THE THESIS

ENTITLED

"DEVELOPMENT AND VALIDATION OF NEWER ANALYTICAL METHODS FOR SOME GROUP OF PHARMACEUTICALS FROM IT'S BULK AND PHARMACEUTICAL DOSAGE FORM"

Submitted to

Sumandeep Vidyapeeth

In partial fulfillment of the requirement for the award of

Doctor of philosophy

In

Pharmaceutical Sciences

By

ASHIM KUMAR SEN

(Registration No: Ph.D. 011 2011)

Under the guidance of

Guide	Co-Guide
Dr. R. Balaraman, PhD, FAMS	Dr. A. K. Seth, M. Pharm., PhD
Professor	HOD/Director
Department of Pharmacy	Department of Pharmacy
Sumandeep Vidyapeeth	Sumandeep Vidyapeeth

DEPARTMENT OF PHARMACY
SUMANDEEP VIDYAPEETH
PIPARIA, VADODARA-391760, GUJARAT, INDIA

JUNE, 2015

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Ashim Kumar Sen

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LIST OF SYMBOLS & ABBREVIATION

µ	:	micron
µg	:	Micro gram
µl	:	Micro liter
µm	:	Micro meter
^{0}C	:	Centigrade temperature
Abs.	:	Absorbance
ACN	:	Acetonitrile
ALI	:	Aliskiren hemifumarate
AMLO	:	Amlodipine besilate
ANOVA	:	Analysis of variance
Å	:	Angstrom
API	:	Active pharmaceutical ingredient
AR	:	Analytical reagent
ATS	:	Automatic TLC sampler
BP	:	British Pharmacopoeia
cm	:	Centimeter
conc.	:	Concentration
DS	:	Derivative spectrophotometry
EMR	:	Electro magnetic radiation
FA	:	Fumaric acid
gm	:	Gram
HCT	:	Hydrochlorothiazide
HPLC	:	High performance liquid chromatography
HPTLC	:	High performance thin layer chromatography
ICH	:	International conference on harmonization
id	:	Internal diameter
IP	:	Indian Pharmacopoeia
IS	:	Internal standard
IUPAC	:	International union of pure and applied chemistry
K	:	Capacity factor

LC	:	Liquid chromatography
LC-MS	:	Liquid chromatography and mass spectroscopy
LOD	:	Limit of detection
LOQ	:	Limit of quantitation
M	:	Molar
MeOH	:	Methanol
mg	:	Milli gram
min	:	Minute
ml	:	Milli liter
mm	:	Milli meter
mM	:	Milli molar
N	:	Theoretical plates
ng	:	Nano gram
nm	:	Nano meter
r^2	:	Correlation coefficient
RP-HPLC	:	Reversed phase high performance liquid Chromatography
R_s	:	Resolution
RSD	:	Relative standard deviation
R_t	:	Retention time
S/N	:	Signal / noise
SD	:	Standard deviation
T	:	Tailing factor
TLC	:	Thin layer chromatography
USP	:	United States Pharmacopoeia
UV	:	Ultra violet
v/v/v	:	Volume / volume/ volume
VAL	:	Valsartan
WHO	:	World Health Organization
λ_{max}	:	Absorbance maximum
σ	:	Sigma

1 INTRODUCTION

1.1 Analytical chemistry

Analytical chemistry is a measurement science useful in all fields of science and medicine which consists of powerful ideas and methods[1] and it is deals with the chemical characterization of matter and it describes what is it and how much it is i.e., qualitative and quantitative analysis, respectively. A qualitative analysis gives information regarding the identity of atomic or molecular species or the functional groups in the sample; a quantitative method provides numerical information as to the relative amount of one or more of these components often follows the series of steps summarized in Figure No. 1.1.

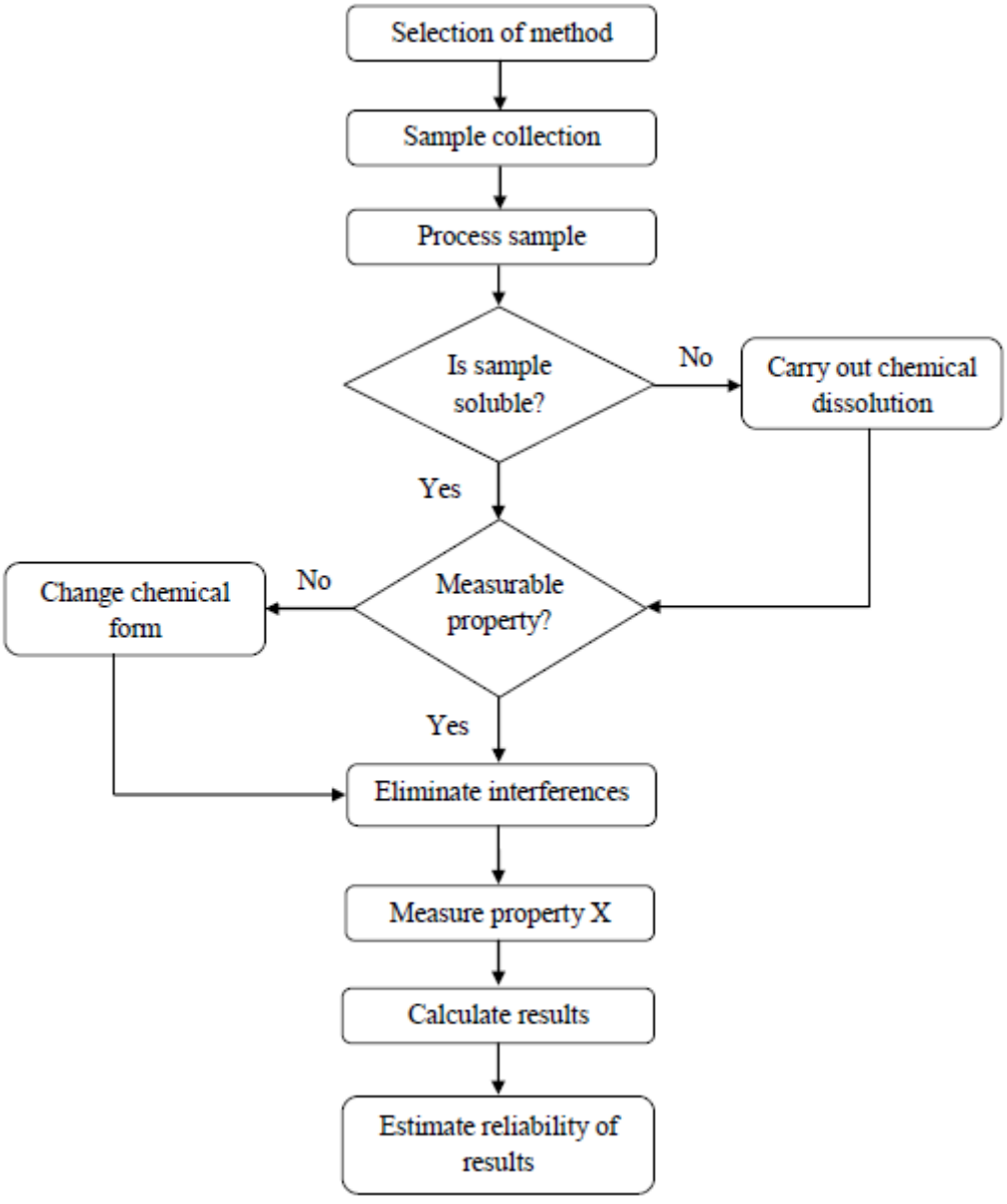


Figure No. 1.1: Steps of quantitative analysis

Analytical chemistry gives information about the chemical composition of natural and artificial materials. Different analytical techniques are employed to identify and quantify the substances which may be present in a material and capable of estimating exact amount of the identified substance. An analytical chemist works to improve the reliability and acceptability of existing techniques to meet the demand for exact measurements which arise constantly in our society. Analytical chemist adapts widely accepted and proven methodologies to know about new materials or to answer new kinds of questions about their contents and their mechanism of reaction or pathway. They carry out research work to discover completely novel principles or techniques of analysis and taking towards major discoveries like lasers and microchip devices for useful purposes. They are serving the need of many fields based on the requirement.

- **In medicine**, analytical chemistry is the basis for diagnostic laboratory tests which helps physician indentifies disease and adding speed to recovery.
- **In industry**, they are responsible for testing raw materials and assuring the quality of finished products for end user.
- **Environmental quality** is often evaluated by testing for unwanted contaminants or pollutant using precise and sensitive technique of analytical chemistry.
- **The nutritional value** or quality of food products such as protein and carbohydrates and trace components such as minerals, vitamins and micronutrients are analyzed by chemical analysis for major components. Even the calories in food products are often calculated from its chemical analysis.

Analytical chemistry plays most important role nearly all aspects of chemistry, such as environmental, agricultural, clinical, forensic, manufacturing, metallurgical and pharmaceutical chemistry. Contaminants and essential nutrients of foods should be analyzed by analytical chemist. The nitrogen content of a fertilizer, carbon monoxide content of air, blood glucose level of diabetics should be monitored by different alalytical techniques.[1-2]

1.2 Analytical methods

Highly specific and sensitive analytical techniques hold the key to design, development, standardization and quality control of medicinal products. They are equally important in drug metabolism and pharmacokinetics studies, which are helpful for assessment of bioavailability and the duration of clinical response. Modern analytical techniques are extremely selective and sensitive, provides precise

information about small samples in presence of other analytes. Now a day's most of the analytical techniques are automated and able to give results very fast. For these reasons analytical techniques are now in widespread use in drug development, manufacturing, stability studies and therapeutic drug monitoring.[3]

1.3 General classification of analytical methods

Analytical methods are often classified as classical/traditional and instrumental method. This classification is largely historical with classical methods and it is replacing by highly sensitive instrumental methods.

1.3.1 Classical methods

In the early days of chemistry, most of the analyses were carried out by separating the components of interest (the analytes) in a sample by precipitation, extraction or distillation. The separated components were then treated with different types of reagents for qualitative analysis based on the nature of analytes that yielded products that could be identified by their colors, odors, boiling and melting points, solubility study, optical activities or refractive indexes. Gravimetric or titrimetric measurements were used to quantify the analytes present in the supplied sample. The quantity of the desired analytes or related substance produced from the analytes under investigation can be determined by gravimetric analysis. In titrimetric analysis, the volume of standard reagent required to react completely with the analyte was measured.

These different types of classical methods for isolating and determining analytes still find use in many laboratories. But, the extent of their application is declining with time.

1.3.2 Instrumental methods

Early in the twentieth century, analytical chemists started exploring to solve analytical problems with the help of information other than those used in classical methods. Thus, physical properties like electromotive force, conductivity, absorption of light, emission of light, fluorescence, mass to charge ratio came in limelight for quantitative estimation of various substances (organic, inorganic and biochemical origin).

Now a day, highly efficient techniques like chromatography & electrophoresis started replacing older methods like distillation, extraction and precipitation for the isolation of components from complex mixtures prior to their qualitative or quantitative

determination. These different types of newer methods for separating and determining chemical entity are known as instrumental methods of analysis collectively.

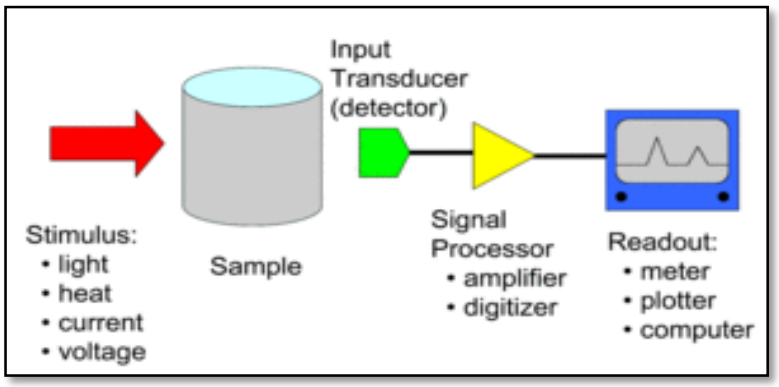


Figure No. 1.2: Block diagram of an analytical instrument

1.3.3 Types of instrumental methods of analysis

Physical and chemical properties are useful for qualitative and quantitative analysis. Table No. 1.1 lists most of the characteristic properties that are currently used for the instrumental methods of analysis.

Table No. 1.1: Chemical and physical properties used in instrumental methods

Characteristic properties	Instrumental methods
Absorption of radiation	Spectrophotometry and photometry (UV, Visible, IR, atomic absorption); photo acoustic spectroscopy, NMR and ESR spectroscopy
Emission of radiation	Emission spectroscopy (fluorescence, phosphorescence luminescence, flame emission spectroscopy)
Scattering of radiation	Turbidimetry, nephelometry, raman spectroscopy
Refraction of radiation	Refractometry, interferometry
Diffraction of radiation	X-ray and electron diffraction method
Rotation of radiation	Polarimetry, optical rotary dispersion, circular dichroism
Electrical potential	Potentiometry, chronopotentiometry
Electrical charge	Coulometry
Electrical current	Amperometry, polarography
Electrical resistance	Conductometry
Mass	Gravimetry (Quartz crystal microbalance)
Mass to charge ratio	Mass spectrometry
Rate of reaction	Kinetic methods
Thermal characteristics	Thermal gravimetry and titrimetry, DSC, DTA, thermal conductometric methods
Radio activity	Activation and isotope dilution methods[4]

Reliable and validated analytical method plays a crucial role throughout the drug development process and these activities are continuous and interconnected. The practice of method validation ensures the performance limits of the measurement. But practically validated method may produce response within known uncertainties. These results are very important steps for drug development process and give knowledge about the product. Lots of time and effort that are put into developing a scientifically-sound, robust and transferrable analytical methods should be aligned with the drug development stage. The resources that are spent on method development and validation must be balanced with regulatory requirements and the probability of commercialized product for end user.

Laboratory resources and method development objectives should be balanced at each stage of drug development process. Method validation is required by regulatory agencies at certain stages of the drug approval process, is defined as the "process of demonstrating that analytical procedures are suitable for their intended use".[5]

Method validation is a continuous process used to confirm that the analytical procedure used for a specific test is suitable for its intended use. Method validation results can be helpful to judge the quality, reliability and consistency of analytical results and it is an integral part of any good analytical practice.

Analytical methods need to be validated or re-validated

- Before their introduction into routine use.
- Whenever the conditions changes for which the method has been validated
- Whenever the method is changed and the change is outside the original scope of the method.

Method validation has received considerable attention in the literature and from industrial committees and regulatory agencies.[6]

Bio-analytical method validation demonstrates that the method is capable of producing reliable results (quantification of desired analytes) with considerable accuracy and precision in presence of other interfering materials present in biological matrix.

Reliable bio-analytical techniques are used in the drug development process to provide valuable support to drug discovery programs on the metabolic fate and pharmacokinetics of chemicals in living cells and in animals. Highly sensitive and selective methods are in use for the quantification of drugs in biological matrices (blood, plasma, serum or urine). The standard calibration curve along with internal

standard is best quantitative approach for biological samples. Calibration standards are used to find out the analyte concentration and blank matrix is used to check any interference between the analytes and matrix. Quality control samples are used to check the accuracy and precision of the developed method. A bio-analytical method should be passed through all the validation parameters as per regulatory guidelines before applying for the drugs and metabolites for their quantitative analysis. As the developed method used in the preclinical, bioavailability, bioequivalence and pharmacokinetic studies, the developed method should have high reproducibility and reliability.

Highly efficient and reliable chromatographic techniques (HPLC or GC) have been widely used for the determination of small molecules in biological sample. In this regard, high performance liquid chromatography coupled with quadrupole mass spectrometry (LC/MS/MS) is the best choice. Developed method is validated to ensure that the method will continue to produce accurate and reproducible results during the sample analysis. The validation process is carried out using a control matrix spiked with the standard compounds to be quantified.[7-9]

The stability-indicating assay method is used to check the stability of a product, which has immense prospect in pharmaceutical industry. With the introduction of "International Conference on Harmonization" (ICH) guidelines, the requirement of establishment of stability-indicating assay method (SIAM) has become more clearly mandated. The guidelines clearly requires the data of forced decomposition studies under a variety of conditions, like acidic or basic pH, photo degradation, oxidation, dry heat, humidity etc. and separation of drug from degradation products.[10]

Impurities in pharmaceutical preparations are the unwanted chemicals that remain present with the active pharmaceutical ingredients (APIs), or evolved during formulation, or upon aging of both API and formulated API's to medicines. The presence of these impurities even in small quantity may influence the safety and efficacy of the pharmaceutical substance. Impurity profiling (i.e., identity as well as the amount of impurity in the pharmaceutical product), is now getting more attention from regulatory authorities. The different official monographs, such as United States Pharmacopoeia (USP), British Pharmacopoeia are slowly fixing permissible limits of impurities present in the API's or pharmaceutical formulations.[11]

More importantly various authorities like ICH, USFDA, Canadian Drug and Health Agency are working on the purity requirements and the identification of impurities in

Active Pharmaceutical Ingredient's (API's). Impurity profiling is getting considerable attention in pharmaceutical research, which establishes biological safety of an individual impurity. Various chromatographic and spectroscopic techniques (TLC, HPTLC, HPLC, AAS etc.) are used alone or in combination for identification and characterization of impurities. Conventional Liquid Chromatography, specifically, HPLC has been used in field of impurity profiling due to the sensitivity, cost per analysis and range of detectors available. Moreover, TLC is most commonly used techniques for the isolation of impurities due to the ease of operation and low operational cost as compared to HPLC. Advanced technique like HPTLC with widely accepted automation technology becoming more powerful tool for the impurity isolation.[12]

1.3.4 Ultraviolet-visible absorption spectrophotometry

Ultraviolet-visible spectrophotometry is one of the most frequently used techniques in pharmaceutical analysis. Ultraviolet (190-380 nm) and visible (380-800 nm) spectrophotometry is involves in the measurement of absorbed radiation by a substance in solution and the instrument used for the purpose is called ultraviolet/visible spectrophotometer. Absorption of light in both the ultraviolet and visible regions of the light matches that required to induce in the molecule an electronic transition and its associated vibrational and rotational transition.

The UV radiation has sufficient energy to excite valence electrons in many atoms or molecules. Consequently UV is involved with electronic excitation. Sometimes because of this electronic excitation, ultraviolet spectroscopy is also known as electronic spectroscopy. The measurement of absorption of ultraviolet radiation provides a convenient means for the analysis of numerous inorganic and organic species.

Absorption of light in ultraviolet region of the electromagnetic spectrum occurs when the energy of light matches that required to induce in the molecule an electronic transition and its associated vibrational and rotational transitions.

A compound or drug possess conjugated double bond, absorbs UV radiation at a specific wavelength and this character of drug is specific for a fixed solvent system.

The wavelength at which maximum absorption occurs is called λ_{max}. It is independent of concentration.

The drugs are quantitatively analyzed by ultraviolet analytical method; it is governed by Beer's & Lambert's law, which is represented as

$$A = abc$$

Where,

A = absorbance

a = absorptivity

b = path length

c = concentration

This relationship exists between the absorbance and the concentration.

1.3.5 Spectrophotometric multi-component analysis

The various spectroscopic techniques used for multi-component analysis are as follows

- Simultaneous equation method (vierodt's method)
- Absorbance ratio method
- Geometric correction method
- Orthogonal polynomial method
- Difference spectrophotometry
- Derivative spectrophotometry
- Two wavelength method
- Absorption factor method (absorption correction method)

The basis of all the spectrophotometric techniques for multi component samples is the property that at all wavelengths,

a. The absorbance of a solution is the sum of absorbances of the individual components or
b. The measured absorbance is the difference between the total absorbance of the solution in the sample cell and that of the solution in the reference cell.

1.3.5.1 Simultaneous equation method (vierodt's method)

If any sample contains two absorbing species (X and Y) and both of them absorbs at the λ_{max} of the other, simultaneous equation (Vierodt's method) method is a suitable technique for the determination of both the drugs simultaneously using the following formula:

$$Cx = \frac{\text{A2 ay1} - \text{A1ay2}}{\text{ax2 ay1} - \text{ax1ay2}}$$

$$Cx = \frac{\text{A1 ax2} - \text{A2 ax1}}{\text{ax2 ay1} - \text{ax1ay2}}$$

Where, Cx and Cy are the concentrations of X and Y, ax_1 and ax_2 are absorptivities of X at λ_1 and λ_2, respectively. ay_1 and ay_2 are absorptivities of Y at λ_1 and λ_2, respectively. A_1 and A_2 are the absorbances of formulation at λ_1 and λ_2.

Absorptivity = absorbance / concentration (gm/100 ml)

Criteria for getting maximum precision using this method are:

- The λ_{max} of X and Y are dissimilar
- Two components do not interact chemically

1.3.5.2 Absorbance ratio method

Absorbance ratio method (Q analysis) is a modification of simultaneous equation (Vierodt's method). This method is depends on the property that, a ratio of absorbance at any two wavelengths is a constant value, does not depend on concentration or pathlength for a substance which obeys Beer's law at all the wavelengths. For example, two different dilutions of the same substance give the same absorbance ratio is referred to as 'Q value' in USP. Two components can be measured simultaneously, using this method where absorbances are measured at two wavelengths (one is the λ_{max} of one components and other being a wavelength of equal absorptivity, i.e. iso-absorptive point).

$$\text{Cx} = \frac{Qm - Qy}{\text{Qx} - \text{Qy}} \times \frac{\text{A1}}{ax1}$$

$$\text{Cy} = \frac{Qm - Qx}{\text{Qy} - \text{Qx}} \times \frac{\text{A1}}{ay1}$$

Where, Cx and Cy are the concentrations of X and Y, ax_1 and ax_2 are absorptivities of X at λ_1 and λ_2, respectively. ay_1 and ay_2 are absorptivities of Y at λ_1 and λ_2, respectively. A_1 and A_2 are the absorbances of formulation at λ_1 and λ_2.

Absorptivity = absorbance / concentration (gm/100 ml)

$$Qm = \frac{A2}{A1} \qquad Qx = \frac{ax2}{ax1} \qquad Qy = \frac{ay2}{ay1}$$

1.3.5.3 Derivative spectrophotometry

Derivative UV spectroscopy has been widely used as a tool for quantitative analysis and quality control. It involves the transformation of a normal UV spectrum to its 1st, 2nd or higher derivative spectrum. The transformations take place in the derivative spectra as compared to normal UV spectra can be described by reference to a Gaussian band, which represents an ideal absorption band.

The 1st derivative (D1) spectrum is a plot of the rate of change of absorbance with wavelength against wavelength or a plot of dA/dλ versus λ. The 2nd derivative (D2) spectrum is a plot of the curvature of the D° spectrum against wavelength or a plot of $d2/d\lambda_2$ versus λ.

These spectral transformations offer two main advantages on derivative spectrophotometry are

a) Derivative spectrum shows improvement in resolution of two overlapping bands than the fundamental spectrum and may allow the accurate determination of the λ_{max} of the individual bands.
b) Derivative spectrophotometry enhances bandwidth discrimination.

For the purpose quantitative analysis, 2nd and 4th derivative methods are most widely used derivative orders.[3]

1.3.6 Chromatography

Chromatography is a group of powerful separation techniques useful in all fields of science. In this technique mixture of compounds can be separated into individual analytes under study using two phases, i.e. mobile and stationary phase. The technique "Chromatography" was invented by Russian botanist Mikhail Tswett and used the technique for separating various plant pigments. A glass column packed with calcium carbonate was used as stationary phase for separating chlorophylls and xanthophylls from plant pigments. Separated compounds formed colored bands on the column and acquired the name chromatography (Chroma means "color" and graphein means "write"). There are various advanced chromatographic techniques, which are most reliable and widely used for the analysis of multi component drugs in their formulation namely,

- High performance liquid chromatography (HPLC)
- High performance thin layer chromatography (HPTLC)
- Gas chromatography (GC)

High performance liquid chromatographic separation is based on interaction and differential partition of the sample between the mobile phase and stationary phase. The commonly used chromatographic methods can be roughly divided into the following groups,

- Normal phase
- Reverse phase
- Ion-exchange
- Ion pair/affinity
- Chiral
- Size exclusion

These techniques are preferred because of its improved performance as compared to classical column chromatography in terms of specificity, rapidity, sensitivity, accuracy, convenience, ease of automation and the cost per analysis.

Advancement in column technology, high pressure pumping system and highly sensitive and compatible detectors have transformed liquid column chromatography into a high speed, efficient, accurate and highly resolved method of separation.

Apart from individual separation method, some highly efficient hybrid techniques (LC-MS, GC-MS, CE-MS, LC-NMR etc.) are becoming popular for widespread use in drug development, manufacturing, stability studies and therapeutic drug monitoring.

1.3.6.1 High performance thin layer chromatography

High performance thin layer chromatography (HPTLC) is advanced and automated version of thin layer chromatography (TLC). TLC is one of the most frequently employed tools for the identification and quantification of various drugs and its formulations. Moreover, it can be used for the analysis of materials in pure state, formulation or biological samples. Perhaps this is the most useful techniques under planar chromatography.

1.3.6.1.1 Principle

The principle of separation involved in HPTLC is adsorption. Here liquid mobile phase flows through the solid stationary phase (TLC plate) due to the capillary action. The separation of components is achieved based on the affinity differences between analytes under investigation towards stationary phase.

1.3.6.1.2 Steps of HPTLC separation

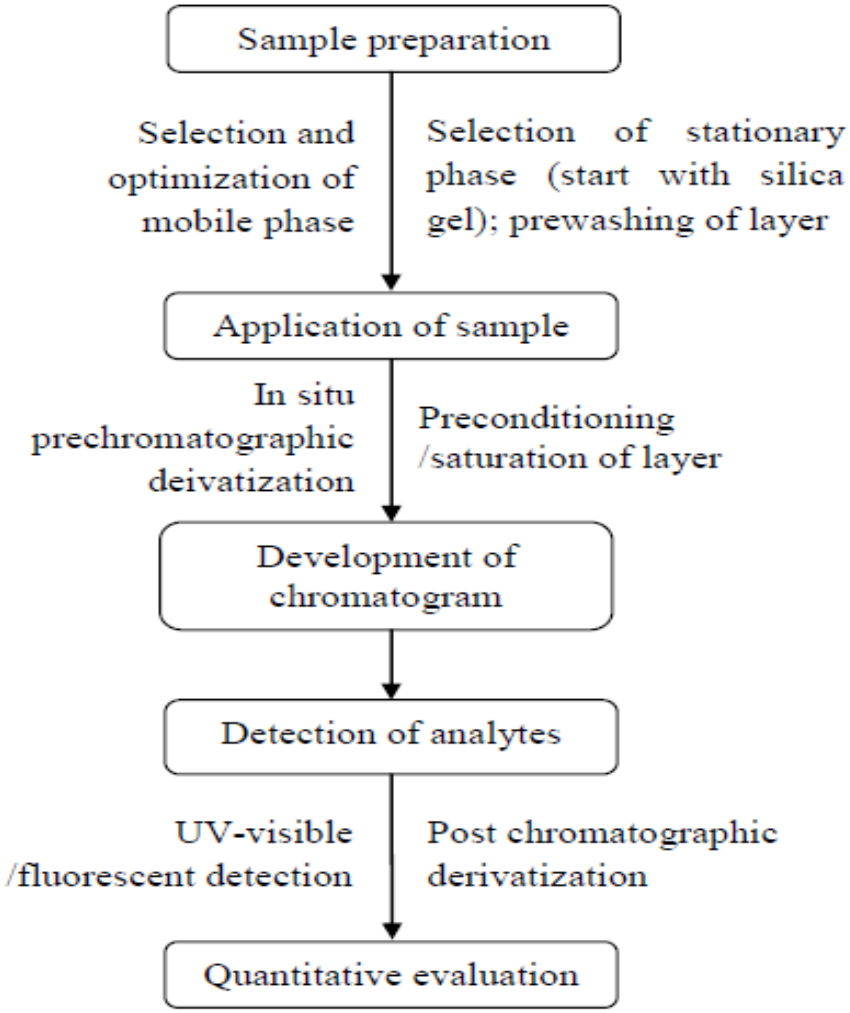


Figure No. 1.3: Steps of HPTLC analysis

1.3.6.1.3 Selection of HPTLC plates and sorbents for the technique

Availability of pre-coated TLC plates along with different support materials and different sorbents made this technique very versatile. HPTLC requires uniformly distributed high quality and lower particle size silica (5 μ) as stationary phase to maintain its efficiency. High performance grade silica is much more efficient and gives reproducible results compared to conventional grade of silica.

1.3.6.1.4 Pretreatments of the TLC plate

TLC plates used for this technique should be free from any type of impurities. Supplied pre-coated plates may be contaminated due to the adsorption of atmospheric materials. This may leads to unwanted peaks, increase in base line noise and reduced sample signal. For this purpose prewashing is required with methanol. Moreover, some other steps like activation (110-120°C for 30 min), conditioning and equilibration of plates are necessary to get optimum efficiency and reproducibility.

1.3.6.1.5 Sample preparation technique

Sample preparation for HPTLC is not challenging unlike other type of chromatographic techniques. Samples should dissolve in a non polar and volatile solvent to avoid spreading of spots and can be applied on the plate directly. Sample and standard compound should be dissolved using same solvent to monitor any chromatographic changes.

1.3.6.1.6 Application of sample on pre-coated plates

Sample application is one of the most important steps for better separation and quantification by HPTLC. Usually analyte concentration ranging from 0.1-1 μg/ml is preferred. Too much concentration may leads to poor separation and band broadening. Moreover, application process should not make any changes to the pre-coated layer to avoid unevenly shaped spots. Better separation can be achieved by spotting the samples as band on the TLC plate.

1.3.6.1.7 Optimization of mobile phase

Mobile phase Optimization is necessary to achieve better separation of analyte. Mobile phase selection for simple mixture can be done easily by trial and error method. R_f value ranged from 0.2-0.8 can be a better choice. Polar stationary and non-polar mobile phase is used for nonmal phase TLC and non-polar stationary phase and polar mobile phase is used for RP-TLC.

1.3.6.1.8 Chamber saturation

Chamber saturation is pre-requisites for HPTLC separation. Reproducible results can be obtained by fixing the saturation time, though saturation time may vary depending on the chamber size and volume of mobile phase available. R_f value may increase if chamber saturation is not up to the mark and may decrease if over saturation occurs.

1.3.6.1.9 Chromatographic development and drying

Ascending, descending, two dimensional, horizontal, multiple over run gradient, radial and anti radial are the most common modes of chromatographic development. Rectangular shaped twin trough glass chambers are preferred in ascending development. But now a day's different shape of chambers and automated multiple development chambers are frequently employed for carrying out different types of TLC analysis. Developed plate is removed from development tank as quickly as possible followed by drying and detection.

1.3.6.1.10 Detection and visualization

Detection and quantification of drug using advanced scanner within a short span of time made this technique very versatile. The technique is non-destructive in nature and UV/fluorimetric detector can be used for the identification and quantification of different compounds at 254 and 366 nm.

1.3.6.1.11 Quantification

HPTLC is capable enough to separate various compounds from a mixture and complete recovery of sample is possible. Sample and standard can be spotted and chromatographed using similar condition to find out the analyte concentration.

1.3.6.1.12 Advantages of HPTLC

There are several advantages of HPTLC are

1. Easy to learn and operate, availability of pre-coated plates made this technique very fast.
2. Time required for interpretation of data is very less.
3. Low maintenance cost, lower analysis time and solvent consumption.
4. Qualitative and quantitative analysis can be done simultaneously.
5. Wide range of stationary phase and mobile phase made enormous scope for this technique.

6. Finger print analysis can be done to check purity and identity along with adulteration and substitution.
7. Multi component analysis along with wide applicability made this technique very versatile.
8. Permanent documentation is possible alongside chromatogram.
9. Post-chromatographic derivatization can be done for analyzing non UV absorbing materials.
10. Filtration and degassing is not required for solvents used in this technique.
11. Dirty samples can be applied on the plate directly without filtration.
12. Mobile phases with varying pH can be employed unlike HPLC where high pH solutions may damage the column.[13-14]

1.3.6.2 High performance liquid chromatography

HPLC can be defined as a "separation technique used for the separation of the compounds of mixtures by their continuous distribution between two phases, one of which is moving past the other." In order to recognize the role of individual molecules, HPLC which is an superior form of liquid chromatography is widely used in separation of high molecular weight and complex mixture of molecules present in most widely used system because the specificity and precision of the HPLC method is excellent.

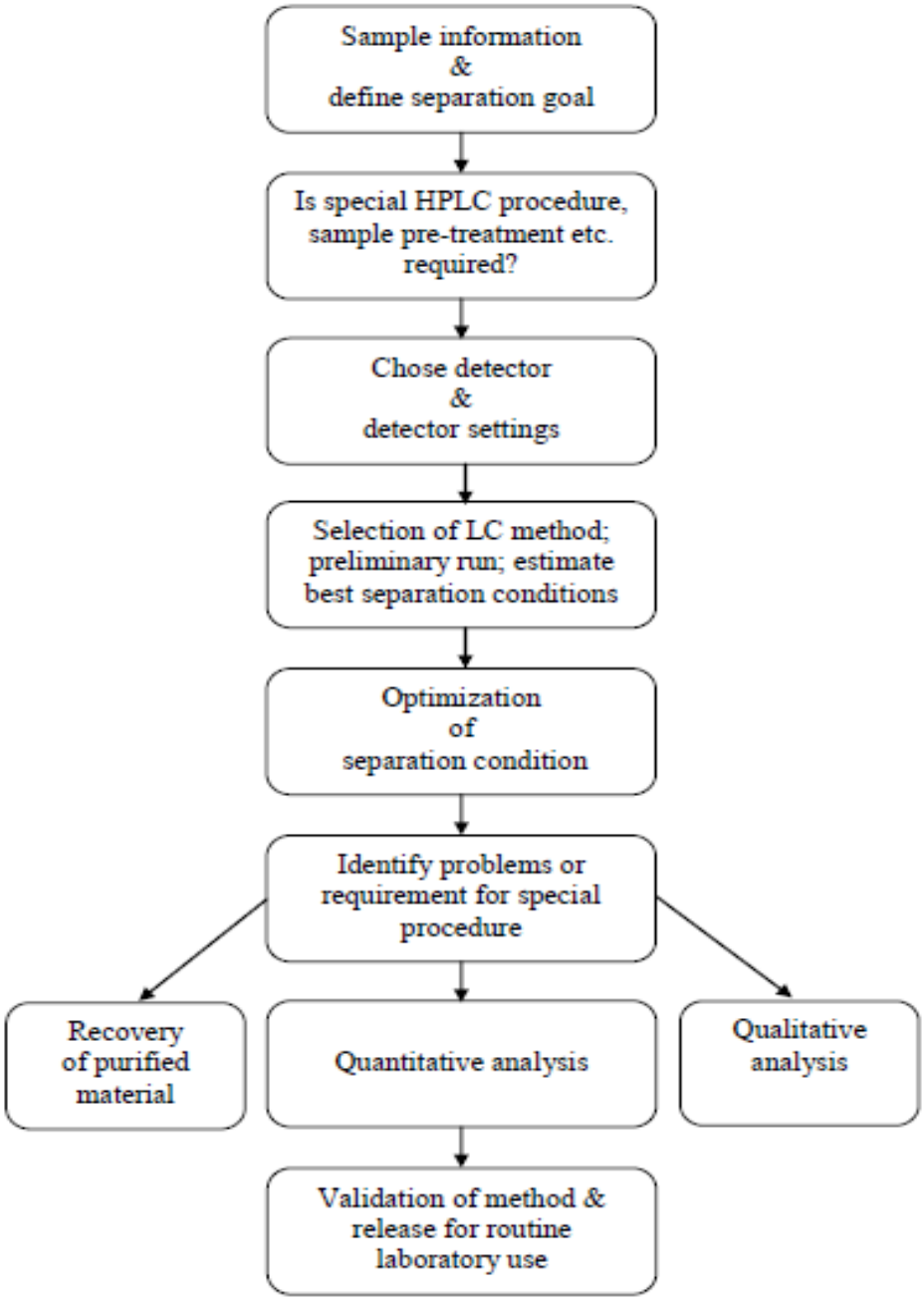


Figure No. 1.4: Steps involved in HPLC method development

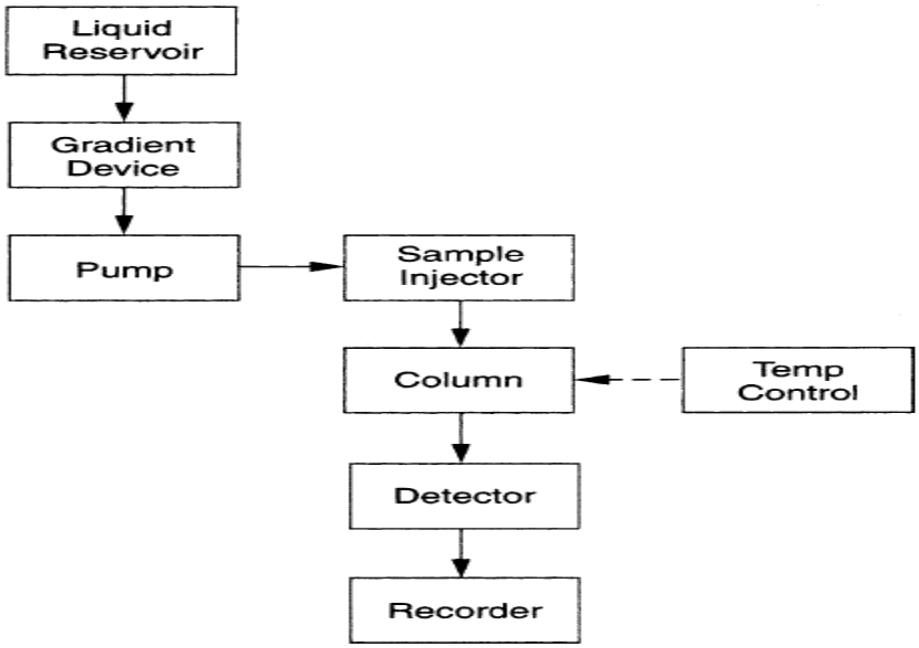


Figure No. 1.5: A schematic of HPLC equipment

HPLC techniques have the following advantages

a. HPLC has high resolving power.
b. Quantitative measurement is accurate.
c. Reproducible and repetitive analysis using the same column.
d. Data handling and analytical procedure is automatic.
e. Column effluents are continuously monitored.
f. Speed of separation is high. **[15]**

Mainly two types of detectors are used in HPLC.

HPLC column can be called as the heart of the chromatographic system. There is necessity of high efficiency columns for the better separation of components. The pharmaceutical industry is the primary driving force for HPLC columns towards higher speed, and better peak shapes and high resolution for analytes. In addition to this, QC laboratories have demanded that column batch-to-batch reproducibility should be improved. Starting from the 1970s to 1990s, "standard" particle sizes of the column packing material has been gradually reducing from 10 to 3 μm and also quality of column packing materials have been steadily improved. In the late 1980s, the introduction of type B silica materials which have high-purity (with low metallic content) was a huge step. This resulted in reduced silanol activity and improved lot-to-lot consistency. Nowadays all modern silica-based columns use the high-purity silica. [16-17]

1.3.6.2.1 UV detector

This is one of the widely used detectors in HPLC system; UV detector can monitor several wavelengths simultaneously by applying a scanning program with multiple wavelengths. UV detectors are able to detect all UV-absorbing components in case they are present in adequate quantity. UV detector is less sensitive compared to PDA detector.

1.3.6.2.2 PDA Detector

PDA detector is used widely nowadays. The advantage of PDA is when it is used a wavelength range can be programmed and in a single analysis all the compounds that absorb within the given range can be identified. PDA detector can also analyze peak purity by matching spectra within a peak.

Accuracy is attainable in HPLC only if wide-ranging system suitability tests are performed before the HPLC analysis. HPLC is widely used for assay of several drugs in pharmaceutical formulations and in biological fluids.

However, there are some limitations of HPLC which include lack of long term reproducibility because of the nature of column packing, price of columns and solvents. Nowadays liquid chromatography combined with mass spectrometry (LC–MS) is widely used. In many stages of quality control and assurance within the pharmaceutical industry this technique is the method-of-choice for analytical support. Recently HPLC-MS has been widely used for assay of drugs. This instrument is also useful in analyzing the impurities of degradation products and pharmaceuticals.[18-19]

The "revolution" in ultra-high pressure LC (UHPLC) started in the year 1997. The first commercial UHPLC system was introduced in the 2004. The transformation from HPLC to UHPLC has been very fast. System pressure of UHPLC is high which allow the use of columns packed with particles with smaller size (2 μm) for faster analyses and it results in superior separations of complex samples. [20-21]

The main benefit of UHPLC versus conventional HPLC is faster analysis and reduction of analysis time up to tenfold with similar resolution. This benefit of "faster analysis with good resolution" provides the primary incentive for most users to consider the purchase of the more expensive UHPLC equipment. [21-22]

1.3.7 Development and validation of analytical methods

Development and validation of an analytical method is an essential process in the drug discovery. Without validated analytical method a drug cannot enter into the market although the drug shows good potency. This is done to make sure the quality and safety of the drug.

1.3.8 Method validation parameters

1.3.8.1 Accuracy

Accuracy can be defined as the "closeness of the measured value to the true value". If a method has to be highly accurate the measured value of analyzed method should be identical to the standard value. Recovery studies are carried out to check the accuracy of the method.

Accuracy can be determined by three ways

1. Comparison to a reference standard
2. Standard addition method
3. Recovery of standard drug added to blank matrix

Recovery studies give the information of whether tablet excipients interfere with the drug spectra.

1.3.8.2 Precision

Precision is defined as "the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogenous sample".

According to ICH guidelines precision can be divided in to three types

- Repeatability
- Intermediate precision
- Reproducibility

Repeatability is the "precision of a method under the same operation conditions over a short period of time."

Intermediate precision is the "agreement of complete measurements when the same method is applied many times within the same laboratory".

Reproducibility checks the precision between laboratories and is often determined in mutual studies or method transfer experiments.

A method may found to be precise if the %RSD<2.

1.3.8.3 Linearity and range

Linearity of a method can be defined as a "measure of how well a calibration plot of response vs. concentration approximates a straight line. Measurements are performed at several analyte concentrations". Using the values obtained from linearity data a calibration curve can be plotted and slope, intercept and correlation coefficient values of the calibration curve provide the desired information regarding linearity of the method.

The lower and upper concentration in which a method shows optimum linearity, precision and accuracy is termed as analytical range of a method.

1.3.8.4 Limit of detection and Limit of quantification

The LOD can be defined as "the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value". The LOD is often calculated base on the signal-to-noise ratio (2-3).

The LOQ of an individual analytical method can be defined as "the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy".

1.3.8.5 Specificity

Specificity can be defined as "the ability to assess unequivocally the analyte in the presence of components which may be expected to be present." The method should be able to detect and quantify desired analytes in presence of different impurities, degradant product and matrix. Assuring specificity of a method is the primary steps in developing and validating a good analytical method.

1.3.8.6 Robustness

The robustness of an analytical procedure is a "measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage". The method should be able to resist its critical parameters within the acceptable limit even after small variation in method.

1.3.8.7 System suitability parameters

System suitability tests should be performed prior to analysis of sample to confirm that the instrument is in appropriate condition for the analysis to be performed. The system should generate data within the acceptable range. To check the suitability of

instrument and method, standard solutions are analyzed repeatedly and parameters like peak area reproducibility, plate number (N), tailing factor, resolution etc. are checked. At least two parameters should be checked to establish the suitability of a method.

Column efficiency (N)

Column efficiency is called as number of theoretical plates. It measures that the band spreading number of theoretical plate is higher. If it is higher it indicates good column and system performance.

Column performance can be defined on terms of values of N.

$$N = 16(t_R/w)2 \text{ or } 3500 \text{ L (cm)}/ \text{ dp } (\mu m)$$

Plate height, H = N /L L=length

Capacity factor (K')

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

$$K^1 = \frac{t_R - t_0}{t_0}$$

t_R = band retention time

t_0 = column dead volume

Resolution

The quality of separation is usually measured by resolution R, of adjacent bands.

$$R_s = \frac{2(t_2 - t_1)}{W_1 + W_2}$$

t_1 and t_2 are retention times of the first and second adjacent bands. W_1 and W_2 are baseline bandwidths.

Asymmetry

The asymmetry is a tool for quickly determining how much if any, of an eluting peak profile deviates in shape from a normal distribution. The subscript 'x' refers to the percentage of peak height at which the asymmetry is determined.

Eg: A_{10} (determined at 10% peak height)

The equation for determining peak asymmetry is

$$A_x = b/a,$$

Where,

'b'= the distance between the perpendicular connecting the baseline to peak maximum and the latest eluting portion of the curve.

'a'= the distance between the perpendicular connecting the baseline to the peak maximum and the earliest eluting portion of the curve.[15, 23-25]

2. REVIEW OF LITERATURE

ALISKIREN HEMIFUMARATE

A RP-HPLC method was reported for the quantification of related impurities of aliskiren hemifumarate by Sampath et al (2014). The chromatographic method was optimized using buffer (2.72 gm potassium dihydrogen phosphate and 7.0 gm 1-Octane sulphonoic acid sodium salt was taken in to 1000 ml HPLC grade water and pH 2.7 was adjusted with dilute orthophospharic acid) and Acetonitrile, 70:30%v/v as mobile phase-A and Acetonitrile: water, 90:10%v/v as mobile phase-B. The flow rate was set at 1 ml/min, wavelength at 230 nm, respectively.[26]

A HPLC method was reported for the determination of aliskiren in human plasma through derivatization with 1-naphthyl isocyanate by Belal et al (2013). The separation was achieved on a C18 column using a mobile phase consisting of acetonitrile/water/phosphoric acid (45:55:0.01, v/v/v, pH 3.2) in a flow rate of 1ml/min with UV detection at 230 nm. Caffeine was used as an internal standard. The factors influencing the derivatization reaction yields were carefully studied and optimized. The method was linear over the concentration range of 5-400 ng/ml with a detection limit of 0.5 ng/ml and a limit of quantification of 1.0 ng/ml. The relative standard deviation was less than 4.2% for both intra-day assay and inter-day assay results. The percentage recovery was in the range 97.1-98.6%.[27]

There was a spectrophotometric method for the determination of aliskiren (ALS) in tablets by Mai et al (2013). The method is based on the reaction of ALS with o-phtalaldehyde (OPA) and n-acetylcysteine (NAC) in a basic buffer. The detection wavelength was selected as 335 nm. The method was found to be linear between 10-200 μg/ml under optimized reaction conditions. The regression equation for calibration data was: y = 0.0102X + 0.0606 with a correlation coefficient 0.9996. The molar absorptivity (ε) was 6.3×105 L.mol-1.cm-1. The limit of detection and limit of quantification (LOQ) were 2.8 and 8.5 μg/ml, respectively.[28]

Enantio separation of aliskiren hemifumarate was performed by Ashok et al (2012) on an immobilized-type Chiralpak IC chiral stationary phase using polar organic phase and RP- HPLC. The method was developed and validated using a mixture of acetonitrile and n-butylamine 100:0.1 (v/v/) as a mobile phase with a flow rate of

1.0 ml/min. Detection wavelength was set at 228 nm. Resolution was greater than 3.0 between the two enantiomers. The method was capable of detecting the R-isomer up to a level of 0.2 µg/ml.[29]

A simple and sensitive method has been reported by Zeynep et al (2012) for the estimation of aliskiren (ALS) in its dosage forms and human plasma. The method was described the reaction of the drug with dansyl chloride in presence of bicarbonate solution of pH 10.5 to give a highly fluorescent derivative which was measured at 501 nm with excitition at 378 nm in dichloromethane. The calibration curves were linear over the concentration ranges of 100-700 and 50-150 ng/ml for standard solution and plasma, respectively. The limits of detection were 27.52 ng/ml in standard solution, 4.91 ng/ml in plasma. The mean recovery of ALS from tablets and plasma was 100.10 and 97.81%, respectively.[30]

A RP-HPLC method was developed and validated for rapid assay of Aliskiren Hemifumarate in pharmaceutical dosage form by Raul SK et al (2012). Isocratic elution at a flow rate of 1.0 ml/min was employed on a Symmetry C18 column at ambient temperature. Acetonitrile: phosphate buffer 60:40 (v/v) was used as mobile phase and 234 nm was set as detection wavelength. The method was Linear in the concentration range of 50-175 µg/ml. The retention time for Aliskiren was 2.28 min.[31]

A simple, accurate, precise and reproducible RP-LC method have reported by Swamy et al (2011) for the quantification of Aliskiren in bulk drug and in Pharmaceutical dosage form. Separation was achieved under optimized chromatographic condition on a Phenomenex Luna C18 (ODS) column (150 X 4.6 mm i.d., particle size 5 µm). The mobile phase consisting of phosphate buffer pH 3.0: Acetonitrile (60:40, v/v). Shimadzu UV-Visible detector SPD-10AVP was used as detector which was set at 293 nm. The retention time of Aliskiren was found to be 5.02 min. The calibration curve was linear in the concentration range of 5-30 µg/ml (r^2-0.9999).[32]

A RP-HPLC method for the determination of Aliskiren hemifumarate in tablet dosage form was reported, Babu et al (2011). Liquid chromatographic separation of

aliskiren was achieved on a Waters Xbridge C18 (150 X 4.6 mm, 5 µm particle size) column consisting a mobile phase of 0.03% trifluroacetic acid (TFA) in water and Acetonitrile (95:5) at a flow rate of 0.8 ml/min. The method was found to be linear in the concentration range of 1-100 µg/ml and the limit of detection and limit of quantification of the method was 0.2 µg/ml and 0.6 µg/ml.[33]

It has been reported that a reversed-phase liquid chromatography (RP-LC) method can be used for the determination of aliskiren in tablet dosage form, Wrasse-Sangoi et al (2011). The LC method is carried out on a Waters XBridge C(18) column (150 4.6 mm i.d.), maintained at 25ºC. The mobile phase consisted of acetonitrile: water (95: 5, v/v)/phosphoric acid (25 mM, pH 3.0) (40:60, v/v), run at a flow rate of 1.0 ml/min, with photodiode array detector set at 229 nm as detection wavelength. The retention of aliskiren was 3.68 min and linear in the range of 10-300 µg/ml (r=0.9999).[34]

An analytical UV spectrophotometric method was developed and validated according to ICH guideline by Wrasse-Sangoi et al (2010) for the determination of aliskiren in commercial formulations. The method was linear in the concentration range between 40 and 100 µg/ml (r^2 = 0.9997, n = 7) and exhibited excellent specificity, accuracy, precision, and robustness. It is a simple, low cost and it has used non polluting reagents. Tthe proposed method was successfully applied for the assay and dissolution studies of aliskiren in tablet formulation and the results were compared with a reported validated RP-LC method, showing non-significant difference (P > 0.05).[35]

AMLODIPINE

Assay of amlodipine besilate was carried out by liquid chromatographic method (Indian Pharmacopoeia-2007, British Pharmacopoeia-2008, United States Pharmacopoeia-2008). A stainless steel column, 15 cm x 3.9 mm, packed with octadecylsilane bonded to porous silica (5 µm) using a mixture of 15 volumes of acetonitrile, 35 volumes of methanol and 50 volumes of a solution prepared by dissolving 7.0 ml of triethylamine in 1000 ml of water and adjust to pH 3.0 with phosphoric acid is used as mobile phase. Flow rate of mobile phase was maintained at 1 ml/min and eluents were monitored at 237 nm. Test solution was prepared by weighing 20 tablets and extracted with mobile phase followed by centrifugation. A

solution containing 0.005 percent w/v of amlodipine besilate RS in the mobile phase was injected using 10 µl loop injector. Test solution and reference solution were injected and contents of the tablets were calculated.[36-38]

There was a simple and sensitive kinetic spectrophotometric method for the determination of amlodipine besylate (AML) by Mahmoud et al (2012). In this method condensation reaction of AML with 7-chloro-4-nitro-2,1,3-benzoxadiazole takes place in an alkaline buffer (pH 8.6), which produces a highly coloured product. The reaction was monitored spectrophometrically, based on the colour change at 470 nm. The conditions were optimized and factors affecting the reaction were studied. The stoichiometry of the reaction was established and the reaction mechanism was postulated. Moreover, both the activation energy and the specific rate constant (at 70˚C) of the reaction were found to be 6.74 kcal $mole^{-1}$ and 3.58s^{-1}, respectively. The initial rate and fixed time methods were utilized for constructing the standard calibration graphs for the determination of AML concentration. The limits of detection and quantification were 0.35 and 1.05 mg/ml under the optimized reaction conditions. The relative standard deviations were found to be 0.85–1.76%. The proposed method was successfully used for the analysis of AML in its pure form and tablets with good accuracy; the recovery percentages ranged from 99.55±1.69% to100.65±1.48%. The results were compared with that of the reported method.[39]

Amlodipine besylate is a potent calcium channel blocker used for the treatment of hypertension, congestive heart failure and angina pectoris. Amlodipine besylate avoids the adverse effect of amlodipine in racemic mixtures. A highly precise and cost effective RP-HPLC method with retention time of 2.60 minutes was reported by Sah et al (2012) for the estimation of amlodipine besylate in tablet formulation. WATERS C18 column 250 mm × 4.6 mm (5µm), with mobile phase as acetonitrile: 70 mM potassium dihydrogen orthophosphate buffer: methanol (15:30:55) and pH adjusted to 3.0 using OPA was used. Mobile phase flow rate was maintained at 1.0 ml/min and detected at 240 nm.[40]

Two simple and sensitive spectrofluorometric methods have been reported by Abdel-Wadood et al (2008) for the determination of amlodipine besylate (AML) in

tablet formulation. The first method was based on the Condensation reaction of AML with ninhydrin and phenylacetaldehyde in buffered medium (pH 7.0) resulting in formation of a green fluorescent product, which exhibits excitation and emission maxima at 375 and 480 nm, respectively. The second method was based on the reaction of AML with 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl) in a buffered medium (pH 8.6) resulting in formation of a highly fluorescent product, which was measured fluorometrically at 535 nm (λex, 480 nm). The factors affecting the reactions were studied and optimized. Under the optimum reaction conditions, linear relationships with good correlation coefficients (0.9949–0.9997) were found between the fluorescence intensity and the concentrations of AML in the concentration range of 0.35–1.8 and 0.55–3.0 µg/ml for ninhydrin and NBD-Cl methods, respectively. The limits of assays detection were 0.09 and 0.16 µg/ml for the first and second method, respectively. The precisions of the methods were satisfactory; the relative standard deviations were ranged from 1.69 to 1.98%. The proposed methods were successfully applied to the analysis of AML in pure and pharmaceutical dosage forms with good accuracy; the recovery percentages ranged from 100.4–100.8±1.70–2.32%. The results were compared with those of the reported method.[41]

Amlodipine besylate is a commonly used antyhipertensive drug acting as calcium antagonist. In this study, a coloured ion-pair complex formation reaction among amlodipine and acid-dye bromophenol blue at pH 3.2 was used for the colorimetric determination of the drug, by Golcu et al (2006). The complex formed was extracted into chloroform and the maximum absorbance of the solution was measured at 414 nm against blank. The calibration curve obeys Beer's law over the concentration range of 6-30 µg/ml and the regression equation was A=0.055C-0.018 (r=0.9997). The recovery of the drug from a commercial tablet was 100.7 % of the label claim with a relative standard deviation of 1.24 %. The results were compared with those of the spectrophotometric method currently used by the manufacturer of the tablets and no significant difference was found.[42]

There was a reported HPLC method for the quantification of amlodipine in plasma, Zarghi et al (2005). The assay enables the measurement of amlodipine for therapeutic drug monitoring with a minimum detectable limit of 0.2 ng ml^{-1}. The

method involves simple, one-step extraction procedure and analytical recovery was about 97%. The separation was performed on an analytical 125 × 4.6 mm i.d. Nucleosil C_8 column. The wavelength was set at 239 nm. The mobile phase was a mixture of 0.01 M sodium dihydrogen phosphate buffer and acetonitrile (63:37, v/v) adjusted to pH 3.5 at a flow rate of 1.5 ml min^{-1}. The calibration curve was linear over the concentration range 0.5–16 ng ml^{-1}. The coefficients of variation for inter-day and intra-day assay were found to be less than 10%.[43]

HYDROCHLOROTHIAZIDE

Hydrochlorothiazide tablet was assayed by UV spectroscopic method (Indian Pharmacopoeia-2007). Twenty tablets were weighed and crushed in to fine powder and quantity equivalent to 20 mg of hydrochlorothiazide was dissolved in 0.1 M sodium hydroxide solution and filtered. Appropriate dilutions were made and absorbance of the resulting solution was measured at 273 nm. Content of the tablet was calculated taking 520 as the value of specific absorbance at 273 nm.[36]

Hydrochlorothiazide was assayed by non aqueous titration (BP-2008), where 0.120 gm equivalent of hydrochlorothiazide was dissolved in 50 ml of dimethyl sulphoxide and titration was carried out against 0.1 M tetrabutylammonium hydroxide in 2-propanol. End point was determined potentiometrically at the second point of inflexion followed by blank titration. Each ml of 0.1 M tetrabutylammonium hydroxide in 2-propanol is equivalent to 14.88 mg of hydrochlorothiazide.[44]

Liquid chromatographic method was used to estimate hydrochlorothiazide (USP 2009), where a degassed mixture of 0.1 M monobasic sodium phosphate and acetonitrile (9:1), pH 3±0.1 with phosphoric acid was used as mobile phase. The concentration of Standard solution was 0.15 mg/ml in mobile phase, used to compare the assay preparation. In assay preparation, 20 tablets were crashed and equivalent quantity of the powder was extracted with mobile phase and filtered. Liquid chromatographic system equipped 254 nm detector and a 25 cm×4.6 mm column that contains packing L1 with 2 ml/min flow rate. Equal volume of standard and assay preparation was injected and chromatograms were recorded. Content of the tablet was calculated based on the peak area obtained.[38]

A stability indicating analytical method was reported by Bhagwate et al (2013), where Hydrochlorothiazide was degraded under different stress conditions as per International Conference on Harmonization. The degraded samples were analyzed in high performance liquid chromatographic (HPLC) system. The Hydrochlorothiazide was well resolved from degradation products using a reversed-phase (C-18) column and a mobile phase consising of Methanol: Buffer pH- 3.2 (60:40 v/v) and flow rate was 1 ml/min, detection wavelength 270 nm and injection volume 20 µl. The method was validated as per ICH guidelines. Results obtained after validation study indicating that the proposed single method allowed analysis of Hydrochlorothiazide in the presence of their degradation products formed under a variety of stress conditions. The developed procedure was also applicable to the determination of stability of the Hydrochlorothiazide in commercial pharmaceutical dosage form.[45]

The method for the estimation of hydrochlorothiazide in tablet dosage form has been reported by Hapse et al (2012). The method showed maximum absorbance at 272 nm in distilled water and in 0.01N NaOH between 200-400 nm. Linearity of hydrochlorothiazide was of between 5.00 µg /ml to 30.00 µg /ml in distilled water and 1.00 µg/ml to 30.00 µg/ml in 0.01N NaOH, with regression equation of y = 0.043x+0.198; (r2 = 0.999) and y=0.059+0.029;(r2 =0.998).[46]

VALSARTAN

Assay of valsartan was carried out by liquid chromatographic method (USP-2009) using a degassed mixture of water, acetonitrile and glacial acetic acid (500:500:1) as mobile phase. Standard solution of valsartan was prepared by dissolving valsartan RS in mobile phase and diluted to get 0.5 mg/ml. For assay preparation, appropriate quantity of the powder was dissolved in mobile phase and filtered. Liquid chromatographic system equipped 273 nm detector and a 12.5 cm×3 mm column that contains 5 µm packing L1 with 0.4 ml/min flow rate. Equal volume of standard and assay preparation was injected separately and chromatograms were recorded. Content of the tablet was calculated by using following formula:$100C(r_u/r_s)$. Where C is the concentration of valsartan RS, r_u and r_s are the peak response obtained from the assay and standard preparation, respectively.[38]

A RP-HPLC method has been reported for the estimation of Valsartan in tablet formulations by Nissankararao et al (2013). The separation was achieved on a X terra, RP-18(100 mm X 4.6 mm 5 μm) using a mobile phase consisting of a degassed mixture of water, Acetonitrile & Glacial acetic acid in the ratio of 550:450:1v/v with a flow rate of 2.0 ml/min. The mobile phase showed the most favourable chromatographic parameter for analysis. The detection of the constituent was done using UV detector at 248 nm. The retention time of valsartan was found to be 2.530 minutes. The linearity range for Valsartan was found to be 4 – 12 μg/ml.[47]

A simple and inexpensive UV spectrophotometric method have been reported by Kalaimagal et al (2012) for the determination of Valsartan in bulk and tablet dosage form. Valsartan is soluble in sodium hydroxide and shows maximum absorption at 249 nm. The drug obeyed Beer's law showing linearity in the range of 5- 30 μg/ml with a correlation coefficient of 0.999. Method A is based on standard absorbance, method B involves estimation by Area under curve (AUC) and method C by derivatisation of Zero order spectrum and method D were Q-Absorbance ratio. All the methods were validated in terms of specificity, limit of detection (LOD), limit of quantification (LOQ), linearity of response, precision and accuracy. Thus the proposed methods could be adopted for routine analysis of bulk drug and its formulation.[48]

Quantification of Valsartan in Tablet Dosage Form was demonstrated by Kendre et al (2012) using a Perkin Elmer HPLC series 200 with software Perkin Elmer total chrome navigator. The C-18 (Kromasil, 250 × 4.6 mm) having particle size of 5 μm was used. The gradient mobile phase system was selected comprising of solution A ACN, solution B phosphate buffer of pH 3.5 added few drops of Triethylamine in buffer solution. The run time of 10 min was selected. The mobile phase composition keep on varying up to 10 min of run time. The flow rate of 1.0 ml/min was used and Perkin Elmer series 200 UV/VIS detector wavelength was set at 250 nm. The retention time of Valsartan was found to be 5.19 min. The percentage recovery was found to be up to 99% to nearly 100% and percentage RSD was found to be less than 2.0%.[49]

A stability-indicating reverse phase high performance liquid chromatography (RP-HPLC) method was reported by Haque et al (2012) for the estimation of Valsartan in tablet dosage form. The method employed, Phenomenox C18, 5 µm,25 cm x 4.6 mm i.d. column in isocratic mode, with mobile phase of methanol & phosphate buffer pH 3.0 in ratio of 65:35(v/v) . The flow rate was 1 ml/min and effluent was monitored at 210 nm. Retention time was found to be 6.22 min. Linear regression analysis data for the calibration plot showed that there was good linear relationship between response and concentration in the range of 10- 100 µg/ml respectively. The LOD and LOQ values for HPLC method were found to be 0.02 and 0.06 µg/ml respectively. No chromatographic interference from tablet excipients was found. The proposed method was successfully used for estimation of Valsartan in tablet dosage form.[50]

There was a reverse phase isocratic RP-HPLC method for determination of Valsartan in tablet dosage form, Vinzuda et al (2010). The method was performed using Thermo-hypersil ODS column (150 mm × 4.6 mm i.d., 5 µm particle size) with mobile phase comprised of water: acetonitrile: glacial acetic acid (500:500:01). The flow rate was set at 1.0 ml/min and effluent was monitored at 273 nm. The retention time of valsartan was found to be 4.6 minute. LOD and LOQ were found to be 2.72 µg/ml and 8.25 µg/ml, respectively. The calibration curve was linear in the concentration range of 40-140 µg/ml with coefficient of correlation 0.9990. The percentage recovery for the valsartan was found to be 99.0-100.2 and the % RSD was found to be less than 2 %. The proposed method was successfully applied for quantitative determination of valsartan in tablet dosage form.[51]

Two UV spectrophotometric methods has been published by Gupta et al (2010) for the estimation of valsartan (VAL) in bulk and tablet dosage form. The zero order spectra of valsartan in methanol shows λ_{max} at 250.0 nm and estimation was carried out by A(1% 1cm) and by comparison with standard (Method I). The second order spectra showed λmax at 241.0 nm where n=2 and estimation were carried out by comparison with standard (Method II). Calibration graphs were found to be linear (r2=0.999) over the concentration range of 10-50 µg/ml.[52]

A method for detection and quantification of valsartan in human plasma has been reported by Perez et al (2007) using an isocratic elution on reversed phase liquid chromatography with ultraviolet detection at a single wavelength (265 nm). Losartan was used as an internal standard. This method involves a solid-phase extraction of drugs (valsartan and losartan) from plasma using C8 cartridges. Separation was achieved on a C18 reversed phase column and the mobile phase consisted of 45% acetonitrile and 55% phosphate buffer (adjusted to pH 2.7 ± 0.1 with phosphoric acid). The assay has been validated over a concentration range of 0.05 to 20 µg/ml with addition of losartan 2.5 µg/ml. Calibration curve was linear in the described concentration range. Determination of valsartan in human plasma by HPLC/UV method was accurate and precise with a quantitation limit of 1.485 µg/ml. The method was sufficiently sensitive for pharmacokinetic studies of valsartan in human plasma.[53]

ALISKIREN + HCT

There was a reported stability-indicating HPLC method for simultaneous determination of aliskiren and hydrochlorothiazide in a combined formulation, Karvelis et al (2014). Chromatographic separation was achieved on a phenyl analytical column with isocratic elution using the mobile phase 0.030 M ammonium acetate-acetonitrile (60 + 40, v/v) at a flow rate of 0.40 ml/min. The UV detector was set at 280 nm. The developed method was linear over the concentration range of 1.5–4.5 and 0.125–0.375 µg/ml for aliskiren and hydrochlorothiazide, respectively. The values of RSD for intraday and interday precision were less than 6.1% and the relative percentage error, E_r, was less than 5% for both drugs. Both the analytes were subjected to stress conditions like acidic and alkaline hydrolysis, oxidation and thermal degradation. The method was applied successfully to the QC and content uniformity tests in combined commercial tablets.[54]

An alternative method for analysis of aliskiren (ALI) and hydrochlorothiazde (HCT) in combined dosage forms by ion-pair RP-HPLC was developed and validated by Belal et al (2013). The pharmaceutical formulations were analyzed using C18 column (250 mm × 4.6 mm, 3 µm) with a mobile phase consisting of 25% methanol, 50% sodium monobasic phosphate aqueous solution containing 6 mM tetrabutylammonium bromide and 25% water at pH 7.2. Isocratic mode of analysis

was performed at a flow rate of 1 ml/min and a column was kept at 30°C under UV detection at 210 nm. Paracetamol was used as internal standard. Developed and validated method was linear over the concentration range of 0.250 to 60 and 0.1 to 10 µg/ml for ALI and HCT, respectively. The limits of detection and quantitation (LOD and LOQ) were found to be 0.075 and 0.198 µg/ml, respectively, for ALI and 0.04 and 0.062 µg/ml, respectively, for HCT. The method proved to be specific, sensitive, precise and accurate with mean recovery values of 101.1 ± 0.32 % and 100.9 ± 0.41 % for ALI and HCT, respectively. The method robustness was evaluated by means of an experimental design. The proposed method was applied successfully to spiked human urine samples with mean recoveries of 98.8 ± 0.36 % and 98.1 ± 0.21 % for ALI and HCT, respectively.[55]

A HPLC and UV-spectrophotometric methods were reported for the simultaneous determination of perindopril and indapamide in bulk and in tablets dosage form by Ezzeldin et al (2013). Chromatographic separation was achieved on Econosphere C-18 column (150 mm x 4.6 mm, 5 µm) using a mobile phase system consisting of acetonitrile: 5mM sodium phosphate buffer (pH 7.5), (50:50) at a flow rate of 0.5 ml/min with UV detection at 202 nm. The methods were found to be linear over the concentration ranges of 1-140 µg/ml and 1-40 µg/ml for perindopril and indapamide, respectively. In UV-spectrophotometric methods, two methods were employed, ratio subtraction and first derivative methods. In Ratio Subtraction method, absorbance readings are taken at two wavelengths 277.48 nm (λ_{max} of aliskiren) and 315 nm (extended spectrum of hydrochlorothiazide) in methanol. In First Derivative method, absorbance readings are taken at two wavelengths 237.2 nm (for aliskiren) and 275.8 nm (for hydrochlorothiazide) in methanol. The applied spectrophotometric methods were found to be rapid, specific, precise and accurate over the concentration range of 5-150 µg/ml and 1 – 41 µg/ml for aliskiren and hydrochlorothiazide respectively.[56]

A study on high performance liquid chromatographic (HPLC) and spectrophotometric methods was reported for the simultaneous determination of Aliskiren (ALK) and Hydrochlorothiazide (HCT) combination in bulk powder and in tablets dosage form, Ezzeldin et al (2013). Determination of ALK and HCT was achieved by chromatographic separation on Econosphere C-18 column using a

mobile phase consisting of water (pH 7.5): acetonitrile (50:50) at a flow rate of 0.5 mL.min−1 and UV detection at 208 nm. Method validation parameters were found to be acceptable over the concentrations range of 5-150 μgmL^{-1}, 1-50 μg.mL^{-1} for ALK and HCT respectively. Regarding the spectrophotometric methods, two methods were employed. Simultaneous Equation method, absorbance readings are taken at two wavelengths 277.48 nm.[57]

There was a stability-indicating MEKC method for the simultaneous determination of aliskiren (ALI) and hydrochlorothiazide (HCTZ) in pharmaceutical formulations using ranitidine as an internal standard by Sangoi et al (2011). The method employed 47 mM Tris buffer and 47 mM anionic detergent SDS solution at pH 10.2 as the background electrolyte. MEKC method was performed on a fused-silica capillary (40 cm) at 28°C. Applied voltage was 26 kV (positive polarity) and photodiode array (PDA) detector was set at 217 nm. The method was linear over the concentration range of 5–100 and 60–1200 μg/mL for HCTZ and ALI, respectively (r^2>0.9997).[58]

ALISKIREN + VAL

It has been reported that the ratio spectra derivative spectrophotometric method can be used for the simultaneous determination of Aliskiren (ALS) and Valsartan (VAL) in their fixed dosage forms, Parmar et al (2014). The method depends on the use of the first derivative of the ratio-spectra obtained by dividing the absorption spectrum of binary mixtures by a standard spectrum of one of the compounds. The first derivative amplitudes at 289 nm and 245 nm were selected for the determination of ALS and VAL respectively. The wavelength interval was selected as Δλ= 4 nm. Methanol: water (50:50) was used as the diluent. Both the drugs, Aliskiren and Valsartan showed linearity in the range of 50-200 μgmL^{-1} and 5-24 μgmL^{-1} respectively. The method was applied to the assay of in-house formulation, which was found in the range of 98.0% to 102.0% of the labeled value for both Aliskiren and Valsartan.[59]

A simple, rapid, economical, precise and accurate UV Spectroscopic method for simultaneous determination of aliskiren hemifumarate (ALH) and valsartan (VAL) has been reported by Tandel et al (2013). For ratio spectra derivative method two

λ_{max} 241.50 nm and 259.76 nm were selected for ALH and VAL respectively. Methanol was used as solvent. Beer's law was obeyed in the concentration range of 10-35 µg/ml for ALH and 10.6-37.1 µg/ml for VAL. For above method correlation co-efficient was close to 1 proving the good linearity between concentration of drug and response. Above method was validated as per ICH guideline. The results of accuracy were found to be 98-102% for the method. The method is precise as the results of reproducibility and repeatability are under the acceptance criteria (%RSD <2).[60]

An isocratic High Performance Liquid Chromatographic (HPLC) method was reported for the determination of Aliskiren Hemifumarate (ALSK) and Valsartan (VAL) in bulk drug by Ghosh et al (2013). The Method employs Waters HPLC system on C8 Column (4.6 x 250 mm, 5 µm) and flow rate of 1 ml/min with a load of 10µl. The Detection was carried out at 220 nm. mobile phase used as Acetonitrile and Phosphate buffer and Methanol was used as mobile phase in the composition of 45:40:15, phosphate buffer (0.02Mm) adjusted the pH to 4 with Orthophosphoric acid within a short runtime of 8 min. The retention times of Aliskiren (ALSK) was 3.407 min, Valsartan (VAL) was 4.268 min. The method was validated according to the regulatory guidelines with respect to specificity, precision, accuracy, linearity and robustness etc.[61]

A stability indicating RP-HPLC method has been developed and Subsequently validated for simultaneous estimation of Aliskiren (ALN) and Valsartan (VAL) from their combination dosage form by Kumaraswamy et al (2012). Water's HPLC equipped with UV-Visible and Diode Array detectors, with Empower-2 software was used. Column used was XTerra® RP8, 5 µm, 100 mm × 4.6 mm i.d., at ambient temperature. Mobile phase consisting of 0.05M Ammonium Acetate and 0.5% TEA buffer having pH 5.5and Acetonitrile in the ratio of 68:32 v/v at a flow rate of 1.0 ml/min and UV detection was carried out at 238 nm and 271 nm for ALN and VAL, respectively. ALN, VAL and their combined dosage form were exposed to thermal, photolytic, oxidative, acid-base hydrolytic stress conditions, the stressed samples were analyzed by proposed method. Peak purity results suggested no other co-eluting, interfering peaks from excipients, impurities, or degradation products due to variable stress condition, and the method is specific for the estimation of ALN and

VAL in presence of their degradation products and impurities within 6 minutes. The retention time of Aliskiren and Valsartan were 1.98 and 4.03 minutes respectively. The method was found to be linear over the range of 1-20 µg per ml for Aliskiren and 1.6-240 µg per ml for Valsartan.[62]

Aliskiren Hemifumarate and Valsartan belong to a group of Anti-hypertensive drugs. A Simple, Rapid, Specific and economic RP-HPLC method has been developed for assaying both the drugs in combinational dosage form by Chokshi et al (2012). Method involves elution of Aliskiren Hemifumarate and Valsartan in Hyper ODS2, Column C18, 250 x 4.6 mm (5 µm) using mobile phase composition of Acetonitrile: 0.05M Potassium dihydrogen phosphate buffer, pH 3.5 adjusted with O-Phosphoric acid (45:55, v/v), at flow rate 1ml/min and analytes were monitored at 224 nm. Method shows good linearity over the concentration range of 10-50 µg/ml for both the drugs.[63]

ALISKIREN + AMLO

Studies have stated that a reversed-phase high-performance liquid chromatography (HPLC) method can be used for the simultaneous determination of amlodipine besylate and aliskiren hemifumarate by Ozdemir et al (2014). The separation was achieved on RP-18 column (250×4.6 mm, 3 µ) using a mobile phase consisting of triethylamine-orthophosphoric acid buffer (50 mM, pH 3.0), acetonitrile and methanol (50:40:10, v/v/v) at a flow rate of 1 ml/min. The degree of linearity of the calibration curves, the percent recovery values of amlodipine and aliskiren and the limits of detection (LOD) and quantification (LOQ) for the HPLC method were determined. The linearity of the method was found to be in the concentration range of 5.0–50.0 mg/ml for aliskiren hemifumarate and 2.65–26.50 mg/ml for amlodipine besylate. LOD and LOQ values were 0.51, 0.95, 1.70 and 3.18 mg/ml for amlodipine besylate and aliskiren hemifumarate.[64]

The present study describes the stability indicating RP-HPLC method for simultaneous estimation of aliskiren hemifumarate and amlodipine besylate in pharmaceutical dosage forms by Runja et al (2014). The proposed RP-HPLC method was developed by using waters 2695 separation module equipped with PDA detector and chromatographic separation was carried on C-8 Inertsil ODS (150 ×

4.6 mm, 5 μ) column at a flow rate of 1 ml/min and the run time was 10 min. The mobile phase consisted of phosphate buffer and acetonitrile in the ratio of 40:60% v/v and pH was adjusted to 3 with orthophosphoric acid and eluents were monitored using PDA detector at 237 nm. The retention time of aliskiren and amlodipine was found to be 3.98 and 5.14 min, respectively. A linearity response was observed in the concentration range of 30-225 μg/m for aliskiren and 2-15 μg/ml for amlodipine, respectively. Limit of detection and limit of quantification for aliskiren are 0.161 μg/ml and 0.489 μg/ml and for amlodipine are 0.133 μg/ml and 0.404 μg/ml, respectively.[65]

An isocratic reverse phase high performance liquid chromatography (RP-HPLC) method was developed and validated for simultaneous estimation of Aliskiren and Amlodipine in tablet dosage form by Divya et al (2013). The estimation was carried out by using the Kromasil, ODS 3V (250 x 4.6 mm, 5 µm) C18 column with 5 µm particle size. Injection volume was 20 µl is injected and eluted with the mobile phase, Phosphate buffer: Acetonitrile in the ratio of 60: 40, which is pumped at a flow rate of 1.0 ml/min. Detection was carried out at 237 nm using photodiode Array (PDA) detector. The retention time for Aliskiren and Amlodipine was 3.9 and 5.0 mins. Aliskiren shows linearity in the range of 25-150 µg/ml and Amlodipine shows linearity in the range of 2.5-15 µg/ml and correlation co-efficients were found to be 0.9979 and 0.9973. The accuracy studies were shown as % recovery for Aliskiren and Amlodipine at 50%, 100% and 150%. The limit of % recovered shown is in the range of 98-102% and the results obtained were found to be within the limits. Limit of detection of 1.37741µg/ml & 4.17396µg/ml & limit of quantification of 0.73967µg/ml and 2.24142µg/ml for Aliskiren & Amlodipine, respectively.[66]

The present article describes about a simple, unique and selective HPLC-PDA method for the simultaneous estimation of aliskiren (ALS) and amlodipine (AML) in human plasma by Mannemala et al (2014). Extraction of the sample was accomplished by protein precipitation technique. Plasma proteins were precipitated by employing acetonitrile containing hydrochlorothiazide as internal standard. The compounds were analyzed by HPLC by using PDA detector on a Hibar C_{18}

(250 × 4.6 mm) column with a mobile phase comprising acetonitrile and phosphate buffer (pH 4.2 and 25 mM; 60:40 v/v) with a flow rate of 0.8 ml/min. Different sample pretreatment techniques were evaluated but protein precipitation was found to be satisfactory, offering good recovery values of 97.11-98.45% for ALS and 97.5-99.12% for AML. The within-day precisions for ALS were 96.66, 99.16 and 99.41% at 90, 240 and 480 ng/ml, respectively, and for AML they were 97.27, 99.54 and 99.31% at 3.3, 8.8 and 17.6 ng/ml, respectively. The between-day precisions for ALS were 96.66, 99.16 and 99.41% at 90, 240 and 480 ng/ml, respectively and the between-day precisions for AML were 98.18, 99.20 and 99.40% at 3.3, 8.8 and 17.6 ng/ml, respectively. The limit of quantitation was 30 and 1.0 ng/ml for ALS and AML, respectively. Different constituents of plasma proteins did not interfere with the absolute recovery of ALS and AML.[67]

A cost effective reverse phase high performance liquid chromatography (RP-HPLC) method have been reported for simultaneous estimation of Aliskiren and Amlodipine in pharmaceutical dosage forms by Vemula et al (2013). Separation was carried out iso-cratically at 30°C ± 0.5°C on an Water's X-bridge C-18 column (4.6 x 150 mm, 5 µ particle size) with a mobile phase composed of acetonitrile - phosphate buffer (pH-2.5) (40:60, v/v) at a flow rate of 1.0 ml/min. Detection was carried out using a PDA detector at 230 nm. The retention times for Aliskiren and Amlodipine were 3.8 min and 5.1 min respectively. The linearity range for Aliskiren and Amlodipine were 18.75-187.5 µg/ml and 1.25-12.5 µg/ml, respectively. The correlation coefficients for both components are close to 1. The relative standard deviations for six replicate measurements of samples in tablets are always less than 2%.[68]

Two simple, sensitive, rapid and accurate analytical methods were reported by Das et al (2012) for the simultaneous determination of aliskiren and amlodipine in marketed formulation of pharmaceutical dosage forms. The Q-analysis based on measurement of absorptivity at 279 nm and 289 nm (as an iso-absorptive point). The second method developed and validated of simultaneous equation using 279/361 nm. Aliskiren and amlodipine at their respective λ_{max} 279 nm and 361 nm and at iso absorptive point 289nm show linearity in a concentration range of 20-100 µg/ml and 5- 25 µg/ml. Recovery studies range from 99.51% for aliskiren and

99.51% for amlodipine in case of simultaneous equation method aliskiren was 100.10% and amlodipine was 100.47%.[69]

The present paper describes simple, accurate, rapid, precise and sensitive UV spectrophotometric absorption correction method for the simultaneous determination of amlodipine and aliskiren in combined tablet dosage form, Patel et al (2013). Methanol was used as solvent. The wavelengths selected for the analysis using absorption correction method were 354.5 nm and 256.0 nm for estimation of amlodipine and aliskiren respectively. Beer's law obeyed in the concentration range of 10-60 μg/mL and 20-120 μg/mL for amlodipine and aliskiren, respectively. The mean percentage drug content for amlodipine and aliskiren were found to be 99.9. ± 1.38 and 99.87 ± 1.25 respectively and the % RSD value was found to be less than 2 which shows the precision of method.[70]

ALISKIREN+ AMLO + HCT

There was a reversed-phase (RP) liquid chromatographic technique for the simultaneous determination of aliskiren (ALS), amlodipine (AMD) and hydrochlorothiazide (HCT) in spiked human plasma and urine, Zeynep et al (2015). The method employs a gradient elution using 10 mM orthophosphoric acid containing 0.1% triethylamine (pH 2.5, v/v) and acetonitrile and an RP-C_{18} column (4.6 mm × 250 mm, 5 μm, Phenomenex) at 1 ml/min of flow rate, with a UV-PDA detector at 271 nm. The linear ranges were 0.01–10 μg/ml in plasma and 0.05–10 μg/ml in urine for both ALS and AMD. The linearty of HCT was in the range of 0.0125–2.5 μg/ml in plasma and urine. Correlation coefficients (r^2) were higher than 0.9983 for all of the analytes, indicating good linear relationship. The method validation was performed with respect to linearity, recovery, accuracy, precision and stability. The developed method could be applied in the routine clinical analysis.[71]

The present paper describes a highly sensitive UPLC-MS/MS method for simultaneous estimation of aliskiren hemifumarate (ALS), amlodipine besylate (AML) and hydrochlorothiazide (HCZ) in spiked human plasma using valsartan as an internal standard (IS) by Ebid et al (2015). Liquid-liquid extraction was carried out for purification and pre-concentration of analytes. The mobile phase consisted of 0.1% formic acid in ammonium acetate buffer (0.02 M, pH 3.5) and methanol

(25:75, v/v), flowing through XBridge BEH (50 × 2.1 mm ID, 5 µm) C18 column, at a flow rate of 0.6 ml/min. Multiple reaction monitoring (MRM) transitions were measured using an electrospray source in the positive ion mode for ALS and AML, whereas HCZ and IS were measured in negative ion mode. Validation of the method was performed as per US-FDA guidelines with linearity in the range of 2.0-400.0, 0.3-25.0 and 5.0-400.0 ng/ml for ALS, AML and HCZ, respectively. In human plasma, ALS, AML and HCZ were stable for at least 1 month at -70 ± 5°C and for at least 6 h at ambient temperature. After extraction from plasma, the reconstituted samples of ALS, AML and HCZ were stable in the autosampler at ambient temperature for 6 h. The LC-MS/MS method is suitable for bioequivalence and pharmacokinetic studies of this combination.[72]

A stability indicating method had been reported for simultaneous quantitative determination of Aliskiren hemifumarate, Amlodipine besylate and Hydrochlorothiazide in bulk and pharmaceutical dosage form, Salim et al (2014). The chromatographic separation was achieved with Inertsil ODS (150×4.6 mm) and 5 µm particle size column. The optimized mobile phase consisting of phosphate buffer pH 3.1: Acetonitrile (70:30%v/v). The flow rate was 1.0 ml/min and eluents were monitored at 236 nm using PDA detector. The retention time of Hydrochlorothiazide, Aliskiren and Amlodipine were found to be 3.057, 5.330 and 6.973 respectively. The percentage recoveries for three molecules were found to be in the range of 99-101%. The calibration curve was constructed between peak area vs concentration and demonstrated good linear in the range of 75-450 µg/ml for Aliskiren, 2.5 – 15.0 µg/ml for Amlodipine and 6.25-37.5 µg/ml for Hydrochlorothiazide. Degradation studies were studied for Aliskiren, Amlodipine and Hydrochlorothiazide under various stress conditions such as acid hydrolysis, base hydrolysis, oxidation, thermal, photochemical and UV. All the degradation peaks were resolved effectively using developed method with different retention times.[73]

A simple, accurate, precise, economical and reproducible method was reported for simultaneous estimation of aliskiren, amlodipine and hydrochlorothiazide in Combined Pharmaceutical Dosage Forms, Patel et al (2014). The excipients in the commercial tablet preparation did not interfere with the assay. The λ_{max} for

aliskiren, amlodipine and hydrochlorothiazide were 252 nm, 360 nm and 271 nm respectively. At 360 nm, Amlodipine showed some absorbance while aliskiren and hydrochlorothiazide showed zero absorbance so that amlodipine was estimated at 360 nm. While at 252 nm and 271 nm aliskiren and hydrochlorothiazide were determine by simultaneous estimation method after eliminating the absorbent of Amlodipine at this wavelength. Linearity in concentration range of 4-28 µg/ml, 4-28 µg/ml and 20 - 120 µg/ml with the mean recoveries were 99.97 ± 0.82, 99.93 ± 0.88 and 100.14 ± 0.81 % for ALK, AML and HTZ, respectively.[74]

A study on RP-High Performance Liquid Chromatography (HPLC) method for the simultaneous estimation of Aliskiren (ALS), Amlodipine (AML) and Hydrochlorothiazide (HCTZ) combined dosage form has been reported by Renukapally et al (2014). The components were well separated using Hypersil BDS, 250 x 4.6 mm, 5 µ column using Acetonitrile:1ml TEA in 1000 potassium phosphate buffer 0.01M (40:60% v/v) as mobile phase at a flow rate of 1.0 ml/min. The eluents were detected at 228 nm using UV detector. The retention time of HCTZ 3.3 min, ALS was found to be 5.9 min and that of AML was 8.0 min. The linearity was observed between 6.25-37.5 µg/ml, 75-450 µg/ml and 2.5-15 µg/ml for HCTZ, ALS and AML respectively. The marketed dosage form was analysed by using the developed method. The mean recoveries were 100±2% for three compounds. The method was validated for system suitability, specificity, linearity, accuracy, precision, ruggedness and robustness as per ICH guidelines and the results were found to be within the limits. The developed method was used for the stability studies (short, long and auto sampler) and forced degradation studies (acidic, alkaline, oxidative and photolytic). This validated method can be used for the routine quality control testing of HCTZ, ALS and AML combined dosage form.[75]

A research article have stated simultaneous estimation of Aliskiren Hemifumarate, amlodipine besylate and hydrochlorothiazide in the Bulk and Pharmaceutical Dosage Forms using losartan as an internal standard by Prathyusha et al (2014). The chromatographic separation of the three drugs was achieved on a reverse phase Inertsil-ODS, C18, 100X 4.6 mm, 5 µm column using 0.1 M Ammonium acetate buffer (pH adjusted to 5 using formic acid) and Acetonitrile in the ratio of 65:35 v/v with flow rate of 1.0 ml/min with injection volume 20 µl and the detection was

carried out at 232 nm. The retention time of aliskiren hemifumarate (ALSK), amlodipine besylate (AMLO) and hydrochlorothiazide (HCT) were found to be 3.90, 5.22 and 1.91 min, respectively. The drug products were subjected to stress conditions like acidic, alkaline, oxidation, UV and Thermal conditions. The degradation products were well resolved from ALSK, AMLO and HCT peaks, thus indicating the stability-indicating nature of the method. The linear regression analysis data for the calibration plots showed good linear relationship in the concentration range of 37.5-225.00 µg/ml for aliskiren hemifumarate, 3.125-18.75 µg/ml for hydrochlorothiazide and 1.25-7.50 µg/ml for amlodipine besylate. The developed method was successfully validated in accordance to ICH guidelines. Hence, this method can be conveniently adopted for the routine analysis in quality control laboratories.[76]

The analysis of aliskiren hemifumarate and hydrochlorothiazide and amlodipine by improved High Performance liquid chromatography method with UV detector and data handling system is investigated. The improved HPLC-UV detector method for the separation and quantification of aliskiren hemifumarate and hydrochlorothiazide and amlodipine is described by Alagar et al (2012). Samples are analysed by means of reverse phase(RP) HPLC using a Waters C8 column (250A-4.6mm,5 µ particle size) and the mobile phase used as 20 mM potassium dihydrogen phosphate buffer of pH adjusted to 6.5 ± 0.1 with sodium hydroxide (NaOH) (A)and methanol(B) and acetonitrile (C). A: B: C ratio was 55:10:35 v/v for the initial 18.0 min and the flow rate is 1.2 ml/min and the column temperature is set at ambient temperature and wavelength fixed at 220 nm UV-detection. The retention times of aliskiren hemifumarate and hydrochlorothiazide and amlodipine were 3.24 min, 12.01 min and 14.89 min, respectively. The described method of aliskiren hemifumarate and hydrochlorothiazide and amlodipine is linear over a range of 150 to 750 µg/ml for Aliskiren, 12.5-62.5 µg/ml for hydrochlorothiazide and 5 to 25 µg/ml amlodipine. The method precision for the determination of assay was below 2.0 %RSD. The percentage recoveries of active pharmaceutical ingredient (API) from pharmaceutical dosage forms ranged from 99%-101%.[77]

ALISKIREN+ OTHERS

A simple, rapid, precise and accurate high performance liquid chromatography method was reported for simultaneous estimation of enalapril and aliskiren in synthetic mixture, Sharma et al (2014). The separation was obtained using a mobile phase consisting of acetonitrile and water in ratio of 80:20 and adjusting pH 4.0 with orthophosphoric acid (10%) using Phenomenex-luna C18 (250 × 4.6 mm, 5 µm) column. The flow rate 1.0 ml/min and UV detection at 210 nm was employed. The retention time for enalapril and aliskiren was 2.63 min and 7.25 min respectively. Linearity for enalapril and aliskiren was found to be in the range of 2-10 µg/ml and 15-75 µg/ml respectively. The method was validated as per the ICH guidelines and the results were within the acceptance criteria for precision, linearity, specificity, stability of solution and robustness.[78]

Studies have stated that a simple, precise, fast and gradient, high performance liquid chromatographic (HPLC) method for the determination of aliskiren, ramipril, valsartan and hydrochlorothiazide in solid dosage forms was developed, Pachauri et al (2010). The quantitative determination of analyte(s) was performed on a Purosphere Star RP 18e analytical column (250×4.6 mm) with 0.2 %v/v TEA buffer (pH: 3.0): ACN as mobile phase, at a flow rate of 1.0 ml min-1. Detection was made by extracting PDA spectra at 215 nm.[79]

Assay of valsartan and hydrochlorothiazide tablets was carried out by liquid chromatographic method (USP-2009) in gradient mode using variable mixture of solution A (Mixture of water, acetonitrile and trifluoroacetic acid; 90:10:0.1) and solution B (Mixture of acetonitrile, water and trifluoroacetic acid; 90:10:0.1). Standard solution was prepared by dissolving appropriate quantity of hydrochlorothiazide and valsartan (in ratio) in to a volumetric flask using diluents (acetonitrile & water, 1:1). Appropriate dilutions were made to get the solution having known concentration of about 0.2mg/ml of valsartan. Equivalent quantity of powdered drug (20 tablets) was transferred to a volumetric flask, diluents were added and sonicated to extract the drugs. Appropriate dilutions were made to get the solution having known concentration of about 0.2mg/ml of valsartan. Liquid chromatographic system equipped 265 nm detector and a 12.5 cm×3 mm column that contains 5 µm packing L1 with 0.4 ml/min flow rate. Equal volume of standard

and assay preparation was injected separately and chromatograms were recorded. Content of the tablet was calculated by using following formula: $LC_s/C_u \times r_u/r_s$. Where, L is the labelled quantity in mg; C_s is the concentration in mg/ml of standard preparation; Cu is the concentration of corresponding analyte in assay preparation; ru and rs are the peak response obtained from the assay and standard preparation, respectively.[38]

Accurate, sensitive and reproducible reversed-phase high-performance liquid chromatography (RP-HPLC), high performance thin layer chromatography (HPTLC) and ultraviolet (UV) spectrophopometric methods were reported by Sharma et al (2014) for the concurrent estimation of amlodipine besylate (AMLO), hydrochlorothiazide (HCTZ) and valsartan (VALS) in bulk and combined tablet formulation. In RP-HPLC method, separation was achieved on a C18 column using potassium dihydrogen orthophosphate buffer (50 mM, pH 3.7) with 0.2% triethylamine as the modifier and acetonitrile in the ratio of 56:44 (v/v) as the mobile phase. Quantification was achieved using a photodiode array detector at 232 nm over a concentration range of 2-25 µg/ml for AMLO, 5-45 µg/ml for HCTZ and 20-150 µg/ml for VALS. For the HPTLC method, the drugs were separated by using ethyl acetate-methanol-toluene-ammonia (7.5:3:2:0.8, v/v/v/v) as the mobile phase. Quantification was achieved using UV detection at 242 nm over a concentration range of 100-600 ng/spot for AMLO, 150-900 ng/spot for HCTZ and 1,200-3,200 ng/spot for VALS. The UV-spectrophotometric simultaneous equation method was based on the measurement of absorbance at three wavelengths; i.e., at 237.6 nm (λmax of AMLO), 270.2 nm (λmax of HCTZ) and 249.2 nm (λmax of VALS) in methanol. Quantification was achieved over the concentration range of 2-20 µg/ml for AMLO, 5-25 µg/ml HCTZ and 10-50 µg/ml for VALS.[80]

There was a simple, sensitive and specific liquid chromatographic method by Tengli et al (2013) for the simultaneous estimation of hydrochlorothiazide, amlodipine and losartan in tablet dosage form and telmisartan as an internal standard. Separation was achieved using a phenomenex luna 5µ CN 100R, 250 × 4.60 mm 5 µ size column, ambient temperature with a low pressure gradient mode with mobile phase containing acetonitrile, water and 0.4% of potassium dihydrogen phosphate buffer pH 2.7 adjusted with orthophosphoric acid (45:35:20). The flow rate was 1 ml/min

and eluent were monitored at 230 nm. The selected chromatographic conditions were found to effectively separate hydrochlorothiazide, amlodipine and losartan with retention time of 3.9, 4.9 and 5.8 min respectively. The linearity range of hydrochlorothiazide, amlodipine and losartan found in the range of 12.5–62.5 μg/ml, 2.5–12.5 μg/ml and 50–250 μg/ml, respectively.[81]

The present paper describes a simple, precise, accurate and economic simultaneous UV spectrophotometric method for the estimation of amlodipine besylate, valsartan and hydrochlorothiazide in combination in bulk mixture and tablet, Galande et al (2012). The estimation was based upon measurement of absorbance at absorbance maxima of 359 nm, 317 nm and 250 nm for amlodipine besylate, hydrochlorothiazide and valsartan in methanol, respectively in bulk mixture and tablet. The Beer Lambert's law obeyed in the concentration range 5-25 μg/ml, 10-50 μg/ml and 5-25 μg/ml for amlodipine besylate, hydrochlorothiazide and valsartan, respectively. The estimation of bulk mixture and tablet was carried out by simultaneous equation, Q-analysis and area under curve method for estimation of amlodipine besylate and hydrochlorothiazide and standard curve method for estimation of valsartan. The results were found to be in the range of 99.6±1.52% to 102±0.51%.[82]

The novel triple combination between Amlodipine (AML), Hydrochlorothiazide (HCT), and Valsartan (VAL) provides a new option for treating hypertension. A HPLC method was reported for their simultaneous determination in pharmaceutical combinations, employing experimental design strategies, Silvana et al (2011). The drugs were separated on a C18 column at 30°C, using a 38:62 (v/v) mixture of 30 mM phosphate buffer (pH 5.5) and MeOH as mobile phase, delivered at 1.0 ml/min. Detection was performed at 234 nm. Despite the wide difference in analytes' concentrations, the method showed good linearity ($r2 > 0.995$) in the ranges 7.0–13.0 μg/ml, 17.6–32.8 μg/ml and 226.2–420.2 μg/ml for AML, HCT, and VAL, respectively, being specific (peak purity >0.999), accurate (bias of analyte recoveries <2.0%), and precise (inter- and intra-day variations <2%). It was also robust to small changes in flow rate (±0.05 ml/min), pH (±0.1 unit) and proportion of MeOH (±3%) in the mobile phase.[83]

Researcher (Varghese et al, 2011) suggested a HPLC and HPTLC-densitometric methods can be used for the simultaneous determination of amlodipine (AML), valsartan (VAL), and hydrochlorothiazide (HYD) in combined tablet dosage form. Method A, the gradient RP-HPLC analysis was performed on a Phenomenex Luna C18 (4.60 mm × 150 mm, 5 µ particle size) column, using a mobile phase consisting of 10 mM ammonium acetate buffer (pH 6.7) and methanol in solvent gradient elution for 20 min at a flow rate of 1 ml/min. Quantification was carried out using a photodiode array UV detector at 238 nm. The employment of a diode array detector allowed selectivity confirmation by peak purity evaluation. Method B, the HPTLC analysis was carried out on an aluminum-backed sheet of silica gel 60F254 layers using chloroform: glacial acetic acid:n-butyl acetate (8:4:2, v/v/v) as the mobile phase. Quantification was achieved with UV densitometry at 320 nm.[84]

A reproducible HPLC method was reported for the simultaneous determination of amlodipine and valsartan in their combined dosage forms and for drug dissolution studies by Mustafa et al (2010). A C18 column (ODS 2, 10 µm, 200 x 4.6 mm) and a mobile phase of phosphate buffer (pH 3.6, 0.01 mol L-1): acetonitrile: methanol (46:44:10 v/v/v) mixture were used for separation and quantification. Analyses were run at a flow-rate of 1 ml/min and at ambient temperature. The injection volume was 20 µl and the ultraviolet detector was set at 240 nm. Under these conditions, amlodipine and valsartan were eluted at 7.1 min and 3.4 min, respectively. Total run time was shorter than 9 min. The developed method was validated according to the literature and found to be linear within the range 0.1-50 µg/ml for amlodipine, and 0.05-50 µg/ml for valsartan.[85]

Two simple, precise and reproducible UV spectrophotometric methods, simultaneous equation method and Q-value analysis methos were reported by Meyyanathan et al (2010) for the simultaneous estimation of nebivolol hydrochloride and valsartan used as cardiovascular drugs available in capsule dosage form and nebivolol hydrochloride with hydrochlorothiazide used as antihistaminic, H blocker available in tablet dosage form. The methods are based on the measurement of absorbance of nebivolol hydrochloride and hydrochlorothiazide at 270.4 nm, 280.2 nm and 270 nm and measurement of absorbance of nebivolol HCl and valsartan at 246.6 nm, 280.2 nm and 275 nm, respectively. These methods

obeyed Beer's law in the concentration range of 0.5 - 2.5 µg/ml for nebivolol HCl, 1.0 – 20 µg/ml for valsartan and 1.0–3.0 µg /ml for hydrochlorothiazide.[86]

Estimation of valsartan, amlodipine and hydrochlorothiazide (HCT) were reported by Sharma et al (2010) using RP-HPLC method in human plasma. VAL, AML and HCT were resolved using a Gemini C18 column and gradient mobile phase starting from 20 % acetonitrile and 80 % 10 mmol L-1 ammonium formate (V/V, pH 3.5 ± 0.2, by formic acid) to 70 % acetonitrile and 30 % 10 mmol L-1 ammonium formate, over 20 minutes, with a flow rate of 1 ml/min. The samples were purified by protein precipitation and extraction. Telmisartan was used as internal standard. The method was validated according to USFDA and EMEA guidelines with good reproducibility and linear responses R = 0.9985 (VAL), 0.9964 (AML), and 0.9971 (HCT). RSD of intra and inter-day precision ranged 2.2-8.1 and 4.6-11.7%, respectively, for all three drugs. Mean extraction recoveries of three QCs for the triple drug combination were 76.5 (VAL), 72.0 (AML) and 73.0 (HCT) % for human plasma.[87]

The present article describes a rapid high performance thin layer chromatographic method for the simultaneous estimation of valsartan and hydrochlorothiazide in combined dosage forms, Shah et al (2009). The stationary phase used was precoated silica gel 60F254. The mobile phase used was a mixture of chloroform: methanol: toluene: glacial acetic acid (6:2:1:0.1 v/v/v/v). The detection of spot was carried out at 260 nm. The method was validated in terms of linearity, accuracy, precision and specificity. The calibration curve was found to be linear between 300 to 800 ng/spot for valsartan and 100 to 600 ng/spot for hydrochlorothiazide. The limit of detection and the limit of quantification for the valsartan were found to be 100 and 300 ng/spot respectively and for hydrochlorothiazide 30 and 100 ng/spot respectively.[88]

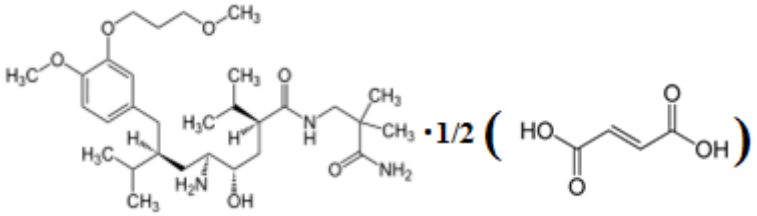
A High performance liquid chromatographic method was published for simultaneous determination of valsartan and hydrochlorothiazide in tablets by Tian et al (2008). A column having 200×4.6 mm i.d. in isocratic mode with mobile phase containing methanol: acetonitrile: water: isopropyl alcohol (22:18:68:2; adjusted to pH 8.0 using triethylamine; v/v) was used. The flow rate was 1.0 ml/min and effluents were monitored at 270 nm. The retention time (min) and linearity range

(μg/ml) for valsartan and hydrochlorothiazide were (3.42, 8.43) and (5-150, 78-234), respectively.[89]

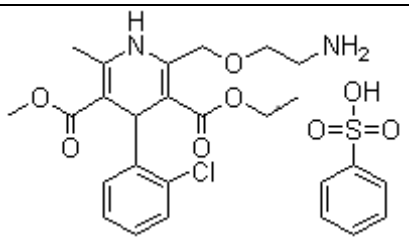
There was a new, simple, accurate, and precise high-performance thin-layer chromatographic (HPTLC) method for simultaneous analysis of valsartan and hydrochlorothiazide in tablet formulations by Kadam et al (2007). Standard and sample solutions of valsartan and hydrochlorothiazide were applied on pre-coated silica gel G 60 F254 HPTLC plates and the plates were developed with chloroform–ethyl acetate–acetic acid, 5:5:0.2 (v/v), as mobile phase. UV detection was performed densitometrically at 248 nm. The retention factors of valsartan and hydrochlorothiazide were 0.27 and 0.56, respectively. The linear range was 800–5600 ng/per spot for valsartan and 125–875 ng/per spot for hydrochlorothiazide; the correlation coefficients, r, were 0.9998 and 0.9988, respectively.[90]

3 Drug Profile[91-6]

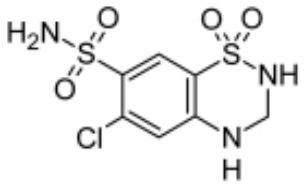
3.1 Aliskiren hemifumarate (Drug 1)

Chemical Structure	
Molecular Formula	$C_{30}H_{53}N_3O_6 \cdot 0.5\ C_4H_4O_4$
Molecular Weight	609.8 g/mol (Free base-551.8 g/mol)
Chemical Name	(2S,4S,5S,7S)-N-(2-methylpropyl) 5amino-4-hydroxy-2,7diisopropyl-8-[4-methoxy-3-(3-methoxypropoxy)phenyl]-octamide hemifumarate.
Description	Aliskiren hemifumarate is a white to slightly yellowish powder and highly hygroscopic in nature.
Solubility	It is highly soluble in water, freely soluble in methanol, ethanol and isopropanol.
Melting Range	76-78˚C
Pka value	9.79
Dose	150/300 mg
Category	Antihypertensive

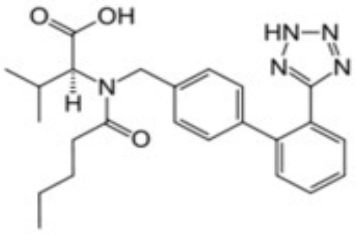
3.2 Amlodipine besilate (Drug 2)

Chemical Structure	
Molecular Formula	$C_{20}H_{25}ClN_2O_5 \bullet C_6H_6O_3S$
Molecular Weight	567.1 g/mol
Chemical Name	3-ethyl 5-methyl (±)-2-[(2-aminoethoxy)methyl]-4-(ochlorophenyl)- 1,4-dihydro-6-methyl-3,5-pyridinedicarboxylate, monobenzenesulfonate.
Description	Amlodipine besilate is a white to pale yellow crystalline powder.
Solubility	It is freely soluble in methanol, sparingly soluble in ethanol, slightly soluble in water and 2-propanol.
Melting Range	178-179˚C
Pka value	9.45
Dose	2.5/5/10 mg
Category	Antihypertensive

3.3 Hydrochlorothiazide (Drug 3)

Chemical Structure	
Molecular Formula	$C_7H_8ClN_3O_4S_2$
Molecular Weight	297.73
Chemical Name	6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide.
Description	HCTZ, USP is a white, or practically white, practically odorless, crystalline powder.
Solubility	It is slightly soluble in water; freely soluble in sodium hydroxide solution, n-butylamine and dimethylformamide; sparingly soluble in methanol; insoluble in ether, chloroform and dilute mineral acids.
Melting Range	266-268˚C
Pka value	7.9
Dose	12.5/25 mg
Category	Antihypertensive

3.4 Valsartan (Drug 4)

Drug 4	**Valsartan**
Chemical Structure	
Molecular Formula	$C_{24}H_{29}N_5O_3$
Molecular Weight	435.52
Chemical Name	N-(l-oxopentyl)-N-[[2'-(1H-tetrazol-5-yl) [1,1'-biphenyl]-4-yl]methyl]-L-valine.
Description	Valsartan is a white to practically white fine powder
Solubility	Soluble in ethanol and methanol; slightly soluble in water
Melting Range	116-117˚C
Pka value	4.37
Dose	160/320 mg
Category	Antihypertensive

4 AIM AND OBJECTIVES OF THE STUDY

Hypertension is one of the most common and powerful risk factors for cardiovascular diseases. Blood pressure control is prerequisite for the management of cardiovascular diseases and complications. More than one medication is required for effective control of blood pressure of cardiovascular patients. ALI (aliskiren hemifumarate) is the first and only representative of a new class of non peptide, low molecular weight; orally active transition state rennin inhibitor. ALI shows effective control of blood pressure and cardiovascular diseases when combined with AMLO (amlodipine besilate), HCT (hydrochlorothiazide) and VAL (valsartan). Literature survey reveals various analytical methods for the estimation of ALI alone and with other drugs in combinations like AMLO, HCT and VAL by UV spectroscopy, HPLC and electrophoresis. There was no reported HPTLC method for the estimation of ALI alone or in combination with other drugs. Still there was a scope for developing more sensitive methods for the determination of ALI in combination with other drugs in their tablet dosage form which can cover up the lacuna of some existing methods. Therefore, aim of the present work was to develop and validate some simpler, sensitive, precise, accurate and cost effective UV spectroscopic, RP-HPLC and HPTLC method compared to existing methods for the determination of ALI, AMLO, VAL and HCT in various tablet formulation.

The objective of the present study was to develop and validate newer analytical methods as per ICH guidelines [24] as follows:

1. To develop and validate a simultaneous equation method for the quantitative determination of aliskiren hemifumarate and hydrochlorothiazide in tablets.
2. To develop and validate an absorbance ratio (Q analysis) method for the simultaneous determination of aliskiren hemifumarate and hydrochlorothiazide in tablets.
3. To develop and validate a first-derivative (zero crossing) spectroscopic method for the simultaneous determination of aliskiren hemifumarate and hydrochlorothiazide in tablets.
4. To develop and validate a highly sensitive RP-HPLC method for the simultaneous analysis of aliskiren hemifumarate and hydrochlorothiazide in tablet formulation.

5. To develop and validate a simultaneous equation method for the determination of aliskiren hemifumarate and valsartan in combined tablet dosage form.
6. To develop and validate an absorbance ratio (Q analysis) method for the simultaneous determination of aliskiren hemifumarate and valsartan in tablets.
7. To develop and validate a first-derivative (zero crossing) spectroscopic method for the simultaneous estimation of aliskiren hemifumarate and valsartan in tablets.
8. To develop and validate a RP-HPLC method for the simultaneous determination of aliskiren hemifumarate and valsartan in tablets.
9. To develop and validate a HPTLC method for the simultaneous determination of aliskiren hemifumarate and valsartan in tablet dosage form.
10. To develop and validate a simultaneous equation method for the estimation of aliskiren hemifumarate and amlodipine besilate in tablets.
11. To develop and validate an absorbance ratio (Q analysis) method for the simultaneous determination of aliskiren hemifumarate and amlodipine besilate in tablets.
12. To develop and validate a first-derivative (zero crossing) spectroscopic method for the simultaneous determination of aliskiren hemifumarate and amlodipine besilate in tablets.
13. To develop and validate a RP-HPLC method for the simultaneous determination of aliskiren hemifumarate, amlodipine besilate and hydrochlorothiazide in combined tablet dosage form.

5. MATERIALS AND METHODS

5.1 Materials

5.1.1 Pure drugs (Reference substance)

5.1.1.1 Aliskiren hemifumarate: Aliskiren hemifumarate reference standard was provided by Jubilant Life Sciences Ltd., Noida, Uttar Pradesh, India.

5.1.1.2 Amlodipine besilate: Amlodipine besilate was received as gift sample from IPCA laboratories, Mumbai, Maharashtra, India.

5.1.1.3 Hydrochlorothiazide: Hydrochlorothiazide was obtained from Glenmark Pharmaceuticals Ltd., Mumbai, Maharashtra, India.

5.1.1.4 Valsartan: Valsartan was received as gift sample from Torrent Pharmaceuticals Limited, Ahmedabad, Gujarat, India.

5.1.2 Marketed formulations (Samples)

5.1.2.1 Formulation 1: RASILEZ HCT Tablet (Aliskiren hemifumarate 300 mg and hydrochlorothiazide 25 mg)

5.1.2.2 Formulation 2: VALTURNA Tablet (Aliskiren hemifumarate 150 mg and valsartan 160 mg)

5.1.2.3 Formulation 3: TEKAMLO Tablet (Aliskiren hemifumarate 300 mg and amlodipine besilate 10 mg)

5.1.2.4 Formulation 4: AMTURNIDE Tablet (Aliskiren hemifumarate 300 mg, amlodipine besilate 10 mg and hydrochlorothiazide 25 mg)

5.1.3 Solvents & chemicals

i. Methanol: AR grade (Loba Chemie Pvt. Ltd., Mumbai, India.) & HPLC grade (Merck Specialities Private Limited, Mumbai, India).
ii. Ethanol: AR grade, SDFCL, Mumbai, India.
iii. Acetonitrile: AR grade (Loba Chemie Pvt. Ltd., Mumbai, India.) & HPLC grade (Merck Specialities Private Limited, Mumbai, India).
iv. Water: HPLC grade, Millipore Direct Q3, Millipore India, Bangalore, India.
v. Chloroform: AR grade, SDFCL, Mumbai, India.
vi. Ethyl acetate: AR grade, SDFCL, Mumbai, India.

vii. Toluene: AR grade, SDFCL, Mumbai, India.
viii. Ammonia solution: AR grade, SDFCL, Mumbai, India.
ix. Glacial acetic acid: AR grade, SDFCL, Mumbai, India.
x. Diethylether: AR grade, SDFCL, Mumbai, India.
xi. Acetone: AR grade, SDFCL, Mumbai, India.
xii. Triethylamine: HPLC grade, Loba Chemie Pvt. Ltd., Mumbai, India.
xiii. Orthophosphoric acid: HPLC grade, Loba Chemie Pvt. Ltd., Mumbai, India.
xiv. Fumaric acid: HPLC grade, Himedia, Mumbai, India.

5.1.4 Instruments and equipments

5.1.4.1 UV-visible spectrophotometer

Model	:	UV–1800 (UV Probe)
Manufacturer	:	Shimadzu Corporation, Kyoto, Japan

5.1.4.2 HPTLC

Manufacturer	:	Camag, Muttenz, Switzerland
Applicator	:	Linomat 5
Scanner	:	Camag 3
Software	:	WinCATS
Hamilton syringe	:	100 µl
UV cabinet	:	254 nm & 366 nm
Pre-coated TLC plates	:	Silica gel 60 GF_{254} aluminium backed layer(20 µm)

5.1.4.3 RP-HPLC

Liquid Chromatograph	:	UFLC Prominence, LC 20 AD (Binary pump)
Manufacturer	:	Shimadzu Corporation, Kyoto, Japan
Detector	:	SPD M 20 A
Software	:	LC Solution
Column	:	Enable C_{18} - 250 mm × 4.6 mm, 5 µm, 120 Å
Hamilton syringe	:	25 µl

5.1.4.4 Digital balance

Model	:	Adventurer Pro AVG264C (0.0001 gm to 260 gm)
Manufacturer	:	Ohaus Corporation, Pine Brook, NJ, USA

5.1.4.5 Water purifier (HPLC grade water)

Model	:	Millipore Direct Q3
Manufacturer	:	Millipore India, Bangalore, India

5.1.4.6 Digital pH meter

Model	:	S901
Manufacturer	:	Systonic, Delhi, India

5.1.4.7 TOSHCON Ultrasonic cleaner (Sonicator)

Model	:	SW 4
Manufacturer	:	Toshniwal Instruments Mfg. Pvt. Ltd., Ajmer, India.

5.2 METHODS

Formulation 1

Method 1

5.2.1 *"Development and validation of simultaneous equation method for the simultaneous determination of aliskiren hemifumarate and hydrochlorothiazide in tablets"*

5.2.1.1 Selection of solvent

Based on the literature survey and solubility studies methanol was selected as solvent for further studies**.**

5.2.1.2 Preparation of standard solution

Stock solution of ALI and HCT were prepared by weighing accurately 11.052 mg of aliskiren hemifumarate (11.052 mg of aliskiren hemifumarate is equivalent to 10 mg of aliskiren) and 10 mg of hydrochlorothiazide standard drug which was then transferred to a 10 ml volumetric flask separately and diluted to 10 ml with methanol to get the concentration of the drugs 1000 µg/ml. Further dilutions were made to get the desired concentration with methanol.

5.2.1.3 Selection of wavelength

Standard stock solutions of ALI and HCT were further diluted separately with methanol to get the drug solutions containing 60 µg/ml of ALI and 5 µg/ml of HCT, respectively. Both the solutions were scanned in the UV region (200-400 nm) and spectra were recorded. Based on the spectral pattern, *simultaneous equation method* was selected for the estimation of both the drugs.

5.2.1.4 Determination of absorptivity value

Different concentrations of ALI (6-300 µg/ml) and HCT (0.5-25 µg/ml) were prepared from respective stock solutions. The absorbances were noted at 271 and 280 nm for both the drugs. The absorptivity values were calculated for ALI and HCT at both the wavelengths by using following formula:

Absorptivity = absorbance / concentration (gm/100 ml)

Absorptivity value of individual solution was calculated and average absorptivity value at specific wavelength of particular drug was used for calculating the concentration of drug.

5.2.1.5 Preparation of sample solution

Twenty tablets of Rasilez HCT (300 mg ALI and 25 mg of HCT) were accurately weighed and average weight was calculated. All the tablets were crushed to fine powder and quantity equivalent to 60 mg of ALI and 5 mg of HCT were weighed and transferred to a 50 ml volumetric flask. Flasks were vortexed after adding 30 ml of methanol and shaken for 10 minutes and volume was made up to the mark with methanol. Contents were filtered through whatman filter paper no 1 and suitable aliquots were prepared to get desired concentrations (eg. ALI 120 µg/ml and HCT 10 µg/ml).

5.2.1.6 Analysis of sample solution

After scanning the sample solution (Formulation) between 200 to 400 nm, absorbances were noted at 271 and 280 nm. The concentrations of drugs in sample/formulation were determined by the simultaneous equation method using the following formula:

$$Cx = \frac{A_2\, a_{y1} - A_1\, a_{y2}}{a_{x2}\, a_{y1} - a_{x1}\, a_{y2}}$$

$$Cy = \frac{A_1\, a_{x2} - A_2\, a_{x1}}{a_{x2}\, a_{y1} - a_{x1}\, a_{y2}}$$

Where Cx and Cy are the concentrations of ALI and HCT, ax_1 and ax_2 are absorptivities of ALI at 271 nm and 280 nm, respectively. ay_1 and ay_2 are absorptivities of HCT at 271 nm and 280 nm, respectively. A_1 and A_2 are the absorbances of formulation at 271 and 280 nm.

5.2.1.7 Validation of the method

The developed method was validated in accordance with "*International Conference on Harmonization"* guidelines for validation of analytical procedures.

5.2.1.8 Specificity

To check the interference between tablet excipients used in the marketed formulation and drug substance, specificity study was performed. Tablet excipients were mixed in

proportion (as per marketed formulation) and diluted using methanol. All the solutions (Placebo, standard and sample solution) were scanned in the UV region and compared with standard spectra to evaluate the interference between excipients and drugs.

5.2.1.9 Linearity and range

Linearity and range of the method was checked by analyzing all the standard solutions separately, containing ALI (6, 12, 60, 120, 180, 240 and 300 µg/ml) and HCT (0.5, 1, 5, 10, 15, 20 and 25 µg/ml) in methanol and absorbances were measured at 271 nm and 280 nm, respectively. Calibration graphs were plotted using absorbances of standard drug solutions versus concentration. Results were subjected to regression analysis by least squares method to calculate the values of slope, intercept and correlation coefficient.

5.2.1.10 Precision

Precision of the proposed method was evaluated based on the following parameters

5.2.1.10.1 Repeatability of measurement

Repeatability of the method was checked by analyzing standard solutions (ALI 60 & 120 µg/ml; HCT 5 & 10 µg/ml) six times by measuring the absorbances of both the drugs at 271 and 280 nm and %RSD was calculated.

5.2.1.10.2 Intra-day precision

Intra-day precision was carried out by analyzing standard solutions (ALI 60 & 120 µg/ml; HCT 5 & 10 µg/ml) in triplicate at two different concentration levels for three times on the same day within the linearity range and % RSD was calculated.

5.2.1.10.3 Inter-day precision

Inter-day precision was determined by repeated analysis of standard solutions (ALI 60 & 120 µg/ml; HCT 5 & 10 µg/ml) in triplicate at two different concentration levels within the linearity range on three different days. Percentage RSD was calculated and results are tabulated.

5.2.1.11 Accuracy

In order to ensure the suitability and reliability of the proposed method, recovery studies were carried out by standard addition method. To an equivalent quantity of pre-analyzed

sample solution (Formulation, ALI: 60, 90 and 120 µg/ml; HCT: 5, 7.5 and 10 µg/ml), a known concentration of standard ALI and HCT were added at 50, 100 and 150% level and the resulting solutions were reanalyzed by the proposed method and % recovery was calculated. The result of the accuracy study was assessed based on the percentage of standard ALI and HCT recovered from the formulation by using following formula:-

$$\%\ \text{Recovery} = (\text{Amount of drug found after addition of standard drug} - \text{Amount of drug found before addition of standard drug}) / (\text{Amount of standard drug added}) \times 100$$

5.2.1.12 LOD and LOQ

Sensitivity of the proposed method was determined in terms of LOD and LOQ. The limit of detection and limit of quantification of ALI and HCT were calculated using the following equation as per ICH guidelines.

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

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

Where σ = The standard deviation of the response, S = The slope of the calibration curve

5.2.1.13 Robustness

Capability of an analytical method to remain unaffected by incorporating small and premeditated deviation in the analytical parameters are refers to the robustness of a procedure. The method should be able to resist its critical parameters within the acceptable limit so it can be used for routine laboratory purpose. Robustness of the method was checked on the basis of slight alteration (±1 nm) in the wave length of measurement at 271 nm and 280 nm and % RSD was calculated.

5.2.1.14 Stability of the solution

Stability of the solutions were checked by observing any changes in terms of absorbance and the spectral pattern compared to freshly prepared solutions by keeping the solutions at room temperature and analyzing at a frequent interval.

5.2.1.15 Analysis of marketed formulation using developed method

Marketed formulation was extracted and diluted to get desired concentrations (eg. ALI 120 µg/ml and HCT 10 µg/ml) as described earlier in sample preparation. Absorbance was measured and percentage assay was calculated solving simultaneous equation method. In order to check the applicability of the method, standard stock solutions of both the drugs (ALI and HCT) were mixed according to the available strength of the marketed formulations and analyzed using developed simultaneous equation method.

Available strengths (mg): ALI + HCT: 150/300 + 12.5/25.

Method 2

5.2.2 *"Development and validation of absorbance ratio (Q analysis) method for the simultaneous determination of aliskiren hemifumarate and hydrochlorothiazide in tablets"*

5.2.2.1 Selection of solvent

Based on the literature survey and solubility studies methanol was selected as solvent for further studies**.**

5.2.2.2 Preparation of standard solution

Stock solution of ALI and HCT were prepared by weighing accurately 11.052 mg of aliskiren hemifumarate (11.052 mg of aliskiren hemifumarate is equivalent to 10 mg of aliskiren) and 10 mg of hydrochlorothiazide standard drug which was then transferred to a 10 ml volumetric flask separately and diluted to 10 ml with methanol to get the concentration of the drugs 1000 µg/ml. Further dilutions were made to get the desired concentration with methanol.

5.2.2.3 Selection of wavelength

Standard stock solution of ALI and HCT were further diluted separately with methanol to get the drug solutions containing 60 µg/ml of ALI and 5 µg/ml of HCT, respectively. Both the solutions were scanned in the UV region (200-400 nm) and spectra were recorded. Based on the spectral pattern, *absorbance ratio method (Q analysis)* was selected for the estimation of both the drugs. From the overlain spectra, 255 nm (isobestic point) and 271 nm (λ_{max} of HCT) were selected for further studies.

5.2.2.4 Determination of absorptivity value

Different concentrations of ALI (6-300 µg/ml) and HCT (0.5-25 µg/ml) were prepared from respective stock solutions. The absorbances were noted at 255 and 271 nm for both the drugs. The absorptivity values were calculated for ALI and HCT at both the wavelengths by using the following formula:

Absorptivity = absorbance/concentration (gm/100 ml)

Absorptivity value of individual solution was calculated and average absorptivity value at specific wavelength of particular drug was used for calculating the concentration of drug.

5.2.2.5 Preparation of sample solution

Twenty tablets of Rasilez HCT (300 mg ALI and 25 mg of HCT) were accurately weighed and average weight was calculated. All the tablets were crushed to fine powder and quantity equivalent to 60 mg of ALI and 5 mg of HCT were weighed and transferred to a 50 ml volumetric flask. Flasks were vortexed after adding 30 ml of methanol and shaken for 10 minutes and volume was made up to the mark with methanol. Contents were filtered through whatman filter paper no 1 and suitable aliquots were prepared to get desired concentrations (eg. ALI 120 µg/ml and HCT 10 µg/ml).

5.2.2.6 Analysis of sample solution

After scanning the sample solution (Formulation) between 200 to 400 nm, absorbances were noted at 255 and 271 nm. The concentrations of drugs in sample/formulation were determined by the absorbance ratio method using the following formula:

$$\mathrm{Cx} = \frac{Qm - Qy}{\mathrm{Qx} - \mathrm{Qy}} \times \frac{\mathrm{A1}}{ax1}$$

$$\mathrm{Cy} = \frac{Qm - Qx}{\mathrm{Qy} - \mathrm{Qx}} \times \frac{\mathrm{A1}}{ay1}$$

Where, ax_1 and ax_2 are absorptivities of ALI at 255 nm and 271 nm, respectively. ay_1 and ay_2 are absorptivities of HCT at 255 nm and 271 nm, respectively.

A1 and A2 are the absorbances of mixture at 255 nm and 271 nm. Cx and Cy are the concentrations of ALI and HCT, respectively in sample solution.

$$Qm = \frac{A2}{A1} \qquad Qx = \frac{ax2}{ax1} \qquad Qy = \frac{ay2}{ay1}$$

5.2.2.7 Validation of the method

The developed method was validated in accordance with "*International Conference on Harmonization"* guidelines for validation of analytical procedures.

5.2.2.8 Specificity

To check the interference between tablet excipients used in the marketed formulation and drug substance, specificity study was performed. Tablet excipients were mixed in proportion (as per marketed formulation) and diluted using methanol. All the solutions (Placebo, standard and sample solution) were scanned in the UV region and compared with standard spectra to evaluate the interference between excipients and drugs.

5.2.2.9 Linearity and range

Linearity and range of the method was checked by analyzing all the standard solutions separately, containing ALI (6, 12, 60, 120, 180, 240 and 300 µg/ml) and HCT (0.5, 1, 5, 10, 15, 20 and 25 µg/ml) were prepared in methanol and absorbances were measured at 255 nm and 271 nm, respectively. Calibration graphs were plotted using absorbances of standard drug solutions versus concentration. Results were subjected to regression analysis by the least squares method to calculate values of slope, intercept and correlation coefficient.

5.2.2.10 Precision

Precision of the proposed method was evaluated based on the following parameters

5.2.2.10.1 Repeatability of measurement

Repeatability of the method was checked by analyzing standard solutions (ALI 60 & 120 µg/ml; HCT 5 & 10 µg/ml) six times by measuring the absorbances of both the drugs at 255 and 271 nm and %RSD was calculated.

5.2.2.10.2 Intra-day precision

Intra-day precision was carried out by analyzing standard solutions (ALI 60 & 120 µg/ml; HCT 5 & 10 µg/ml) in triplicate at two different concentration levels for three times on the same day within the linearity range and % RSD was calculated.

5.2.2.10.3 Inter-day precision

Inter-day precision was determined by repeated analysis of standard solutions (ALI 60 & 120 µg/ml; HCT 5 & 10 µg/ml) in triplicate at two different concentration levels within

the linearity range on three different days. Percentage RSD was calculated and results are tabulated.

5.2.2.11 Accuracy

In order to ensure the suitability and reliability of the proposed method, recovery studies were carried out by standard addition method. To an equivalent quantity of pre-analyzed sample solution (Formulation, ALI: 60, 90 and 120 µg/ml; HCT: 5, 7.5 and 10 µg/ml), a known concentration of standard ALI and HCT were added at 50, 100 and 150% level and the resulting solutions were reanalyzed by the proposed method and % recovery was calculated. The result of the accuracy study was assessed based on the percentage of standard ALI and HCT recovered from the formulation by using following formula

$$\%\ \text{Recovery} = (\text{Amount of drug found after addition of standard drug} - \text{Amount of drug found before addition of standard drug}) / (\text{Amount of standard drug added}) \times 100$$

5.2.2.12 LOD and LOQ

Sensitivity of the proposed method was determined in terms of LOD and LOQ. The limit of detection and limit of quantification of ALI and HCT were calculated using the following equation as per ICH guidelines.

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

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

Where σ = The standard deviation of the response, S = The slope of the calibration curve

5.2.2.13 Robustness

Capability of an analytical method to remain unaffected by incorporating small and premeditated deviation in the analytical parameters are refers to the robustness of a procedure. The method should be able to resist its critical parameters within the acceptable limit so it can be used for routine laboratory purpose. Robustness of the method was checked on the basis of slight alteration (±1 nm) in the wave length of measurement at 255 nm and 271 nm and % RSD was calculated.

5.2.2.14 Stability of the solution

Stability of the solutions were checked by observing any changes in terms of absorbance and the spectral pattern compared with freshly prepared solutions by keeping the solutions at room temperature and analyzing at a frequent interval.

5.2.2.15 Analysis of marketed formulation using developed method

Marketed formulation was extracted and diluted to get desired concentrations (eg. ALI 120 µg/ml and HCT 10 µg/ml) as described earlier in sample preparation. Absorbance was measured and percentage assay was calculated solving absorbance ratio (Q analysis) method.In order to check the applicability of the method, standard stock solutions of both the drugs (ALI and HCT) were mixed according to the available strength of the marketed formulations and analyzed using developed absorbance ratio (Q analysis) method.

Available strengths (mg): ALI + HCT: 150/300 + 12.5/25

Method 3

5.2.3 *"Development and validation of first-derivative (Zero crossing) spectroscopic method for the simultaneous determination of aliskiren hemifumarate and hydrochlorothiazide in tablets"*

Derivative UV spectroscopy has been widely used as a tool for quantitative analysis and quality control. This technique has various advantages over the conventional absorbancy methods, such as the discrimination of the sharp spectral features over the large bands and the enhancement of the resolution of the overlapping spectra. A derivative spectrum shows better resolution of overlapping bands than the fundamental spectrum and may permit the accurate determination of the λ_{max} of the individual bands. Secondly, derivative spectrophotometry discriminates in favour of substances of narrow spectral bandwidth against broad bandwidth substances. All the amplitudes in the derivative spectrum are proportional to the concentration of the analyte provided that Beer's law is obeyed by the fundamental spectrum. In derivative spectral method, firstly UV spectrum of drug would be recorded and processed to get derivative spectrum. At the zero crossing point of one drug, the second drug would be measured which gives a reasonable means of estimating drug without interference of additives or impurities and thereby improves the sensitivity of the method.

5.2.3.1 Selection of solvent

Based on the literature survey and solubility studies methanol was selected as solvent for further studies**.** The UV spectra of ALI and HCT were recorded individually in methanol. All the spectra were processed to obtain their derivative spectra. In methanol derivative spectra of ALI and HCT showed favourable zero crossing points and good linearity. Hence, methanol was selected as solvent for further studies.

5.2.3.2 Preparation of standard solution

Stock solution of ALI and HCT were prepared by weighing accurately 11.052 mg of aliskiren hemifumarate (11.052 mg of aliskiren hemifumarate is equivalent to 10 mg of aliskiren) and 10 mg of hydrochlorothiazide standard drug which was then transferred to a 10 ml volumetric flask separately and diluted to 10 ml with methanol to get the

concentration of the drugs 1000 µg/ml. Further dilutions were made to get the desired concentration with methanol.

5.2.3.3 Selection of wavelength

Standard stock solutions of ALI and HCT were further diluted separately with methanol to get the drug solutions containing 60 µg/ml of ALI and 5 µg/ml of HCT, respectively. Both the solutions were scanned in the UV region (200-400 nm) and spectra were recorded. The spectra of ALI and HCT were converted into first and second derivative spectra. Based on the spectral pattern and zero crossing points first derivative method was selected for the study. First derivative spectra showed typical zero-crossing points at 280.20 nm for ALI and 241 nm for HCT. From the overlain spectra, 241 nm and 280.20 nm were selected for further studies.

5.2.3.4 Preparation of calibration curve

A calibration curve was plotted for both ALI and HCT in the range of 6 to 300 µg/ml and 0.5 to 25 µg/ml, respectively. Results were subjected to regression analysis by least square method to calculate the values of slope, intercept and correlation coefficient.

5.2.3.5 Preparation of sample solution

Twenty tablets of Rasilez HCT (300 mg of ALI and 25 mg of HCT) were accurately weighed and average weight was calculated. All the tablets were crushed to fine powder and quantity equivalent to 60 mg of ALI and 5 mg of HCT were weighed and transferred to a 50 ml volumetric flask. Flasks were vortexed after adding 30 ml of methanol and shaken for 10 minutes and volume was made up to the mark with methanol. Contents were filtered through whatman filter paper no 1 and suitable aliquots were prepared to get desired concentrations (eg. ALI 120 µg/ml and HCT 10 µg/ml).

5.2.3.6 Analysis of sample solution

All the solutions were scanned in the UV region (200-400 nm) and spectra were recorded and converted into their 1^{st} derivative spectra and amplitude was measured. The concentration of drugs in sample/formulation was determined by using the regression equation.

5.2.3.7 Validation of the method

The developed method was validated in accordance with "*International Conference on Harmonization"* guidelines for validation of analytical procedures.

5.2.3.8 Specificity

To check the interference between tablet excipients used in the marketed formulation and drug substance, specificity study was performed. Tablet excipients were mixed in proportion (as per marketed formulation) and diluted using methanol. All the solutions (Placebo, standard and sample solution) were scanned in the UV region and compared with standard spectra to evaluate the interference between excipients and drugs**.**

5.2.3.9 Linearity and range

Standard stock solutions of ALI and HCT were further diluted separately with methanol to get a series of drug solutions containing 6-300 µg/ml of ALI and 0.5-25 µg/ml of HCT. All the solutions were scanned in the UV region (200-400 nm) and spectra were recorded and converted into their 1^{st} derivative spectra. Linearity and range of the method was checked by measuring 1^{st} derivative signal and plotting calibration curve for both the drugs separately, containing ALI (6, 12, 60, 120, 180, 240 and 300 µg/ml) and HCT (0.5, 1, 5, 10, 15, 20 and 25 µg/ml) at 241 nm and 280.20 nm, respectively.

5.2.3.10 Precision

Precision of the proposed method was evaluated based on the following parameters

5.2.3.10.1 Repeatability of measurement

Repeatability of the method was checked by analyzing standard solutions (ALI: 60 & 120 µg/ml; HCT: 5 & 10 µg/ml) six times by measuring the 1^{st} derivative signal of ALI at 241 nm and HCT at 280.20 nm and %RSD was calculated.

5.2.3.10.2 Intra-day precision

Intra-day precision was carried out by analyzing standard solutions in triplicate at two different concentration levels (ALI: 60 & 120 µg/ml; HCT: 5 & 10 µg/ml) for three times on the same day within the linearity range and % RSD was calculated.

5.2.3.10.3 Inter-day precision

Inter-day precision was determined by repeated analysis of standard solutions in triplicate at two different concentration levels (ALI: 60 & 120 µg/ml; HCT: 5 & 10 µg/ml) within the linearity range on three different days. Percentage RSD was calculated.

5.2.3.11 Accuracy

In order to ensure the suitability and reliability of the proposed method, recovery studies were carried out by standard addition method. To an equivalent quantity of pre-analyzed sample solution (Formulation, ALI: 60, 90 and 120 µg/ml; HCT: 5, 7.5 and 10 µg/ml), a known concentration of standard ALI and HCT were added at 50, 100 and 150% level and the resulting solutions were reanalyzed and % recovery was calculated. The result of the recovery study was assessed based on the percentage of standard ALI and HCT recovered from the formulation by using following formula

$$\%\ \text{Recovery} = (\text{Amount of drug found after addition of standard drug} - \text{Amount of drug found before addition of standard drug}) / (\text{Amount of standard drug added}) \times 100$$

5.2.3.12 LOD and LOQ

Sensitivity of the proposed method was determined in terms of LOD and LOQ. The limit of detection and limit of quantification of ALI and HCT were calculated using the following equation as per ICH guidelines.

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

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

Where σ = Standard deviation of the response, S = Slope of the calibration curve

5.2.3.13 Robustness

Capability of an analytical method to remain unaffected by incorporating small and premeditated deviation in the analytical parameters are refers to the robustness of a procedure. The method should be able to resist its critical parameters within the acceptable limit so it can be used for routine laboratory purpose. Robustness of the

method was checked on the basis of slight alteration (±1 nm) in the wave length of measurement at 241 nm and 280.20 nm.

5.2.3.14 Stability of the solution

Stability of the solutions were checked by observing any changes in the spectral pattern compared with freshly prepared solutions by keeping the solutions at room temperature and analyzing at a frequent interval.

5.2.3.15 Analysis of marketed formulation using developed method

Marketed formulation was extracted and diluted to get desired concentrations (eg. ALI 120 µg/ml and HCT 10 µg/ml) as described earlier in sample preparation. All the solutions were scanned in the UV region (200-400 nm) and spectra were recorded and converted into their 1^{st} derivative spectra. The concentration of drugs in sample/formulation was determined by using the regression equation and percentage assay was calculated.

In order to check the applicability of the method, standard stock solutions of both the drugs (ALI and HCT) were mixed according to the available strengths of the marketed formulations and analyzed using developed *first-derivative (Zero crossing) spectroscopic* method.

Available strengths (mg): ALI + HCT: 150/300 + 12.5/25

Method 4

5.2.4 *"Development and validation of RP-HPLC method for the simultaneous determination of aliskiren hemifumarate and hydrochlorothiazide in tablets"*

5.2.4.1 Selection of mode of chromatographic method

Based on the literature survey RP-HPLC mode was selected.

5.2.4.2 Selection of column

Based on the literature survey C_{18} column was selected.

5.2.4.3 Selection of wavelength

Selectivity of HPLC method that uses UV detector depends on proper selection of wavelength. A wavelength which gives good response for both the drugs has to be selected. Overlain UV spectra of both the drugs were taken in RP-HPLC system and 280 nm was selected as the wavelength for study.

5.2.4.4 Trials for selection of mobile phase

Based on the literature survey different mobile phases with different compositions were tried and suitable mobile phase was selected for further studies.

5.2.4.5 Optimization of separation conditions[71, 97]

Strength of buffer, mobile phase composition, pH, flow rate etc. was varied to get optimum chromatographic conditions.

5.2.4.6 Preparation of standard solution

The stock solution of HCT was prepared by weighing accurately 10 mg of HCT standard drug which was transferred to a 10 ml volumetric flask and diluted to 10 ml with mobile phase to get the concentration of the drug 1000 µg/ml. The mixed standard stock solution of ALI and HCT were prepared by weighing accurately, 13.26 mg (13.26 mg of aliskiren hemifumarate is equivalent to 12 mg of aliskiren) of ALI, which was then mixed with 1 ml of HCT standard solution (1000 µg/ml) in to a 10 ml volumetric flask and diluted to 10 ml with mobile phase to get the concentration of the drugs 1200 and 100 µg/ml, respectively.

5.2.4.7 Preparation of sample solution

Twenty tablets of Rasilez HCT (300 mg of ALI and 25 mg of HCT) were accurately weighed and average weight was calculated. All the tablets were crushed to fine powder and quantity equivalent to 60 mg ALI and 5 mg of HCT were weighed and transferred to a 50 ml volumetric flask. Flasks were vortexed after adding 30 ml of mobile phase and shaken for 10 minutes and volume was made up to the mark with mobile phase. Contents were filtered through 0.45 µm membrane filter and suitable aliquots were prepared to get desired concentrations (eg. ALI 120 µg/ml and HCT 10 µg/ml).

5.2.4.8 Validation of chromatographic method

Developed method was validated according to ICH guidelines using following parameters

5.2.4.9 Specificity

To check the interference between tablet excipients used in the marketed formulation and drug substance, specificity study was performed. Tablet excipients were mixed in proportion (as per marketed formulation) and diluted using mobile phase. All the solutions (Placebo, mixed standard and sample solution) were injected and compared with the standard to evaluate the interference between excipients and drug peaks.

5.2.4.10 Linearity and range

Linearity of the method was checked by analyzing mixed standard solutions containing ALI (1.2, 6, 12, 60, 120, 180 and 240 µg/ml) and HCT (0.1, 0.5, 1, 5, 10, 15 and 20 µg/ml) in mobile phase. Calibration graphs were plotted using peak areas of standard drugs versus concentration. Results were subjected to regression analysis by the least squares method to calculate the values of slope, intercept and correlation coefficient.

5.2.4.11 Precision

The precision of the method was checked by carrying out repeatability, intra-day and inter-day precision.

5.2.4.11.1 Repeatability of measurement

To check the repeatability of the method a standard mixed solution (ALI: 60 & 120 µg/ml; HCT: 5 & 10 µg/ml) was injected 6 times and % RSD was calculated.

5.2.4.11.2 Intra-day precision

Intra-day precision was carried out by analyzing three replicate injections at two different concentration levels (ALI: 60 & 120 µg/ml; HCT: 5 & 10 µg/ml) on the same day within the linearity range and % RSD was calculated.

5.2.4.11.3 Inter-day precision

Inter-day precision was studied by comparing the results on three different days analyzing two replicate injections at two different concentration levels (ALI: 60 & 120 µg/ml; HCT: 5 & 10 µg/ml) within the linearity range and % RSD was calculated and results are presented in.

5.2.4.12 Accuracy

In order to ensure the suitability and reliability of the proposed method, recovery studies were carried out by standard addition method. To an equivalent quantity of pre-analyzed sample solution (Formulation, ALI: 24, 48 and 72 µg/ml; HCT: 2, 4 and 6 µg/ml), a known concentration of standard ALI and HCT were added at 50, 100 and 150% level and the resulting solutions were reanalyzed by the proposed method and % recovery was calculated. The result of the accuracy study was assessed based on the percentage of standard ALI and HCT recovered from the formulation by using following formula

$$\%\ \text{Recovery} = (\text{Amount of drug found after addition of standard drug} - \text{Amount of drug found before addition of standard drug}) / (\text{Amount of standard drug added}) \times 100$$

5.2.4.13 LOD and LOQ

Sensitivity of the proposed method was determined in terms of LOD and LOQ. The limit of detection and limit of quantification of ALI and HCT were calculated using the following equation as per ICH guidelines.

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

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

Where σ = The standard deviation of the response, S = The slope of the calibration curve

5.2.4.14 Robustness

Capability of an analytical method to remain unaffected by incorporating small and premeditated deviation in the analytical parameters are refers to the robustness of a procedure. The method should be able to resist its critical parameters within the acceptable limit so it can be used for routine laboratory purpose. Robustness of the method was checked on the basis of slight alteration in the organic phase (90 ± 2%v/v), buffer strength (0.2 ± 0.1%v/v), buffer pH (6 ± 0.2 unit), flow rate (1 ± 0.1 ml/min) etc.

5.2.4.15 Stability of the solution

Stability of the solutions were checked by observing any changes in the chromatographic pattern compared with freshly prepared solutions by keeping the solutions at room temperature and analyzing at a frequent interval.

5.2.4.16 System suitability test

System suitability tests were performed to confirm that the instrument was in appropriate condition for the analysis to be performed. Six replicates of the standard solution was injected and chromatograms were recorded to confirm the suitability of the chromatograph (Peak area reproducibility, retention time, no of theoretical plates, resolution, tailing factor).

5.2.4.17 Analysis of marketed formulation by developed method

Marketed formulation was extracted and diluted to get desired concentrations (ALI 60 & 120 µg/ml and HCT 5 & 10 µg/ml) as described earlier in sample preparation. All the

solutions were injected and chromatograms were recorded. Based on the peak area of analytes, percentage assay of the formulation was calculated using developed method.

In order to check the applicability of the method, standard stock solutions of both the drugs (ALI and HCT) were mixed according to the available strengths of the marketed formulations and analyzed using developed method.

Available strengths (mg): ALI + HCT: 150/300 + 12.5/25

Formulation 2

Method 5

5.2.5 *"Development and validation of simultaneous equation method for the simultaneous determination of aliskiren hemifumarate and valsartan in tablets"*

5.2.5.1 Selection of solvent

Based on the literature survey and solubility studies methanol was selected as solvent for further studies**.**

5.2.5.2 Preparation of standard solution

Stock solution of ALI and VAL were prepared by weighing accurately 11.052 mg of aliskiren hemifumarate (11.052 mg of aliskiren hemifumarate is equivalent to 10 mg of aliskiren) and 10.67 mg of valsartan standard drug which was then transferred to a 10 ml volumetric flask separately and diluted to 10 ml with methanol to get the concentration of the drugs 1000 and 1067 µg/ml, respectively. Further dilutions were made to get the desired concentration with methanol.

5.2.5.3 Selection of wavelength

Standard stock solutions of ALI and VAL were further diluted separately with methanol to get the drug solutions containing 15 µg/ml of ALI and 16 µg/ml of VAL, respectively. Both the solutions were scanned in the UV region (200-400 nm) and spectra were recorded. Based on the spectral pattern, *simultaneous equation method* was selected for the estimation of both the drugs. From the overlain spectra, 250 nm and 280 nm were selected for further studies.

5.2.5.4 Determination of absorptivity value

Different concentrations of ALI (1-30 µg/ml) and VAL (1.067-32 µg/ml) were prepared from respective stock solutions. The absorbances were noted at 250 and 280 nm for both the drugs. The absorptivity values were calculated for ALI and of VAL at both the wavelengths by using the following formula and tabulated.

Absorptivity = absorbance / concentration (gm/100 ml)

Absorptivity value of individual solution was calculated and average absorptivity value at specific wavelength of particular drug was used for calculating the concentration of drug.

5.2.5.5 Preparation of sample solution

Twenty tablets of Valturna (300 mg of ALI and 320 mg of VAL) were accurately weighed and average weight was calculated. All the tablets were crushed to fine powder and quantity equivalent to 5 mg of ALI and 5.33 mg of VAL were weighed and transferred to a 50 ml volumetric flask. Flasks were vortexed after adding 30 ml of methanol and shaken for 10 minutes and volume was made up to the mark with methanol. Contents were filtered through whatman filter paper no 1 and diluted to get desired concentrations (ALI 15 µg/ml and VAL 16 µg/ml).

5.2.5.6 Analysis of sample solution

After scanning the sample solution (Formulation) between 200 to 400 nm, absorbances were noted at 250 and 280 nm. The concentration of drugs in sample/formulation were determined by the simultaneous equation method using the following formula:

$$Cx = \frac{A_2\, a_{y1} - A_1\, a_{y2}}{a_{x2}\, a_{y1} - a_{x1}\, a_{y2}}$$

$$Cy = \frac{A_1\, a_{x2} - A_2\, a_{x1}}{a_{x2}\, a_{y1} - a_{x1}\, a_{y2}}$$

Where C_x and C_y are the concentrations of ALI and VAL, ax_1 and ax_2 are absorptivities of ALI at 250 nm and 280 nm, respectively. ay_1 and ay_2 are absorptivities of VAL at 250 nm and 280 nm, respectively. A_1 and A_2 are absorbances of mixture at 250 and 280 nm.

5.2.5.7 Validation of the method

The developed method was validated in accordance with *International Conference on Harmonization* guidelines for validation of analytical procedures.

5.2.5.8 Specificity

To check the interference between tablet excipients used in the marketed formulation and drug substance, specificity study was performed. Tablet excipients were mixed in proportion (as per marketed formulation) and diluted using methanol. All the solutions

(Placebo, standard and sample solution) were scanned in the UV region and compared with standard spectra to evaluate the interference between excipients and drugs.

5.2.5.9 Linearity and range

Linearity and range of the method was checked by analyzing all the standard solutions separately, containing ALI (1, 5, 10, 15, 20, 25 and 30 µg/ml) and VAL (1.067, 5.33, 10.67, 16, 21.33, 26.67 and 32 µg/ml) in methanol and absorbances were measured at 250 nm and 280 nm, respectively. Calibration graphs were plotted using absorbances of standard drug solutions versus concentration. Results were subjected to regression analysis by the least squares method to calculate the values of slope, intercept and correlation coefficient.

3.2.5.10 Precision

Precision of the proposed method was evaluated based on the following parameters

3.2.5.10.1 Repeatability of measurement

To check the repeatability of the measurement a standard solution (ALI: 10 & 20 µg/ml; VAL: 10.67 & 21.33 µg/ml) of both the drugs were subjected to six times analysis and %RSD was calculated.

5.2.5.10.2 Intra-day precision

Intra-day precision was carried out by analyzing standard solutions (ALI: 10 & 20 µg/ml; VAL: 10.67 & 21.33 µg/ml) in triplicate at two different concentration levels for three times on the same day within the linearity range and % RSD was calculated.

5.2.5.10.3 Inter-day precision

Inter-day precision was determined by repeated analysis of standard solutions (ALI: 10 & 20 µg/ml; VAL: 10.67 & 21.33 µg/ml) in triplicate at two different concentration levels within the linearity range on three different days. Percentage RSD was calculated and results are tabulated.

5.2.5.11 Accuracy

In order to ensure the suitability and reliability of the proposed method, recovery studies were carried out by standard addition method. To an equivalent quantity of pre-analyzed sample solution (Formulation, ALI: 6, 8 and 10 µg/ml; VAL: 6.3996, 8.5328 and 10.6667 µg/ml), a known concentration of standard ALI and VAL were added at 50, 100 and 150% level and the resulting solutions were reanalyzed and % recovery was calculated. The result of the accuracy study was assessed based on the percentage of standard ALI and VAL recovered from the formulation by using following formula:

$$\%\ \text{Recovery} = (\text{Amount of drug found after addition of standard drug} - \text{Amount of drug found before addition of standard drug}) / (\text{Amount of standard drug added}) \times 100$$

5.2.5.12 LOD and LOQ

The limit of detection and limit of quantification of ALI and VAL were calculated using the following equation as per ICH guidelines.

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

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

Where σ = The standard deviation of the response, S = The slope of the calibration curve.

5.2.5.13 Robustness

Capability of an analytical method to remain unaffected by incorporating small and premeditated deviation in the analytical parameters are refers to the robustness of a procedure. The method should be able to resist its critical parameters within the acceptable limit so it can be used for routine laboratory purpose. Robustness of the method was checked on the basis of slight alteration (±1 nm) in the wave length of measurement at 250 nm and 280 nm.

5.2.5.14 Stability of the solution

Stability of the solutions were checked by observing any changes in the spectral pattern compared with freshly prepared solutions by keeping the solutions at room temperature and analyzing at frequent intervals.

5.2.5.15 Analysis of marketed formulation using developed method:

Marketed formulation was extracted and diluted to get desired concentrations (ALI 15 µg/ml and VAL 16 µg/ml) as described earlier in sample preparation. Absorbance was measured and percentage assay was calculated solving simultaneous equation method**.**

Method 6

5.2.6 *"Development and validation of absorbance ratio (Q analysis) method for the simultaneous determination of aliskiren hemifumarate and valsartan in tablets"*

5.2.6.1 Selection of solvent

Based on the literature survey and solubility studies methanol was selected as solvent for further studies**.**

5.2.6.2 Preparation of standard solution

Stock solution of ALI and VAL were prepared by weighing accurately 11.052 mg of ALI (11.052 mg of aliskiren hemifumarate is equivalent to 10 mg of aliskiren) and 10.67 mg of VAL standard drug which was transferred to a 10 ml volumetric flask separately and diluted to 10 ml with methanol to get the concentration of the drugs 1000 and 1067 µg/ml, respectively. Further dilutions were made to get the desired concentration with methanol.

5.2.6.3 Selection of wavelength

Standard stock solutions of ALI and VAL were further diluted separately with methanol to get the drug solutions containing 15 µg/ml of ALI and 16 µg/ml of VAL, respectively. Both the solutions were scanned in the UV region (200-400 nm) and spectra were recorded. Based on the spectral pattern, *absorbance ratio method (Q analysis)* was selected for the estimation of both the drugs. From the overlain spectra, 282 nm (isobestic point) and 250 nm (λ_{max} of VAL) were selected for further studies.

5.2.6.4 Determination of absorptivity value

Different concentrations of ALI (1-30 µg/ml) and VAL (1.067-32 µg/ml) were prepared from respective stock solutions. The absorbances were noted at 250 and 282 nm for both the drugs. The absorptivity values were calculated for ALI and VAL at both the wavelengths by using the following formula and tabulated.

Absorptivity = absorbance / concentration (gm/100 ml)

Absorptivity value of individual solution was calculated and average absorptivity value at specific wavelength of particular drug was used for calculating the concentration of drug.

5.2.6.5 Preparation of sample solution:

Twenty tablets of Valturna (300 mg ALI and 320 mg of VAL) were accurately weighed and average weight was calculated. All the tablets were crushed to fine powder and quantity equivalent to 5 mg of ALI and 5.33 mg of VAL were weighed and transferred to a 50 ml volumetric flask. Flasks were vortexed after adding 30 ml of methanol and shaken for 10 minutes and volume was made up to the mark with methanol. Contents were filtered through whatman filter paper no 1 and diluted to get desired concentrations (ALI 15 µg/ml and VAL 16 µg/ml).

5.2.6.6 Analysis of sample solution

After scanning the sample solution (Formulation) between 200 to 400 nm, absorbances were noted at 250 and 282 nm. The concentrations of drugs in sample/formulation were determined by the absorbance ratio method using the following formula

$$\mathrm{Cx} = \frac{Qm - Qy}{\mathrm{Qx} - \mathrm{Qy}} \times \frac{\mathrm{A1}}{ax1}$$

$$\mathrm{Cy} = \frac{Qm - Qx}{\mathrm{Qy} - \mathrm{Qx}} \times \frac{\mathrm{A1}}{ay1}$$

Where, ax_1 and ax_2 are absorptivities of ALI at 250 nm and 282 nm, respectively. ay_1 and ay_2 are absorptivities of VAL at 250 nm and 282 nm, respectively.

A1 and A2 are the absorbances of mixture at 250 nm and 282 nm. Cx and Cy are the concentrations of ALI and VAL, respectively in sample solution.

$$Qm = \frac{A2}{A1} \qquad Qx = \frac{ax2}{ax1} \qquad Qy = \frac{ay2}{ay1}$$

5.2.6.7 Validation of the method

The developed method was validated in accordance with "International Conference on Harmonization" guidelines for validation of analytical procedures.

5.2.6.8 Specificity

To check the interference between tablet excipients used in the marketed formulation and drug substance, specificity study was performed. Tablet excipients were mixed in proportion (as per marketed formulation) and diluted using methanol. All the solutions

(Placebo, standard and sample solution) were scanned in the UV region and compared with standard spectra to evaluate the interference between excipients and drugs**.**

5.2.6.9 Linearity and range

Linearity and range of the method was checked by analyzing all the standard solutions separately, containing ALI (1, 5, 10, 15, 20, 25 and 30 µg/ml) and VAL (1.067, 5.33, 10.67, 16, 21.33, 26.67 and 32 µg/ml) in methanol and absorbances were measured at 250 nm and 282 nm, respectively. Calibration graphs were plotted using absorbabances of standard drug solutions versus concentration. Results were subjected to regression analysis by the least squares method to calculate the values of slope, intercept and correlation coefficient.

5.2.6.10 Precision

Precision of the proposed method was evaluated based on the following parameters

5.2.6.10.1 Repeatability of measurement

To check the repeatability of the measurement a standard solution (ALI: 10 & 20 µg/ml; VAL: 10.67 & 21.33 µg/ml) of both the drugs were subjected to six time analysis and %RSD was calculated.

5.2.6.10.2 Intra-day precision

Intra-day precision was carried out by analyzing standard solutions (ALI: 10 & 20 µg/ml; VAL: 10.67 & 21.33 µg/ml) in triplicate at two different concentration levels for three times on the same day within the linearity range.

5.2.6.10.3 Inter-day precision

Inter-day precision was determined by repeated analysis of standard solutions (ALI: 10 & 20 µg/ml; VAL: 10.67 & 21.33 µg/ml) in triplicate at two different concentration levels within the linearity range on three different days and percentage RSD was calculated.

5.2.6.11 Accuracy

In order to ensure the suitability and reliability of the proposed method, recovery studies were carried out by standard addition method. To an equivalent quantity of pre-analyzed

sample solution (Formulation, ALI: 6, 8 and 10 µg/ml; VAL: 6.3996, 8.5328 and 10.6667 µg/ml), a known concentration of standard ALI and VAL were added at 50, 100 and 150% level and the resulting solutions were reanalyzed and % recovery was calculated. The result of the accuracy study was assessed based on the percentage of standard ALI and VAL recovered from the formulation by using following formula

$$\%\ \text{Recovery} = (\text{Amount of drug found after addition of standard drug} - \text{Amount of drug found before addition of standard drug}) / (\text{Amount of standard drug added}) \times 100$$

5.2.6.12 LOD and LOQ

The limit of detection and limit of quantification of ALI and VAL were calculated using the following equation as per ICH guidelines.

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

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

Where σ = The standard deviation of the response, S = The slope of the calibration curve

5.2.6.13 Robustness

Capability of an analytical method to remain unaffected by incorporating small and premeditated deviation in the analytical parameters are refers to the robustness of a procedure. The method should be able to resist its critical parameters within the acceptable limit so it can be used for routine laboratory purpose. Robustness of the method was checked on the basis of slight alteration (±1 nm) in the wave length of measurement at 250 nm and 282 nm and % RSD was calculated**.**

5.2.6.14 Stability of the solution

Stability of the solutions were checked by observing any changes in the spectral pattern compared with freshly prepared solutions by keeping the solutions at room temperature and analyzing at a frequent interval.

5.2.6.15 Analysis of marketed formulation using developed method

Marketed formulation was extracted and diluted to get desired concentrations (ALI 15 µg/ml and VAL 16 µg/ml) as described earlier in sample preparation. Absorbance was measured and percentage assay was calculated solving absorbance ratio (Q analysis) method.

Method 7

5.2.7 *"Development and validation of first-derivative (Zero crossing) spectroscopic method for the simultaneous determination of aliskiren hemifumarate and valsartan in tablets"*

Derivative UV spectroscopy has been widely used as a tool for quantitative analysis and quality control. This technique has various advantages over the conventional absorbancy methods, such as the discrimination of the sharp spectral features over the large bands and the enhancement of the resolution of the overlapping spectra. A derivative spectrum shows better resolution of overlapping bands than the fundamental spectrum and may permit the accurate determination of the λ_{max} of the individual bands. Secondly, derivative spectrophotometry discriminates in favour of substances of narrow spectral bandwidth against broad bandwidth substances. All the amplitudes in the derivative spectrum are proportional to the concentration of the analyte provided that Beer's law is obeyed by the fundamental spectrum. In derivative spectral method, firstly UV spectrum of drug would be recorded and processed to get derivative spectrum. At the zero crossing point of one drug, the second drug would be measured which gives a reasonable means of estimating drug without interference of additives or impurities and thereby improves the sensitivity of the method.

5.2.7.1 Selection of solvent

By checking solubility in different solvents methanol was selected as solvent for further studies**.** The UV spectra of ALI and VAL were recorded individually in methanol. All the spectra were processed to obtain their derivative spectra. In methanol derivative spectra of ALI and VAL showed favourable zero crossing points and good linearity was observed. Hence, methanol was selected as solvent for further studies.

5.2.7.2 Preparation of standard solution

Stock solution of ALI and VAL were prepared by weighing accurately 11.052 mg of ALI (11.052 mg of aliskiren hemifumarate is equivalent to 10 mg of aliskiren) and 10.67 mg of VAL standard drug which was then transferred to a 10 ml volumetric flask separately and diluted to 10 ml with methanol to get the concentration of the drugs 1000 µg/ml and

1067 µg/ml, respectively. Further dilutions were made to get the desired concentration with methanol.

5.2.7.3 Selection of wavelength

Standard stock solutions of ALI and VAL were further diluted separately with methanol to get the drug solutions containing 10 µg/ml of ALI and 10.67 µg/ml of VAL, respectively. Both the solutions were scanned in the UV region (200-400 nm) and spectra were recorded. The spectra of ALI and VAL were recorded and these were converted into first and second derivative spectra. Based on the spectral pattern and zero crossing points first derivative method was selected for the study. First derivative spectra showed typical zero-crossing points at 280.30 nm for ALI and 244 nm for VAL. From the overlain spectra, 244 nm and 280.30 nm were selected for further studies.

5.2.7.4 Preparation of calibration curve

Calibration curves were plotted for both ALI and VAL in the range 1 to 30 µg/ml and 1.0666 to 32 µg/ml, respectively). Results were subjected to regression analysis by least square method to calculate the values of slope, intercept and correlation coefficient.

5.2.7.5 Preparation of sample solution

Twenty tablets of Valturna (300 mg of ALI and 320 mg of VAL) were accurately weighed and average weight was calculated. All the tablets were crushed to fine powder and quantity equivalent to 5 mg of ALI and 5.33 mg of VAL were weighed and transferred to a 50 ml volumetric flask. Flasks were vortexed after adding 30 ml of methanol and shaken for 10 minutes and volume was made up to the mark with methanol. Contents were filtered through whatman filter paper no 1 and diluted to get desired concentrations (ALI 15 µg/ml and VAL 16 µg/ml).

5.2.7.6 Analysis of sample solution

All the solutions were scanned in the UV region (200-400 nm) and spectrums were recorded and converted into their derivative spectra and amplitude was measured. The concentration of drugs in sample/formulation was determined by using the regression equation.

5.2.7.7 Validation of the method

The developed method was validated in accordance with "International Conference on Harmonization" guidelines for validation of analytical procedures.

5.2.7.8 Specificity

To check the interference between tablet excipients used in the marketed formulation and drug substance, specificity study was performed. Tablet excipients were mixed in proportion (as per marketed formulation) and diluted using methanol. All the solutions (Placebo, standard and sample solution) were scanned in the UV region and compared with standard spectra to evaluate the interference between excipients and drugs**.**

5.2.7.9 Linearity and range

Standard stock solutions of ALI and VAL were further diluted separately with methanol to get a series of drug solutions containing 1-30 µg/ml for ALI and 1.067-32 µg/ml for VAL. All the solutions were scanned in the UV region (200-400 nm) and spectra were recorded and converted into their derivative spectra. Linearity and range of the method was checked by measuring 1st derivative signal and plotting calibration curve for both the drugs separately, containing ALI (1, 5, 10, 15, 20, 25 and 30 µg/ml) and VAL (1.067, 5.33, 10.67, 16, 21.33, 26.67 and 32 µg/ml) at 244 nm and 280.30 nm, respectively.

5.2.7.10 Precision

Precision of the proposed method was evaluated based on the following parameters

5.2.7.10.1 Repeatability of measurement

To check the repeatability of the measurement a standard solution (ALI: 10 & 20 µg/ml; VAL: 10.67 & 21.33 µg/ml) of both the drugs were subjected to six time analysis and % RSD was calculated.

5.2.7.10.2 Intra-day precision

Intra-day precision was carried out by analyzing standard solutions (ALI: 10 & 20 µg/ml; VAL: 10.67 & 21.33 µg/ml) in triplicate at two different concentration levels for three times on the same day within the linearity range and % RSD was calculated.

5.2.7.10.3 Inter-day precision

Inter-day precision was determined by repeated analysis of standard solutions (ALI: 10 & 20 µg/ml; VAL: 10.67 & 21.33 µg/ml) in triplicate at two different concentration levels within the linearity range on three different days and percentage RSD was calculated.

5.2.7.11 Accuracy

In order to ensure the suitability and reliability of the proposed method, recovery studies were carried out by standard addition method. To an equivalent quantity of pre-analyzed sample solution (Formulation, ALI: 6, 8 and 10 µg/ml; VAL: 6.3996, 8.5328 and 10.6667 µg/ml), a known concentration of standard ALI and VAL were added at 50, 100 and 150% level and the resulting solutions were reanalyzed and % recovery was calculated. The result of the accuracy study was assessed based on the percentage of standard ALI and VAL recovered from the formulation by using following formula

$$\%\ \text{Recovery} = (\text{Amount of drug found after addition of standard drug} - \text{Amount of drug found before addition of standard drug}) / (\text{Amount of standard drug added}) \times 100$$

5.2.7.12 LOD and LOQ

The limit of detection and limit of quantification of ALI and VAL were calculated using the following equation as per ICH guidelines.

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

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

Where σ = The standard deviation of the response, S = The slope of the calibration curve

5.2.7.13 Robustness

Capability of an analytical method to remain unaffected by incorporating small and premeditated deviation in the analytical parameters are refers to the robustness of a procedure. The method should be able to resist its critical parameters within the acceptable limit so it can be used for routine laboratory purpose. Robustness of the

method was checked on the basis of slight alteration (±1 nm) in the wave length of measurement at 244 nm and 280.30 nm**.**

5.2.7.14 Stability of the solution

Stability of the solutions were checked by observing any changes in the spectral pattern compared with freshly prepared solutions by keeping the solutions at room temperature and analyzing at a frequent interval.

5.2.7.15 Analysis of marketed formulation using developed method

Marketed formulation was extracted and diluted to get desired concentrations (ALI 15 µg/ml and VAL 16 µg/ml) as described earlier in sample preparation. All the solutions were scanned in the UV region (200-400 nm) and spectra were recorded and converted into their 1^{st} derivative spectra. The concentration of drugs in sample/formulation was determined by using the regression equation and percentage assay was calculated.

METHOD 8

5.2.8 *"Development and validation of RP-HPLC method for the simultaneous determination of aliskiren hemifumarate and valsartan in tablets"*

5.2.8.1 Selection of mode of chromatographic method

Based on the literature survey RP-HPLC mode was selected.

5.2.8.2 Selection of column

Based on the literature survey C_{18} column was selected

5.2.8.3 Selection of wavelength

Selectivity of HPLC method that uses UV detector depends on proper selection of wavelength. A wavelength which gives good response for both the drugs has to be selected. Overlain UV spectra of both the drugs were taken in RP-HPLC system and 280 nm was selected as the wavelength for study.

5.2.8.4 Trials for selection of mobile phase

Based on the literature survey different mobile phases with different compositions were tried and suitable mobile phase was selected for further studies.

5.2.8.5 Optimization of separation conditions

Strength of buffer, mobile phase composition, pH, flow rate etc. were varied to achieve optimum separation of all the analytes under study.

5.2.8.6 Preparation of standard solution

Stock solution containing 1000 µg/ml of ALI and 1067 µg/ml of VAL was prepared by weighing 11.052 mg (11.052 mg of aliskiren hemifumarate is equivalent to 10 mg of aliskiren) of aliskiren hemifumarate and 10.67 mg of valsartan in to a 10 ml volumetric flask separately with the help of mobile phase and sonicated. Both the solutions were mixed and diluted proportionally with mobile phase to get desired mixed standard solution of varying concentration for analysis.

5.2.8.7 Preparation of sample solution

Twenty tablets of Valturna (300 mg of ALI and 320 mg of VAL) were accurately weighed and average weight was calculated. All the tablets were crushed to fine powder and quantity equivalent to 5 mg ALI and 5.33 mg of VAL were weighed and transferred to a 50 ml volumetric flask. Flasks were vortexed after adding 30 ml of mobile phase and shaken for 10 minutes and volume was made up to the mark with mobile phase. Contents were filtered through 0.45 µm membrane filter and suitable aliquots were prepared to get desired concentrations (eg. ALI 15 µg/ml and VAL 16 µg/ml).

5.2.8.8 Validation of chromatographic method

Developed method was validated according to ICH guidelines using following parameters

5.2.8.9 Specificity

To check the interference between tablet excipients used in the marketed formulation and drug substance, specificity study was performed. Tablet excipients were mixed in proportion (as per marketed formulation) and diluted using mobile phase. All the solutions (Placebo, mixed standard and sample solution) were injected and compared with the standard to evaluate the interference between excipients and drug peaks.

5.2.8.10 Linearity and range

Linearity of the method was checked by analyzing mixed standard solutions containing ALI (0.5, 1, 5, 10, 15, 20, 25 and 30 µg/ml) and VAL (0.53, 1.067, 5.33, 10.67, 16, 21.33, 26.67 and 32 µg/ml) were prepared in mobile phase. Column was equilibrated for 15 minutes with the mobile phase before injecting the solutions. Calibration graphs were plotted using peak areas of standard drugs versus concentration. Results were subjected to regression analysis by the least squares method to calculate values of slope, intercept and correlation coefficient.

5.2.8.11 Precision

The precision of the method was checked by carrying out repeatability, intra-day and inter-day precision.

5.2.8.11.1 Repeatability of measurement

To check the repeatability of the method a standard mixed solution (ALI: 5 & 10 µg/ml; VAL: 5.33 & 10.67 µg/ml) was injected 6 times and % RSD was calculated.

5.2.8.11.2 Intra-day precision

Intra-day precision was carried out by analyzing three replicate injections at two different concentration levels (ALI: 5 & 10 µg/ml; VAL: 5.33 & 10.67 µg/ml) on the same day within the linearity range and % RSD was calculated.

5.2.8.11.3 Inter-day precision

Inter-day precision was studied by comparing the results on three different days analyzing six replicate injections at two different concentration levels (ALI: 5 & 10 µg/ml; VAL: 5.33 & 10.67 µg/ml) within the linearity range and % RSD was calculated.

5.2.8.12 Accuracy

In order to ensure the suitability and reliability of the proposed method, recovery studies were carried out by standard addition method. To an equivalent quantity of pre-analyzed sample solution (Formulation, ALI: 4, 6 and 8 µg/ml; VAL: 4.2664, 6.3996 and 8.5328 µg/ml), a known concentration of standard ALI and VAL were added at 50, 100 and 150% level and resulting solutions were reanalyzed and % recovery was calculated. The result of the accuracy study was assessed based on the percentage of standard ALI and VAL recovered from the formulation by using following formula:

$$\%\ \text{Recovery} = (\text{Amount of drug found after addition of standard drug} - \text{Amount of drug found before addition of standard drug}) / (\text{Amount of standard drug added}) \times 100$$

5.2.8.13 LOD and LOQ

The limit of detection and limit of quantification of ALI and VAL were calculated using the following equation as per ICH guidelines.

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

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

Where σ = The standard deviation of the response, S = The slope of the calibration curve.

5.2.8.14 Robustness

Capability of an analytical method to remain unaffected by incorporating small and premeditated deviation in the analytical parameters are refers to the robustness of a procedure. The method should be able to resist its critical parameters within the acceptable limit so it can be used for routine laboratory purpose. Robustness of the method was checked on the basis of slight alteration in the organic phase (75 ± 2%v/v), buffer strength (0.2 ± 0.1%v/v), pH (6 ± 0.2 unit), flow rate (1 ± 0.1 ml/min).

5.2.8.15 Stability of the solution

Stability of the solutions were checked by observing any changes in the chromatographic pattern compared with freshly prepared solutions by keeping the solutions at room temperature and analyzing at a frequent interval.

5.2.8.16 System suitability test

System suitability tests were performed to confirm that the instrument was in appropriate condition for the analysis to be performed. Six Replicates of the standard solution was injected and chromatograms were recorded to confirm the suitability of the chromatograph, (Peak area reproducibility, retention time, no of theoretical plates, resolution, tailing factor).

5.2.8.17 Analysis of marketed formulation using developed method

Marketed formulation was extracted and diluted to get desired concentrations (ALI 10 & 15 µg/ml and VAL 10.67 & 16 µg/ml) as described earlier in sample preparation. All the solutions were injected and chromatograms were recorded. Based on the peak area of analytes, percentage assay of the formulation was calculated.

Method 9

5.2.9 *"Development and validation of HPTLC method for the simultaneous determination of aliskiren hemifumarate and valsartan in tablets"*

5.2.9.1 Selection of mode of chromatographic method

Based on the literature survey Pre-coated silica gel $60F_{254}$ on aluminium sheets were selected for study.

5.2.9.2 Selection of solvent

Based on the literature survey and solubility study, methanol was selected as solvent for further studies.

5.2.9.3 Selection of wavelength

UV spectra of drugs on pre-coated plate were recorded and 281 nm was selected as wavelength of detection.

5.2.9.4 Trials for selection of mobile phase

Initially different solvents like chloroform, methanol, acetonitrile, toluene, ethyl acetate, diethyl ether, propanol etc. were used as individual solvent to develop TLC plates (previously spotted with a fixed concentration of both the drugs). Moreover, extensive literature survey was carried out to get information about previously reported methods of other drugs. Finally based on the literature survey and preliminary trials, different mobile phases with different compositions were tried and suitable mobile phase was selected for further studies**.**

5.2.9.5 Optimization of separation conditions [98-102]

Parameters like mobile phase composition, saturation time, development distance, volume of mobile phase, activation time etc. were varied and optimum chromatographic condition was selected.

5.2.9.6 Preparation of standard solution

Stock solution containing 1000 µg/ml of ALI and 1067 µg/ml of VAL was prepared by weighing 11.052 mg (11.052 mg of aliskiren hemifumarate is equivalent to 10 mg of

aliskiren) of aliskiren hemifumarate and 10.67 mg of valsartan in to a 10 ml volumetric flask separately using methanol and sonicated. Working standard solution (ALI 100 µg/ml and VAL 106.7 µg/ml) was prepared by mixing 1 ml of each stock solution and volume was made up to 10 ml with methanol.

5.2.9.7 Preparation of sample solution

Twenty tablets of Valturna (300 mg ALI and 320 mg of VAL) were accurately weighed and average weight was calculated. All the tablets were crushed to fine powder and quantity equivalent to 5 mg of ALI and 5.33 mg of VAL were weighed and transferred to a 50 ml volumetric flask. Flasks were vortexed after adding 30 ml of methanol and shaken for 10 minutes and volume was made up to the mark with methanol. Contents were filtered through whatman filter paper and used for analysis (ALI 100 µg/ml and VAL 106.7 µg/ml).

5.2.9.8 Validation of chromatographic method[98-102]

Developed method was validated according to ICH guidelines using following parameters.

5.2.9.9 Specificity

Specificity of the method was checked by analyzing chromatographic peaks of drugs for peak purity. The peak purity of both the drugs ALI and VAL were assessed by comparing their respective spectra at peak start, peak apex and peak end positions of the spot.

5.2.9.10 Linearity and range

Linearity of the method was checked by applying different volumes, i.e. 0.5, 1, 2, 4, 6, 8 and 10 µl (ALI: 50-1000 ng/band; VAL: 53.33-1067 ng/band) of mixed working standard solution (ALI 100 µg/ml and VAL 106.7 µg/ml). The developed plate was analysed and chromatograms were recorded. Calibration curves were plotted using peak area versus concentration (ng/band). Results were subjected to regression analysis by the least squares method to calculate the values of slope, intercept and correlation coefficient.

5.2.9.11 Precision

The precision of the method was checked by carrying out repeatability, intra-day and inter-day precision.

5.2.9.11.1 Repeatability of measurement

To check the repeatability of the method a standard mixed solution (ALI: 400 & 600 ng/band; VAL: 426.67 & 640 ng/band) was spotted on the TLC plate six times within the linearity range, chromatograms were recorded and %RSD was calculated.

5.2.9.11.2 Intra-day precision

Intra-day precision studies were performed by spotting two different volume of the mixed standard solution (ALI: 400 & 600 ng/spot; VAL: 426.67 & 640 ng/spot) in triplicate on the same day within the linearity range and % RSD was calculated.

5.2.9.11.3 Inter-day precision

Inter-day precision studies were performed by spotting two different volume of the mixed standard solution (ALI: 400 & 600 ng/spot; VAL: 426.67 & 640 ng/spot) in triplicate on three different days within the linearity range and % RSD was calculated.

5.2.9.12 Accuracy

In order to ensure the suitability and reliability of the proposed method, recovery studies were carried out by standard addition method. To an equivalent quantity of pre-analyzed sample solution (Formulation, ALI: 200, 300 and 400 ng/band; VAL: 213.33, 320 and 426.67 ng/band), a known concentration of standard ALI and VAL were added at 50, 100 and 150% level and the resulting solutions were reanalyzed and % recovery was calculated. The result of the accuracy study was assessed based on the percentage of standard ALI and VAL recovered from the formulation by using following formula

$$\%\ \text{Recovery} = (\text{Amount of drug found after addition of standard drug} - \text{Amount of drug found before addition of standard drug}) / (\text{Amount of standard drug added}) \times 100$$

5.2.9.13 LOD and LOQ

The limit of detection and limit of quantification of ALI and VAL were calculated using the following equation as per ICH guidelines.

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

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

Where σ = The standard deviation of the response, S = The slope of the calibration curve.

5.2.9.14 Robustness

Capability of an analytical method to remain unaffected by incorporating small and premeditated deviation in the analytical parameters are refers to the robustness of a procedure. The method should be able to resist its critical parameters within the acceptable limit so it can be used for routine laboratory purpose. Robustness of the method was checked on the basis of slight alteration in the mobile phase composition (±0.1 ml), volume of mobile phase(20±5 ml), saturation time (20±5 min), development distance (80 ± 5 mm), time from spotting to chromatography, time (15±10 min) from chromatography to scanning (15±10 min) etc.

5.2.9.15 Stability of the solution

Stability of the solutions were checked by observing any changes in the chromatographic pattern compared with freshly prepared solutions by keeping the solutions at room temperature and analyzing at a frequent interval.

5.2.9.16 Analysis of marketed formulation using developed method

Marketed formulation was extracted and diluted to get desired concentrations (ALI 100 µg/ml and VAL 106.7 µg/ml) as described earlier in sample preparation. Different volumes were applied on the TLC plate and developed. Percentage assay of the formulation was calculated using developed method.

Formulation 3

Method 10

5.2.10 *"Development and validation of simultaneous equation method for the simultaneous determination of aliskiren hemifumarate and amlodipine besilate in tablets"*

5.2.10.1 Selection of solvent

Based on the literature survey and solubility studies methanol was selected as solvent for further studies**.**

5.2.10.2 Preparation of standard solution

Stock solution of ALI and AMLO were prepared by weighing accurately 11.052 mg of ALI (11.052 mg of aliskiren hemifumarate is equivalent to 10 mg of aliskiren) and 10 mg of AMLO standard drug which was then transferred to a 10 ml volumetric flask separately and diluted to 10 ml with methanol to get the concentration of the drugs 1000 µg/ml. Further dilutions were made to get the desired concentration with methanol.

5.2.10.3 Selection of wavelength

Standard stock solutions of ALI and AMLO were further diluted separately with methanol to get the drug solutions containing 20 µg/ml of ALI and 20 µg/ml of AMLO, respectively. Both the solutions were scanned in the UV region (200-400 nm) and spectra were recorded. Based on the spectral pattern, *simultaneous equation method* was selected for the estimation of both the drugs. From the overlain spectra, 237 nm and 280 nm were selected for further studies**.**

5.2.10.4 Determination of absorptivity value

Different concentrations of ALI (1-50 µg/ml) and AMLO (1-50 µg/ml) were prepared from respective stock solutions. The absorbances were noted at 237 and 280 nm for both the drugs. The absorptivity values were calculated for ALI and of AMLO at both the wavelengths by using the following formula and tabulated.

Absorptivity = absorbance/concentration (gm/100 ml)

Absorptivity value of individual solution was calculated and average absorptivity value at specific wavelength of particular drug was used for calculating the concentration of drug.

5.2.10.5 Preparation of sample solution

Twenty tablets of Tekamlo (300 mg ALI and 10 mg of AMLO) were accurately weighed and average weight was calculated. All the tablets were crushed to fine powder and quantity equivalent to 150 mg of ALI and 5 mg AMLO were weighed and transferred to a 50 ml volumetric flask. Flask was vortexed after adding 30 ml of methanol and shaken for 10 minutes and volume was made up to the mark with methanol and filtered through whatman filter paper no 1. One ml of the above mentioned solution was transferred to a 10 ml standard flask and 2.9 mg of AMLO standard was added and mixed to maintain the concentration of both the drugs same. Volume was made up to the mark with methanol and diluted with the same solvent to get desired concentration (ALI & AMLO 20 µg/ml).

5.2.10.6 Analysis of sample solution

After scanning the sample solution (Formulation) between 200 to 400 nm, absorbances were noted at 237 and 280 nm. The concentration of drugs in sample/formulation were determined by the simultaneous equation method using the following formula:

$$Cx = \frac{A_2\, a_{y1} - A_1\, a_{y2}}{a_{x2}\, a_{y1} - a_{x1}\, a_{y2}}$$

$$Cy = \frac{A_1\, a_{x2} - A_2\, a_{x1}}{a_{x2}\, a_{y1} - a_{x1}\, a_{y2}}$$

Where C_x and C_y are the concentrations of ALI and AMLO, ax_1 and ax_2 are absorptivities of ALI at 237 nm and 280 nm, respectively. ay_1 and ay_2 are absorptivities of AMLO at 237 nm and 280 nm, respectively. A_1 and A_2 are absorbances of mixture at 237 and 280 nm.

5.2.10.7 Validation of the method

The developed method was validated in accordance with "*International Conference on Harmonization"* guidelines for validation of analytical procedures.

5.2.10.8 Specificity

To check the interference between tablet excipients used in the marketed formulation and drug substance, specificity study was performed. Tablet excipients were mixed in proportion (as per marketed formulation) and diluted using methanol. All the solutions (Placebo, standard and sample solution) were scanned in the UV region and compared with standard spectra to evaluate the interference between excipients and drugs**.**

5.2.10.9 Linearity and range

Linearity and range of the method was checked by analyzing all the standard solutions separately, containing ALI (1, 5, 10, 20, 30, 40 and 50 µg/ml) and AMLO (1, 5, 10, 20, 30, 40 and 50 µg/ml) in methanol at 237 nm and 280 nm, respectively. Calibration graphs were plotted using absorbances of standard drug solutions versus concentration. Results were subjected to regression analysis by the least squares method to calculate the values of slope, intercept and correlation coefficient.

5.2.10.10 Precision

The precision of the method was checked by carrying out repeatability of measurement, intra-day and inter-day precision.

5.2.10.10.1 Repeatability of measurement

To check the repeatability of the measurement a standard solution (ALI 10 & 20 µg/ml; AMLO 10 & 20 µg/ml) of both the drugs were subjected to six times analysis and %RSD was calculated.

5.2.10.10.2 Intra-day precision

Intra-day precision was carried out by repeated measurement of the absorbance of standard solutions in triplicate at two different concentration levels (ALI 10 & 20 µg/ml; AMLO 10 & 20 µg/ml) for three times on the same day within the linearity range.

5.2.10.10.3 Inter-day precision

Inter-day precision was studied by comparing the results on three different days taking three replicate measurement at two different concentration levels (ALI 10 & 20 µg/ml;

AMLO 10 & 20 µg/ml) within the linearity range. Percentage RSD was calculated and results are tabulated.

5.2.10.11 Accuracy

In order to ensure the suitability and reliability of the proposed method, recovery studies were carried out by standard addition method. To an equivalent quantity of pre-analyzed sample solution (Formulation, ALI: 10, 15 and 20 µg/ml; AMLO: 10, 15 and 20 µg/ml, pure AMLO standard drug was added to maintain the same concentration for both the analytes), a known concentration of standard ALI and AMLO were added at 50, 100 and 150% level and resulting solutions were reanalyzed and % recovery was calculated. The result of the accuracy study was assessed based on the percentage of standard ALI and AMLO recovered from the formulation by using following formula

$$\%\ \text{Recovery} = (\text{Amount of drug found after addition of standard drug} - \text{Amount of drug found before addition of standard drug}) / (\text{Amount of standard drug added}) \times 100$$

5.2.10.11 LOD and LOQ

The limit of detection and limit of quantification of ALI and AMLO were calculated using the following equation as per ICH guidelines.

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

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

Where σ = The standard deviation of the response, S = The slope of the calibration curve

5.2.10.12 Robustness

Capability of an analytical method to remain unaffected by incorporating small and premeditated deviation in the analytical parameters are refers to the robustness of a procedure. The method should be able to resist its critical parameters within the acceptable limit so it can be used for routine laboratory purpose. Robustness of the method was checked on the basis of slight alteration (±1 nm) in the wave length of measurement at 237 nm and 280 nm.

5.2.10.13 Stability of the solution

Stability of the solutions were checked by observing any changes in the spectral pattern compared with freshly prepared solutions by keeping the solutions at room temperature and analyzing at a frequent interval.

5.2.10.14 Analysis of marketed formulation using developed method

Marketed formulation was extracted and diluted to get desired concentrations (ALI & AMLO 20 µg/ml) as described earlier in sample preparation. Absorbance was measured and percentage assay was calculated solving simultaneous equation method. In order to check the applicability of the method, standard stock solutions of both the drugs (ALI and AMLO) were mixed according to the available strength of the marketed formulations and analyzed using developed simultaneous equation method.

Available strengths (mg): ALI + AMLO: 150/300 + 5/10

Method 11

5.2.11 "Development and validation of absorbance ratio (Q analysis) method for the simultaneous determination of aliskiren hemifumarate and amlodipine besilate in tablets"

5.2.11.1 Selection of solvent

Based on the literature survey and solubility studies methanol was selected as solvent for further studies**.**

5.2.11.2 Preparation of standard solution

Stock solution of ALI and AMLO were prepared by weighing accurately 11.052 mg of ALI (11.052 mg of aliskiren hemifumarate is equivalent to 10 mg of aliskiren) and 10 mg of AMLO standard drug which was transferred to a 10 ml volumetric flask separately and diluted to 10 ml with methanol to get the concentration of the drugs 1000 µg/ml. Further dilutions were made to get the desired concentration with methanol.

5.2.11.3 Selection of wavelength

Standard stock solutions of ALI and AMLO were further diluted separately with methanol to get the drug solutions containing 20 µg/ml of ALI and 20 µg/ml of AMLO, respectively. Both the solutions were scanned in the UV region (200-400 nm) and spectra were recorded. Based on the spectral pattern, *absorbance ratio method (Q analysis)* was selected for the estimation of both the drugs. From the overlain spectra, 271 nm (isobestic point) and 237 nm (λ_{max} of AMLO) were selected for further studies.

5.2.11.4 Determination of absorptivity value

Different concentrations of ALI and AMLO (1-50 µg/ml) were prepared from respective stock solutions separately. The absorbances were noted at 237 and 271 nm for both the drugs. The absorptivity values were calculated for ALI and of AMLO at both the wavelengths by using the following formula.

Absorptivity = absorbance/concentration (gm/100 ml)

Absorptivity value of individual solution was calculated and average absorptivity value at specific wavelength of particular drug was used for calculating the concentration of drug.

5.2.11.5 Preparation of sample solution

Twenty tablets of Tekamlo (300 mg of ALI and 10 mg of AMLO) were accurately weighed and average weight was calculated. All the tablets were crushed to fine powder and quantity equivalent to 150 mg of ALI and 5 mg of AMLO were weighed and transferred to a 50 ml volumetric flask. Flask was vortexed after adding 30 ml of methanol and shaken for 10 minutes and volume was made up to the mark with methanol and filtered through whatman filter paper no 1. One ml of the above mentioned solution was transferred to a 10 ml standard flask and 2.9 mg of AMLO standard was added and mixed to maintain the concentration of both the drugs same. Volume was made up to the mark with methanol and diluted with the same solvent to get desired concentration (ALI & AMLO 20 µg/ml).

5.2.11.6 Analysis of sample solution

After scanning the sample solution (Formulation) between 200 to 400 nm, absorbances were noted at 237 and 271 nm. The concentrations of drugs in sample/formulation were determined by the absorbance ratio method using the following formula

$$\mathrm{Cx} = \frac{Qm - Qy}{\mathrm{Qx} - \mathrm{Qy}} \times \frac{\mathrm{A1}}{ax1}$$

$$\mathrm{Cy} = \frac{Qm - Qx}{\mathrm{Qy} - \mathrm{Qx}} \times \frac{\mathrm{A1}}{ay1}$$

Where, ax_1 and ax_2 are absorptivities of ALI at 237 nm and 271 nm, respectively. ay_1 and ay_2 are absorptivities of AMLO at 237 nm and 271 nm, respectively.

A1 and A2 are the absorbances of mixture at 237 nm and 271 nm. Cx and Cy are the concentrations of ALI and AMLO, respectively in sample solution.

$$Qm = \frac{A2}{A1} \qquad Qx = \frac{ax2}{ax1} \qquad Qy = \frac{ay2}{ay1}$$

5.2.11.7 Validation of the method

The developed method was validated in accordance with *"International Conference on Harmonization"* guidelines for validation of analytical procedures.

5.2.11.8 Specificity

To check the interference between tablet excipients used in the marketed formulation and drug substance, specificity study was performed. Tablet excipients were mixed in proportion (as per marketed formulation) and diluted using methanol. All the solutions (Placebo, standard and sample solution) were scanned in the UV region and compared with standard spectra to evaluate the interference between excipients and drugs.

5.2.11.9 Linearity and range

Linearity and range of the method was checked by analyzing all the standard solutions separately, containing ALI (1, 5, 10, 20, 30, 40 and 50 µg/ml) and AMLO (1, 5, 10, 20, 30, 40 and 50 µg/ml), in methanol and absorbance was noted at 237 nm and 271 nm, respectively. Calibration graphs were plotted using absorbances of standard drug solutions versus concentration. Results were subjected to regression analysis by the least squares method to calculate values of slope, intercept and correlation coefficient.

5.2.11.10 Precision

The precision of the method was checked by carrying out repeatability of measurement, intra-day and inter-day precision.

5.2.11.10.1 Repeatability of measurement

To check the repeatability of the measurement a standard solution (ALI 10 & 20 µg/ml; AMLO 10 & 20 µg/ml) of both the drugs were subjected to six time analysis and %RSD was calculated.

5.2.11.10.2 Intra-day precision

Intra-day precision was carried out by repeated measurement of the absorbance of standard solutions (ALI 10 & 20 µg/ml; AMLO 10 & 20 µg/ml) in triplicate at two different concentration levels for three times on the same day within the linearity range.

5.2.11.10.3 Inter-day precision

Inter-day precision was studied by comparing the results on three different days taking three replicate measurement at two different concentration (ALI 10 & 20 µg/ml; AMLO 10 & 20 µg/ml) levels within the linearity range. Percentage RSD was calculated.

5.2.11.11 Accuracy

In order to ensure the suitability and reliability of the proposed method, recovery studies were carried out by standard addition method. To an equivalent quantity of pre-analyzed sample solution (Formulation, ALI: 10, 15 and 20 µg/ml; AMLO: 10, 15 and 20 µg/ml, pure AMLO standard drug was added to maintain the same concentration for both the analytes), a known concentration of standard ALI and AMLO were added at 50, 100 and 150% level and the resulting solutions were reanalyzed and % recovery was calculated. The result of the accuracy study was assessed based on the percentage of standard ALI and AMLO recovered from the formulation by using following formula

$$\%\ \text{Recovery} = (\text{Amount of drug found after addition of standard drug} - \text{Amount of drug found before addition of standard drug}) / (\text{Amount of standard drug added}) \times 100$$

5.2.11.12 LOD and LOQ

The limit of detection and limit of quantification of ALI and AMLO were calculated using the following equation as per ICH guidelines.

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

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

Where σ = The standard deviation of the response, S = The slope of the calibration curve.

5.2.11.13 Robustness

Capability of an analytical method to remain unaffected by incorporating small and premeditated deviation in the analytical parameters are refers to the robustness of a procedure. The method should be able to resist its critical parameters within the acceptable limit so it can be used for routine laboratory purpose. Robustness of the method was checked on the basis of slight alteration (±1 nm) in the wave length of measurement at 237 nm and 271 nm.

3.2.11.14 Stability of the solution

Stability of the solutions were checked by observing any changes in the spectral pattern compared with freshly prepared solutions by keeping the solutions at room temperature and analyzing at frequent intervals.

5.2.11.15 Analysis of marketed formulation using developed method

Marketed formulation was extracted and diluted to get desired concentrations (ALI & AMLO 20 µg/ml) as described earlier in sample preparation. Absorbance was measured and percentage assay was calculated solving simultaneous equation method. In order to check the applicability of the method, standard stock solutions of both the drugs (ALI and AMLO) were mixed according to the available strength of the marketed formulations and analyzed using developed simultaneous equation method.

Available strengths (mg): ALI + AMLO: 150/300 + 5/10

Method 12

5.2.12 *"Development and validation of first-derivative (Zero crossing) spectroscopic method for the simultaneous determination of aliskiren hemifumarate and amlodipine besilate in tablets"*

Derivative UV spectroscopy has been widely used as a tool for quantitative analysis and quality control. This technique has various advantages over the conventional absorbancy methods, such as the discrimination of the sharp spectral features over the large bands and the enhancement of the resolution of the overlapping spectra. A derivative spectrum shows better resolution of overlapping bands than the fundamental spectrum and may permit the accurate determination of the λ_{max} of the individual bands. Secondly, derivative spectrophotometry discriminates in favour of substances of narrow spectral bandwidth against broad bandwidth substances. All the amplitudes in the derivative spectrum are proportional to the concentration of the analyte provided that Beer's law is obeyed by the fundamental spectrum. In derivative spectral method, firstly UV spectrum of drug would be recorded and processed to get derivative spectrum. At the zero crossing point of one drug, the second drug would be measured which gives a reasonable means of estimating drug without interference of additives or impurities and thereby improves the sensitivity of the method.

5.2.12.1 Selection of solvent

By checking solubility in different solvents methanol was selected as solvent for further studies**.** The UV spectra of ALI and AMLO were recorded individually in methanol. All the spectra were processed to obtain their derivative spectra. In methanol derivative spectra of ALI and AMLO showed favourable zero crossing points and good linearity was observed. Hence, methanol was selected as solvent for further studies.

5.2.12.2 Preparation of standard solution

Stock solution of ALI and AMLO were prepared by weighing accurately 11.052 mg of ALI (11.052 mg of aliskiren hemifumarate is equivalent to 10 mg of aliskiren) and 10 mg of AMLO standard drug which was transferred to a 10 ml volumetric flask separately and

diluted to 10 ml with methanol to get the concentration of both the drugs 1000 µg/ml. Further dilutions were made to get the desired concentration with methanol.

5.2.12.3 Selection of wavelength

Standard stock solutions of ALI and AMLO were further diluted separately with methanol to get the drug solutions containing 10 µg/ml of ALI and 10 µg/ml of AMLO, respectively. Both the solutions were scanned in the UV region (200-400 nm) and spectra were recorded. The spectra of ALI and AMLO were recorded and converted into first and second derivative spectra. Based on the spectral pattern and zero crossing points first derivative method was selected for the study. First derivative spectra were showed typical zero-crossing points at 254 nm for ALI and 237 nm for AMLO. From the overlain spectra, 237 nm and 254 nm were selected for further studies.

5.2.12.4 Preparation of calibration curve

Calibration graphs were plotted for both ALI and AMLO in the range of 0.5-50 µg/ml using 1st derivative signal of standard drug solutions versus concentration. Results were subjected to regression analysis by the least squares method to calculate the values of slope, intercept and correlation coefficient.

5.2.12.5 Preparation of sample solution

Twenty tablets of Tekamlo (300 mg of ALI and 10 mg of AMLO) were accurately weighed and average weight was calculated. All the tablets were crushed to fine powder and quantity equivalent to 150 mg ALI and 5 mg AMLO were weighed and transferred to a 50 ml volumetric flask. Flask was vortexed after adding 30 ml of methanol and shaken for 10 minutes and volume was made up to the mark with methanol and filtered through whatman filter paper no 1. One ml of the above mentioned solution was transferred to a 10 ml standard flask and 2.9 mg of AMLO standard was added and mixed to maintain the concentration of both the drugs same. Volume was made up to the mark with methanol and diluted with the same solvent to get desired concentration (ALI & AMLO 20 µg/ml).

5.2.12.6 Analysis of sample solution

All the solutions were scanned in the UV region (200-400 nm) and spectrums were recorded and converted into their derivative spectra. The concentration of drugs in sample/formulation was determined by using the regression equation.

5.2.12.7 Validation of the method

The developed method was validated in accordance with International Conference on Harmonization guidelines for validation of analytical procedures.

5.2.12.8 Specificity

To check the interference between tablet excipients used in the marketed formulation and drug substance, specificity study was performed. Tablet excipients were mixed in proportion (as per marketed formulation) and diluted using methanol. All the solutions (Placebo, standard and sample solution) were scanned in the UV region and compared with standard spectra to evaluate the interference between excipients and drugs.

5.2.12.9 Linearity and range

Standard stock solutions of ALI and AMLO were further diluted separately with methanol to get a series of drug solutions containing 0.5-50 µg/ml for both the drugs. All the solutions were scanned in the UV region (200-400 nm) and spectrums were recorded and converted into their 1^{st} derivative spectra. Linearity and range of the method was checked by measuring 1^{st} derivative signal and plotting calibration curve for both the drugs separately, containing ALI and AMLO (0.5, 5, 10, 20, 30, 40 and 50 µg/ml) at 237 nm and 254 nm, respectively.

5.2.12.10 Precision

The precision of the method was checked by carrying out repeatability of measurement, intra-day and inter-day precision.

5.2.12.10.1 Repeatability of measurement

To check the repeatability of the measurement a standard solution (ALI 10 & 20 µg/ml; AMLO 10 & 20 µg/ml) of both the drugs were subjected to six time analysis and %RSD was calculated.

5.2.12.10.2 Intra-day precision

Intra-day precision was carried out by repeated measurement of the absorbance of standard solutions in triplicate at two different concentration levels (ALI and AMLO 10 & 20 µg/ml) for three times on the same day within the linearity range.

5.2.12.10.3 Inter-day precision

Inter-day precision was studied by comparing the results on three different days taking three replicate measurement at two different concentration (ALI and AMLO 10 & 20 µg/ml) levels within the linearity range. Percentage RSD was calculated and results are tabulated.

5.2.12.11 Accuracy

In order to ensure the suitability and reliability of the proposed method, recovery studies were carried out by standard addition method. To an equivalent quantity of pre-analyzed sample solution (Formulation, ALI: 10, 15 and 20 µg/ml; AMLO: 10, 15 and 20 µg/ml, pure AMLO standard drug was added to maintain the same concentration for both the analytes), a known concentration of standard ALI and AMLO were added at 50, 100 and 150% level and the resulting solutions were reanalyzed and % recovery was calculated. The result of the accuracy study was assessed based on the percentage of standard ALI and AMLO recovered from the formulation by using following formula

$$\%\ \text{Recovery} = (\text{Amount of drug found after addition of standard drug} - \text{Amount of drug found before addition of standard drug}) / (\text{Amount of standard drug added}) \times 100$$

5.2.12.12 LOD and LOQ

The limit of detection and limit of quantification of ALI and AMLO were calculated using the following equation as per ICH guidelines.

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

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

Where σ = The standard deviation of the response, S = The slope of the calibration curve

5.2.12.13 Robustness

Capability of an analytical method to remain unaffected by incorporating small and premeditated deviation in the analytical parameters are refers to the robustness of a procedure. The method should be able to resist its critical parameters within the acceptable limit so it can be used for routine laboratory purpose. Robustness of the method was checked on the basis of slight alteration (±1 nm) in the wave length of measurement at 237 nm and 254 nm.

5.2.12.14 Stability of the solution

Stability of the solutions were checked by observing any changes in the spectral pattern compared with freshly prepared solutions by keeping the solutions at room temperature and analyzing at frequent intervals.

5.2.12.15 Analysis of marketed formulation using developed method

Marketed formulation was extracted and diluted to get desired concentrations (ALI & AMLO 20 µg/ml) as described earlier in sample preparation. All the solutions were scanned in the UV region (200-400 nm) and spectra were recorded and converted into their 1^{st} derivative spectra. The concentration of drugs in sample/formulation was determined by using the regression equation and percentage assay was calculated.

In order to check the applicability of the method, standard stock solutions of both the drugs (ALI and AMLO) were mixed according to the available strength of the marketed formulations and analyzed using developed *first-derivative (Zero crossing) spectroscopic* method.

Available strengths (mg): ALI + AMLO: 150/300 + 5/10

Formulation 4

Method 13

5.2.13 *"Development and validation of RP-HPLC method for the simultaneous determination of aliskiren hemifumarate, amlodipine besilate and hydrochlorothiazide in tablets"*

5.2.13.1 Selection of mode of chromatographic method

Based on the literature survey RP-HPLC mode was selected.

5.2.13.2 Selection of column

Based on the literature survey C_{18} column was selected.

5.2.13.3 Selection of wavelength

UV spectra of all the drugs were taken in RP-HPLC system and from the overlain spectra, 237 nm was selected as the wavelength**.**

5.2.13.4 Trials for selection of mobile phase

Based on the literature survey different mobile phases with different compositions were tried and suitable mobile phase was selected for further studies.

5.2.13.5 Optimization of separation conditions

Strength of buffer, mobile phase composition, pH, flow rate etc. were varied and tried to achieve optimum separation condition.

5.2.13.6 Preparation of standard solution

The stock solution of AMLO and HCT were prepared by weighing accurately 10 mg of each drug which was then transferred to a 10 ml volumetric flask separately and diluted to 10 ml with mobile phase to get the concentration of the drugs 1000 µg/ml. The mixed standard stock solution (ALI 1500 µg/ml, AMLO 50 µg/ml and HCT 125 µg/ml) of ALI, AMLO and HCT was prepared by weighing accurately16.78 mg (16.78 mg of aliskiren hemifumarate is equivalent to 15 mg of aliskiren) of ALI, which was mixed with 0.5 ml of AMLO and 1.25 ml of HCT stock solution in to a 10 ml volumetric flask and volume was made up to 10 ml with mobile phase.

5.2.13.7 Preparation of sample solution

Twenty tablets of Amturnide (300 mg ALI, 10 mg AMLO and 25 mg of HCT) were accurately weighed and average weight was calculated. All the tablets were crushed to fine powder and quantity equivalent to 120 mg ALI, 4 mg AMLO and 10 mg HCT were transferred to a 100 ml volumetric flask. Flasks were vortexed after adding 50 ml of mobile phase and shaken for 10 minutes. Contents were filtered through 0.45 µm membrane filter and diluted to get desired concentration (ALI 120 µg/ml, AMLO 4 µg/ml and HCT 10 µg/ml).

5.2.13.8 Validation of chromatographic method

Developed method was validated according to ICH guidelines using following parameters

5.2.13.9 Specificity

To check the interference between tablet excipients used in the marketed formulation and drug substance, specificity study was performed. Tablet excipients were mixed in proportion (as per marketed formulation) and diluted using mobile phase. All the solutions (Placebo, mixed standard and sample solution) were injected and compared with the standard to evaluate the interference between excipients and drug peaks.

5.2.13.10 Linearity and range

Linearity of the method was checked by analyzing mixed standard solutions containing ALI (7.5, 15, 60, 120, 180, 240 and 300 µg/ml), AMLO (0.25, 0.5, 2, 4, 6, 8 and 10 µg/ml) and HCT (0.625, 1.25, 5, 10, 15, 20 and 25 µg/ml) in mobile phase. Column was equilibrated for 15 minutes with the mobile phase before injecting the solutions. Calibration graphs were plotted using peak areas of standard drugs versus concentration. Results were subjected to regression analysis by the least squares method to calculate the values of slope, intercept and correlation coefficient are shown in.

5.2.13.11 Precision

The precision of the method was checked by carrying out repeatability, intra-day and inter-day precision.

5.2.13.11.1 Repeatability of measurement

To check the repeatability of the method a standard mixed solution (ALI: 60 & 120 µg/ml; AMLO 2 & 4 µg/ml; HCT: 5 & 10 µg/ml) were injected 6 times and % RSD was calculated.

5.2.13.11.2 Intra-day precision

Intra-day precision was carried out by analyzing six replicate injections at two different concentration levels (ALI: 60 & 120 µg/ml; AMLO 2 & 4 µg/ml; HCT: 5 & 10 µg/ml) on the same day within the linearity range and % RSD was calculated.

5.2.13.11.3 Inter-day precision

Inter-day precision was studied by comparing the results on three different days analyzing three replicate injections at two different concentration (ALI: 60 & 120 µg/ml; AMLO 2 & 4 µg/ml; HCT: 5 & 10 µg/ml) levels within the linearity range and % RSD was calculated.

5.2.13.12 Accuracy

In order to ensure the suitability and reliability of the proposed method, recovery studies were carried out by standard addition method. To an equivalent quantity of pre-analyzed sample solution (Formulation, ALI: 60, 120 and 180 µg/ml; AMLO: 2, 4 and 6 µg/ml HCT: 5, 10 and 15 µg/ml), a known concentration of standard ALI, AMLO and HCT were added at 50, 100 and 150% level and the resulting solutions were reanalyzed and % recovery was calculated. The result of the accuracy study was assessed based on the percentage of standard ALI, AMLO and HCT recovered from the formulation by using following formula

$$\%\ \text{Recovery} = (\text{Amount of drug found after addition of standard drug} - \text{Amount of drug found before addition of standard drug}) / (\text{Amount of standard drug added}) \times 100$$

5.2.13.13 LOD and LOQ

The limit of detection and limit of quantification of ALI, AMLO and HCT were calculated using the following equation as per ICH guidelines.

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

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

Where σ = The standard deviation of the response, S = The slope of the calibration curve

5.2.13.14 Robustness

Capability of an analytical method to remain unaffected by incorporating small and premeditated deviation in the analytical parameters are refers to the robustness of a procedure. The method should be able to resist its critical parameters within the acceptable limit so it can be used for routine laboratory purpose. Robustness of the method was checked on the basis of slight alteration in organic phase (90 ± 2%v/v), buffer (0.2 ± 0.1%v/v), pH (6 ± 0.2 unit), flow rate (1 ± 0.1 ml/min).

5.2.13.15 Stability of the solution

Stability of the solutions were checked by observing any changes in the chromatographic pattern compared with freshly prepared solutions by keeping the solutions at room temperature and analyzing at frequent intervals.

5.2.13.16 System suitability test

System suitability tests were performed to confirm that the instrument was in appropriate condition for the analysis to be performed. Six Replicates of the standard solution was injected and chromatograms were recorded to confirm the suitability of the chromatograph (Peak area reproducibility, no of theoretical plates, retention time, resolution, tailing factor).

5.2.13.17 Analysis of marketed formulation using developed method

Marketed formulation was extracted and diluted to get desired concentrations (ALI 120 µg/ml, AMLO 4 µg/ml and HCT 10 µg/ml) as described earlier in sample preparation. All the solutions were injected and chromatograms were recorded. Based on the peak

area of analytes, percentage assay of the formulation was calculated using developed method.

In order to check the applicability of the method, standard stock solutions of both the drugs (ALI, AMLO and HCT) were mixed according to the available strengths of the marketed formulations and analyzed using developed method.

Available strengths (mg): ALI + AMLO + HCT: 150/300 + 5/10 + 12.5/25

6. RESULTS AND DISCUSSION

METHOD 1

6.1 *"Development and validation of simultaneous equation method for the simultaneous determination of aliskiren hemifumarate and hydrochlorothiazide in tablets"*

Estimation of ALI and HCT was achieved by simultaneous equation method using Shimadzu UV-1800 (UV Pro), double beam UV-visible spectrophotometer.

6.1.1 Selection of solvent

Based on the literature survey and solubility studies methanol was selected as solvent, which showed higher absorbance and distinct λ_{max} for both the drugs**.**

6.1.2 Selection of wavelength

Standard solutions of ALI (60 µg/ml) and HCT (5 µg/ml) were separately scanned in the UV region (200-400 nm) and spectra were recorded using methanol as blank. Their overlain spectra are shown in Figure No. 6.1.1.

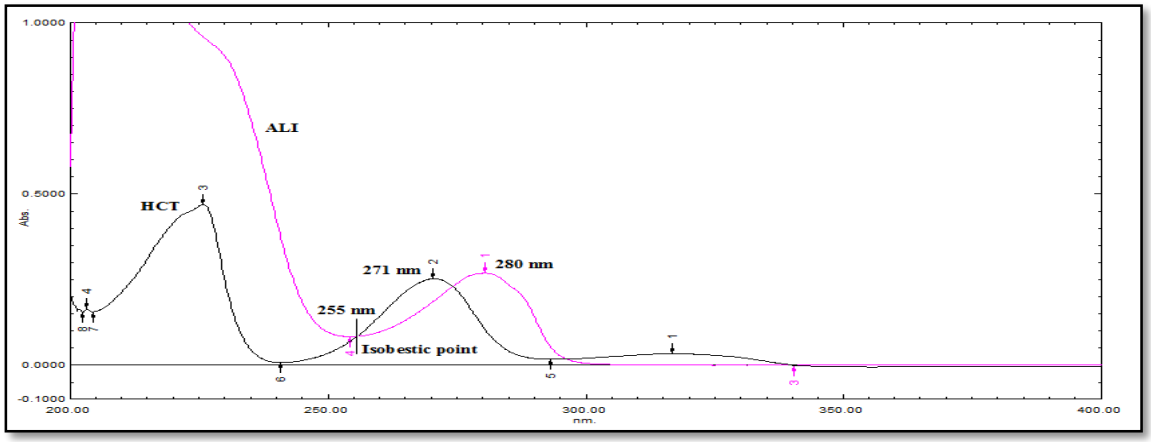


Figure No. 6.1.1: Overlain UV spectra of ALI (60 µg/ml) and HCT (5 µg/ml)

From the overlain spectra, initially different wavelengths were tried for the study. But finally 271 nm (λ_{max} of HCT) and 280 nm (λ_{max} of ALI) were selected, which showed good linearity and hence used for simultaneous estimation of ALI and HCT by *simultaneous equation method*.

6.1.3 Determination of absorptivity value

The developed method was found to be linear in the concentration range of 6-300 µg/ml for ALI and 0.5-25 µg/ml for HCT. Absorbances were measured at 271 nm and 280 nm for both the drugs and absorptivity values were calculated and presented in Table No. 6.1.1 & 6.1.2.

Table No. 6.1.1: Absorbances and absorptivities of ALI at selected wavelengths

Conc. (µg/ml)	ALI					
	271 nm			280 nm		
	Abs.*	Absorptivity	Avg. Absorptivity	Abs.*	Absorptivity	Avg. Absorptivity
6	0.0210	34.9833	(ax_1) **35.2830**	0.0290	48.2500	(ax_2) **49.6197**
12	0.0406	33.8569		0.0582	48.5278	
60	0.2055	34.2556		0.2988	49.7972	
120	0.4222	35.1847		0.5913	49.2750	
180	0.6480	35.9981		0.9080	50.4426	
240	0.8752	36.4681		1.2223	50.9284	
300	1.0870	36.2344		1.5035	50.1167	

**average of six determinations*

Table No. 6.1.2: Absorbances and absorptivities of HCT at selected wavelengths

Conc. (µg/ml)	HCT					
	271 nm			280 nm		
	Abs.*	Absorptivity	Avg. Absorptivity	Abs.*	Absorptivity	Avg. Absorptivity
0.5	0.0295	590.6667	(ay_1) **590.5593**	0.0134	267.0000	(ay_2) **268.1950**
1	0.0586	586.3333		0.0272	271.8333	
5	0.2963	592.6667		0.1357	271.4000	
10	0.5881	588.0500		0.2651	265.0833	
15	0.8869	591.2667		0.3963	264.2000	
20	1.1872	593.5917		0.5380	269.0083	
25	1.4784	591.3400		0.6721	268.8400	

**average of six determinations*

6.1.4 Validation of the method

The developed method was validated in accordance with "*International Conference on Harmonization"* guidelines for validation of analytical procedures.

6.1.5 Specificity

Overlain spectra of placebo and drug solutions indicate that there was no interference between excipients and standard drugs (Figure No. 6.1.2).

6.1.6 Linearity and range

ALI and HCT were found to be linear in the concentration range of 6-300 µg/ml and 0.5-25 µg/ml, respectively. Overlain spectra of ALI and HCT are shown in Figure No. 6.1.3-6.1.5.

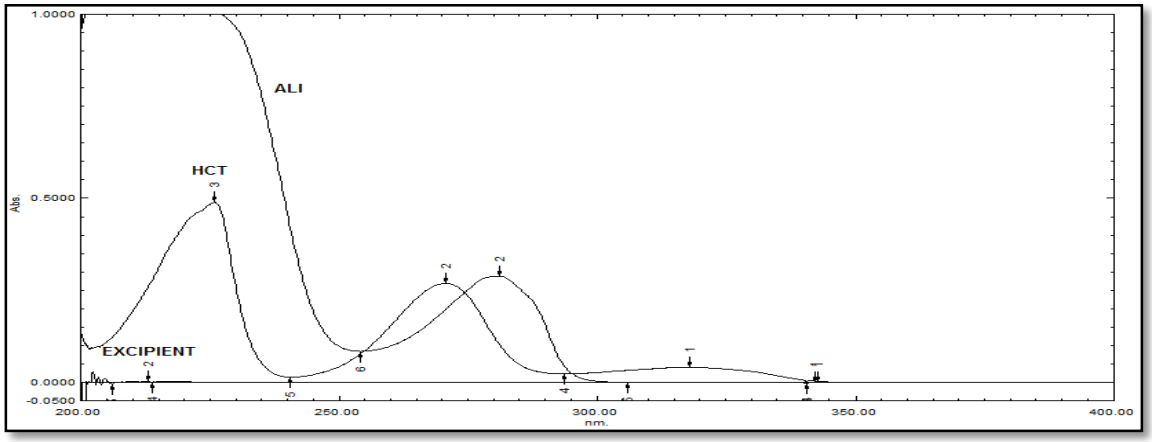


Figure No. 6.1.2: Overlain UV spectra of formulation excipients and standard drugs

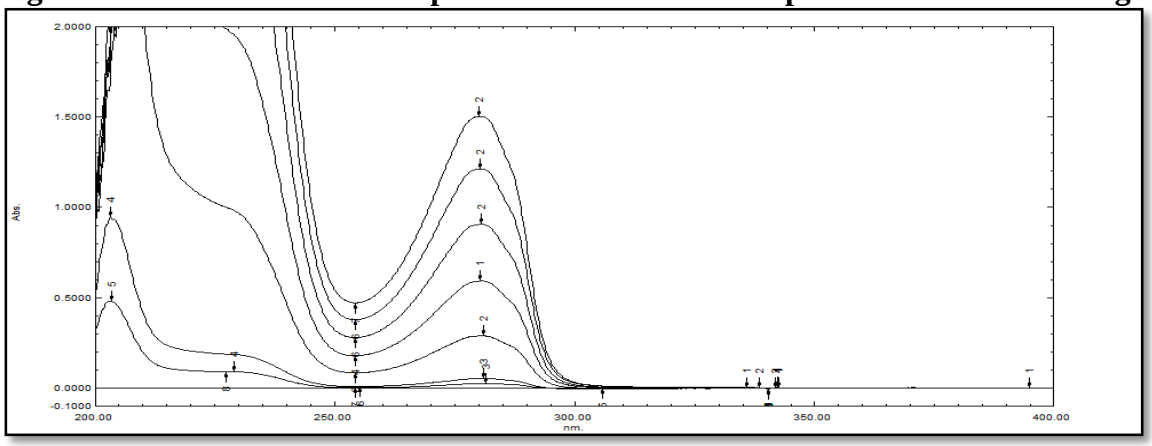


Figure No. 6.1.3: Overlain UV spectra of ALI (6-300 µg/ml)

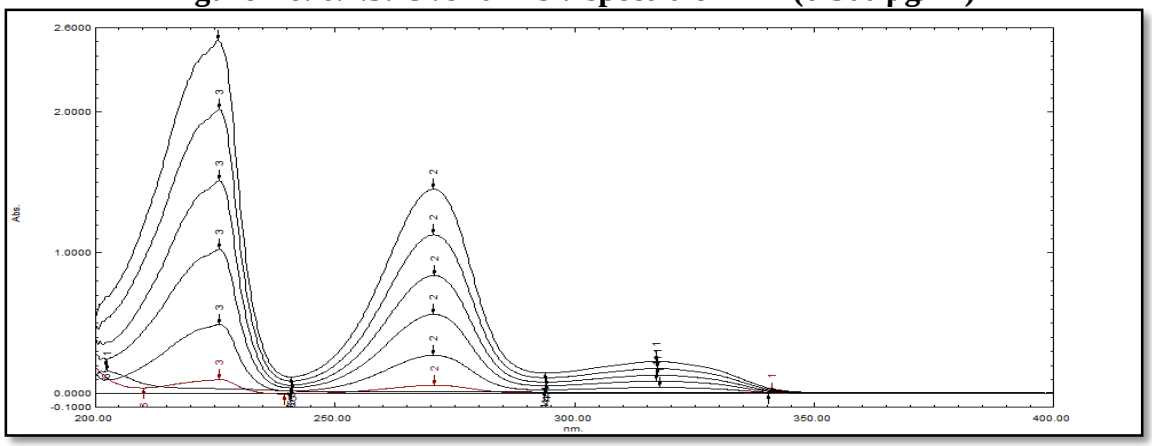


Figure No. 6.1.4: Overlain UV spectra of HCT (0.5-25 µg/ml)

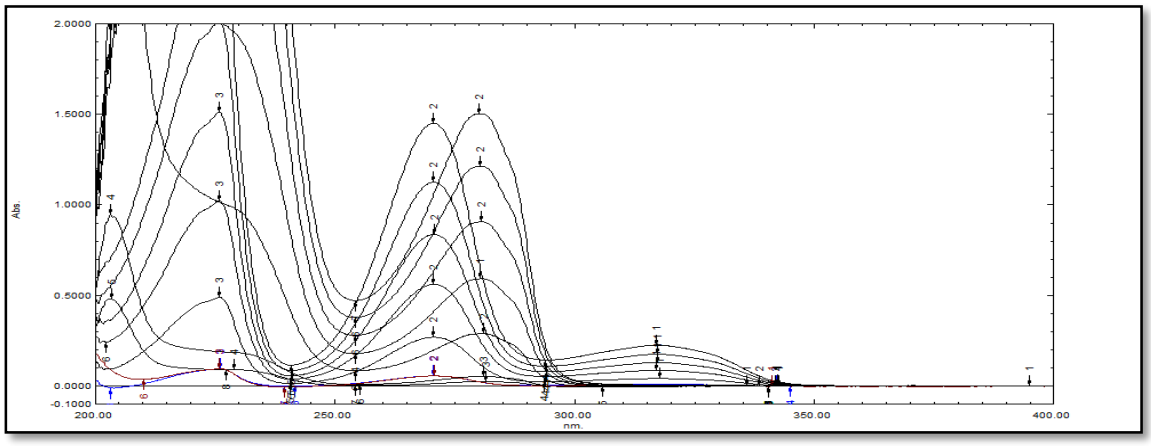


Figure No. 6.1.5: Overlain UV spectra of ALI (6-300 µg/ml) & HCT (0.5-25 µg/ml)

Calibration graphs (Figure No. 6.1.6 - 6.1.9) were plotted using absorbance of standard drug versus concentration. Results were subjected to regression analysis by least square method to calculate the values of slope, intercept and correlation coefficient of ALI and HCT at 271 and 280 nm are shown in Table No. 6.1.8.

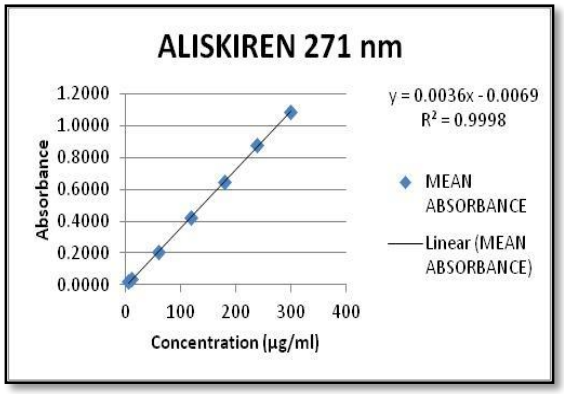


Figure No. 6.1.6: Calibration graph of ALI (6-300 µg/ml) at 271 nm

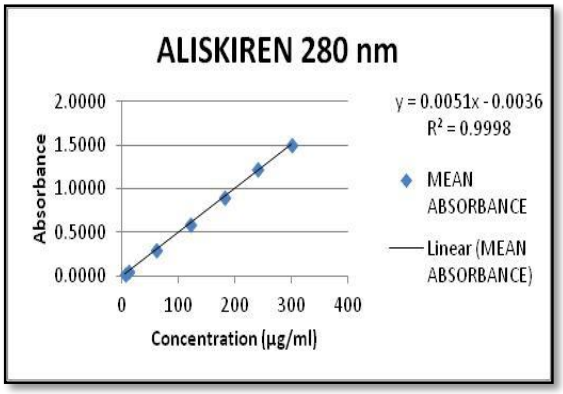


Figure No. 6.1.7: Calibration graph of ALI (6-300 µg/ml) at 280 nm

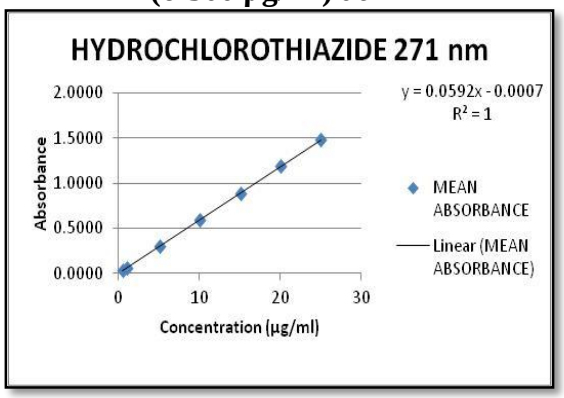


Figure No. 6.1.8: Calibration graph of HCT (0.5-25 µg/ml) at 271 nm

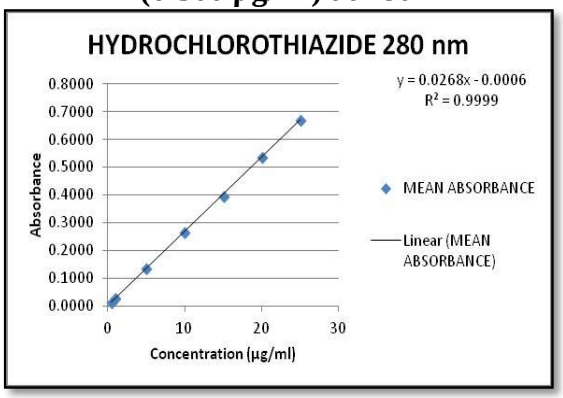


Figure No. 6.1.9: Calibration graph of HCT (0.5-25 µg/ml) at 280 nm

6.1.7 Precision

Results of precision studies expressed in % RSD follows ICH guideline acceptable limits (<2), which shows good repeatability, low intra and inter-day variability, indicating an excellent precision of the developed method (Table No. 6.1.3 - 6.1.5).

Table No. 6.1.3: Results of repeatability of measurement

Repeatability					
Sr. No.	**Conc.**	**Absorbance**			
		ALI		**HCT**	
		271 nm	**280 nm**	**271 nm**	**280 nm**
1	ALI 60 µg/ml HCT 5 µg/ml	0.2041	0.2996	0.2945	0.1356
2		0.2019	0.2978	0.2975	0.1346
3		0.1998	0.2987	0.2968	0.1356
4		0.2045	0.2991	0.2984	0.1358
5		0.2034	0.2986	0.2945	0.1349
6		0.2018	0.2979	0.2916	0.1328
Mean±SD*		**0.2026±0.0018**	**0.2988±0.0007**	**0.2956±0.0025**	**0.1349±0.0011**
% RSD		**0.8682**	**0.2219**	**0.8465**	**0.8314**
1	ALI 120 µg/ml HCT 10 µg/ml	0.4175	0.5884	0.5815	0.2638
2		0.4167	0.5987	0.5839	02618
3		0.4195	0.5965	0.5817	0.2648
4		0.4157	0.5987	0.5896	0.2618
5		0.4169	0.5982	0.5861	0.2637
6		0.4189	0.5945	0.5891	0.2658
Mean±SD*		**0.4175±0.0014**	**0.5958±0.0040**	**0.5853±0.0035**	**0.2636±0.0016**
% RSD		**0.3420**	**0.6693**	**0.6061**	**0.6071**
Mean % RSD		**0.6051**	**0.4504**	**0.7263**	**0.7193**

**mean±SD, (n= 6) number of determination*

Table No. 6.1.4: Results of intra-day precision

Intra-day precision					
Sr. No.	**Conc.**	**Absorbance**			
		ALI		**HCT**	
		271 nm	**280 nm**	**271 nm**	**280 nm**
1	ALI 60 µg/ml HCT 5 µg/ml	0.2012	0.2987	0.2945	0.1368
2		0.2014	0.2969	0.2968	0.1348
3		0.2004	0.2987	0.2948	0.1365
4		0.2033	0.2987	0.2938	0.1365
5		0.2021	0.2965	0.2958	0.1389
6		0.2018	0.2985	0.2919	0.1348
7		0.2033	0.2965	0.2968	0.1348
8		0.2011	0.2982	0.2918	0.1349
9		0.2054	0.2928	0.2937	0.1364
Mean±SD*		**0.2022±0.0015**	**0.2973±0.0019**	**0.2944±0.0019**	**0.1360±0.0014**
% RSD		**0.7604**	**0.6481**	**0.6300**	**1.0239**

1	ALI 120 µg/ml HCT 10 µg/ml	0.4235	0.5984	0.5894	0.2639
2		0.4215	0.5987	0.5845	0.2648
3		0.4209	0.5964	0.5846	0.2637
4		0.4215	0.5942	0.5835	0.2648
5		0.4254	0.5924	0.5879	0.2638
6		0.4155	0.5967	0.5819	0.2618
7		0.4244	0.5964	0.5816	0.2646
8		0.4245	0.5941	0.5864	0.2679
9		0.4218	0.5976	0.5818	0.2619
Mean±SD*		**0.4221±0.0031**	**0.5950±0.0039**	**0.5846±0.0028**	**0.2641±0.0018**
% RSD		**0.7380**	**0.6538**	**0.4782**	**0.6836**
Mean % RSD		**0.7492**	**0.6510**	**0.5541**	**0.8538**

**mean±SD, (n=3) number of determination*

Table No. 6.1.5: Results of inter-day precision

Inter-day precision						
Sr. No.		**Conc.**	**Absorbance**			
			ALI		**HCT**	
			271 nm	**280 nm**	**271 nm**	**280 nm**
1	Day 1	ALI 60 µg/ml HCT 5 µg/ml	0.2015	0.2986	0.2945	0.1356
2			0.2044	0.2978	0.2975	0.1348
3			0.2054	0.2987	0.2987	0.1368
4	Day 2		0.2018	0.2891	0.2987	0.1357
5			0.2024	0.2986	0.2946	0.1394
6			0.2045	0.2979	0.2919	0.1346
7	Day 3		0.2021	0.2941	0.29378	0.1358
8			0.2035	0.2987	0.29458	0.1346
9			0.2071	0.2973	0.2934	0.1385
Mean±SD*			**0.2036±0.0019**	**0.2968±0.0032**	**0.2953±0.0024**	**0.1362±0.0017**
% RSD			**0.9233**	**1.0848**	**0.8216**	**1.2648**
1	Day 1	ALI 120 µg/ml HCT 10 µg/ml	0.4215	0.5878	0.5874	0.2658
2			0.4265	0.5956	0.5894	0.2685
3			0.4217	0.5905	0.5864	0.2679
4	Day 2		0.4232	0.5987	0.5867	0.2649
5			0.4235	0.5882	0.58621	0.2689
6			0.4235	0.5945	0.5865	0.2618
7	Day 3		0.4215	0.5864	0.5864	0.2689
8			0.4265	0.5982	0.5861	0.2684
9			0.4321	0.5967	0.5812	0.2639
Mean±SD*			**0.4244±0.0035**	**0.5930±0.0048**	**0.5863±0.0022**	**0.2666±0.0026**
% RSD			**0.8130**	**0.8046**	**0.3672**	**0.9653**
Mean % RSD			**0.8682**	**0.9447**	**0.5944**	**1.1150**

**mean±SD, (n= 3) number of determination*

6.1.8 Accuracy

The results of recovery studies ranged from 98-101% for both the drugs showing the accuracy of the method (Table No. 6.1.6). This indicates that there is no interference from tablet excipients.

Table No. 6.1.6: Results of recovery studies

Accuracy (% Recovery)				
ALI				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard drug added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	60	30	29.8039	99.3462
50	90	45	44.7898	99.5330
50	120	60	60.1437	100.2394
100	60	60	60.1950	100.3250
100	90	90	89.8489	99.8321
100	120	120	120.2034	100.1695
150	60	90	90.1478	100.1642
150	90	135	134.6590	99.7474
150	120	180	180.1386	100.0770
Mean±SD*				**99.9371±0.3403**
% RSD				**0.3405**
HCT				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard drug added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	5	2.5	2.4310	97.2403
50	7.5	3.75	3.6627	97.6708
50	10	5	4.8811	97.6227
100	5	5	4.8994	97.9872
100	7.5	7.5	7.3882	98.5100
100	10	10	9.8183	98.1834
150	5	7.5	7.3854	98.4725
150	7.5	11.25	11.1338	98.9670
150	10	15	14.7887	98.5915
Mean±SD*				**98.1384±0.5543**
% RSD				**0.5648**

**mean±SD, (n= 3) number of determination*

6.1.9 LOD and LOQ

The values of LOD and LOQ were found to be very low which proves the sensitivity of the proposed method is shown in Table No. 6.1.8.

6.1.10 Robustness

The proposed method was checked for robustness study, but no significant changes (% RSD<2) found in absorption, indicating that the method is robust (Table No. 6.1.7).

Table No. 6.1.7: Results of robustness study

Parameter	**Drugs**			
Wavelengths	**ALI**		**HCT**	
(271 & 280 ±1 nm)	**Assay (%)***	**% RSD**	**Assay (%)***	**% RSD**
270 & 279 nm	98.2155	**1.6984**	101.5432	**1.7442**
271 & 280 nm	99.2554		99.0223	
272 & 281 nm	101.5255		98.2125	

** (n= 3) number of determination*

6.1.11 Stability of the solution

Solution stability was performed at room temperature and it was found to be stable up to two days.

Table No. 6.1.8: Summary of validation parameters for the proposed method

Parameters	**ALI**		**HCT**	
Detection wavelengths (nm)	271	280	271	280
Linearity range (µg/ml)	6-300		0.5-25	
Correlation coefficient	0.9998	0.9998	1	0.9999
Regression equation	y = 0.0036x - 0.0069	y = 0.0051x - 0.0036	y = 0.0592x - 0.0007	y = 0.0268x - 0.0006
Precision (%RSD)				
Repeatability of measurement (n=6)	0.6051	0.4456	0.7236	0.7193
Intra-day (n=3)	0.7492	0.6510	0.5541	0.8538
Inter-day (n=3)	0.8682	0.9447	0.5944	1.1150
*Accuracy**				
% Recovery (n=3)	99.9371±0.3403		98.1384±0.5543	
%RSD (n=3)	0.3405		0.5648	
Specificity	No interference			
LOD (µg/ml)	1.2617	0.6392	0.0468	0.0530
LOQ (µg/ml)	3.8233	1.9371	0.1413	0.1605

**mean±SD, n= number of determinations*

6.1.12 Analysis of marketed formulation

The proposed method was successfully applied for the quantitative determination of ALI and HCT in commercial formulation (Rasilez HCT tablet: 300 mg of ALI and 25 mg of HCT). Six replicate determinations were carried out and the experimental values were found to be within 97 and 100 % for both the drugs and hence the developed method can be used for the simultaneous determination of both the drugs in combined formulation (Table No. 6.1.9). Overlain spectra of standard drugs and formulation are shown in Figure No. 6.1.10.

Table No. 6.1.9: Results of formulation analysis

Drugs	Amount (mg/tablet)		% Drug found*	% RSD
	Labelled	Found		
ALI	300	296.87	98.9582±0.8194	0.8281
HCT	25	24.70	98.8147±1.2010	1.2155

**mean ± SD, (n=6) average of six determinations*

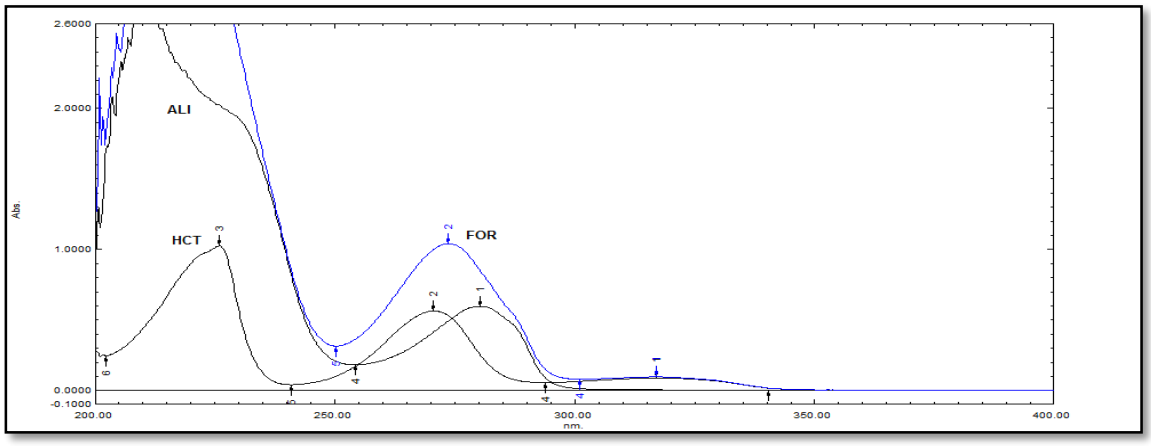


Figure No. 6.1.10: Overlain spectra of standard ALI (120 µg/ml), HCT (10 µg/ml) & formulation (120 & 10 µg/ml)

In order to check the applicability of the method, all the available strength (ratio) of marketed formulations were analyzed using standard drug solution in optimum ratio.

Available strength (mg)

ALI + HCT: 150/300 + 12.5/25

Study (% assay) suggests that, the proposed method can be applied to all the formulations of different strengths available in the market.

METHOD 2

6.2 *"Development and validation of absorbance ratio (Q analysis) method for the simultaneous determination of aliskiren hemifumarate and hydrochlorothiazide in tablets"*

Estimation of ALI and HCT was achieved by absorbance ratio method using Shimadzu UV-1800 (UV Pro), double beam UV-visible spectrophotometer.

6.2.1 Selection of solvent

Based on the literature survey and solubility studies methanol was selected as solvent, which showed higher absorbance and distinct λ_{max} for both the drugs**.**

6.2.2 Selection of wavelength

Standard solutions of ALI (60 µg/ml) and HCT (5 µg/ml) were separately scanned in the UV region (200-400 nm) and spectra were recorded using methanol as blank. Their overlain spectra are shown in Figure No. 6.2.1.

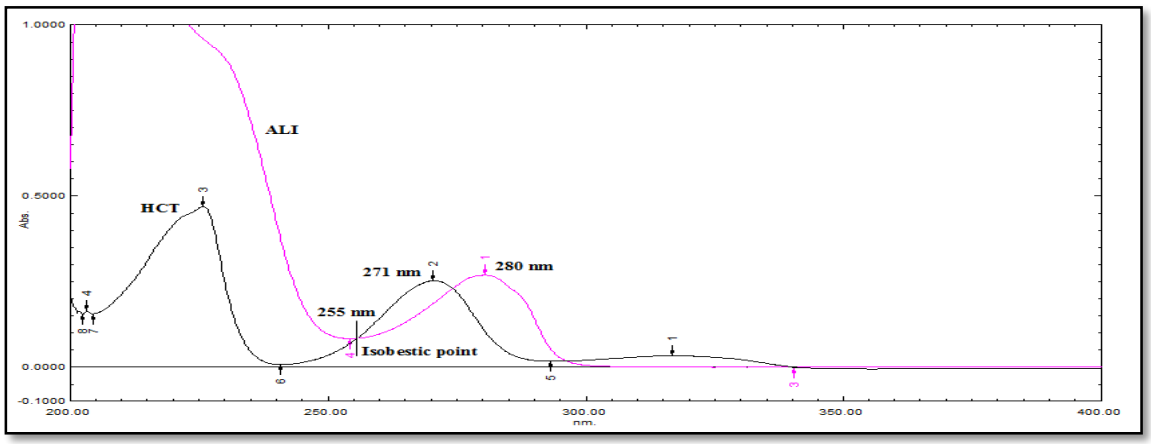


Figure No. 6.2.1: Overlain UV spectra of ALI (60 µg/ml) and HCT (5 µg/ml)

From the overlain spectra, initially different wavelengths were tried for the study. But, finally 255 (isobestic point) nm and 271 nm (λ_{max} of HCT) were selected, which showed good linearity and hence used for the simultaneous estimation by *absorption ratio (Q analysis)* method.

6.2.3 Determination of absorptivity value

The developed method was found to be linear in the concentration range of 6-300 µg/ml for ALI and 0.5-25 µg/ml for HCT. Absorbances were measured at 255 nm and 271 nm for both the drugs and absorptivity values were calculated and presented in Table No. 6.2.1 & 6.2.2.

Table No. 6.2.1: Absorbances and absorptivities of ALI at selected wavelength

Conc. (µg/ml)	ALI					
	255 nm			271 nm		
	Abs.*	Absorptivity	Avg. Absorptivity	Abs.*	Absorptivity	Avg. Absorptivity
6	0.0088	14.6111	(ax_1) **15.1100**	0.0210	34.9833	(ax_2) **35.2830**
12	0.0174	14.5278		0.0406	33.8569	
60	0.0880	14.6717		0.2055	34.2556	
120	0.1778	14.8125		0.4222	35.1847	
180	0.2808	15.6019		0.6480	35.9981	
240	0.3789	15.7876		0.8752	36.4681	
300	0.4727	15.7572		1.0870	36.2344	

**average of six determinations*

Table No. 6.2.2: Absorbances and absorptivities of HCT at selected wavelength

Conc. (µg/ml)	HCT					
	255 nm			271 nm		
	Abs.*	Absorptivity	Avg. Absorptivity	Abs.*	Absorptivity	Avg. Absorptivity
0.5	0.0094	188.3333	(ay_1) **187.6291**	0.0295	590.6667	(ay_2) **590.5593**
1	0.0185	184.8333		0.0586	586.3333	
5	0.0927	185.3933		0.2963	592.6667	
10	0.1891	189.0833		0.5881	588.0500	
15	0.2808	187.2222		0.8869	591.2667	
20	0.3789	189.4517		1.1872	593.5917	
25	0.4727	189.0867		1.4784	591.3400	

**average of six determinations*

6.2.4 Validation of the method:

The developed method was validated in accordance with "*International Conference on Harmonization"* guidelines for validation of analytical procedures.

6.2.5 Specificity

Overlain spectra of placebo and drug solution indicate that there was no interference between excipients and standard drugs (Figure No. 6.2.2)

6.2.6 Linearity and range

ALI and HCT were found to be linear in the concentration range of 6-300 µg/ml and 0.5-25 µg/ml, respectively. Overlain spectra of ALI and HCT are shown in Figure No. 6.2.3-6.2.5.

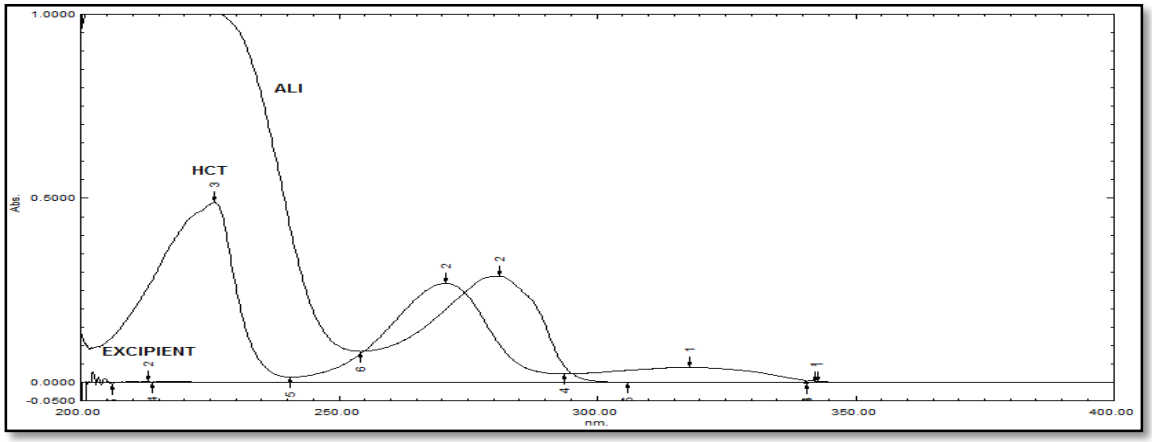


Figure No. 6.2.2: Overlain UV spectra of formulation excipients and standard drugs

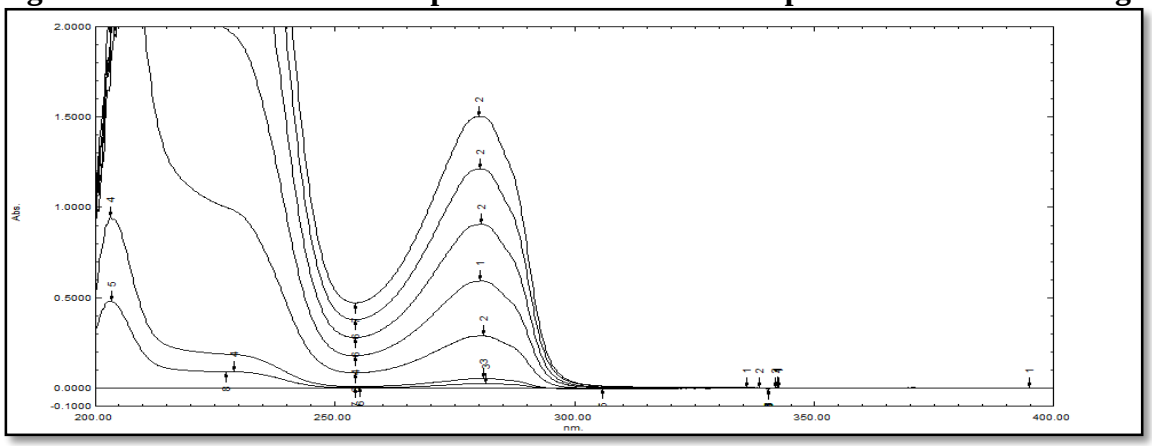


Figure No. 6.2.3: Overlain UV spectra of ALI (6-300 µg/ml)

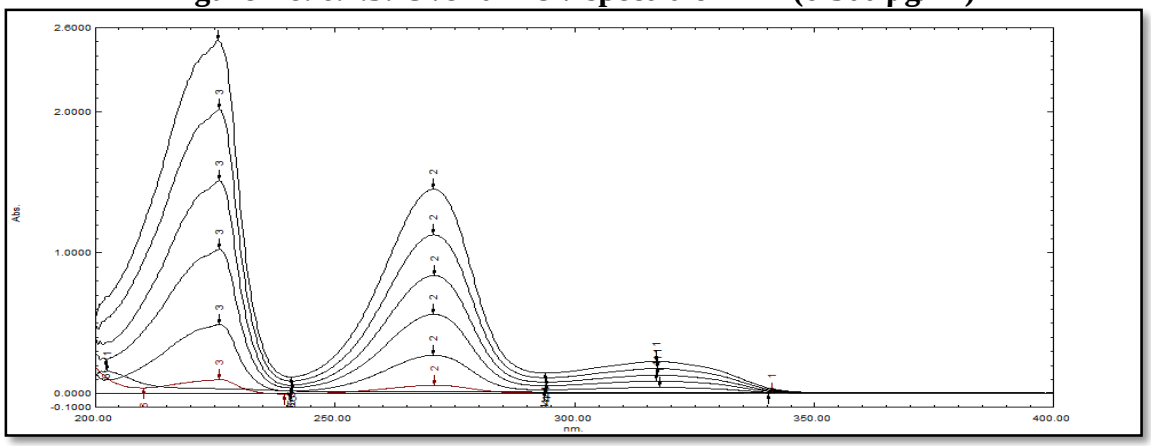


Figure No. 6.2.4: Overlain UV spectra of HCT (0.5-25 µg/ml)

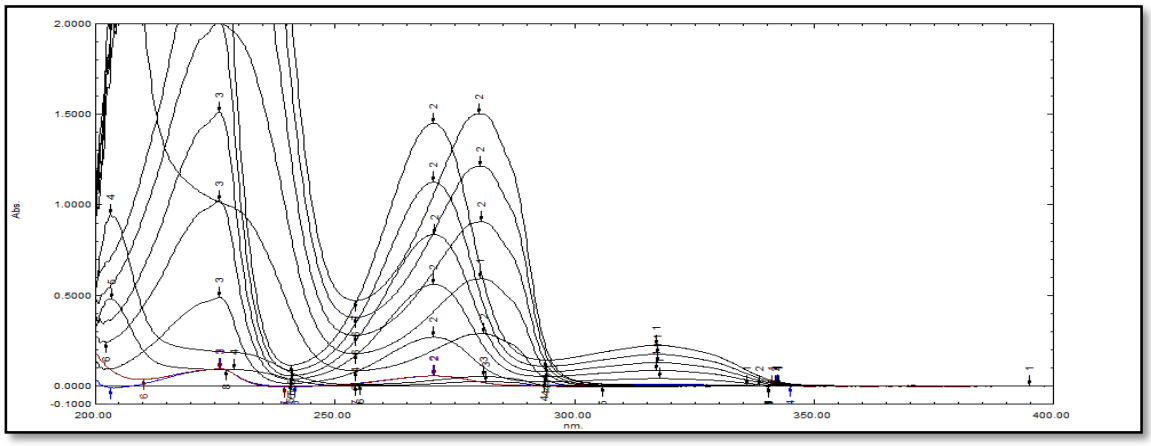


Figure No. 6.2.5: Overlain UV spectra of ALI (6-300 µg/ml) & HCT (0.5-25 µg/ml)

Calibration graphs (Figure No. 6.2.6 - 6.2.9) were plotted using absorbance of standard drug versus concentration. Results were subjected to regression analysis by least square method to calculate the values of slope, intercept and correlation coefficient of ALI and HCT at 255 nm and 271 nm are shown in Table No. 6.2.8.

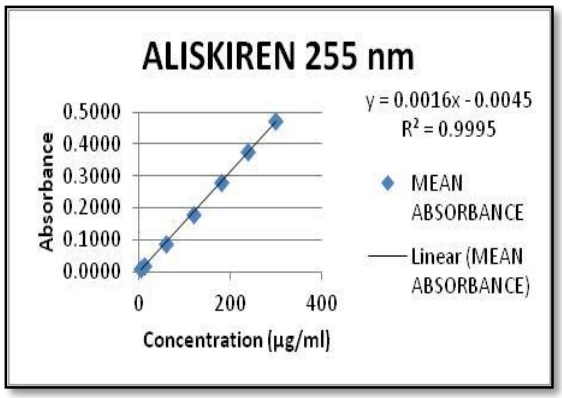


Figure No. 6.2.6: Calibration graph of ALI (6-300 µg/ml) at 255 nm

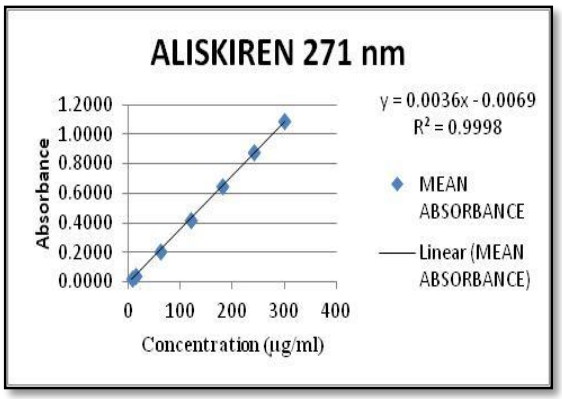


Figure No. 6.2.7: Calibration graph of ALI (6-300 µg/ml) at 271 nm

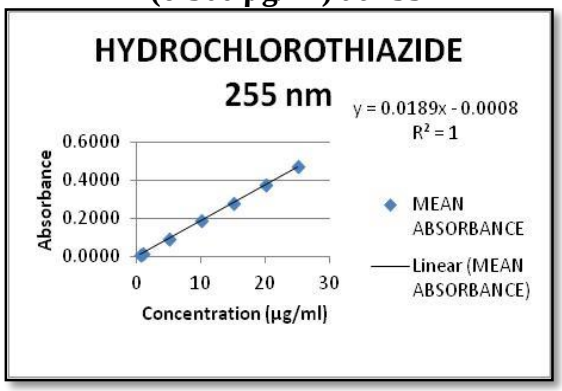


Figure No. 6.2.8: Calibration graph of HCT (0.5-25 µg/ml) at 255 nm

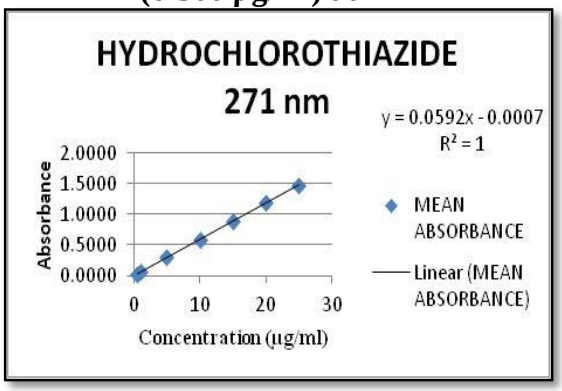


Figure No. 6.2.9: Calibration graph of HCT (0.5-25 µg/ml) at 271 nm

6.2.7 Precision

Results of precision studies expressed in %RSD follows ICH guideline acceptable limits (<2), which shows good repeatability and low inter-day variability, indicating an excellent precision of the developed method (Table No. 6.2.3 - 6.2.5).

Table No. 6.2.3: Results of repeatability of measurement

Repeatability					
Sr. No.	**Conc.**	**Absorbance**			
		ALI		**HCT**	
		255 nm	**271 nm**	**255 nm**	**271 nm**
1	ALI 60 µg/ml HCT 5 µg/ml	0.0871	0.2041	0.0871	0.2945
2		0.0882	0.2019	0.0882	0.2975
3		0.0885	0.1998	0.0885	0.2968
4		0.0869	0.2045	0.0869	0.2984
5		0.0881	0.2034	0.0881	0.2945
6		0.0876	0.2018	0.0876	0.2916
Mean±SD*		**0.0877±0.0006**	**0.2026±0.0018**	**0.0877±0.0006**	**0.2956±0.0025**
% RSD		**0.7304**	**0.8682**	**0.7304**	**0.8465**
1	ALI 120 µg/ml HCT 10 µg/ml	0.1784	0.4175	0.1784	0.5815
2		0.1764	0.4167	0.1764	0.5839
3		0.1768	0.4195	0.1768	0.5817
4		0.1758	0.4157	0.1758	0.5896
5		0.1768	0.4169	0.1768	0.5861
6		0.1765	0.4189	0.1765	0.5891
Mean±SD*		**0.1768±0.0009**	**0.4175±0.0014**	**0.1768±0.0009**	**0.5853±0.0035**
% RSD		**0.4937**	**0.3420**	**0.4937**	**0.6061**
Mean % RSD		**0.6121**	**0.6051**	**0.6121**	**0.7263**

**mean±SD, (n= 6) number of determinations*

Table No. 6.2.4: Result of intra-day precision

Intra-day precision					
Sr. No.	**Conc.**	**Absorbance**			
		ALI		**HCT**	
		255 nm	**271 nm**	**255 nm**	**271 nm**
1	ALI 60 µg/ml HCT 5 µg/ml	0.0876	0.2012	0.0876	0.2945
2		0.0875	0.2014	0.0875	0.2968
3		0.0888	0.2004	0.0888	0.2948
4		0.0894	0.2033	0.0894	0.2938
5		0.0871	0.2021	0.0871	0.2958
6		0.0872	0.2018	0.0872	0.2919
7		0.0885	0.2033	0.0885	0.2968
8		0.0867	0.2011	0.0867	0.2918
9		**0.0885**	**0.2054**	**0.0885**	**0.2937**
Mean±SD*		**0.0879±0.0009**	**0.2022±0.0015**	**0.0879±0.0009**	**0.2944±0.0019**
% RSD		**1.0327**	**0.7604**	**1.0327**	**0.6300**

1	ALI 120 µg/ml HCT 10 µg/ml	0.1757	0.4235	0.1757	0.5894
2		0.1768	0.4215	0.1768	0.5845
3		0.1768	0.4209	0.1768	0.5846
4		0.1786	0.4215	0.1786	0.5835
5		0.1764	0.4254	0.1764	0.5879
6		0.1778	0.4155	0.1778	0.5819
7		0.1761	0.4244	0.1761	0.5816
8		0.1779	0.4245	0.1779	0.5864
9		0.1759	0.4218	0.1759	0.5818
Mean±SD*		**0.1769±0.0010**	**0.4221±0.0030**	**0.1769±0.0010**	**0.5846±0.0028**
% RSD		**0.5671**	**0.7013**	**0.5671**	**0.4782**
Mean % RSD		**0.7999**	**0.7308**	**0.7999**	**0.5541**

**mean±SD, (n= 3) number of determinations*

Table No. 6.2.5: Results of inter-day precision

Inter-day precision						
Sr. No.		**Conc.**	**Absorbance**			
			ALI		**HCT**	
			255 nm	**271 nm**	**255 nm**	**271 nm**
1	Day 1	ALI 10 µg/ml AMLO 10 µg/ml	0.0875	0.2015	0.0875	0.2945
2			0.0889	0.2044	0.0889	0.2975
3			0.0878	0.2054	0.0878	0.2987
4	Day 2		0.0888	0.2018	0.0888	0.2987
5			0.0875	0.2024	0.0875	0.2946
6			0.0878	0.2045	0.0878	0.2919
7	Day 3		0.0889	0.2021	0.0889	0.2938
8			0.0878	0.2035	0.0878	0.2946
9			0.0884	0.2071	0.0884	0.2934
Mean±SD*			**0.0882±0.0006**	**0.2036±0.0019**	**0.0882±0.0006**	**0.2953±0.0024**
% RSD			**0.6738**	**0.9233**	**0.6738**	**0.8216**
1	Day 1	ALI 20 µg/ml AMLO 20 µg/ml	0.1775	0.4215	0.1775	0.5874
2			0.1759	0.4265	0.1759	0.5894
3			0.1767	0.4217	0.1767	0.5864
4	Day 2		0.1761	0.4232	0.1761	0.5867
5			0.1768	0.4235	0.1768	0.58621
6			0.1775	0.4235	0.1775	0.5865
7	Day 3		0.1781	0.4215	0.1781	0.5864
8			0.1769	0.4265	0.1769	0.5861
9			0.1789	0.4321	0.1789	0.5812
Mean±SD*			**0.1772±0.0010**	**0.4244±0.0035**	**0.1772±0.0010**	**0.5863±0.0022**
% RSD			**0.5378**	**0.8130**	**0.5378**	**0.3672**
Mean % RSD			**0.6058**	**0.8682**	**0.6058**	**0.5944**

**mean±SD, (n= 3) number of determinations*

6.2.8 Accuracy

The results of recovery studies ranged from 98-101% for both the drugs showing the accuracy of the method (Table No. 6.2.6). This indicates that there is no interference from tablet excipients.

Table No. 6.2.6: Results of recovery studies

Accuracy (% Recovery)				
ALI				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard drug added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	60	30	29.6435	98.8116
50	90	45	44.6341	99.1870
50	120	60	59.6248	99.3747
100	60	60	59.3225	98.8709
100	90	90	90.3940	100.4378
100	120	120	119.2153	99.3461
150	60	90	88.9802	98.8669
150	90	135	133.5110	98.8971
150	120	180	180.7422	100.4123
Mean±SD*				**99.3560±0.6419**
% RSD				**0.6461**
HCT				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard drug added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	5	2.5	2.5134	100.5341
50	7.5	3.75	3.7197	99.1927
50	10	5	4.9327	98.6532
100	5	5	5.0725	101.4497
100	7.5	7.5	7.3670	98.2270
100	10	10	9.9678	99.6784
150	5	7.5	7.5618	100.8235
150	7.5	11.25	11.3555	100.9377
150	10	15	14.7077	98.0511
Mean±SD*				**99.7275±1.2642**
% RSD				**1.2677**

**mean±SD, (n= 3) number of determination*

6.2.9 LOD and LOQ

The values of LOD and LOQ were found to be very low which proves the sensitivity of the method is shown in Table No. 6.2.8.

6.2.10 Robustness

The proposed method was checked for robustness study, but no significant changes (% RSD<2) found in absorption, indicating that the proposed method is robust (Table No. 6.2.7)

Table No. 6.2.7: Results of robustness study

Parameter	Drugs			
Wavelengths (255 & 271 ±1 nm)	ALI		HCT	
	Assay (%)*	% RSD	Assay (%)*	% RSD
254 & 270 nm	97.2545	1.9622	98.2455	1.6606
255 & 271 nm	99.1245		100.1214	
256 & 272 nm	101.1455		96.8655	

** (n= 3) number of determination*

6.2.11 Stability of the solution

Solution stability was performed at room temperature and it was found to be stable up to two days.

Table 6.2.8: Summary of validation parameters for the proposed method

Parameters	ALI		HCT	
Detection wavelengths (nm)	255	271	255	271
Linearity range (µg/ml)	6-300		0.5-25	
Correlation coefficient	0.9995	0.9998	1	1
Regression equation	y = 0.0016x - 0.0045	y = 0.0036x - 0.0069	y = 0.0189x - 0.0008	y = 0.0592x - 0.0007
Precision (%RSD)				
Repeatability of measurement (n=6)	0.6121	0.6051	0.6121	0.7263
Intra-day (n=3)	0.7999	0.7308	0.7999	0.5541
Inter-day (n=3)	0.6058	0.8682	0.6058	0.5944
Accuracy				
% Recovery (n=3)	99.3560±0.6419		99.7275±1.2642	
%RSD (n=3)	0.6461		1.2677	
Specificity	No interference			
LOD (µg/ml)	1.4349	1.2617	0.0859	0.0468
LOQ (µg/ml)	4.3481	3.8233	0.2604	0.1419

**mean±SD, n= number of determinations*

6.2.12 Analysis of marketed formulation

The proposed method was successfully used for the quantitative determination of ALI and HCT in commercial formulation (Rasilez HCT tablet: 300 mg of ALI and 25 mg of HCT). Six replicate determinations were carried out and experimental values were found to be within 97-100% for both the drugs and hence the developed method can be used for the simultaneous determination of both the drugs in combined formulation (Table No. 6.2.9). Overlain spectra of standard drugs and formulation are showed in Figure No. 6.2.10.

Table No. 6.2.9: Result of formulation analysis

Drugs	Amount (mg/tablet)		% Drug found*	% RSD
	Labelled	Found		
ALI	300	295.29	98.4291±1.1807	1.1995
HCT	25	24.47	97.8766±1.1160	1.1402

**mean ± SD (n=6) average of six determinations*

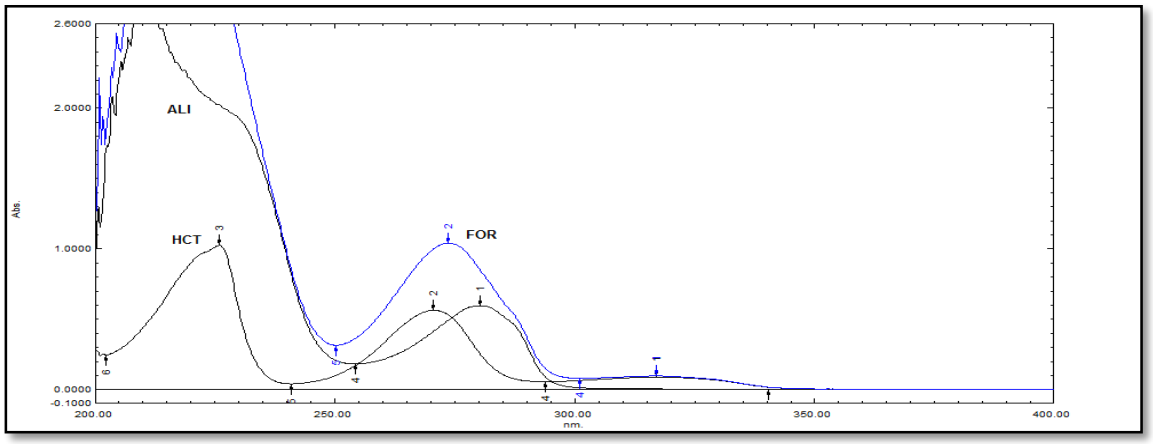


Figure No. 6.2.10: Overlain UV spectra of standard ALI (120 µg/ml), HCT (10 µg/ml) & formulation (120 & 10 µg/ml)

In order to check the applicability of the method, all the available strength (ratio) of marketed formulations were analyzed using standard drug solution in optimum ratio.

Available strength (mg)

ALI + HCT: 150/300 + 12.5/25

Study (% assay) suggests that, the proposed method can be applied to all the formulations of different strengths available in the market.

METHOD 3

6.3 *"Development and validation of first-derivative (Zero crossing) spectroscopic method for the simultaneous determination of aliskiren hemifumarate and hydrochlorothiazide in tablets"*

Estimation of ALI and HCT was achieved by first derivative spectroscopic method using Shimadzu UV-1800 (UV Pro), double beam UV-visible spectrophotometer.

6.3.1 Selection of solvent

Based on the literature survey and solubility studies methanol was selected as solvent, which showed higher derivative signal and favourable zero crossing points for both the drugs.

6.3.2 Selection of wavelength

Standard solutions of ALI (60 µg/ml) and HCT (5 µg/ml) were separately scanned in the UV region (200-400 nm) and spectra were recorded using methanol as blank. Both the spectra were converted into first and second derivative spectra. Based on the spectral pattern and zero crossing points, first derivative method was selected for the study. First derivative spectra were showed typical zero-crossing points at 280.20 nm for ALI and 241 nm for HCT. From the overlain spectra, 241 nm and 280.20 nm were selected for further studies are shown in Figure No. 6.3.1.

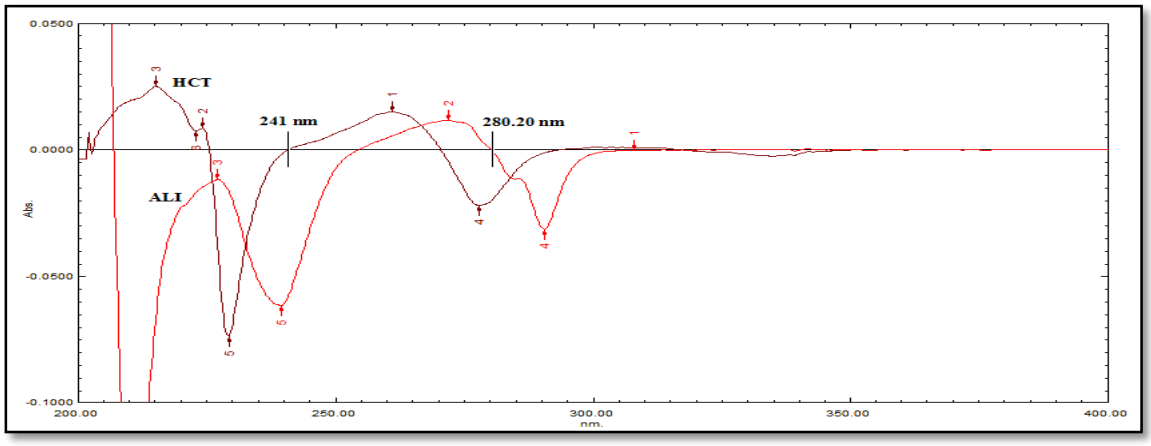


Figure No. 6.3.1: Overlain 1st derivative UV spectra of ALI (60 µg/ml) and HCT (5 µg/ml)

At 241 nm, HCT showed zero absorbance but ALI had considerable absorbance. Similarly at 280.20 nm, ALI showed zero absorbance but HCT had considerable absorbance (Table No. 6.3.1).

Table No. 6.3.1: Selection of zero crossing points for ALI & HCT

Drugs	Zero crossing point (nm)	Detection wavelength (nm)
ALI	280.2	241
HCT	241	280.2

6.3.3 Preparation of calibration curve

A calibration curve (Figure No. 6.3.6 - 6.3.7) was plotted for both ALI and HCT in the range of 6 to 300 µg/ml and 0.5 to 25 µg/ml, respectively (Table No. 6.3.2). Results were subjected to regression analysis by least square method to calculate the values of slope, intercept and correlation coefficient are shown in Table No. 6.3.8.

Table No. 6.3.2: Linearity data of 1st derivative UV spectroscopic method

Sr. No.	ALI at 241 nm			HCT at 280.2 nm		
	Conc. (µg/ml)	1st Derivative signal*	% RSD	Conc. (µg/ml)	1st Derivative signal*	% RSD
1	6	0.0065	1.1551	0.5	0.0025	1.0523
2	12	0.0129	0.8162	1	0.0047	0.9823
3	60	0.0632	0.3416	5	0.0216	0.9578
4	120	0.1264	0.4345	10	0.0444	0.8460
5	180	0.1919	0.3913	15	0.0654	0.9784
6	240	0.2478	0.5359	20	0.0881	0.9277
7	300	0.3043	0.8235	25	0.1139	0.7612

**average of six determinations*

6.3.4 Validation of the method:

The developed method was validated in accordance with "*International Conference on Harmonization"* guidelines for validation of analytical procedures.

6.3.5 Specificity

Overlain spectra of tablet excipients and drug solution indicate that there was no interference between excipients and standard drugs (Figure No. 6.3.2).

6.3.6 Linearity and range

ALI and HCT were found to be linear in the concentration range of 6-300 µg/ml and 0.5-25 µg/ml, respectively. Overlain spectra of ALI and HCT are shown in Figure No. 6.3.3-6.3.5.

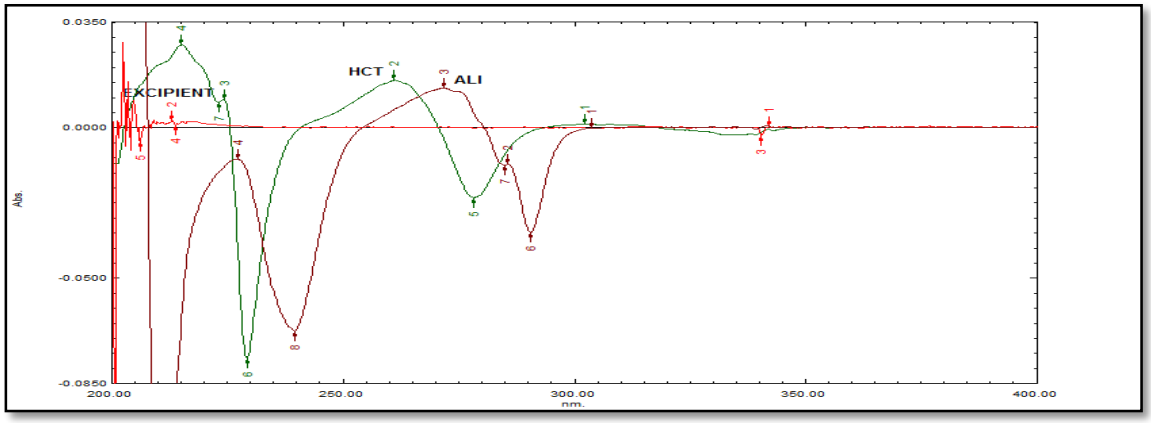


Figure No. 6.3.2: Overlain UV 1st derivative spectra of formulation excipients and standard drugs

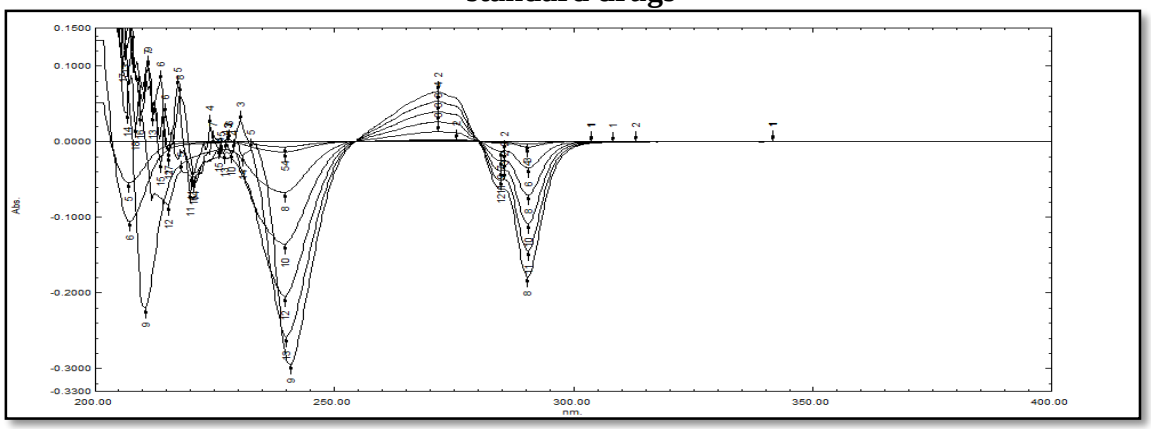


Figure No. 6.3.3: Overlain UV 1st derivative spectra of ALI (6-300 µg/ml)

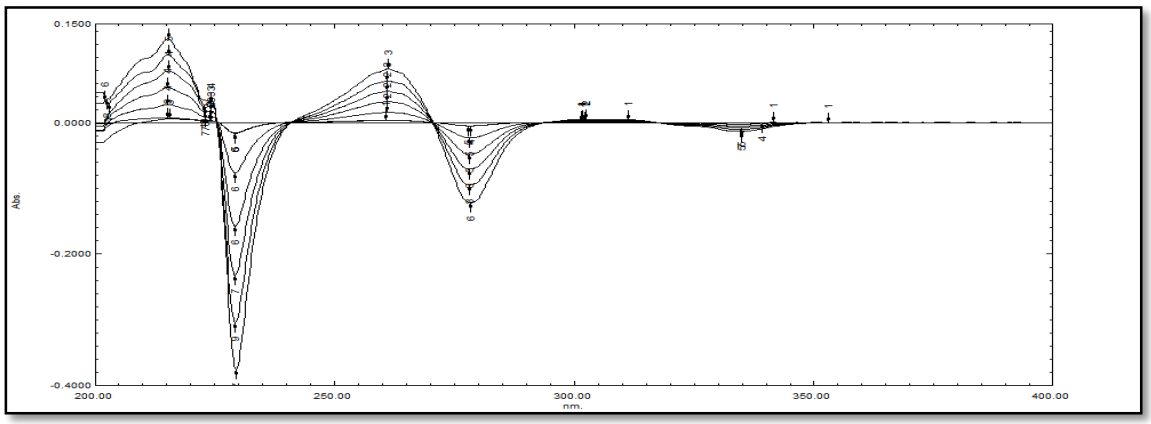


Figure No. 6.3.4: Overlain UV 1st derivative spectra of HCT (0.5-25 µg/ml)

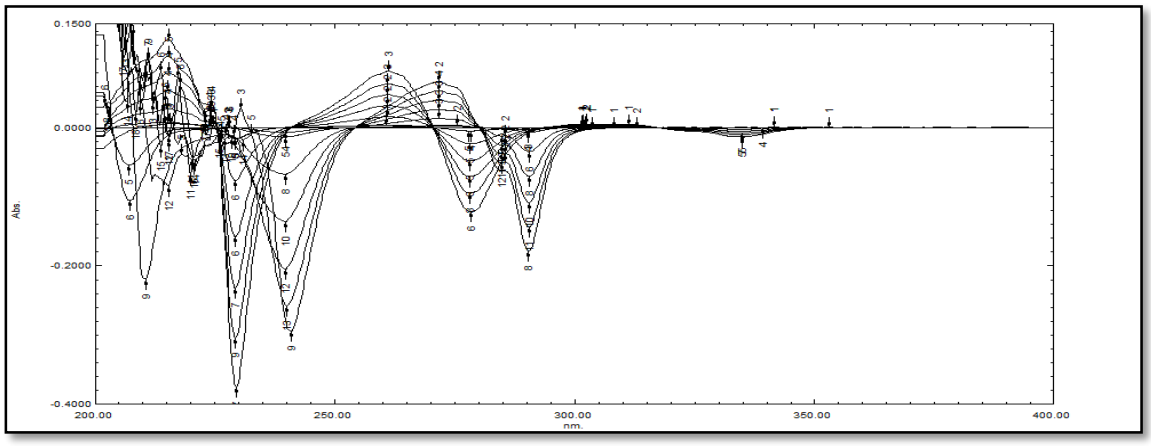


Figure No. 6.3.5: Overlain UV 1st derivative spectra of ALI (6-300 µg/ml) & HCT (0.5-25 µg/ml)

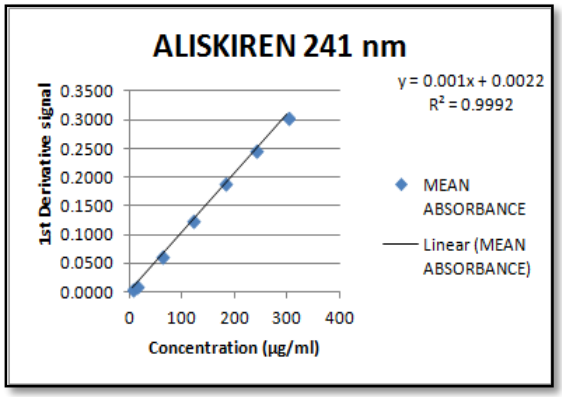


Figure No. 6.3.6: Calibration graph of ALI (6-300 µg/ml) at 241 nm

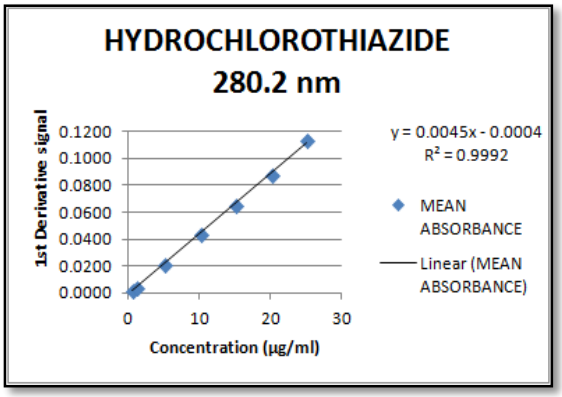


Figure No. 6.3.7: Calibration graph of HCT (0.5-25 µg/ml) at 280.2 nm

6.3.7 Precision

Results of precision studies expressed in % RSD follows ICH guideline acceptable limits (<2), which shows good repeatability and low inter-day variability, indicating an excellent precision of the developed method (Table No. 6.3.3, 6.3.4 & 6.3.5).

Table No. 6.3.3: Results of repeatability of measurement

Repeatability			
Sr. No.	Conc.	1st derivative signal	
		ALI (241 nm)	HCT (280.2 nm)
1	ALI 60 µg/ml	0.0635	0.0216
2		0.0634	0.0211
3		0.0641	0.0216
4	HCT 5 µg/ml	0.0632	0.0219
5		0.0648	0.0218
6		0.0634	0.0221

Mean±SD*		**0.0637±0.0006**	**0.0217±0.0003**
% RSD		**0.9501**	**1.5820**
1	ALI 120 µg/ml	0.1245	0.0446
2		0.1256	0.0447
3		0.1257	0.0448
4		0.1236	0.0457
5	HCT 10 µg/ml	0.1254	0.0438
6		0.1258	0.0447
Mean±SD*		**0.1251±0.0009**	**0.0447±0.0006**
% RSD		**0.6969**	**1.3523**
Mean % RSD		**0.8235**	**1.4671**

**mean±SD, (n= 6) number of determination*

Table No. 6.3.4: Results of intra-day precision

Intra-day precision			
Sr. No.	**Conc.**	**1st derivative signal**	
		ALI	**HCT**
		241 nm	**280.2 nm**
1	ALI 60 µg/ml HCT 5 µg/ml	0.0654	0.0217
2		0.0645	0.0216
3		0.0647	0.0217
4		0.0649	0.0218
5		0.0649	0.0216
6		0.0654	0.0216
7		0.0658	0.0217
8		0.0638	0.0213
9		0.0645	0.0213
Mean±SD*		**0.0649±0.0006**	**0.0216±0.0002**
% RSD		**0.9241**	**0.8170**
1	ALI 120 µg/ml HCT 10 µg/ml	0.1254	0.0457
2		0.1236	0.0454
3		0.1248	0.0445
4		0.1257	0.0457
5		0.1256	0.0453
6		0.1258	0.0455
7		0.1256	0.0457
8		0.1247	0.0458
9		0.1233	0.0452
Mean±SD*		**0.1249±0.0009**	**0.0454±0.0004**
% RSD		**0.7466**	**0.8860**
Mean % RSD		**0.8354**	**0.8515**

**mean±SD, (n=3) number of determinations*

Table No. 6.3.5: Results of inter-day precision

Inter-day precision				
Sr. No.		Conc.	1st derivative signal	
			ALI	HCT
			241 nm	280.2 nm
1	Day 1	ALI 60 µg/ml HCT 5 µg/ml	0.0648	0.0215
2			0.0638	0.0217
3			0.0647	0.0218
4	Day 2		0.0635	0.0217
5			0.0648	0.0213
6			0.0638	0.0215
7	Day 3		0.0638	0.0214
8			0.0639	0.0216
9			0.0647	0.0218
Mean±SD			**0.0642±0.0005**	**0.0216±0.0002**
% RSD			**0.8315**	**0.8170**
1	Day 1	ALI 120 µg/ml HCT 10 µg/ml	0.1247	0.0457
2			0.1235	0.0457
3			0.1258	0.0456
4	Day 2		0.1254	0.0463
5			0.1245	0.0453
6			0.1267	0.0467
7	Day 3		0.1236	0.0458
8			0.1258	0.0461
9			0.1247	0.0458
Mean±SD			**0.1250±0.0011**	**0.0459±0.0004**
% RSD			**0.8469**	**0.9080**
Mean % RSD			**0.8392**	**0.8625**

**mean±SD, (n= 3) number of determination*

6.3.8 Accuracy

The results of recovery studies ranged from 98-102% for both the drugs showing the accuracy of the method (Table No. 6.3.6). This indicates that there is no interference from tablet excipients.

Table No. 6.3.6: Results of recovery studies

Accuracy (% Recovery)				
ALI				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard drug added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	60	30	29.60	98.6667
50	90	45	45.60	101.3333
50	120	60	60.90	101.5000
100	60	60	60.30	100.5000
100	90	90	89.30	99.2222

100	120	120	121.90	101.5833
150	60	90	89.20	99.1111
150	90	135	131.40	97.3333
150	120	180	181.60	100.8889
Mean±SD*				**100.0154±1.4940**
% RSD				**1.4938**
HCT				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard drug added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	5	2.5	2.4667	98.6667
50	7.5	3.75	3.7222	99.2593
50	10	5	4.9556	99.1111
100	5	5	5.0444	100.8889
100	7.5	7.5	7.4333	99.1111
100	10	10	10.1778	101.7778
150	5	7.5	7.4444	99.2593
150	7.5	11.25	11.4111	101.4321
150	10	15	14.8444	98.9630
Mean±SD*				**99.8299±1.1870**
% RSD				**1.1890**

**mean±SD, (n= 3) number of determination*

6.3.9 LOD and LOQ

The values of LOD and LOQ were found to be very low which proves the sensitivity of the method is shown in Table No. 6.3.8.

6.3.10 Robustness

The proposed method was checked for robustness study, but no significant changes (% RSD<2) found in 1st derivative signal, indicating that the proposed method is robust (Table No. 6.3.7).

Table No. 6.3.7: Results of robustness study

Parameter		**Drugs**			
Wavelengths (241 & 280.2 ±1 nm)		**ALI**		**HCT**	
ALI	**HCT**	**Assay (%)***	**% RSD**	**Assay (%)***	**% RSD**
240	279.20	97.6585	**1.5887**	101.6541	**1.7333**
241	280.20	98.3687		99.2545	
242	281.20	100.6645		98.2987	

**(n= 3) number of determination*

6.3.11 Stability of the solution

Solution stability was performed at room temperature and it was found to be stable up to two days.

Table No. 6.3.8: Summary of validation parameters for the proposed method

Parameters	ALI	HCT
Detection wavelengths (nm)	241	280.20
Linearity range (µg/ml)	6-300	0.5-25
Correlation coefficient	0.9992	0.9992
Regression equation	y = 0.001x + 0.0022	y = 0.0045x - 0.0004
Precision (%RSD)		
Repeatability of measurement (n=6)	0.8235	1.4671
Intra-day (n=3)	0.8354	0.8515
Inter-day (n=3)	0.8392	0.8625
Accuracy		
% Recovery (n=3)	100.01±1.4940	99.83±1.1870
%RSD (n=3)	1.4938	1.1890
Specificity	No interference	
LOD (µg/ml)	0.8868	0.1197
LOQ (µg/ml)	2.6874	0.3629

**mean±SD, n= number of determinations*

6.3.12 Analysis of marketed formulation

The proposed method was successfully used for the quantitative determination of ALI and HCT in commercial formulation (Rasilez HCT tablet: 300 mg ALI and 25 mg of HCT). Six replicate determinations were carried out and experimental values were found to be 98-102% for both the drugs and hence the developed method can be used for the simultaneous determination of both the drugs in combined formulation (Table No. 6.3.9). Overlain spectra of standard drugs and formulation are shown in Figure No. 6.3.8.

Table No. 6.3.9: Results of formulation analysis

Drugs	Amount (mg/tablet)		% Drug found*	% RSD
	Labelled	Found		
ALI	300	295.72	98.5751±1.3886	1.4087
HCT	25	24.48	97.9277±1.1159	1.1395

**mean ± SD (n=6) average of six determinations*

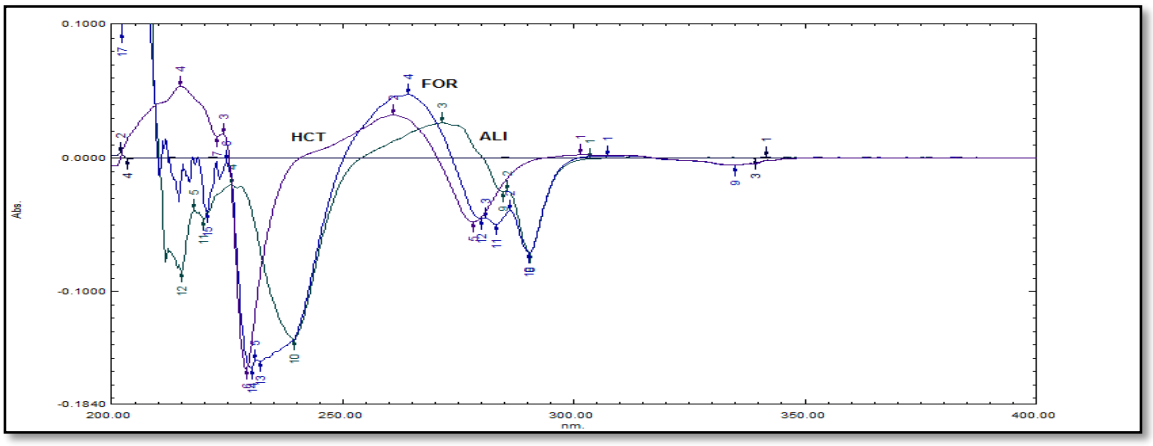


Figure No. 6.3.8: Overlain UV 1st derivative spectra of standard ALI (120 µg/ml), HCT (10 µg/ml) & formulation (120 & 10 µg/ml)

In order to check the applicability of the method, all the available strength (ratio) of marketed formulations were analyzed using standard drug solution in optimum ratio.

Available strength (mg)

ALI + HCT: 150/300 + 12.5/25

Study (% assay) suggests that, the proposed method can be applied to all the formulations of different strengths available in the market.

METHOD 4

6.4 *"Development and validation of RP-HPLC method for the simultaneous determination of aliskiren hemifumarate and hydrochlorothiazide in tablets"*

6.4.1 Selection of mode of chromatographic method

Reverse phase chromatography is the first choice for most regular samples. Compared to other form of liquid chromatography, reverse phase chromatography is more convenient and rugged and it produces more satisfactory results in final separation. Reverse phase chromatographic technique was selected since both the drugs are polar in nature.

6.4.2 Selection of column

High performance RPC columns are efficient, stable, reproducible and compatible with wide variety of samples. Moreover, detection of analyte is easier in RPC with UV detector because of the solvents used. Based on the literature survey C_{18} column was selected.

6.4.3 Selection of wavelength

UV spectra of both the drugs were taken in RP-HPLC system and from the overlain spectra, 280 nm was selected as the wavelength for study, Figure No. 6.4.1.

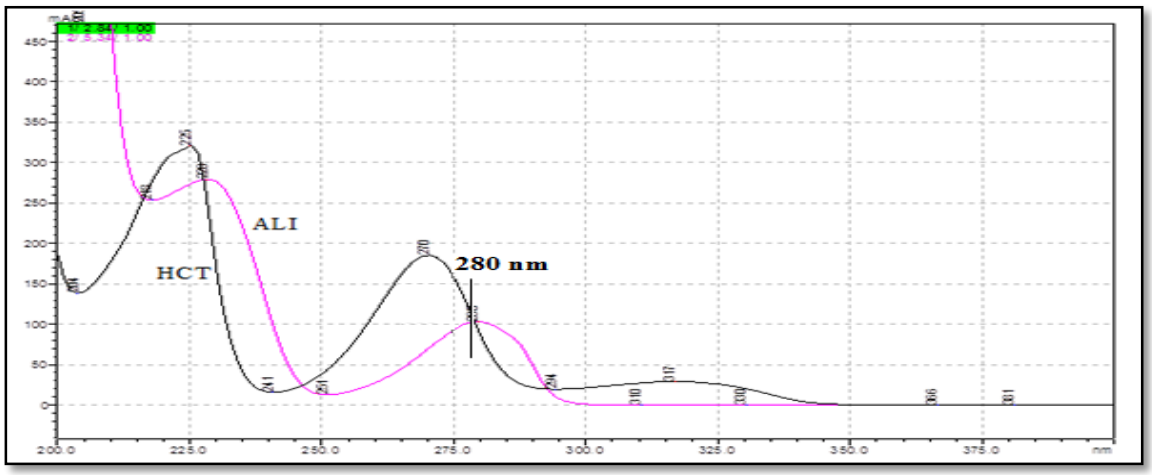


Figure No. 6.4.1: Overlain UV spectra of standard ALI and HCT on RP-HPLC

6.4.4 Trials for selection of mobile phase

Based on the literature survey different mobile phases with different compositions were tried and suitable mobile phase was selected for further studies (Table No. 6.4.1, Figure No. 6.4.2-6.4.10).

Initial condition:

Stationary phase	:	Enable C_{18} column (250 × 4.6 mm, 5 µ)
Flow rate	:	1 ml/ minute
Operating temperature	:	Room temperature
Selected wavelength	:	280 nm

Table No. 6.4.1: Trials for selection of mobile phase

Sr. No.	Mobile Phase	Observation	Remarks	Fig. No.
1	20 mM Sodium phosphate (pH 3): Acetonitrile (65:35 %v/v)	Broad peak with tailing	Not satisfactory	6.4.2
2	20 mM Phosphate buffer (pH 4.6): Methanol (25:75 %v/v)	Fronting was observed	Not satisfactory	6.4.3
3	0.2% TEA (pH 3): Acetonitrile (50:50 %v/v)	Tailing	Not satisfactory	6.4.4
4	0.1% TEA (pH 3): Methanol (50:50 %v/v)	Merged with split peak	Not satisfactory	6.4.5
5	0.2% TEA pH 3: Methanol (70:30 %v/v)	Less separation	Not satisfactory	6.4.6
6	0.2% TEA (pH 3): Methanol (30:70 %v/v)	Tailing with more run time	Not satisfactory	6.4.7
7	0.2% TEA (pH 3): Methanol (20:80 %v/v)	Overlapping peaks with tailing	Not satisfactory	6.4.8
8	0.2% TEA (pH 5): Methanol (10:90 %v/v)	Good peaks with less separation	Not satisfactory	6.4.9
9	**0.2% TEA (pH 6): Methanol (10:90 %v/v)**	**Optimum peak shapes with resolution**	**Satisfactory**	**6.4.10**

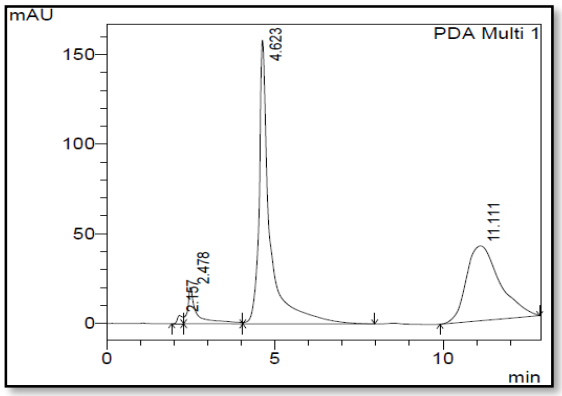


Figure No. 6.4.2: 20 mM sodium phosphate (pH 3): Acetonitrile (65:35 %v/v)

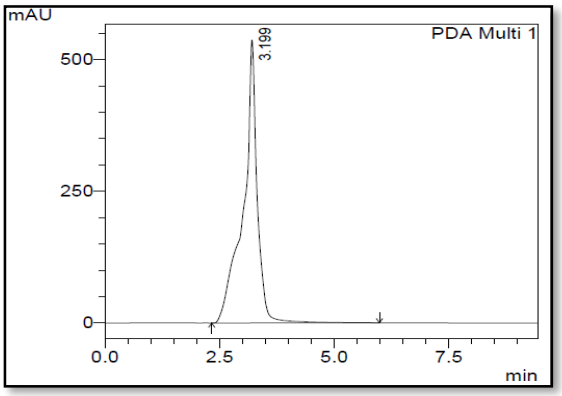


Figure No. 6.4.3: 20 mM Phosphate buffer (pH 4.6): Methanol (25:75 %v/v)

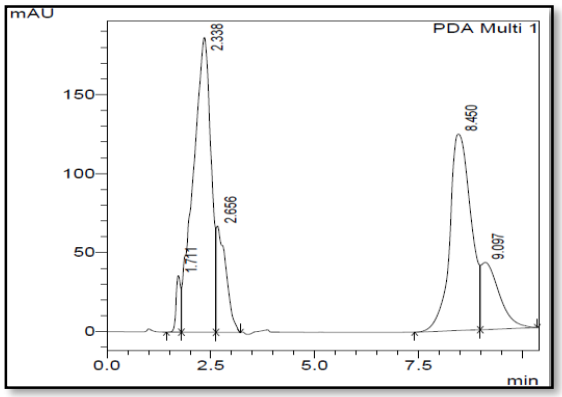


Figure No. 6.4.4: 0.2% TEA (pH 3): Acetonitrile (50:50 %v/v)

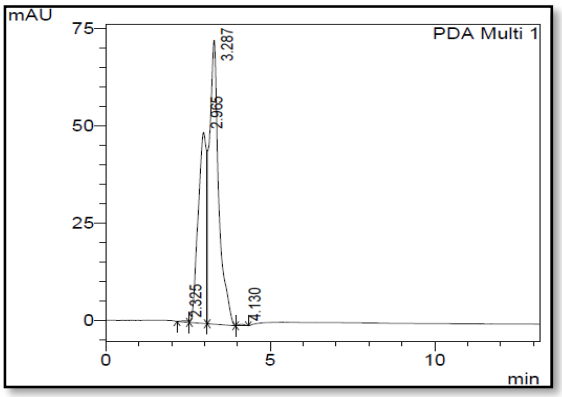


Figure No. 6.4.5: 0.1% TEA (pH 3): Methanol (50:50 %v/v)

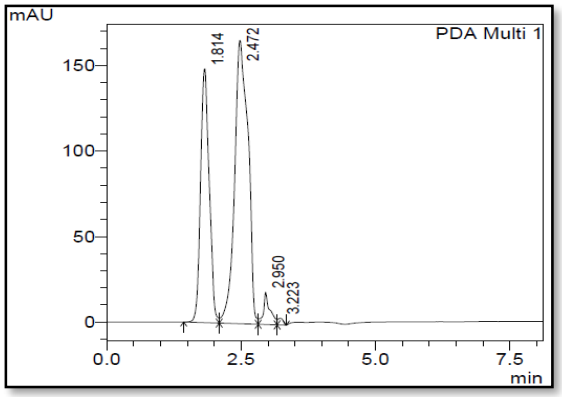


Figure No. 6.4.6: 0.2% TEA (pH 3): Methanol (70:30 %v/v)

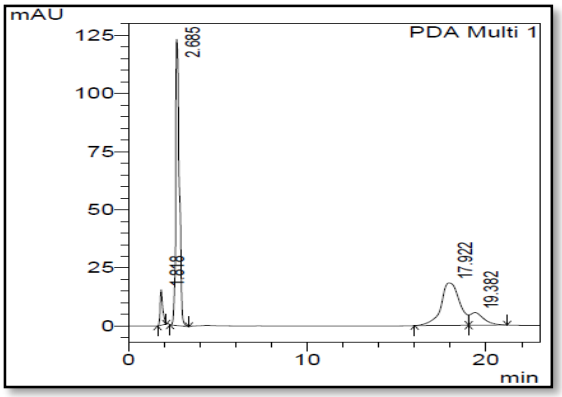


Figure No. 6.4.7: 0.2% TEA (pH 3): Methanol (30:70 %v/v)

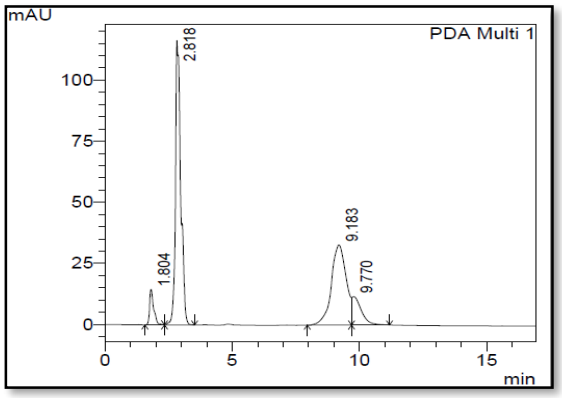


Figure No. 6.4.8: 0.2% TEA (pH 3): Methanol (20:80 %v/v)

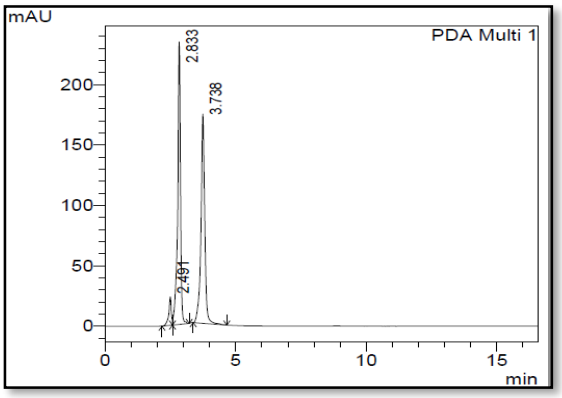


Figure No. 6.4.9: 0.2% TEA (pH 5): Methanol (10:90 %v/v)

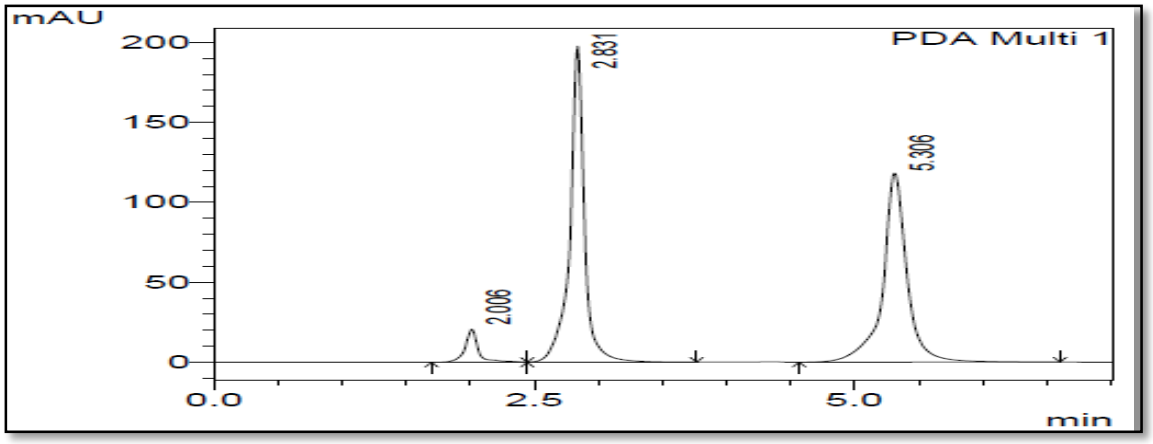


Figure No. 6.4.10: 0.2% TEA (pH 6): Methanol (10:90 %v/v)

6.4.5 Optimization of separation condition

The chromatographic conditions were optimized to achieve the best resolution, peak shape, theoretical plate for all the analytes under investigation. Initially several proportion of buffer (acetate, phosphate etc.), acetonitrile and methanol were tried to achieve optimum separation of all the analytes under study. Based on the preliminary trials triethylamine in water and methanol in combination was selected for further studies. Strength of buffer (0.1-0.3%), mobile phase composition, pH (3-7), flow rate (0.8-1.2) etc. were varied to get optimum chromatographic conditions which can produce acceptable results based on the peak parameters. Finally the separation of components were achieved on Enable C_{18} column with mobile phase consisting of 0.2% triethylamine in water (pH 6 was adjusted with orthophosphoric acid) and methanol (10:90%v/v) at a

flow rate of 1 ml/min was employed with PDA detection at 280 nm which gave satisfactory separation and peak symmetry. The optimized RP-HPLC method was validated and successfully applied for the quantitative determination of ALI and HCT in commercial formulation (Rasilez HCT tablet: 300 mg of ALI and 25 mg of HCT).

6.4.6 Fixed chromatographic condition

Stationary phase	: Enable C_{18} column (250x 4.6 mm, 5 µm, 120 Å)
Mobile phase	: 0.2%v/v triethylamine in water (pH 6 with orthophosphoric acid) and methanol (10:90 % v/v)
Detection wavelength	: 280 nm
Flow rate	: 1 ml/ minute
Operating pressure	: 110 kgf
Temperature	: Room temperature

The retention time of ALI and HCT and FA were found to be 5.315, 2.824 and 1.988 min, respectively, are shown in Figure No. 6.4.11.

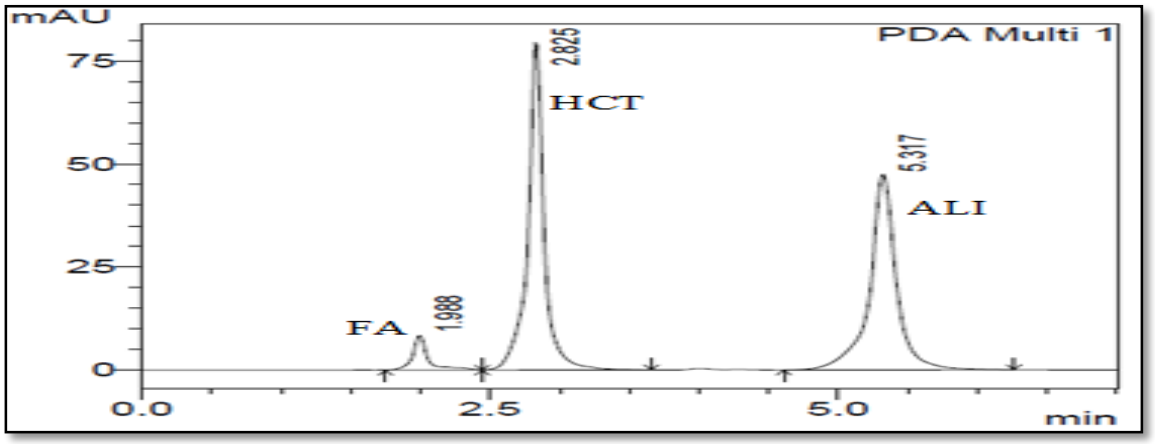


Figure No. 6.4.11: RP-HPLC chromatogram of ALI (120 µg/ml) and HCT (10 µg/ml) using fixed chromatographic condition

6.4.7 Validation of the method

The developed method was validated in accordance with "*International Conference on Harmonization"* guidelines for validation of analytical procedures.

6.4.8 Specificity

No interfering peaks were found within the stipulated run time, which shows the specificity of the method (Figure No. 6.4.12).

6.4.9 Linearity and range

ALI and HCT were found to be linear in the concentration range of 1.2-240 µg/ml and 0.1-20 µg/ml, respectively (Table No. 6.4.2; Figure No. 6.4.16-6.4.22). Calibration curves (Figure No. 6.4.23 & 6.4.24) were plotted using peak area of standard drug versus concentration. Results were subjected to regression analysis by the least squares method to calculate the values of slope, intercept and correlation coefficient (Table No. 6.4.8).

Table No. 6.4.2: Data for calibration curve (ALI: 1.2-240 & HCT: 0.1-20 µg/ml)

Sr. No.	ALI			HCT		
	Conc. (µg/ml)	Peak Area*	% RSD	Conc. (µg/ml)	Peak Area*	% RSD
1	1.2	5935.00	1.4429	0.1	7371.50	1.2706
2	6	33707.33	0.5941	0.5	35470.67	0.8723
3	12	68407.83	0.7617	1	69718.67	1.0745
4	60	327125.33	0.1314	5	332922.83	0.7500
5	120	631882.50	0.0569	10	647513.67	0.5956
6	180	926186.67	0.1133	15	957209.83	0.3001
7	240	1286307.67	0.3336	20	1295919.00	0.4984

**average of six determinations*

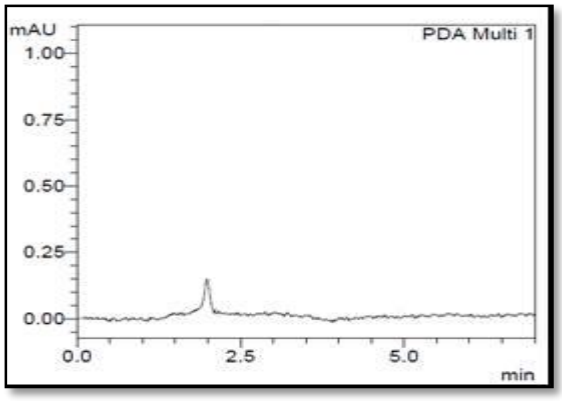


Figure No. 6.4.12: Chromatogram of excipients used in the formulation

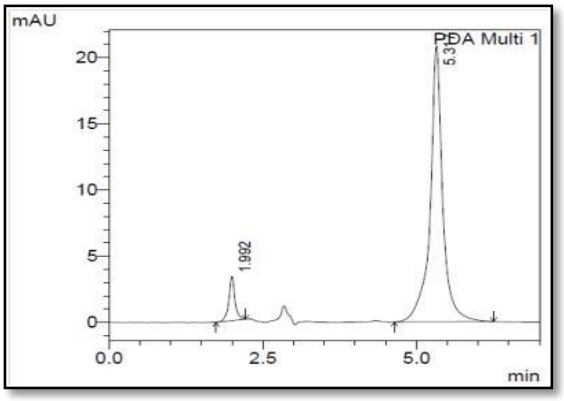


Figure No. 6.4.13: Standard chromatogram of ALI (10 µg/ml)

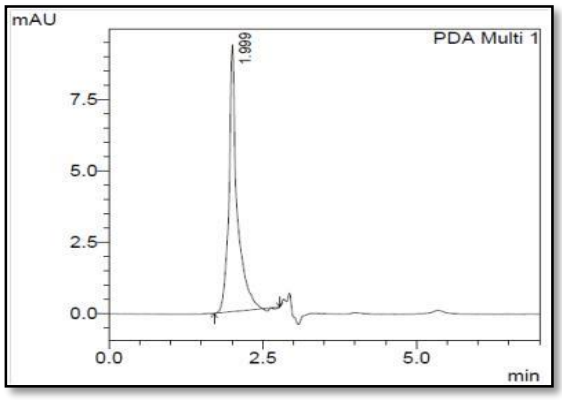


Figure No. 6.4.14: Standard chromatogram of FA (10 µg/ml)

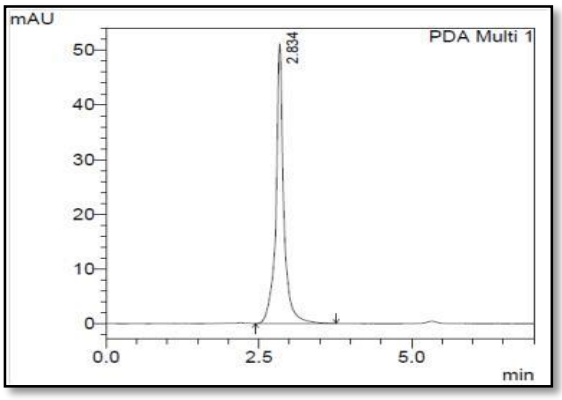


Figure No. 6.4.15: Standard chromatogram of HCT(10 µg/ml)

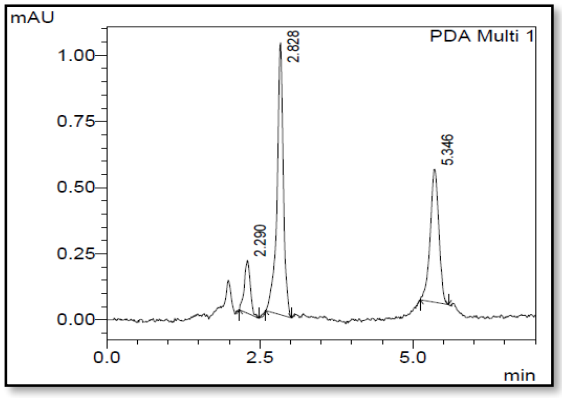


Figure No. 6.4.16: Standard chromatogram of ALI (1.2 µg/ml) and HCT (0.1 µg/ml)

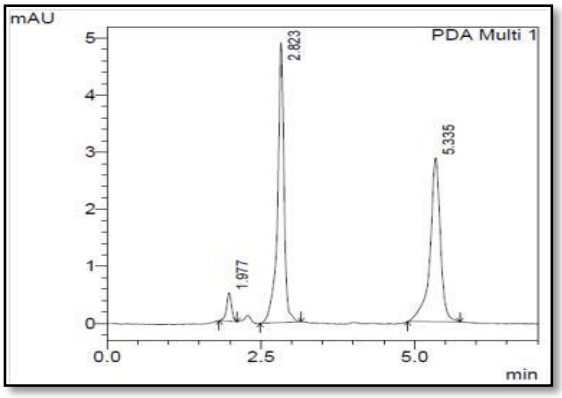


Figure No. 6.4.17: Standard chromatogram of ALI (6 µg/ml) and HCT (0.5 µg/ml)

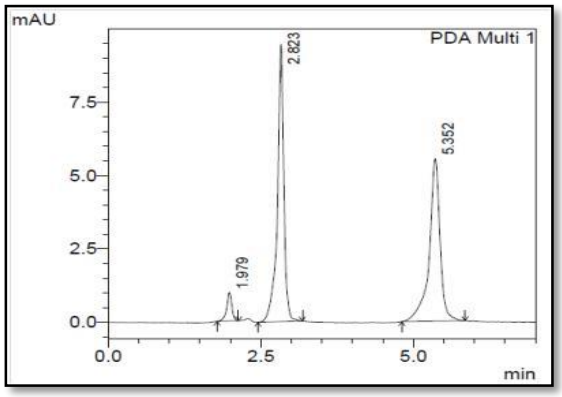


Figure No. 6.4.18: Standard chromatogram of ALI (12 µg/ml) and HCT (1 µg/ml)

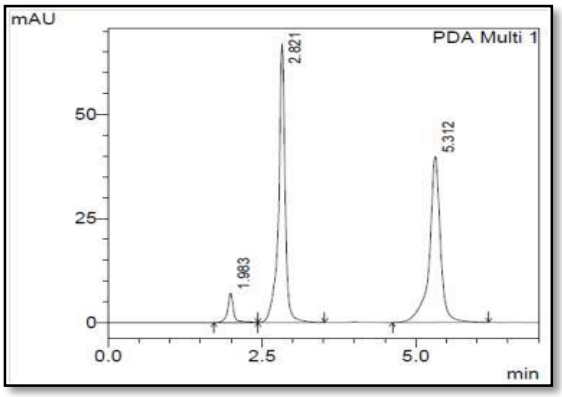


Figure No. 6.4.19: Standard chromatogram of ALI (60 µg/ml) and HCT (5 µg/ml)

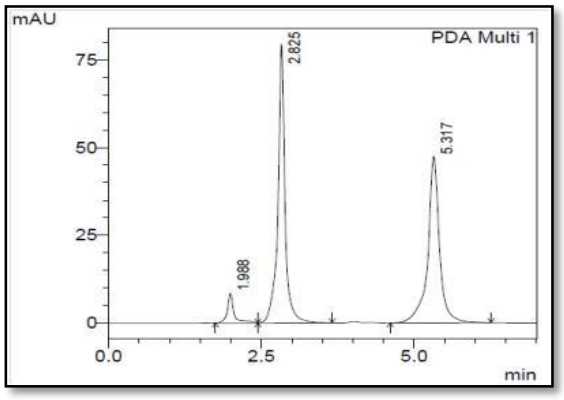


Figure No. 6.4.20: Standard chromatogram of ALI (120 µg/ml) and HCT (10 µg/ml)

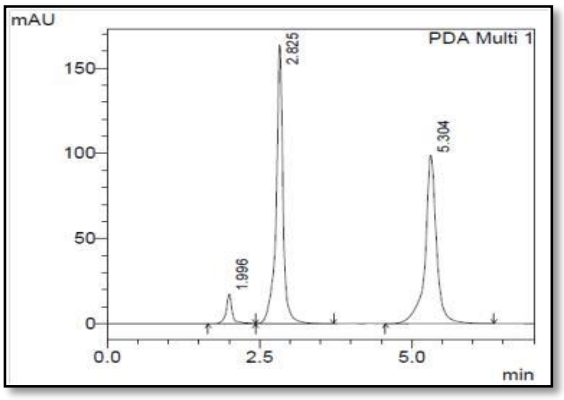


Figure No. 6.4.21: Standard chromatogram of ALI (180 µg/ml) and HCT (15 µg/ml)

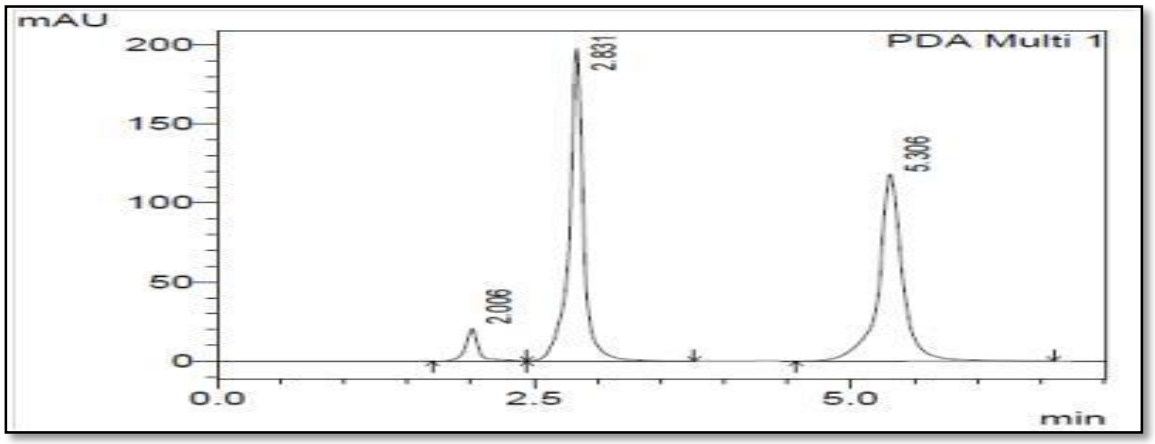


Figure No. 6.4.22: Standard chromatogram of ALI (240 µg/ml) and HCT (20 µg/ml)

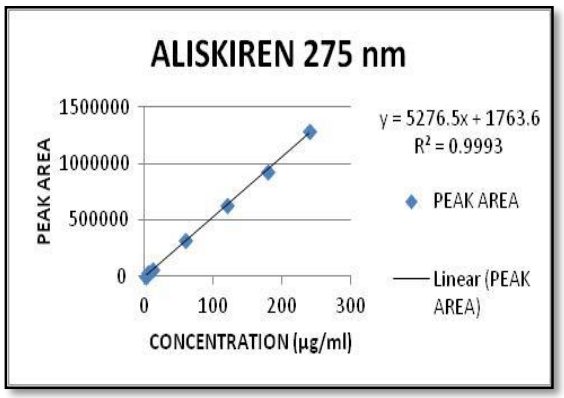


Figure No. 6.4.23: Calibration graph of ALI (1.2-240 µg/ml)

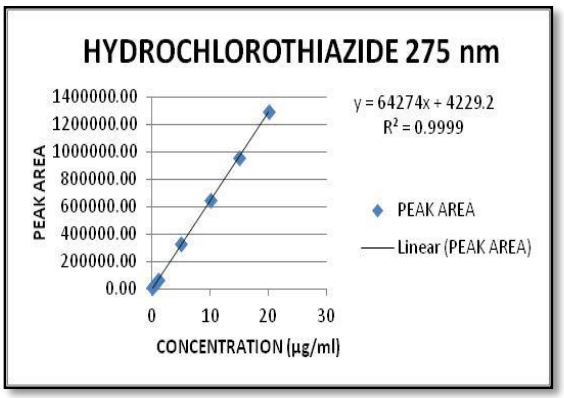


Figure No. 6.4.24: Calibration graph of HCT (0.1-20 µg/ml)

6.4.10 Precision

The precision of the method was checked by carrying out repeatability, intra-day and inter-day precision. Results of precision studies expressed in %RSD follows ICH guideline acceptable limits (% RSD<2), which shows good repeatability and low inter-day variability, indicating an excellent precision of the developed method (Table No. 6.4.3, 6.4.4 & 6.4.5).

Table No. 6.4.3: Results of repeatability of measurement

Repeatability								
Sr. No.	ALI				HCT			
	Conc. (µg/ml)	Peak area	Mean ± SD	% RSD	Conc. (µg/ml)	Peak area	Mean ± SD	% RSD
1	60	327132	326758.00 ± 931.0895	0.2849	5	333054	332922.83 ± 2496.9719	0.7500
2		326342				331835		
3		326734				337843		
4		328365				331734		
5		326342				331034		
6		325633				332037		
1	120	631899	632814.50 ± 622.4303	0.0984	10	648634	647513.67 ± 3856.9109	0.5956
2		633425				654732		
3		632341				645302		
4		633452				646595		
5		633092				645837		
6		632678				643982		
Mean % RSD				**0.1917**	**Mean % RSD**			**0.6728**

**mean±SD, (n= 6) number of determination*

Table No. 6.4.4: Results of intra-day precision

Inter-day precision								
Sr. No.	ALI				HCT			
	Conc. (µg/ml)	Peak area	Mean ± SD	% RSD	Conc. (µg/ml)	Peak area	Mean ± SD	% RSD
1	60	327132	327167.33 ± 466.4260	0.1426	5	337843	333127.56 ± 2738.2978	0.8220
2		326843				331734		
3		327679				331034		
4		326532				332037		
5		327543				333054		
6		327023				331835		
7		327679				337843		
8		326532				331734		
9		327543				331034		

1	120	631899	631887.67 ± 289.7361	0.0459	10	645302	646335.00 ± 6733.2116	1.0418
2		632011				646595		
3		631784				645837		
4		631256				643982		
5		632002				654732		
6		632343				645302		
7		631899				631899		
8		632011				648634		
9		631784				654732		
Mean % RSD				**0.0942**	**Mean % RSD**			**0.9319**

**mean±SD, (n= 3) number of determination*

Table No. 6.4.5: Results of inter-day precision

Inter-day precision									
Sr. No.		**ALI**				**HCT**			
		Conc. (µg/ml)	**Peak area**	**Mean ± SD**	**% RSD**	**Conc. (µg/ml)**	**Peak area**	**Mean ± SD**	**% RSD**
1	**1**	60	327132	327060.56 ± 686.4596	0.2099	5	331835	332250.67 ± 3852.2259	1.1594
2			326843				337843		
3			327676				331734		
4	**2**		325574				326532		
5			327543				327543		
6			327023				332037		
7	**3**		327679				333054		
8			326532				331835		
9			327543				337843		
1	**1**	120	631899	631976.56 ± 215.8119	0.0341	10	643982	644761.56 ± 8441.3433	1.3092
2			632011				648634		
3			631784				654732		
4	**2**		632256				631256		
5			632002				646595		
6			632343				645837		
7	**3**		631699				654732		
8			632011				645302		
9			631784				631784		
Mean % RSD					**0.1220**	**Mean % RSD**			**1.2343**

**mean±SD, (n=3) number of determination*

6.4.11 Accuracy

The results of recovery studies ranged from 98-100% for both the drugs showing the accuracy of the method. This indicates that there is no interference from tablet excipients (Table No. 6.4.6).

Table No. 6.4.6: Results of recovery studies

Accuracy (% Recovery)				
ALI				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard drug added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	24	12	11.8984	99.1534
50	48	24	23.7142	98.8091
50	72	36	35.6139	98.9275
100	24	24	23.7883	99.1179
100	48	48	47.6351	99.2399
100	72	72	72.8482	101.1780
150	24	36	36.5335	101.4821
150	48	72	71.4138	99.1858
150	72	108	106.1969	98.3305
Mean±SD				**99.4916±1.0810**
% RSD				**1.0866**
HCT				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard drug added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	2	1	0.9964	99.6406
50	4	2	1.9866	99.3310
50	6	3	3.0219	100.7302
100	2	2	2.0132	100.6581
100	4	4	4.0477	101.1914
100	6	6	5.9764	99.6074
150	2	3	2.9834	99.4477
150	4	6	6.0061	100.1011
150	6	9	8.8283	98.0918
Mean±SD				**99.8666±0.9296**
% RSD				**0.9308**

**mean±SD, (n=3) number of determination*

6.4.12 LOD and LOQ

The values of LOD and LOQ were found to be 0.3376 and 1.0230 µg/ml for ALI, 0.0263 and 0.0797 µg/ml for HCT, proves the sensitivity of the developed method (Table No. 6.4.8).

6.4.13 Robustness

The proposed method was checked through all the parameters described earlier under robustness studies. But there were no considerable variations in the chromatographic pattern after introducing small changes in experimental condition, indicates that the developed method is robust (Table No. 6.4.7).

Table No. 6.4.7: Results of robustness study

Sr. No.	Modification	ALI		HCT	
		R_t	Peak area	R_t	Peak area
1	Organic phase (90 ± 2%v/v)	5.254	325454	2.325	324545
		5.328	326857	2.284	323125
		5.415	328455	2.354	328465
% RSD* (<2)		**1.5113**	**0.4593**	**1.5153**	**0.8500**
2	Strength of buffer (0.2 ± 0.1%v/v)	5.225	324565	2.295	326555
		5.345	328451	2.231	322451
		5.412	326454	2.251	325455
% RSD* (<2)		**1.7784**	**0.5952**	**1.4494**	**0.6540**
3	Effect of pH (6 ± 0.2 unit)	5.243	324754	2.264	326454
		5.315	325625	2.234	325456
		5.423	328454	2.194	323454
% RSD* (<2)		**1.7007**	**0.5929**	**1.5744**	**0.4699**
4	Effect of flow rate (1 ± 0.1 ml/min)	5.345	325645	2.394	325454
		5.268	327454	2.345	326487
		5.164	324684	2.311	328545
% RSD* (<2)		**1.7272**	**0.4315**	**1.7755**	**0.4815**

**%RSD of three observation*

6.4.14 Stability of the solution

Solution stability was performed at room temperature and it was found to be stable up to two days.

Table No. 6.4.8: Summary of validation parameters for the RP-HPLC method

Parameters	ALI	HCT
Linearity range (µg/ml)	1.2-240	0.1-20
Correlation coefficient	0.9993	0.9999
Regression equation	y = 5276.5x + 1763.6	y = 64274x + 4229.2
Precision (%RSD)		
Repeatability of injection (n=6)	0.1917	0.6728
Intra-day (n=3)	0.0942	0.9319
Inter-day (n=3)	0.1220	1.2343
*Accuracy**		
% Recovery (n=3)	99.4916±1.0810	99.8666±0.9296
%RSD (n=3)	1.0866	0.9308
Specificity	No interference	
LOD (µg/ml)	0.3376	0.0263
LOQ (µg/ml)	1.0230	0.0797

**mean±SD, n= number of determinations*

6.4.15 System suitability test

System suitability tests were performed and results showed that the parameters tested were within the acceptable limit as per the ICH guidelines, indicating that the developed method is suitable for the analysis to be performed (Table No. 6.4.9).

Table No. 6.4.9: Results of system suitability studies

Parameters	Drugs		Acceptance criteria
	ALI*	HCT*	
Peak area reproducibility **%RSD**	68258.57±634.53 0.9296	663692.57±5370.09 0.8091	**% RSD <2**
Retention time (Rt) min **%RSD**	5.315±0.0264 0.4975	2.824±0.0027 0.0950	**% RSD< 2**
Resolution (Rs)	9.553±0.1132	4.403±0.0549	**>2**
No. of theoretical plate (N)	4877±36.20	3552±43.58	**>2000**
Tailing factor	0.857±0.0155	0.847±0.0099	**< 2**

**mean ± SD, (n=6) values of six determination*

6.4.16 Analysis of marketed formulation

The proposed method was successfully used for the quantitative determination of ALI and HCT in commercial formulation (Rasilez HCT tablet: 300 mg ALI and 25 mg of HCT). Six replicate determinations were carried out and experimental values were found to be within 98-101% for both the drugs are presented in Table No. 6.4.11. Therefore, the proposed method can be successfully applied for the quantitative analysis of ALI and HCT in tablet formulation. Chromatogram of formulation is shown in Figure No. 6.4.26.

Table No. 6.4.10: Results of formulation analysis

Drugs	Amount (mg/tablet)		% Drug found*	% RSD
	Labelled	Found		
ALI	300	297.84	99.2797±0.7106	0.7157
HCT	25	24.74	98.9493±0.2601	0.2628

**mean ± SD (n=6) average of six determinations*

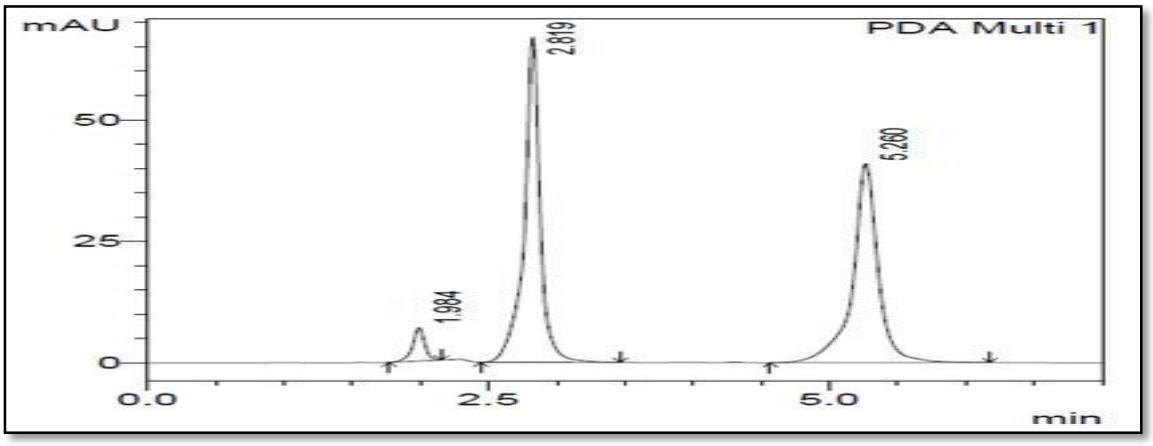


Figure No. 6.4.25: Chromatogram of formulation ALI (120 µg/ml) and HCT (10 µg/ml)

In order to check the applicability of the method, all the available strength (ratio) of marketed formulations were analyzed using standard drug solution in optimum ratio.

Available strength (mg)

ALI + HCT: 150/300 + 12.5/25

Study (% assay) suggests that, the proposed method can be applied to all the formulations of different strengths available in the market.

METHOD 5

6.5 *"Development and validation of simultaneous equation method for the simultaneous determination of aliskiren hemifumarate and valsartan in tablets"*

Estimation of ALI and VAL was achieved by simultaneous equation method using Shimadzu UV-1800 (UV Pro), double beam UV-visible spectrophotometer.

6.5.1 Selection of solvent

Based on the literature survey and solubility studies methanol was selected as solvent, which showed higher absorbance and distinct λ_{max} for both the drugs**.**

6.5.2 Selection of wavelength

Standard solutions of ALI (15 µg/ml) and VAL (16 µg/ml) were separately scanned in the UV region (200-400 nm) and spectra were recorded using methanol as blank. Their overlain spectra are shown in Figure No. 6.5.1.

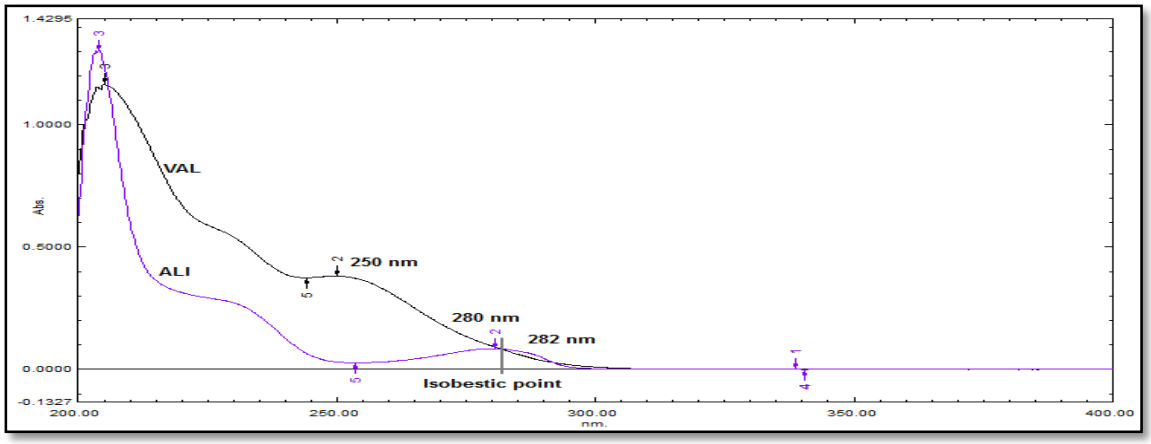


Figure No. 6.5.1: Overlain UV spectra of ALI (15 µg/ml) and VAL (16 µg/ml)

From the overlain spectra, 250 nm (λ_{max} of VAL) and 280 nm (λ_{max} of ALI) were selected for further studies, which showed good linearity and hence used for simultaneous estimation of ALI and VAL by *simultaneous equation* method.

6.5.3 Determination of absorptivity value

The developed method was found to be linear in the concentration range of 1-30 µg/ml for ALI and 1.067-32 µg/ml for VAL. Absorbances were measured at 250 nm and 280

nm for both the drugs and absorptivity values were calculated and presented in Table No. 6.5.1 & 6.5.2.

Table No. 6.5.1: Absorbances and absorptivities of ALI at selected wavelength

Conc. (µg/ml)	ALI					
	250 nm			280 nm		
	Abs.*	Absorptivity	Avg. Absorptivity	Abs.*	Absorptivity	Avg. Absorptivity
1	0.0018	18.0000	(ax_1) **19.5218**	0.0052	51.500	(ax_2) **55.0824**
5	0.0098	19.5333		0.0275	55.0333	
10	0.0206	20.6167		0.0556	55.5667	
15	0.0304	20.2556		0.0847	56.4333	
20	0.0395	19.7500		0.1124	56.1833	
25	0.0484	19.3467		0.1389	55.5600	
30	0.0575	19.1500		0.1659	55.3000	

**average of six determinations*

Table No. 6.5.2: Absorbances and absorptivities of VAL at selected wavelength

Conc. (µg/ml)	VAL					
	250 nm			280 nm		
	Abs.*	Absorptivity	Avg. Absorptivity	Abs.*	Absorptivity	Avg. Absorptivity
1.067	0.0256	239.8587	(ay_1) **241.6187**	0.0063	58.5974	(ay_2) **61.1153**
5.33	0.1281	240.0953		0.0325	60.8754	
10.67	0.2586	242.4703		0.0642	60.2191	
16	0.3878	242.3958		0.0959	59.9583	
21.33	0.5136	240.7269		0.1284	60.1642	
26.67	0.6493	243.4819		0.1651	61.8939	
32	0.7754	242.3021		0.2115	66.0990	

**average of six determinations*

6.5.4 Validation of the method

The developed method was validated in accordance with "*International Conference on Harmonization"* guidelines for validation of analytical procedures.

6.5.5 Specificity

Overlain spectra of tablet excipients and drug solution indicate that there was no interference between excipients and standard drugs (Figure No. 6.5.2).

6.5.6 Linearity and range

The developed method was found to be linear in the concentration range of 1-30 µg/ml for ALI and 1.067-32 µg/ml for VAL, respectively. Overlain spectra of ALI and VAL are shown in Figure No. 6.5.3-6.5.5.

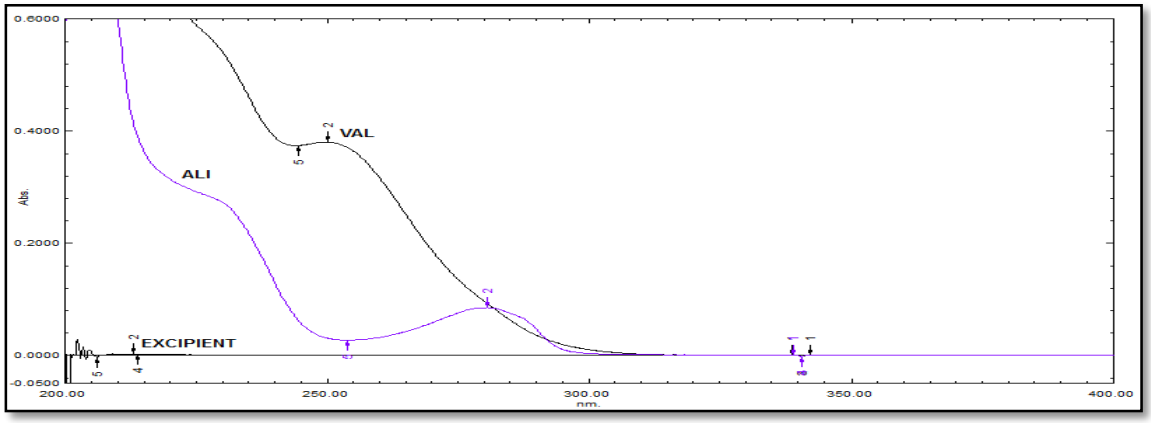


Figure No. 6.5.2: Overlain UV spectra of formulation excipients and standard drugs

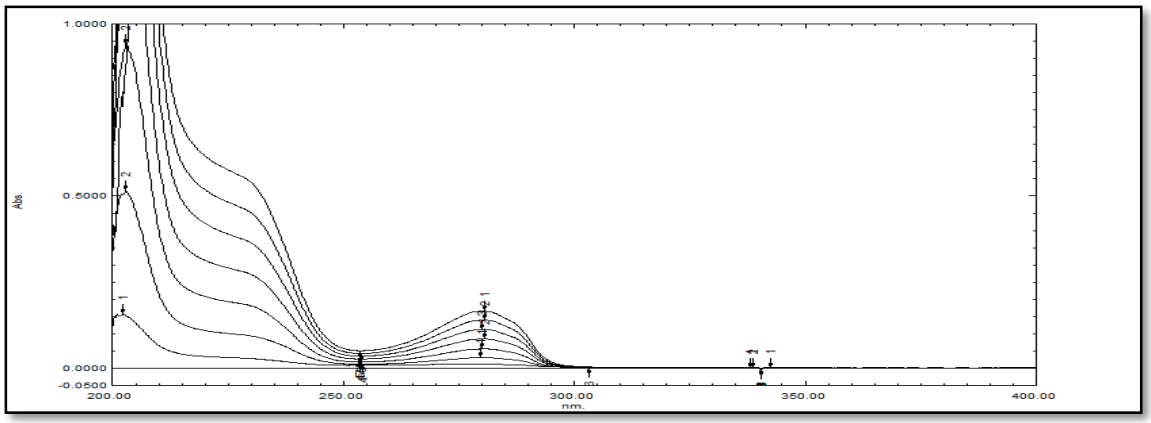


Figure No. 6.5.3: Overlain UV spectra of ALI (1-30 µg/ml)

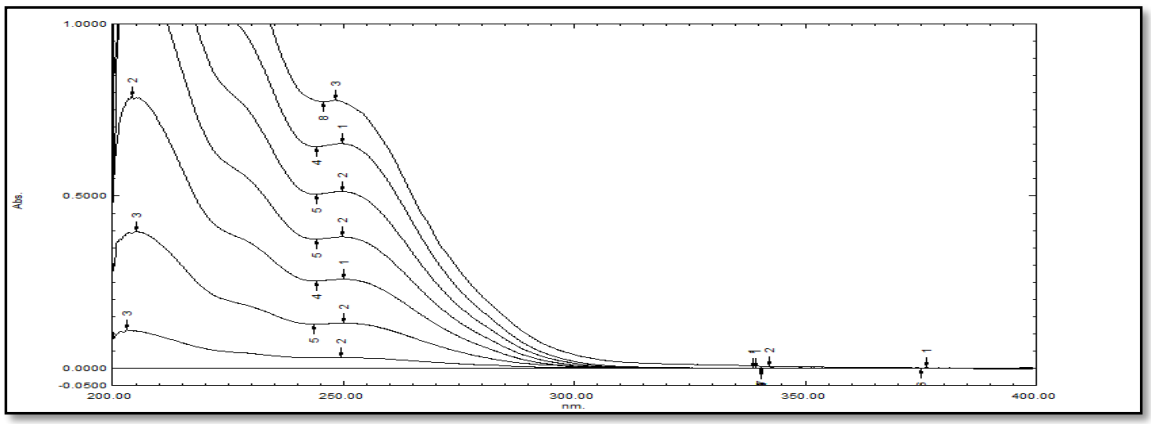


Figure No. 6.5.4: Overlain UV spectra of VAL (1.067-32 µg/ml)

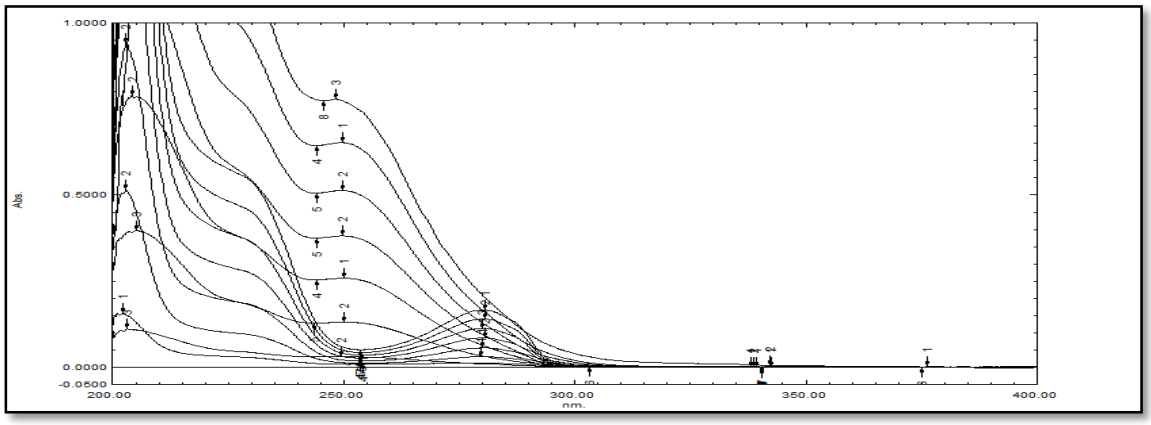


Figure No. 6.5.5: Overlain UV spectra of ALI (1-30 µg/ml) & VAL (1.067-32 µg/ml)

Calibration graphs (Figure No. 6.5.6-6.5.9) were plotted using absorbance of standard drug versus concentration. Results were subjected to regression analysis by least square method to calculate the values of slope, intercept and correlation coefficient of ALI and VAL at 250 and 280 nm are shown in Table No. 6.5.8.

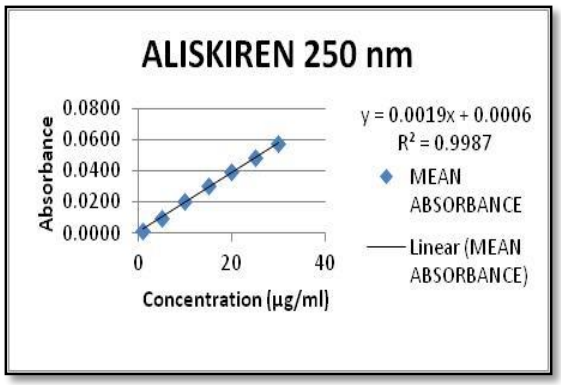


Figure No. 6.5.6: Standard calibration graph of ALI (1-30 µg/ml) at 250 nm

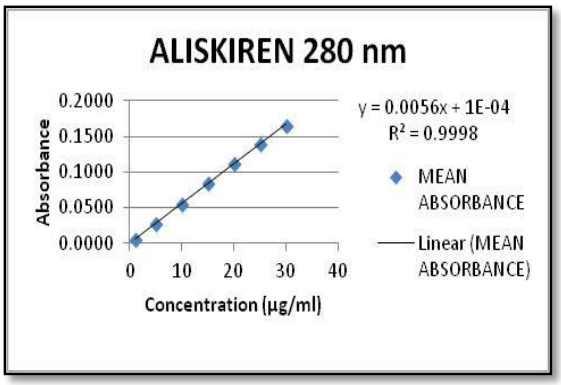


Figure No. 6.5.7: Standard calibration graph of ALI (1-30 µg/ml) at 280 nm

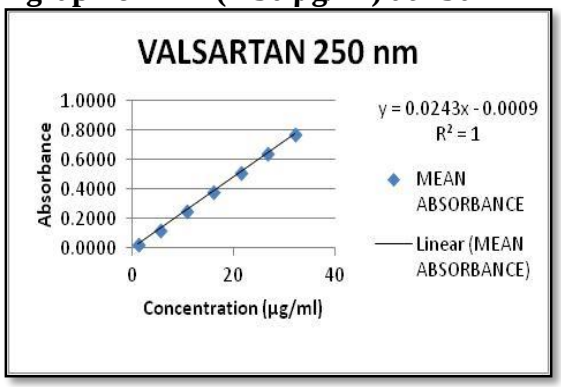


Figure No. 6.5.8: Standard calibration graph of VAL (1.067-32 µg/ml) at 250 nm

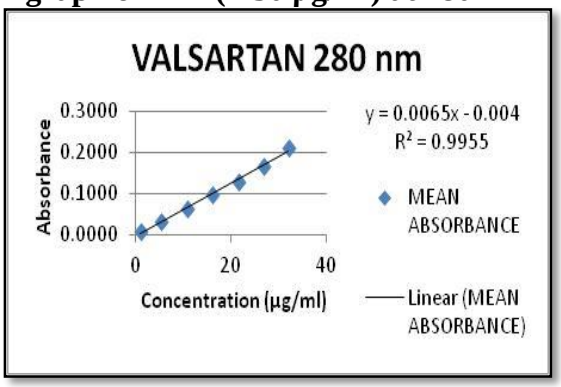


Figure No. 6.5.9: Standard calibration graph of VAL (1.067-32 µg/ml) at 280 nm

6.5.7 Precision

Results of precision studies expressed in %RSD follows ICH guideline acceptable limits (<2), which shows good repeatability and low intra and inter-day variability, indicating an excellent precision of the developed method (Table No. 6.5.3-6.5.5).

Table No. 6.5.3: Results of repeatability of measurement

Repeatability					
Sr. No.	Conc.	Absorbance			
		ALI		VAL	
		250 nm	280 nm	250 nm	280 nm
1	ALI 10 µg/ml VAL 10.67 µg/ml	0.0211	0.0551	0.2585	0.0648
2		0.0208	0.0558	0.2589	0.0651
3		0.0212	0.0561	0.2569	0.0638
4		0.0215	0.0563	0.2614	0.0639
5		0.0209	0.0565	0.2621	0.0651
6		0.0209	0.0554	0.2654	0.0638
Mean±SD*		**0.0211±0.0003**	**0.0559±0.0005**	**0.2605±0.0031**	**0.0644±0.0006**
% RSD		**1.2256**	**0.9650**	**1.1755**	**1.0081**
1	ALI 20 µg/ml VAL 21.33 µg/ml	0.0398	0.1162	0.5145	0.1276
2		0.0395	0.1157	0.5167	0.1284
3		0.0389	0.1152	0.5169	0.1299
4		0.0396	0.1145	0.5181	0.1258
5		0.0395	0.1123	0.5162	0.1268
6		0.0385	0.1114	0.5142	0.1275
Mean±SD*		**0.0393±0.0005**	**0.1142±0.0019**	**0.5151±0.0015**	**0.1277±0.0014**
% RSD		**1.2569**	**1.6972**	**0.2897**	**1.0962**
Mean % RSD		**1.2413**	**1.3311**	**0.7326**	**1.0521**

**mean±SD, (n= 6) number of determination*

Table No. 6.5.4: Results of intra-day precision

Intra-day precision					
Sr. No.	Conc.	Absorbance			
		ALI		VAL	
		250 nm	280 nm	250 nm	280 nm
1	ALI 10 µg/ml VAL 10.67 µg/ml	0.0205	0.0563	0.2641	0.0624
2		0.0208	0.0565	0.2615	0.0651
3		0.0209	0.0554	0.2657	0.0638
4		0.0208	0.0552	0.2585	0.0641
5		0.0205	0.0557	0.2589	0.0646
6		0.0207	0.0565	0.2569	0.0658
7		0.0212	0.0562	0.2614	0.0647
8		0.0214	0.0568	0.2582	0.0651
9		0.0212	0.0556	0.2579	0.0652
Mean±SD*		**0.0209±0.0003**	**0.0560±0.0006**	**0.2603±0.0030**	**0.0645±0.0010**
% RSD		**1.5222**	**1.0009**	**1.1616**	**1.5496**

1	ALI 20 µg/ml VAL 21.33 µg/ml	0.0375	0.1132	0.5214	0.1256
2		0.0374	0.1118	0.5216	0.1272
3		0.0385	0.1123	0.5217	0.1248
4		0.0388	0.1124	0.5155	0.1268
5		0.0395	0.1127	0.5141	0.1268
6		0.0389	0.1121	0.5134	0.1276
7		0.0386	0.1135	0.5227	0.1284
8		0.0385	0.1124	0.5324	0.1299
9		0.0381	0.1141	0.5360	0.1256
Mean±SD*		**0.0384±0.0007**	**0.1127±0.0007**	**0.5221±0.0078**	**0.1270±0.0016**
% RSD		**1.7400**	**0.6546**	**1.4895**	**1.2328**
Mean % RSD		**1.6311**	**0.8278**	**1.3256**	**1.3912**

**mean±SD, (n= 3) number of determination*

Table No. 6.5.5: Results of inter-day precision

Inter-day precision						
Sr. No.		**Conc.**	**Absorbance**			
			ALI		**VAL**	
			250 nm	**280 nm**	**250 nm**	**280 nm**
1	Day 1	ALI 10 µg/ml VAL 10.67 µg/ml	0.0214	0.0551	0.2582	0.0643
2			0.0213	0.0565	0.2579	0.0657
3			0.0211	0.0565	0.2587	0.0638
4	Day 2		0.0208	0.0568	0.2561	0.0658
5			0.0205	0.0563	0.2658	0.0658
6			0.0209	0.0565	0.2585	0.0651
7	Day 3		0.0208	0.0554	0.2589	0.0638
8			0.0209	0.0552	0.2569	0.0641
9			0.0209	0.0557	0.2614	0.0646
Mean±SD*			**0.0210±0.0003**	**0.0560±0.0007**	**0.2592±0.0029**	**0.0648±0.0008**
% RSD			**1.3093**	**1.1607**	**1.1150**	**1.3003**
1	Day 1	ALI 20 µg/ml VAL 21.33 µg/ml	0.0375	0.1145	0.5421	0.1238
2			0.0384	0.1124	0.5155	0.1254
3			0.0376	0.1128	0.5141	0.1276
4	Day 2		0.0372	0.1131	0.5134	0.1284
5			0.0388	0.1135	0.5241	0.1299
6			0.0375	0.1118	0.5212	0.1267
7	Day 3		0.0389	0.1123	0.5123	0.1258
8			0.0375	0.1124	0.5246	0.1261
9			0.0375	0.1131	0.5236	0.1256
Mean±SD*			**0.0379±0.0006**	**0.1129±0.0008**	**0.5212±0.0093**	**0.1266±0.0018**
% RSD			**1.6893**	**0.7056**	**1.7760**	**1.4326**
Mean % RSD			**1.4993**	**0.9332**	**1.4455**	**1.3664**

**mean±SD, (n= 3) number of determination*

6.5.8 Accuracy

The results of recovery studies ranged from 98-101% for both the drugs showing the accuracy of the method (Table No. 6.5.6). This indicates that there is no interference from tablet excipients.

Table No. 6.5.6: Results of recovery studies

Accuracy (% Recovery)				
ALI				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard drug added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	6	3	2.9655	98.8498
50	8	4	3.9405	98.5136
50	10	5	4.9055	98.1100
100	6	6	5.8757	97.9279
100	8	8	7.9120	98.9000
100	10	10	10.1631	101.6313
150	6	9	8.9949	99.9432
150	8	12	11.7650	98.0415
150	10	15	14.9123	99.4156
Mean±SD*				**99.0370±1.1781**
% RSD				**1.1895**
VAL				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard drug added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	6.3996	3.1998	3.1562	98.6380
50	8.5328	4.2664	4.2401	99.3839
50	10.6666	5.3333	5.3962	101.1785
100	6.3996	6.3996	6.3327	98.9544
100	8.5328	8.5328	8.6426	101.2868
100	10.6666	10.6666	10.5419	98.8308
150	6.3996	9.5994	9.5626	99.6162
150	8.5328	12.7992	12.6582	98.8983
150	10.6666	15.9999	16.0023	100.0150
Mean±SD*				**99.6447±0.9978**
% RSD				**1.0013**

**mean±SD, (n= 3) number of determination*

6.5.9 LOD and LOQ

The values of LOD and LOQ were found to be very low which proves the sensitivity of the method is shown in Table No. 6.5.8.

6.5.10 Robustness

The proposed method was checked for robustness study, but no significant changes (% RSD<2) found in absorption, indicating that the method is robust (Table No. 6.5.7).

Table No. 6.5.7: Results of robustness study

Parameter	Drugs			
Wavelengths	**ALI**		**VAL**	
(250 & 280 ±1 nm)	**Assay (%)***	**% RSD**	**Assay (%)***	**% RSD**
249 & 279 nm	97.2689	**1.8986**	100.2678	**1.5075**
250 & 280 nm	98.6548		98.2579	
251 & 281 nm	100.9871		97.3649	

** (n= 3) number of determination*

6.5.11 Stability of the solution

Solution stability was performed at room temperature and it was found to be stable up to two days.

Table No. 6.5.8: Summary of validation parameters for the proposed method

Parameters	**ALI**		**VAL**	
Detection wavelengths (nm)	250	280	250	280
Linearity range (µg/ml)	1-30		1.067-32	
Correlation coefficient	0.9987	0.9998	1	0.9955
Regression equation	y = 0.0019x + 0.0006	y = 0.0056x + 0.0001	y = 0.0243x - 0.0009	y = 0.0065x - 0.004
Precision (%RSD)				
Repeatability of measurement (n=6)	1.2413	1.3311	0.7326	1.0521
Intra-day (n=3)	1.6311	0.8278	1.3256	1.3912
Inter-day (n=3)	1.4993	0.9332	1.4455	1.3664
Accuracy				
% Recovery (n=3)	99.0370±1.1781		99.6447±0.9978	
%RSD (n=3)	1.1895		1.0013	
Specificity	No interference			
LOD (µg/ml)	0.2127	0.0811	0.0477	0.2869
LOQ (µg/ml)	0.6446	0.2459	0.1445	0.8693

**mean±SD, n= number of determinations*

6.5.12 Analysis of marketed formulation

The proposed method was successfully used for the quantitative determination of ALI and VAL in commercial formulation (Valturna tablet: 300 mg ALI and 320 mg of VAL). Six replicate determinations were carried out and experimental values were found to be between 98 and 102% for both the drugs and hence the developed method can be used for

the simultaneous determination of both the drugs in combined formulation are presented in Table No. 6.5.9. Overlain spectra of standard drugs and formulation are showed in Figure No. 6.5.10.

Table No. 6.5.9: Result of formulation analysis

Drugs	Amount (mg/tablet)		% Drug found*	% RSD
	Labelled	Found		
ALI	300	296.02	98.6740±1.2584	1.2754
VAL	320	313.21	97.8782±1.1735	1.1989

**mean ± SD (n=6) average of six determinations*

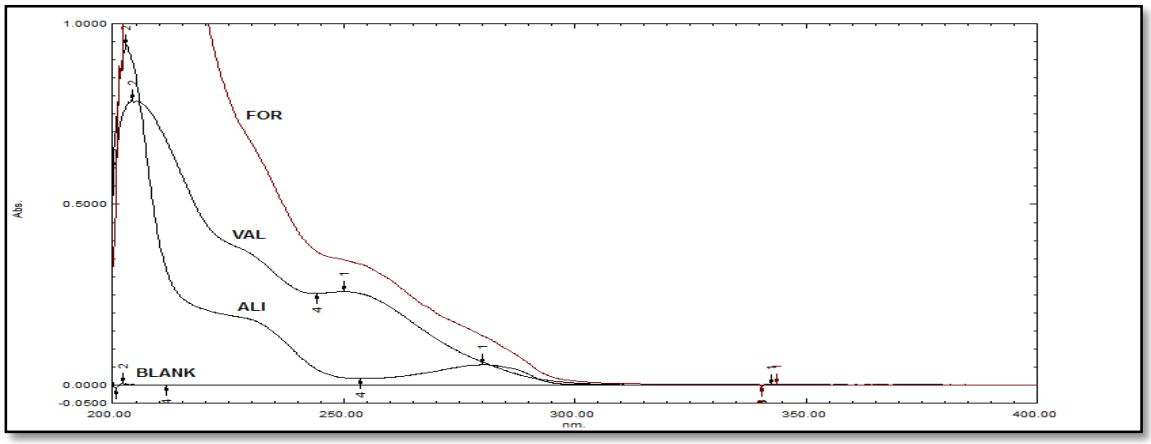


Figure No. 6.5.10: Overlain UV spectra of standard ALI (10 µg/ml), VAL (10.67 µg/ml) & formulation (10 & 10.67 µg/ml)

Results of formulation analysis (% assay) suggest that, the proposed method can be applied successfully for the quantitative determination of ALI and VAL in tablet dosage form.

METHOD 6

6.6 *"Development and validation of absorbance ratio (Q analysis) method for the simultaneous determination of aliskiren hemifumarate and valsartan in tablets"*

Estimation of ALI and VAL was achieved by absorbance ratio method using Shimadzu UV-1800 (UV Pro), double beam UV-visible spectrophotometer.

6.6.1 Selection of solvent

Based on the literature survey and solubility studies methanol was selected as solvent, which showed higher absorbance and distinct λ_{max} for both the drugs**.**

6.6.2 Selection of wavelength

Standard solutions of ALI (15 µg/ml) and VAL (16 µg/ml) were separately scanned in the UV region (200-400 nm) and spectra were recorded using methanol as blank. Their overlain spectra are shown in Figure No. 6.6.1.

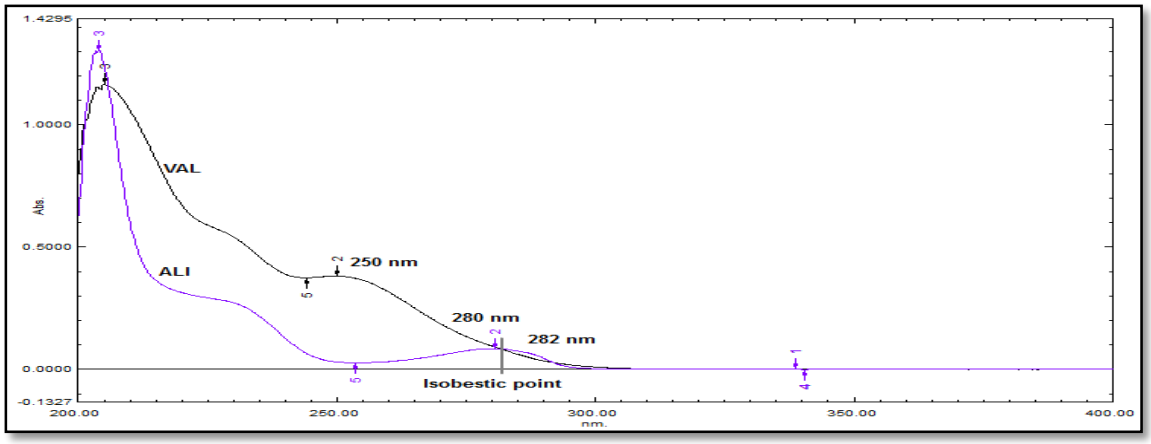


Figure No. 6.6.1: Overlain UV spectra of ALI (15 µg/ml) and VAL (16 µg/ml)

From the overlain spectra, initially different wavelengths were tried for the study. But finally 250 nm (λ_{max} of VAL) and 282 nm (isobestic point) were selected, which showed good linearity and hence used for simultaneous estimation of ALI and VAL by *absorbance ratio (Q analysis)* method.

6.6.3 Determination of absorptivity value

The developed method was found to be linear in the concentration range of 1-30 µg/ml for ALI and 1.067-32 µg/ml for VAL, respectively. Absorbances were measured at 250

nm and 282 nm for both the drugs and absorptivity values were calculated and presented in Table No. 6.6.1 & 6.6.2.

Table No. 6.6.1: Absorbances and absorptivities of ALI at selected wavelength

Conc. (µg/ml)	ALI					
	250 nm			282 nm		
	Abs.*	Absorptivity	Avg. Absorptivity	Abs.*	Absorptivity	Avg. Absorptivity
1	0.0018	18.0000		0.0052	52.3333	
5	0.0098	19.5333		0.0277	55.4667	
10	0.0206	20.6167	(ax_1)	0.0555	55.4667	(ax_2)
15	0.0304	20.2556	**19.5217**	0.0838	55.8678	**54.9086**
20	0.0395	19.7500		0.1110	55.4833	
25	0.0484	19.3467		0.1377	55.0867	
30	0.0575	19.1500		0.1640	54.6556	

**average of six determinations*

Table No. 6.6.2: Absorbances and absorptivities of VAL at selected wavelength

Conc. (µg/ml)	VAL					
	250 nm			282 nm		
	Abs.*	Absorptivity	Avg. Absorptivity	Abs.*	Absorptivity	Avg. Absorptivity
1.067	0.0256	239.8587		0.0052	49.0656	
5.33	0.1281	240.0953		0.0277	52.0003	
10.67	0.2586	242.4703	(ay_1)	0.0555	52.0003	(ay_2)
16	0.3878	242.3958	**241.6187**	0.0838	52.3760	**51.4773**
21.33	0.5136	240.7269		0.1110	52.0157	
26.67	0.6493	243.4819		0.1377	51.6439	
32	0.7754	242.3021		0.1640	51.2396	

**average of six determinations*

6.6.4 Validation of the method

The developed method was validated in accordance with "*International Conference on Harmonization"* guidelines for validation of analytical procedures.

6.6.5 Specificity

Overlain spectra of tablet excipients and drug solutions indicate that there was no interference between excipients and standard drugs (Figure No. 6.6.2).

6.6.6 Linearity and range

The developed method was found to be linear in the concentration range of 1-30 µg/ml for ALI and 1.067-32 µg/ml for VAL, respectively. Overlain spectra of ALI and VAL are shown in Figure No. 6.6.3-6.6.5.

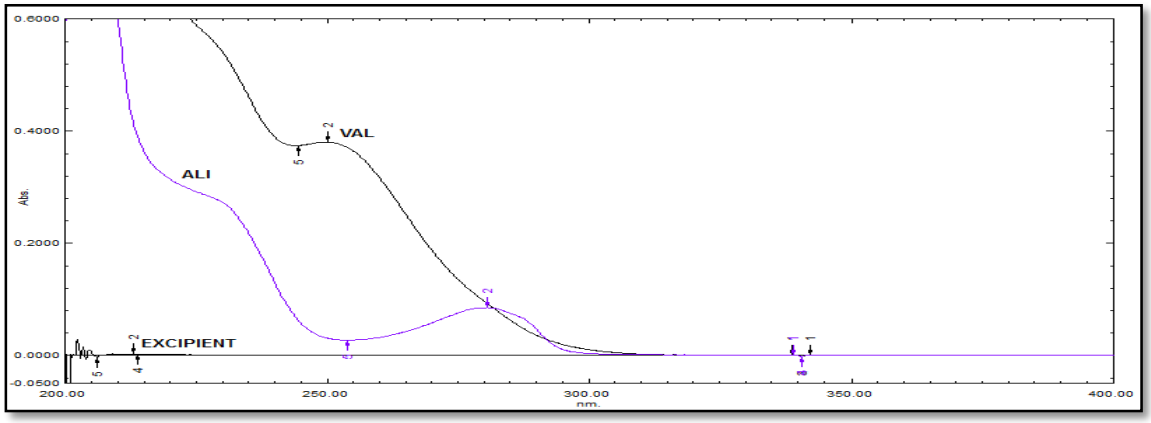


Figure No. 6.6.2: Overlain UV spectra of formulation excipients and standard drugs

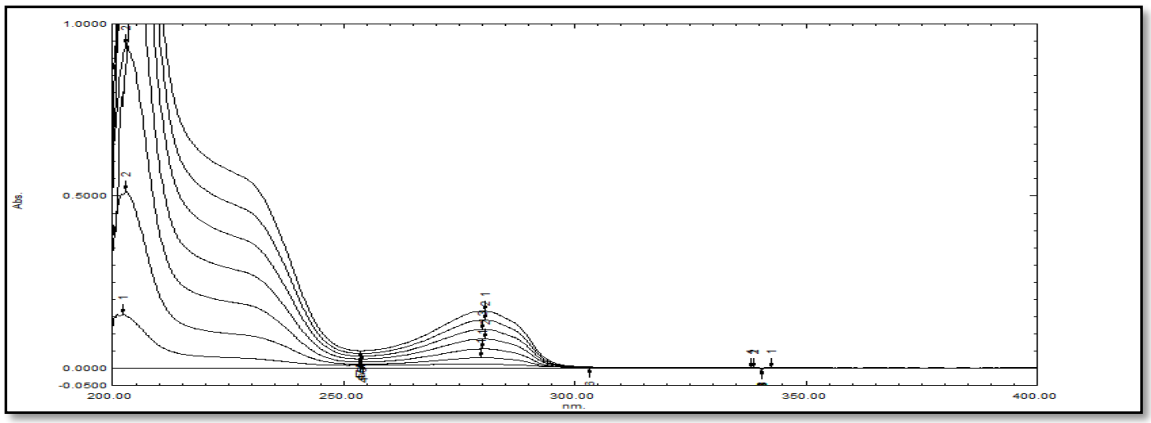


Figure No. 6.6.3: Overlain UV spectra of ALI (1-30 µg/ml)

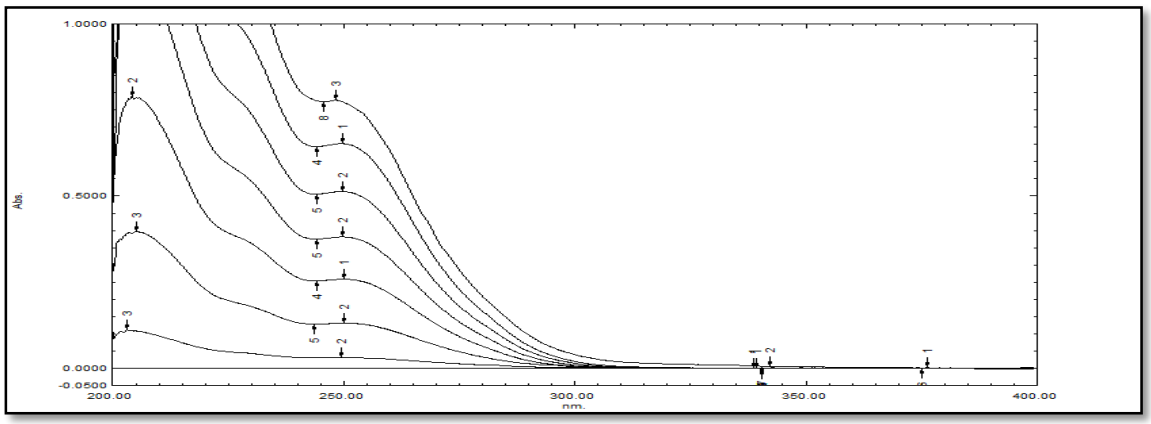


Figure No. 6.6.4: Overlain UV spectra of VAL (1.067-32 µg/ml)

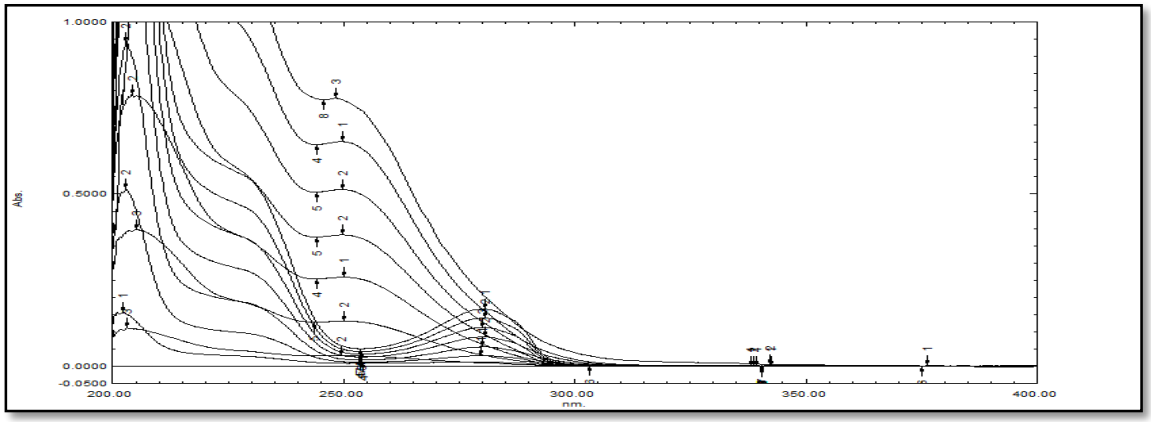


Figure No. 6.6.5: Overlain UV spectra of ALI (1-30 µg/ml) & VAL (1.067-32 µg/ml)

Calibration graphs (Figure No. 6.6.6-6.6.9) were plotted using absorbance of standard drug versus concentration. Results were subjected to regression analysis by least square method to calculate the values of slope, intercept and correlation coefficient of ALI and VAL at 250 and 282 nm are shown in Table No. 6.6.8.

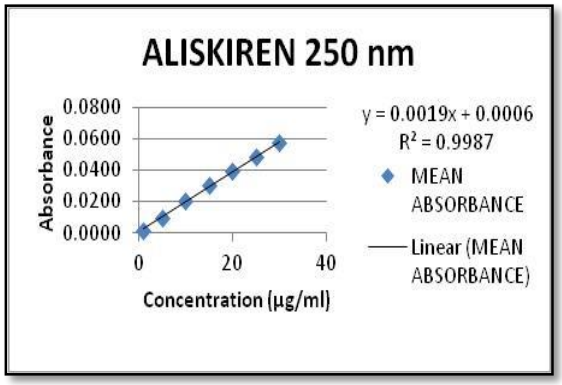


Figure No. 6.6.6: Standard calibration graph of ALI (1-30 µg/ml) at 250 nm

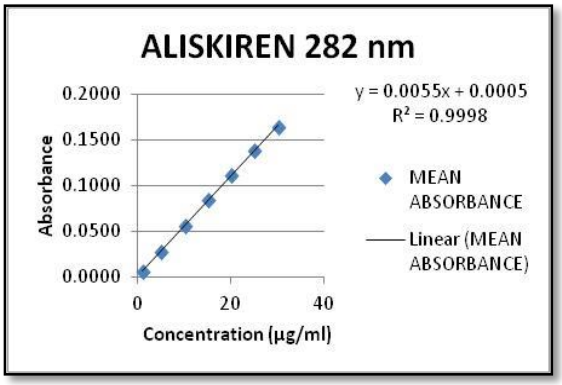


Figure No. 6.6.7: Standard calibration graph of ALI (1-30 µg/ml) at 282 nm

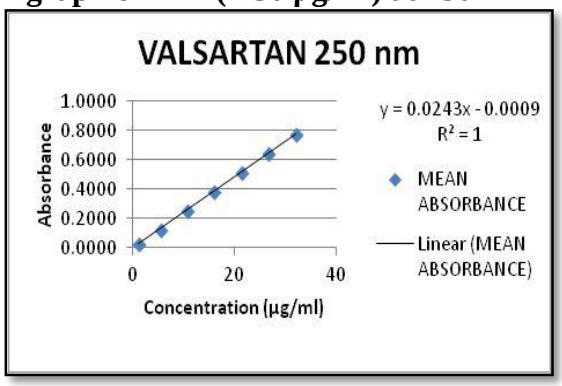


Figure No. 6.6.8: Standard calibration graph of VAL (1.07-32 µg/ml) at 250 nm

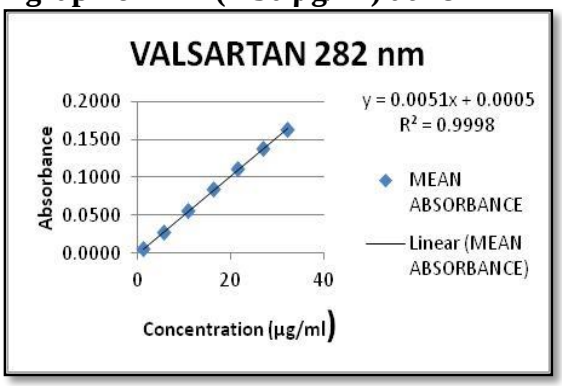


Figure No. 6.6.9: Standard calibration graph of VAL (1.07-32 µg/ml) at 282 nm

6.6.7 Precision

Results of precision studies expressed in %RSD follows ICH guideline acceptable limits (%RSD<2), which shows good repeatability and low inter-day variability, indicating an excellent precision of the developed method **(**Table No. 6.6.3, 6.6.4 & 6.6.5).

Table No. 6.6.3: Results of repeatability of measurement

Repeatability					
Sr. No.	Conc.	Absorbance			
		ALI		VAL	
		250 nm	282 nm	250 nm	282 nm
1	ALI 10 µg/ml	0.0211	0.0565	0.2585	0.0562
2		0.0208	0.0571	0.2589	0.0558
3		0.0212	0.0548	0.2569	0.0548
4	VAL 10.67 µg/ml	0.0215	0.0567	0.2614	0.0561
5		0.0209	0.0562	0.2621	0.0537
6		0.0209	0.0558	0.2654	0.0553
Mean±SD*		**0.0211±0.0003**	**0.0562±0.0008**	**0.2605±0.0031**	**0.0553±0.0009**
% RSD		**1.2256**	**1.4390**	**1.1755**	**1.7166**
1	ALI 20 µg/ml	0.0398	0.1108	0.5145	0.1108
2		0.0395	0.1154	0.5167	0.1112
3		0.0389	0.1127	0.5169	0.1121
4	VAL 21.33 µg/ml	0.0396	0.1138	0.5181	0.1142
5		0.0395	0.1157	0.5162	0.1134
6		0.0385	0.1109	0.5142	0.1125
Mean±SD*		**0.0393±0.0005**	**0.1132±0.0021**	**0.5161±0.0015**	**0.1124±0.0013**
% RSD		**1.2569**	**1.8840**	**0.2897**	**1.1489**
Mean % RSD		**1.2413**	**1.6615**	**0.7326**	**1.4328**

**mean±SD, (n= 6) number of determination*

Table No. 6.6.4: Results of intra-day precision

Intra-day precision					
Sr. No.	Conc.	Absorbance			
		ALI		VAL	
		250 nm	282 nm	250 nm	282 nm
1	ALI 10 µg/ml	0.0205	0.0564	0.2641	0.0554
2		0.0208	0.0562	0.2615	0.0556
3		0.0209	0.0558	0.2657	0.0562
4	VAL 10.67 µg/ml	0.0208	0.0548	0.2585	0.0558
5		0.0205	0.0556	0.2589	0.0548
6		0.0207	0.0578	0.2569	0.0548
7		0.0212	0.0563	0.2614	0.0561
8		0.0214	0.0564	0.2582	0.0553
9		0.0212	0.0548	0.2579	0.0551
Mean±SD*		**0.0209±0.0003**	**0.0560±0.0009**	**0.2603±0.0030**	**0.0555±0.0005**
% RSD		**1.5222**	**1.6423**	**1.1616**	**0.9288**

1	ALI 20 µg/ml VAL 21.33 µg/ml	0.0375	0.1109	0.5214	0.1128
2		0.0374	0.1112	0.5216	0.1134
3		0.0385	0.1108	0.5217	0.1131
4		0.0388	0.1121	0.5155	0.1106
5		0.0395	0.1114	0.5141	0.1102
6		0.0389	0.1134	0.5134	0.1109
7		0.0386	0.1137	0.5227	0.1127
8		0.0385	0.1146	0.5324	0.1138
9		0.0381	0.1128	0.5360	0.1134
Mean±SD*		**0.0384±0.0007**	**0.1123±0.0014**	**0.5221±0.0078**	**0.1123±0.0014**
% RSD		**1.7400**	**1.2181**	**1.4895**	**1.2181**
Mean % RSD		**1.6311**	**1.4302**	**1.3256**	**1.0734**

**mean±SD, (n= 3) number of determination*

Table No. 6.6.5: Results of inter-day precision

Inter-day precision						
Sr. No.		**Conc.**	**Absorbance**			
			ALI		**VAL**	
			250 nm	**282 nm**	**250 nm**	**282 nm**
1	Day 1	ALI 10 µg/ml VAL 10.67 µg/ml	0.0214	0.0568	0.2582	0.0553
2			0.0213	0.0568	0.2579	0.0551
3			0.0211	0.0561	0.2587	0.0552
4	Day 2		0.0208	0.0562	0.2561	0.0553
5			0.0205	0.0558	0.2658	0.0567
6			0.0209	0.0568	0.2585	0.0558
7	Day 3		0.0208	0.0556	0.2589	0.0548
8			0.0209	0.0554	0.2569	0.0561
9			0.0209	0.0568	0.2614	0.0541
Mean±SD*			**0.0210±0.0003**	**0.0563±0.0006**	**0.2592±0.0029**	**0.0554±0.0008**
% RSD			**1.3093**	**1.0099**	**1.1150**	**1.3597**
1	Day 1	ALI 20 µg/ml VAL 21.33 µg/ml	0.0375	0.1135	0.5187	0.1135
2			0.0384	0.1137	0.5155	0.1126
3			0.0376	0.1109	0.5141	0.1106
4	Day 2		0.0372	0.1112	0.5134	0.1102
5			0.0388	0.1108	0.5241	0.1109
6			0.0375	0.1121	0.5212	0.1137
7	Day 3		0.0389	0.1128	0.5123	0.1126
8			0.0375	0.1134	0.5246	0.1125
9			0.0375	0.1141	0.5236	0.1124
Mean±SD*			**0.0379±0.0006**	**0.1125±0.0013**	**0.5186±0.0049**	**0.1121±0.0013**
% RSD			**1.6893**	**1.1418**	**0.9510**	**1.1180**
Mean % RSD			**1.4993**	**1.0759**	**1.0330**	**1.2389**

**mean±SD, (n= 3) number of determination*

6.6.8 Accuracy

The results of recovery studies ranged from 98-102% for both the drugs showing the accuracy of the method (Table No. 6.6.6). This indicates that there is no interference from tablet excipients.

Table No. 6.6.6: Results of recovery studies

Accuracy (% Recovery)				
ALI				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard drug added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	6	3	3.0418	101.3928
50	8	4	3.9887	99.7186
50	10	5	5.0594	101.1883
100	6	6	5.8776	97.9604
100	8	8	8.0754	100.9429
100	10	10	9.9906	99.9061
150	6	9	8.9412	99.3465
150	8	12	11.9315	99.4291
150	10	15	15.2216	101.4771
Mean±SD*				**100.1513±1.1841**
% RSD				**1.1824**
VAL				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard drug added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	6.3996	3.1998	3.2725	102.2713
50	8.5328	4.2664	4.2775	100.2592
50	10.6666	5.3333	5.3231	99.8095
100	6.3996	6.3996	6.4046	100.0780
100	8.5328	8.5328	8.5133	99.7720
100	10.6666	10.6666	10.6445	99.7927
150	6.3996	9.5994	9.5728	99.7231
150	8.5328	12.7992	12.8950	100.7486
150	10.6666	15.9999	16.1972	101.2332
Mean±SD*				**100.4097±0.8172**
% RSD				**0.8139**

**mean±SD, (n= 3) number of determination*

6.6.9 LOD and LOQ

The values of LOD and LOQ were found to be very low which proves the sensitivity of the method is shown in Table No. 6.6.8.

6.6.10 Robustness

The proposed method was checked for robustness study, but no significant changes (% RSD<2) found in absorption (Table No. 6.6.7).

Table No. 6.6.7: Results of robustness study

Parameter	Drugs			
Wavelengths (250 & 282±1 nm)	ALI		VAL	
	Assay (%)*	% RSD	Assay (%)*	% RSD
249 & 281 nm	97.9878	1.3847	99.5454	1.6515
250 & 282 nm	100.5688		98.2985	
251 & 283 nm	98.4763		101.5647	

** (n= 3) number of determination*

6.6.11 Stability of the solution

Solution stability was performed at room temperature and it was found to be stable up to two days.

Table No. 6.6.8: Summary of validation parameters for the proposed method

Parameters	ALI		VAL	
Detection wavelengths (nm)	250	282	250	282
Linearity range (µg/ml)	1-30		1.0666-32	
Correlation coefficient	0.9987	0.9998	1	0.9998
Regression equation	y = 0.0019x + 0.0006	y = 0.0055x + 0.0005	y = 0.0243x - 0.0009	y = 0.0051x + 0.0005
Precision (%RSD)				
Repeatability of measurement (n=6)	1.2413	1.6615	0.7326	1.4328
Intra-day (n=3)	1.6311	1.4302	1.3256	1.0734
Inter-day (n=3)	1.4993	1.0759	1.0330	1.2389
Accuracy				
% Recovery (n=3)	100.1513±1.1841		100.4097±0.8172	
%RSD (n=3)	1.1824		0.8139	
Specificity	No interference			
LOD (µg/ml)	0.2127	0.1588	0.0477	0.1697
LOQ (µg/ml)	0.6446	0.4814	0.1446	0.5142

**mean±SD, n= number of determinations*

6.6.12 Analysis of marketed formulation

The proposed method was successfully used for the quantitative determination of ALI and VAL in commercial formulation (Valturna tablet: 300 mg ALI and 320 mg of VAL). Six replicate determinations were carried out and experimental values were found to be within 98-102%v/v for both the drugs and hence the developed method can be used for the simultaneous determination of both the drugs in combined formulation are presented in Table No. 6.6.9. Overlain spectra of standard drugs and formulation are showed in Figure No. 6.6.10.

Table No. 6.6.9: Results of formulation analysis

Drugs	Amount (mg/tablet)		% Drug found*	% RSD
	Labelled	Found		
ALI	300	293.80	97.9346±1.4524	1.4830
VAL	320	313.69	98.0297±1.0448	1.0658

**mean±SD (n=6) average of six determinations*

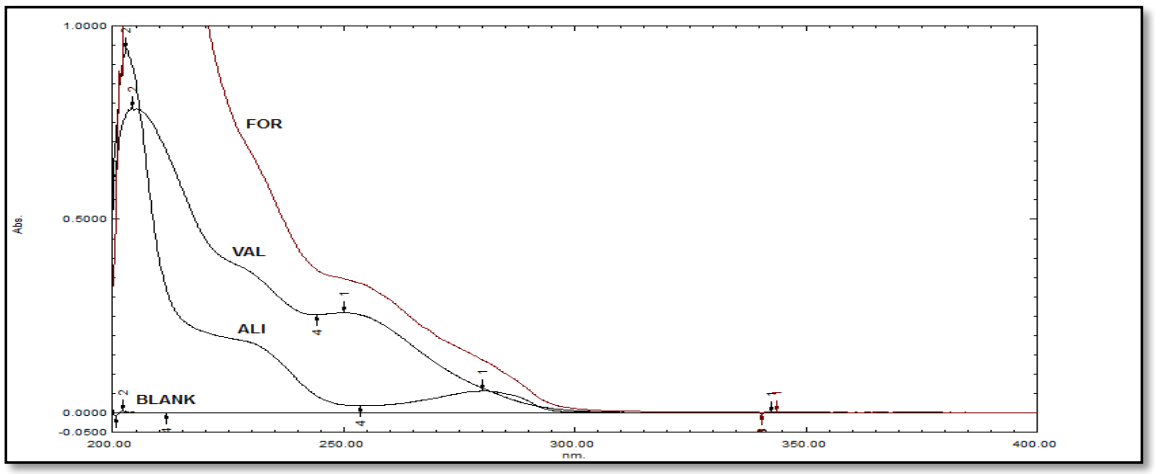


Figure No. 6.6.10: Overlain UV spectra of standard ALI (10 µg/ml), VAL (10.67 µg/ml) & formulation (10 & 10.67 µg/ml)

Results of formulation analysis (% assay) suggest that, the proposed method can be applied successfully for the quantitative determination of ALI and VAL in tablet dosage form.

METHOD 7

6.7 *"Development and validation of first-derivative (Zero crossing) spectroscopic method for the simultaneous determination of aliskiren hemifumarate and valsartan in tablets"*

Estimation of ALI and VAL was achieved by first derivative spectroscopic method using Shimadzu UV-1800 (UV Pro), double beam UV-visible Spectrophotometer.

6.7.1 Selection of solvent

Based on the literature survey and solubility studies methanol was selected as solvent, which showed higher absorbance and favourable zero crossing points for both the drugs**.**

6.7.2 Selection of wavelength

Standard solutions of ALI (15 µg/ml) and VAL (16 µg/ml) were separately scanned in the UV region (200-400 nm) and spectra were recorded using methanol as blank. Both the spectra were converted into first and second derivative spectra. Based on the spectral pattern and zero crossing points, first derivative method was selected for the study. First derivative spectra showed typical zero-crossing points at 280.30 nm for ALI and 244 nm for VAL. From the overlain spectra, 244 nm and 280.30 nm were selected for further studies are shown in Figure No. 6.7.1.

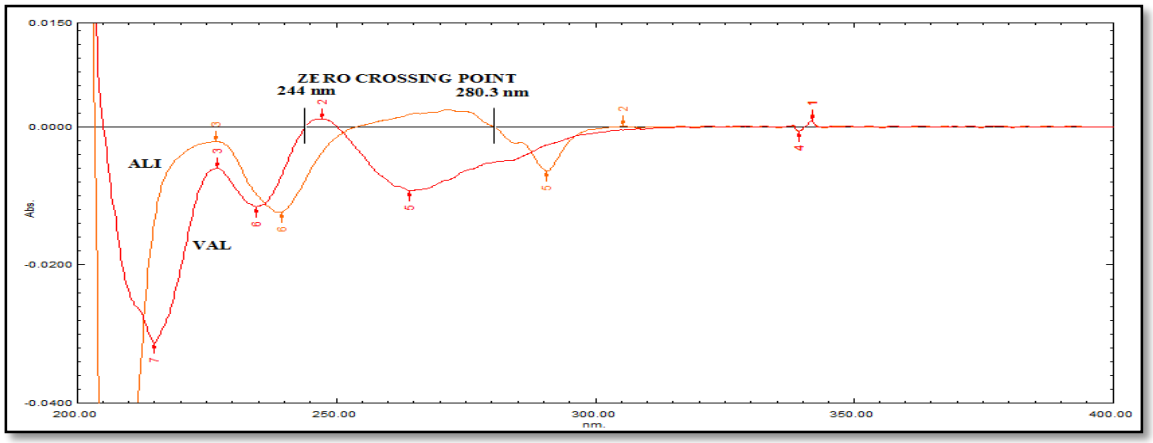


Figure No. 6.7.1: Overlain 1st derivative UV spectra of ALI (10 µg/ml) and VAL (10.67 µg/ml)

At 244 nm, VAL showed zero absorbance but ALI had considerable absorbance. Similarly at 280.30 nm, ALI showed zero absorbance but VAL had considerable absorbance (Table No. 6.7.1).

Table No. 6.7.1: Selection of zero crossing points for ALI & VAL

Drugs	Zero crossing point (nm)	Detection wavelength (nm)
ALI	280.30	244
VAL	244	280.30

6.7.3 Preparation of calibration curve

Calibration curves (Table No. 6.7.2; Figure No. 6.7.6 & 6.7.7) were plotted for both ALI and VAL in the range 1 to 30 µg/ml and 1.067 to 32 µg/ml, respectively. Results were subjected to regression analysis by least square method to calculate the values of slope, intercept and correlation coefficient are shown in Table No. 6.7.8.

Table No. 6.7.2: Linearity data of 1st derivative UV spectroscopic method

Sr. No.	ALI at 244 nm			VAL at 280.3 nm		
	Conc. (µg/ml)	1st derivative signal*	% RSD	Conc. (µg/ml)	1st derivative signal*	% RSD
1	1	0.0008	1.2392	1.07	0.0006	1.3306
2	5	0.0039	1.1889	5.33	0.0027	1.5425
3	10	0.0078	1.1567	10.67	0.0048	1.7903
4	15	0.0116	1.2708	16.00	0.0070	1.4515
5	20	0.0157	0.9867	21.33	0.0091	0.7992
6	25	0.0195	1.1059	26.67	0.0112	0.7031
7	30	0.0233	0.9787	32.00	0.0134	0.7901

(n=6) average of six determinations

6.7.4 Validation of the method

The developed method was validated in accordance with "*International Conference on Harmonization"* guidelines for validation of analytical procedures.

6.7.5 Specificity

Overlain spectra of tablet excipients and drug solutions indicate that there was no interference between excipients and standard drugs (Figure No. 6.7.2).

6.7.6 Linearity and range

ALI and VAL were found to be linear in the concentration range of 1-30 µg/ml and 1.067-32 µg/ml, respectively. Overlain spectra of ALI and VAL are shown in Figure No. 6.7.3-6.7.5.

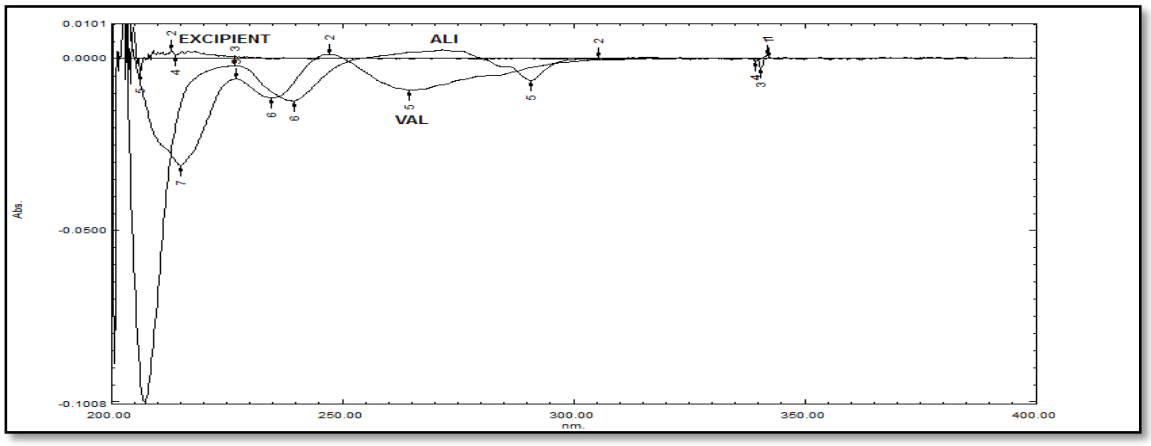


Figure No. 6.7.2: Overlain UV 1st derivative spectra of formulation excipient and standard drugs

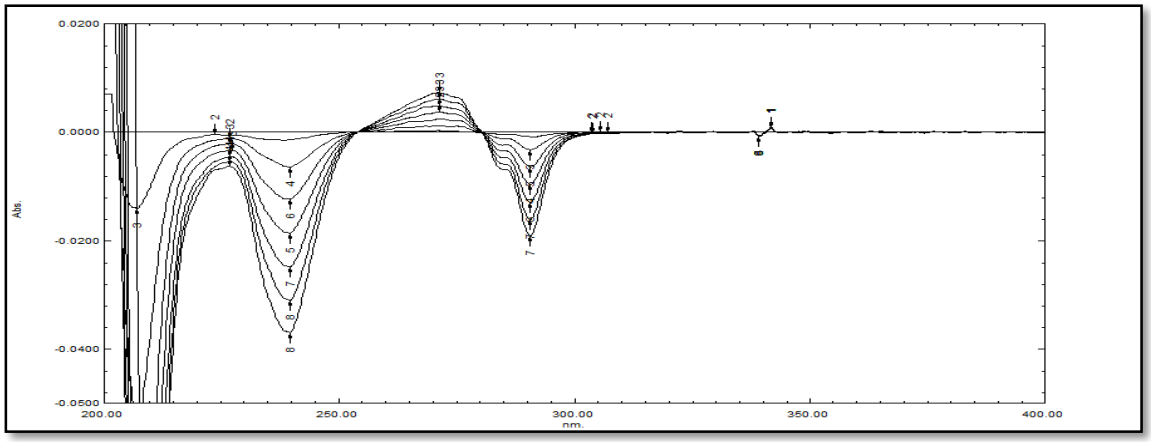


Figure No. 6.7.3: Overlain 1st derivative UV spectra of ALI (1-30 µg/ml)

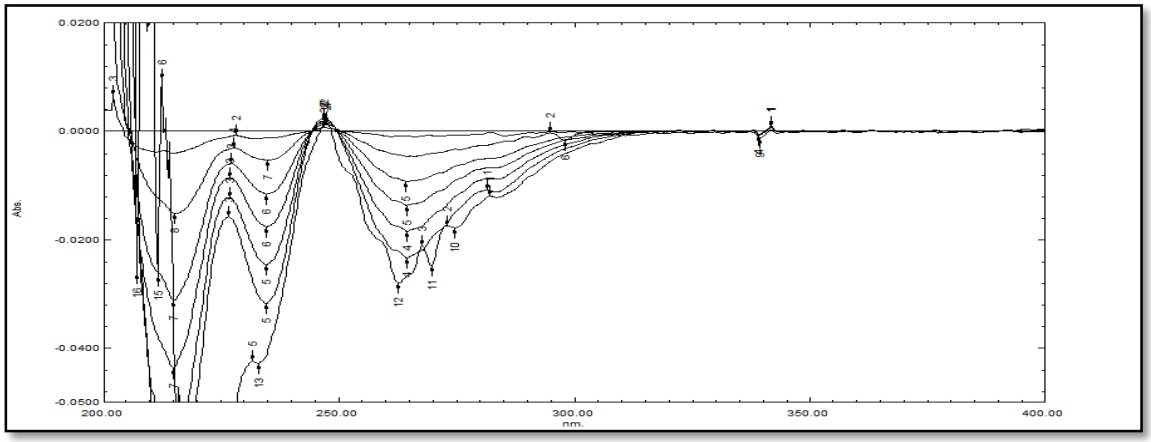


Figure No. 6.7.4: Overlain 1st derivative UV spectra of VAL (1.067-32 µg/ml)

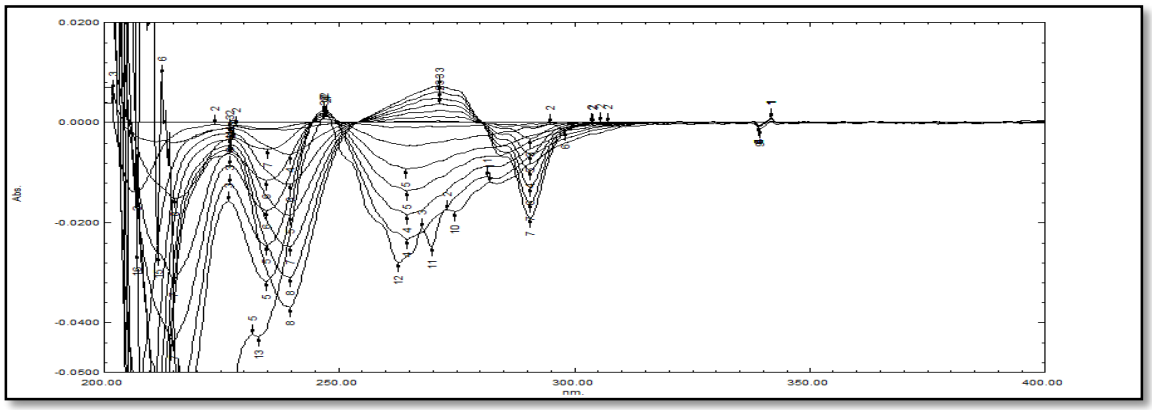


Figure No. 6.7.5: Overlain 1st derivative UV spectra of ALI (1-30 µg/ml) & VAL (1.067-32 µg/ml)

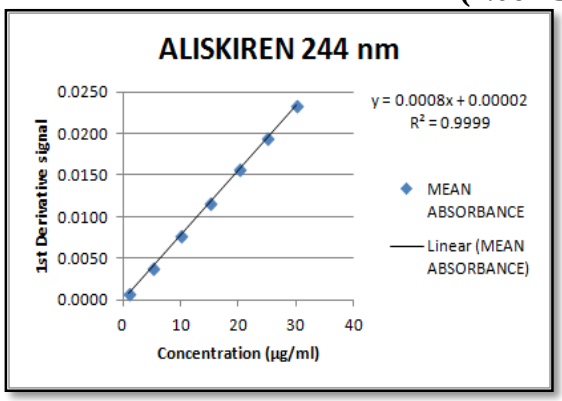


Figure No. 6.7.6: Standard calibration curve of ALI (1-30 µg/ml)

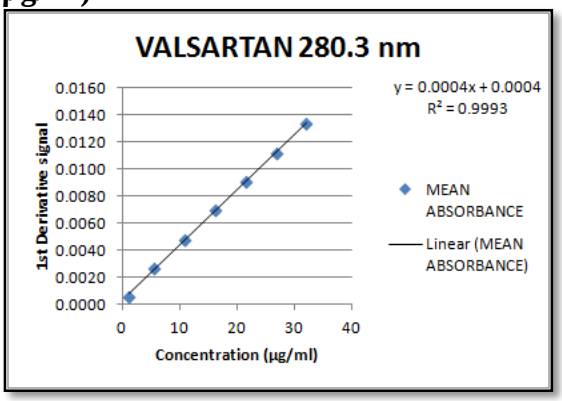


Figure No. 6.7.7: Standard calibration curve of VAL (1.067-32 µg/ml)

6.7.7 Precision

Results of precision studies expressed in % RSD follows ICH guideline acceptable limits (%RSD<2), which shows good repeatability and low inter-day variability, indicating an excellent precision of the developed method (Table No. 6.7.3, 6.7.4 & 6.7.5).

Table No. 6.7.3: Results of repeatability of measurement

Repeatability			
Sr. No.	**Conc.**	**1st derivative signal**	
		ALI (244 nm)	**VAL (280.30 nm)**
1	ALI 10 µg/ml	0.0077	0.0046
2		0.0079	0.0046
3		0.0078	0.0045
4	VAL 10.67 µg/ml	0.0079	0.0046
5		0.0076	0.0045
6		0.0077	0.0046
Mean±SD*		**0.0078±0.0001**	**0.0046±0.00005**
% RSD		**1.4674**	**1.0309**

1	ALI 20 µg/ml VAL 21.33 µg/ml	0.0156	0.0090
2		0.0158	0.0091
3		0.0158	0.0092
4		0.0161	0.0092
5		0.0155	0.0091
6		0.0157	0.0093
Mean±SD*		**0.0158±0.0002**	**0.0092±0.0001**
% RSD		**1.3166**	**0.9802**
Mean % RSD		**1.3920**	**1.0055**

**mean±SD, (n= 6) number of determination*

Table No. 6.7.4: Results of intra-day precision

Intra-day precision			
		1st derivative signal	
Sr. No.	**Conc.**	**ALI (244 nm)**	**VAL (280.30 nm)**
1	ALI 10 µg/ml VAL 10.67 µg/ml	0.0078	0.0048
2		0.0077	0.0046
3		0.0076	0.0048
4		0.0078	0.0047
5		0.0079	0.0047
6		0.0079	0.0046
7		0.0079	0.0048
8		0.0079	0.0047
9		0.0078	0.0046
Mean±SD*		**0.0078±0.0001**	**0.0047±0.0001**
% RSD		**1.2518**	**1.8740**
1	ALI 20 µg/ml VAL 21.33 µg/ml	0.0158	0.0093
2		0.0159	0.0093
3		0.0161	0.0093
4		0.0156	0.0090
5		0.0158	0.0091
6		0.0158	0.0092
7		0.0158	0.0092
8		0.0162	0.0093
9		0.0165	0.0093
Mean±SD*		**0.0159±0.0003**	**0.0092±0.0001**
% RSD		**1.7208**	**0.9705**
Mean % RSD		**1.4863**	**1.4222**

**mean±SD, (n= 3) number of determination*

Table No. 6.7.5: Results of inter-day precision

Inter-day precision				
Sr. No.		Conc.	1st derivative signal	
			ALI (244 nm)	VAL (280.30 nm)
1	Day 1	ALI 10 µg/ml VAL 10.67 µg/ml	0.0078	0.0049
2			0.0079	0.0048
3			0.0077	0.0047
4	Day 2		0.0076	0.0048
5			0.0077	0.0046
6			0.0079	0.0049
7	Day 3		0.0079	0.0048
8			0.0079	0.0048
9			0.0078	0.0048
Mean±SD*			**0.0078±0.0001**	**0.0048±0.0001**
% RSD			**1.3572**	**1.9629**
1	Day 1	ALI 20 µg/ml VAL 21.33 µg/ml	0.0155	0.0092
2			0.0155	0.0093
3			0.0161	0.0093
4	Day 2		0.0162	0.0092
5			0.0163	0.0090
6			0.0158	0.0091
7	Day 3		0.0158	0.0092
8			0.0158	0.0093
9			0.0159	0.0093
Mean±SD*			**0.0159±0.0003**	**0.0092±0.0001**
% RSD			**1.7752**	**1.0142**
Mean % RSD			**1.5662**	**1.4886**

**mean±SD, (n= 3) number of determination*

6.7.8 Accuracy

The results of recovery studies ranged from 98-102% for both the drugs showing the accuracy of the method (Table No. 6.7.6). This indicates that there is no interference from tablet excipients.

Table No. 6.7.6: Results of recovery studies

Accuracy (% Recovery)				
ALI				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard drug added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	6	3	2.9750	99.1667
50	8	4	3.9750	99.3750
50	10	5	4.9750	99.5000
100	6	6	6.1000	101.6667
100	8	8	7.9750	99.6875
100	10	10	9.8500	98.5000
150	6	9	9.1000	101.1111
150	8	12	11.9750	99.7917
150	10	15	14.7250	98.1667
Mean±SD*				**99.6628±1.1223**
% RSD				**1.1261**
VAL				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard drug added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	6.3996	3.1998	3.1004	96.8936
50	8.5328	4.2664	4.2172	98.8468
50	10.6666	5.3333	5.3334	100.0019
100	6.3996	6.3996	6.3504	99.2312
100	8.5328	8.5328	8.7172	102.1611
100	10.6666	10.6666	10.5834	99.2200
150	6.3996	9.5994	9.6004	100.0104
150	8.5328	12.7992	12.4672	97.4061
150	10.6666	15.9999	16.0834	100.5219
Mean±SD*				**99.3659±1.5903**
% RSD				**1.6004**

**mean±SD, (n= 3) number of determination*

6.7.9 LOD and LOQ

The values of LOD and LOQ were found to be very low which proves the sensitivity of the method is shown in Table No. 6.7.8.

6.7.10 Robustness

The proposed method was checked for robustness study, but no significant changes (% RSD<2) found in 1st derivative signal, indicating that the proposed method is robust (Table No. 6.7.7).

Table No. 6.7.7: Results of robustness study

Parameter		Drugs			
Wavelengths (244 & 280.30±1 nm)		ALI		VAL	
ALI	VAL	Assay (%)*	% RSD	Assay (%)*	% RSD
243	279.30	98.5465	**1.5497**	99.1544	**1.5833**
244	280.30	100.5698		101.5475	
245	281.30	97.5641		98.5652	

**mean±SD, (n= 3) number of determination*

6.7.11 Stability of the solution

Solution stability was performed at room temperature and it was found to be stable up to two days.

Table No. 6.7.8: Summary of validation parameters for the proposed method

Parameters	ALI	VAL
Detection wavelengths (nm)	244	280.30
Linearity range (µg/ml)	1-30	1.0666-32
Correlation coefficient	0.9999	0.9996
Regression equation	y = 0.0008x + 0.0005	y = 0.0004x + 0.0003
Precision (%RSD) Repeatability of measurement (n=6) Intra-day (n=3) Inter-day (n=3)	 1.3920 1.4863 1.5662	 1.0055 1.4222 1.4886
Accuracy % Recovery (n=3) %RSD (n=3)	 99.6628±1.1223 1.1261	 99.3659±1.5903 1.6559
Specificity	No interference	
LOD (µg/ml)	0.1132	0.2609
LOQ (µg/ml)	0.3429	0.7906

**mean±SD, n= number of determinations*

6.7.12 Analysis of marketed formulation

The proposed method was successfully used for the quantitative determination of ALI and VAL in commercial formulation (Valturna tablet: 300 mg of ALI and 320 mg of VAL). Six replicate determinations were carried out and experimental values were found to be 98-101% for both the drugs and hence the developed method can be used for the simultaneous determination of both the drugs in combined formulation are presented in

Table No. 6.7.9. Overlain spectra of standard drugs and formulation are showed in Figure No. 6.7.8.

. Table No. 6.7.9: Results of formulation analysis

Drugs	Amount (mg/tablet)		% Drug found*	% RSD
	Labelled	Found		
ALI	300	293.81	97.9385±1.5117	1.5435
VAL	320	313.82	98.0695±1.4984	1.5279

**mean ± SD (n=6) average of six determinations*

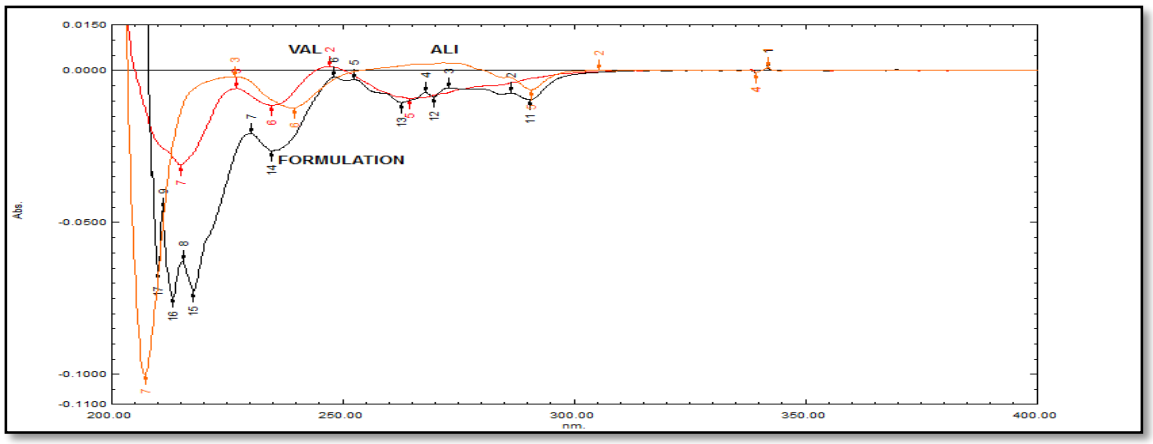


Figure No. 6.7.8: Overlain 1st derivative UV spectra of ALI (10 µg/ml), VAL (10.67 µg/ml) and formulation ALI (10 µg/ml) & VAL (10.67 µg/ml)

Results (% assay) of study suggest that, the proposed method can be successfully applied for the quantitative analysis of ALI and VAL in tablet formulation.

METHOD 8

6.8 *"Development and validation of RP-HPLC method for the simultaneous determination of aliskiren hemifumarate and valsartan in tablets"*

6.8.1 Selection of mode of chromatographic method

Reverse phase chromatography is the first choice for most regular samples. Compared to other form of liquid chromatography, reverse phase chromatography is more convenient and rugged and it produces more satisfactory results in final separation. Reverse phase chromatographic technique was selected since both the drugs are polar in nature.

6.8.2 Selection of column

High performance RPC columns are efficient, stable, reproducible and compatible with wide variety of samples. Moreover, detection of analyte is easier in RPC with UV detector because of the solvents used. Based on the literature survey C_{18} column was selected.

6.8.3 Selection of wavelength

UV spectra of both the drugs were taken in RP-HPLC system and from the overlain spectra, 280 nm was selected as the wavelength for study, Figure No. 6.8.1.

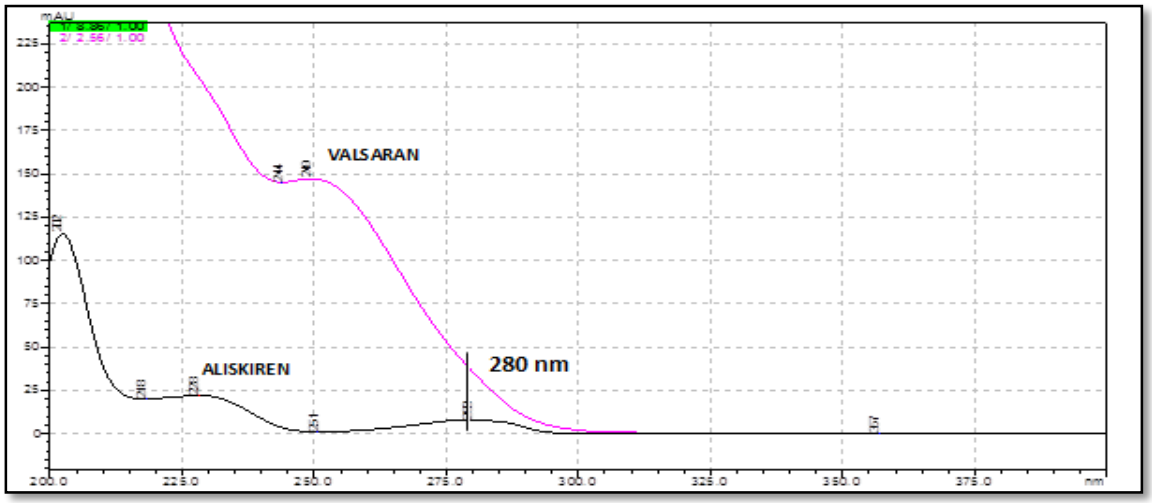


Figure No. 6.8.1: Overlain UV spectra of ALI and VAL on RP-HPLC system

6.8.4 Trials for selection of mobile phase

Based on the literature survey different mobile phases with different compositions were tried and suitable mobile phase was selected for further studies (Table No. 6.8.1, Figure No. 6.8.2-6.8.9).

Initial condition:

Stationary phase	:	Enable C_{18} column (250 × 4.6 mm, 5 µ)
Flow rate	:	1 ml/ minute
Operating temperature	:	Room temperature
Selected wavelength	:	280 nm

Table No. 6.8.1: Trials for selection of mobile phase

Sr. No.	Mobile Phase	Observation	Remarks	Fig. No.
1	0.2% TEA (pH 2.82): Methanol (70:30 %v/v)	Overlapping peaks with no resolution	Not satisfactory	6.8.2
2	0.2% TEA (pH 3): Methanol (20:80 %v/v)	Retention time is same for VAL and FA	Not satisfactory	6.8.3
3	0.2% TEA (pH 3): Methanol (10:90 %v/v)	VAL didn't elute	Not satisfactory	6.8.4
4	0.2% TEA (pH 5): Methanol (10:90 %v/v)	Tailing of VAL	Not satisfactory	6.8.5
5	0.2% TEA (pH 4): Methanol (10:90 %v/v)	Fronting of VAL	Not satisfactory	6.8.6
6	0.2% TEA (pH 6): Methanol (10:90 %v/v)	Retention time is same for VAL and FA	Not satisfactory	6.8.7
7	0.2% TEA (pH 6): Methanol (30:70 %v/v)	Better peak parameters for VAL	Not satisfactory	6.8.8
8	**0.2% TEA (pH 6): Methanol (25:75 %v/v)**	**Optimum peak parameters**	**Satisfactory**	**6.8.9**

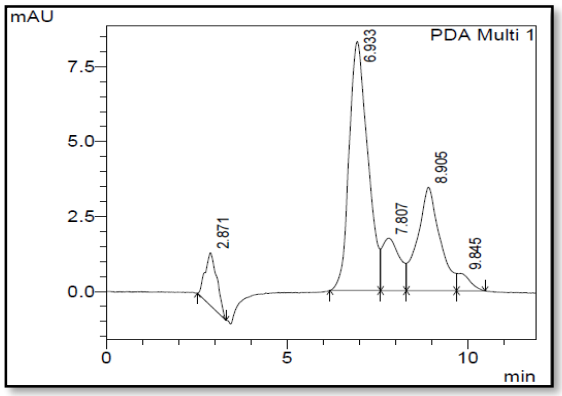


Figure No. 6.8.2: 0.2% TEA (pH 2.82): Methanol (70:30 %v/v)

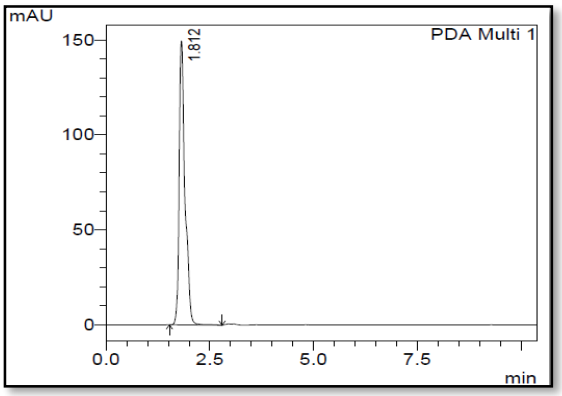


Figure No. 6.8.3: 0.2% TEA (pH 3): Methanol (20:80 %v/v)

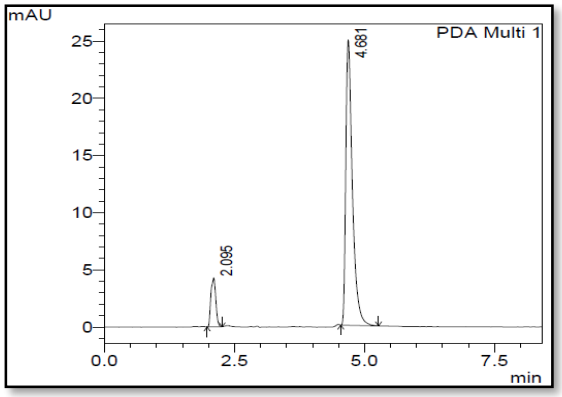


Figure No. 6.8.4: 0.2% TEA (pH 3): Methanol (10:90 %v/v)

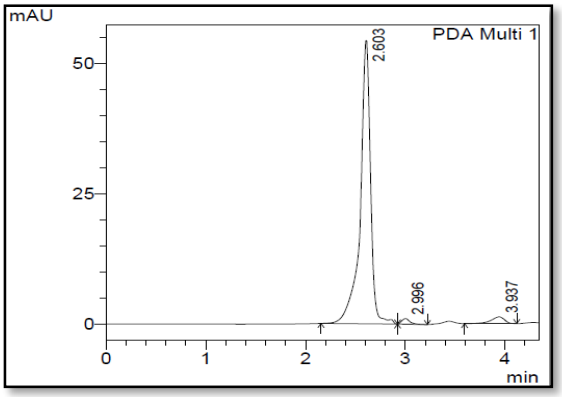


Figure No. 6.8.5: 0.2% TEA (pH 5): Methanol (10:90 %v/v)

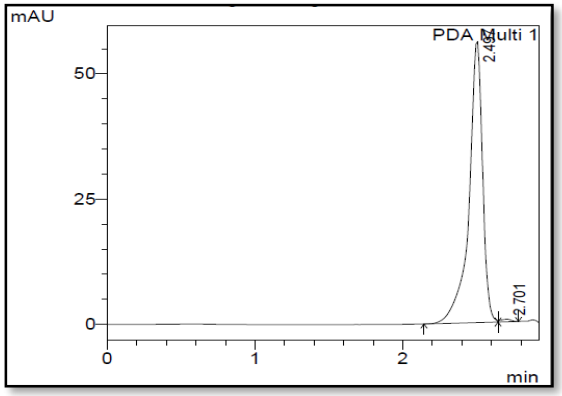


Figure No. 6.8.6: 0.2% TEA (pH 4): Methanol (10:90 %v/v)

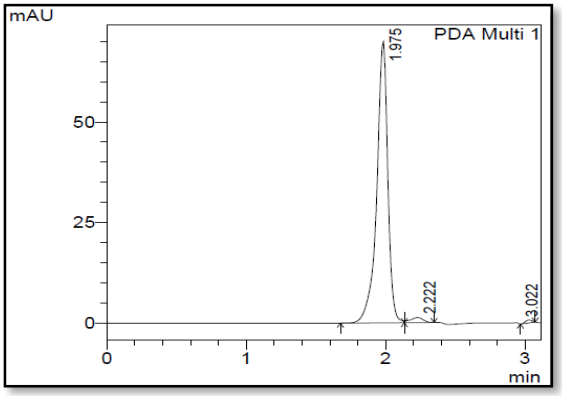


Figure No. 6.8.7: 0.2% TEA (pH 6): Methanol (10:90 %v/v)

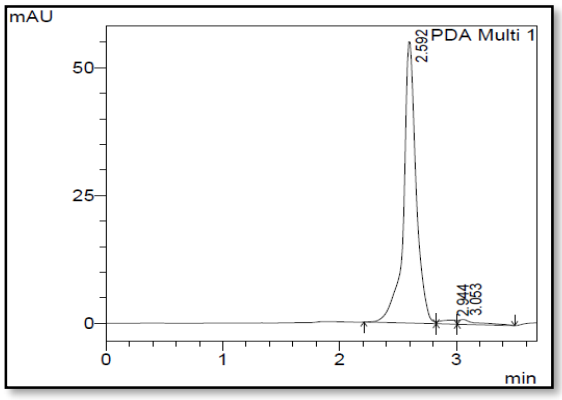


Figure No. 6.8.8: 0.2% TEA (pH 6): Methanol (30:70 %v/v)

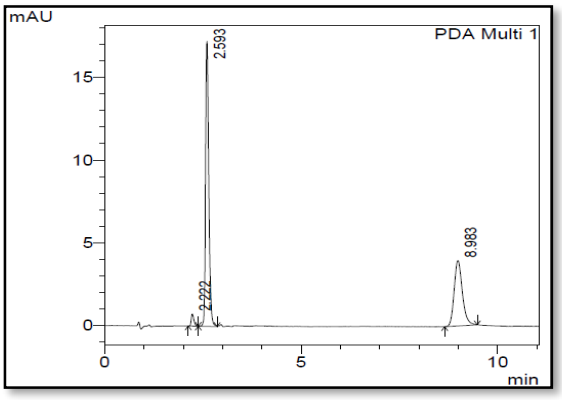


Figure No. 6.8.9: 0.2% TEA (pH 6): Methanol (25:75 %v/v)

6.8.5 Optimization of separation conditions

The chromatographic conditions were optimized to achieve the best resolution, peak shape, theoretical plate for all the analytes under investigation. Initially several proportion of buffer (acetate, phosphate etc.), acetonitrile and methanol were tried to achieve optimum separation of all the analytes under study. Based on the preliminary trials triethylamine in water and methanol in combination was selected for further studies. Strength of buffer (0.1-0.3%), mobile phase composition, pH (3-7), flow rate (0.8-1.2) etc. was varied to get optimum chromatographic conditions which can produce acceptable results based on the peak parameters. Finally the separation of components were achieved on Enable C_{18} column with mobile phase consisting of 0.2% triethylamine in water (pH 6 was adjusted with orthophosphoric acid) and methanol (25:75%v/v) at a flow rate of 1 ml/min was employed with PDA detection at 280 nm which gave satisfactory separation and peak symmetry. The optimized RP-HPLC method was validated and successfully applied for the quantitative determination of ALI and VAL in commercial formulation (Valturna tablet: 300 mg of ALI and 320 mg of VAL).

Fixed chromatographic condition

Stationary phase	**:**	Enable C_{18} column (250 x 4.6 mm, 5 µm, 120 Å)
Mobile phase	**:**	0.2%v/v triethylamine in water (pH 6 with orthophosphoric acid) and methanol
Solvent ratio	**:**	25: 75%v/v
pH	**:**	6
Detection wavelength	**:**	280 nm
Flow rate	**:**	1 ml/ minute
Operating pressure	**:**	150 kgf
Temperature	**:**	Room temperature

The retention time of ALI and VAL were found to be 8.877 and 2.558 min, respectively, are shown in Figure No. 6.8.10.

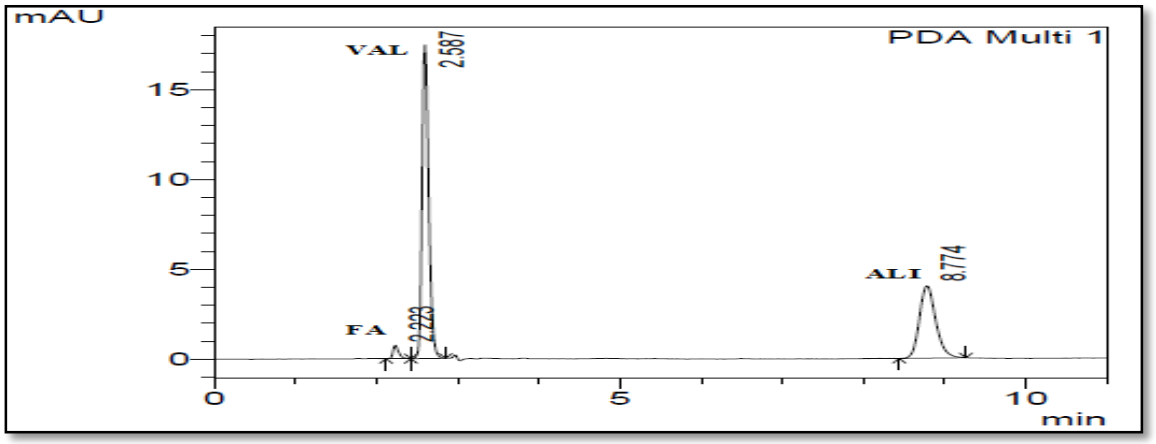


Figure No. 6.8.10: RP-HPLC chromatogram of ALI (10 µg/ml) and VAL (10.67 µg/ml)

6.8.6 Validation of the method

The developed method was validated in accordance with "*International Conference on Harmonization"* guidelines for validation of analytical procedures.

6.8.7 Specificity

No interfering peaks were found within the stipulated run time, which shows the specificity of the method (Figure No. 6.8.11).

6.8.8 Linearity and range

ALI and VAL were found to be linear in the concentration range of 0.50-30 µg/ml and 0.53-32 µg/ml, respectively (Table No. 6.8.2; Figure No. 6.8.15-6.8.21). Calibration curves (Figure No. 6.4.22 & 6.4.23) were plotted using peak area of standard drug versus concentration. Results were subjected to regression analysis by the least squares method to calculate the values of slope, intercept and correlation coefficient (Table No. 6.8.8).

Table No. 6.8.2: Data for calibration curve (ALI: 0.5-30 & VAL: 0.53-32 µg/ml)

Sr. No.	ALI			VAL		
	Conc. (µg/ml)	Peak Area*	% RSD	Conc. (µg/ml)	Peak Area*	% RSD
1	0.5	3017.33	1.3743	0.53	5856.83	0.6339
2	5	26982.50	1.2433	5.33	47457.17	0.8803
3	10	56915.67	0.9650	10.67	97726.50	0.5548
4	15	81873.33	0.7220	16.00	143333.33	0.5898
5	20	113065.17	0.9889	21.33	193897.50	0.4186
6	25	139176.00	1.6622	26.67	242327.00	0.6973
7	30	165990.67	0.7776	32.00	293742.83	1.0637

**(n=6) average of six determinations*

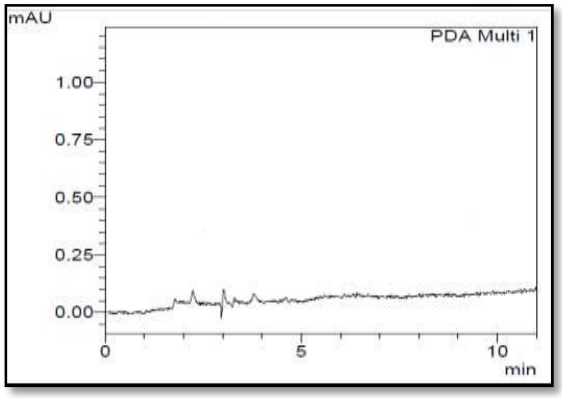


Figure No. 6.8.11: Chromatogram of tablet excipients at 280 nm

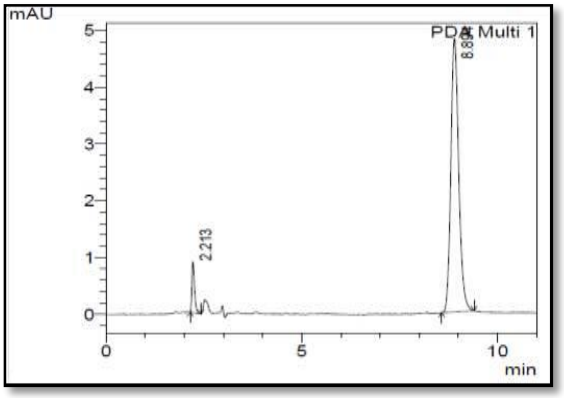


Figure No. 6.8.12: Standard chromatogram of ALI (10 µg/ml) at 280 nm

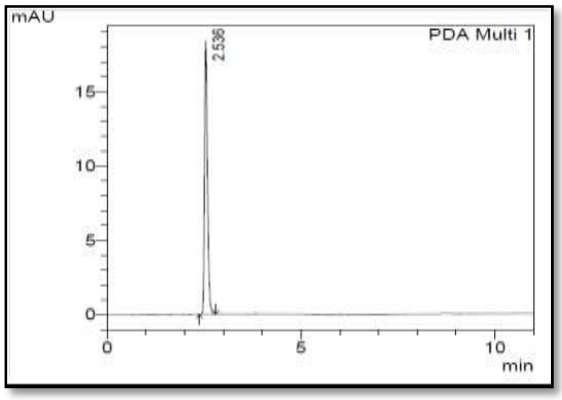


Figure No. 6.8.13: : Standard chromatogram of VAL (10.67 µg/ml) at 280 nm

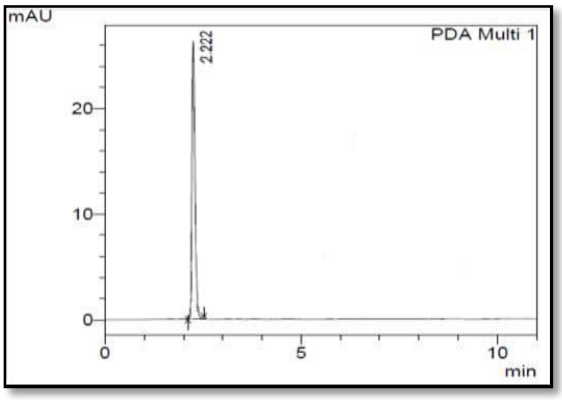


Figure No. 6.8.14: Standard chromatogram of FA at 280 nm

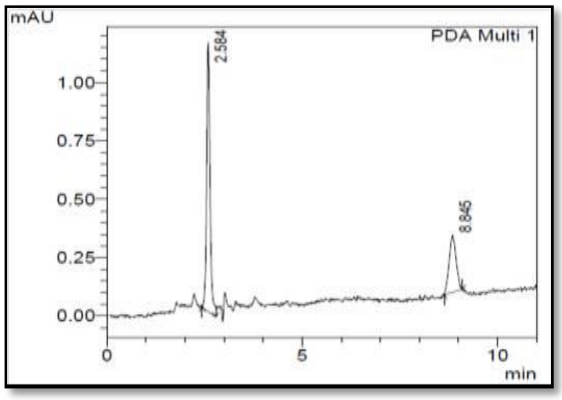


Figure No. 6.8.15: Standard chromatogram of ALI (0.5 µg/ml) & VAL (0.53 µg/ml) at 280 nm

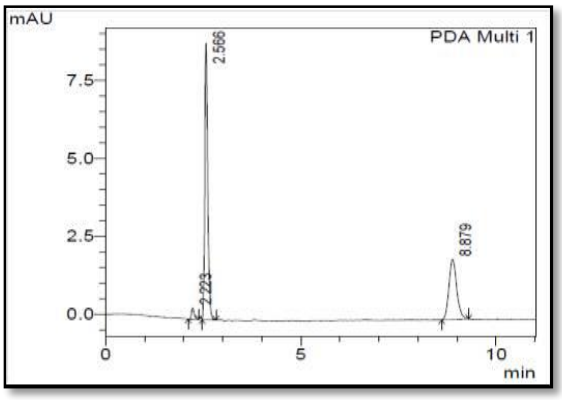


Figure No. 6.8.16: Standard chromatogram of ALI (5 µg/ml) & VAL (5.33 µg/ml) at 280 nm

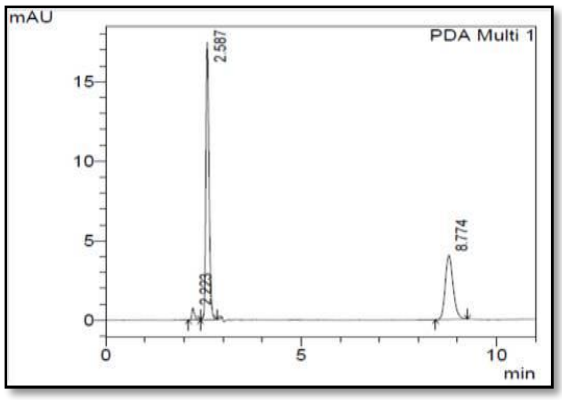


Figure No. 6.8.17: Standard chromatogram of ALI (10 µg/ml) & VAL (10.67 µg/ml) at 280 nm

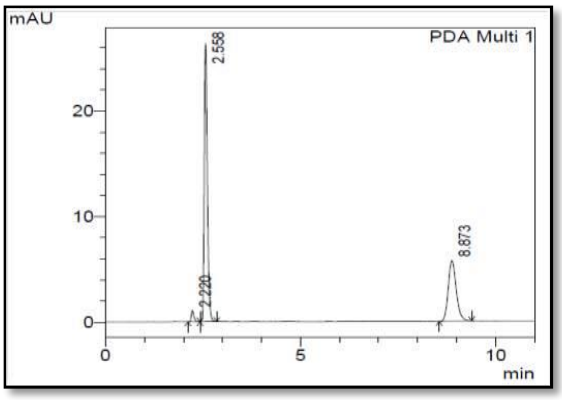


Figure No. 6.8.18: Standard chromatogram of ALI (15 µg/ml) & VAL (16 µg/ml) at 280 nm

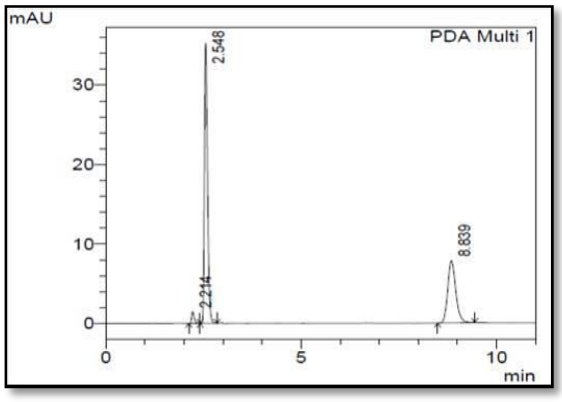


Figure No. 6.8.19: Standard chromatogram of ALI (20 µg/ml) & VAL (21.33 µg/ml) at 280 nm

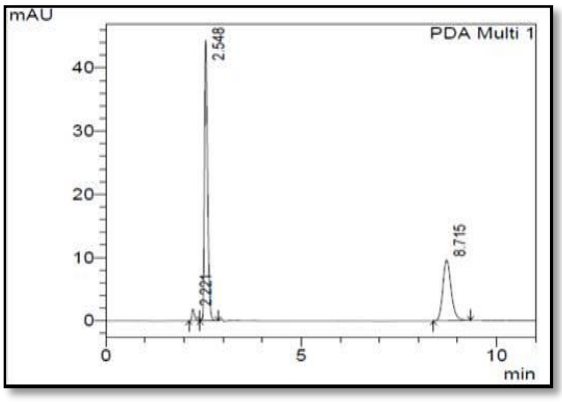


Figure No. 6.8.20: Standard chromatogram of ALI (25 µg/ml) & VAL (26.67 µg/ml) at 280 nm

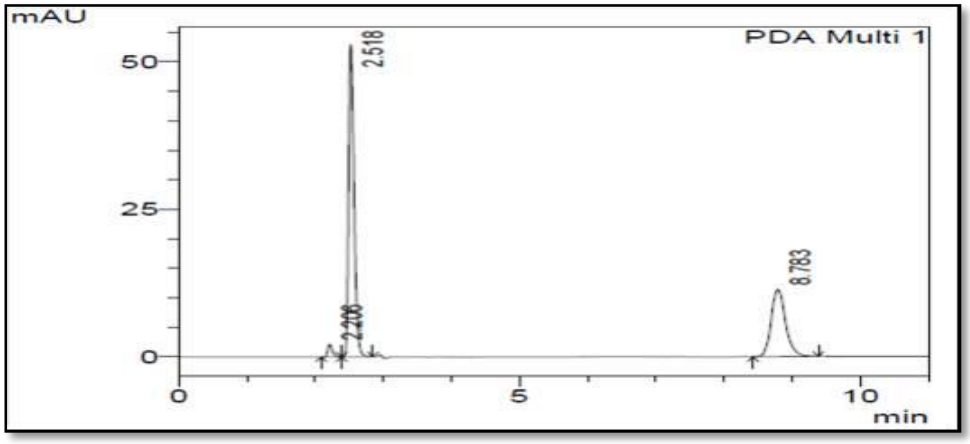


Figure No. 6.8.21: Standard chromatogram of ALI (30 µg/ml) & VAL (32 µg/ml) at 280 nm

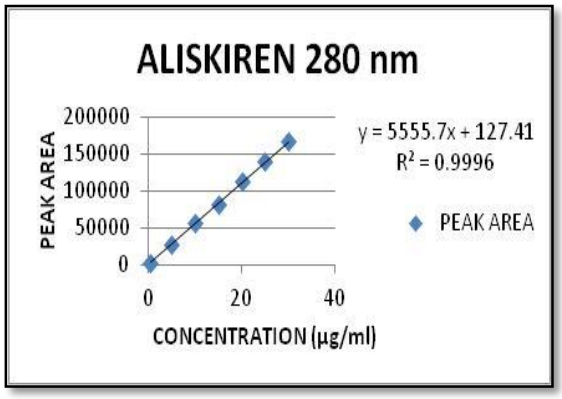


Figure No. 6.8.22: Standard calibration graph of ALI (0.5-30 µg/ml) at 280 nm

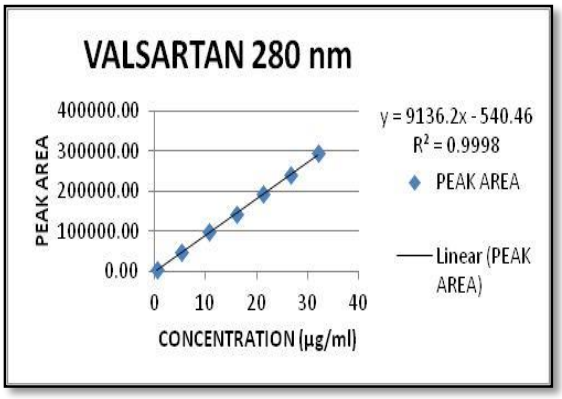


Figure No. 6.8.23: Standard calibration graph of VAL (0.53-32 µg/ml) at 280 nm

6.8.9 Precision

The precision of the method was checked by carrying out repeatability, intra-day and inter-day precision. Results of precision studies expressed in %RSD follows ICH guideline acceptable limits (% RSD<2), which shows good repeatability and low inter-day variability, indicating an excellent precision of the developed method (Table No. 6.8.3, 6.8.4 & 6.8.5).

Table No. 6.8.3 Results of repeatability of injection

Repeatability								
Sr. No.	ALI				VAL			
	Conc. (µg/ml)	Peak area	Mean ± SD	% RSD	Conc. (µg/ml)	Peak area	Mean ± SD	% RSD
1	5	26607	26756.50 ± 247.8780	0.9264	5.33	47453	47436.67 ± 756.5497	1.5949
2		26384				47434		
3		27035				48757		
4		26749				47564		
5		27018				46837		
6		26746				46575		
1	10	56349	57057.83 ± 534.6264	0.9370	10.67	97635	97922.67 ± 450.1736	0.4597
2		57038				98464		
3		56784				97363		
4		57493				98464		
5		57842				97763		
6		56841				97847		
Mean % RSD				**0.9317**	**Mean % RSD**			**1.0273**

**mean±SD, (n= 6) number of determination*

Table No. 6.8.4: Results of intra-day precision

Intra-day precision								
Sr. No.	ALI				VAL			
	Conc. (µg/ml)	Peak area	Mean ± SD	% RSD	Conc. (µg/ml)	Peak area	Mean ± SD	% RSD
1	5	26758	27039.11 ± 231.2166	0.8551	5.33	47636	47960.33 ± 581.4377	1.2123
2		27084				47464		
3		27094				48464		
4		26749				47464		
5		26859				48746		
6		27094				48565		
7		27048				47363		
8		27173				48477		
9		27493				47464		

1	10	56831	56962.33 ± 389.1818	0.6832	10.67	97846	97974.44 ± 649.4837	0.6629
2		57048				98474		
3		57493				97488		
4		56748				96773		
5		57489				98373		
6		57264				97353		
7		56839				98737		
8		56472				98363		
9		56477				98363		
Mean % RSD				**0.7692**	**Mean % RSD**			**0.9376**

**mean±SD, (n= 3) number of determination*

Table No. 6.8.5: Results of inter-day precision

Inter-day precision									
Sr. No.		**ALI**				**VAL**			
		Conc. (µg/ml)	**Peak area**	**Mean ± SD**	**% RSD**	**Conc. (µg/ml)**	**Peak area**	**Mean ± SD**	**% RSD**
1	**1**	5	26785	26888.44 ± 300.2408	1.1166	5.33	47645	48116.22 ± 569.4587	1.1835
2			27127				47464		
3			27463				47575		
4	**2**		26874				48747		
5			26564				48747		
6			26754				48373		
7	**3**		26473				47444		
8			27053				48577		
9			26903				48474		
1	**1**	10	56473	56917.56 ± 531.4843	0.9338	10.67	97343	97952.89 ± 543.8356	0.5552
2			56473				98535		
3			57038				97363		
4	**2**		57163				97464		
5			56483				98365		
6			56273				97365		
7	**3**		57027				98435		
8			57492				98343		
9			57836				98363		
Mean % RSD					**1.0252**	**Mean % RSD**			**0.8693**

**mean±SD, (n= 3) number of determination*

6.8.10 Accuracy

The results of recovery studies ranged from 99-101% for both the drugs showing the accuracy of the method (Table No. 6.8.6). This indicates that there is no interference from tablet excipients.

Table No. 6.8.6: Results of recovery studies

Accuracy (% Recovery)				
ALI				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard drug added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	4	2	2.0137	100.6875
50	6	3	2.9668	98.8918
50	8	4	4.0094	100.2348
100	4	4	4.0659	101.6469
100	6	6	6.0409	100.6816
100	8	8	8.0908	101.1348
150	4	6	6.0395	100.6588
150	6	9	8.8884	98.7602
150	8	12	12.1432	101.1934
Mean±SD				**100.4322±0.9959**
% RSD				**0.9916**
VAL				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard drug added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	4.2664	2.1332	2.1631	101.4034
50	6.3996	3.1998	3.2041	100.1351
50	8.5328	4.2664	4.2067	98.6005
100	4.2664	4.2664	4.2527	99.6798
100	6.3996	6.3996	6.3181	98.7266
100	8.5328	8.5328	8.4133	98.5990
150	4.2664	6.3996	6.3445	99.1394
150	6.3996	9.5994	9.5260	99.2355
150	8.5328	12.7992	12.7351	99.4990
Mean±SD				**99.4465±0.8980**
% RSD				**0.9030**

**mean±SD, (n=3) number of determination*

6.8.11 LOD and LOQ

The values of LOD and LOQ were found to be very low which proves the sensitivity of the method is shown in Table No. 6.8.8.

6.8.12 Robustness

The proposed method was checked through all the parameters described earlier under robustness studies. But there were no considerable variations in the chromatographic pattern after introducing small changes in experimental condition, indicates that the developed method is robust (Table No. 6.8.7).

The proposed method was checked through all parameters described earlier under robustness studies, but no significant changes (% RSD <2) found in retention time, peak area or symmetry of the peaks (Table No. 6.8.7).

Table No. 6.8.7: Results of robustness study

Sr. No.	Modification	ALI (10 µg/ml)		VAL (10.67 µg/ml)	
		R_t	Peak area	R_t	Peak area
1	Organic phase (75 ± 2%v/v)	8.725	57125	2.492	97869
		8.845	56858	2.551	98156
		8.912	55639	2.562	96546
% RSD (<2)		**1.0733**	**1.4011**	**1.4849**	**0.8806**
2	Strength of buffer (0.2 ± 0.1%v/v)	8.835	57124	2.529	98124
		8.851	56458	2.546	97698
		8.911	56975	2.582	99668
% RSD (<2)		**0.4519**	**0.6148**	**1.0603**	**1.0523**
3	Effect of pH (6 ± 0.2 unit)	8.815	57544	2.493	97587
		8.852	57124	2.542	98245
		8.916	56354	2.583	98769
% RSD (<2)		**0.5767**	**1.0587**	**1.7745**	**0.6031**
4	Effect of flow rate (1 ± 0.1 ml/min)	9.024	58124	2.587	98457
		8.861	56987	2.536	97845
		8.751	57254	2.495	96985
% RSD (<2)		**1.5470**	**1.0348**	**1.8151**	**0.7564**

**mean±SD, (n= 3) number of determination*

6.8.13 Stability of the solution

Solution stability was performed at room temperature and it was found to be stable up to two days.

Table No. 6.8.8: Summary of validation parameters for the proposed RP-HPLC

Parameters	ALI	VAL
Linearity range (µg/ml)	0.5-30	0.5333-32
Correlation coefficient	0.9996	0.9998
Regression equation	y = 5555.7x + 127.41	y = 9136.2x - 540.46
Precision (%RSD)		
Repeatability of injection (n=6)	0.9317	1.0273
Intra-day (n=3)	0.7692	0.9376
Inter-day (n=3)	1.0252	0.8693
*Accuracy**		
% Recovery (n=3)	100.4322±0.9959	99.4465±0.8980
%RSD (n=3)	0.9221	0.9030
Specificity	No interference	
LOD (µg/ml)	0.1461	0.1627
LOQ (µg/ml)	0.4426	0.4930

**mean±SD, n= number of determinations*

6.8.14 System suitability test

System suitability tests were performed and results showed that the parameters tested were within the acceptable limit as per the ICH guidelines, indicating that the developed method is suitable for the analysis to be performed (Table No. 6.8.9).

Table No. 6.8.9: Results of system suitability studies

Parameters	Drugs		Acceptance criteria
	ALI*	VAL*	
Peak area reproducibility %RSD	57267.96±657.82 1.1487	97160.71±1489.27 1.5328	**% RSD< 2**
Retention time (Rt) min %RSD	8.815±0.0642 0.4975	2.558±0.0229 0.8942	**% RSD< 2**
Resolution (Rs)	22.78±0.3370	2.26±0.0357	**>2**
Theoretical plate (N)	8688±162	4001±62	**>2000**
Tailing factor (T)	1.236±0.0109	1.472±0.0249	**< 2**

**mean±SD (n=6) observation of six determinations*

6.8.15 Analysis of marketed formulation

The proposed method was successfully used for the quantitative determination of ALI and VAL in commercial formulation (Valturna tablet: 300 mg ALI and 320 mg of VAL). Six replicate determinations were carried out and experimental values were found to be within 99-101% for both the drugs are presented in Table No. 6.8.10. Chromatogram of formulation is shown in Figure No. 6.8.24.

Table No. 6.8.10: Results of formulation analysis

Drugs	Amount (mg/tablet)		% Drugs found*	% RSD
	Labelled	Found		
ALI	300	299.64	99.8809±0.9389	0.9400
VAL	320	319.86	99.9554±0.5028	0.5030

**mean ± SD (n=6) average of six determinations*

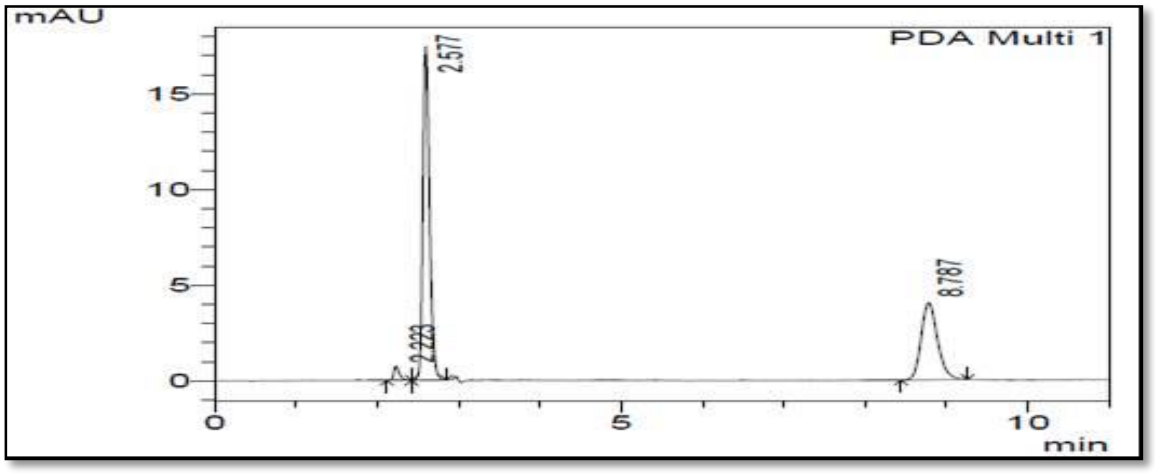


Figure No. 6.8.24: Chromatogram of formulation (ALI 10 µg/ml & VAL 10.67 µg/ml) at 280 nm

Result (% assay) of study suggests that, the proposed method can be applied for the quantitative analysis of ALI and VAL in tablet formulation.

METHOD 9

6.9 *"Development and validation of HPTLC method for the simultaneous determination of aliskiren hemifumarate and valsartan in tablets"*

A simple, precise, rapid and highly sensitive method was developed and validated for the simultaneous estimation of ALI and VAL in tablets using HPTLC. The advantage of HPTLC is that large no of samples can be analyzed simultaneously in a shorter time period, less solvent and sample is required for the analysis. Moreover, less cost per analysis, low maintenance cost and ease of sample preparation makes this technique superior to other type of chromatography.

6.9.1 Selection of mode of chromatographic method

Based on the literature survey pre-coated silica gel $60F_{254}$ on aluminium sheets were selected for study.

6.9.2 Selection of solvent

Based on the literature survey and solubility study, methanol was selected as solvent for further studies.

6.9.3 Selection of wavelength

UV spectra of drugs on pre-coated plates (previously spotted with drugs and developed) were recorded and from the overlain spectra 281 nm was selected as wavelength of detection, Figure No. 6.9.1.

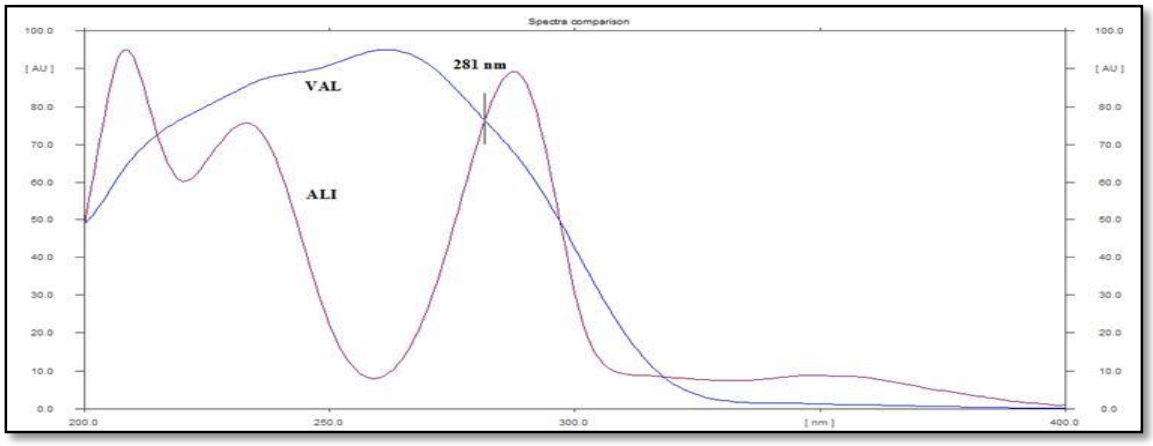


Figure No. 6.9.1: Overlain UV spectra of ALI and VAL on pre-coated TLC plate

6.9.4 Trials for selection of mobile phase

Initially different solvents like chloroform, methanol, acetonitrile, toluene, ethyl acetate, diethyl ether, propanol etc. were used as individual solvent to develop TLC plates (previously spotted with a fixed concentration of both the drugs). Moreover, extensive literature survey was carried out to get information about previously reported methods of other drugs. Finally based on the literature survey and preliminary trials, different mobile phases with different compositions were tried and suitable mobile phase was selected for further studies **(**Table No. 6.9.1).

Table No. 6.9.1: Trials for selection of mobile phase

Sr. No.	Mobile Phase	Observation	Remarks
1	Chloroform: methanol: toluene (5:2:2 v/v/v)	No separation between ALI and FA and tailing	Not satisfactory
2	Chloroform: methanol: toluene: glacial acetic acid (6:2:1:0.1 v/v/v/v)	Tailing	Not satisfactory
3	Methanol: ethyl acetate: glacial acetic acid (1:9:0.5 v/v/v)	No separation between ALI and FA	Not satisfactory
4	Methanol: toluene: ethyl acetate: glacial acetic acid (4:2:3:0.1 v/v/v/v)	ALI and FA separated with tailing	Not satisfactory
5	Methanol: toluene: ethyl acetate: glacial acetic acid (4:2:3:0.2 v/v/v/v)	Less separation with tailing	Not satisfactory
6	Methanol: toluene: ethyl acetate: formic acid (4:2:3:0.1 v/v/v/v)	ALI and FA separated with tailing	Not satisfactory
7	Methanol: toluene: ethyl acetate: acetonitrile: glacial acetic acid (3:4:4:2:0.6 v/v/v/v/v)	Spots were not compact	Not satisfactory
8	Chloroform: methanol: ethyl acetate: toluene: glacial acetic acid (2:2:4:1:0.5 v/v/v/v/v)	Spots were not compact and R_f value of VAL was more than 0.9	Not satisfactory
9	Chloroform: methanol: ethyl acetate: toluene: ammonia: glacial acetic acid (3:3:2:2:0.1:0.1 v/v/v/v/v/v)	Separation was less between ALI and FA	Not satisfactory
10	**Chloroform: methanol: ethyl acetate: toluene: ammonia: glacial acetic acid (3:3:2:2:0.25:0.44 v/v/v/v/v/v)**	**Good separation with symmetrical peaks**	**Satisfactory**

6.9.5 Optimization of separation conditions

The chromatographic conditions were optimized with a view to develop a simultaneous assay method for ALI and VAL. The separations of components were achieved by spotting mixed standard solution on TLC plates (Pre-coated silica gel $60F_{254}$ on aluminium sheets) and run in different individual solvents. Based on the results obtained from initial trials mobile phase system consisting of chloroform: methanol: ethyl acetate: toluene: ammonia: glacial acetic acid was selected because in this system good compact and dense spots were obtained. Different ratios of chloroform: methanol: ethyl acetate: toluene: ammonia: glacial acetic acid like 4:2:2:2:0.25:0.44, 2:4:2:2:0.25:0.44, 3:3:3:1:0.25:0.44, 3:3:1:3:0.25:0.44 v/v/v/v/v/v etc. were tried and the ratio of 3:3:2:2:0.25:0.44 v/v/v/v/v/v was selected because it gave compact spots and good resolution between analytes, good separation from solvent front and sample application position. Moreover, fumaric acid was well separated from ALI and VAL. Other parameters like saturation time, development distance, volume of mobile phase, detection wave length, activation time etc. were varied and optimized to get reproducible Rf values, better resolution, symmetrical peak shape for all the components including fumaric acid, which is separating from ALI hemifumarate. All the components were scanned at 281 nm and reproducible Rf values were found to be 0.5711±0.0078, 0.7911±0.0136 and 0.2843±0.0053 for ALI, VAL and FA, respectively, are shown in Figure No. 6.9.2.

6.9.6 Fixed chromatographic conditions

Stationary Phase	:	Pre-coated silica gel $60F_{254}$ on aluminium sheets
Mobile phase	:	Chloroform: methanol: ethyl acetate: toluene: ammonia: glacial acetic acid (3:3:2:2:0.25:0.44 v/v/v/v/v/v)
Chamber saturation	:	20 minutes
Migration distance	:	80 mm
Band width	:	6 mm
Slit dimension	:	6 X 0.45 mm
Source of radiation	:	Deuterium lamp
Scanning wavelength	:	281 nm

R_f values	:	
Aliskiren	:	0.5711±0.0078
Valsartan	:	0.7911±0.0136
Fumaric acid	:	0.2843±0.0053

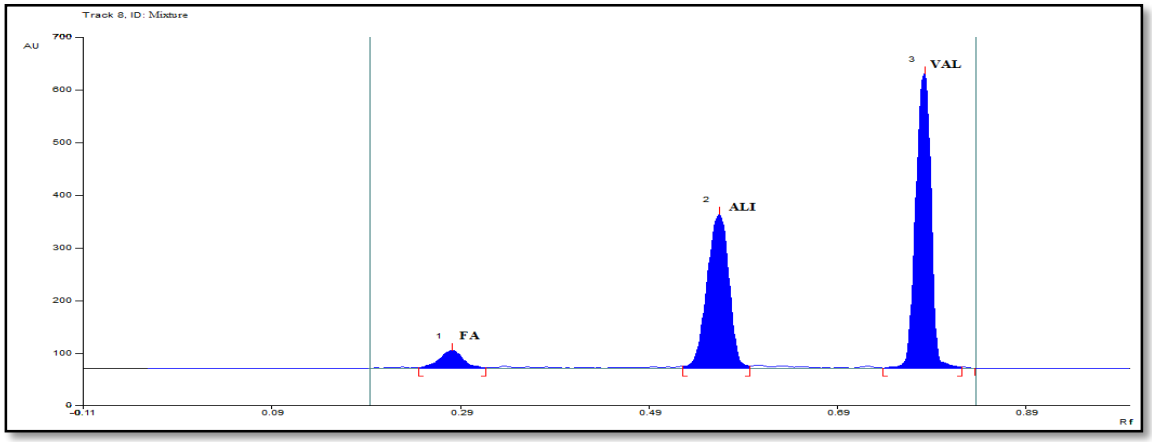


Figure No. 6.9.2: Chromatogram of standard mixture of ALI (400 ng/band) and VAL (426.8 ng/band)

6.9.7 Validation of the method

The developed method was validated in accordance with "*International Conference on Harmonization"* guidelines for validation of analytical procedures.

6.9.8 Specificity

The peak purity of both the drugs ALI and VAL was assessed by comparing their respective spectra at peak start, peak apex and peak end positions of the spot. The good correlation among spectra acquired at start (s), apex (m) and end (e) of the peaks indicatives of peak purity for both ALI {correlation r(s, m) = 0.9999, r (m, e) = 0.9998} and VAL {correlation r(s, m) = 0.9999, r(m, e) = 0.9994}. It can be concluded that no impurities or degradation products migrated with the peaks obtained from standard solutions of the drugs.

6.9.9 Linearity and range

ALI and VAL were found to be linear in the concentration range of 50-1000 ng/band and 53.33-1067 ng/band, respectively (Table No. 6.9.2; Figure No. 6.9.8-6.9.14). Linearity of

the method was established by plotting standard calibration curves (Figure No. 6.9.15 and 6.9.16) using peak area versus concentration (ng/band). Results were subjected to regression analysis by the least squares method to calculate the values of slope, intercept and correlation coefficient **(**Table No. 6.9.8).

Table No. 6.9.2: Linearity data of ALI (50-1000 ng/band) and VAL (53.33-1067 ng/band) at 281 nm

Sr. No.	ALI*		VAL*	
	Concentration (ng/band)	Peak Area	Concentration (ng/band)	Peak Area
1	50	1222.92	53.33	1061.42
2	100	2025.47	106.67	1925.70
3	200	3123.55	213.33	3499.60
4	400	5614.82	426.67	7461.20
5	600	7813.48	640.00	11362.13
6	800	10210.08	853.33	16029.42
7	1000	12584.85	1066.67	20041.30

**(n=6) Average of six determinations*

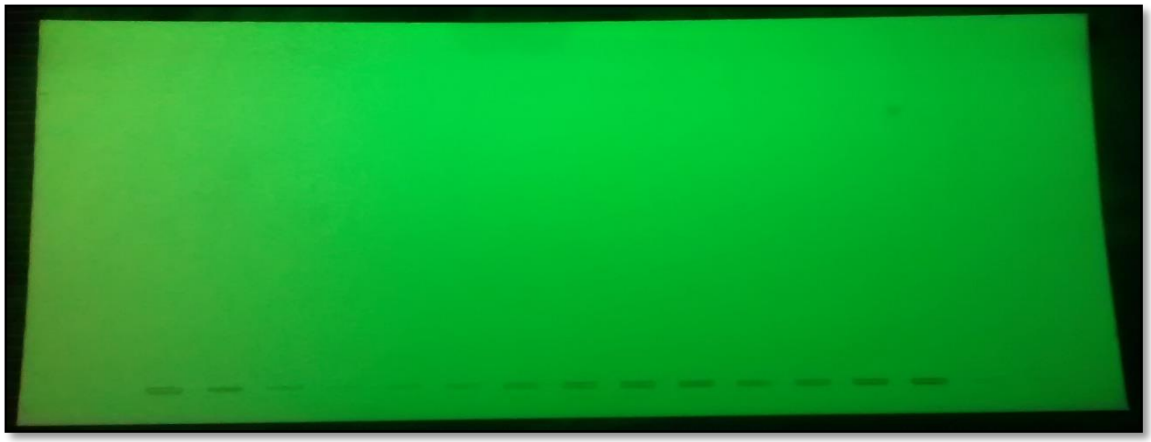


Figure No. 6.9.3: Spotted HPTLC plate for linearity study

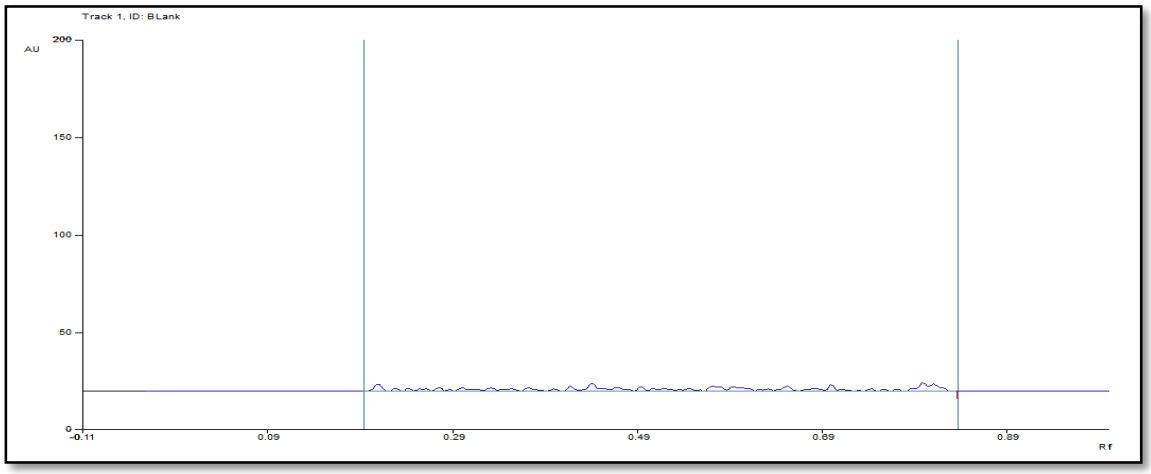


Figure No. 6.9.4: Chromatogram of methanol (diluent/blank) at 281 nm

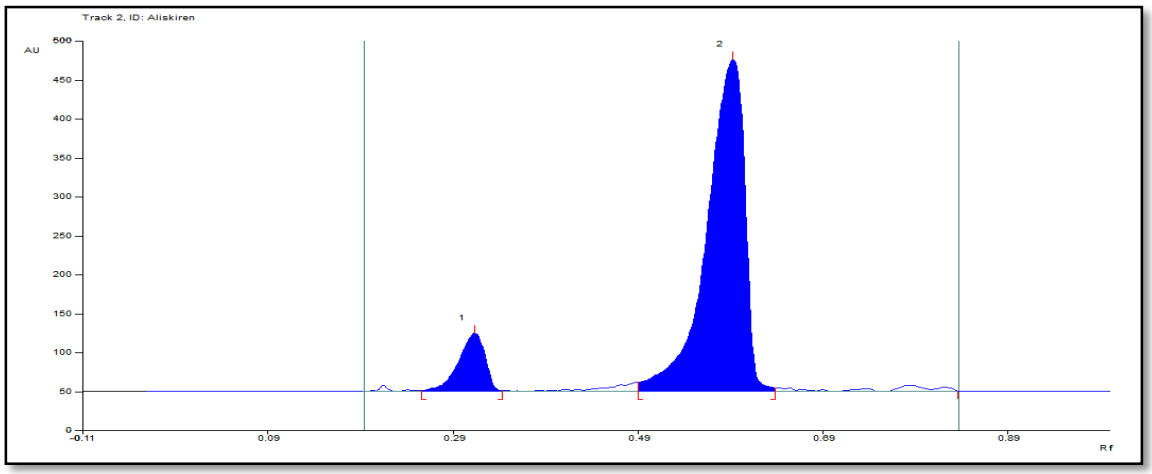


Figure No. 6.9.5: Standard chromatogram of ALI (1000 ng/band) at 281 nm

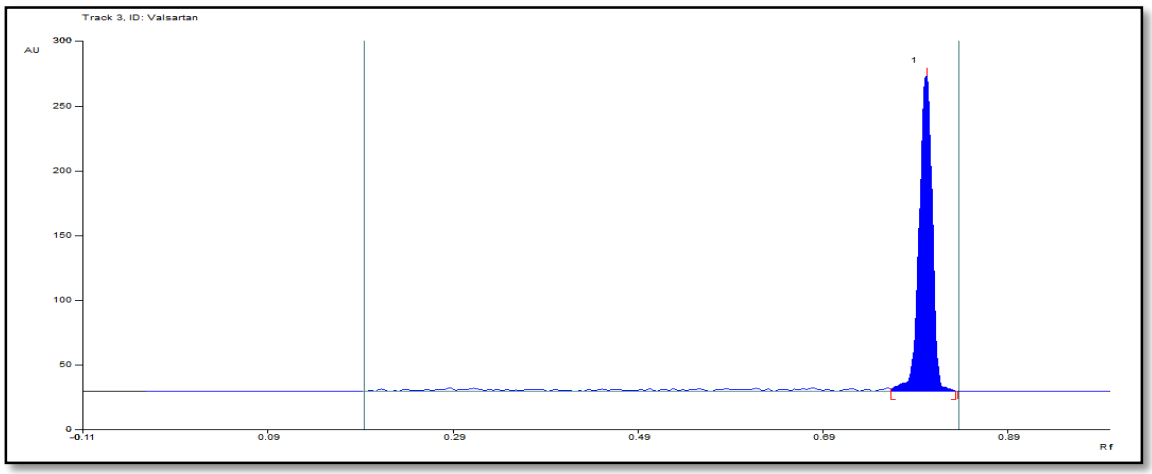


Figure No. 6.9.6: Standard chromatogram of VAL (1067 ng/band) at 281 nm

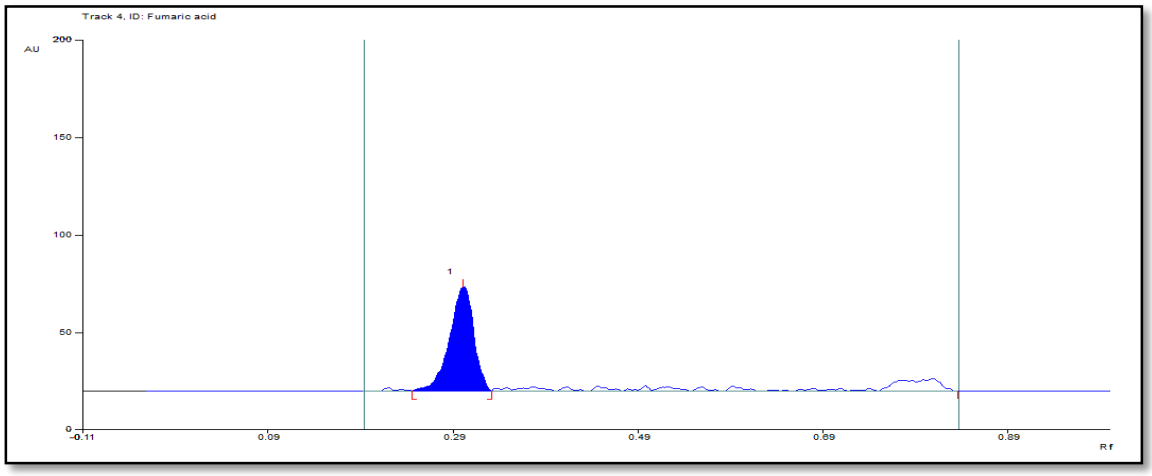


Figure No. 6.9.7: Standard chromatogram of benzoic acid (500 ng/band) at 281 nm

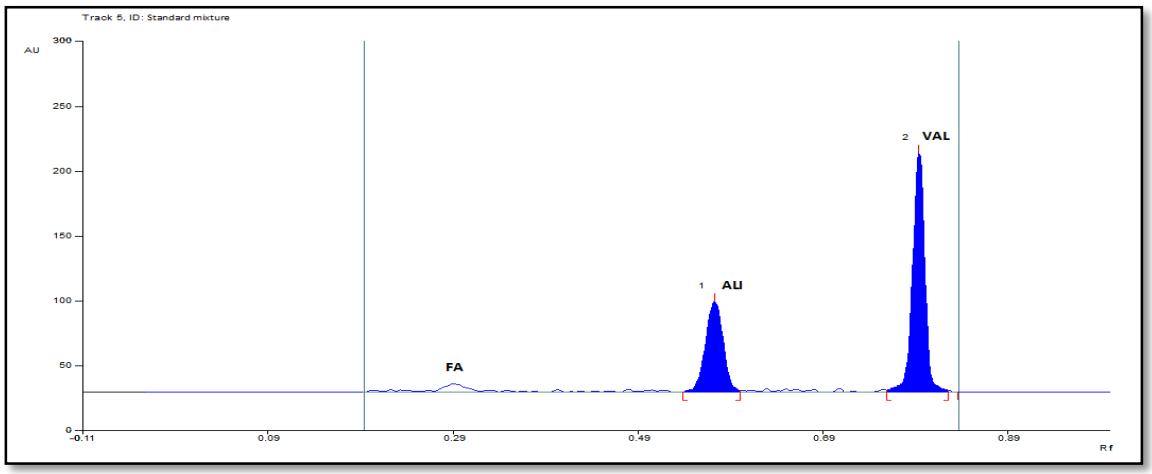


Figure No. 6.9.8: Standard chromatogram of ALI (50 ng/band) and VAL (53.33 ng/band

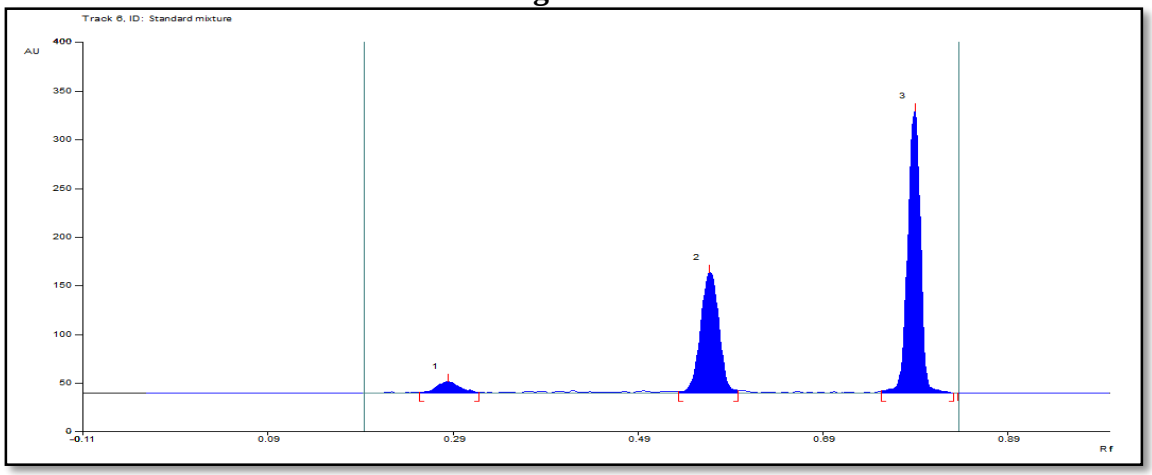


Figure No. 6.9.9: Standard chromatogram of ALI (100 ng/band) and VAL (106.67 ng/band

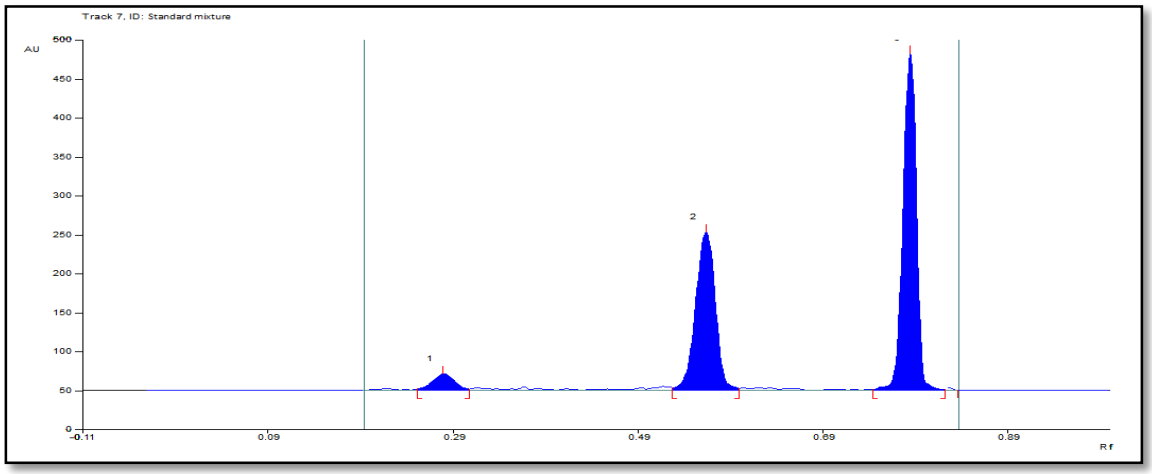


Figure No. 6.9.10: Standard chromatogram of ALI (200 ng/band) and VAL (213.33 ng/band)

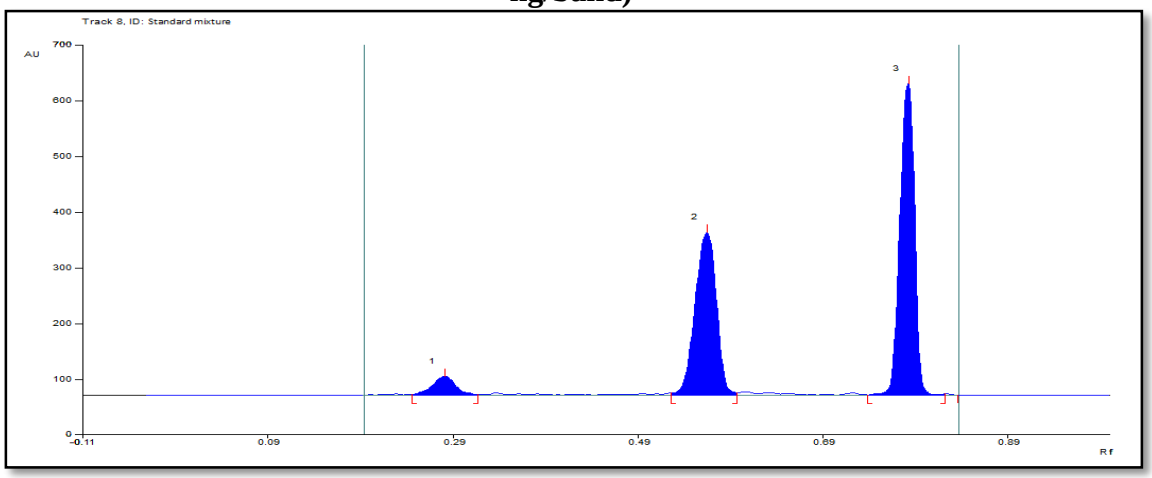


Figure No. 6.9.11: Standard chromatogram of ALI (400 ng/band) and VAL (426.67 ng/band)

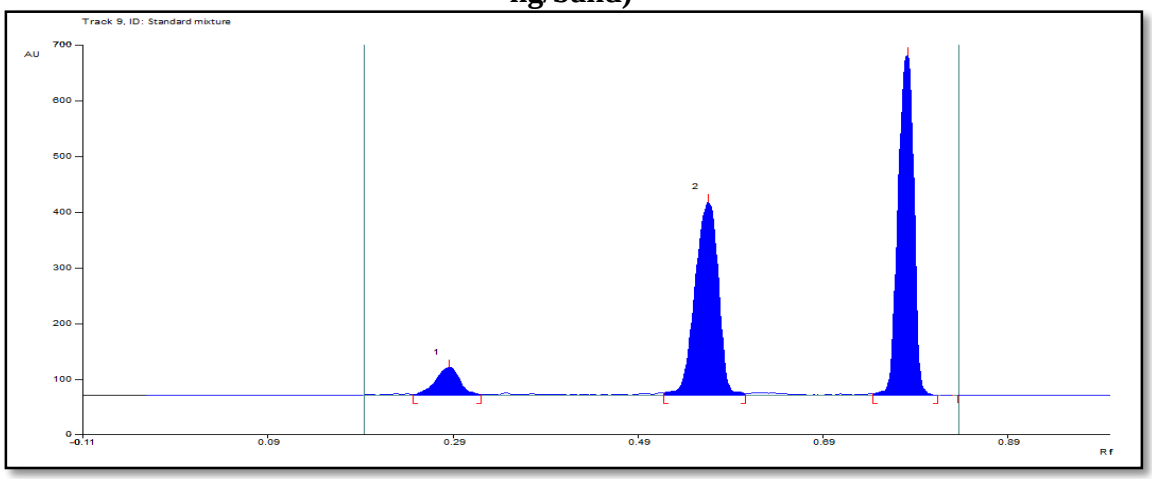


Figure No. 6.9.12: Standard chromatogram of ALI (600 ng/band) and VAL (640 ng/band)

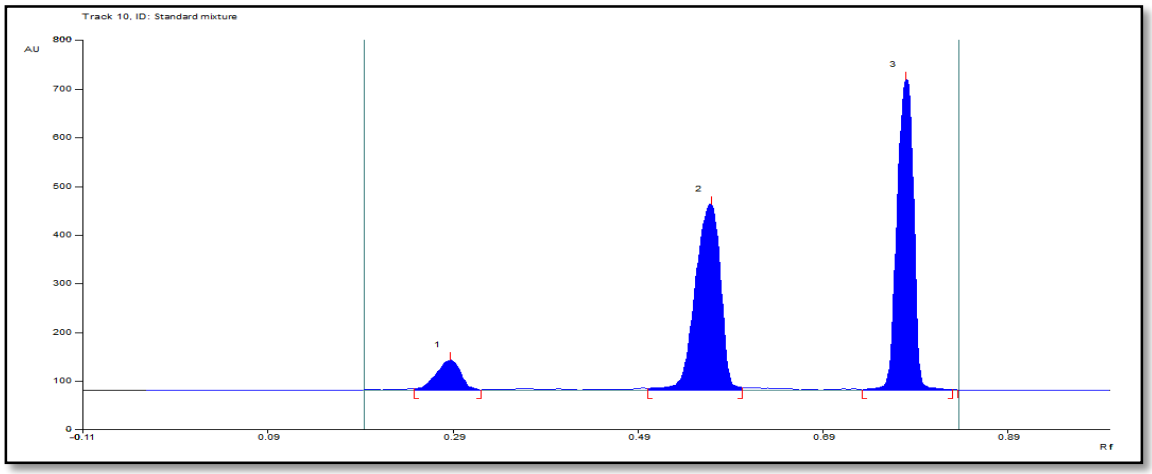


Figure No. 6.9.13: Standard chromatogram of ALI (800 ng/band) and VAL (853.33 ng/band)

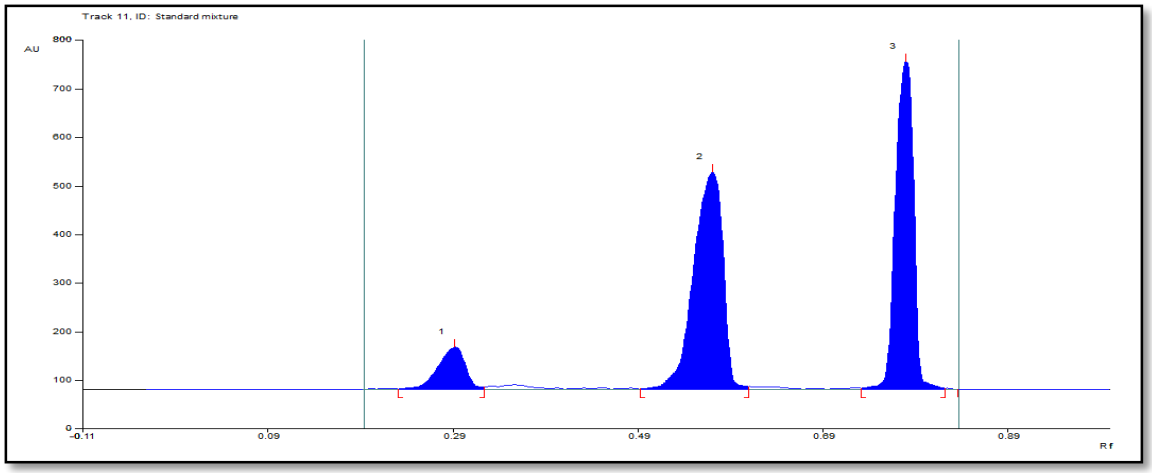


Figure No. 6.9.14: Standard chromatogram of ALI (1000 ng/band) and VAL (1067 ng/band)

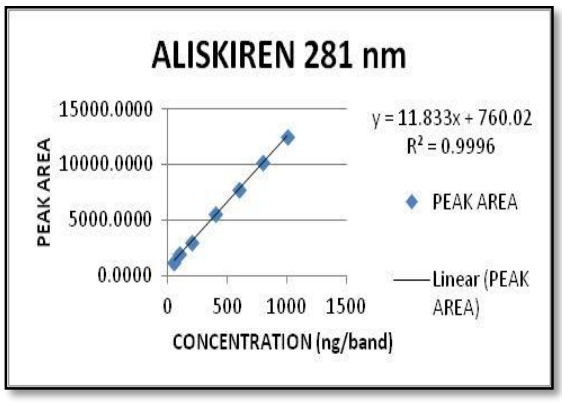


Figure No. 6.9.15: Standard calibration graph of ALI (50-1000 ng/band) at 281 nm

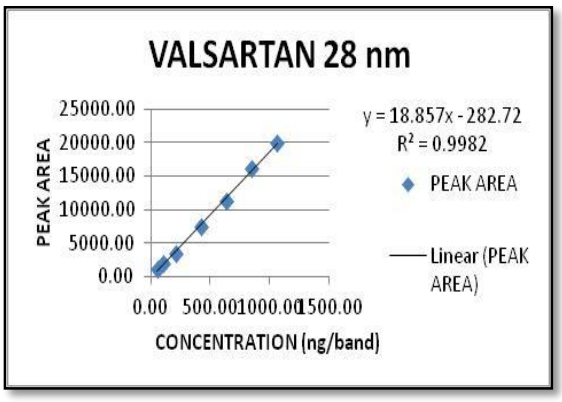


Figure No. 6.9.16: Standard calibration graph of VAL (53.33-1067 ng/band) at 281 nm

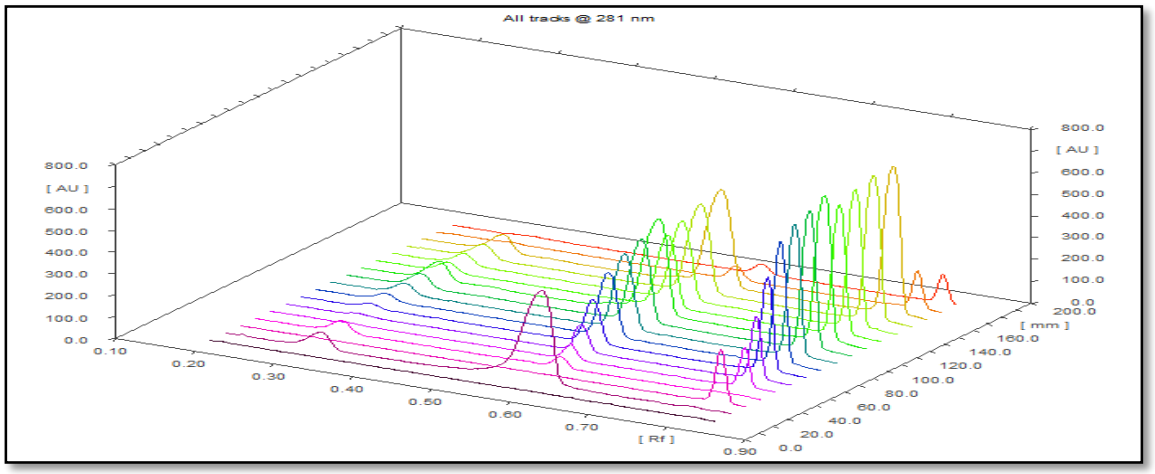


Figure No. 6.9.17: 3D overlain chromatograms of ALI, VAL & FA

6.9.10 Precision

The precision of the method was checked by carrying out repeatability of sample application, intra-day and inter-day precision. Results of precision studies expressed in % RSD follows ICH guideline acceptable limits (%RSD<2), which shows good repeatability, low intra and inter-day variability, indicating an excellent precision of the developed method (Table No. 6.9.3, 6.9.4 & 6.9.5).

Table No. 6.9.3: Results of repeatability of sample application

Repeatability								
Sr. No.	ALI				VAL			
	Conc. (ng/band)	Peak area	Mean ± SD	% RSD	Conc. (ng/band)	Peak area	Mean ± SD	% RSD
1	400	5536.2	5590.42 ± 98.8093	1.7675	426.67	7486.6	7475.38 ± 51.1007	0.6836
2		5625.0				7521.8		
3		5684.3				7385.3		
4		5587.2				7465.0		
5		5684.0				7468.2		
6		5425.8				7525.4		
1	600	7856.1	7852.40 ± 63.3427	0.8067	640	11317.2	11357.18 ± 76.2512	0.6714
2		7782.4				11425.2		
3		7789.0				11354.0		
4		7865.0				11452.2		
5		7865.6				11354.8		
6		7956.3				11239.7		
Mean % RSD				**1.2871**	**Mean % RSD**			**0.6775**

**mean±SD, (n= 6) number of determination*

Table No. 6.9.4: Results of intra-day precision

Intra-day precision								
Sr. No.	ALI				VAL			
	Conc. (ng/band)	Peak area	Mean ± SD	% RSD	Conc. (ng/band)	Peak area	Mean ± SD	% RSD
1	400	5483.7	5615.81 ± 65.3030	1.1628	426.67	7465.0	7513.63 ± 103.8429	1.3821
2		5674.3				7468.2		
3		5618.0				7486.6		
4		5578.0				7521.8		
5		5587.7				7385.3		
6		5645.1				7465.0		
7		5684.3				7687.3		
8		5587.2				7459.3		
9		5684.0				7684.2		
1	600	7865.6	7832.59 ± 63.3742	0.8091	640	11345.6	11512.66 ± 100.8108	0.8757
2		7956.3				11402.8		
3		7825.3				11584.6		
4		7856.1				11654.3		
5		7754.0				11587.8		
6		7768.0				11468.0		
7		7854.2				11498.0		
8		7845.6				11596.0		
9		7768.2				11476.8		
Mean % RSD				**0.9860**	**Mean % RSD**			**1.1289**

**mean±SD, (n=3) number of determination*

Table No. 6.9.5: Results of inter-day precision

Inter-day precision									
Sr. No.		ALI				VAL			
		Conc. (ng/band)	Peak area	Mean ± SD	% RSD	Conc. (ng/band)	Peak area	Mean ± SD	% RSD
1	**1**	400	5578.0	5653.43 ± 56.7179	1.0032	426.67	7548.2	7497.43 ± 60.0987	0.8016
2			5587.7				7567.5		
3			5645.1				7486.6		
4	**2**		5684.3				7521.8		
5			5587.2				7385.3		
6			5684.0				7465.0		
7	**3**		5674.5				7465.0		
8			5725.6				7468.2		
9			5714.5				7569.3		
1	**1**		7856.3				11248.5		
2			7769.4				11397.2		
3			7865.6				11489.7		
4			7956.3				11358.8		

5	**2**	600	7825.3	7832.18 ± 74.1789	0.9471	640	11345.6	11415.56 ± 106.2714	0.9309
6			7856.1				11402.8		
7	**3**		7825.6				11584.6		
8			7851.0				11547.0		
9			7684.0				11365.8		
Mean % RSD					**0.9752**	**Mean % RSD**			**0.8663**

**mean±SD, (n=3) number of determination*

6.9.11 Accuracy

The results of recovery studies ranged from 98-101% for both the drugs showing the accuracy of the method (Table No. 6.9.6). This indicates that there is no interference from tablet excipients.

Table No. 6.9.6: Results of recovery studies

Accuracy (% Recovery)				
ALI				
Recovery level (%)	**Initial amount of formulation (ng/band)**	**Standard added (ng/band)**	**Recovered (ng/band)**	**% Recovered**
50	200	100	98.2152	98.2152
50	300	150	150.5761	100.3841
50	400	200	203.0142	101.5071
100	200	200	203.2762	101.6381
100	300	300	304.3833	101.4611
100	400	400	402.6255	100.6564
150	200	300	300.8339	100.2780
150	300	450	461.5454	102.5656
150	400	600	607.5439	101.2573
Mean±SD				**100.8848±1.2272**
% RSD				**1.2165**
VAL				
Recovery level (%)	**Initial amount of formulation (ng/band)**	**Standard added (ng/band)**	**Recovered (ng/band)**	**% Recovered**
50	213.3333	106.6667	106.4037	99.7535
50	320.0000	160.0000	162.3504	101.4690
50	426.6666	213.3333	211.2652	99.0306
100	213.3333	213.3333	207.1726	97.1122
100	320.0000	320.0000	321.1561	100.3613
100	426.6666	426.6666	419.3736	98.2907
150	213.3333	320.0000	325.7387	101.7933
150	320.0000	479.9999	481.8922	100.3942
150	426.6666	639.9999	635.6169	99.3152
Mean±SD				**99.7244±1.4878**
% RSD				**1.4919**

**mean±SD, (n=3) number of determination*

6.9.12 LOD and LOQ

The values of LOD and LOQ were found to be very low which proves the sensitivity of the method is shown in Table No. 6.9.8.

6.9.13 Robustness

The proposed method was checked for robustness study, but no significant changes (% RSD<2) found in peak parameters (R_f value and peak area reproducibility), shown in Table No. 6.9.7.

Table No. 6.9.7: Results of robustness study

Sr. No.	Modification	ALI*		VAL*	
		R_f value	**Peak area**	**R_f value**	**Peak area**
1	M/P composition (± 0.1 ml)	0.5727 ± 0.0047	5643.2667 ± 53.4008	0.7907 ± 0.0108	7481.5667 ± 46.2511
% RSD (<2)		**0.8252**	**0.9463**	**1.3556**	**0.6182**
2	Volume of M/P (20 ±5 ml)	0.5783 ± 0.0031	5649.1667 ± 77.5746	0.7830 ± 0.0075	7538.1333 ± 90.3395
% RSD (<2)		**0.5283**	**1.3732**	**0.9642**	**1.1984**
3	Chamber saturation time (20 ± 5 min)	0.5683 ± 0.0042	5685.8333 ± 47.0696	0.7927 ± 0.0097	7605.3000 ± 49.3927
% RSD (<2)		**0.7326**	**0.8278**	**1.2253**	**0.6495**
4	Development distance (80 ± 5 mm)	0.5703 ± 0.0071	5736.5667 ± 105.0784	0.7827 ± 0.0095	7642.2000 ± 99.9011
% RSD (<2)		**1.2439**	**1.8317**	**1.2144**	**1.3072**
5	Time from spotting to chromatography (15±10 min)	0.5673 ± 0.0060	5667.1333 ± 51.3471	0.7820 ± 0.0125	7786.4667 ± 76.6317
% RSD (<2)		**1.0625**	**0.9061**	**1.5972**	**0.9842**
6	Time from chromatography to scanning (15±10 min)	0.5797 ± 0.0064	5741.5000 ± 66.3514	0.7893 ± 0.0067	7836.3333 ± 58.8736
% RSD (<2)		**1.0956**	**1.1556**	**0.8435**	**0.7513**

**Mean±SD, (n=3) number of determinations*

6.9.14 Stability of the solution

Solution stability was performed at room temperature and it was found to be stable up to two days.

Table No. 6.9.8: Summary of validation parameters for the proposed method

Parameters	ALI	VAL
Linearity range (ng/band)	50-1000	53.33-1067
Correlation coefficient	0.9996	0.9982
Regression equation	y = 11.833x + 760.02	y = 18.857x - 282.72
Precision (%RSD)		
Repeatability of sample application (n=6)	1.2871	0.6775
Intra-day (n=3)	0.9860	1.1289
Inter-day (n=3)	0.9752	0.8663
*Accuracy**		
% Recovery (n=3)	100.8848±1.2272	99.7244±1.4878
%RSD (n=3)	1.2165	1.4919
Specificity	No interference	
LOD (ng/band)	5.8346	5.5271
LOQ (ng/band)	17.6807	16.7489

**mean±SD, n= number of determinations*

6.9.15 Analysis of marketed formulation

The proposed method was successfully used for the quantitative determination of ALI and VAL in commercial formulation (Valturna tablet: 300 mg of ALI and 320 mg of VAL). Six replicate determinations were carried out and experimental values were found to be within 98-102% for both the drugs are presented in Table No. 6.9.10. Chromatogram of formulation is shown in Figure No. 6.9.18.

Table No. 6.9.10: Results of formulation analysis

Drugs	Amount (mg/tablet)		%Drug found*	% RSD
	Labelled	Found		
ALI	300	299.64	99.8805±0.7297	0.7306
VAL	320	315.44	98.5739±0.5716	0.5799

**mean ± SD (n=6) average of six determinations*

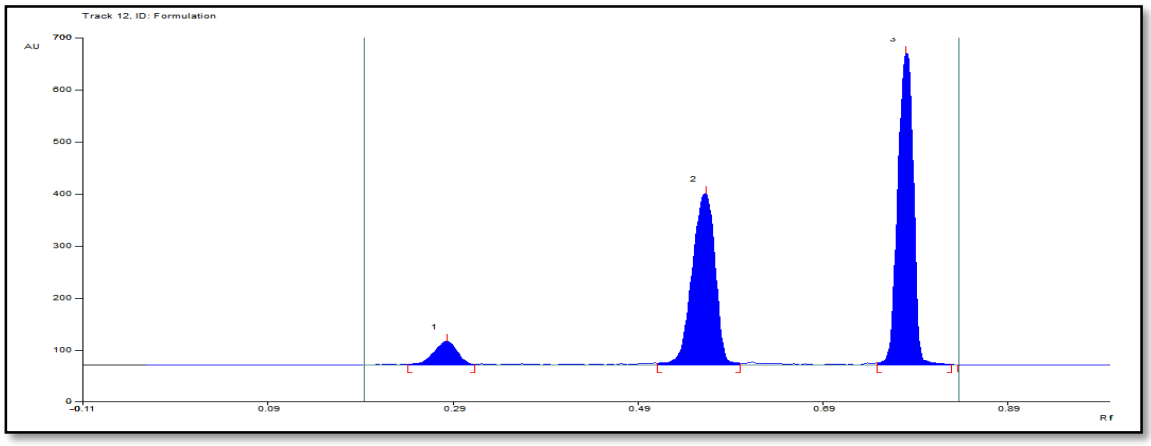


Figure No. 6.9.18: Chromatogram of formulation ALI (500 ng/band) and VAL (533.33 ng/band)

Result (% assay) of study suggests that, the proposed method can be applied for the quantitative analysis of ALI and VAL in tablet formulation.

METHOD 10

6.10 *"Development and validation of simultaneous equation method for the simultaneous determination of aliskiren hemifumarate and amlodipine besilate in tablets"*

Estimation of ALI and AMLO was achieved by simultaneous equation method using Shimadzu UV-1800 (UV Pro), double beam UV-visible Spectrophotometer.

6.10.1 Selection of solvent

Based on the literature survey and solubility studies methanol was selected as solvent, which showed higher absorbance and distinct λ_{max} for both the drugs.

6.10.2 Selection of wavelength

Standard solutions of ALI (20 µg/ml) and AMLO (20 µg/ml) were separately scanned in the UV region (200-400 nm) and spectra were recorded using methanol as blank. Their overlain spectra are shown in Figure No. 6.10.1.

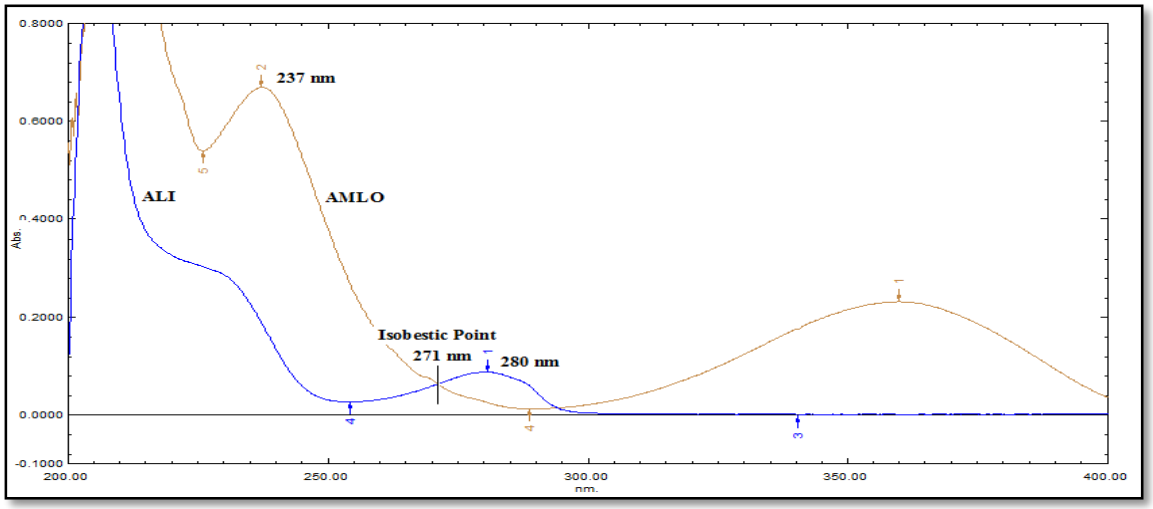


Figure No. 6.10.1: Overlain UV spectra of ALI (20 µg/ml) and AMLO (20 µg/ml)

From the overlain spectra, 237 nm (λ_{max} of AMLO) and 280 nm (λ_{max} of ALI) were selected for further studies, which showed good linearity and hence used for simultaneous estimation of ALI and AMLO by *simultaneous equation* method.

6.10.3 Determination of absorptivity value

The developed method was found to be linear in the concentration range of 1-50 µg/ml for both the drugs. Absorbances were measured at 237 nm and 280 nm for both the drugs and absorptivity values were calculated and presented in Table No. 6.10.1 & 6.10.2.

Table No. 6.10.1: Absorbances and absorptivities of ALI at selected wavelength

Conc. (µg/ml)	ALI					
	237 nm			280 nm		
	Abs.*	Absorptivity	Avg. Absorptivity	Abs.*	Absorptivity	Avg. Absorptivity
1	0.0097	96.5000	(ax_1) **96.4366**	0.0045	44.8333	(ax_2) **43.7706**
5	0.0483	96.6000		0.0213	42.5667	
10	0.0955	95.5000		0.0436	43.6000	
20	0.1915	95.7333		0.0872	43.5792	
30	0.2922	97.3889		0.1336	44.5278	
40	0.3893	97.3208		0.1762	44.0542	
50	0.4801	96.0133		0.2162	43.2333	

**average of six determinations*

Table No. 6.10.2: Absorbances and absorptivities of AMLO at selected wavelength

Conc. (µg/ml)	AMLO					
	237 nm			280 nm		
	Abs.*	Absorptivity	Avg. Absorptivity	Abs.*	Absorptivity	Avg. Absorptivity
1	0.0327	326.5000	(ay_1) **332.3540**	0.0015	15.1450	(ay_2) **13.3937**
5	0.1674	334.8667		0.0065	13.0570	
10	0.3384	338.4000		0.0122	12.1750	
20	0.6666	333.2917		0.0268	13.4167	
30	0.9890	329.6794		0.0393	13.1111	
40	1.3296	332.4083		0.0530	13.2375	
50	1.6567	331.3320		0.0681	13.6133	

**average of six determinations*

6.10.4 Validation of the method

The developed method was validated in accordance with "*International Conference on Harmonization"* guidelines for validation of analytical procedures.

6.10.5 Specificity

Overlain spectra of tablet excipients and drug solution indicate that there was no interference between excipients and standard drugs (Figure No. 6.10.2).

6.10.6 Linearity and range

ALI and AMLO were found to be linear in the concentration range of 1-50 µg/ml. Overlain spectra of ALI and AMLO are shown in Figure No. 6.10.3-6.10.5.

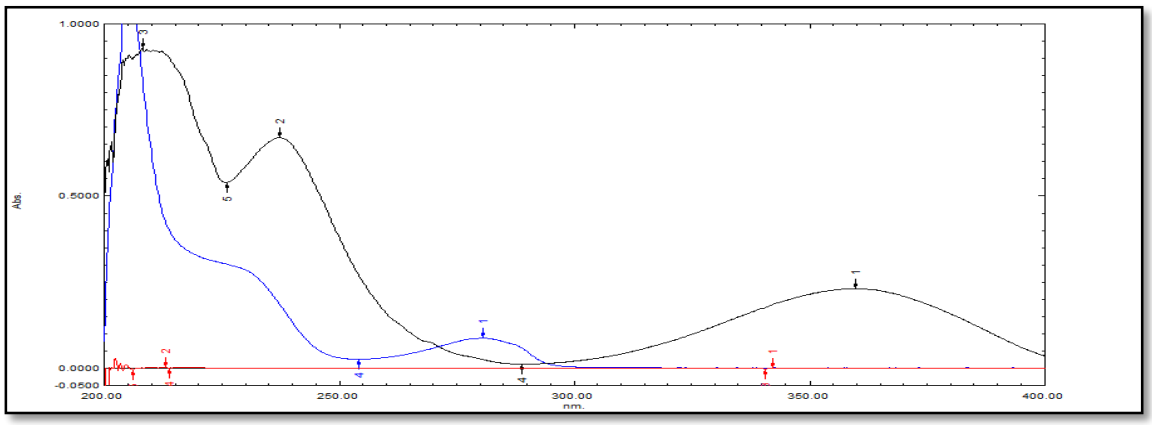


Figure No. 6.10.2: Overlain UV spectra of formulation excipients and standard drugs

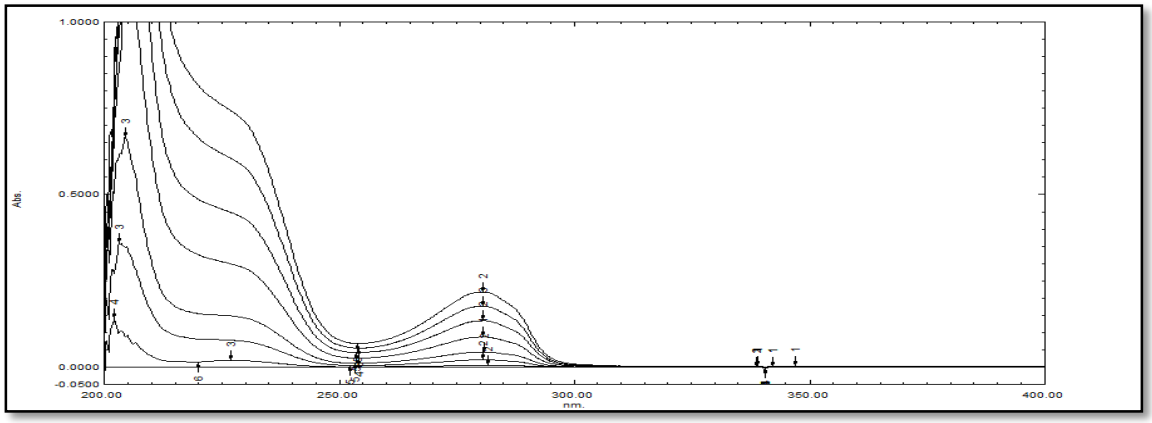


Figure No. 6.10.3: Overlain UV spectra of ALI (1-50 µg/ml)

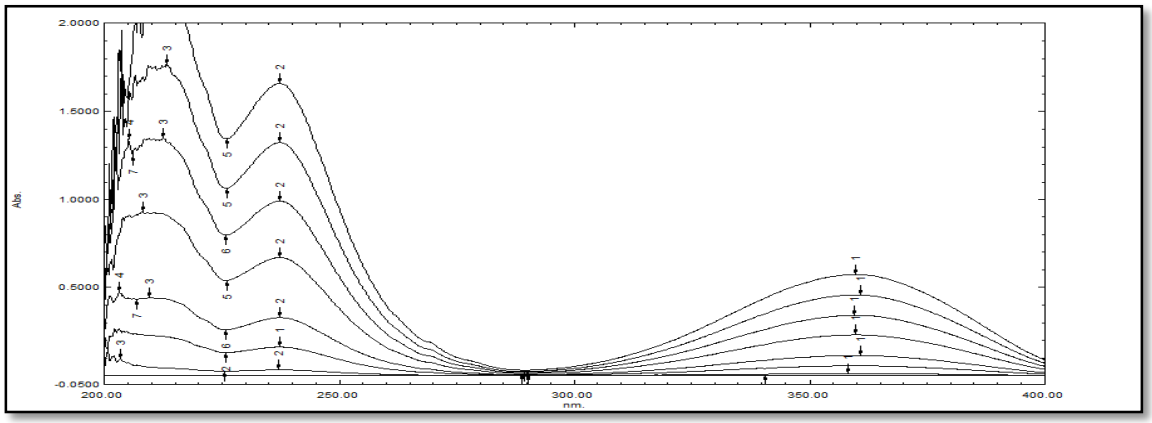


Figure No. 6.10.4: Overlain UV spectra of AMLO (1-50 µg/ml)

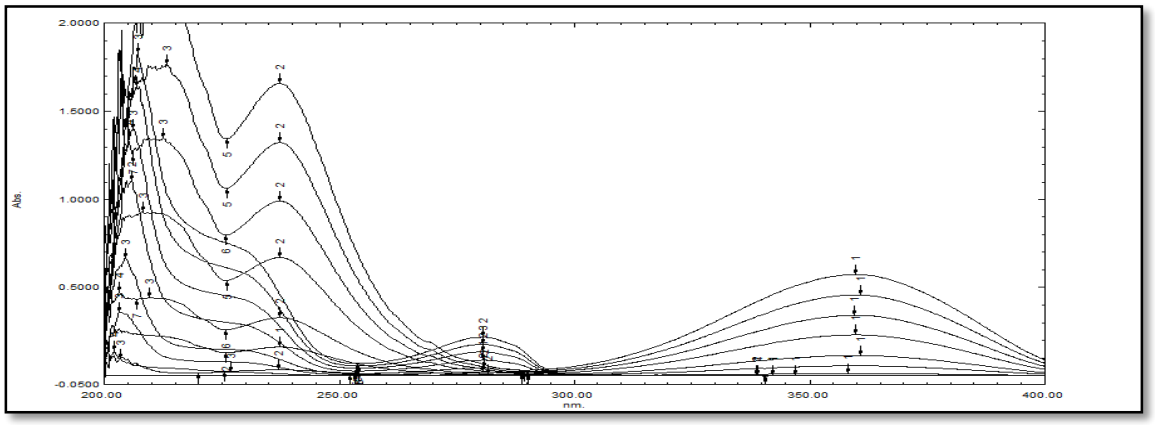


Figure No. 6.10.5: Overlain UV spectra of ALI & AMLO (1-50 µg/ml)

Calibration graphs (Figure No. 6.10.6-6.10.9) were plotted using absorbance of standard drug versus concentration. Results were subjected to regression analysis by least square method to calculate the values of slope, intercept and correlation coefficient of ALI and AMLO at 237 and 280 nm are shown in Table No. 6.10.8.

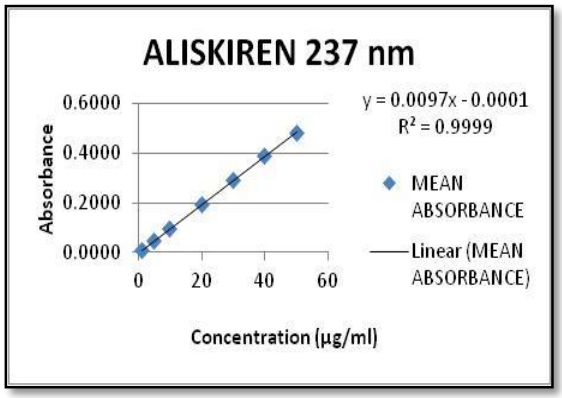


Figure No. 6.10.6: Calibration graph of ALI (1-50 µg/ml) at 237 nm

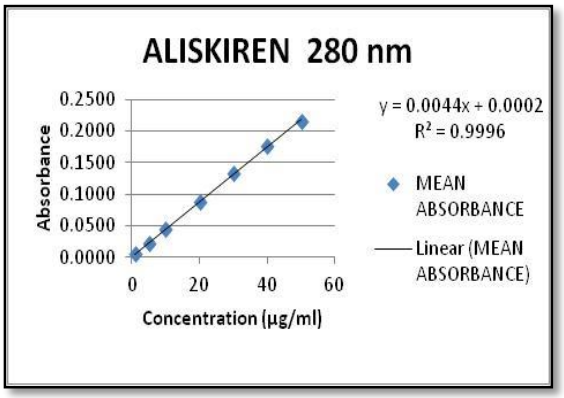


Figure No. 6.10.7: Calibration graph of ALI (1-50 µg/ml) at 280 nm

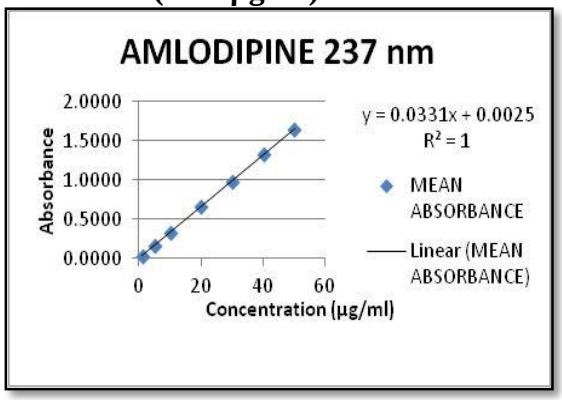


Figure No. 6.10.8: Calibration graph of AMLO (1-50 µg/ml) at 237 nm

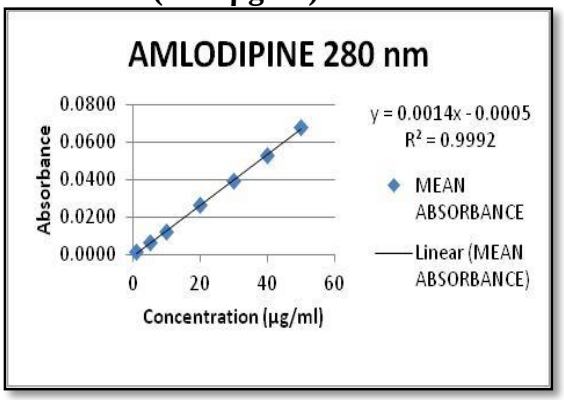


Figure No. 6.10.9: Calibration graph of AMLO (1-50 µg/ml) at 280 nm

6.10.7 Precision

Results of precision studies expressed in % RSD follows ICH guideline acceptable limits (% RSD<2), which shows good repeatability and low inter-day variability, indicating an excellent precision of the developed method (Table No. 6.10.3, 6.10.4 & 6.10.5).

Table No. 6.10.3: Results of repeatability of measurement

Repeatability					
Sr. No.	Conc.	Absorbance			
		ALI		AMLO	
		237 nm	280 nm	237 nm	280 nm
1	ALI 10 µg/ml	0.0948	0.0431	0.3345	0.0121
2		0.0961	0.0441	0.3365	0.0121
3		0.0956	0.0435	0.3347	0.0120
4	AMLO 10 µg/ml	0.0947	0.0436	0.3345	0.0124
5		0.0943	0.0442	0.3375	0.0124
6		0.0965	0.0435	0.3372	0.0122
Mean±SD*		**0.0953±0.0009**	**0.0437±0.0004**	**0.3358±0.0014**	**0.0122±0.0002**
% RSD		**0.9112**	**0.9478**	**0.4196**	**1.3719**
1	ALI 20 µg/ml	0.1924	0.0857	0.6645	0.0271
2		0.1954	0.0851	0.6687	0.0265
3		0.1953	0.0875	0.6648	0.0267
4	AMLO 20 µg/ml	0.1928	0.0852	0.6682	0.0268
5		0.1943	0.0846	0.6653	0.0271
6		0.1947	0.0853	0.6623	0.0271
Mean±SD*		**0.1942±0.0013**	**0.0856±0.0010**	**0.6656±0.0024**	**0.0269±0.0003**
% RSD		**0.6554**	**1.1774**	**0.3630**	**0.9532**
Mean % RSD		**0.7833**	**1.0626**	**0.3913**	**1.1625**

**mean±SD, (n= 6) number of determination*

Table No. 6.10.4: Results of intra-day precision

Intra-day precision					
Sr. No.	Conc.	Absorbance			
		ALI		AMLO	
		237 nm	280 nm	237 nm	280 nm
1	ALI 10 µg/ml	0.0965	0.0437	0.3372	0.0124
2		0.0965	0.0436	0.3407	0.0120
3		0.0954	0.0439	0.3356	0.0124
4		0.0961	0.0443	0.3391	0.0123
5		0.0945	0.0439	0.3395	0.0124
6	AMLO 10 µg/ml	0.0965	0.0435	0.3457	0.0124
7		0.0950	0.0445	0.3370	0.0124
8		0.0954	0.0435	0.3345	0.0121
9		0.0961	0.0438	0.3348	0.0121
Mean±SD*		**0.0958±0.0007**	**0.0439±0.0003**	**0.3382±0.0035**	**0.0123±0.0002**
% RSD		**0.7651**	**0.7908**	**1.0426**	**1.2956**

1	ALI 20 µg/ml AMLO 20 µg/ml	0.1943	0.0853	0.6648	0.0258
2		0.1947	0.0865	0.6682	0.0264
3		0.1935	0.0846	0.6653	0.0263
4		0.1954	0.0842	0.6623	0.0266
5		0.1956	0.0865	0.6657	0.0268
6		0.1935	0.0857	0.6721	0.0261
7		0.1947	0.0854	0.6628	0.0261
8		0.1935	0.0856	0.6748	0.0258
9		0.1937	0.0846	0.6674	0.0261
Mean±SD*		**0.1943±0.0008**	**0.0854±0.0008**	**0.6670±0.0041**	**0.0262±0.0003**
% RSD		**0.4265**	**0.9567**	**0.6217**	**1.2901**
Mean % RSD		**0.5958**	**0.8737**	**0.8322**	**1.2928**

**mean±SD, (n= 3) number of determination*

Table No. 6.10.5: Results of inter-day precision

Inter-day precision						
Sr. No.		**Conc.**	**Absorbance**			
			ALI		**AMLO**	
			237 nm	**280 nm**	**237 nm**	**280 nm**
1	Day 1	ALI 10 µg/ml AMLO 10 µg/ml	0.0953	0.0435	0.3391	0.0121
2			0.0965	0.0446	0.3395	0.0123
3			0.0935	0.0428	0.3348	0.0124
4	Day 2		0.0943	0.0438	0.3346	0.0124
5			0.0948	0.0437	0.3374	0.0121
6			0.0957	0.0436	0.3372	0.0121
7	Day 3		0.0954	0.0439	0.3457	0.0120
8			0.0935	0.0441	0.3356	0.0124
9			0.0947	0.0438	0.3351	0.0121
Mean±SD*			**0.0949±0.0010**	**0.0438±0.0005**	**0.3377±0.0035**	**0.0122±0.0002**
% RSD			**1.0491**	**1.1026**	**1.0403**	**1.3026**
1	Day 1	ALI 20 µg/ml AMLO 20 µg/ml	0.1953	0.0847	0.6718	0.0262
2			0.1928	0.0845	0.6638	0.0258
3			0.1943	0.0853	0.6778	0.0263
4	Day 2		0.1947	0.0835	0.6608	0.0261
5			0.1935	0.0846	0.6748	0.0258
6			0.1934	0.0852	0.6682	0.0264
7	Day 3		0.1947	0.0865	0.6653	0.0268
8			0.1964	0.0857	0.6673	0.0261
9			0.1937	0.0867	0.6687	0.0263
Mean±SD*			**0.1943±0.0011**	**0.0852±0.0010**	**0.6687±0.0054**	**0.0262±0.0003**
% RSD			**0.5683**	**1.1744**	**0.8015**	**1.1764**
Mean % RSD			**0.8087**	**1.1385**	**0.9209**	**1.2395**

**mean±SD, (n= 3) number of determination*

6.10.8 Accuracy

The results of recovery studies ranged from 98-101% for both the drugs showing the accuracy of the method (Table No. 6.10.6). This indicates that there is no interference from tablet excipients.

Table No. 6.10.6: Results of recovery studies

Accuracy (% Recovery)				
ALI				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard drug added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	10	5	5.0001	100.0030
50	15	7.5	7.3375	97.8338
50	20	10	10.0852	100.8524
100	10	10	9.9075	99.0751
100	15	15	14.9154	99.4357
100	20	20	20.1399	100.6997
150	10	15	15.0782	100.5211
150	15	22.5	22.8258	101.4480
150	20	30	29.6237	98.7458
Mean±SD*				**99.8461±1.1625**
% RSD				**1.1643**
AMLO				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard drug added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	10	5	5.0395	100.7899
50	15	7.5	7.4936	99.9149
50	20	10	10.1747	101.7470
100	10	10	9.9823	99.8227
100	15	15	14.9833	99.8886
100	20	20	19.8733	99.3663
150	10	15	15.0352	100.2346
150	15	22.5	22.5058	100.0258
150	20	30	30.2430	100.8099
Mean±SD*				**100.2889±0.7149**
% RSD				**0.7129**

**mean±SD, (n= 3) number of determination*

6.10.9 LOD and LOQ

The values of LOD and LOQ were found to be very low which proves the sensitivity of the method is shown in Table No. 6.10.8.

6.10.10 Robustness

The proposed method was checked for robustness study, but no significant changes (% RSD<2) found in absorption, indicating that the method is robust (Table No. 6.10.7).

Table No. 6.10.7: Results of robustness study

Parameter	Drugs			
Wavelengths (237 & 280 ±1 nm)	ALI		AMLO	
	Assay (%)*	% RSD	Assay (%)*	% RSD
236 & 279 nm	98.6554	1.4898	101.2154	1.7709
237 & 280 nm	99.4876		102.3545	
238 & 281 nm	101.5465		98.8545	

**mean±SD, (n= 3) number of determination*

6.10.11 Stability of the solution

Solution stability was performed at room temperature and it was found to be stable up to two days.

Table No. 6.10.8: Summary of validation parameters for the proposed method

Parameters	ALI		AMLO	
Detection wavelengths (nm)	237	280	237	280
Linearity range (µg/ml)	1-50		1-50	
Correlation coefficient	0.9999	0.9996	1	0.9992
Regression equation	y = 0.0097x - 0.0001	y = 0.0044x + 0.0002	y = 0.0331x - 0.0025	y = 0.0014x - 0.0005
Precision (%RSD)				
Repeatability of measurement (n=6)	0.7833	1.0626	0.3913	1.1625
Intra-day (n=3)	0.5958	0.8737	0.8322	1.2928
Inter-day (n=3)	0.8087	1.1385	0.9209	1.2395
Accuracy				
% Recovery (n=3)	99.8461±1.1625		100.2889±0.7149	
%RSD (n=3)	1.1643		0.7129	
Specificity	No interference			
LOD (µg/ml)	0.2329	0.1959	0.0959	0.1796
LOQ (µg/ml)	0.7059	0.5938	0.2908	0.5442

**mean±SD, n= number of determinations*

6.10.12 Analysis of marketed formulation

The proposed method was successfully used for the quantitative determination of ALI and AMLO in commercial formulation (Tekamlo tablet: 300 mg ALI and 10 mg of AMLO). Six replicate determinations were carried out and experimental values were found to be within 99 to 101 % for ALI and AMLO and hence the developed method can be used for the simultaneous determination of both the drugs in combined formulation (Table No. 6.10.9). Overlain spectra of standard drugs and formulation are showed in Figure No. 6.10.10.

Table No. 6.10.9: Result of formulation analysis

Drugs	Amount (mg/tablet)		% Drug found*	% RSD
	Labelled	Found		
ALI	300	294.83	98.2774±1.2008	1.2218
AMLO	10	9.78	97.8377±0.7151	0.7309

**mean ± SD (n=6) average of six determinations*

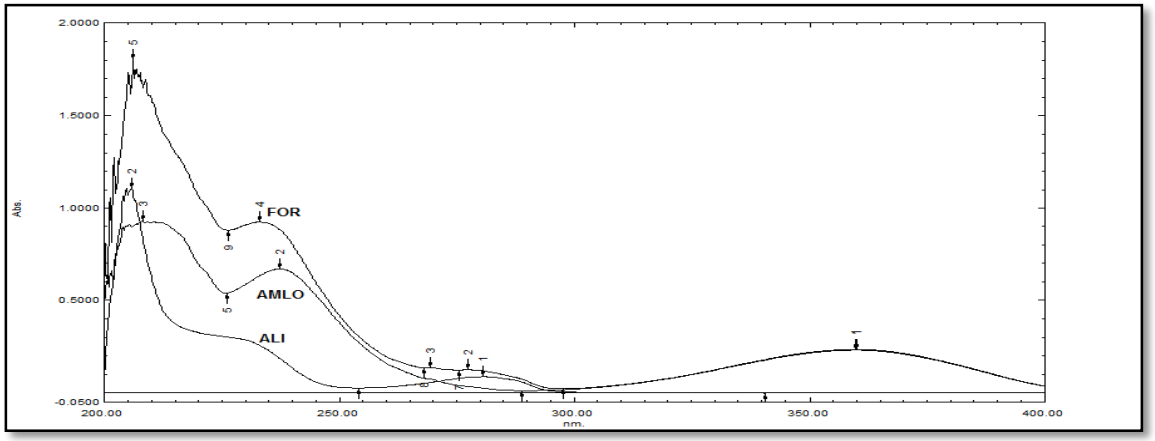


Figure No. 6.10.10: Overlain UV spectra of ALI, AMLO (20 µg/ml) and formulation (ALI & AMLO 20 µg/ml)

In order to check the applicability of the method, all the available strength (ratio) of marketed formulations were analyzed using standard drug solution in optimum ratio.

Available strength (mg)

ALI + AMLO: 150/300 + 5/10

Study (% assay) suggests that, the proposed method can be applied to all the formulations of different strengths available in the market.

METHOD 11

6.11 *"Development and validation of absorbance ratio (Q analysis) method for the simultaneous determination of aliskiren hemifumarate and amlodipine besilate in tablets"*

Estimation of ALI and AMLO was achieved by absorbance ratio method using Shimadzu UV-1800 (UV Pro), double beam UV-visible spectrophotometer.

6.11.1 Selection of solvent

Based on the literature survey and solubility studies methanol was selected as solvent, which showed higher absorbance and distinct λ_{max} for both the drugs**.**

6.11.2 Selection of wavelength

Standard solutions of ALI and AMLO (20 µg/ml) were separately scanned in the UV region (200-400 nm) and spectra were recorded using methanol as blank. Their overlain spectra are shown in Figure No. 6.11.1.

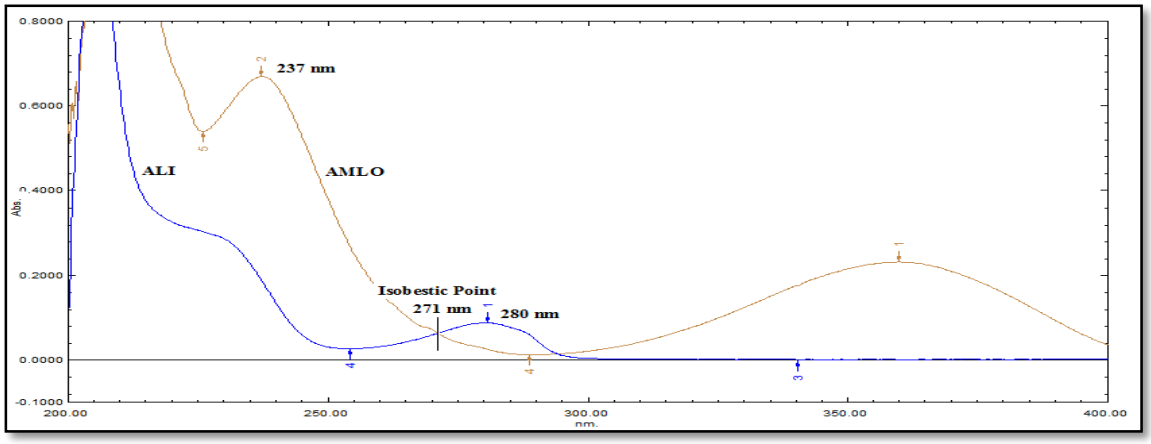


Figure No. 6.11.1: Overlain UV spectra of ALI & AMLO (20 µg/ml)

From the overlain spectra, 237 nm (λ_{max} of AMLO) and 271 nm (isobestic point) were selected for further studies, which showed good linearity and hence used for simultaneous estimation by *absorption ratio (Q analysis)* method.

6.11.3 Determination of absorptivity value

The developed method was found to be linear in the concentration range of 1-50 µg/ml for both the drugs. Absorbances were measured at 237 nm and 271 nm for both the drugs and absorptivity values were calculated and presented in Table No. 6.11.1 & 6.11.2.

Table No. 6.11.1: Absorbances and absorptivities of ALI at selected wavelength

Conc. (µg/ml)	ALI					
	237 nm			271 nm		
	Abs.*	Absorptivity	Avg. Absorptivity	Abs.*	Absorptivity	Avg. Absorptivity
1	0.0097	96.5000	(ax_1) **96.4366**	0.0030	30.4000	(ax_2) **31.1918**
5	0.0483	96.6000		0.0157	31.3667	
10	0.0955	95.5000		0.0308	30.7833	
20	0.1915	95.7333		0.0621	31.0500	
30	0.2922	97.3889		0.0959	31.9500	
40	0.3893	97.3208		0.1262	31.5417	
50	0.4801	96.0133		0.1563	31.2510	

**average of six determinations*

Table No. 6.11.2: Absorbances and absorptivities of AMLO at selected wavelength

Conc. (µg/ml)	AMLO					
	237 nm			271 nm		
	Abs.*	Absorptivity	Avg. Absorptivity	Abs.*	Absorptivity	Avg. Absorptivity
1	0.0327	326.5000	(ay_1) **332.3540**	0.0030	30.0000	(ay_2) **31.1918**
5	0.1674	334.8667		0.0157	31.3667	
10	0.3384	338.4000		0.0308	30.7833	
20	0.6666	333.2917		0.0621	31.0500	
30	0.9890	329.6794		0.0959	31.9500	
40	1.3296	332.4083		0.1262	31.5417	
50	1.6567	331.3320		0.1563	31.2510	

**average of six determinations*

6.11.4 Validation of the method

The developed method was validated in accordance with "*International Conference on Harmonization"* guidelines for validation of analytical procedures.

6.11.5 Specificity

Overlain spectra of placebo and drug solution indicate that there was no interference between excipients and standard drugs (Figure No. 6.11.2).

6.11.6 Linearity and range

ALI and AMLO were found to be linear in the concentration range of 1-50 µg/ml. Overlain spectra of ALI and AMLO are shown in Figure No. 6.11.3-6.11.5.

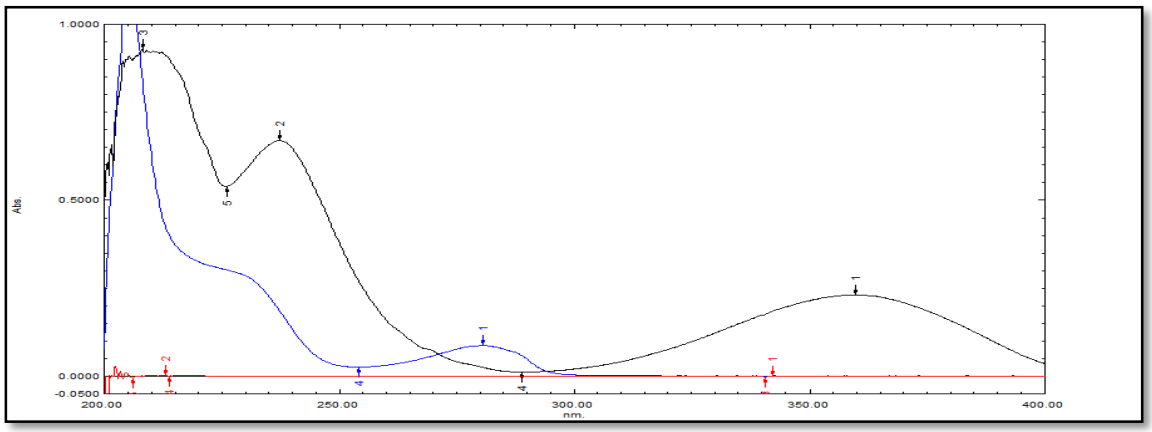


Figure No. 6.11.2: Overlain UV spectra of formulation excipients and standard drugs

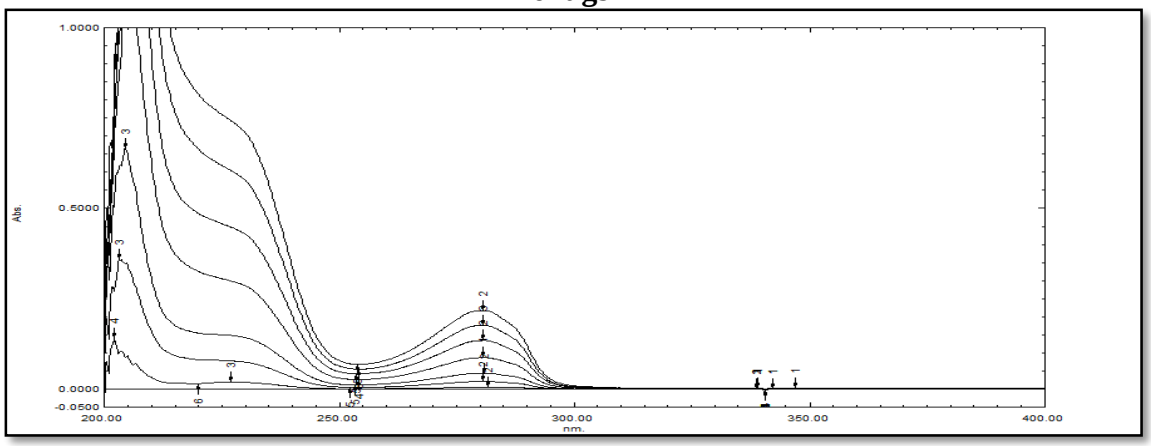


Figure No. 6.11.3: Overlain UV spectra of ALI (1-50 µg/ml)

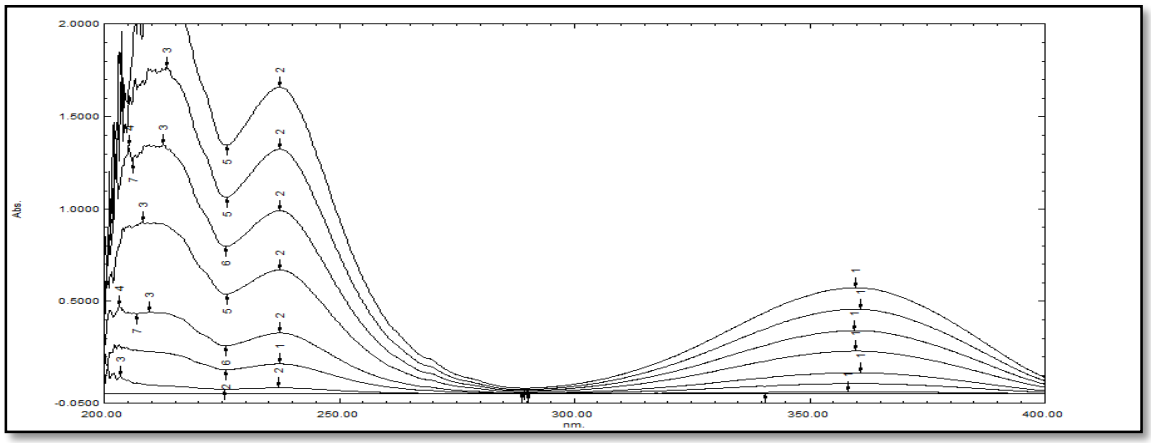


Figure No. 6.11.4: Overlain UV spectra of AMLO (1-50 µg/ml)

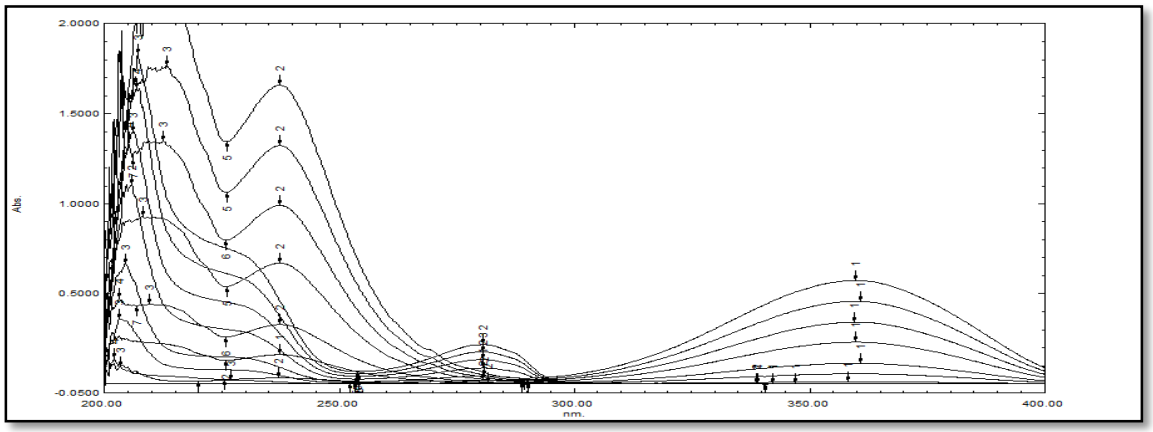


Figure No. 6.11.5: Overlain UV spectra of ALI & AMLO (1-50 µg/ml)

Calibration graphs (Figure No. 6.11.6-6.11.9) were plotted using absorbance of standard drug versus concentration. Results were subjected to regression analysis by least square method to calculate the values of slope, intercept and correlation coefficient of ALI and AMLO at 237 nm and 271 nm are shown in Table No. 6.11.8.

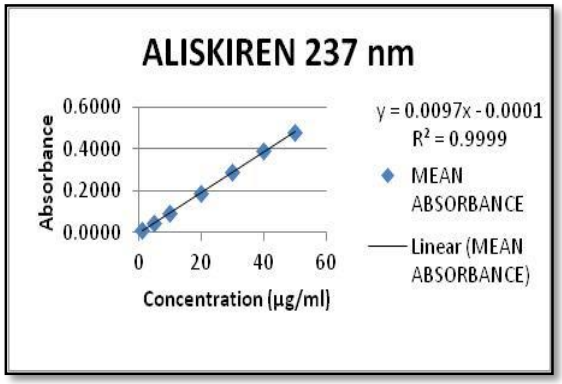


Figure No. 6.11.6: Calibration graph of ALI (1-50 µg/ml) at 237 nm

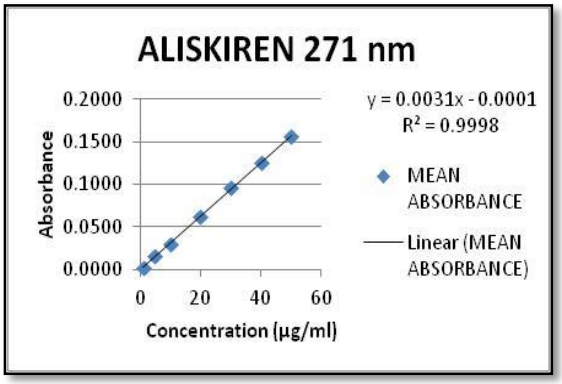


Figure No. 6.11.7: Calibration graph of ALI (1-50 µg/ml) at 271 nm

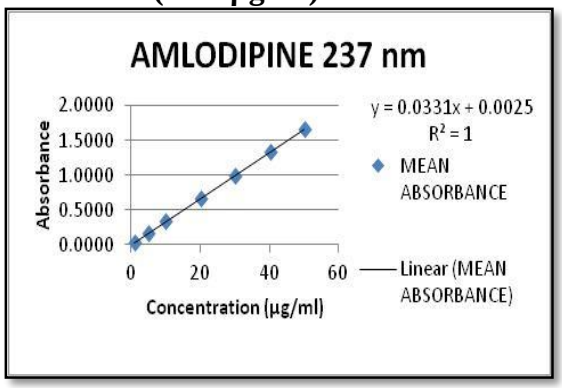


Figure No. 6.11.8: Calibration graph of AMLO (1-50 µg/ml) at 237 nm

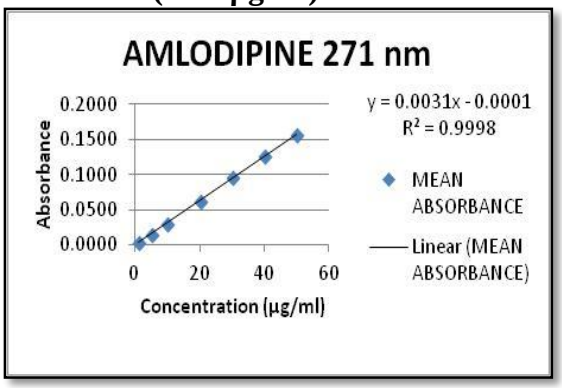


Figure No. 6.11.9: Calibration graph of AMLO (1-50 µg/ml) at 271 nm

6.11.7 Precision

Results of precision studies expressed in % RSD follows ICH guideline acceptable limits (% RSD<2), which shows good repeatability and low inter-day variability, indicating an excellent precision of the developed method (Table No. 6.11.3, 6.11.4 & 6.11.5).

Table No. 6.11.3: Results of repeatability of measurement

Repeatability					
Sr. No.	**Conc.**	**Absorbance**			
		ALI		**AMLO**	
		237 nm	**271 nm**	**237 nm**	**271 nm**
1	ALI 10 µg/ml AMLO 10 µg/ml	0.0948	0.0307	0.3345	0.0307
2		0.0961	0.0311	0.3365	0.0311
3		0.0956	0.0306	0.3347	0.0306
4		0.0947	0.0313	0.3345	0.0313
5		0.0943	0.0302	0.3375	0.0302
6		0.0965	0.0308	0.3372	0.0308
Mean±SD*		**0.0953±0.0009**	**0.0308±0.0004**	**0.3363±0.0016**	**0.0309±0.0004**
% RSD		**0.9112**	**1.2567**	**0.4680**	**1.2913**
1	ALI 20 µg/ml AMLO 20 µg/ml	0.1924	0.0615	0.6645	0.0615
2		0.1954	0.0628	0.6687	0.0628
3		0.1953	0.0621	0.6648	0.0621
4		0.1928	0.0618	0.6682	0.0618
5		0.1943	0.0619	0.6653	0.0619
6		0.1947	0.0622	0.6623	0.0622
Mean±SD*		**0.1942±0.0013**	**0.0621±0.0004**	**0.6657±0.0021**	**0.0621±0.0006**
% RSD		**0.6554**	**0.7117**	**0.3149**	**1.0101**
Mean % RSD		**0.7833**	**0.9842**	**0.3914**	**1.1507**

**mean±SD, (n= 6) number of determination*

Table No. 6.11.4: Results of intra-day precision

Intra-day precision					
Sr. No.	**Conc.**	**Absorbance**			
		ALI		**AMLO**	
		237 nm	**271 nm**	**237 nm**	**271 nm**
1	ALI 10 µg/ml AMLO 10 µg/ml	0.0965	0.0302	0.3372	0.0302
2		0.0965	0.0309	0.3407	0.0309
3		0.0954	0.0302	0.3356	0.0302
4		0.0961	0.0308	0.3391	0.0308
5		0.0945	0.0309	0.3395	0.0309
6		0.0965	0.0313	0.3457	0.0313
7		0.0950	0.0305	0.3370	0.0305
8		0.0954	0.0309	0.3345	0.0309
9		0.0961	0.0311	0.3348	0.0311
Mean±SD*		**0.09580±0.0007**	**0.0308±0.0004**	**0.3382±0.0035**	**0.0308±0.0004**
% RSD		**0.7651**	**1.2393**	**1.0426**	**1.2393**

1	ALI 20 µg/ml AMLO 20 µg/ml	0.1943	0.0608	0.6648	0.0608
2		0.1947	0.0614	0.6682	0.0614
3		0.1935	0.0625	0.6653	0.0625
4		0.1954	0.0611	0.6623	0.0611
5		0.1956	0.0616	0.6657	0.0616
6		0.1935	0.0615	0.6721	0.0615
7		0.1947	0.0628	0.6628	0.0628
8		0.1935	0.0621	0.6748	0.0621
9		0.1937	0.0634	0.6674	0.0634
Mean±SD*		**0.1943±0.0008**	**0.0619±0.0009**	**0.6670±0.0041**	**0.0619±0.0009**
% RSD		**0.4265**	**1.3764**	**0.6217**	**1.3764**
Mean % RSD		**0.5958**	**1.3078**	**0.8322**	**1.3078**

**mean±SD, (n= 3) number of determination*

Table No. 6.11.5: Results of inter-day precision

Inter-day precision						
Sr. No.		**Conc.**	**Absorbance**			
			ALI		**AMLO**	
			237 nm	**271 nm**	**237 nm**	**271 nm**
1	Day 1	ALI 10 µg/ml AMLO 10 µg/ml	0.0953	0.0309	0.3391	0.0309
2			0.0965	0.0311	0.3395	0.0311
3			0.0935	0.0304	0.3348	0.0304
4	Day 2		0.0943	0.0315	0.3346	0.0315
5			0.0948	0.0314	0.3374	0.0314
6			0.0957	0.0302	0.3372	0.0302
7	Day 3		0.0954	0.0308	0.3457	0.0308
8			0.0935	0.0309	0.3356	0.0309
9			0.0947	0.0318	0.3351	0.0318
Mean±SD*			**0.0949±0.0010**	**0.0310±0.0005**	**0.3377±0.0035**	**0.0310±0.0005**
% RSD			**1.0491**	**1.6606**	**1.0403**	**1.6606**
1	Day 1	ALI 20 µg/ml AMLO 20 µg/ml	0.1953	0.0624	0.6718	0.0624
2			0.1928	0.0625	0.6638	0.0625
3			0.1943	0.0611	0.6778	0.0611
4	Day 2		0.1947	0.0615	0.6608	0.0615
5			0.1935	0.0628	0.6748	0.0628
6			0.1934	0.0621	0.6682	0.0621
7	Day 3		0.1947	0.0625	0.6653	0.0625
8			0.1964	0.0611	0.6673	0.0611
9			0.1937	0.0635	0.6687	0.0635
Mean±SD*			**0.1943±0.0011**	**0.0622±0.0008**	**0.6687±0.0054**	**0.0622±0.0008**
% RSD			**0.5683**	**1.2944**	**0.8015**	**1.2944**
Mean % RSD			**0.8087**	**1.4775**	**0.9209**	**1.4775**

**mean±SD, (n= 3) number of determination*

6.11.8 Accuracy

The results of recovery studies ranged from 98-102% for both the drugs showing the accuracy of the method. This indicates that there is no interference from tablet excipients (Table No. 6.11.6).

Table No. 6.11.6: Results of recovery studies

Accuracy (% Recovery)				
ALI				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard drug added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	10	5	5.0006	100.0115
50	15	7.5	7.5962	101.2821
50	20	10	9.8288	98.2882
100	10	10	10.0776	100.7760
100	15	15	15.0085	100.0564
100	20	20	20.5744	102.8720
150	10	15	15.0105	100.0700
150	15	22.5	23.1587	102.9277
150	20	30	30.6774	102.2581
Mean±SD*				**100.9491±1.5409**
% RSD				**1.5265**
AMLO				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard drug added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	10	5	5.0394	100.7875
50	15	7.5	7.5118	100.1580
50	20	10	10.2190	102.1901
100	10	10	9.9329	99.3292
100	15	15	14.9111	99.4076
100	20	20	19.7352	98.6759
150	10	15	14.9706	99.8039
150	15	22.5	22.2136	98.7273
150	20	30	29.6694	98.8982
Mean±SD*				**99.7753±1.1409**
% RSD				**1.1435**

**mean±SD, (n= 3) number of determination*

6.11.9 LOD and LOQ

The values of LOD and LOQ were found to be very low which proves the sensitivity of the method is shown in Table No. 6.11.8.

6.11.10 Robustness

The proposed method was checked for robustness study, but no significant changes (% RSD<2) found in absorption, indicating that the proposed method is robust. (Table No. 6.11.7)

Table No. 6.11.7: Results of robustness study

Parameter	Drugs			
Wavelengths (237 & 280 ±1 nm)	ALI		HCT	
	Assay (%)*	% RSD	Assay (%)*	% RSD
236 & 279 nm	98.3255	**1.7410**	97.6875	**1.4639**
237 & 280 nm	99.7587		99.4545	
238 & 281 nm	96.3554		100.5684	

** (n= 3) number of determination*

6.11.11 Stability of the solution

Solution stability was performed at room temperature and it was found to be stable up to two days.

Table No. 6.11.8: Summary of validation parameters for the proposed method

Parameters	ALI		AMLO	
Detection wavelengths (nm)	237	271	237	271
Linearity range (µg/ml)	1-50			
Correlation coefficient	0.9999	0.9998	1	0.9998
Regression equation	y = 0.0097x - 0.0001	y = 0.0031x - 0.0001	y = 0.0331x - 0.0025	y = 0.0031x - 0.0001
Precision (%RSD)				
Repeatability of measurement (n=6)	0.7833	0.9842	0.3914	1.1507
Intra-day (n=3)	0.5958	1.3078	0.8322	1.3078
Inter-day (n=3)	0.8087	1.4775	0.9209	1.4775
Accuracy				
% Recovery (n=3)	100.9491±1.5409		99.7753±1.1409	
%RSD (n=3)	1.5265		1.1435	
Specificity	No interference			
LOD (µg/ml)	0.2329	0.1197	0.0959	0.1197
LOQ (µg/ml)	0.7059	0.3629	0.2908	0.3629

**mean±SD, n= number of determinations*

6.11.12 Analysis of marketed formulation

The proposed method was successfully used for the quantitative determination of ALI and AMLO in commercial formulation (Tekamlo tablet: 300 mg of ALI and 10 mg of AMLO). Six replicate determinations were carried out and experimental values were found to be within 97-101%v/v for both the drugs and hence the developed method can be used for the simultaneous determination of both the drugs in combined formulation **(**Table No. 6.11.9). Overlain spectra of standard drugs and formulation are shown in Figure No. 6.11.10.

Table No. 6.11.9: Result of formulation analysis

Drugs	Amount (mg/tablet)		% Drug found*	% RSD
	Labelled	Found		
ALI	300	295.37	98.4581±1.1763	1.1947
AMLO	10	9.97	99.7429±1.2965	1.2999

**mean ± SD (n=6) average of six determinations*

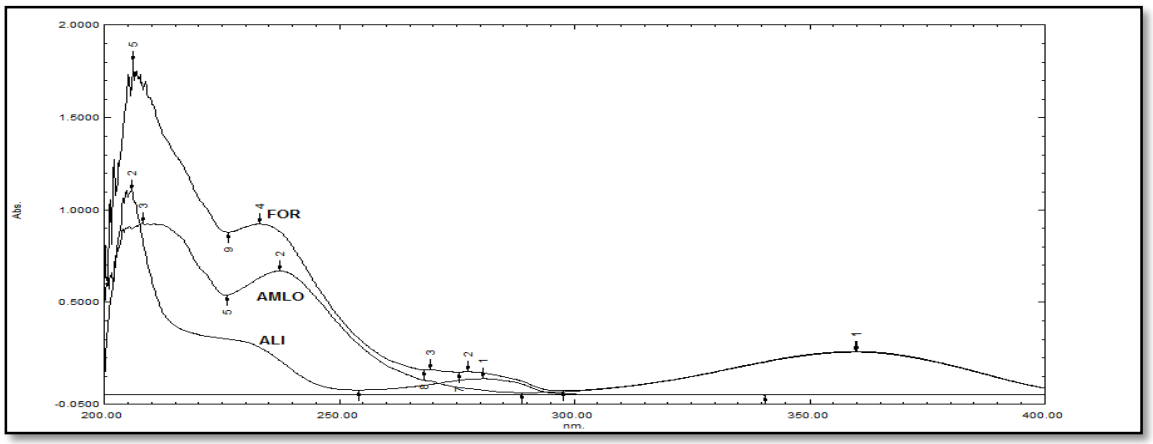


Figure No. 6.11.10: Overlain UV spectra of ALI, AMLO (20 µg/ml) and formulation (ALI & AMLO-20 µg/ml)

In order to check the applicability of the method, all the available strength (ratio) of marketed formulations were analyzed using standard drug solution in optimum ratio.

Available strength (mg)

ALI + AMLO: 150/300 + 5/10

Study (%assay) suggests that, the proposed method can be applied to all the formulations of different strengths available in the market.

METHOD 12

6.12 *"Development and validation of first-derivative (Zero crossing) spectroscopic method for the simultaneous determination of aliskiren hemifumarate and amlodipine besilate in tablets"*

Estimation of ALI and AMLO was achieved by first derivative spectroscopic method using Shimadzu UV-1800 (UV Pro), double beam UV-visible spectrophotometer.

6.12.1 Selection of solvent

Based on the literature survey and solubility studies methanol was selected as solvent, which showed higher derivative signal and favourable zero crossing points for both the drugs**.**

6.12.2 Selection of wavelength

Standard solutions of ALI & AMLO (10 µg/ml) were separately scanned in the UV region (200-400 nm) and spectra were recorded using methanol as blank. Both the spectra were converted into first and second derivative spectra. Based on the spectral pattern and zero crossing points, first derivative method was selected for the study. First derivative spectra showed typical zero-crossing points at 254 nm for ALI and 237 nm for AMLO. From the overlain spectra, 237 nm and 254 nm were selected for further studies are shown in Figure No. 6.12.1.

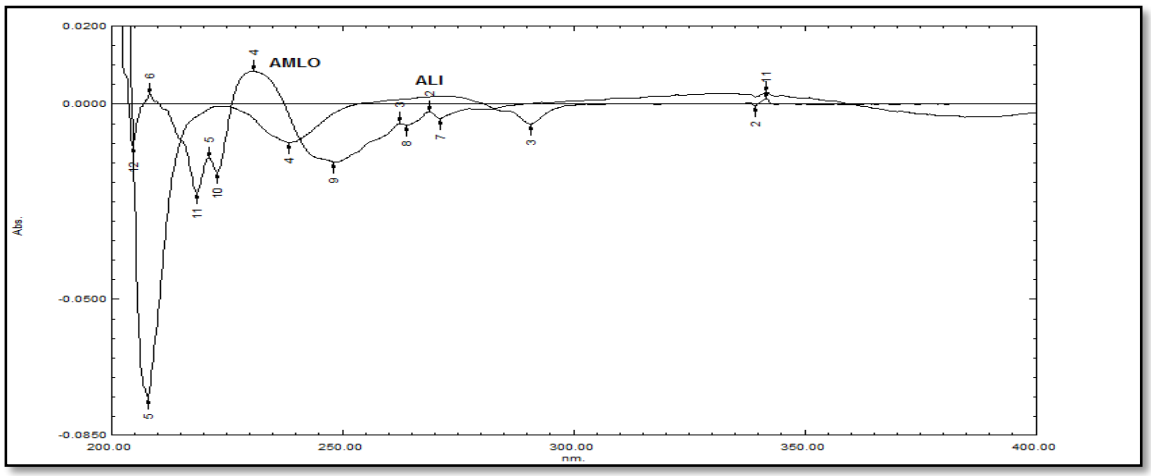


Figure No. 6.12.1: Overlain 1st derivative UV spectra of ALI and AMLO (10 µg/ml)

At 254 nm, ALI showed zero absorbance but AMLO had considerable absorbance. Similarly at 237 nm, AMLO showed zero absorbance but ALI had considerable absorbance.

Table No. 6.12.1: Selection of zero crossing points for ALI & AMLO

Drugs	Zero crossing point (nm)	Detection wavelength (nm)
ALI	254	237
AMLO	237	254

6.12.3 Preparation of calibration curve

A calibration curve (Figure 6.12.6-6.12.7) was plotted for both ALI and AMLO in the range of 0.5 to 50 µg/ml (Table No. 6.12.2). Results were subjected to regression analysis by least square method to calculate the values of slope, intercept and correlation coefficient of both the drugs are shown in Table No. 6.12.8.

Table No. 6.12.2: Linearity data of 1st derivative signal of ALI & AMLO at selected wavelengths

Sr. No.	ALI at 237 nm			AMLO at 254 nm		
	Conc. (µg/ml)	1st derivative signal *	% RSD	Conc. (µg/ml)	1st derivative signal *	% RSD
1	0.5	0.0010	0.8325	0.5	0.0007	1.2963
2	5	0.0052	1.5602	5	0.0056	1.5972
3	10	0.0095	0.9415	10	0.0116	0.9459
4	20	0.0185	1.0481	20	0.0234	0.7355
5	30	0.0278	0.5298	30	0.0349	0.5924
6	40	0.0374	0.8948	40	0.0474	1.7897
7	50	0.0458	0.8782	50	0.0589	1.1160

**average of six determinations*

6.12.4 Validation of the method

The developed method was validated in accordance with "*International Conference on Harmonization"* guidelines for validation of analytical procedures.

6.12.5 Specificity

Overlain spectra of tablet excipients and drug solution indicate that there was no interference between excipients and standard drugs (Figure No. 6.12.2).

6.12.6 Linearity and range

ALI and AMLO were found to be linear in the concentration range of 0.5-50 µg/ml. Overlain spectra of ALI and AMLO are shown in Figure No. 6.12.3-6.12.5.

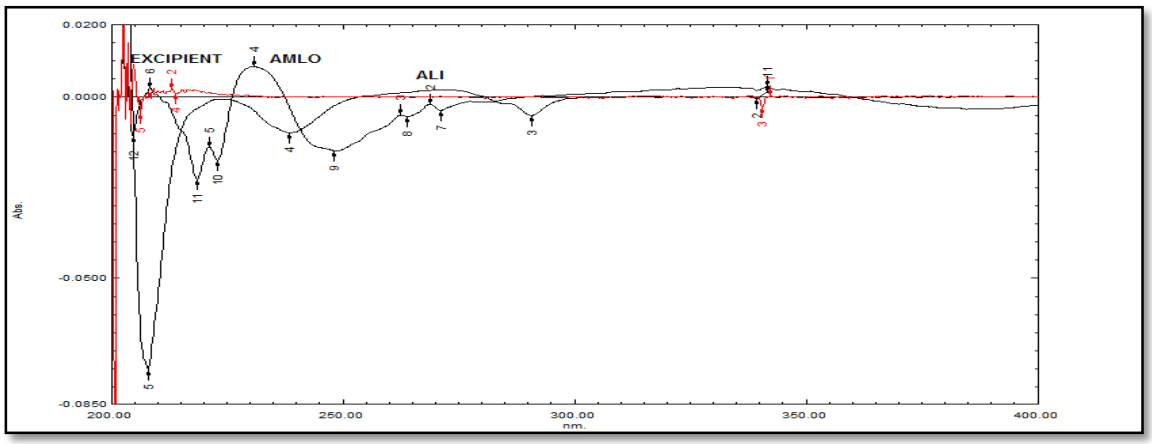


Figure No. 6.12.2: Overlain 1st derivative UV spectra of formulation excipients and standard drugs

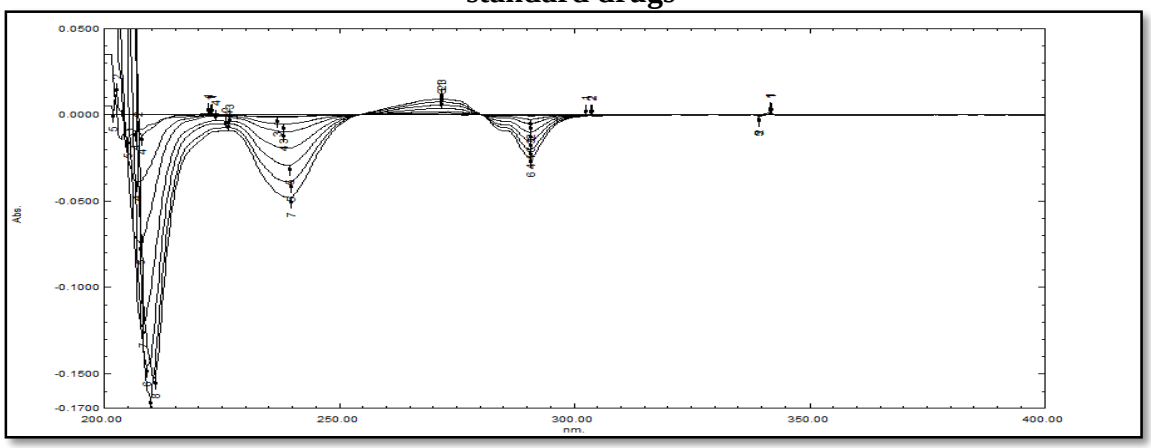


Figure No. 6.12.3: Overlain 1st derivative UV spectra of ALI (0.5-50 µg/ml)

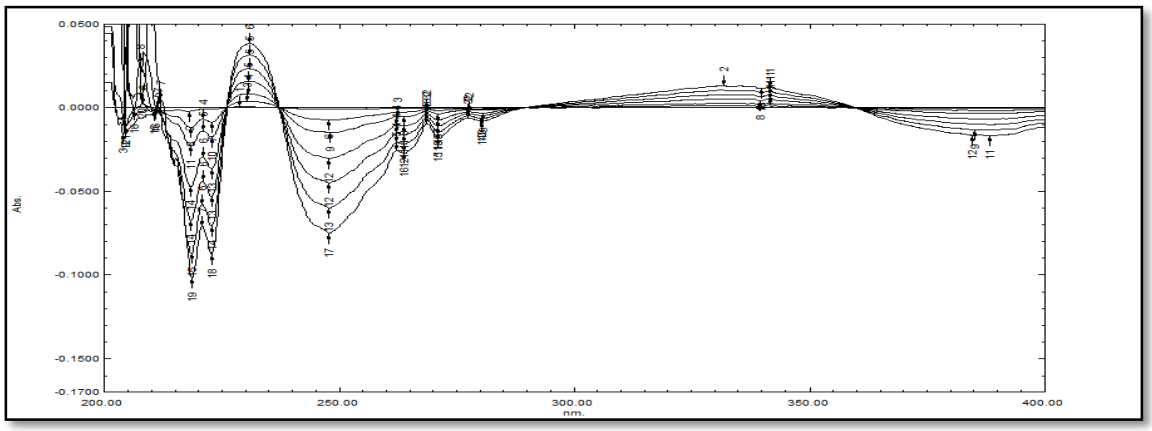


Figure No. 6.12.4: Overlain 1st derivative UV spectra of AMLO (0.5-50 µg/ml)

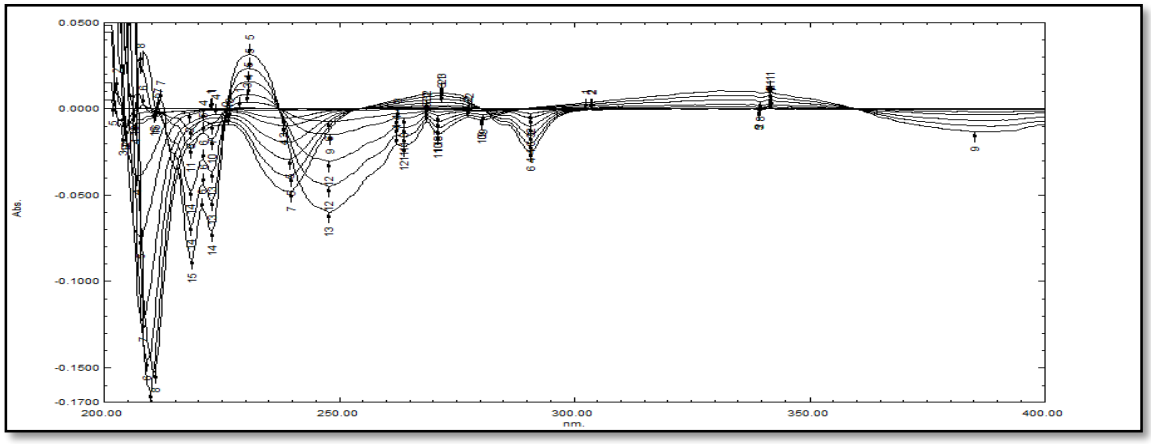


Figure No. 6.12.5: Overlain 1st derivative UV spectra of ALI & AMLO (0.5-50 µg/ml)

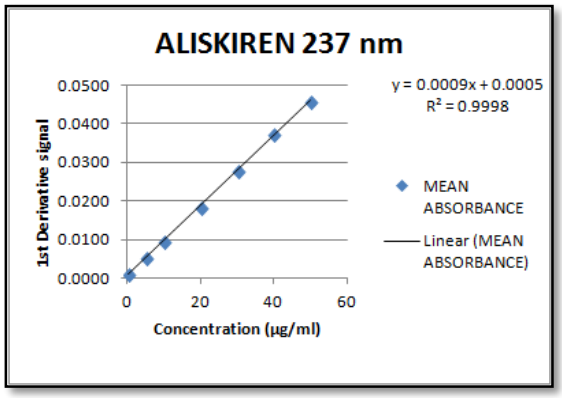


Figure No. 6.12.6: Calibration graph of ALI (0.5-50 µg/ml) at 237 nm

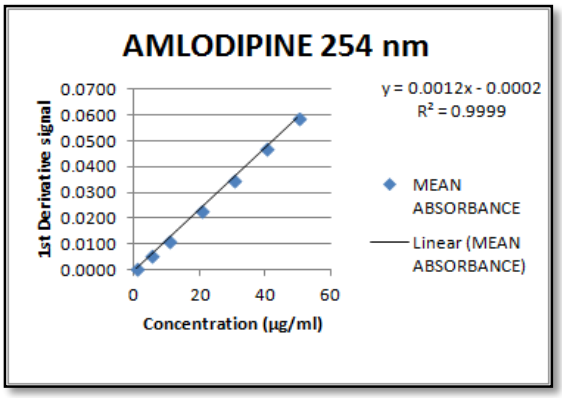


Figure No. 6.12.7: Calibration graph of AMLO (0.5-50 µg/ml) at 254 nm

6.12.7 Precision

Results of precision studies expressed in % RSD follows ICH guideline acceptable limits (% RSD<2), which shows good repeatability and low inter-day variability, indicating an excellent precision of the developed method (Table No. 6.12.3, 6.12.6 & 6.12.5).

Table No. 6.12.3: Results of repeatability of measurement

Repeatability			
Sr. No.	Conc.	1st derivative signal	
		ALI (237 nm)	AMLO (254 nm)
1	ALI 10 µg/ml	0.0098	0.0118
2		0.0095	0.0117
3		0.0095	0.0114
4		0.0096	0.0117

5	AMLO 10 µg/ml	0.0094	0.01159
6		0.0095	0.0119
Mean±SD*		**0.0096±0.0001**	**0.0117±0.0002**
% RSD		**1.4434**	**1.4831**
1	ALI 20 µg/ml	0.0185	0.0231
2		0.0186	0.0235
3		0.0187	0.0236
4		0.0186	0.0234
5	HCT 20 µg/ml	0.0189	0.0241
6		0.0185	0.0242
Mean±SD*		**0.0186±0.0002**	**0.0237±0.0004**
% RSD		**0.8080**	**1.7889**
Mean % RSD		**1.1257**	**1.6360**

**mean±SD, (n= 6) number of determination*

Table No. 6.12.4: Results of intra-day precision

Intra-day precision			
		1st derivative signal *	
Sr. No.	**Conc.**	**ALI (237 nm)**	**AMLO (254 nm)**
1	ALI 10 µg/ml AMLO 10 µg/ml	0.0094	0.0114
2		0.0095	0.0117
3		0.0096	0.0116
4		0.0094	0.0117
5		0.0095	0.0113
6		0.0094	0.0114
7		0.0096	0.0117
8		0.0097	0.0116
9		0.0091	0.0115
Mean±SD*		**0.0095±0.0002**	**0.0115±0.0002**
% RSD		**1.8296**	**1.3001**
1	ALI 20 µg/ml AMLO 20 µg/ml	0.0187	0.0238
2		0.0186	0.0239
3		0.0189	0.0231
4		0.0184	0.0235
5		0.0182	0.0236
6		0.0183	0.0234
7		0.0183	0.0238
8		0.0184	0.0241
9		0.0185	0.0238
Mean±SD*		**0.0185±0.0002**	**0.0237±0.0003**
% RSD		**1.2034**	**1.2676**
Mean % RSD		**1.5165**	**1.2839**

**mean±SD, (n=3) number of determinations*

Table No. 6.12.5: Results of inter-day precision

Inter-day precision				
Sr. No.		**Conc.**	**1st derivative signal ***	
			ALI	**AMLO**
			237 nm	**254 nm**
1		ALI 10 µg/ml AMLO 10 µg/ml	0.0094	0.0114
2			0.0094	0.0113
3			0.0095	0.0114
4			0.0094	0.0117
5			0.0095	0.0116
6			0.0096	0.0117
7			0.0095	0.0118
8			0.0096	0.0116
9			0.0098	0.0117
Mean±SD*			**0.0095±0.0001**	**0.0116±0.0002**
% RSD			**1.3670**	**1.4811**
1		ALI 20 µg/ml AMLO 20 µg/ml	0.0187	0.0237
2			0.0186	0.0237
3			0.0189	0.0234
4			0.0183	0.0231
5			0.0185	0.0235
6			0.0185	0.0236
7			0.0184	0.0229
8			0.0182	0.0241
9			0.0189	0.0242
Mean±SD*			**0.0186±0.0002**	**0.0236±0.0004**
% RSD			**1.3231**	**1.7841**
Mean % RSD			**1.3451**	**1.6326**

**mean±SD, (n=3) number of determinations*

6.12.8 Accuracy

The results of recovery studies ranged from 98-102% for both the drugs showing the accuracy of the method (Table No. 6.12.6). This indicates that there is no interference from tablet excipients.

Table No. 6.12.6: Results of recovery studies

Accuracy (% Recovery)				
ALI				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard drug added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	10	5	5.1111	102.2222
50	15	7.5	7.4444	99.2593
50	20	10	10.1111	101.1111
100	10	10	9.8889	98.8889
100	15	15	14.7778	98.5185
100	20	20	20.3333	101.6667
150	10	15	15.2222	101.4815
150	15	22.5	22.4444	99.7531
150	20	30	30.3333	101.1111
Mean±SD				**100.4458±1.3523**
% RSD				**1.3463**
AMLO				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard drug added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	10	5	4.9167	98.3333
50	15	7.5	7.4167	98.8889
50	20	10	10.1667	101.6667
100	10	10	10.2500	102.5000
100	15	15	14.9167	99.4444
100	20	20	20.3333	101.6667
150	10	15	15.2500	101.6667
150	15	22.5	22.8333	101.4815
150	20	30	29.9167	99.7222
Mean±SD				**100.5967±1.4988**
% RSD				**1.4899**

**mean±SD, (n= 3) number of determination*

6.12.9 LOD and LOQ

The values of LOD and LOQ were found to be very low which proves the sensitivity of the method is shown in Table No. 6.12.8.

6.12.10 Robustness

The proposed method was checked for robustness study, but no significant changes (% RSD<2) found in 1st derivative signal, indicating that the proposed method is robust (Table No. 6.12.7).

Table No. 6.12.7: Results of robustness study

Parameter		Drugs			
Wavelengths(±1 nm)		ALI		AMLO	
ALI	AMLO	Assay (%)*	% RSD	Assay (%)*	% RSD
236	253	98.6554	**1.5534**	98.3651	**1.6710**
237	254	96.6558		101.6554	
238	255	99.6554		100.5478	

**(n= 3) number of determination*

6.12.11 Stability of the solution

Solution stability was performed at room temperature and it was found to be stable up to two days.

Table No. 6.12.8: Summary of validation parameters for the proposed method

Parameters	ALI	AMLO
Detection wavelengths (nm)	237	254
Linearity range (µg/ml)	0.5-50	
Correlation coefficient	0.9998	0.9999
Regression equation	y = 0.0009x + 0.0005	y = 0.0012x - 0.0002
Precision (%RSD)		
Repeatability of measurement (n=6)	1.1257	1.6360
Intra-day (n=3)	1.5165	1.2839
Inter-day (n=3)	1.3451	1.6326
Accuracy		
% Recovery (n=3)	100.4458±1.3523	100.5967±1.4988
%RSD (n=3)	1.3463	1.4899
Specificity	No interference	
LOD (µg/ml)	0.1366	0.1296
LOQ (µg/ml)	0.4141	0.3928

**mean±SD, n= number of determinations*

6.12.12 Analysis of marketed formulation

The proposed method was successfully used for the quantitative determination of ALI and AMLO in commercial formulation (Tekamlo tablet: 300 mg of ALI and 10 mg of AMLO). Six replicate determinations were carried out and experimental values were found to be 97-100% for both the drugs and hence the developed method can be used for the simultaneous determination of both the drugs in combined formulation (Table No.

6.12.9). Overlain spectra of standard drugs and formulation are showed in Figure No. 6.12.8.

. Table No. 6.12.9: Results of formulation analysis

Drugs	Amount (mg/tablet)		% Drug* found	% RSD
	Labelled	Found		
ALI	300	295.98	98.6617±1.5378	1.5586
AMLO	10	9.91	99.1293±1.0521	1.0614

**mean ± SD (n=6) average of six determinations*

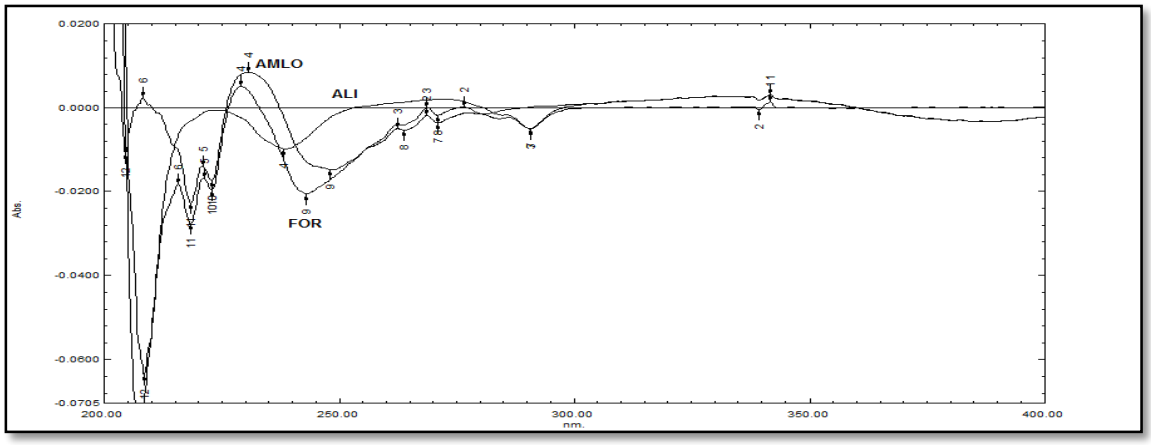


Figure No. 6.12.8: Overlain 1st UV spectra of standard ALI & AMLO (20 µg/ml) & FOR (20 & 20 µg/ml)

In order to check the applicability of the method, all the available strength (ratio) of marketed formulations were analyzed using standard drug solution in optimum ratio.

Available strength (mg)

ALI + AMLO: 150/300 + 5/10

Study (% assay) suggests that, the proposed method can be applied to all the formulations of different strengths available in the market.

Method 13

6.13 *"Development and validation of RP-HPLC method for the simultaneous determination of aliskiren hemifumarate, amlodipine besilate and hydrochlorothiazide in tablets"*

6.13.1 Selection of mode of chromatographic method

Reverse phase chromatography is the first choice for most regular samples. Compared to other form of liquid chromatography, reverse phase chromatography is more convenient and rugged and it produces more satisfactory results in final separation. Reverse phase chromatographic technique was selected since both the drugs are polar in nature.

6.13.2 Selection of column

High performance RPC columns are efficient, stable, reproducible and compatible with wide variety of samples. Moreover, detection of analyte is easier in RPC with UV detector because of the solvents used. Based on the literature survey C_{18} column was selected.

6.13.3 Selection of wavelength

UV spectra of all the drugs were taken in RP-HPLC system and from the overlain spectra, 237 nm was selected as the wavelength for study, Figure No. 6.13.1.

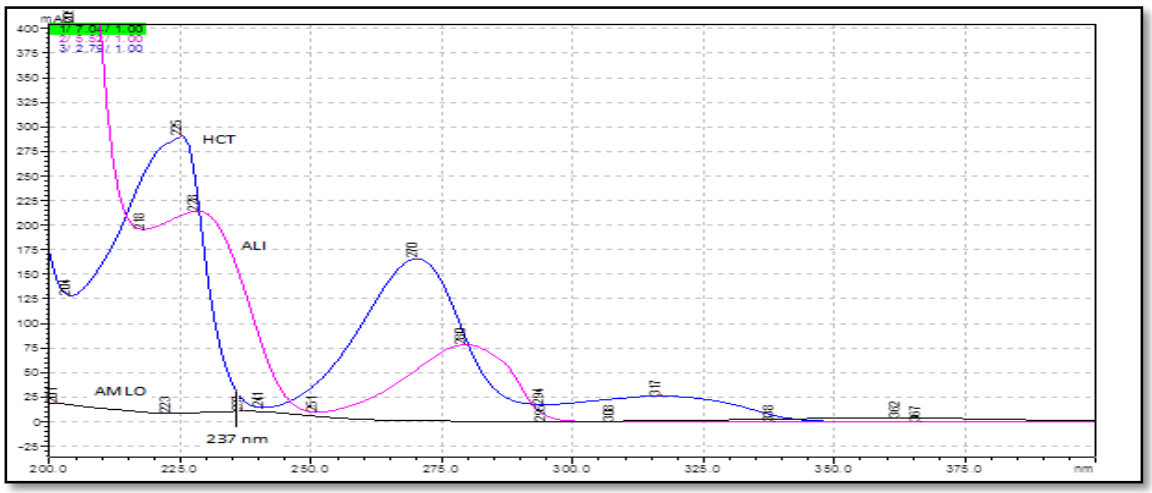


Figure No. 6.13.1: Overlain UV Spectra of ALI, AMLO and HCT on HPLC system

6.13.4 Trials for selection of mobile phase

Based on the literature survey different mobile phases with different compositions were tried and suitable mobile phase was selected for further studies (Table No 6.13.1; Figure No. 6.13.2-6.13.11).

Initial condition:

Stationary phase	:	Enable C_{18} column (250 × 4.6 mm, 5 µ)
Flow rate	:	1 ml/ minute
Operating temperature	:	Room temperature
Selected wavelength	:	237 nm

Table No. 6.13.1: Trials for selection of mobile phase

Sr. No.	Mobile Phase	Observation	Remarks	Fig. No.
1	Sodium phosphate (pH 3): Acetonitrile (60:40 %v/v)	Overlapping peaks without separation	Not satisfactory	6.13.2
2	Sodium phosphate (pH 3): Acetonitrile (65:35 %v/v)	Broad peak with tailing	Not satisfactory	6.13.3
3	20 mM Phosphate buffer (pH 4.6): Methanol (25:75 %v/v)	Fronting with split peak of ALI	Not satisfactory	6.13.4
4	0.2% TEA (pH 3): Acetonitrile (50:50 %v/v)	Overlapping peaks with bad shape	Not satisfactory	6.13.5
5	0.1% TEA (pH 3): Methanol (50:50 %v/v)	All the drugs merged together (no separation)	Not satisfactory	6.13.6
6	0.2% TEA pH 3: Methanol (70:30 %v/v)	Peak shape was not good with less separation	Not satisfactory	6.13.7
7	0.2% TEA (pH 3): Methanol (30:70 %v/v)	Broad peaks with less separation	Not satisfactory	6.13.8
8	0.2% TEA (pH 3): Methanol (20:80 %v/v)	Overlapping peaks with tailing	Not satisfactory	6.13.9
9	0.2% TEA (pH 5): Methanol (10:90 %v/v)	Good peaks with less separation	Improved	6.13.10
10	**0.2% TEA (pH 6): Methanol (10:90 %v/v)**	**Optimum peak parameters**	**Satisfactory**	**6.13.11**

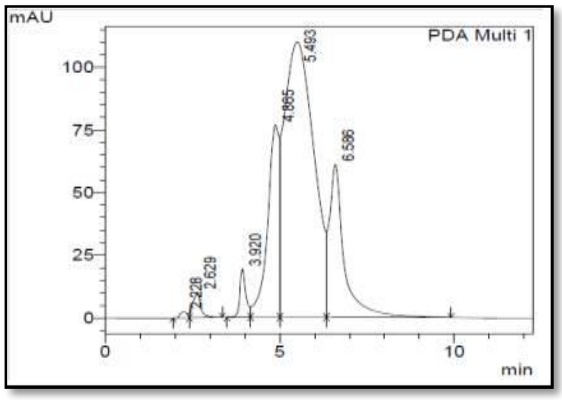


Figure No. 6.13.2: Sodium phosphate (pH 3): acetonitrile (60:40 %v/v)

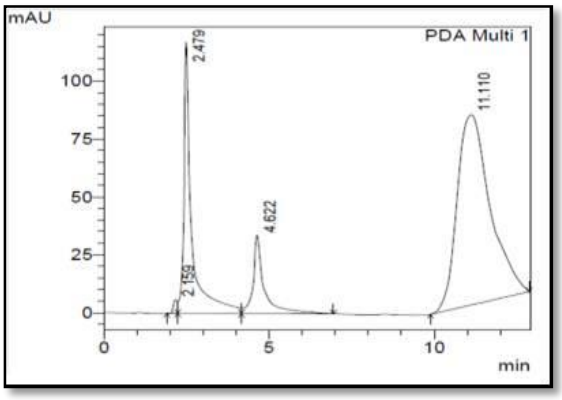


Figure No. 6.13.3: Sodium phosphate (pH 3): acetonitrile (65:35 %v/v)

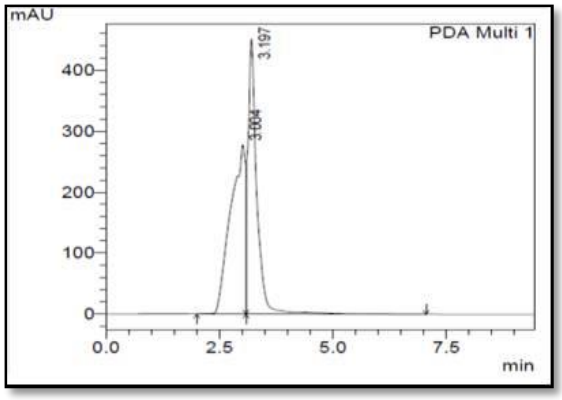


Figure No. 6.13.4: 20 mM Phosphate buffer (pH 4.6): methanol (25:75 %v/v)

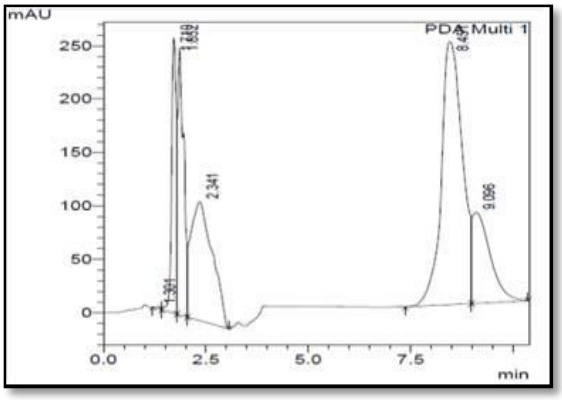


Figure No. 6.13.5: 0.2% TEA (pH 3): acetonitrile (50:50 %v/v)

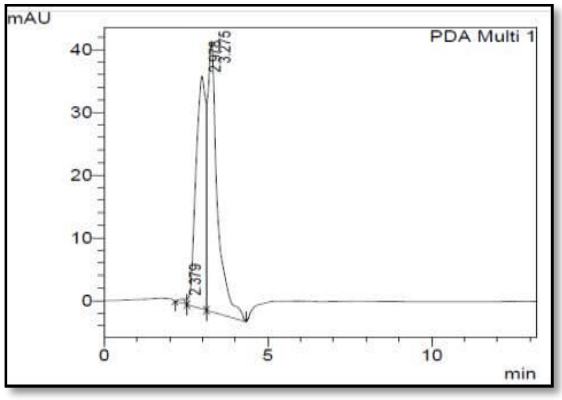


Figure No. 6.13.6: 0.1% TEA (pH 3): methanol (50:50 %v/v)

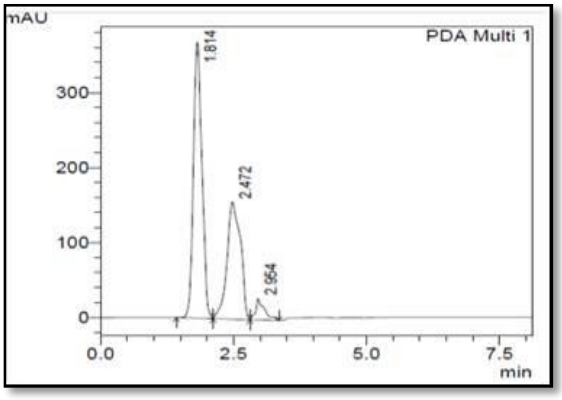


Figure No. 6.13.7: 0.2% TEA pH 3: methanol (70:30 %v/v)

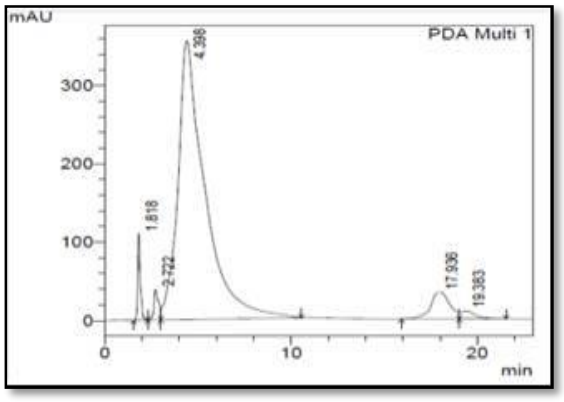


Figure No. 6.13.8: 0.2% TEA (pH 3): methanol (30:70 %v/v)

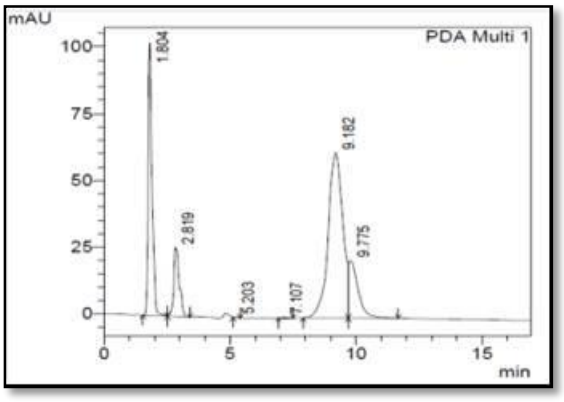


Figure No. 6.13.9: 0.2% TEA (pH 3): methanol (20:80 %v/v)

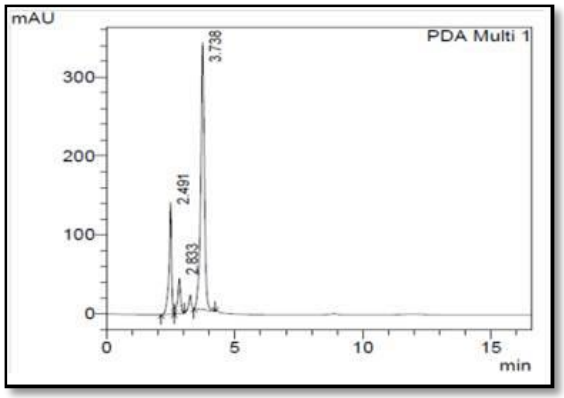


Figure No. 6.13.10: 0.2% TEA (pH 5): methanol (10:90 %v/v)

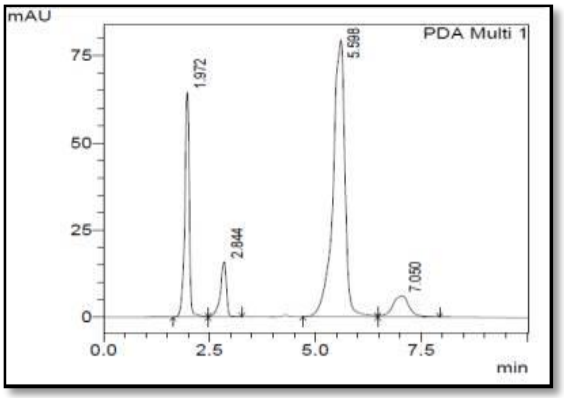


Figure No. 6.13.11: 0.2% TEA (pH 6): methanol (10:90 %v/v)

6.13.5 Optimization of separation conditions

The chromatographic conditions were optimized to achieve the best resolution, peak shape, theoretical plate for all the analytes under investigation. Initially several proportion of buffer (acetate, phosphate etc.), acetonitrile and methanol were tried to achieve optimum separation of all the analytes under study. Based on the preliminary trials triethylamine in water and methanol in combination was selected for further studies. Strength of buffer (0.1-0.3%), mobile phase composition, pH (3-7), flow rate (0.8-1.2) etc. were varied to get optimum chromatographic conditions which can produce acceptable results based on the peak parameters. Finally the separation of components were achieved on Enable C_{18} column with mobile phase system consisting of 0.2% triethylamine in water (pH 6 was adjusted with orthophosphoric acid) and methanol

(10:90%v/v) at a flow rate of 1 ml/min was employed with PDA detection at 237 nm which gave satisfactory separation and peak symmetry. The optimized RP-HPLC method was validated and successfully applied for the quantitative determination of ALI, AMLO and HCT in commercial formulation (Amturnide tablet: 300 mg of ALI, 10 mg of AMLO and 25 mg of HCT).

6.13.6 Fixed chromatographic condition

Stationary phase	:	Enable C_{18} column (250 x 4.6 mm, 5 µm, 120 Å)
Mobile phase	:	0.2%v/v triethylamine in water (pH 6 with OPA) and methanol
Solvent ratio	:	10: 90%v/v
Detection wavelength	:	237 nm
Flow rate	:	1 ml/ minute
Operating pressure	:	109 kgf
Temperature	:	Room temperature

The retention time of ALI, AMLO and HCT were found to be 5.520±0.0229, 6.952±0.0539 and 2.794±0.0071 min, respectively, are shown in Figure No. 6.13.12.

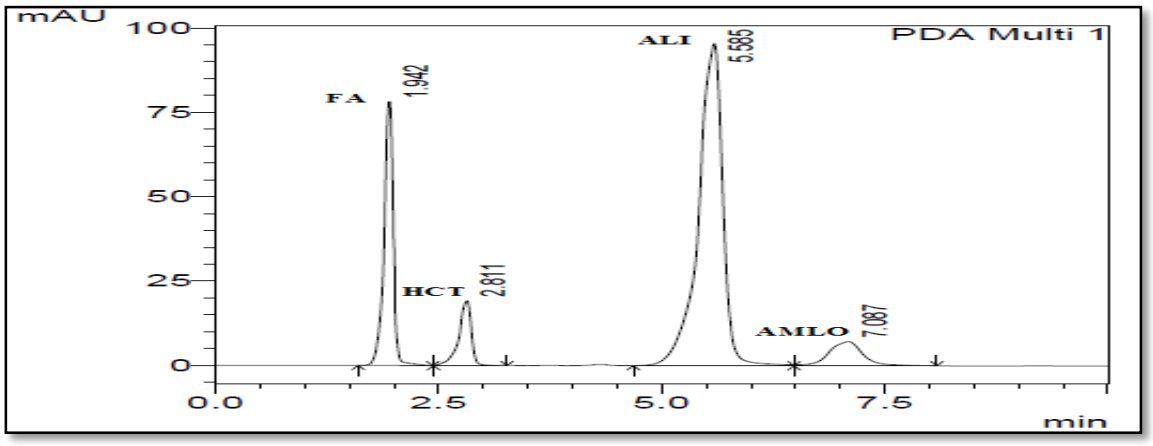


Figure No. 6.13.12: RP-HPLC chromatogram of ALI (180 µg/ml), AMLO (6 µg/ml) and HCT (15 µg/ml)

6.13.7 Validation of the method

The developed method was validated in accordance with "*International Conference on Harmonization"* guidelines for validation of analytical procedures.

6.13.8 Specificity

No interfering peaks were found within the stipulated run time, which shows the specificity of the method (Figure No. 6.13.13).

6.13.9 Linearity and range

ALI, AMLO and HCT were found to be linear in the concentration range of 7.5-300, 0.25-10 and 0.625-25 µg/ml, respectively (Table No. 6.13.2, Figure No. 6.13.18-6.13.24). Calibration curves (Figure No. 6.13.25-6.13.27) were plotted between peak area and concentration. Results were subjected to regression analysis by the least squares method to calculate the values of slope, intercept and correlation coefficient (Table No. 6.13.8).

Table No. 6.13.2: Data for calibration curve (ALI: 7.5-300, AMLO: 0.25-10 & HCT: 0.625-25 µg/ml)

Sr. No.	ALI			AMLO			HCT		
	Conc. (µg/ml)	Peak Area*	% RSD	Conc. (µg/ml)	Peak Area*	% RSD	Conc. (µg/ml)	Peak Area*	% RSD
1	7.5	72073.17	0.4558	0.25	7057.17	1.6447	0.625	7468.50	1.3046
2	15	147511.67	0.6444	0.5	14148.17	1.5329	1.25	15434.67	1.0854
3	60	595866.33	0.8470	2	58303.17	0.9826	5	62110.00	0.5788
4	120	1201673.67	0.5708	4	116715.17	0.5660	10	122239.50	0.4841
5	180	1818290.83	0.4130	6	174657.83	0.4451	15	187314.67	0.2863
6	240	2428581.50	0.4347	8	235194.67	0.3198	20	249369.83	0.3265
7	300	2997150.50	0.4332	10	295860.67	0.3820	25	313203.33	1.0559

**average of six determinations*

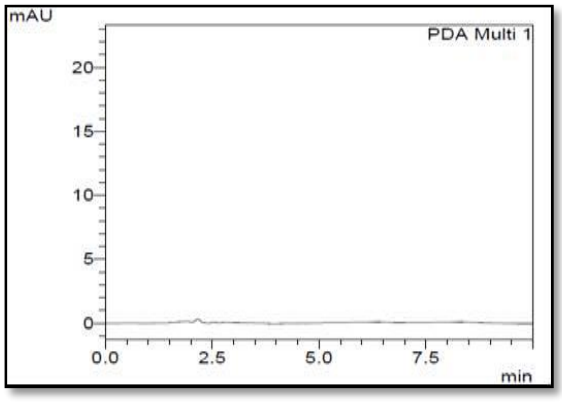


Figure No. 6.13.13: Chromatogram of excipients used in tablet formulation

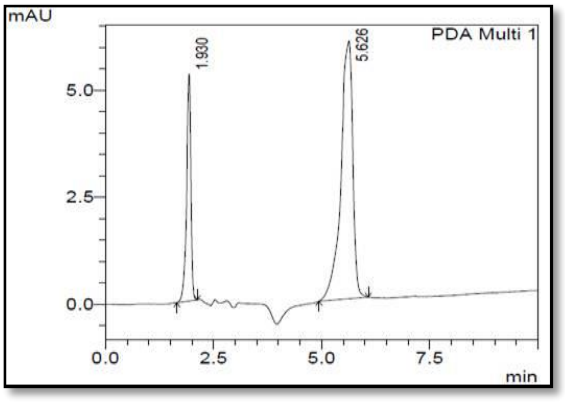


Figure No. 6.13.14: Chromatogram of ALI (10 µg/ml)

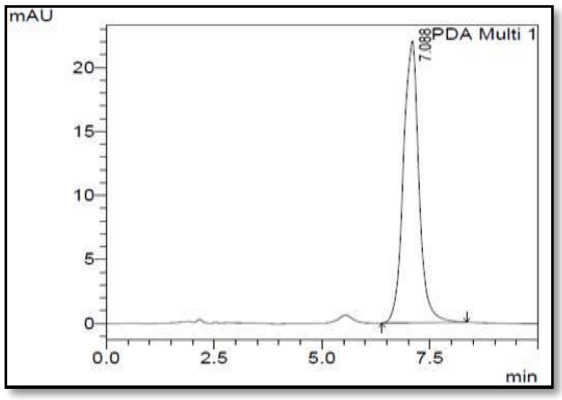


Figure No. 6.13.15: Chromatogram of AMLO (20 µg/ml)

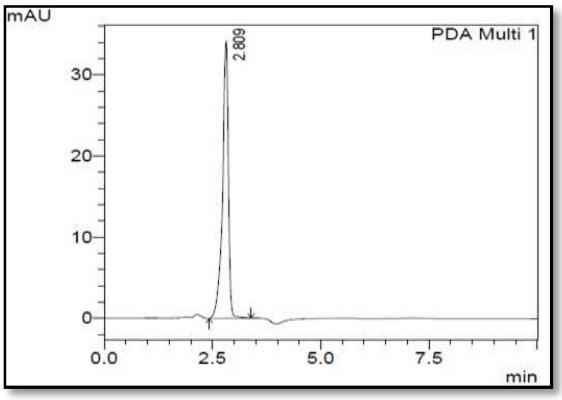


Figure No. 6.13.16: Chromatogram of HCT (25 µg/ml)

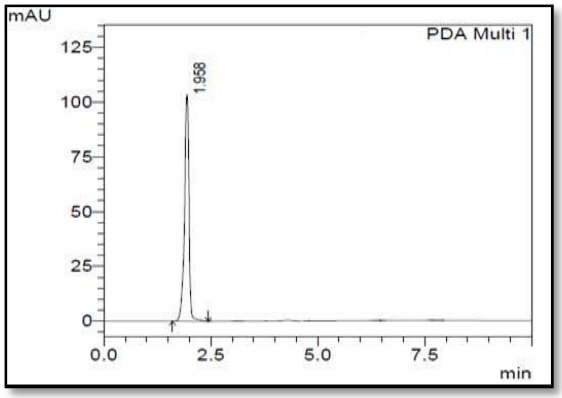


Figure No. 6.13.17: Chromatogram of benzoic acid

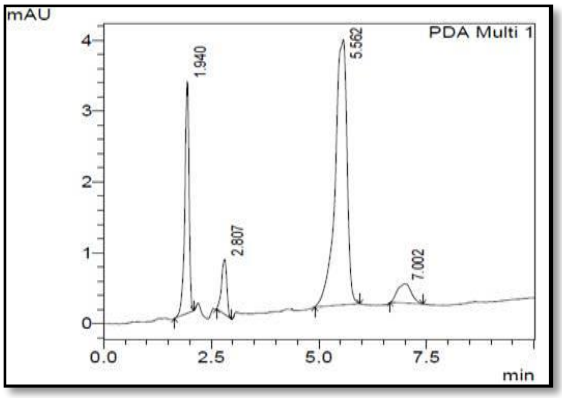


Figure No. 6.13.18: Standard chromatogram of ALI, AMLO & HCT (7.5, 0.25 & 0.625 µg/ml)

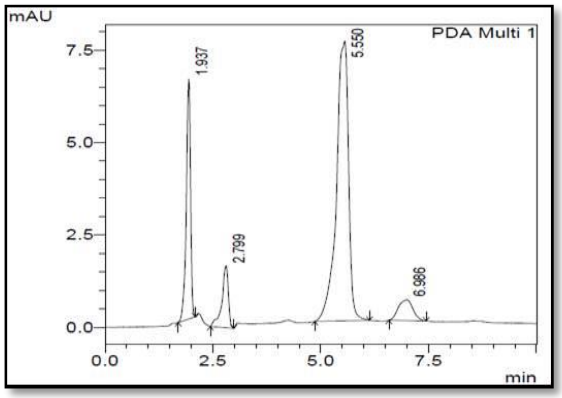


Figure No. 6.13.19: Standard chromatogram of ALI, AMLO & HCT (15, 0.5 & 1.25 µg/ml)

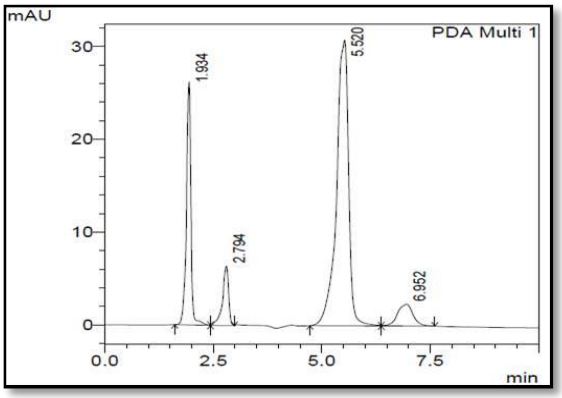


Figure No. 6.13.20: Standard chromatogram of ALI, AMLO & HCT (60, 2 & 5 µg/ml)

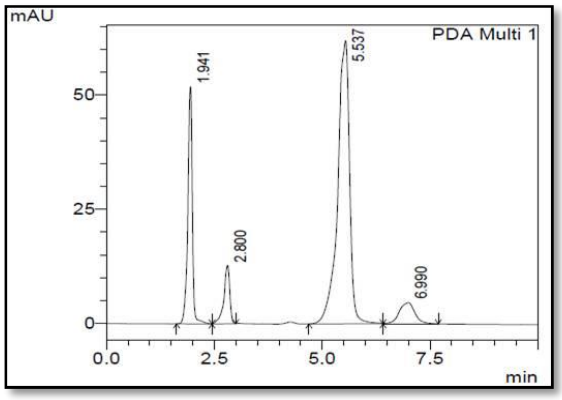


Figure No. 6.13.21: Standard chromatogram of ALI, AMLO & HCT (120, 4 & 10 µg/ml)

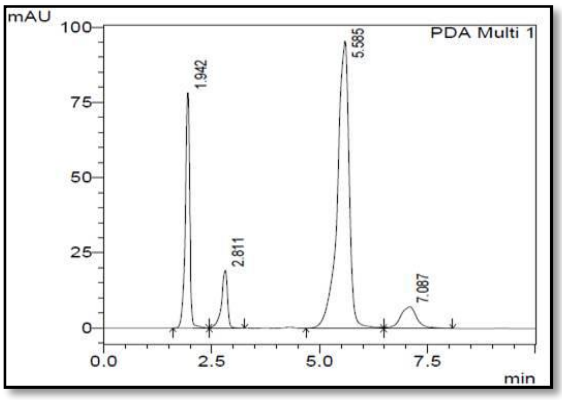


Figure No. 6.13.22: Standard chromatogram of ALI, AMLO & HCT (180, 6 & 15 µg/ml)

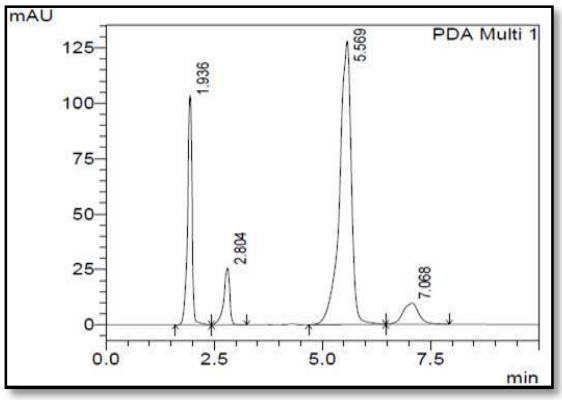


Figure No. 6.13.23: Standard chromatogram of ALI, AMLO & HCT (240, 8 & 20 µg/ml)

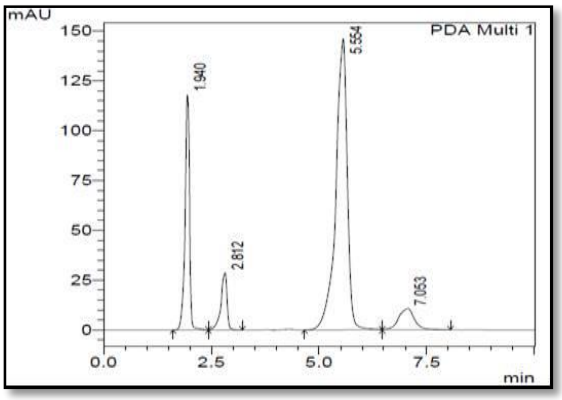


Figure No. 6.13.24: Standard chromatogram of ALI, AMLO & HCT (300, 10 & 25 µg/ml)

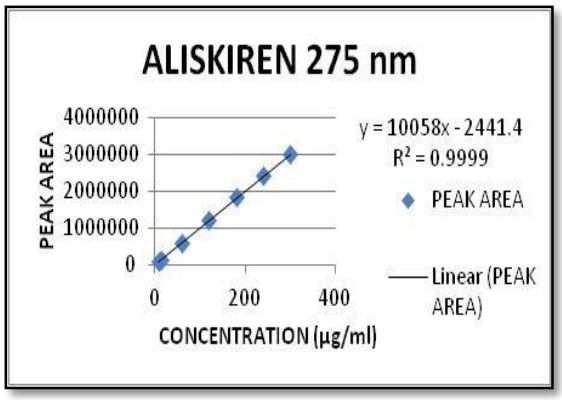


Figure No. 6.13.25: Calibration curve of ALI (7.5-300 µg/ml)

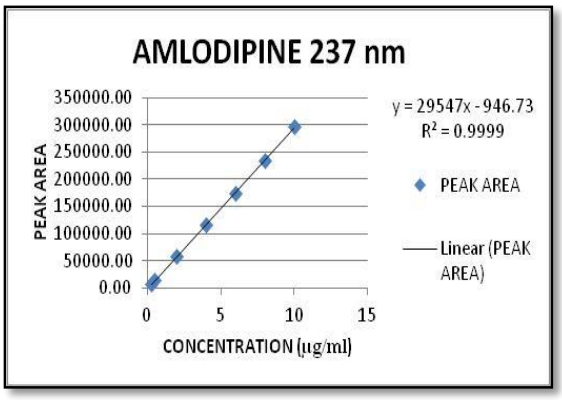


Figure No. 6.13.26: Calibration curve of AMLO (0.25-10 µg/ml)

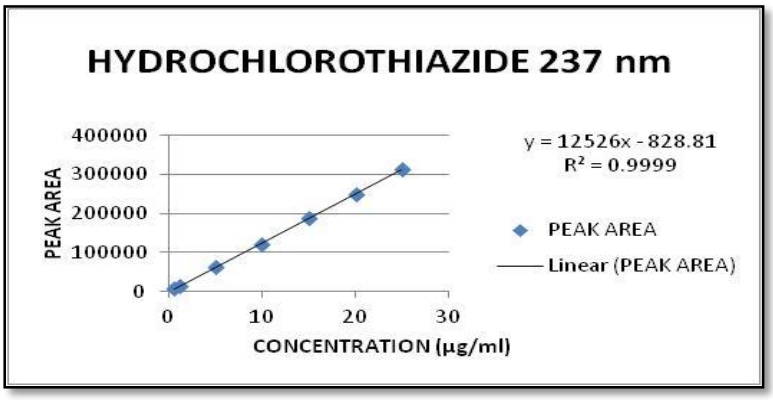


Figure No. 6.13.27: Calibration curve of HCT (0.625-25 µg/ml)

6.13.10 Precision

The precision of the method was checked by carrying out repeatability, intra-day and inter-day precision. Results of precision studies expressed in % RSD follows ICH guideline acceptable limits (%RSD <2), which shows good repeatability and low inter-day variability, indicating an excellent precision of the developed method (Table No. 6.13.3, 6.13.4 & 6.13.5).

Table No. 6.13.3: Results of repeatability of measurement

Repeatability				
Sr. No.	**Conc.**	**ALI**	**AMLO**	**HCT**
		Peak area	**Peak area**	**Peak area**
1	**ALI** 60 µg/ml **AMLO** 2 µg/ml **HCT** 5 µg/ml	595096	58475	61839
2		602484	58023	61901
3		597588	57174	62322
4		590449	58339	60937
5		610646	58574	61636
6		601984	58435	61847
Mean±SD*		**599707.83±6985.99**	**58170.00±523.19**	**61747.00±456.34**
% RSD		**1.1649**	**0.8994**	**0.7390**
1	**ALI** 120 µg/ml **AMLO** 4 µg/ml **HCT** 10 µg/ml	1197431	116435	121737
2		1204456	117012	122173
3		1194343	116546	121838
4		1203837	117038	122377
5		1210021	116903	123001
6		1207478	115984	121646
Mean±SD*		**1202927.67±5968.14**	**116653.00±411.37**	**122128.67±509.01**
% RSD		**0.4961**	**0.3526**	**0.4168**
Mean % RSD		**0.8305**	**0.6260**	**0.5779**

**mean±SD, (n= 6) number of determination*

Table No. 6.13.4: Results of intra-day precision

Intra-day precision				
Sr. No.	**Conc.**	**ALI**	**AMLO**	**HCT**
		Peak area	**Peak area**	**Peak area**
1	**ALI** 60 µg/ml **AMLO** 2 µg/ml **HCT** 5 µg/ml	603445	58394	61836
2		591443	58939	61943
3		593747	59177	61796
4		600646	58947	61955
5		596464	59174	61736
6		592370	58744	61973
7		596466	59372	61972
8		601759	58673	61077
9		590303	58747	61888
Mean±SD*		**596293.67±4760.65**	**58907.44±302.72**	**61797.33±282.80**
% RSD		**0.7984**	**0.5139**	**0.4576**
1	**ALI** 120 µg/ml **AMLO** 4 µg/ml **HCT** 10 µg/ml	1192021	116737	121838
2		1203301	117298	122938
3		1193737	116823	120983
4		1201292	117383	121882
5		1205459	117377	123777
6		1202828	117377	124737
7		1192392	117238	120838
8		1208383	116837	122828
9		1202127	117366	123838
Mean±SD*		**1200171.11±5975.69**	**117159.56±275.75**	**122628.78±1344.39**
% RSD		**0.4979**	**0.2354**	**1.0963**
Mean % RSD		**0.6481**	**0.3746**	**0.7770**

**mean±SD, (n=3) number of determinations*

Table No. 6.13.5: Results of inter-day precision

Intra-day precision					
Sr. No.		**Conc.**	**ALI**	**AMLO**	**HCT**
			Peak area	**Peak area**	**Peak area**
1	I	**ALI** 60 µg/ml **AMLO** 2 µg/ml **HCT** 5 µg/ml	597383	58747	61827
2			601646	59247	61973
3			610838	58937	61938
4	II		598373	58747	61636
5			589363	58747	61273
6			593646	58747	62087
7	III		593773	58877	62173
8			607837	58377	62273
9			603838	59377	62373
Mean±SD*			**599633.00±7043.60**	**58867.00±297.32**	**61950.33±339.94**
% RSD			**1.1747**	**0.5051**	**0.5487**
1	I	**ALI** 120 µg/ml **AMLO** 4 µg/ml **HCT** 10 µg/ml	1203424	117366	123737
2			1216535	116963	120988
3			1193737	117355	121737
4	II		1207474	117938	123737
5			1210838	118178	122882
6			1186467	119011	123727
7	III		1226461	116737	123272
8			1196368	119737	122727
9			1217373	119377	122887
Mean±SD*			**1206519.67±12813.38**	**118073.56±1084.59**	**122854.89±951.57**
% RSD			**1.0620**	**0.9186**	**0.7745**
Mean % RSD			**1.1183**	**0.7118**	**0.6616**

**mean±SD, (n= 3) number of determination*

6.13.11 Accuracy

The results of recovery studies ranged from 98-102% for all the drugs showing the accuracy of the method. This indicates that there is no interference from tablet excipients (Table No. 6.13.6).

Table No. 6.13.6: Results of recovery studies

Accuracy (% Recovery)				
ALI				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard drug added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	60	30	29.7997	99.3324
50	120	60	60.8435	101.4058
50	180	90	91.9334	102.1483
100	60	60	60.2134	100.3557
100	120	120	120.8250	100.6875
100	180	180	178.1533	98.9740
150	60	90	89.2678	99.1865
150	120	180	177.6839	98.7133
150	180	270	267.5377	99.0881
Mean±SD*				**99.9879±1.2164**
% RSD				**1.2165**
AMLO				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard drug added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	2	1	1.0088	100.8809
50	4	2	1.9771	98.8531
50	6	3	2.9837	99.4565
100	2	2	1.9602	98.0087
100	4	4	3.9503	98.7573
100	6	6	5.9920	99.8670
150	2	3	3.0488	101.6282
150	4	6	5.9704	99.5072
150	6	9	9.2927	103.2518
Mean±SD*				**100.0234±1.6347**
% RSD				**1.6343**
HCT				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard drug added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	5	2.5	2.5696	102.7852
50	10	5	5.0660	101.3192
50	15	7.5	7.4999	99.9991
100	5	5	4.9884	99.7688
100	10	10	10.1226	101.2264
100	15	15	15.0183	100.1220
150	5	7.5	7.3835	98.4461
150	10	15	14.9016	99.3439
150	15	22.5	22.4355	99.7134
Mean±SD*				**100.3027±1.2832**
% RSD				**1.2793**

**mean±SD, (n= 3) number of determination*

6.13.12 LOD and LOQ

The values of LOD and LOQ were found to be very low which proves the sensitivity of the proposed method is shown in Table No. 6.13.8.

6.13.13 Robustness

The proposed method was checked through all the parameters described earlier under robustness studies. But there were no considerable variations in the chromatographic pattern after introducing small changes in experimental condition, indicates the developed method is robust (Table No. 6.13.7).

Table No. 6.13.7: Results of robustness studies

Sr. No.	Modification	ALI (60 µg/ml)		AMLO (2 µg/ml)		HCT (5 µg/ml)	
		R_t	Peak area	R_t	Peak area	R_t	Peak area
1	Organic phase (90 ± 2%v/v)	5.454	584677	6.987	57468	2.825	62457
		5.528	596458	7.054	58143	2.884	61867
		5.615	586485	7.224	56984	2.854	62457
% RSD* (<2)		**1.4567**	**1.1923**	**1.7236**	**1.0118**	**1.0336**	**0.5471**
2	Strength of buffer (0.2 ± 0.1%v/v)	5.555	585633	6.985	58645	2.892	63124
		5.567	594575	7.154	57865	2.847	62867
		5.591	575456	7.124	59411	2.838	62545
% RSD* (<2)		**0.3290**	**1.6346**	**1.2722**	**1.3182**	**1.0119**	**0.4616**
3	Effect of pH (6 ± 0.2 unit)	5.264	595555	7.012	58645	2.837	63124
		5.394	586657	7.125	58469	2.884	62845
		5.391	575473	7.234	59631	2.921	63024
% RSD* (<2)		**1.3871**	**1.7175**	**1.5583**	**1.0630**	**1.4614**	**0.2244**
4	Effect of flow rate (1 ± 0.1 ml/min)	5.345	596554	6.898	58364	2.846	62865
		5.346	584557	7.012	58345	2.793	63145
		5.412	579198	7.125	59698	2.823	63455
% RSD* (<2)		**0.7153**	**1.5146**	**1.6187**	**1.3192**	**0.9422**	**0.4673**

** (n=3) number of determination*

6.13.14 Stability of the solution

Solution stability was performed at room temperature and it was found to be stable up to two days.

Table No. 6.13.8: Summary of validation parameters for the proposed RP-HPLC Method

Parameters	ALI	AMLO	HCT
Linearity range (µg/ml)	7.5-300	0.25-10	0.625-25
Correlation coefficient	0.9999	0.9999	0.9999
Regression equation	y = 10058x - 2441.4	y = 29547x - 946.73	y = 12526x - 828.81
Precision (%RSD)			
Repeatability of injection (n=6)	0.8305	0.6260	0.5779
Intra-day (n=3)	0.6481	0.3746	0.7770
Inter-day (n=3)	1.1183	0.7118	0.6616
Accuracy			
% Recovery (n=3)	99.9879±1.2164	100.0234±1.6347	100.3027±1.2832
%RSD (n=3)	1.2165	1.6343	1.2793
Specificity	No interference		
LOD (µg/ml)	0.8957	0.0448	0.0903
LOQ (µg/ml)	2.7142	0.1358	0.2736

**mean±SD, n= number of determinations*

6.13.15 System suitability test

System suitability tests were performed and results shown that the parameters tested were within the acceptable limit as per the ICH guidelines, indicating that the developed method is suitable for the analysis to be performed (Table No. 6.13.9).

Table No. 6.13.9: Results of system suitability studies

Parameters	Drugs			Acceptance criteria
	ALI*	AMLO*	HCT*	
Peak area reproducibility	97292.71 ± 703.68	147607.57 ± 916.27	122390 ± 1056.19	
%RSD	0.7233	0.6207	0.8630	**% RSD <2**
Retention time (Rt) min	5.552±0.0229	7.023±0.0539	2.803±0.0071	
%RSD	0.4133	0.7673	0.2529	**% RSD< 2**
Resolution (Rs)	8.061±0.0432	2.790±0.0360	3.952±0.0428	**>2**
Theoretical plate (N)	2571±25	2081±53	2151±24	**>2000**
Tailing factor	0.753±0.0070	0.985±0.0169	0.730±0.0159	**< 2**

**mean ± SD, (n=6) average of six determinations*

6.13.16 Analysis of marketed formulation

The proposed method was successfully used for the quantitative determination of ALI, AMLO and HCT in commercial formulation (Amturnide tablet: 300 mg of ALI, 10 mg of AMLO and 25 mg of HCT). Six replicate determinations were carried out and experimental values were found to be within 97-101% for all the drugs are presented in Table No. 6.13.10. Therefore the proposed method can be successfully applied for the quantitative analysis of ALI, AMLO and HCT in tablet formulation. Chromatogram of formulation is showed in Figure No. 6.13.28.

Table No. 6.13.10: Results of formulation analysis

Drugs	Amount (mg/tablet)		% Drug found*	% RSD
	Labelled	Found		
ALI	300	300.24	100.0803±0.9966	0.9958
AMLO	10	9.92	99.1893±1.0516	1.0602
HCT	12.5	12.27	98.1985±1.0723	1.0920

**mean ± SD (n=6) values of six determination*

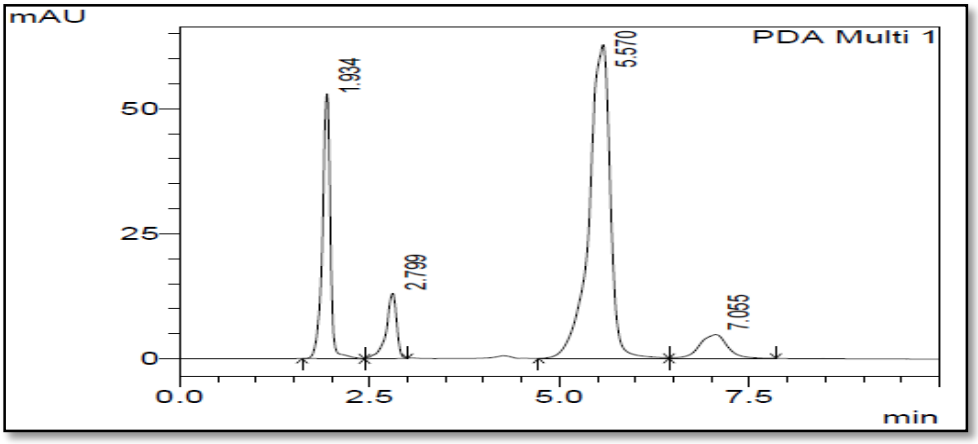


Figure No. 6.13.28: Chromatogram of formulation: ALI (120 µg/ml), AMLO (4 µg/ml) and HCT (10 µg/ml)

In order to check the applicability of the method, all the available strength (ratio) of marketed formulations were analyzed using standard drug solution in optimum ratio.

Available strength (mg)

ALI + AMLO + HCT: 150/300 + 5/10 + 12.5/25

Study (% assay) suggests that, the proposed method can be applied to all the formulations of different strengths available in the market.

6.14 STATISTICAL ANALYSIS

Five different methods namely RP-HPLC, HPTLC, simultaneous equation, absorbance ratio and first derivative spectroscopic methods were developed and validated for three different formulations. Statistical analysis was performed to assess the effect of all the developed methods based on assay results obtained. Statistical significance between all the methods were tested using one-way ANOVA followed by Bonferroni multiple comparison tests (95% confidence level) as appropriate using computer based fitting program (Prism, Graphpad version 5, Graphpad software Inc). Significants level was set at $p<0.05$ for all the tests. Results of ANOVA are presented in Table No. 6.14.1-3. The results of assay reveal that there was no significant difference between all the methods for formulation 1, 2 & 3.

Table No. 6.14.1: Results of statistical comparison using one way ANOVA & Bonferroni multiple comparison test for formulation 1 (ALI & HCT tablet)

Drugs	RP-HPLC	Simultaneous Method	Absorbance Ratio Method	First Derivative Method
ALI	99.2797±0.7106	98.9582±0.8194	98.4291±1.1807	98.5751±1.3886
HCT	98.9493±0.2601	98.8147±1.2010	97.8766±1.1160	97.9277±1.1159
All values are expressed in Mean±SD (n=6)				

Table No. 6.14.2: Results of statistical comparison using one way ANOVA & Bonferroni multiple comparison test for formulation 2 (ALI & VAL tablet)

Drugs	RP-HPLC	HPTLC	Simultaneous Method	Absorbance Ratio Method	First Derivative Method
ALI	99.8809 ± 0.9389	99.8805 ± 0.7297	98.6740 ± 1.2584	97.9346 ± 1.4524	97.9385 ± 1.5117
VAL	99.9554 ± 0.5028	98.5739 ± 0.5716	97.8782 ± 1.1735	98.0297 ± 1.0448	98.0695 ± 1.4984
All values are expressed in Mean±SD (n=6)					

Table No. 6.14.3: Results of statistical comparison using one way ANOVA & Bonferroni multiple comparison test for formulation 3 (ALI & AMLO tablet)

Drugs	Simultaneous Method	Absorbance Ratio Method	First Derivative Method
ALI	98.2774±1.2008	98.4581±1.1763	98.6617±1.5378
AMLO	97.8377±0.7151	99.7429±1.2965	99.1293±1.0521
All values are expressed in Mean±SD (n=6)			

7. CONCLUSION

Different methods namely RP-HPLC, HPTLC, simultaneous equation, abosorbance ratio and first derivative spectroscopic methods were developed for simultaneous determination of ALI & HCT; ALI & VAL; ALI & AMLO; ALI, AMLO & HCT in combined tablet dosage form. All the developed methods were validated as per ICH guidelines. Proposed methods are found to be simple, sensitive, precise, accurate and cost effective. The advantages of proposed methods are as follows; all the developed UV spectrophotometric methods are very simple, requires little sample preparation procedure, wide concentration range with high sensitivity, method describes standard and sample preparation procedure based on the form of analytes under investigation, i.e. aliskiren (13.26 mg of aliskiren hemifumarate is equivalent to 12 mg of aliskiren); RP-HPLC separation was achieved using C18 column (most widely used), 0.2% triethylamine in water (pH 6 with orthophosphoric acid) and methanol as mobile phase, which can be afforded by all the laboratories; fumaric acid was well separated from both the analytes. Moreover, based on the sensitivity and resolution aspect, all the RP-HPLC and HPTLC methods can be used for the analysis of ALI and other drugs in biological fluids or in pharmacokinetic and stability studies. Statistical analysis was performed to assess the effect of all the developed methods based on assay results obtained. Statistical significance between all the methods were tested using one-way ANOVA followed by Bonferroni multiple comparison tests (95% confidence level) as appropriate using computer based fitting program (Prism, Graphpad version 5, Graphpad software Inc). Significants level was set at $p<0.05$ for all the tests. The results reveal that there is no statistical significant difference between all the methods for formulation 1, 2 & 3. Therefore, all the methods can be used successfully for routine analysis in tablet dosage form.

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