completely evaporated prior to detection step,
4. Even corrosive and UV-absorbing mobile phases can be employed,
5. Requires no regeneration of chromatographic (sorbent) layer,
6. Possibility of sequential *in situ* spectra recording for positive identification of fractions,
7. Preservation of total sample on layer without time constrains.

Modern apparatus like video scanners, densitometers and new chromatographic chambers, more effective elution efficacy, high-resolution sorbents with selective particle size or chemically modified surface together make HPTLC an important alternative analytical technique, easy to combine with other analytical methods. Owing to the advantages of HPTLC it would be worthwhile to develop methods using HPTLC for the medicinally important antihypertensive combinations.

The recommendation for first-line therapy to treat hypertension remains either a beta blocker or a diuretic given in low dosage. The rationale for combining beta blockers with diuretics is two-fold: beta blockers blunt the increase in the plasma renin level that is induced by diuretics and diuretics decrease the sodium and water retention that is caused by beta blockers. Several beta blockers and diuretic combination therapies available to control, lower and/or maintain blood pressure include:

1. Atenolol and Indapamide

   Atenolol belongs to beta blocker group, a class of drugs used primarily in cardiovascular diseases such as hypertension. Indapamide is a diuretic generally used in the treatment of hypertension and edema caused by congestive heart failure. The

combination of atenolol and indapamide is used in the therapy of arterial hypertension.

2. Propranolol HCl and Hydrochlorothiazide

   Propranolol HCl and Hydrochlorothiazide are beta-blocker and diuretic, respectively. Their combination works by decreasing the force and slowing down the heartbeat, helping the heart beat more regularly thus, reducing the amount of work the heart has to perform. It also increases the elimination of excess fluid which helps in lowering the blood pressure.

3. Bisoprolol fumarate and Hydrochlorothiazide

   Bisoprolol is a beta-adrenergic receptor blocking agent used for treating high blood pressure and angina. Hydrochlorothiazide is a diuretic used for treating high blood pressure as well as accumulation of fluid. It works by blocking salt and fluid reabsorption in the kidneys thereby, causing increased urine output.

4. Nebivolol HCl and Hydrochlorothiazide

   Nebivolol HCl exerts its actions by exhibiting a high selectivity for beta-1 adrenergic receptors and by reducing the peripheral vascular resistance via modulation of nitrous oxide release. Hydrochlorothiazide inhibits the reabsorption of sodium and chloride at the beginning of distal convoluted tubule. It causes natriuretic effect mainly by decreasing sodium and chloride reabsorption via inhibition of a specific $Na^{+}Cl^{-}$ co-transporter.

Considering the above facts the following drug combinations were taken for the study:

Sr. No.	Combination	Brand name	Manufacturer	Label Claim
1	Atenolol and Indapamide	Aten-D Tablet	Zydus Cadila Health care Ltd.	Atenolol 5.0 mg Indapamide 2.5 mg
2	Propranolol HCl and Hydrochlorothiazide	Ciplar-H Tablet	Cipla Ltd	Propranolol HCl 40 mg Hydrochlorothiazide 25 mg
3	Bisoprolol fumarate and Hydrochlorothiazide	Lodoz 2.5	Merck India Ltd.	Bisoprolol fumarate 2.5mg Hydrochlorothiazide 6.25mg
4	Nebivolol HCl and Hydrochlorothiazide	Nebilong -H	Micro Labs Ltd. (Carsyon)	Nebivolol HCl 5.0 mg Hydrochlorothiazide 12.5 mg

Literature survey revealed that various methods have been reported for estimation of Atenolol, Indapamide, Propranolol HCl, Hydrochlorothiazide, Bisoprolol fumarate and Nebivolol HCl individually as well as in combination with other drugs. Few HPTLC methods have been reported for combination of Bisoprolol fumarate and Hydrochlorothiazide as well as Nebivolol HCl and Hydrochlorothiazide combination. Higher Rf values and linearity at higher concentration instigates the need for other reliable and rugged methods for the simultaneous analysis of drugs from above combinations. Further, there are no reported HPTLC methods for the simultaneous estimation of Atenolol and Indapamide and Propranolol HCl and Hydrochlorothiazide in bulk and in pharmaceutical dosage form. Hence it was decided to develop new HPTLC methods for these combinations.

7.1 Simultaneous analysis of Atenolol and Indapamide by HPTLC

Atenolol, 4-(2-hydroxy-3-[(1-methylethyl) amino] propoxy] benzene acetamide, is β 1 selective (cardioselective) β adrenergic receptor blocking agent whereas Indapamide, Benzamide,3-(aminosulfonyl)-4-chloro-*N*-(2,3-dihydro-2-methyl-1*H*-indol-1-yl)-4-chloro -*N*-(2-methyl-1-indolinyl)-3-sulfamoylbenzamide, is classed as a diuretic and antihypertensive agent.

The drug profiles and literature reviews of Indapamide (IND) and Atenolol (ATN) are given in 3.2 and 3.7.

7.1.1 Experimental:

- **Chemicals and reagents:**

  Reference standards of Atenolol (99.7%) and Indapamide (99.4%) were procured from Emcure Pharmaceuticals Ltd., Pune, India. The tablets Aten-D (Atenolol-50 mg and Indapamide- 2.5 mg) of Zydus Cadila health care Ltd. is procured from the market. Toluene, ethyl acetate, methanol and ammonia were of AR grade and were purchased from Merck Chemicals, Mumbai, India.

- **Preparation of standard stock solution-I:**

  50 mg of Atenolol (ATN) and 25 mg of Indapamide (IND) were accurately weighed and transferred to a 25 mL volumetric flask and dissolved in methanol. Volume was made up to the mark with methanol to get a **Standard stock solution I** containing ATN 2 mg/mL or 2000 µg/mL and IND 1 mg/mL or 1000 µg/mL, respectively.

- **Working standard solution-II**

  1 ml of the standard stock solution-I was diluted to 10 mL with methanol to get a working standard solution-II of ATN (0.2 mg/mL or 200 µg/mL) and IND (0.1 mg/mL or 100 µg/mL).

- **Chromatography:**

  The Camag TLC consisted of Linomat V sample applicator (Camag, Muttenz, Switzerland). Camag TLC scanner III controlled by WinCATS software (V 3.15, Camag) was used for sample application and quantitative evaluation. Chromatography was performed on Merck silica gel 60 F_{254} precoated aluminum TLC plate (10 cm × 10 cm with 250 µm thickness), with toluene: ethyl acetate:

methanol: ammonia in the ratio of 5:3:3:0.1 v/v/v/v as mobile phase. Samples were applied as bands 6 mm long at 6 mm interval under a stream of nitrogen with a Camag 100 microlitre syringe (Hamilton, Bonded, Switzerland). The slit dimension was 5 mm × 0.45 mm. Ascending development to a distance of 8 cm was performed in a 20 min. presaturated 10 cm × 10 cm twin trough TLC developing chamber (Camag). Densitometric scanning was performed in absorbance mode at wavelength of 229 nm.

- **Analytical Method Validation:**

  Validation was done as per ICH guidelines. The developed method was validated with respect to parameters such as linearity, LOD and LOQ, precision, accuracy and specificity.

- **Linearity:**

  Calibration solutions of ATN and IND were prepared by suitably diluting the standard stock solution-II 1-6 µL (200-1200 ng/spot) for ATN and (100-600 ng/spot) for IND was applied on TLC plate with the help of microliter syringe, using Linomat V sample applicator. Each concentration spotted three times on the plate. The plate was developed as per mentioned chromatographic conditions. After the development the plate was removed from the chamber and dried under a current of hot air. The UV response of the drug was monitored using the densitometer set at 229 nm. Data peak area of each band was recorded. The data of peak area versus the concentration of respective drug was plotted and drug concentration (ng/spot) against the area (AU) was found to be linear in the range of 200 - 1200 ng/spot for ATN and 100 - 600 ng/spot for IND.

- **Limit of detection and limit of quantitation:**

  LOD and LOQ were experimentally verified by spotting a series of dilute standard solutions of known concentrations of ATN and IND until the average responses were approximately 3 or 10 times the standard deviation of the responses for three replicate determinations.

- **Precision:**

  The precision of the method was determined by intra-day and inter-day studies. Intra-day variations were performed by analysis of three different concentrations (400, 800 and 1200 ng/spot for ATN and 200, 400, 600 ng/spot for IND) of the drugs

three times on the same day. The inter-day variations were similarly evaluated over a period of 3 days.

- **Repeatability of measurement:**

  1µL of the standard solution-II ATN (0.2 mg/mL or 200 µg/mL) and IND (0.1 mg/mL or 100 µg/mL) were spotted on a TLC plate, developed, dried and the spots were scanned seven times without changing the plate position and % RSD for measurement of peak area for both the drugs were calculated to determine the instrumental precision

- **Repeatability of sample application:**

  1µL of the standard solution-II ATN (0.2 mg/mL or 200 µg/mL) and IND (0.1 mg/mL or 100 µg/mL) were applied six times on a TLC plate, developed, dried and the spots were scanned and % RSD for measurement of peak area for both the drugs were calculated to determine the repeatability.

- **Robustness:**

  Robustness of the method was performed by making small deliberate changes in chromatographic conditions. Mobile phases having different compositions like toluene: ethyl acetate: methanol: ammonia (5.1: 3: 3: 0.1 v/v/v/v) and (5: 2.9: 2.9: 0.1 v/v/v/v) were tried and chromatograms were run. The amount of mobile phase was varied over the range of ± 1 %. The development distance was varied from 7.5 and 8.5 cm. Duration of saturation time of chamber was varied as 25 and 30 min. The time from spotting to chromatography and from chromatography to scanning was varied from +10 min. The robustness of the method was determined at three different concentration levels 400, 800 and 120 ng/spot and 200, 400 and 600 ng/spot for Atenolol and Indapamide, respectively.

- **Accuracy:**

  Accuracy was done in terms of recovery studies. Recovery studies were carried out by standard addition method. The analyzed samples were spiked with extra 80 %, 100 % and 120 % of standard drugs. The mixtures were reanalyzed by the proposed method. The experiment was conducted in triplicate.

- **Analysis of marketed formulation:**

  Twenty tablets of the pharmaceutical formulation Aten-D (containing 5 mg Atenolol and 2.5 mg Indapamide) were accurately weighed and average weight determined.

They were crushed to a fine powder and an amount of the powder equivalent to 5 mg ATN and 2.5 mg IND was weighed and transferred into 25 mL volumetric flask and dissolved in 15 mL of methanol, sonicated for 15 min. and diluted to mark with same solvent and filtered through whatman filter paper no.1. The sample solution was further diluted with methanol.

A fixed volume of 1µL of the standard solution-II and sample solutions were spotted as sharp bands on the chromatoplate. The chromatoplate was then developed in a twin trough chamber containing the mobile phase and developed bands of the drugs were scanned at 229 nm. The analysis was repeated in triplicate. The possibility of excipients interference with the analysis was examined.

- **Specificity:**

  The specificity of the method was ascertained by analyzing standard drugs and samples extracted from formulations. The spots for ATN and IND were confirmed by comparing the Rf of the spot with those of the standard.

7.1.2. Results and Discussions:

- **HPTLC method development:**

  HPTLC method was optimized with a view to develop a simultaneous determination for ATN and IND from tablet formulations. The mixed standard stock solution (200 ng/band of ATN and 100 ng/band of IND) were taken and 1µL samples were spotted on to TLC plates and run in different solvent systems. After many trials it was found that toluene: ethyl acetate: methanol: ammonia in the ratio of (5:3:3:0.1 v/v/v/v) gave a good resolution, sharp and symmetrical peaks with Rf of 0.27 ± 0.01 for ATN and 0.71 ± 0.02 for IND, respectively. The plate was scanned from 200-400 nm, a typical spectrum of ATN and IND are as shown in Fig. 7.01. Wavelength of 229 nm was selected for densitometric evaluation, since both the drugs show good absorption.

- **Validation:**

  Using optimised chromatographic conditions the HPTLC method developed was validated in terms of linearity, LOD and LOQ, precision, robustness and accuracy.

- **Specificity:**

  The method is specific for ATN and IND, since it resolved the peak of ATN (R_f =

0.27 ± 0.01) and IND (R_f = 0.71 ± 0.02) in presence of other excipients in the formulation (Fig. 7.02).

- **Linearity:**

  Linear regression data for the calibration plots revealed good linear relationships between response and concentration over the ranges 200– 1200 ng/spot for ATN and 100–600 ng/spot for IND. The linear regression equations were Y = 2.709X + 168.3 (r^2 = 0.9965) for ATN and Y = 9.163X + 47.15 (r^2 = 0.9999) for IND. The residuals plot shows a random pattern, indicating a good fit for a linear model. The calibration curves and residuals plots are given in Fig. 7.03 and Fig. 7.04 and Table 7.01 (A), and Table 7.01 (B) for ATN and IND, respectively.

- **Limits of Detection and Quantitation:**

  The results of the LOD and LOQ were found to be 35 ng/spot and 125 ng/spot for ATN and 25 ng/spot and 80 ng/spot for IND, respectively.

- **Precision:**

  The precision of the developed method was demonstrated by repeatability and intermediate precision studies. This was done by three replicate analysis of the composite sample. The precision of the method was expressed as relative standard deviation (RSD, %). The % RSD of intra-day and inter-day precision values was found to be 0.35 and 0.55% for ATN and 0.75 and 1.18 % for IND, respectively, indicating good precision (Table 7.02).

- **Instrumental precision:**

  The % RSD for 200 ng/spot of ATN and 100 ng/spot of IND (n=6) was found to be 0.425 and 0.735, respectively.

- **Repeatability of sample application:**

  1 µL of the standard solution II ATN (0.2 mg/mL or 200 µg/mL) and IND (0.1 mg/mL or 100 µg/mL) were applied seven times on a TLC plate, developed, dried and the spots were scanned. The mean % for peak area was found to be 0.87 for ATN and 1.03 for IND, respectively.

- **Robustness:**

  The robustness of the method was found out by testing the effect of small deliberate changes in the chromatographic conditions. The method was found to be robust enough that the peak area was not apparently affected by small variation in the

chromatographic conditions and there were no significant changes in the retention times of ATN and IND. The low values of the % RSD indicate the robustness of the method, as shown in Table 7.03.

- **Accuracy:**

  Accuracy of the method was evaluated by carrying out recovery study. A known concentration of the standard drug was added to a preanalysed tablet sample at three different levels namely 80 %, 100 % and 120 %. Each level was repeated three times. Total amount of the drug was determined by the proposed method. The percentage recovery was calculated. The mean % recoveries were 99.95 ± 0.18 for ATN and 99.67 ± 0.68 for IND, respectively (Table 7.04).

- **Analysis of marketed formulation:**

  Two spots at Rf of 0.27 (ATN) and 0.71 (IND) were observed in the chromatogram of the drug samples extracted from conventional tablets. There was no interference from the excipients commonly present in the conventional tablets. The % drug content found for ATN and IND were 99.95 % and 99.68%, respectively. This indicates that there is no degradation of ATN and IND in the marketed formulations that were analyzed by this method. The low % RSD value indicated the suitability of the method for simultaneous analysis of ATN and IND in pharmaceutical dosage forms (Table 7.05).

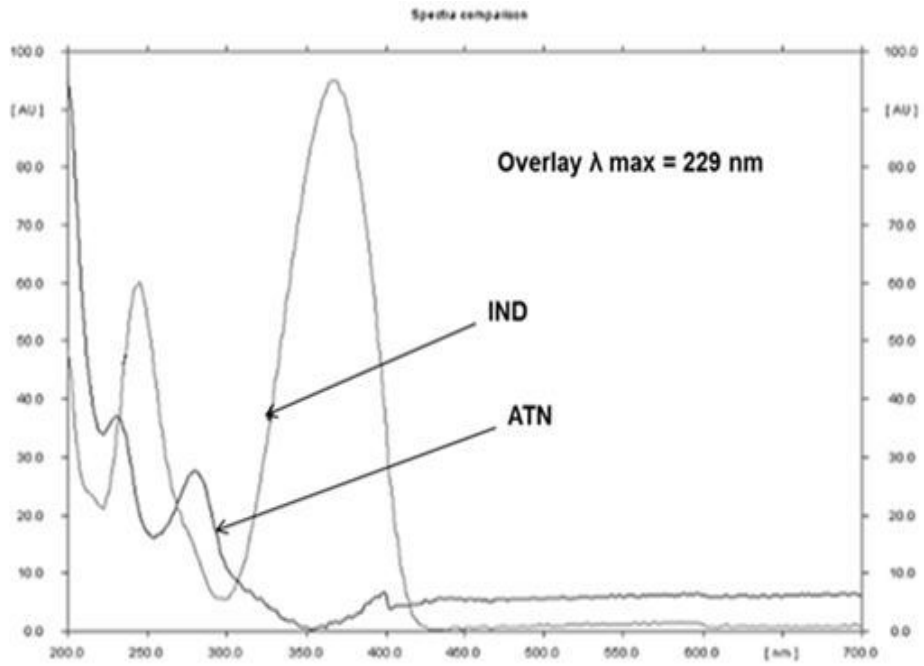

Fig. 7.01. *In situ* overlay absorption spectra of ATN and IND

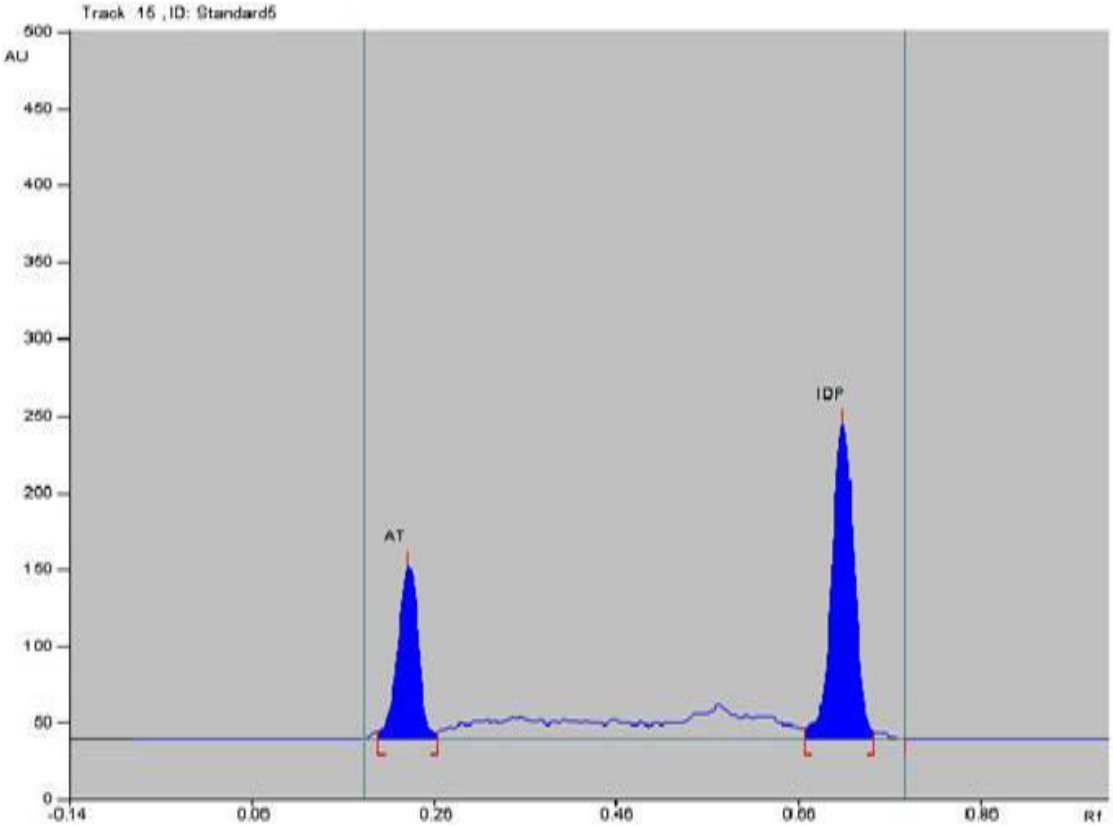

Fig.7.02. Typical densitogram of ATN and IND

Table 7.01 (A): Data for ATN from linearity study (n= 3)

Conc. (ng/spot)	Mean peak area ± SD	RSD (%)
200	782.66 ± 9.313	1.19
400	1179.95 ± 12.86	1.09
600	1727.50 ± 17.79	1.03
800	2392.60 ± 27.52	1.15
1000	2889.08 ± 35.25	1.22
1200	3417.12 ± 36.56	1.07
Regression coefficient r^2		**0.9965**
Slope		**2.709**
Intercept		**168.3**

Table 7.01 (B): Data for IND from linearity study (n= 3)

Conc. (ng/spot)	Mean peak area ± SD	RSD (%)
100	978.5 ± 10.50	1.07
200	1850.2 ± 20.91	1.13
300	2812.2 ± 33.18	1.18
400	3709.7 ± 42.29	1.14
500	4626.8 ± 47.19	1.02
600	5546.8 ± 67.12	1.21
Regression coefficient r^2		**0.9999**
Slope		**9.163**
Intercept		**47.15**

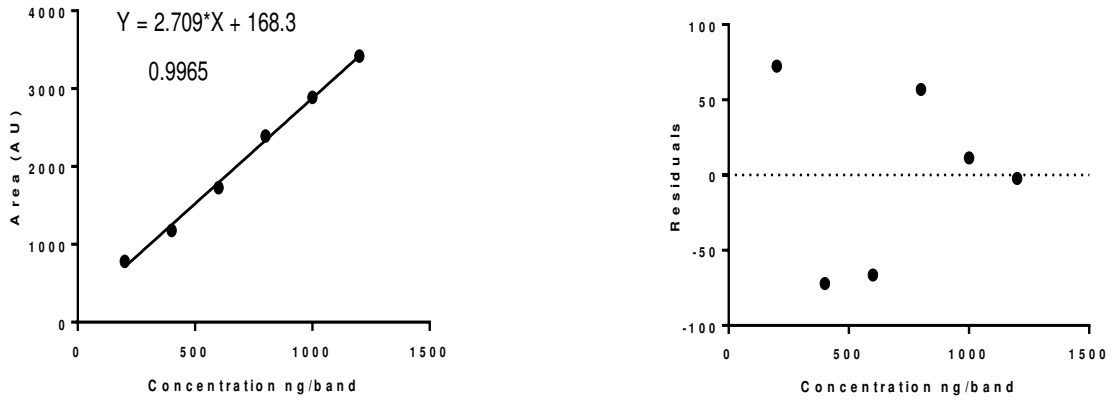

Fig.7.03. Calibration curve and residual plot for ATN

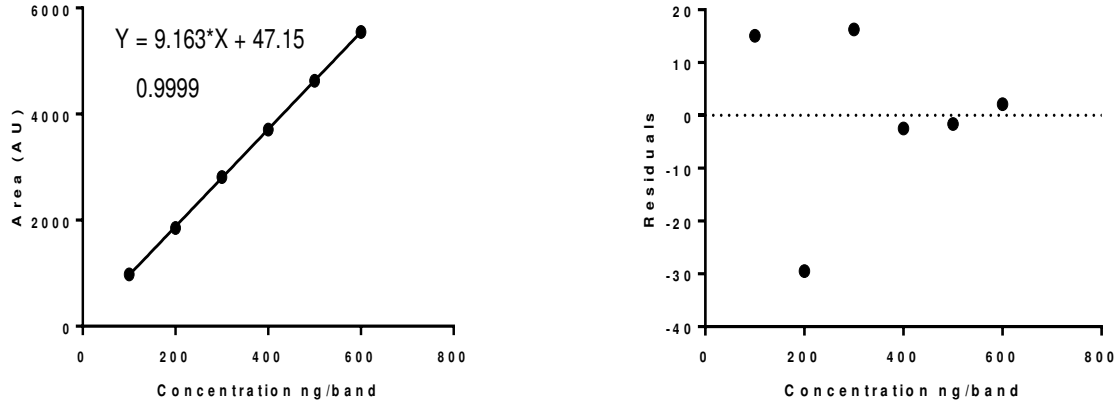

Fig.7.04. Calibration curve and residual plot for IND

Table 7.02: Precision studies of ATN and IND

Conc. (ng/spot)	Intra-day precision (n=3)			Inter-day precision (n=3)		
	Measured Conc. ± SD	(%) RSD	Recovery (%)	Measured Conc. ±SD	(%) RSD	Recovery (%)
Atenolol						
400	400.24 ± 3.00	0.75	100.06	398.72 ± 0.76	0.19	99.68
800	797.76 ± 1.36	0.17	99.72	796.48 ± 1.99	0.25	99.56
1200	1198.56 ± 1.56	0.13	99.88	1200.72 ± 14.65	1.22	100.06
Indapamide						
200	198.56 ± 1.27	0.64	99.28	199.34 ± 2.27	1.14	99.67
400	400.12 ± 3.56	0.89	100.03	399.6 ± 4.08	1.02	99.90
600	596.82 ± 4.24	0.71	99.47	599.88 ± 8.34	1.39	99.98

Table 7.03: Robustness evaluation of ATN and IND (n=3)

Parameter	ATN		IND	
	SD of the peak area	% RSD	SD of the peak area	% RSD
A: Mobile phase composition (± 0.1mL)				
Toluene: ethyl acetate: methanol: ammonia (5.1: 3: 3: 0.1 v/v/v/v)	22.22	1.36	21.42	0.80
Toluene: ethyl acetate: methanol: ammonia (5: 2.9: 2.9: 0.1 v/v/v/v)	12.42	0.75	20.23	1.47
B: Mobile phase volume (± 1mL)				
10. 1 mL	11.13	1.01	10.48	0.96
12. 1 mL	13.51	1.03	18.15	1.07
C: Development distance (± 0.5 cm)				
7.5 cm	11.28	1.28	19.45	1.30
8.5 cm	9.39	1.85	12.68	0.68
D: Duration of saturation (± 5min.)				
25 min	14.15	0.81	12.09	1.05
30 min	14.40	1.06	13.59	1.14
Time from application to chromatography (+10 min)	15.56	0.95	13.83	1.18
Time from chromatography to scanning (+10 min)	18.40	0.89	14.64	0.94

Table 7.04: Recovery study of ATN and IND by HPTLC (n=3)

Label claim (mg/tablet)	Amount Added (%)	Total amount (mg)	Amount recovered (mg)	Recovery (%)	Mean (%) Recovery (± SD)
ATN 50	80	90	89.793	99.77	99.95 ± 0.18
	100	100	100.13	100.13	
	120	110	109.94	99.95	
IND 2.5	80	4.5	4.473	99.40	99.67 ± 0.68
	100	5.0	4.9585	99.17	
	120	5.5	5.525	100.45	

Table 7.05: Assay results of ATN and IND in tablets (n= 3)

Aten-D tablet contains	Label claim (mg/tablet)	Amount found (mg ± SD)	RSD (%)	Recovery (%)
ATN	50 mg	49.975 ± 0.58	1.16	99.95
IND	2.5 mg	2.492 ± 0.027	1.10	99.68

7.1.3. Conclusion:

The proposed HPTLC method has been developed and validated as per ICH guidelines for the simultaneous determination of ATN and IND in pharmaceutical dosage form. The % RSD for all parameters was found to be less than 2, which indicates the validity of the method and assay results obtained by this method are in good agreement. The Statistical analysis proves that the method is repeatable and selective for the simultaneous determination of ATN and IND as bulk drug and in pharmaceutical dosage form. The results revealed no interference of excipients. The proposed method can be used for routine quantitative estimation of ATN and IND in Pharmaceutical preparation.

7.2 Simultaneous analysis of Propranolol HCl and Hydrochlorothiazide by HPTLC

Propranolol hydrochloride, (+)-1-(isopropylamino)-3-(1-naphthyloxy)-2-propanol hydrochloride, is a non-selective beta blocker mainly used in the treatment of hypertension. Hydrochlorothiazide chemically, 6-Chloro-3, 4-dihydro-2H-l, 2, 4-benzothiadiazine-7-sulfonamide 1, 1-dioxide, is a thiazide diuretic used as antihypertensive agent.

The drug profiles and literature reviews of Hydrochlorothiazide (HCTZ) and Propranolol hydrochloride (PHCl) are given in 3.5 and 3.11.

7.2.1 Experimental:

- **Chemicals and reagents:**

  Reference standards of Propranolol hydrochloride (100 %) and Hydrochlorothiazide (99.97%) were procured from Piramal Healthcare Ltd. Mumbai and Emcure Pharmaceuticals Ltd., Pune, India. The tablets Ciplar-H (Propranolol HCl-40 mg and Hydrochlorothiazide- 25 mg) of Cipla Ltd. is procured from the market. Chloroform, ethyl acetate and methanol were of AR grade and were purchased from Merck Chemicals, Mumbai, India.

- **Preparation of standard stock solution-I:**

  40 mg of Propranolol hydrochloride (PHCl) and 25 mg of Hydrochlorothiazide (HCTZ) were accurately weighed and transferred to a 25 mL volumetric flask and dissolved in methanol. Volume was made up to the mark with methanol to get a **Standard stock solution I** containing PHCl 1.6 mg/mL or 1600 µg/mL and HCTZ 1 mg/mL or 1000 µg/mL, respectively.

- **Working standard solution-II:**

  1 ml of the standard stock solution-I was diluted to 10 mL with methanol to get a working standard solution-II of PHCl (0.16 mg/mL or 160 µg/mL) and HCTZ (0.1 mg/mL or 100 µg/mL).

- **Chromatography:**

  The Camag TLC consisted of Linomat V sample applicator (Camag, Muttenz, Switzerland). Camag TLC scanner III controlled by WinCATS software (V 3.15, Camag) was used for sample application and quantitative evaluation.

Chromatography was performed on Merck silica gel 60 F_{254} precoated aluminum TLC plate (10 cm × 10 cm with 250 µm thickness), with chloroform: ethyl acetate: methanol in the ratio of 4: 4: 2 v/v/v as mobile phase. Samples were applied as bands 6 mm long at 6 mm interval under a stream of nitrogen with a Camag 100 microlitre syringe (Hamilton, Bonded, Switzerland). The slit dimension was 5 mm × 0.45 mm. Ascending development to a distance of 8 cm was performed in a 20 min. presaturated 10 cm × 10 cm twin trough TLC developing chamber (Camag). Densitometric scanning was performed in absorbance mode at wavelength of 274nm.

- **Analytical Method Validation:**

  Validation was done as per ICH guidelines. The developed method was validated with respect to parameters such as linearity, LOD and LOQ, precision, accuracy and specificity.

- **Linearity:**

  Standard solution-II 1-6 µL (160-960 ng/spot) for PHCl and (100-600 ng/spot) for HCTZ was applied on TLC plate with the help of microliter syringe, using Linomat V sample applicator. Each concentration spotted three times on the plate. The plate was developed as per mentioned chromatographic conditions. After the development the plate was removed from the chamber and dried under a current of hot air. The UV response of the drug was monitored using the densitometer set at 274 nm. Peak area was recorded for the each concentration level of PHCl and HCTZ. The data of peak area versus the concentration of respective drug was plotted and drug concentration (ng/spot) against the area (AU) was found to be linear in the range of 160 - 960 ng/spot for PHCl and 100 - 600 ng/spot for HCTZ.

- **Limit of detection and limit of quantitation:**

  LOD and LOQ were experimentally verified by spotting a series of dilute standard solutions of known concentrations of PHCl and HCTZ until the average responses were approximately 3 or 10 times the standard deviation of the responses for three replicate determinations.

- **Precision:**

  The precision of the method was assesses by intra-day and inter-day studies. Intra-day variations were performed by analysis of three different concentrations (320, 640 and 960 ng/spot for PHCl and 200, 400 and 600 ng/spot for HCTZ) of the drugs

three times on the same day. The inter-day variations were similarly evaluated over a period of 3 days.

- **Repeatability of measurement:**

  1 µL of the standard solution-II PHCl (0.16 mg/mL or 160 µg/mL) and HCTZ (0.1 mg/mL or 100 µg/mL) were spotted on a TLC plate, developed, dried and the spots were scanned seven times without changing the plate position and % RSD for measurement of peak area for both the drugs were calculated to determine the instrumental precision

- **Repeatability of sample application:**

  1 µL of the standard solution-II PHCl (0.16 mg/mL or 160 µg/mL) and HCTZ (0.1 mg/mL or 100 µg/mL) were applied six times on a TLC plate, developed, dried and the spots were scanned and % RSD for measurement of peak area for both the drugs were calculated to determine the repeatability.

- **Robustness:**

  Robustness of the method was performed by making small deliberate changes in chromatographic conditions. Following the introduction of small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different compositions like chloroform: ethyl acetate: methanol (4.1: 4.1: 2 v/v/v) and (3.9: 3.9: 2 v/v/v) were tried and chromatograms were run. The amount of mobile phase was varied over the range of ± 1 %. The development distance was varied from 7.5 and 8.5 cm. Duration of saturation time of chamber was varied as 25 and 30 min. The time from spotting to chromatography and from chromatography to scanning was varied from +10 min. The robustness of the method was determined at three different concentration levels 320, 640 and 960 ng/spot and 200, 400 and 600 ng/spot for PHCl and HCTZ, respectively.

- **Accuracy:**

  Accuracy was done in terms of recovery studies. Recovery studies were carried out by standard addition method. The analyzed samples were spiked with extra 80 %, 100 % and 120 % of standard drugs. The mixtures were reanalyzed by the proposed method. The experiment was conducted in triplicate.

- **Analysis of marketed formulation:**

  Twenty tablets of the pharmaceutical formulation Ciplar-H (containing 40 mg Propranolol HCl and 25 mg Hydrochlorothiazide) were accurately weighed and average weight determined. They were crushed to a fine powder and an amount of the powder equivalent to 40 mg PHCl and 25 mg HCTZ was weighed and transferred into 25 mL volumetric flask and dissolved in 15 mL of methanol, sonicated for 15 min. and diluted to mark with same solvent and filtered through whatman filter paper no.1. The sample solution was further diluted with methanol.

  A fixed volume of 1µl of the standard solution-II and sample solutions were spotted as sharp bands on the chromatoplate. The chromatoplate was then developed in a twin trough chamber containing the mobile phase and developed bands of the drugs were scanned at 274 nm. The analysis was repeated in triplicate. The possibility of excipients interference with the analysis was examined.

- **Specificity:**

  The specificity of the method was ascertained by analyzing standard drugs and samples extracted from formulations. The spots for PHCl and HCTZ were confirmed by comparing the Rf of the spot with those of the standard.

7.2.2. Results and Discussions:

- **HPTLC method development:**

  HPTLC method was optimized with a view to develop a simultaneous determination for PHCl and HCTZ from tablet formulations. The mixed standard stock solution (160 ng/band of PHCl and 100 ng/band of HCTZ) and were taken and 1µL samples were spotted on to TLC plates and run in different solvent systems. After many trials it was found that chloroform: ethyl acetate: methanol in the ratio of 4: 4: 2 (v/v) gave a good resolution, sharp and symmetrical peaks with Rf of 0.27 ± 0.02 for PHCl and 0.56 ± 0.02 for HCTZ, respectively. The plate was scanned from 200-400 nm, a typical spectrum of Propranolol HCl and Hydrochlorothiazide are as shown in Fig. 7.05. Wavelength of 274 nm was selected for densitometric evaluation, since both the drugs show good absorption.

- **Validation:**

  Using optimised chromatographic conditions the HPTLC method developed was validated in terms of linearity, LOD and LOQ, precision, robustness and accuracy.

- **Specificity:**

  The method is specific for PHCl and HCTZ, since it resolved the peak of PHCl (R_f = 0.27 ± 0.02) and HCTZ (R_f = 0.56 ± 0.02) in presence of other excipients in the formulation (Fig. 7.06).

- **Linearity:**

  Linear regression data for the calibration plots revealed good linear relationships between response and concentration over the ranges 160– 960 ng/spot for PHCl and 100–600 ng/spot for HCTZ. The linear regression equations were Y = 7.252X + 2805 (r^2 = 0.9998) for PHCl and Y = 12.38X + 1628 (r^2 = 0.9998) for HCTZ. The residuals plot shows a random pattern, indicating a good fit for a linear model. The calibration curves and residuals plots are given in Fig. 7.07 and Fig. 7.08 and Table 7.06 (A), and Table 7.06 (B) for PHCl and HCTZ, respectively.

- **Limits of Detection and Quantitation:**

  The results of the LOD and LOQ were found to be 80 ng/spot and 135 ng/spot for PHCl and 40 ng/spot and 60 ng/spot for HCTZ, respectively.

- **Precision:**

  The precision of the developed method was demonstrated by repeatability and intermediate precision studies. This was done by three replicate analysis of the composite sample. The precision of the method was expressed as relative standard deviation (RSD, %). The % RSD of intra-day and inter-day precision values was found to be 1.107 and 1.12% for PHCl and 1.167 and 1.107 % for HCTZ, respectively, indicating good precision (Table 7.07).

- **Instrumental precision:**

  The % RSD for 160 ng/spot of PHCl and 100 ng/spot of HCTZ (n=6) was found to be 0.941 and 0.872, respectively.

- **Repeatability of sample application:**

  1.0 µL of the standard solution-II PHCl (0.16 mg/mL or 160 µg/mL) and HCTZ (0.1 mg/mL or 100 µg/mL) were applied seven times on a TLC plate, developed, dried

and the spots were scanned. The mean % for peak area was found to be 0.641 for PHCl and 0.982 for HCTZ, respectively.

- **Robustness:**

  The robustness of the method was found out by testing the effect of small deliberate changes in the chromatographic conditions. The method was found to be robust enough that the peak area was not apparently affected by small variation in the chromatographic conditions and there were no significant changes in the retention times of PHCl and HCTZ. The low values of the % RSD indicate the robustness of the method, as shown in Table 7.08.

- **Accuracy:**

  Accuracy of the method was evaluated by carrying out recovery study. A known concentration of the standard drug was added to a preanalysed tablet sample at three different levels namely 80 %, 100 % and 120 %. Each level was repeated three times. Total amount of the drug was determined by the proposed method. The percentage recovery was calculated. The mean % recoveries were 99.63 ± 0.127 for PHCl and 99.15 ± 0.116 for HCTZ, respectively (Table 7.09).

- **Analysis of marketed formulation:**

  Two spots at Rf of 0.27 PHCl and 0.56 HCTZ were observed in the chromatogram of the drug samples extracted from conventional tablets. There was no interference from the excipients commonly present in the conventional tablets. The % drug content found for PHCl and HCTZ were 99.55 % and 99.60%, respectively. This indicates that there is no degradation of PHCl and HCTZ in the marketed formulations that were analyzed by this method. The low % RSD value indicated the suitability of the method for simultaneous analysis of PHCl and HCTZ in pharmaceutical dosage forms (Table 7.10).

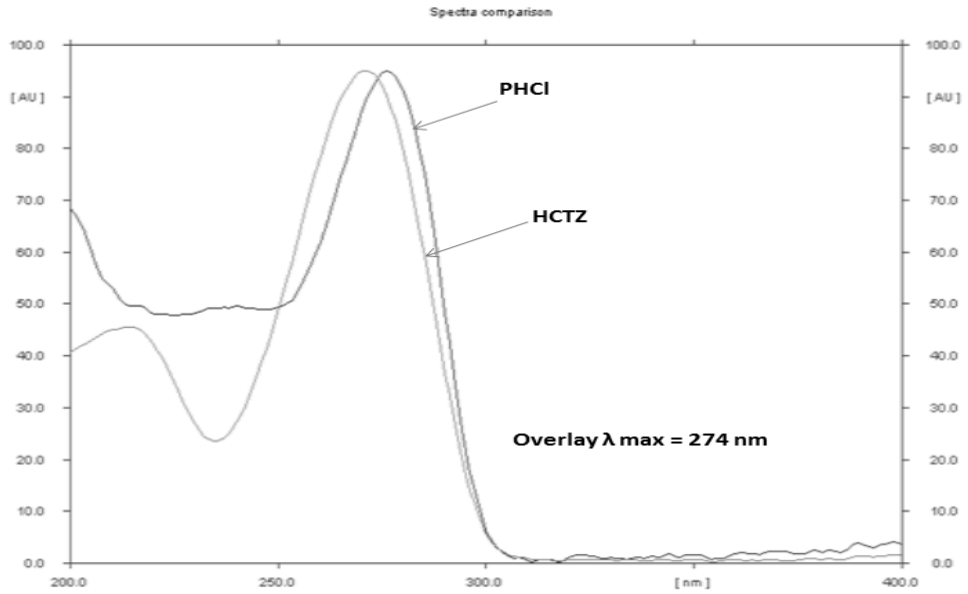

Fig.7.05. *In situ* overlay absorption spectra of PHCl and HCTZ

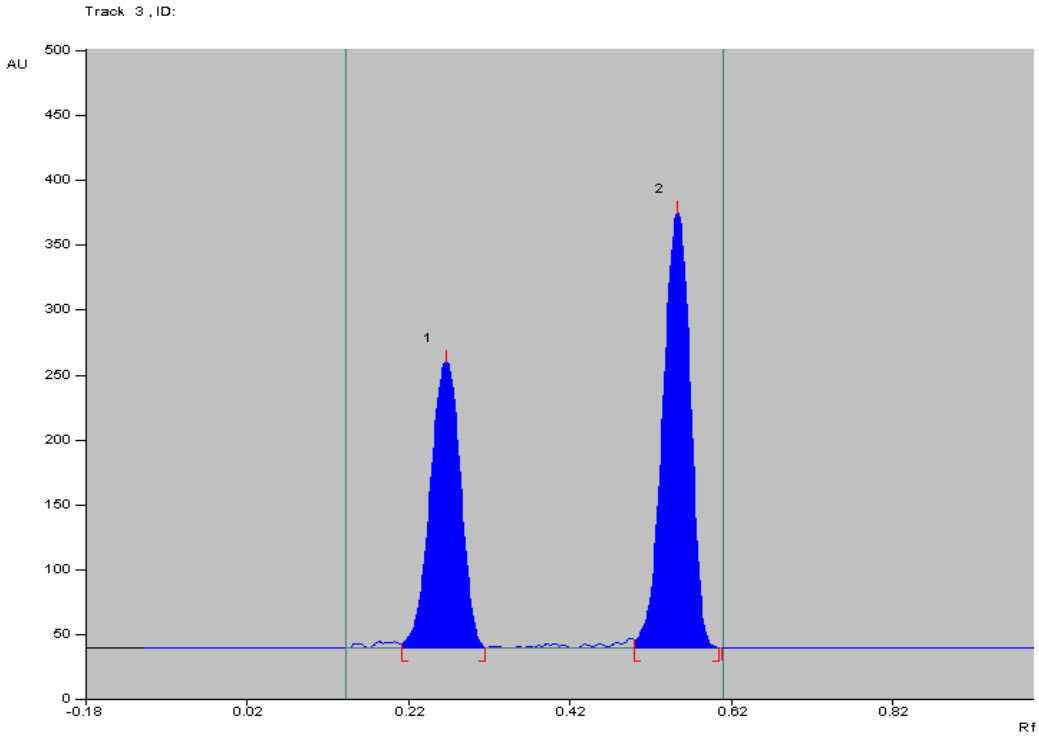

Fig.7.06. Typical densitogram of PHCl (peak 1) and HCTZ (peak 2)

Table 7.06 (A): Data for PHCl from linearity study (n= 3)

Conc. (ng/spot)	Mean peak area ± SD	RSD (%)
160	3946.4 ± 43.41	1.10
320	5105.2 ± 51.56	1.01
480	6346.5 ± 71.72	1.13
640	7455.9 ± 87.23	1.17
800	8579.4 ± 87.51	1.02
960	9762.4 ± 114.22	1.17
Regression coefficient r^2		**0.9998**
Slope		**7.252**
Intercept		**2805**

Table 7.06 (B): Data for HCTZ from linearity study (n= 3)

Conc. (ng/spot)	Mean peak area ± SD	RSD (%)
100	2829.1 ± 32.25	1.14
200	4114.1 ± 44.43	1.08
300	5397.8 ± 59.92	1.11
400	6574.2 ± 72.32	1.10
500	7810.5 ± 96.70	1.23
600	9043.3 ± 91.34	1.01
Regression coefficient r^2		**0.9998**
Slope		**12.38**
Intercept		**1628**

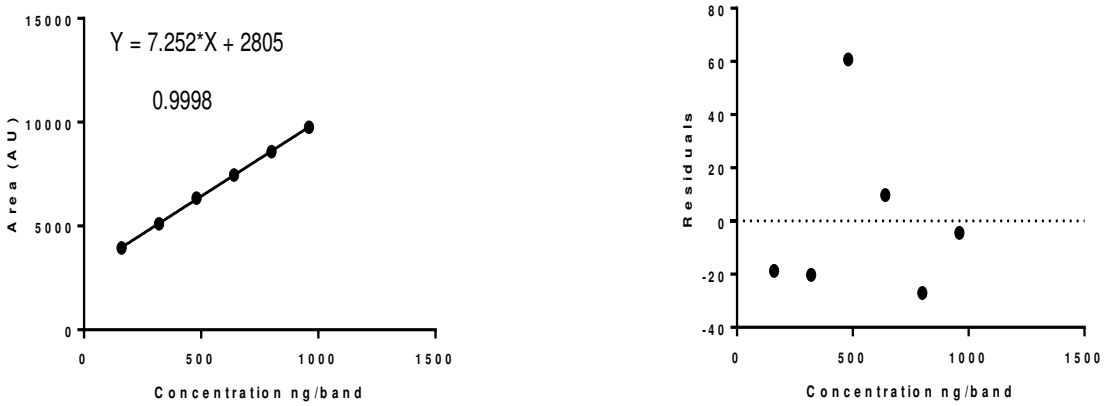

Fig.7.07. Calibration curve and residual plot for PHCl

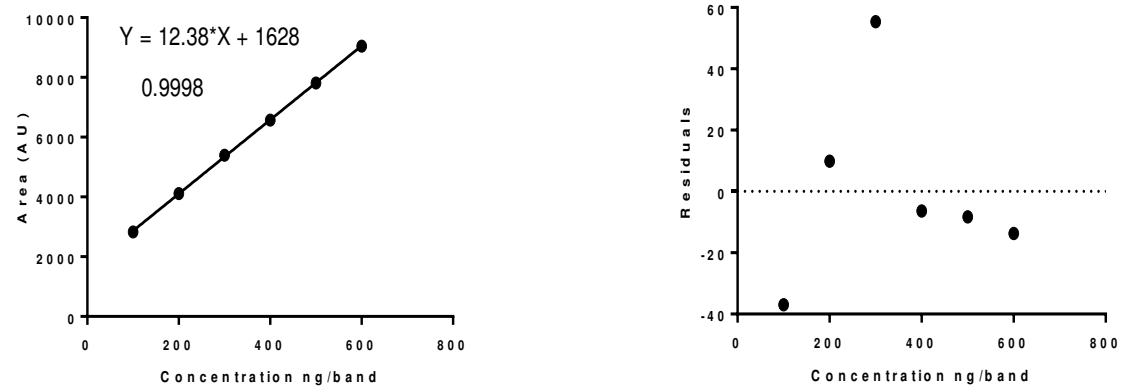

Fig. 7.08. Calibration curve and residual plot for HCTZ

Table 7.07: Precision studies of PHCl and HCTZ

Conc. (ng/spot)	Intra-day precision (n=3)			Inter-day precision (n=3)		
	Measured Conc. ± SD	(%) RSD	Recovery (%)	Measured Conc. ±SD	(%) RSD	Recovery (%)
Propranolol hydrochloride						
320	318.56 ± 3.50	1.10	99.55	318.34 ± 3.565	1.12	99.48
640	638.10 ± 7.00	1.09	99.70	638.01 ± 6.70	1.05	99.69
960	959.02 ± 10.84	1.13	99.90	958.88 ± 11.41	1.19	99.88
Hydrochlorothiazide						
200	198.24 ± 2.24	1.13	99.12	198.72 ± 2.34	1.18	99.36
400	398.76 ± 4.90	1.23	99.69	396.48 ± 4.36	1.10	99.12
600	598.56 ± 6.82	1.14	99.76	597.72 ± 6.22	1.04	99.62

Table 7.08: Robustness evaluation of PHCl and HCTZ (n=3)

Parameter	PHCl		HCTZ	
	SD of the peak area	% RSD	SD of the peak area	% RSD
A: Mobile phase composition (± 0.1 mL)				
Chloroform: ethyl acetate: methanol (4.1: 4.1: 2 v/v/v)	15.033	1.16	10.02	1.05
Chloroform: ethyl acetate: methanol (3.9: 3.9: 2 v/v/v)	20.82	1.25	15.20	1.32
B: Mobile phase volume (± 1 mL)				
11. 0 mL	7.12	1.01	10.69	0.96
12. 0 mL	10.11	1.03	13.18	1.07
C: Development distance (± 0.5 cm)				
7.5 cm	9.28	1.24	10.13	1.10
8.5 cm	11.39	1.04	10.66	1.18
D: Duration of saturation (± 5 min.)				
25 min	10.21	1.26	11.09	1.15
30 min	12.08	1.16	13.98	1.18
Time from application to chromatography (+10 min)	10.69	1.14	12.83	1.13
Time from chromatography to scanning (+10 min)	13.10	0.96	11.64	1.02

Table 7.09: Recovery study of PHCL and HCTZ (n=3)

Label claim (mg/tablet)	Amount Added (%)	Total amount (mg)	Amount recovered (mg)	Recovery (%)	Mean (%) Recovery (± SD)
PHCl 40	80	72	71.63	99.49	99.63 ± 0.127
	100	80	79.79	99.74	
	120	88	87.69	99.65	
HCTZ 25	80	45	44.58	99.07	99.15 ± 0.116
	100	50	49.64	99.28	
	120	55	54.50	99.09	

Table 7.10: Assay results of PHCl and HCTZ in tablets (n= 3)

Ciplar-H tablet contains	Label claim (mg/tablet)	Amount found (mg ± SD)	RSD (%)	Recovery (%)
PHCl	40 mg	39.82 ± 0.44	1.10	99.55
HCTZ	25 mg	24.90 ± 0.28	1.14	99.60

7.2.3. Conclusion:

In the present study a HPTLC method has been developed for simultaneous estimation of Propranolol HCl and hydrochlorothiazide in bulk and its formulation. The proposed method was sufficiently sensitive and reproducible for the analysis of the PHCl and HCTZ tablet dosage form. The additives usually present in the pharmaceutical formulations of the assayed samples did not interfere with the determination of PHCl and HCTZ. The method is simple, precise, specific, and accurate and can be used for the routine simultaneous analysis of the PHCl and HCTZ in pharmaceutical preparations.

7.3 Simultaneous analysis of Bisoprolol Fumarate and Hydrochlorothiazide by HPTLC

Bisoprolol fumarate, (±)-1-[4-[[2-(1-Methylethoxy) ethoxy] methyl] phenoxy]-3[(1-methylethyl) amino]-2-propanol (E)-2-butenedioate (2:1) (salt), is a synthetic, beta1-selective (cardioselective) adrenoceptor blocking agent. Hydrochlorothiazide chemically, 6-Chloro-3, 4-dihydro-2H-l, 2, 4-benzothiadiazine-7-sulfonamide 1, 1-dioxide, a thiazide diuretic used as antihypertensive agent.

The drug profiles and literature reviews of Hydrochlorothiazide (HCTZ) and Bisoprolol fumarate (BSF) are given in 3.5 and 3.12.

7.3.1 Experimental:

- **Chemicals and reagents:**

  Reference standards of Bisoprolol fumarate (100 %) and Hydrochlorothiazide (99.97%) were procured from Unichem Laboratories Ltd, Mumbai and Emcure Pharmaceuticals Ltd., Pune, India. The tablets Lodoz 2.5 (Bisoprolol fumararte-2.5 mg and Hydrochlorothiazide- 6.25 mg) of Merck Ltd. is procured from the market. Ethyl acetate, methanol and ammonia were of AR grade and were purchased from Merck Chemicals, Mumbai, India.

- **Preparation of standard stock solution-I:**

  150 mg of Bisoprolol fumarate (BSF) and 100 mg of Hydrochlorothiazide (HCTZ) were accurately weighed and transferred to a 100 mL volumetric flask and dissolved in methanol. Volume was made up to the mark with methanol to get a **Standard stock solution-I** containing BSF 1.5 mg/mL or 1500 µg/mL and HCTZ 1 mg/mL or 1000 µg/mL, respectively.

- **Working standard solution-II**

  1 mL of the standard stock solution-I was diluted to 10 mL with methanol to get a working standard solution-II of BSF (0.15mg/mL or 150 µg/mL) and HCTZ (0.1 mg/mL or 100 µg/mL).

- **Chromatography:**

  The Camag TLC consisted of Linomat V sample applicator (Camag, Muttenz, Switzerland). Camag TLC scanner III controlled by WinCATS software (V 3.15,

Camag) was used for sample application and quantitative evaluation. Chromatography was performed on Merck silica gel 60 F_{254} precoated aluminum TLC plate (10 cm × 10 cm with 250 µm thickness), with Ethyl acetate: methanol: ammonia 10:0.5:0.5 (v/v/v) as mobile phase. Samples were applied as bands 6 mm long at 6 mm interval under a stream of nitrogen with a Camag 100 microlitre syringe (Hamilton, Bonded, Switzerland). The slit dimension was 5 mm × 0.45 mm. Ascending development to a distance of 8 cm was performed in a 20 min. presaturated 10 cm × 10 cm twin trough TLC developing chamber (Camag). Densitometric scanning was performed in absorbance mode at wavelength of 225 nm.

- **Analytical Method Validation**

  Validation was done as per ICH guidelines. The developed method was validated with respect to parameters such as linearity, LOD and LOQ, precision, accuracy and specificity.

- **Linearity:**

  Standard standard solution-II 1-6 µL (150-900 ng/spot) for BSF and (100-600 ng/spot) for HCTZ was applied on TLC plate with the help of microliter syringe, using Linomat V sample applicator. Each concentration was spotted three times on the plate. The plate was developed as per mentioned chromatographic conditions. After the development the plate was removed from the chamber and dried under a current of hot air. The UV response of the drug was monitored using the densitometer set at 225 nm. Peak area was recorded for the each concentration level of BSF and HCTZ. The data of peak area versus the concentration of respective drug was plotted and drug concentration (ng/spot) against the area (AU) was found to be linear in the range of 150-900 ng/spot for BSF and 100-600 ng/spot for HCTZ.

- **Limit of detection and limit of quantitation:**

  LOD and LOQ were experimentally verified by spotting a series of dilute standard solutions of known concentrations of BSF and HCTZ until the average responses were approximately 3 or 10 times the standard deviation of the responses for three replicate determinations.

- **Precision:**

  The precision of the method was assessed by intra-day and inter-day studies. Intra-day variations were performed by analysis of three different concentrations (300, 600 and 900 ng/spot for BSF and 200, 400 and 600 ng/spot for HCTZ) of the drugs three times on the same day. The inter-day variations were similarly evaluated over a period of 3 days.

- **Repeatability of measurement:**

  1µL of the standard solution-II BSF (0.15 mg/mL or 150 µg/mL) and HCTZ (0.1 mg/mL or 100 µg/mL) were spotted on a TLC plate, developed, dried and the spots were scanned seven times without changing the plate position and % RSD for measurement of peak area for both the drugs were calculated to determine the instrumental precision

- **Repeatability of sample application:**

  1µL of the standard solution II BSF (0.15 mg/mL or 150 µg/mL) and HCTZ (0.1 mg/mL or 100 µg/mL) were applied six times on a TLC plate, developed, dried and the spots were scanned and % RSD for measurement of peak area for both the drugs were calculated to determine the repeatability.

- **Robustness:**

  Robustness of the method was performed by making small deliberate changes in chromatographic conditions. Following the introduction of small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different compositions like ethyl acetate: methanol: ammonia (10: 1: 0.5 v/v/v) and (9.9: 0.5: 0.5 v/v/v) were tried and chromatograms were run. The amount of mobile phase was varied over the range of ± 1 mL. The development distance was varied from 7.5 and 8.5 cm. Duration of saturation time of chamber was varied as 25 and 30 min. The time from spotting to chromatography and from chromatography to scanning was varied from +10 min. The robustness of the method was determined at three different concentration levels 300, 600 and 900 ng/spot and 200, 400 and 600 ng/spot for BSF and HCTZ, respectively.

- **Accuracy:**

  Accuracy was done in terms of recovery studies. Recovery studies were carried out by standard addition method. The analysed samples were spiked with extra 80 %,

100 % and 120 % of standard drugs. The mixtures were reanalysed by the proposed method. The experiment was conducted in triplicate.

- **Analysis of marketed formulation:**

  Twenty tablets of the pharmaceutical formulation Lodoz 2.5 (containing 2.5 mg Bisoprolol fumarate and 6.25 mg Hydrochlorothiazide) were accurately weighed and average weight determined. They were crushed to a fine powder and an amount of the powder equivalent to 2.5 mg BSF and 6.25 mg HCTZ was weighed and transferred into 25 mL volumetric flask and dissolved in 15 mL of methanol, sonicated for 15 min. and diluted to mark with same solvent and filtered through whatman filter paper no.1. The sample solution was further diluted with methanol.

  A fixed volume of 1µL of the standard solution-II and sample solutions were spotted as sharp bands on the chromatoplate. The chromatoplate was then developed in a twin trough chamber containing the mobile phase and developed bands of the drugs were scanned at 225 nm. The analysis was repeated in triplicate. The possibility of excipients interference with the analysis was examined.

- **Specificity:**

  The specificity of the method was ascertained by analyzing standard drugs and samples extracted from formulations. The spots for BSF and HCTZ were confirmed by comparing the Rf of the spot with those of the standard.

- **Comparison of the proposed method with the reported method:**

  The proposed method was compared with the reported method from the literature:

  Development and Validation of TLC-Densitometry Method for Simultaneous Estimation of Bisoprolol Fumarate and Hydrochlorothiazide in Bulk and Tablets[8]

  Stationary Phase: Precoated silica gel aluminum plate 60 F 254

  Mobile phase: Chloroform: ethanol: glacial acetic acid (5:1.5:0.2 v/v/v)

  Detector wavelength: 225 nm

7.3.2. Results and Discussions:

- **HPTLC method development:**

  HPTLC method was optimized with a view to develop a simultaneous determination for BSF and HCTZ from tablet formulation. The mixed standard stock solution (150 ng/spot of BSF and 100 ng/spot of HCTZ) and were taken and 1µL samples were spotted on to TLC plates and run in different solvent systems. After several trials it was found that ethyl acetate: methanol: ammonia 10:0.5:0.5 (v/v/v) gave a good resolution, sharp and symmetrical peaks with Rf of 0.60 ± 0.03 for BSF and 0.38 ± 0.03 for HCTZ, respectively. The plate was scanned from 200-400 nm, a typical spectrum of BSF and HCTZ are as shown in Fig. 7.09. Wavelength of 225 nm was selected for densitometric evaluation, since both the drugs show good absorption.

- **Validation:**

  Using optimised chromatographic conditions the HPTLC method developed was validated in terms of linearity, LOD and LOQ, precision, robustness and accuracy.

- **Specificity:**

  The method is specific for BSF and HCTZ, since it resolved the peak of BSF (R_f = 0.60 ± 0.03) and HCTZ (R_f = 0.38 ± 0.03) in presence of other excipients in the formulation (Fig. 7.10).

- **Linearity:**

  Linear regression data for the calibration plots revealed good linear relationships between response and concentration over the ranges 150– 960 ng/spot for BSF and 100–600 ng/spot for HCTZ. The linear regression equations were Y = 3.611X - 236.6 (r2 = 0.9992) for BSF and Y = 3.215X + 326 (r2 = 0.9991) for HCTZ. The residuals plot shows a random pattern, indicating a good fit for a linear model. The calibration curve and residuals plots are given in Fig. 7.11 and Fig. 7.12 and Table 7.11 (A), and Table 7.11 (B) for BSF and HCTZ, respectively.

- **Limits of Detection and Quantitation:**

  The results of the LOD and LOQ were found to be 50 ng/spot and 100 ng/spot for BSF and 25 ng/spot and 50 ng/spot for HCTZ, respectively.

- **Precision:**

  The precision of the developed method was demonstrated by intra-day precision and

Inter-day precision studies. This was done by three replicate analysis of the composite sample. The precision of the method was expressed as relative standard deviation (RSD, %). The % RSD of intra-day and inter-day precision values were found to be 0.76 and 0.483% for BSF and 0.513 and 1.037 % for HCTZ, respectively, indicating good precision (Table 7.12).

- **Instrumental precision:**

  The % RSD for 150 ng/spot of BSF and 100 ng/spot of HCTZ (n=6) was found to be 0.841 and 0.560, respectively.

- **Repeatability of sample application:**

  1 µL of the standard solution-II BSF (0.15 mg/mL or 150 µg/mL) and HCTZ (0.1 mg/mL or 100 µg/mL) were applied seven times on a TLC plate, developed, dried and the spots were scanned. The mean % RSD for peak area was found to be 0.721 for BSF and 0.657 for HCTZ, respectively.

- **Robustness:**

  The robustness of the method was found out by testing the effect of small deliberate changes in the chromatographic conditions. The method was found to be robust enough that the peak area was not apparently affected by small variation in the chromatographic conditions and there were no significant changes in the retention times of BSF and HCTZ. The low values of the % RSD indicate the robustness of the method, as shown in Table 7.13.

- **Accuracy:**

  Accuracy of the method was evaluated by carrying out recovery study. A known concentration of the standard drug was added to a preanalysed tablet sample at three different levels namely 80 %, 100 % and 120 %. Each level was repeated three times. Total amount of the drug was determined by the proposed method. The percentage recovery was calculated. The mean % recoveries were 99.33 ± 0.125 for BSF and 99.60 ± 0.04 for HCTZ, respectively (Table 7.14).

- **Analysis of marketed formulation:**

  Two spots at Rf of 0.60 BSF and 0.38 HCTZ were observed in the chromatogram of the drug samples extracted from conventional tablets. There was no interference from the excipients commonly present in the conventional tablets. The % drug content found for BSF and HCTZ were 99.45 % and 100.25%, respectively. This

indicates that there is no degradation of BSF and HCTZ in the marketed formulations that were analysed by this method. The low % RSD value indicated the suitability of the method for simultaneous analysis of BSF and HCTZ in pharmaceutical dosage forms (Table 7.15).

- **Comparison of the proposed method with the reported method:**

  The proposed method was compared with the reported method. Linearity concentration range of the proposed method is 150 – 900 ng/spot for BSF and 100-600 ng/spot for HCTZ as against 200-1200 ng/spot for BSF and 100-800 ng/spot for HCTZ of the reported method. Accuracy of the proposed method is comparable to the reported method with low % RSD. (Table 7.16)

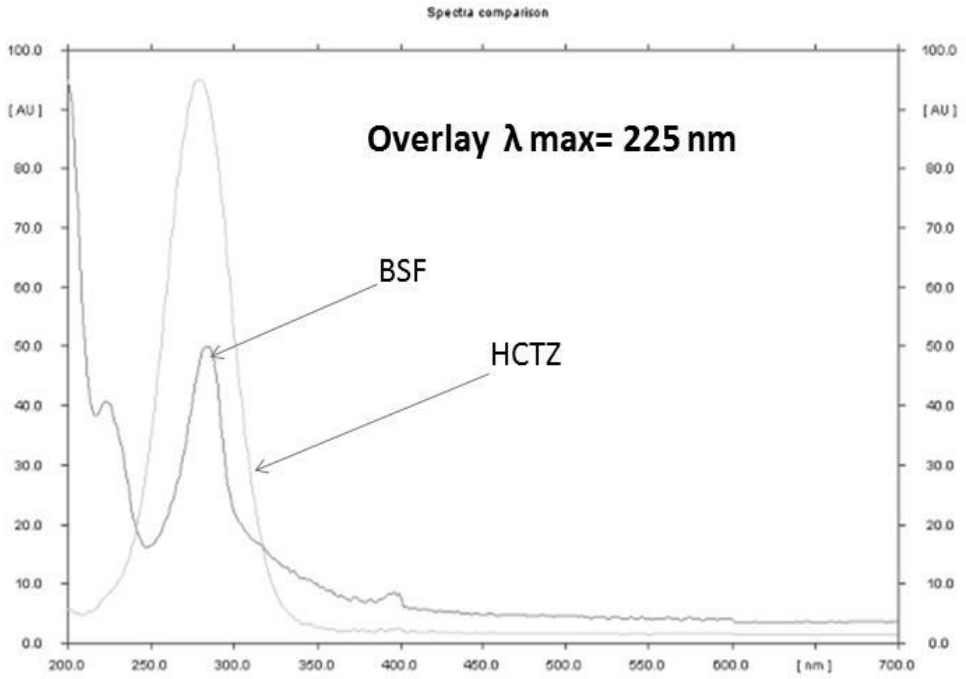

Fig.7.09. *In situ* overlay absorption spectra of BSF and HCTZ

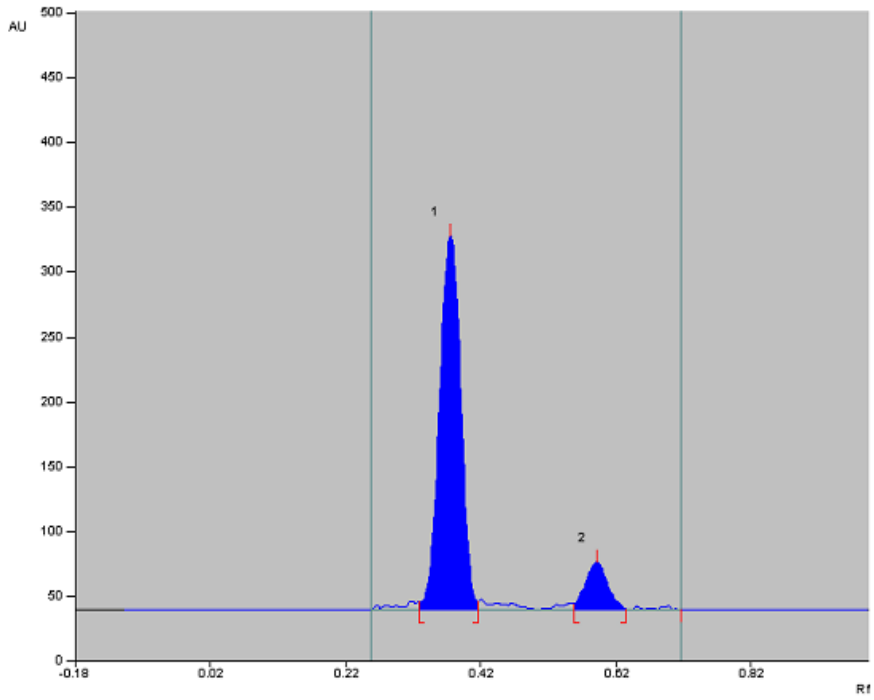

Fig.7.10. Typical densitogram of BSF (peak 2) and HCTZ (peak 1)

Table 7.11 (A): Data for BSF from linearity study (n= 3)

Conc. (ng/spot)	Mean peak area ± SD	RSD (%)
150	335.4 ± 3.69	1.10
300	795.6 ± 8.20	1.03
450	1409 ± 15.92	1.13
600	1918.4 ± 21.29	1.11
750	2486.5 ± 30.83	1.24
900	3010.7 ± 35.23	1.17
Regression coefficient r^2		0.9992
Slope		3.611
Intercept		- 236.6

Table 7.11 (B): Data for HCTZ from linearity study (n= 3)

Conc. (ng/spot)	Mean peak area ± SD	RSD (%)
100	639.67 ± 6.525	1.02
200	976.33 ± 11.42	1.17
300	1272.90 ± 14.26	1.12
400	1637.60 ± 18.01	1.10
500	1945.80 ± 19.65	1.01
600	2235.67 ± 23.25	1.04
Regression coefficient r^2		0.9991
Slope		3.215
Intercept		326

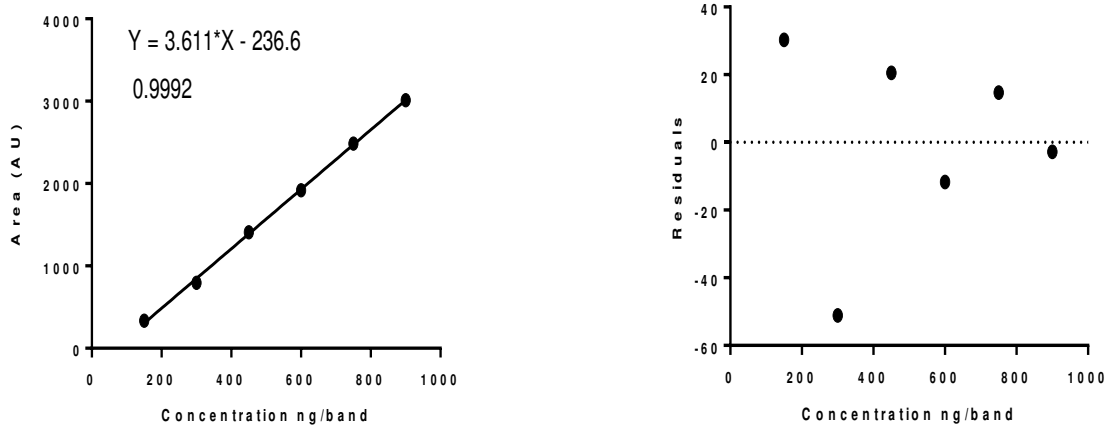

Fig.7.11. Calibration curve and residual plot for BSF

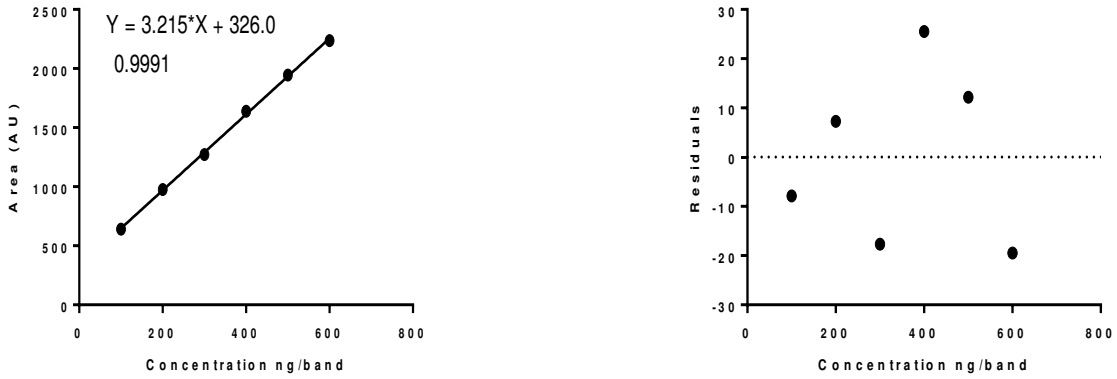

Fig.7.12. Calibration curve and residual plot for HCTZ

Table 7.12: Precision studies of BSF and HCTZ

Conc. (ng/spot)	Intra-day precision (n=3)			Inter-day precision (n=3)		
	Measured Conc. ± SD	(%) RSD	Recovery (%)	Measured Conc. ±SD	(%) RSD	Recovery (%)
Bisoprolol fumarate						
300	298.50 ± 1.40	0.47	99.50	298.71 ± 0.63	0.21	99.57
600	596.70 ± 4.83	0.81	99.45	594.60 ± 4.22	0.71	99.10
900	894.51 ± 9.03	1.01	99.39	897.03 ± 4.75	0.53	99.67
Hydrochlorothiazide						
200	201.70 ± 2.12	1.05	100.85	198.64 ± 1.91	0.96	99.32
400	407.08 ± 1.27	0.31	101.77	397.72 ± 3.14	0.79	99.43
600	593.64 ± 1.07	0.18	98.94	598.26 ± 8.14	1.36	99.71

Table 7.13: Robustness evaluation of BSF and HCTZ (n=3)

Parameter	BSF		HCTZ	
	SD of peak area	RSD (%)	SD of peak area	RSD (%)
A: Mobile phase composition (± 0.1 mL)				
Ethyl acetate: methanol: ammonia (10: 1: 0.5 v/v/v)	5.78	0.85	7.41	1.16
Ethyl acetate: methanol: ammonia (9.9: 0.5: 0.5 v/v/v)	7.80	0.75	8.30	0.85
B: Mobile phase volume (± 1 mL)				
10 mL	6.18	1.01	6.41	1.15
11 mL	7.95	1.04	7.85	1.09
C: Development distance(± 0.5 cm)				
7 cm	6.43	1.32	8.00	1.20
7.5 cm	6.87	0.86	8.34	0.97
8 cm	5.30	0.76	7.20	0.64
D: Duration of saturation (± 5 min.)				
20 min	5.26	0.72	7.43	0.52
25 min	6.54	1.09	7.98	1.28
30 min	6.98	1.45	8.37	1.13
E: Time from application to chromatography (+ 10)	6.75	1.20	8.91	1.43
F: Time from chromatography to scanning (+ 10)	7.10	0.95	8.53	1.39

Table 7.14: Recovery study of BSF and HCTZ (n=3)

Label claim (mg/tablet)	Amount Added (%)	Total amount (mg)	Amount recovered (mg)	Recovery (%)	Mean (%) Recovery (± SD)
BSF 2.5	80	4.5	4.47	99.33	99.33 ± 0.125
	100	5	4.96	99.20	
	120	5.5	5.47	99.45	
HCTZ 6.26	80	11.25	11.21	99.64	99.60 ± 0.04
	100	12.50	12.45	99.60	
	120	13.75	13.69	99.56	

Table 7.15: Assay results of BSF and HCTZ in tablets (n= 3)

LODOZ 2.5 tablet contains	Label claim (mg/tablet)	Amount found (mg ± SD)	RSD (%)	Recovery (%)
BSF	2.5 mg	2.4862 ± 0.278	1.12	99.45
HCTZ	6.25 mg	6.266 ± 0.0739	1.18	100.25

Table 7.16: Comparison of the proposed method with the reported methods:

Parameter	Reported method		Proposed method	
	BSF	HCTZ	BSF	HCTZ
Mobile Phase	chloroform: ethanol: glacial acetic acid (5:1.5:0.2 v/v)		**Ethyl acetate: methanol: ammonia (10:0.5:0.5 v/v)**	
Detection wavelength (nm)	225		**225**	
Retention factor (Rf)	0.62	0.40	**0.60**	**0.38**
Linearity range (ng/spot)	200-1200	100-800	**150-900**	**100-600**
LOD (ng/spot) and LOQ (ng/spot)	34.59 104.54	16.25 50	**50** **100**	**25** **50**
% Recovery (n=3)	99.75	99.34	**99.33**	**99.60**
Precision (% RSD) Intra-day (n=3) Inter-day (n=3)	 0.68 0.88	 1.55 1.086	 **0.76** **0.483**	 **0.513** **1.037**

7.3.3. Conclusion:

The present study demonstrates a HPTLC method for simultaneous estimation of BSF and HCTZ in bulk drug and pharmaceutical dosage form. The method was validated for linearity, accuracy, precision and specificity. The method has been validated according to ICH guidelines. Low % RSD values indicate that the method is precise, accurate and robust method. The proposed method can be used for the routine analysis of BSF and HCTZ from its dosage forms.

7.4 Simultaneous analysis of Nebivolol HCl and Hydrochlorothiazide by HPTLC

Nebivolol hydrochloride, (1RS, 1′RS)-1, 1′-[(2RS, 2′SR)-bis (6-fluoro-3, 4-dihydro-2H-1-benzopyran-2-yl)]- 2,2′-iminodiethanol hydrochloride, is a highly cardioselective vasodilatory beta1 receptor blocker used in treatment of hypertension. Hydrochlorothiazide chemically, 6-Chloro-3, 4-dihydro-2H-l, 2, 4-benzothiadiazine-7-sulfonamide 1, 1-dioxide, is a thiazide diuretic used as antihypertensive agent.

The drug profiles and literature reviews of Nebivolol HCl (NEB) and Hydrochlorothiazide (HCTZ) are given in 3.1 and 3.5.

7.4.1 Experimental:

- **Chemicals and reagents:**
  Reference standards of Nebivolol HCl (99.3 %) and Hydrochlorothiazide (99.97%) were procured from Torrent Pharmaceuticals Ltd. and Emcure Pharmaceuticals Ltd respectively. The tablets Nebilong-H (Nebivolol HCl-5.0 mg and Hydrochlorothiazide- 12.5mg) of Micro lab. Ltd. was procured from the market. Chloroform, toluene, methanol and ammonia were of AR grade purity (E-Merck Ltd).
- **Preparation of standard stock solution-I:**
  150 mg of Nebivolol HCl (NEB) and 50 mg of Hydrochlorothiazide (HCTZ) were accurately weighed and transferred to a 50 mL volumetric flask and dissolved in methanol. Volume was made up to the mark with methanol to get a **Standard stock solution-I** containing NEB 3.0 mg/mL or 3000 µg/mL and HCTZ 1.0 mg/mL or 1000 µg/mL, respectively.
- **Working standard solution-II**
  0.3 ml of the standard stock solution-I was diluted to 10 mL with methanol to get a combined working standard solution-II of NEB (0.09 mg/mL or 90 µg/mL) and HCTZ (0.03 mg/mL or 30 µg/mL).

- **Chromatography:**

  The Camag TLC consisted of Linomat V sample applicator (Camag, Muttenz, Switzerland). Camag TLC scanner III controlled by WinCATS software (V 3.15, Camag) was used for sample application and quantitative evaluation. Chromatography was performed on Merck silica gel 60 F_{254} precoated aluminum TLC plate (10 cm × 10 cm with 250 µm thickness), with chloroform: toluene: methanol: ammonia 5:3:2:0.1 (v/v) as mobile phase. Samples were applied as bands 6 mm long at 6 mm interval under a stream of nitrogen with a Camag 100 microlitre syringe (Hamilton, Bonded, Switzerland). The slit dimension was 5 mm × 0.45 mm. Ascending development to a distance of 8 cm was performed in a 20 min. presaturated 10 cm × 10 cm twin trough TLC developing chamber (Camag). Densitometric scanning was performed in absorbance mode at wavelength of 278 nm.

- **Analytical Method Validation**

  Validation was done as per ICH guidelines. The developed method was validated with respect to parameters such as linearity, LOD and LOQ, precision, accuracy and specificity.

  - **Linearity:**

    Calibration solutions of mixture of NEB and HCTZ were prepared by suitably diluting the standard stock solution - II (2 – 7 ml) in methanol. 1µL of standard solution of NEB and HCTZ were spotted as sharp bands to the precoated TLC plates. The chamber was saturated with mobile phase for 20 minutes. The mobile phase was allowed to travel for 80 mm above the application of band. After the development the plate was removed from the chamber and dried under a current of hot air. The UV response of the drug was monitored using the densitometer set at 278 nm. Data peak area of each band was recorded. The data of peak area versus the concentration of respective drug was plotted and drug concentration (ng/spot) against the peak area (AU) was found to be linear in the range of 180 - 630 ng/spot for NEB and 60 - 210 ng/spot for HCTZ.

  - **Limit of detection and limit of quantitation:**

    LOD and LOQ were experimentally verified by spotting a series of dilute standard solutions of known concentrations of NEB and HCTZ until the average responses

were approximately 3 or 10 times the standard deviation of the responses for three replicate determinations.

- **Precision:**

  The precision of the method was verified by intra-day and inter-day studies. Intra-day variations were performed by analysis of three different concentrations (270 ng/spot, 450 ng/spot and 630 ng/spot for NEB and 90 ng/spot, 150 ng/spot and 210 ng/spot for HCTZ) three times on the same day. The inter-day variations were similarly evaluated over a period of 3 days.

- **Repeatability of measurement:**

  2 µL of the standard solution-II NEB (0.09 mg/mL or 90 µg/mL) and HCTZ (0.03 mg/mL or 30 µg/mL) were spotted on a TLC plate, developed, dried and the spots were scanned seven times without changing the plate position and % RSD for measurement of peak area for both the drugs were calculated to determine the instrumental precision

- **Repeatability of sample application:**

  2 µL of the standard solution-II NEB (0.09 mg/mL or 90 µg/mL) and HCTZ (0.03 mg/mL or 30 µg/mL) were applied six times on a TLC plate, developed, dried and the spots were scanned and % RSD for measurement of peak area for both the drugs were calculated to determine the repeatability.

- **Robustness:**

  Robustness of the method was performed by making small deliberate changes in chromatographic conditions. Mobile phases having different compositions like chloroform: toluene: methanol: ammonia (5.1: 3: 2: 0.1 v/v/v) and (5: 2.9: 1.9: 0.1 v/v/v) were tried and chromatograms were run. The amount of mobile phase was varied over the range of ± 1 mL. The development distance was varied from 7.5 and 8.5 cm. Duration of saturation time of chamber was varied as 25 and 30 min. The time from spotting to chromatography and from chromatography to scanning was varied from +20 min. The robustness of the method was determined at three different concentration levels 270, 450 and 630 ng/spot and 90, 150 and 210 ng/spot for NEB and HCTZ, respectively.

- **Accuracy:**

  Accuracy was done in terms of recovery studies. Recovery studies were carried out by standard addition method. The analysed samples were spiked with extra 80 %, 100 % and 120 % of standard drugs. The mixtures were reanalysed by the proposed method. The experiment was conducted in triplicate.

- **Analysis of marketed formulation:**

  Twenty tablets of the pharmaceutical formulation Nebilong-H (containing 5 mg Nebivolol HCl and 12.5 mg Hydrochlorothiazide) were accurately weighed and average weight determined. They were crushed to a fine powder and an amount of the powder equivalent to 5 mg NEB and 12.5 mg HCTZ was weighed and transferred into 25 mL volumetric flask and dissolved in 15 mL of methanol, sonicated for 15 min. and diluted to mark with same solvent and filtered through whatman filter paper no.1. The sample solution was further diluted with methanol.

  A fixed volume of 0.1 µL of the standard solution-I and sample solutions were spotted as sharp bands on the chromatoplate. The chromatoplate was then developed in a twin trough chamber containing the mobile phase and developed bands of the drugs were scanned at 278 nm. The analysis was repeated in triplicate. The possibility of excipients interference with the analysis was examined.

- **Specificity:**

  The specificity of the method was ascertained by analyzing standard drugs and samples extracted from formulations. The spots for NEB and HCTZ were confirmed by comparing the Rf of the spot with those of the standard.

- **Comparison of the proposed method with the reported methods:**

  The proposed method was compared with the following reported method from the literature:

1. A validated HPTLC method for simultaneous quantification of nebivolol and hydrochlorothiazide in bulk and tablet formulation[9]

   Stationary Phase: Precoated silica gel aluminum plate 60 F_{254}

   Mobile phase: 1, 4 dioxane: toluene: triethylamine (5:3:0.1)

   Detector wavelength: 280 nm.

2. Simultaneous estimation of nebivolol hydrochloride and hydrochlorothiazide in tablets by TLC-densitometry[10]

   Stationary Phase: Precoated silica gel aluminum plate 60 F 254

   Mobile phase: Toluene: ethyl acetate: methanol: ammonia (3:2.7:1.7:0.1 v/v/v)

   Detector wavelength: 281 nm.

7.4.2. Results and Discussions:

- **HPTLC method development:**

  HPTLC method was optimized with a view to develop a simultaneous determination for NEB and HCTZ from tablet formulation. 1µL samples of the individual mixed standard stock solutions (180 ng/spot of NEB and 60 ng/spot of HCTZ) were spotted on to TLC plates and run in different solvent systems. After many trials it was found that chloroform: toluene: methanol: ammonia 5:3:2:0.1 (v/v) gave a good resolution, sharp and symmetrical peaks with Rf of 0.42 ± 0.02 for NEB and 0.30 ± 0.02 for HCTZ, respectively. The plate was scanned from 200-400 nm, a typical spectrum of NEB and HCTZ are as shown in Fig. 7.13. Wavelength of 278 nm was selected for densitometric evaluation, since both the drugs show good absorption.

- **Validation:**

  Using optimised chromatographic conditions the HPTLC method developed was validated in terms of linearity, LOD and LOQ, precision, robustness and accuracy.

- **Specificity:**

  The method is specific for NEB and HCTZ, since it resolved the peak of NEB (R_f = 0.42 ± 0.02) and HCTZ (R_f = 0.30 ± 0.02) in presence of other excipients in the formulation (Fig. 7.14).

- **Linearity:**

  Linear regression data for the calibration plots revealed good linear relationships between response and concentration over the ranges 180– 630 ng/spot for NEB and 60–210 ng/spot for HCTZ. The linear regression equations were Y = 3.011X + 120.8 (r^2 = 0.9992) for NEB and Y = 28.60X – 618.2 (r^2 = 0.9989) for HCTZ. The residuals plot shows a random pattern, indicating a good fit for a linear model. The calibration curves and residuals plots are given in Fig. 7.15 and Fig. 7.16 and Table 7.17 (A), and Table 7.17 (B) for NEB and HCTZ, respectively.

- **Limits of Detection and Quantitation:**

  The results of the LOD and LOQ were found to be 40 ng/spot, 160 ng/spot for NEB and 30 ng/spot, 50 ng/spot HCTZ, respectively.

- **Precision:**

  The precision of the developed method was demonstrated by intra-day and inter-day precision studies. This was done by three replicate analysis of the sample. The precision of the method was expressed as relative standard deviation (RSD, %). The % RSD of intra-day and inter-day precision values was found to be 1.31 and 1.39 % for NEB and 1.08 and 1.32 % for HCTZ, respectively, indicating good precision (Table 7.18).

- **Instrumental precision:**

  The % RSD for 180 ng/spot of NEB and 60 ng/spot of HCTZ (n=6) was found to be 1.031 and 1.011, respectively.

- **Repeatability of sample application:**

  2.0 µL of the standard solution-II NEB (0.09 mg/mL or 90 µg/mL) and HCTZ (0.03 mg/mL or 30 µg/mL) were applied seven times on a TLC plate, developed, dried and the spots were scanned. The mean % for peak area was found to be 0.983 for NEB and 1.120 for HCTZ, respectively.

- **Robustness:**

  The robustness of the method was found out by testing the effect of small deliberate changes in the chromatographic conditions. The method was found to be robust enough that the peak area was not apparently affected by small variation in the chromatographic conditions and there were no significant changes in the retention times of NEB and HCTZ. The low values of the % RSD indicate the robustness of the method, as shown in Table 7.19.

- **Accuracy:**

  Accuracy of the method was evaluated by carrying out recovery study. A known concentration of the standard drug was added to a preanalysed tablet sample at three different levels namely 80 %, 100 % and 120 %. Each level was repeated three times. Total amount of the drug was determined by the proposed method. The percentage recovery was calculated. The mean % recoveries were 99.93 ± 0.324 for NEB and 99.98 ± 0.50 for HCTZ, respectively (Table 7.20).

- **Analysis of marketed formulation:**

  Two spots at Rf of 0.42 (NEB) and 0.30 (HCTZ) were observed in the chromatogram of the drug samples extracted from conventional tablets. There was no interference from the excipients commonly present in the conventional tablets. The % drug content found for NEB and HCTZ were 99.89 % and 99.34 %, respectively. This indicates that there is no degradation of NEB and HCTZ in the marketed formulations that were analysed by this method. The low % RSD value indicated the suitability of the method for simultaneous analysis of NEB and HCTZ in pharmaceutical dosage forms (Table 7.21).

- **Comparison of the proposed method with the reported methods:**

  The proposed method was compared with the reported methods. Linearity concentration range of the proposed method is 180 – 630 ng/spot for NEB and 60-210 ng/spot for HCTZ as against 500-5000 ng/spot for NEB and 400-6000 ng/spot for HCTZ from the reported methods. LOD and LOQ for NEB and HCTZ of the proposed method are lower than the reported methods. The proposed method uses easily available solvents in the mobile phase which gives well resolved peaks with Rf values of 0.42 for NEB and 0.30 for HCTZ. (Table 7.22)

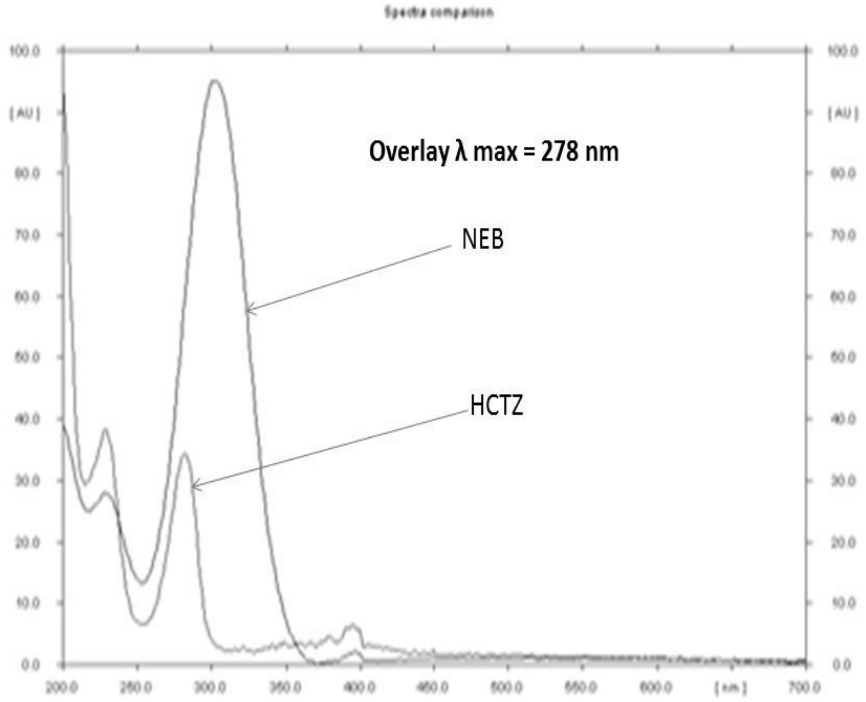

Fig.7.13. I*n situ* overlay absorption spectra of NEB and HCTZ

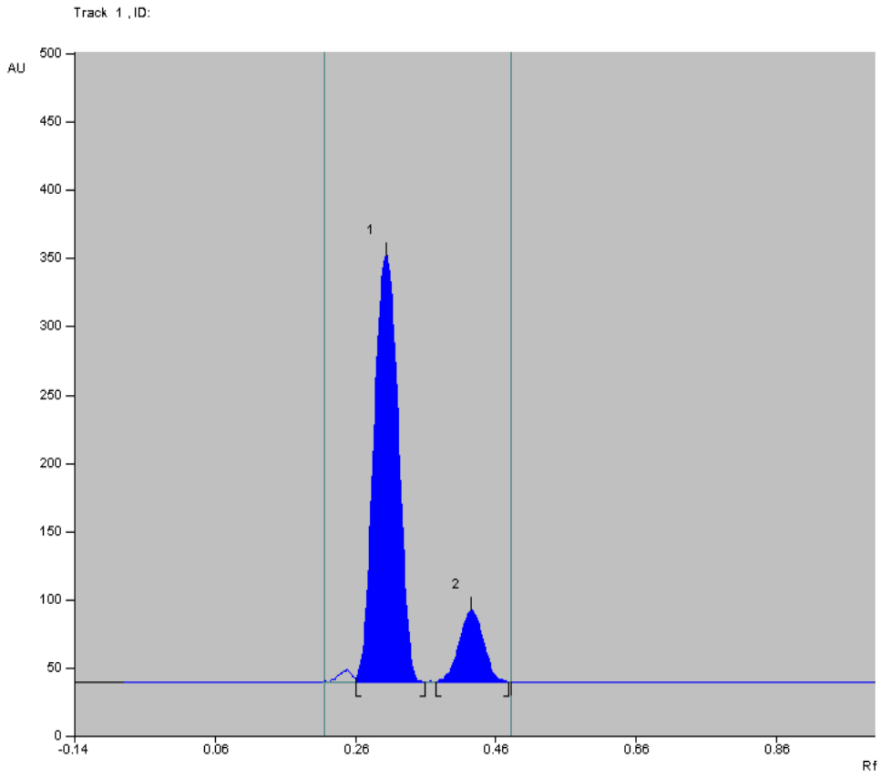

Fig.7.14. Typical densitogram of NEB (Peak 2) and HCTZ (Peak 1)

Table 7.17 (A): Data for NEB from linearity study (n= 3)

Conc. (ng/spot)	Mean peak area ± SD	RSD (%)
180	680 ± 7.48	1.10
270	915.8 ± 9.43	1.03
360	1199.5 ± 13.55	1.13
450	1468.5 ± 16.30	1.11
540	1765.2 ± 21.89	1.24
630	2013.8 ± 23.56	1.17
Regression coefficient r^2		0.9992
Slope		3.011
Intercept		120.8

Table 7.17 (B): Data for HCTZ from linearity study (n= 3)

Conc. (ng/spot)	Mean peak area ± SD	RSD (%)
60	1165.2 ± 11.89	1.02
90	1920.5 ± 22.47	1.17
120	2767.8 ± 31.00	1.12
150	3671.4 ± 40.39	1.10
180	4485.1 ± 45.30	1.01
210	5453.5 ± 56.72	1.04
Regression coefficient r^2		0.9990
Slope		28.60
Intercept		– 618.2

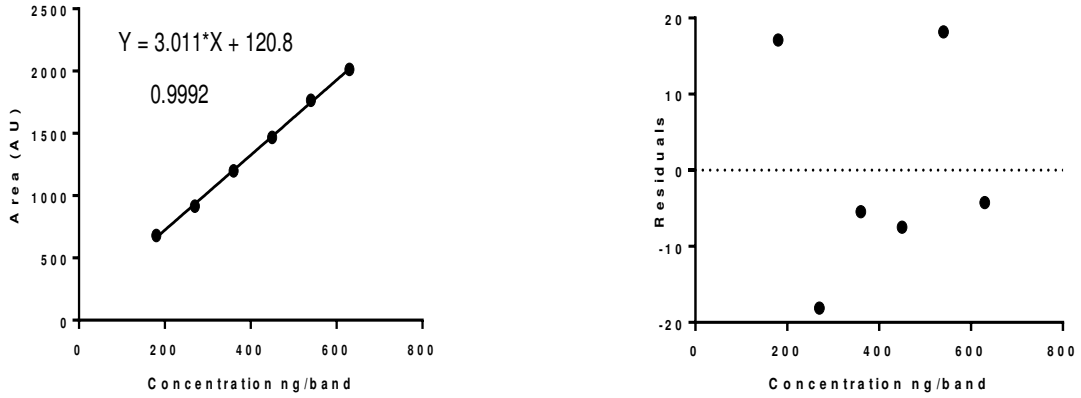

Fig.7.15. Calibration curve and residual plot for NEB

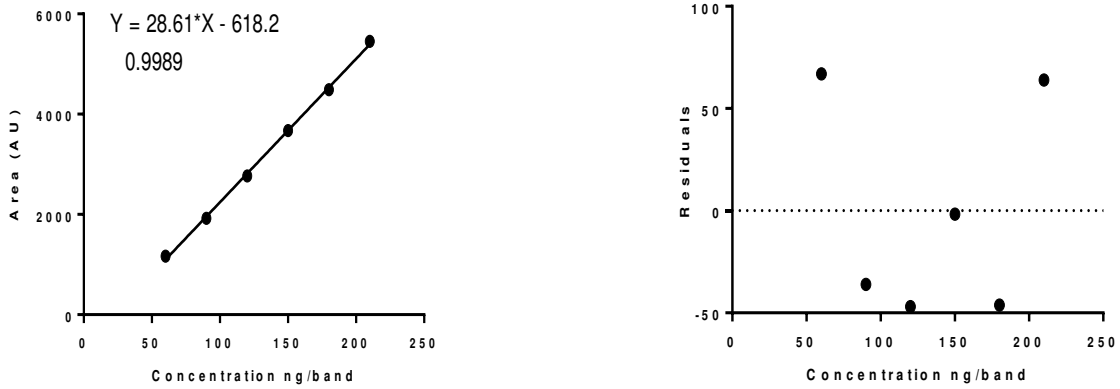

Fig. 7.16. Calibration curve and residual plot for HCTZ

Table 7.18: Precision studies of NEB and HCTZ

Conc. (ng/spot)	Intra-day precision (n=3)			Inter-day precision (n=3)		
	Measured Conc. ± SD	(%) RSD	Recovery (%)	Measured Conc. ±SD	(%) RSD	Recovery (%)
Nebivolol HCl						
270	269.51 ± 2.99	1.11	99.82	272.16 ± 3.84	1.41	100.8
450	445.73 ± 6.73	1.51	99.05	449.87 ± 6.93	1.54	99.97
630	632.77 ± 8.290	1.31	100.44	636.93 ± 7.71	1.21	101.1
Hydrochlorothiazide						
90	90.153 ± 1.12	1.24	100.17	90.27 ± 1.30	1.44	100.3
150	148.76 ± 1.52	1.02	99.17	149.10 ± 1.94	1.30	99.39
210	213.15 ± 2.10	0.98	101.5	212.52 ± 2.57	1.21	101.2

Table 7.19: Robustness evaluation of NEB and HCTZ (n=3)

Parameter	NEB		HCTZ	
	SD of peak area	RSD (%)	SD of peak area	RSD (%)
A: Mobile phase composition (± 0.1mL)				
Chloroform: toluene: methanol: ammonia (5.1: 3: 2: 0.1 v/v/v/v)	4.074	0.98	4.93	0.56
Chloroform: toluene: methanol: ammonia (5: 2.9: 1.9: 0.1 v/v/v/v)	5.13	0.76	6.38	0.81
B: Mobile phase volume(± 1mL)				
9.1 mL	6.10	0.83	5.41	0.75
11.1 mL	6.23	0.94	6.20	0.91
C: Development distance(± 0.5 cm)				
7.5 cm	4.43	0.64	5.14	0.73
8.5 cm	4.87	0.72	5.84	0.97
D: Duration of saturation (± 5 min.)				
25 min	4.11	0.97	6.98	0.81
30 min	4.98	0.54	6.37	0.57
E: Time from application to chromatography (+ 10)	5.75	0.58	6.91	0.64
F: Time from chromatography to scanning (+ 10)	5.16	0.91	6.51	0.83

Table 7.20: Recovery study of NEB and HCTZ (n=3)

Label claim (mg/tablet)	Amount Added (%)	Total amount (mg)	Amount recovered (mg)	Recovery (%)	Mean (%) Recovery (± SD)
NEB 5.0	80 100 120	9 10 11	8.975 10.03 10.973	99.72 100.3 99.76	99.93 ± 0.324
HCTZ 12.5	80 100 120	22.5 25 27.5	22.401 24.962 27.65	99.56 99.85 100.53	99.98 ± 0.50

Table 7.21: Assay of NEB and HCTZ in tablets (n= 3)

Nebilong tablet contains	Label claim (mg/tablet)	Amount found (mg ± SD)	RSD (%)	Recovery (%)
NEB	5.0 mg	4.9945 ± 0.047	0.94	99.89
HCTZ	12.5 mg	12.417 ± 0.073	0.59	99.34

Table 7.22: Comparison of the proposed method with the reported methods:

Parameter	Reported method 1		Reported method 2		Proposed method	
	NEB	**HCTZ**	**NEB**	**HCTZ**	**NEB**	**HCTZ**
Mobile Phase	1,4 dioxane: toluene: triethylamine (5:3:0.1)		Toluene:ethyl acetate: methanol: ammonia (3:2.7:1.7:0.1 v/v/v)		chloroform: toluene: methanol: ammonia 5:3:2:0.1	
Detection wavelength (nm)	282		281		**278**	
Retention factor (Rf)	0.75	0.43	0.68	0.38	**0.42**	**0.30**
Linearity range (ng/spot)	1000-5000	400-2000	500-3000	1000-6000	**180-630**	**60-210**
LOD (ng/spot) and LOQ(ng/spot)	43 130	25 76	73.58 223.09	101.12 309.46	**40** **160**	**30** **50**
% Recovery (n=3)	100.06	99.97	100.2	100.38	**99.93**	**99.98**
Precision (% RSD) Intra-day (n=3) Inter-day (n=3)	 0.04 0.04	 0.047 0.06	 1.44 1.78	 0.52 0.45	 **1.31** **1.39**	 **1.08** **1.32**

7.4.3. Conclusion:

The developed HPTLC method is simple, precise, specific and accurate for simultaneous estimation of NEB and HCTZ in bulk and tablet dosage form. The statistical analysis proves that the method is precise, selective and robust for the analysis of NEB and HCTZ indicating non-interference of excipients in the estimation. The proposed method could be applied for routine analysis in a quality control laboratory. As the linearity concentration range of the proposed method is 180 – 630 ng/spot for NEB and 60-210 ng/spot for HCTZ as against 500-5000 ng/spot for NEB and 400-6000 ng/spot for HCTZ of the reported methods, the proposed method can be adopted for the simultaneous estimation of these drugs.

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Pharmaceutical analytical chemistry is an important part in monitoring the quality of pharmaceutical products for safety and efficacy. With the advancement in synthetic organic chemistry and other branches of chemistry including bioanalytical sciences and biotechnology, the scope of analytical chemistry has enhanced to, much higher levels. The emphasis in current use of analytical methods particularly involving advance analytical technology has made it possible not only to evaluate the potency of active ingredients in dosage forms and APIs but also to characterize, elucidate, identify and quantify impurities. Pharmacopoeias rely more on instrumental techniques rather than the classical wet chemistry method.

Drug analysis plays an important role in the development of drugs, their manufacture and therapeutic use. Pharmaceutical industries rely upon quantitative chemical analysis to ensure that the raw material used and the final products obtained meet the required specifications. The number of drugs and drug formulations introduced into the market has been increasing at an alarming rate. These drugs or formulations may be either new entities or novel dosage forms or multicomponent dosage forms.

Among the life threatening diseases, Hypertension is one of the major public health problems of worldwide distribution and is a major risk factor for cardiovascular disease morbidity and mortality. Combination pharmacotherapy is a valuable therapeutic strategy in achieving the target blood pressure for patients with risk factors for cardiovascular diseases.

Fixed dose combination therapy is increasing, particularly for meeting the more stringent blood pressure goals recommended for hypertensive patients with concurrent medical problems such as diabetes and renal diseases. Advantage of combination therapy include higher response rates, including additive effects at lower doses; the potential of the drugs to attenuate or cancel compensatory hemodynamic changes induced by single agent therapy; the potential to attenuate or cancel adverse reactions induced by single agents; the potential to provide beneficial effects on target organ damage through effects unrelated to antihypertensive action; and the patients perceived benefits of greater convenience, lower cost and better compliance

Fixed dose combination concept is used as an alternative for the patients who fail to lower their blood pressure and associated multiple risk factors with formulations containing two or more drugs in combinations. The fixed dose combination concept aims to reduce multiple risk factors simultaneously such as blood pressure; low density lipoprotein

cholesterol and platelet function through the administration of a single formulation that combines various effective medications at lower doses. The fixed dose combination has two or three BP-lowering agents and has been shown to be superior to the combination of these antihypertensive with statins and aspirin.

Considering all these views the following antihypertensive combinations that are presently being marketed were selected for the study.

Two components formulation

- Nebivolol hydrochloride and Indapamide
- Amlodipine besylate and Indapamide
- Ramipril and Amlodipine besylate
- Amlodipine besylate and Hydrochlorothiazide
- Atenolol and Indapamide
- Propranolol hydrochloride and Hydrochlorothiazide
- Bisoprolol fumarate and Hydrochlorothiazide
- Nebivolol hydrochloride and Hydrochlorothiazide

Three components formulation

- Amlodipine besylate, Losartan potassium and Hydrochlorothiazide
- Ramipril, Losartan potassium and Hydrochlorothiazide
- Atenolol, Hydrochlorothiazide and Losartan potassium

More than three components formulation

- Atorvastatin calcium, Losartan potassium, Atenolol and Aspirin
- Aspirin, Atenolol, Hydrochlorothiazide, Ramipril and Simvastatin

In the present study, HPLC methods were developed for the simultaneous analysis of following antihypertensive combinations containing two components (Four formulations), three components (Three formulations), four and five components (One each).

- Nebivolol hydrochloride and Indapamide
- Amlodipine besylate and Indapamide
- Ramipril and Amlodipine besylate
- Amlodipine besylate and Hydrochlorothiazide
- Amlodipine besylate, Losartan potassium and Hydrochlorothiazide

- Ramipril, Losartan potassium and Hydrochlorothiazide
- Atenolol, Hydrochlorothiazide and Losartan potassium
- Atorvastatin calcium, Losartan potassium, Atenolol and Aspirin
- Aspirin, Atenolol, Hydrochlorothiazide, Ramipril and Simvastatin

HPTLC methods were developed for two component cardiac formulations. (Four formulations)

All the methods described in the thesis are simple, rapid and reliable. The developed methods were validated statistically for linearity, precision, specificity, accuracy and ruggedness. The methods could be used not only for quality control but also for process development of bulk drugs.

Simultaneous analysis of Nebivolol hydrochloride and Indapamide by HPLC:

An RP-HPLC method for the simultaneous estimation of Nebivolol HCl (NEB) and Indapamide (IND) in tablet dosage form was developed and validated by ICH guidelines. The separation of two drugs was achieved on a Hypersil Gold C_{18} column (250 mm X 4.6 mm, 5 µ) as stationary phase with a mobile phase consisting of methanol: water in the ratio of 80:20 v/v at a flow rate of 1 mL/min and UV detection at 265 nm. The retention times of Indapamide and Nebivolol HCl were 3.383 min. and 7.767min, respectively. The linearity of the proposed method was investigated in the range of 10–60 µg/mL for NEB and 3-18 µg/mL for IND is quite lower as compared to reported methods. The LOD and LOQ were found to be 1.0µg/mL, 2.2µg/mL and 0.1µg/mL, 0.5µg/mL for NEB and IND, respectively. The recoveries of Indapamide and Nebivolol HCl were found to be 99.43 % and 99.26%, respectively. All validation parameters were within the acceptable range. The described HPLC method can be useful for routine quality control analysis.

Simultaneous analysis of Amlodipine and Indapamide by HPLC:

A simple, specific, accurate and precise RP-HPLC method has been developed for the simultaneous estimation of Amlodipine (AMLO) and Indapamide (IND) in tablet dosage form by using Hypersil Gold C_{18} column (250 mm X 4.6 mm, 5 µ). The proposed method uses simple mobile phase of methanol: water (80:20 v/v) against buffered mobile phases

used in the reported methods. The flow rate used was1 mL/min at 238 nm. The retention times of Indapamide and Amlodipine were found to be 3.392 min. and 7.550 min, respectively. The linearity of the proposed method was investigated in the range of 1–6 µg/mL for AMLO and 0.5-3µg/mL for IND is quite lower as compared to reported methods. The LOD and LOQ were found to be 0.4 µg/mL, 0.8 µg/mL and 0.1µg/mL, 0.5µg/mL for AMLO and IND, respectively. The recoveries of AMLO and IND were found to be 99.28 % and 99.42%, respectively. Accuracy and precision of the proposed method are comparable to the reported methods with low % RSD. Thus, the proposed RP-HPLC method has its applications in the field of quality control of raw materials, formulations and dissolutions studies.

Simultaneous analysis of Amlodipine and Hydrochlorothiazide by HPLC:

A simple, specific, accurate and precise RP-HPLC method was developed for simultaneous estimation of Amlodipine (AMLO) and Hydrochlorothiazide (HCTZ) in tablet dosage form. Chromatography was performed on a Hypersil Gold C_{18} column (250 mm X 4.6 mm, 5 µ) with a mobile phase consisting of methanol: water in the ratio of 95:5.0 v/v at a flow rate of 0.8 mL/min and UV detection at 230 nm. The retention times of Hydrochlorothiazide and Amlodipine were found to be 3.867 min. and 7.325 min, respectively. Linearity concentration range of the proposed method was 0.5 - 3 µg/mL for AMLO and 1.25 – 7.5 µg/mL for HCTZ which is quite lower as compared to reported method. LOD and LOQ for AMLO and HCTZ of the proposed method are found to be 0.2 µg/mL, 0.5 µg/mL and 0.6µg/mL, 1µg/mL. The recoveries of Amlodipine and Hydrochlorothiazide were found to be 99.23 % and 99.47%, respectively. The low relative standard deviation confirm the suitability of the proposed method for the routine quality control analysis of Amlodipine and Hydrochlorothiazide from bulk drug and in marketed tablets without any interference from excipients.

Simultaneous analysis of Amlodipine and Ramipril by HPLC:

An isocratic RP-HPLC method has been developed for the simultaneous estimation of Amlodipine (AMLO) and Ramipril (RAM) in combined dosage form. Separation was achieved on Hypersil Gold C_{18} column (250 mm X 4.6 mm, 5 µ) using mobile phase consisting of methanol: water in the ratio of 95:5.0 v/v at a flow rate of 1 mL/min and UV

detection at 227nm.The retention times of Ramipril and Amlodipine were 2.630 min. and 7.192 min. respectively. The method was validated as per ICH guidelines. Linearity concentration range of the proposed method was 0.5 - 3 µg/mL for RAM and 1– 6 µg/mL for AMLO. The LOD and LOQ were found to be 0.2 µg/mL and 0.5µg/mL respectively for both AMLO and RAM. The recoveries of AMLO and RAM were found to be 99.30 % and 99.37%, respectively. Validation studies demonstrated that the proposed RP-HPLC method is rapid, selective, requires a simple sample preparation procedure and it is suitable for analysis of AMLO and RAM in their formulation in a single isocratic run, in contrast with previous gradient method. Therefore the developed method can be used for routine analysis. The method can also be applied for bio-analytical work.

Simultaneous analysis of Losartan potassium, Hydrochlorothiazide and Amlodipine besylate by HPLC:

A simple RP-HPLC method has been developed and validated for simultaneous estimation of Losartan potassium (LOS), Hydrochlorothiazide (HCTZ) and Amlodipine besylate (AMLO) from pharmaceutical dosage form. A chromatographic separation was accomplished on Hypersil Gold column C_{18} (250 mm X 4.6 mm, 5 µ) with mobile phase consisting of 95:5 v/v at a flow rate of 0.8 mL/min at a detection wavelength of 230 nm. The retention times of Losartan potassium and Hydrochlorothiazide and Amlodipine were 2.850 min., 3.875 min. and 7.333 min. respectively. Linearity concentration range of the proposed method is 10-60 µg/mL for LOS, 2.5-15µg/mL for HCTZ and 1-6 µg/mL for AMLO which is quiet lower as compared to the reported methods. The LOD and LOQ were found to be 0.6 µg/mL, 1.8 µg/mL and 0.2µg/mL, 0.5µg/mL and 0.4µg/mL, 0.8µg/mL for LOS, HCTZ and AMLO, respectively. The recoveries of LOS, HCTZ and AMLO were found to be 99.52 %, 99.52% and 99.17% respectively. All validation parameters were within the acceptable range. Hence, the proposed method can be useful for routine quality control analysis of these drugs in combination tablets.

Simultaneous analysis of Losartan potassium, Ramipril and Hydrochlorothiazide by HPLC:

This work is concern with application of simple, precise and accurate RP-HPLC method for simultaneous estimation of Losartan potassium (LOS), Ramipril (RAM) and

Hydrochlorothiazide (HCTZ) in combined dosage form. Chromatographic separation was achieved isocratically on Hypersil Gold column C_{18} (250 mm X 4.6 mm, 5 µ) with a mobile phase composed of methanol: water in the ratio of 95:5.0 v/v at flow rate of 1 mL/min. Detection is carried out at 218 nm. The retention times of Losartan potassium, Ramipril and Hydrochlorothiazide were 2.383 min., 2.658 min. and 3.108 min. respectively. The method was found to be linear in the range of 2- 12 µg/mL for LOS, 0.2- 1.2µg/mL for RAM and 0.5–3.0 µg/mL for HCTZ. The proposed method gives better column efficiency and intraday & inter-day precision than the reported method. The LOD and LOQ were found to be 0.6 µg/mL, 1.8 µg/mL and 0.08µg/mL, 0.2µg/mL and 0.2µg/mL, 0.5µg/mL for LOS, RAM and HCTZ, respectively. The recoveries of LOS, RAM and HCTZ were found to be 99.84%, 99.40% and 99.68% respectively.The peaks are well resolved and the run time for the simultaneous analysis for LOS, RAM and HCTZ is less than 7 min. as compared to the reported method which requires more than 12 min.Thus the proposed method was successfully applied for simultaneous determination of LOS, RAM and HCTZ in routine analysis.

Simultaneous analysis of Losartan potassium, Hydrochlorothiazide and Atenolol by HPLC:

A simple, rapid, precise method RP-HPLC method was developed for simultaneous estimation of Losartan potassium (LOS), Hydrochlorothiazide (HCTZ) and Atenolol (ATN) in tablet dosage form. To achieve maximum resolution, methanol: water in the ratio of 95:5.0 v/v was selected as mobile phase, using Hypersil Gold column C_{18} (250 mm X 4.6 mm, 5 µ) at flow rate of 0.8 mL/min. Detection is carried out at 225 nm. In this conditions Losartan potassium, Hydrochlorothiazide and Atenolol were eluted at 2.633 min., 3.883 min. and 7.783 min. respectively. The linearity was found in the concentration range 2-12µg/mL for LOS, 0.5- 3 µg/mL for HCTZ and 2-12µg/mL for ATN, which is quite lower as compared to the reported method. The LOD and LOQ were found to be 0.6 µg/mL, 1.8 µg/mL and 0.2µg/mL, 0.5µg/mL and 0.8µg/mL, 2µg/mL for LOS, HCTZ and ATN respectively. The recoveries of LOS, HCTZ and ATN were found to be 99.64%, 99.50% and 99.39% respectively. All the analytical validation parameters were determined and found within the acceptable limit as per ICH guidelines. Thus, the proposed method can be used

for the routine analysis of LOS, HCTZ and ATN from its dosage forms. The method can also be used for the biological and pharmacokinetic studies.

Simultaneous analysis of Atorvastatin calcium, Losartan potassium, Atenolol and Aspirin by HPLC:

A simple, specific, accurate and precise RP-HPLC method has been developed for the simultaneous estimation of Atorvastatin calcium (ATV), Losartan potassium (LOS), Atenolol (ATN) and Aspirin (ASP) in bulk and tablet dosage form. Separation was achieved on Hypersil Gold column C_{18} (250 mm X 4.6 mm, 5 µ) using methanol: water in the ratio of 60:40 v/v as mobile phase. The flow rate used was1 mL/min at 230 nm. The retention times of Atenolol, Aspirin Losartan potassium, and Atorvastatin calcium were found to be 2.475 min., 2.942, 3.550and5.267 min. respectively. The method was validated as per ICH guidelines. The linearity of the proposed method were investigated in the range of 2–12µg/mL for ATN, 3-18 µg/mL for ASP, 2-12 µg/mL for LOS and 0.4-2.4µg/mL for ATV, which is quite lower as compared to reported methods. The LOD and LOQ were found to be 0.1µg/mL, 0.4µg/mL and 0.3µg/mL, 1.2µg/mL and 0.3 µg/mL, 0.8 µg/mL and 0.1µg/mL, 0.4µg/mL for ATN, ASP, LOS, and ATV respectively. The recoveries of ATN, ASP, LOS, and ATV were found to be 99.36%, 99.15%, 99.32% and 99.18% respectively. Validation studies demonstrated that the proposed RP-HPLC method is simple, rapid and precise. The high accuracy and low % RSD confirm the suitability of the proposed method for the routine quality control analysis in bulk and tablet dosage form. The method can also be adopted for dissolution studies and pharmacokinetic studies.

Simultaneous analysis of Aspirin, Atenolol, Hydrochlorothiazide, Ramipril and Simvastatin by HPLC:

An isocratic RP-HPLC method has been developed for the simultaneous estimation of Aspirin (ASP), Atenolol (ATN), Hydrochlorothiazide (HCTZ), Ramipril (RAM) and Simvastatin (SIM) in capsule dosage form. Separation was achieved on Hypersil Gold column (250 mm X 4.6 mm, 5 µ) using mobile phase consisting of methanol: water in the ratio of 95:5.0 v/vat a flow rate of 1 mL/min and UV detection at 230nm. The retention times of ASP, RAM, HCTZ, SIM and ATN were 1.983 min., 2.525 min., 3.108 min., 3.867 min. and 7.833 min. respectively. The method was validated as per ICH guidelines. The

linearity of the proposed method were investigated in the range of 20–120 µg/mL for ASP, 1-6 µg/mL for RAM, 2.5-15 µg/mL for HCTZ, 4-24 µg/mL for SIM and 10-60 µg/mL for ATN, respectively. The LOD and LOQ were found to be 0.6µg/mL, 1.4µg/mL and 0.2µg/mL, 0.5µg/mL and 0.23 µg/mL, 0.6 µg/mL and 1.32µg/mL, 3.97µg/mL and 1.98µg/mL, 5µg/mL for ASP, RAM, HCTZ, SIM and ATN respectively. The recoveries of ASP, RAM, HCTZ, SIM and ATN were found to be 99.60%, 99.40%, 99.13%, 99.46% and 99.54% respectively. Validation studies demonstrated that the proposed RP-HPLC method is rapid, selective, requires a simple sample preparation procedure and it is suitable for analysis of ASP, RAM, HCTZ, SIM and ATN, in their formulation in a single isocratic run, in contrast with previous gradient methods. Hence, the proposed method can be conveniently adopted for the routine quality control analysis in the multicomponent formulation and also suitable for analysis of dissolution and kinetic studies.

Simultaneous analysis of Atenolol and Indapamide by HPTLC:

A new, simple, accurate and precise HPTLC method has been developed and validated as per ICH guidelines for the simultaneous determination of Atenolol (ATN) and Indapamide (IND) in pharmaceutical dosage form. The separation of these drugs were achieved on aluminium HPTLC plate precoated with silica gel $60F_{254}$, using toluene: ethyl acetate: methanol: ammonia in the ratio of 5:3:3:0.1(v/v/v/v) as mobile phase. Detection was performed densitometrically at 229 nm. The Rf value of ATN and IND were 0.27 and 0.71, respectively. Linearity was observed in the concentration range of 200-1200 ng/spot for ATN and 100- 600 ng/spot for IND. LOD and LOQ were found to be 35ng/spot, 125 ng/spot and 25ng/spot, 80 ng/spot for ATN and IND respectively. Percent recoveries obtained for ATN and IND were 99.95% and 99.67%, respectively. The results revealed no interference of excipients. The proposed method can be used for routine quantitative estimation of Atenolol and Indapamide from their combined dosage form.

Simultaneous analysis of Propranolol HCl and Hydrochlorothiazide by HPTLC:

A new, simple, accurate and precise HPTLC method has been developed and validated for the simultaneous determination of Propranolol HCl (PHCl) and Hydrochlorothiazide (HCTZ) in pharmaceutical dosage form. The separation was achieved on aluminium HPTLC plate precoated with silica gel $60F_{254}$, using chloroform: ethyl

acetate: methanol in the ratio of 4: 4: 2 v/v/v/v as mobile phase. Detection was performed densitometrically at 274 nm. The Rf value of PHCl and HCTZ were found to be 0.27 and 0.56, respectively. Linearity was observed in the concentration range of 160-960 ng/spot for PHCl and 100- 600 ng/spot for HCTZ. LOD and LOQ were found to be 80ng/spot, 135 ng/spot and 40ng/spot, 60 ng/spot for PHCl and HCTZ respectively. Percent recoveries obtained for PHCl and HCTZ were 99.63% and 99.15%, respectively. The Statistical analysis proves that the method is repeatable and selective for the simultaneous determination of PHCl and HCTZ. The proposed method can be used for routine analysis of PHCl and HCTZ from their combined dosage form.

Simultaneous analysis of Bisoprolol fumarate and Hydrochlorothiazide by HPTLC:

A new HPTLC method has been developed and validated for the simultaneous estimation of Bisoprolol fumarate (BSF) and Hydrochlorothiazide (HCTZ) in tablet dosage form. Chromatographic separation was performed on aluminium HPTLC plate precoated with silica gel $60F_{254}$, using ethyl acetate: methanol: ammonia 10:0.5:0.5 (v/v/v) as mobile phase. Detection was carried out densitometrically at 225 nm. The Rf value of BSF and HCTZ were found to be 0.60 and 0.38, respectively. Linearity was observed in the concentration range of 150-900 ng/spot for BSF and 100- 600 ng/spot for HCTZ. LOD and LOQ were found to be 50ng/spot, 100 ng/spot and 25ng/spot, 50 ng/spot for BSF and HCTZ respectively. Percent recoveries obtained for BSF and HCTZ were 99.33% and 99.60%, respectively. All the analytical validation parameters were determined and found within the acceptable limit as per ICH guidelines. The proposed method can be used for the routine analysis of Bisoprolol fumarate and Hydrochlorothiazide from its dosage forms.

Simultaneous analysis of Nebivolol HCl and Hydrochlorothiazide by HPTLC:

A simple, accurate and precise HPTLC method has been developed and validated for the simultaneous determination of Nebivolol HCl (NEB) and Hydrochlorothiazide (HCTZ) in pharmaceutical dosage form. The separation was achieved on aluminium HPTLC plate precoated with silica gel $60F_{254}$, using chloroform: toluene: methanol: ammonia 5:3:2:0.1 (v/v/v/v) as mobile phase. Detection was performed densitometrically at 278 nm. The Rf value of NEB and HCTZ were found to be 0.42 and 0.30 respectively. Linearity was observed in the concentration range of 180-630 ng/spot for NEB and 60- 210 ng/spot for

HCTZ. The method was validated as per ICH guidelines. LOD and LOQ were found to be 40ng/spot, 160 ng/spot and 30ng/spot, 50 ng/spot for NEB and HCTZ respectively. Percent recoveries obtained for NEB and HCTZ were 99.93% and 99.98%, respectively All validation parameters were within the acceptable range. Hence, the proposed method can be useful for routine quality control analysis of these drugs in tablet formulation.

In the present study, HPLC and HPTLC methods were developed and validated as per ICH guidelines for multicomponent analysis of some anti-hypertensive drugs. The developed HPLC and HPTLC methods have been validated for the linearity, accuracy, precision and robustness in order to ascertain the suitability of the analytical method. As the methods are found to be precise, robust and accurate, they may have good commercial value. Thus the newly developed analytical methods can be used in research institutions, industries and testing laboratories for routine analysis.

Publications and Presentations:

Publications:

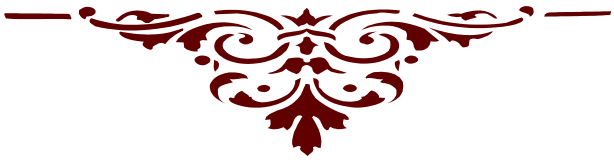
1. Savita S Yadav and Janhavi R Rao, "Simultaneous HPTLC Analysis of Atenolol and Indapamide in Tablet Formulation", International Journal of Comprehensive Pharmacy (IJCP), 9 (2): 2011, 1-4.
2. Savita S Yadav, Janhavi R Rao, "Spectrophotometric estimation of Nebivolol hydrochloride and HPTLC method for simultaneous determination of Nebivolol hydrochloride and Hydrochlorothiazide in bulk drug and in pharmaceutical formulation", Asian Journal of Research in Chemistry, 5(5): 2012, 576-581.
3. Savita S Yadav, Janhavi R Rao, "Simultaneous HPTLC Analysis of Bisoprolol Fumarate And Hydrochlorothiazide In Pharmaceutical Dosage Form", International Journal of Pharmacy and Pharmaceutical Sciences, 2013, 5 (2), 286-290.
4. Savita S Yadav, Janhavi R Rao, "Simultaneous Estimation of Losartan, Hydrochlorothiazide and Atenolol from Solid Dosage Form by RP-HPLC", International Journal of Pharmacy and Pharmaceutical Sciences, 2014, 6 (1), 283-288.
5. Savita S Yadav, Janhavi R Rao, "RP-HPLC Method for Simultaneous Estimation of Losartan, Hydrochlorothiazide and Amlodipine in Tablet Dosage Form", Asian Journal of Pharmaceutical and Clinical Research, 2014, 7(1), 137-140.
6. Savita S Yadav, Janhavi R Rao, "RP-HPLC Method for Simultaneous Estimation of Aspirin, Ramipril, Hydrochlorothiazide, Simvastatin And Atenolol From Pharmaceutical Dosage Form", International Journal of Pharmacy and Pharmaceutical Sciences, 2014, 6(9); 443-448.

Presentations:

1. Savita S Yadav, Anuja Nevase, Janhavi R Rao. Simultaneous Estimation of Losartan, Hydrochlorothiazide and Atenolol from Solid Dosage Form by RP-HPLC, Presented in University of Pune Sponsored Conference on "Drug Discovery-Past, Present and Future" in 10th and 11th Oct. 2013, held at MAEER's Maharashtra Institute of Pharmacy, MIT Campus, Paud Road, Pune.
2. Savita S Yadav, Snehal Chaudhari, Janhavi R Rao, Simultaneous HPTLC Analysis of Bisoprolol Fumarate and Hydrochlorothiazide In Pharmaceutical Dosage Form. Presented in UGC Sponsored National Seminar on "Recent Trends in Chemical and material science" in 12nd and 13th Jan. 2013 held at B.V.D.U. Yashwantrao Mohite College Erandwane, Pune.
3. Savita Yadav and Janhavi Rao, RP-HPLC Method for Simultaneous Estimation of Aspirin, Ramipril, Hydrochlorothiazide, Simvastatin and Atenolol from Tablet Dosage Form, presented in "World Congress on Pharmaceutical Sciences and Chemical Technology" in 16th -18th Dec. 2013 held at Colombo, Sri Lanka.

CHAPTER 1

INTRODUCTION



CHAPTER 2

AIMS AND OBJECTIVES

CHAPTER 3

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CHAPTER 5

DEVELOPMENT OF ANALYTICAL METHODS FOR THE SIMULTANEOUS ANALYSIS OF ANTIHYPERTENSIVE FORMULATIONS CONTAINING THREE COMPONENTS BY HPLC

CHAPTER 6

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SIMULTANEOUS ANALYSIS OF PROPRANOLOL HCL AND HYDROCHLOROTHIAZIDE BY HPTLC

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SIMULTANEOUS ANALYSIS OF ATENOLOL AND INDAPAMIDE BY HPTLC

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SIMULTANEOUS ANALYSIS OF ASPIRIN, RAMIPRIL, HYDROCHLOROTHIAZIDE, SIMVASTATIN AND ATENOLOL BY HPLC

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SIMULTANEOUS ANALYSIS OF LOSARTAN POTASSIUM, ASPIRIN, ATORVASTATIN AND ATENOLOL BY HPLC

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SIMULTANEOUS ANALYSIS OF LOSARTAN POTASSIUM, HYDROCHLOROTHIAZIDE AND ATENOLOL BY HPLC

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SIMULTANEOUS ANALYSIS OF LOSARTAN POTASSIUM, RAMIPRIL AND HYDROCHLOROTHIAZIDE BY HPLC

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SIMULTANEOUS ANALYSIS OF LOSARTAN POTASSIUM, HYDROCHLOROTHIAZIDE AND AMLODIPINE BY HPLC

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SIMULTANEOUS ANALYSIS OF AMLODIPINE BESYLATE AND RAMIPRIL BY HPLC

4.3

SIMULTANEOUS ANALYSIS OF AMLODIPINE BESYLATE AND HYDROCHLOROTHIAZIDE BY HPLC

4.2

SIMULTANEOUS ANALYSIS OF AMLODIPINE BESYLATE AND INDAPAMIDE BY HPLC

4.1

SIMULTANEOUS ANALYSIS OF NEBIVOLOL HYDROCHLORIDE AND INDAPAMIDE BY HPLC