THE THESIS

ENTITLED

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

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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
ALO	:	Alogliptin benzoate
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
BA	:	Benzoic acid
gm	:	Gram
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
MET	:	Metformin hydrochrode
mg	:	Milli gram
min	:	Minute
ml	:	Milli liter
mm	:	Milli meter
mM	:	Milli molar
N	:	Theoretical plates
ng	:	Nano gram
nm	:	Nano meter
PIO	:	Pioglitazone hydrochloride
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
WHO	:	World Health Organization
λ_{max}	:	Absorbance maximum
σ	:	Sigma

1 INTRODUCTION

1.1 General Introduction

Pharmaceutical drugs have the main aim of serving the human and protect them from diseases and to cure potential illness and prevention of different type of diseases. For this, medicines should be free from impurities and other interference which might harm humans and cause serious side effects.

Innovation of a drug molecule is the first step of the drug development process. The drug molecule should have the therapeutic value to check, cure or control diseases. Such drug molecules are otherwise called as active pharmaceutical ingredients (APIs). The synthesis, characterization and analysis of such drug molecules are very essential for identification of drug candidates for further detailed investigations.

The analytical investigation of drug products, bulk drug materials, impurities, intermediates, drug formulations, and degradation products of the drugs, and biological samples which contains the drugs and their metabolites is very significant in the field of pharmaceutical research. With the aim of characterizing bulk drug material quality by setting limits of their API content, analytical assay methods were included in the official monographs. There are wide variety of assay methods in the monographs like titrimetry, chromatography, spectrometry and capillary electrophoresis. Electro analytical methods are also included indifferent monographs.

Analytical techniques described above play a great role from the initial stages of drug development to marketing and post marketing. Analytical techniques helps in understanding the physical and chemical stability of the drug, selection and design of the dosage form, evaluating the stability of the drug molecules, identification and quantitation of the impurities. Quantitative or qualitative analysis of drug and its metabolites are extensively applied in the pharmacokinetic studies. [1]

1.2. Introduction to analytical chemistry

There are wide varieties of analytical techniques used for the qualitative and quantitative analysis of pharmaceutical substances.

Analytical chemistry can be defined as "The resolution of a chemical compound into its proximate or ultimate parts; the determination of its elements or of the foreign substances it may contain". This definition outlines the importance and scope of analytical chemistry in very broad terms. When an analyst being presented a completely unknown sample, the first objective is to ascertain the substances which are present in it. The fundamental problem in the analysis is finding out what impurities are present in the given sample, or finding out certain specified impurities which are absent.

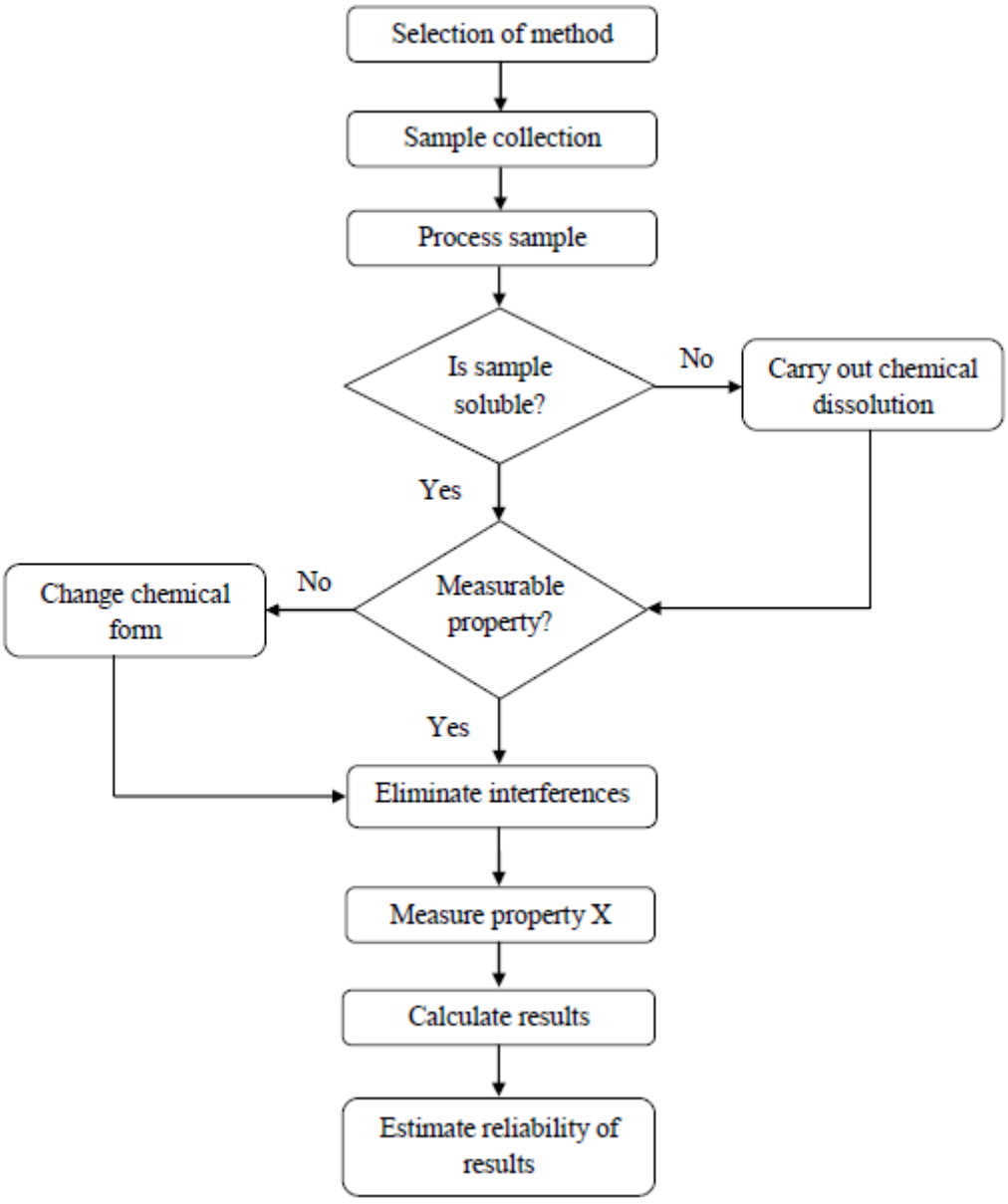


Figure No. 1.1: Steps of quantitative analysis

After determining the nature of constituents in the given sample, the next task of the analyst is to determine how much components are present.

Analytical chemistry has many similarities with respect to other scientific fields. Chemical analysis is a vital tool in academic, industrial, medical and government laboratories because of its interdisciplinary nature.

Analytical chemistry measurements also play a vital role in many research fields like physics, geology, chemistry, biochemistry, biology and other sciences. For example, quantitative analysis of sodium, potassium, and calcium ions by using flame photometric techniques in the body fluids of animals allows physiologists to study importance of these ions in muscle contraction, relaxation and nerve signal conduction. [2]

Quantitative and qualitative analysis also plays an important role in determining the safety and efficacy of medicines. Analytical techniques which are highly specific, accurate and sensitive hold key to the standardization, quality control, design and development of medicinal products. They are also having vital role in pharmacokinetics and in drug metabolism studies. Both of these studies are fundamental to the determination of duration of clinical response and bioavailability.

Modern analytical methods are extremely sensitive, and they require only small amount of sample material to provide precise and detailed information. Because of these reasons analytical methods are in widespread use. They are widely used in monitoring the use of drugs and medicines, product development, and stability studies. [2 -5]

Analytical method development and validation can be defined as "a continuous and interconnected activity conducted throughout the drug development process to verify that a given method measures a parameter as intended and establishes the performance limits of the measurement." Validated methods always produce results within known uncertainties. The results of validated method are crucial for the continuation of drug development, as these methods define the budding knowledge base which supports the product. The time and effort that are put into developing transferrable, robust and scientifically-sound analytical methods have to be in association with the drug development stage.

Effective method development should ensure that the laboratory resources are optimized, and at each stage of drug development the methods meet the objectives required. Method validation, which is required by different regulatory agencies at some or other stages of the drug approval process, can also be defined as the "process

demonstrating that analytical procedures are suitable for their intended use" and to judge the consistency, quality and reliability of analytical method, the results obtained from method validation can be used. Method validation is an integral part of any good analytical practice. [6]

1.3 Bioanalytical method development and validation

Bioanalytical methods are widely used for the estimation of drugs and their metabolites in biological matrices. Highly sensitive, reproducible and selective methods are required in today's drug development environment, for quantifying drugs in matrices such as urine, plasma, serum and blood.

Bioanalytical method validation may be defined as "procedure employed to demonstrate that an analytical method used for quantification of analytes in a biological matrix is reliable and reproducible to achieve its purpose: to quantify the analyte with a degree of accuracy and precision appropriate to the task."

Bioanalytical techniques are used to determine the pharmacokinetics of chemicals which are present in the living cells and also the metabolic processes in animals. These methods are used to provide support to drug discovery programs in the drug development process.

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 validation parameters like accuracy and precision of the method which is developed. 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- 10]

The stability-indicating assay method is used to check the stability of a product, which has immense prospect in pharmaceutical industry. After the introduction of "International Conference on Harmonization" (ICH) guidelines, it is necessary that stability-indicating assay method (SIAM) should be established. The guidelines clearly requires the data of forced decomposition studies under a variety of conditions, like acidic or basic pH, photo degradation, oxidation, humidity and dry heat etc. and also separation of drug from its degradation products.[11]

Impurities in pharmaceutical preparations may be defined as "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 such type of impurities even in small quantity can influence the safety and efficacy of the pharmaceutical substance. Impurity profiling which consists of identity and the amount of impurity in the pharmaceutical product is now getting more attention from various regulatory authorities. There are different official monographs, such as United States Pharmacopoeia (USP), British Pharmacopoeia those are slowly fixing permissible limits of impurities which are present in the API's or pharmaceutical formulations. [12]

More importantly various authorities like USFDA, ICH and Canadian Drug and Health Agency are working on the requirements of purity and the identification of various impurities present in the 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. [13]

1.4 Analytical techniques

Techniques in analytical chemistry can be broadly classified as qualitative and quantitative analysis.

1.4.1 Titrimetric techniques

Titrimetric method of analysis was invented by Gay–Lussac in the year 1835 when the term volumetric method which subsequently leads to the origin of term titration. The assay methods have been modernized nowadays even though it is a very old technique. Non-Aqueous titration methods are used for the determination of very weak acids and also potentiometric titrations for accurate end point detection thereby improving the precision of the different methods.

There are different types of titrimetric analysis:

a. Acid-base titrations
b. Non-aqueous titrations
c. Complexometric titrations
d. Redox titrations
e. Precipitation titrations etc.

1.4.2 Chromatographic techniques

Chromatography is a "technique in which the components of a mixture are separated based on differences in the rates at which they are carried through a fixed or stationary phase by a gaseous or liquid mobile phase."

1.4.2.1 Classification of chromatographic methods

According to principle, chromatographic techniques are basically classified into two:

1.4.2.2 Adsorption Chromatography

Adsorption chromatography was first developed by Russian botanist Tswett in 1906. The principle of separation of compounds by column chromatography is adsorption at the solid-liquid interface. Compounds with least affinity move down the column at a faster rate than those with greater affinity for the adsorbent. Adsorbents of varying degree of capacity are used as stationary phase. They should be chemically inert, insoluble in solvents and active.

Commonly used adsorbents are activated alumina, activated silica, activated charcoal, activated magnesium silicate etc.

1.4.2.3 Partition chromatography

Partition chromatographic separations are based on the partition coefficients of the individual components of a mixture between a liquid stationary phase and a gaseous or liquid mobile phase. Mobile phase is a liquid in case of partition chromatography.

1.4.2.4 Column chromatography

In column chromatography, the stationary phase kept in a narrow tube and mobile phase is forced through the tube under pressure or gravity.eg. GC, HPLC .There are mainly two types of column chromatography.

1.4.2.5 High-performance liquid chromatography (HPLC)

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.

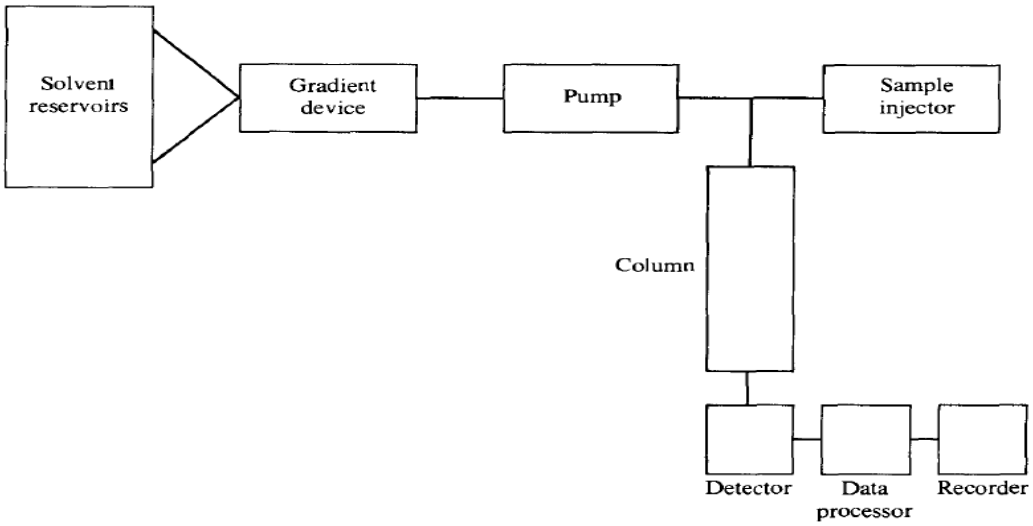


Figure No. 1.2: A schematic of HPLC equipment

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. [1, 2]

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. [14, 15]

Mainly two types of detectors are used in HPLC.

1.4.2.6 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.

1.4.2.7 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. **[1, 16]**

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.

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.

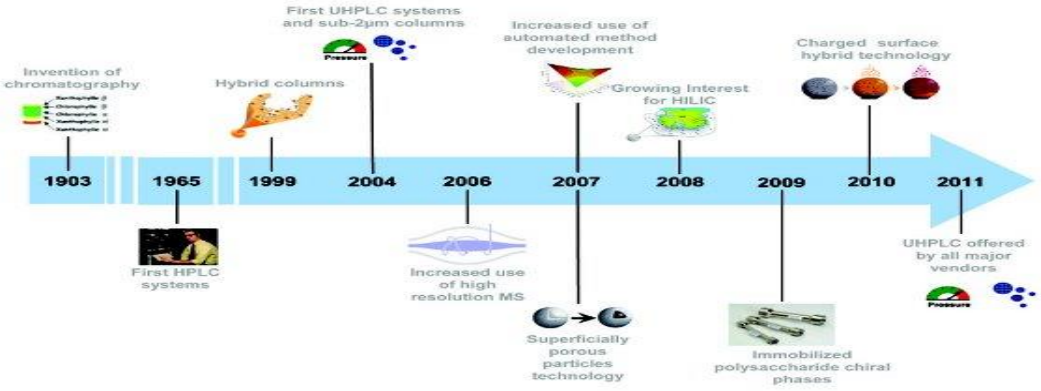


Figure No. 1.3: Recent development in HPLC

Table 1.1 System characteristics of UHPLC [17-20]

System characteristics of UHPLC	Range and Comment
High pressure limit	15,000 to 19,000 psi (1000 to 1250 bar) with flow rate limits of 2 to 5 mL/min. Compatible with conventional and sub-2 µm particle columns.
Low system dispersion	Instrumental bandwidth of 5 to 20 µL (4 σ) depending on configuration. System band broadening reduced by using smaller connection tubing <0.005" I.D. and small UV flow cells (0.5 to 2 µL). Compatible with columns down to 2 to 3 mm ID.
Low gradient dwell volume	100 to 400 µL (higher for quaternary pumps). Compatible to high throughput screening (HTS). Small dwell (mixing) volumes may negatively impact UV detector noise.
Others	Fast injection cycle (~20s) and detector response, and high acquisition rate (>40 pt/s) for HTS. Compatibility to existing HPLC methods desirable (e.g., flow range, column oven size, sample loop).
Benefits of UHPLC	**Comment**
High throughput	Increase throughput by 3 -10 fold vs. conventional HPLC while maintaining similar resolution, e.g., 5 min vs. 20 min purity analysis.
Rapid method development	Fast analysis with short columns is ideal for rapid column and mobile phase screening and method optimization.
High resolution	Increase resolution by up to 3 fold vs. HPLC, e.g., Peak capacities (P_c) 400 – 600 vs. ~200 for HPLC.
Solvent saving	Typical 5 to 15 fold reduction vs. HPLC due to shorter analysis time and use of smaller ID columns.
Higher sensitivity	3 to 10 fold increase of mass sensitivity (reduction of sample amounts injected). Use of long-pathlength UV flow cells (50 – 60 mm) can increase concentration sensitivity up to six times
Higher precision	Significant increase of retention time (2 - 3 fold) and peak area precision (<0.1% RSD achievable at injection volumes >1 µL)
Can be combined with other approaches	UHPLC is compatible with high-temperature LC, 2D-LC, or core shell columns individually or in combination. These are optional rather than alternative approaches.

1.4.3 Planar chromatography

In planar chromatography, mobile phase moves through the stationary phase by capillary action or under the influence of gravity. In this the stationary phase is supported on a flat plate or in the pores of paper.eg. TLC, HPTLC.

1.4.3.1 Thin layer chromatography

TLC finds a lot of application in the field of pharmaceutical analysis. TLC is in widespread use for the detection and quantitation of organic impurities. First the adsorbent should be extremely selective towards the substances being separated. Some adsorbents may be too strongly adsorbing or too weakly adsorbing. Thin layer chromatography is mainly used for the analysis of a wide variety of inorganic and organic substances, because of its distinctive advantages like minimal sample clean-up, wide choice of mobile phases, flexibility in sample distinction, high sample loading capacity and low cost. [1, 5]

1.4.3.2 High performance thin layer chromatography

High performance thin layer chromatography (HPTLC) has been emerged as an important instrument in drug analysis. HPTLC is a fast separation technique and flexible enough to analyze a wide variety of samples.

Advantages of HPTLC

1. Sample and standard can be processed simultaneously using small quantity of mobile phase.
2. Better analytical accuracy and precision.
3. Mobile phases with higher pH(8 and more) can be used unlike HPLC where high pH solutions may damage the column
4. Analysis time is less and less cost per analysis
5. Less amount of mobile phase is required per sample.
6. No need of sample pretreatment.
7. Turbid samples and suspensions can be directly applied without filtration.
8. In HPTLC same chromatogram can be scanned repeatedly by using the same or different parameters
9. Low maintenance cost.
10. Multi component analysis along with wide applicability made this technique very versatile.
11. Permanent documentation is possible alongside chromatogram.
12. Post-chromatographic derivatization can be done for analyzing non UV

absorbing materials.

13. Filtration and degassing is not required for solvents used in this technique. [21,22]

1.4.4 Gas chromatography

Gas chromatography is a powerful separation technique for detection of volatile organic compounds. In this technique, vaporization of the sample is required, which is carried through a prepared column, at a suitable temperature, by a stream of carrier gas which acts as mobile phase. By applying the combination of separation and on-line detection allows accurate quantitative determination of complex mixtures, including small amount of compounds in some specific cases. Gas liquid chromatography commands a substantial role in the analysis of pharmaceutical product. The creation of high-molecular mass products such as polypeptides, or thermally unstable antibiotics confines the scope of this technique. Its main constraint rests in the comparative non-volatility of the drug substances. Because of this limitation derivatization is compulsory. Gas chromatography is also an important tool for analysis of impurities of pharmaceuticals.

1.4.5 Spectroscopic techniques

1.4.5.1 UV-Visible spectrophotometry

Spectrophotometry is the quantitative measurement of the reflection or transmission properties of a material as a function of wavelength and it is one of the frequently employed techniques in pharmaceutical analysis. It involves the measurement of amount of UV or Visible radiation absorbed by a substance in a solution. The method has the advantages of low time and low labor consumption. The precision of these methods is also excellent. The use of UV–Visible spectrophotometry applied in the analysis of pharmaceutical dosage form has increased rapidly over the last few years. There are various methods for analysis of compounds using UV-Visible spectrophotometry. [1, 4]

The spectrophotometric assay not only involves in measurement of absorbance of samples which contains only one absorbing component but also where the

concentration of one or more substances is required in samples known to contain other absorbing substances which potentially interfere the assay.

Various spectrophotometric methods are available for assay of substances in multicomponent samples.

- 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)

1.4.5.2 Simultaneous equation method. (Vierodt's method)

Simultaneous equation method is applicable if a sample contains two absorbing drugs X and Y, each of which absorbs at the λmax of other drug.

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

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

Where, C_x and C_y are the concentrations of X and Y, ax_1 and ax_2 are absorptivities of X at $\lambda_1$and λ_2 nm, respectively. ay_1 and ay_2 are absorptivities of Y at $\lambda_1$and λ_2, respectively. A_1 and A_2 are absorbances of mixture at λ_1 and λ_2, respectively. These two equations are constructed upon the fact that at λ_1 and λ_2 the absorbance of the mixture is the sum of the individual absorbances of X and Y.

1.4.5.3 Absorbance ratio method (Q analysis method)

This method is a modification of the simultaneous equation method. It depends on the fact that, for a substance which obey's Beers law at all wavelengths,

the ratio of absorbances at any two wavelengths is a constant value independent of concentration or path length.

In the absorbance ratio method absorbances are measured at two wavelengths. One being the λ_{max} of one of the components ($\lambda_{2)}$ and other iso absorptive point of the two components ($\lambda_{1)}$. The following equation is used for absorbance ratio method

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

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

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

Where, ax_1 and ax_2 are the absorptivities of X at λ_1 and λ_2. ay_1 and ay_2 are absorptivities of Y at $\lambda_1$and λ_2.

A1 and A2 are the absorbances of mixture at λ_1 and λ_2. Cx and Cy are the concentrations of X and Y, respectively in sample solution.

1.4.5.4 Derivative spectrophotometry

Derivative spectrophotometry involves the conversion of a normal spectrum to its first, second or higher derivative spectrum. In the late 1970's microcomputers were introduced. The introduction of micro computers made it easy to use mathematical methods to generate derivative spectra easily, quickly, and reproducibly. This significantly increased the use of the derivative technique.

The normal absorption spectrum is referred as zero order, fundamental or (D^0) spectrum. The first derivative (D^1) spectrum is a plot of the rate of change of absorbance with wavelength against wavelength. The derivative method has found its applications also in infrared, atomic absorption, fluorescence spectrometry, and fluorimetry. The use of derivative spectrometry is not only restricted to special cases, but also whenever quantitative study of normal spectra is problematic. Disadvantage

is also associated with derivative methods; some form of is required in conjunction with differentiation as differential degrades the signal-to-noise ratio. [1, 5]

1.4.6 Near infrared spectroscopy (NIRS)

Near infrared spectroscopy (NIRS) is a widely used technique. This spectroscopic technique provides multi component analysis of almost any matrix. It is also rapid and non-destructive. NIR spectroscopy has gained a wide appreciation in recent years, within the pharmaceutical industry for product quality control, process monitoring and raw material testing. This technique requires an easy sample preparation and no sample pretreatment. Fiber optic probes are used for separating the sample measurement position. Also a single spectra is required for getting the physical and chemical sample parameters. All these advantages over other analytical techniques are reason for growing pharmaceutical interest in NIR spectroscopy.

1.4.7 Nuclear magnetic resonance spectroscopy (NMR)

NMR is a widely used technique for structural elucidation of compounds. This technique has been recently used for the quantitative analysis to determine impurities present in the drugs, characterization of composition of the drug products and determination of drugs in pharmaceutical formulations and biological fluids.

1.4.8 Fluorimetry and phosphorimetry

Need for a sensitive analytical technique which uses a micro sample is very important in the field of pharmaceutical analysis. Fluorescence spectrometry is one of the techniques that serve the purpose of high sensitivity without the loss of specificity or precision. Fluorimetry and phosphorimetry are used widely nowadays for quantitative analysis of various drugs in pharmaceutical formulations and biological fluids.

1.4.9 Electrochemical methods

Electro chemical methods are those methods which involves electrochemical reactions. The use of electrochemical analysis has increased greatly over the last few

years. Electrochemical methods like potentiometry, conductometry, polarography and amperometry are widely used for quantification of pharmaceuticals. [1]

1.4.10 Factors affecting the choice of analytical methods

The techniques described above have differing degrees of sensitivity, selectivity, cost of analysis, sophistication etc. For a given determination, analyst has to select the best procedure considering all these parameters. For this following criteria's are considered.

a. The type of analysis required: molecular or elemental, occasional or routine.
b. The nature of substance to be analyzed: Hygroscopic substances, radioactive substances, corrosive substances, etc.
c. Interferences from other substances other than the analyte under investigation.
d. Analytical instruments and other facilities available.
e. The concentration range needed for the analysis
f. Accuracy needed for the method.
g. Time required for the analysis. In case of analytical results required faster time should be monitored.
h. Which method of analysis will be preferred for the analyte of interest? Destructive or non destructive? [2]

1.4.11 Analytical method development and method validation

Analytical method development and method validation is an important process in the drug discovery. Without validated analytical method a drug cannot enter into the market although the drug shows good potency. This is to ensure the quality and safety of the drug.

1.4.11.1 Method Validation Parameters

- **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 true value. Accuracy is determined by recoveries studies.

Accuracy can be determined by three ways.

1. Comparison to a reference standard
2. Standard addition of the analyte
3. Recovery of the analyte spiked into blank matrix.

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

- **Precision**

Precision can be 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 there are three types of precision.

1. Repeatability
2. Intermediate precision
3. 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 examines the precision between laboratories and is often determined in collaborative studies or method transfer experiments.

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

- **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 of the calibration curve provides the desired information on linearity.

The range of the method can be defined as the lower and upper concentrations for which the analytical method has adequate accuracy, precision and linearity.

- **Limit of detection and Limit of quantification**

The limit of detection 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 based on acertain signal-to-noise ratio, typically 2or 3.

The quantitation limit of an individual analytical procedure can be defined as "the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy." The quantitation limit is particularly used for the determination of impurities and degradation products.

- **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." Typically these might include impurities, degradants, matrix etc. Assuring specificity is the first step in developing and validating a good method.

- **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 most important aspect of robustness is to develop methods that allow for expected variations in the separation parameters.

- **System suitability parameters**

Each day before starting the sample analysis, the operator must establish that the HPLC system and procedure can provide data of acceptable quality. This purpose is fulfilled with system suitability experiments, which can be defined as "tests to

ensure that the method can generate results of acceptable accuracy and precision." The system suitability parameters are measured after method development and validation have been completed. Parameters that are used to assess system suitability prior to analysis include theoretical plate number (N), resolution *(R,)* tailing factor, *k* and/or α, and relative standard deviation (RSD) of peak height or peak area for repetitive injections. Out of these, at least two criteria are required to demonstrate the system suitability of any method. The RSD of peak height or area of five repetitive injections of a standard solution is normally accepted as one of the standard criteria. For an assay method of a major component, the RSD should typically be less than 1% for these five repetitive injections. **[23-26]**

2. REVIEW OF LITERATURE

ALOGLITIN BENZOATE

There was a simple, precise and economical spectrophotometric method for the estimation of Alogliptin benzoate in bulk and pharmaceutical formulations, by Yadav et al (2014). First order derivative method was used for the quantitative determination of the drug. Alogliptin benzoate shows a sharp peak at 278.0 nm in first order derivative spectrum with n =1. The drug was found to be linear in the concentration range of 2-16 µg/ml with correlation coefficient of 0.9996. **[27]**

There was a reversed-phase liquid chromatographic (RP-LC) method for the determination of alogliptin (ALG) based on isocratic elution. The mobile phase used was potassium dihydrogen phosphate buffer pH (4.6) - acetonitrile (20:80, *v/v*) at a flow rate of 1 ml/min with UV detection at 215 nm, El Bagari et al (2012) Chromatographic separation was achieved on a Symmetry cyanide column (150 mm × 4.6 mm, 5 µm). Linearity, accuracy and precision were found to be acceptable over the concentration range of 5-160 µg/ml for ALG in bulk. **[28]**

METFORMIN HYDROCHLORIDE

An optimized method for determination of Metformin at ppb level in human plasma has been reported by Tache et al (2001) (Derivatization of Metformin with *p*-nitrobenzoyl chloride was carried out in a biphasic system. The derivative extracted in the organic layer dichloromethane was concentrated and analyzed by HPLC-DAD, using an isocratic elution and mobile phase used was water-methanol mixture 65/35 monitored at 280 nm. Good selectivity against co-extracted matrix components was observed. A detection limit of 10 ppb Metformin in human plasma was reached. **[29]**

A simple, rapid and sensitive HPLC method with UV detection for determination of metformin in plasma samples from bioequivalence assays has been reported by Porta et al (2008). Protein precipitation with acetonitrile was used to accomplish sample preparation. A reversed-phase phenyl column at 40°C was used to perform chromatographic separation and mobile phase consisted of a mixture of

phosphate buffer and acetonitrile at flow rate of 1.0 ml/min. Wavelength of detection was 236 nm. [30]

An algorithm for demonstrating the specificity of the analytical method for an analyte of interest against its degradation product by applying chemical (photolytic-hydrolysis, acidic and alkaline hydrolysis and acidic, neutral and alkaline oxidation) and physical (thermal degradation and photolysis) and stress conditions was developed. The authors Tache et al (2007) define specific conditions for testing capacity of the analytical method to distinguish between the analyte and its by-products resulting in different environmental conditions. The case of Metformin hydrochloride HPLC assay method was used as an effective example, it is found that the Metformin recovery is 99.98% by photolysis with white light or UV radiation. A moderate difference in between mild and high temperature application (99.98% in the first case and 86.00% in the second case, respectively) was observed by thermal degradation. [31]

There was a published paper on stability-indicating liquid chromatographic method and validation for the analysis of metformin hydrochloride and its related compound (1-cyanoguanidine) in tablet formulations by Rimawi (2009). A Nova-Pak silica column was used for the method and the chromatography was performed using wavelength of detection at 232 nm. Mobile phase used was a mixture of ammonium dihydrogen phosphate buffer and methanol (21:79, v/v) and Isocratic elution was employed. [32]

There was a simple spectrophotometric method for the estimation of Metformin hydrochloride in bulk and in tablet formulation by Mubeena et al (2010). A yellow chromogen formed by the oxidation of the primary amino group of Metformin hydrochloride using hydrogen peroxide, which is determined spectrophotometrically at 400 nm. The drug showed linearity in the concentration range of 4-26 mcg/ml. The percentage recovery of the drug for the proposed method ranged from 99-101.3% indicating no interference of the tablet excipients. [33]

It has been reported that a reversed-phase high-performance liquid chromatographic (RP-HPLC) method can be used to quantify metformin

hydrochloride in raw material and pharmaceutical formulations using C (18) analytical reverse-phase column by Arayne et al 2006. Diazepam was used as an internal standard. Methanol-water (30:70 v/v),was used as mobile phase which is pumped at a flow rate of 0.5 ml/min at ambient temperature and the retention time was about 4.4 min with symmetrical peaks. Detection wavelength was found be at 233 nm with no interference of commonly used excipients. The method was linear over the concentration range 0.312-5 µg/mL (R2 = 0.9995). The limit of detection of metformin was 0.1 mcg/ml and the limit of quantitation was 0.3 mcg/ml. The results obtained showed a good agreement with the declared contents in pharmaceutical formulations. **[34]**

A simple, accurate, economical and reproducible HPLC method has been published for quantitative estimation of metformin hydrochloride from tablet dosage form and formulated microspheres by Kar et al (2009). The HPLC method used was reverse phase chromatographic method using phenomenex C_{18} column and the mobile phase used was acetonitrile: phosphate buffer (65:35) pH adjusted to 5.75 with o-phosphoric acid and glipizide as internal standard. The method was found to be linear in the concentration range of 0.5-25 µg/ml for metformin hydrochloride. Results of analysis were validated statistically and by recovery studies. **[35]**

A study on optimization of a simple HPLC-UV method for the determination of metformin in human plasma has been reported, Chhetri et al (2014). Ion pair separation followed by UV detection was performed on deproteinized human plasma samples. The column used for the study was discovery reversed phase C-18 column (250×4.6 mm, 5 µm) with UV detection at 233 nm. 34% acetonitrile and 66% aqueous phase was used as mobile phase. Aqueous phase contained 10 mM KH_2PO_4 and 10 Mm sodium lauryl sulfate. Aqueous phase pH was adjusted to 5.2. The mobile phase was run isocratically. The flow rate was at 1.3 ml/min. The method was found to be linear in the concentration range of 0.125–2.5 µg/ml and coefficient coefficient (R^2) was found to be 0.9951. The lowest limit of quantification and detection was 125 and 62 ng/ml respectively. In this method samples are easy to prepare with minimum dilution and is time efficient. **[36]**

A simple, specific, accurate and isocratic reversed phase HPLC method was developed and validated for the determination of Metformin Hydrochloride by Madhukar et al (2011). Separation was achieved with an Intersil-Extend-C 18 HPLC column. The detection was carried out using a PDA detector set at wavelength of 232 nm. The method was linear in the concentration range of 1-250 µg/ml and showed good recoveries hence the method can be applied for quality control of Metformin Hydrochloride. **[37]**

It was stated that a precise, simple, selective, and stability-indicating high-performance thin-layer chromatographic method for analysis of metformin hydrochloride both as a bulk drug and in formulation was developed and validated. Pritam et al (2013). The analysis was carried out on a HPTLC aluminium plates precoated with silica gel 60 F254 as the stationary phase. The solvent system consisted of water: methanol: triethylamine (1:3.5:0.2 v/v). The system was found to give compact spot for metformin hydrochloride (RF value of 0.48±0.02). Densitometric analysis of metformin hydrochloride was carried out at 247 nm. The correlation coefficient was found to be $r^2 = 0.9965 \pm 0.0013$ with respect to peak area in the concentration range 100- 600 ng per spot. Metformin hydrochloride was subjected to hydrolysis (in acid, alkali, and neutral solutions), oxidation and photo-degradation. The drug undergoes degradation under acid, alkali, neutral, H_2O_2, and photolytic conditions. From the degradation studies it was evident that the drug is susceptible to hydrolysis, oxidation and photo degradation. **[38]**

PIOGLITAZONE HYDROCHLORIDE

It has been reported that a RP-HPLC method was developed and validated for the determination of Pioglitazone hydrochloride in pharmaceutical dosage forms, Srinivasulu et al (2010). Chromatography was carried out using C18 column (250x4.6mm) and the mobile phase used was a mixture of Buffer: acetonitrile (55:45%v/v) at a flow rate 1.0 ml/min. The analyte was monitored using UV detector at 254 nm .The retention time was found to be 9.738 min for Pioglitazone HCl. **[39]**

ALO+MET

It has been reported that two simple, precise and economical UV spectrophotometric methods have been developed for the simultaneous estimation of Alogliptin benzoate and Metformin hydrochloride in bulk and pharmaceutical dosage forms. Chirag et al (2014). Method A is simultaneous equation method (Vierodt's Method), which is based on measurement of absorption at 277 nm and 232 nm i.e.λmax of Alogliptin benzoate and Metformin hydrochloride respectively. Method B is Absorbance ratio (Q analysis method) which is based on measurement of absorption at wavelength of 250 nm and 277 nm i.e. isoabsorptive point of Alogliptin benzoate and Metformin hydrochloride and λ max of Alogliptin benzoate respectively. The accuracy of methods were determined by recovery studies and was found to be within range of 98-102% for both Alogliptin benzoate and Metformin hydrochloride. **[40]**

Three simple, rapid, accurate, precise, reliable and economical spectrophotometric methods for simultaneous determination of Alogliptin benzoate (ALO) and Metformin hydrochloride (MET) was reported by Patel et al (2014). The methods described in this paper were simultaneous equation (method A), zero crossing first order derivative (method B) and dual wavelength method (method C). The linearity ranges for ALO and MET were found to be 0.1-0.5 µg/ml and 4-20 µg/ml for all the methods respectively. Values of limit of detection (LOD) were 0.005517 µg/ml, and 0.24018 µg/ml and the values of limit of quantitation (LOQ) were 0.016717 µg/ml and 0.727823 µg/ml for ALO and MET respectively for method A. For method B, LOD values were 0.021481 µg/ml and 0.120206 µg/ ml and LOQ values were 0.065095 µg/ml and 0.364261 µg/ml for ALO and MET, respectively. LOD values were 0.008013 µg/ml and 1.34055 µg/ml and LOQ values were 0.024283 µg/ml and 4.062272 µg/ml for ALO and MET for method C. The precision values were less than 2% R.S.D for all three methods. **[41]**

A RP-HPLC method for two drugs have been developed and validated for simultaneous determination of Alogliptin and Metformin hydrochloride in Tablet dosage form by Praveen et al (2013). Mobile phase used in this method was 0.2% TEA pH adjusted with OPA to 6.0 was used as buffer was prepared by adjusting the

ratio of buffer and methanol as 30:70 v/v + 0.2% triethylamine and a flow rate of 1.0 ml/min. The column used was Agilent C18 with dimension 250 mm lengths, 4.6 mm i.d., 5µ particle size. The optimum wavelength for detection was 254 nm and a run time of 10 minutes was used. The system suitability parameters were found to be within the limits and linearity was observed in the range of 25-150 µg/ml for Alogliptin and Metformin hydrochloride respectively. **[42]**

It was stated that a new reversed-phase high pressure liquid chromatographic (RP-HPLC) method was developed for the determination of Metformin & Alogliptin (ALG) based on isocratic elution using a mobile phase consisting of potassium dihydrogen phosphate buffer [pH 4.0] and acetonitrile [HPLC Grade] (70:30, *v/v*) at a flow rate of 1 mL min^{-1} with UV detection at 235 nm. Sri et al (2013). The chromatography was performed on an X Terra column (250 mm × 4.6 mm, 5 µm). The run time was maintained for 8 mins. The Inter day and intraday precision was found to be within the limits. The Accuracy values were within specified limits (98-102%). Metformin was found to be linear in the concentration range of 300-700 µg/ mL and for alogliptin from 7.5-17.5 µg /ml. The limit of detection for metformin and alogliptin was found to be 0.175 and 0.050 µg/ml respectively. The Limit of Quantification for Metformin and Alogliptin was found to be 0.57and 0.20 µg/ml respectively. **[43]**

New HPLC methods for the determination of alogliptin and metformin combination as well as individually were developed and validated, Thangabalan et al (2014). Two drugs were resolved very efficiently in the proposed HPLC method. The retention time for alogliptin and metformin were found to be 1.727 and 2.900 minutes respectively. The wavelength of detection was found to be 230 nm. The % assay of alogliptin and metformin was found to be 99.74 and 99.31 respectively. **[44]**

Two simple, precise, accurate, and rapid UV Spectrophotometric methods have been developed for simultaneous estimation of Alogliptin benzoate and Meformin hydrochloride in combined tablet dosage form, Pavan et al (2014). The methods described in the article were (A) Q-absorbance and (B) Area under curve method. Method-A involves measurement of absorbance at 251 nm (iso-absorptive point) and 232 nm (λmax of Meformin hydrochloride). Method-B involves

measurement of peak area in the wavelength range 272-282 nm and 227-237 nm for Alogliptin benzoate and Meformin hydrochloride respectively. [45]

A new reversed-phase High Pressure Liquid Chromatographic (RP-HPLC) method was developed for the determination of metformin & alogliptin (ALG) based on isocratic elution and the mobile phase used was potassium dihydrogen phosphate buffer [pH 4.0] and Acetonitrile [HPLC Grade] (70:30, *v/v*) at a flow rate of 1 mL min−1 with UV detection at 235 nm. Satya et al (2013).The chromatographic separation was achieved on an XTerra column (250 mm × 4.6 mm, 5 μm). The Accuracy values were within specified limits (98-102%) The calibration curve for metformin was linear from (300-700 μg mL) and for alogliptin from (7.5-17.5 μg /ml).The limit of detection for metformin and alogliptin was found to be 0.175 and 0.050 μg/ml respectively. The limit of quantification for metformin and alogliptin was found to be 0.57 and 0.20 μg/ml respectively. [46]

ALO+PIO

A new stability- indicating RP-HPLC method has been developed for estimation of Alogliptin and Pioglitazone in bulk and pharmaceutical dosage form by Neelima et al (2014).The proposed method was a precise, sensitive, and accurate RP-HPLC method for the determination of Alogliptin and Pioglitazone. Different combinations of buffer and organic solvents were tried on Hypersil BDS C18 column to optimize the mobile phase. From this, mobile phase containing a mixture of Phosphate Buffer: Acetonitrile in the ratio of 45:55 % v/v was selected at a flow rate of 1.0 ml/min for the development of the method, and the peaks with good shape and resolution were found resulting in short retention time, baseline stability and minimum noise. The retention times of Alogliptine and Pioglitazone were found to be 3.42 and 5.24 min respectively. [47]

A simple, accurate and precise high performance liquid chromatographic (HPLC) method has been developed for simultaneous determination of pioglitazone and alogliptin in bulk and dosage form, Raval et al (2014).The method has been validated as per ICH guidelines. The column used for separation is BDS hypersil C_{18} 250mm × 4.6mm, 5μ (particle size) column with flow rate 1.0 mL per minute in

isocratic mode and the mobile phase used was buffer pH 3.5: Methanol (70:30). Column oven temperature was maintained at 25°C and detection wavelength was 271 nm. Pioglitazone and alogliptin were found to be linear in the concentration range of 3.75-18.75 µg/ml and 6.25-31.25 µg/ml respectively [48]

It was reported that a simple, accurate, precise and rapid RP-HPLC method was developed and validated as per ICH guidelines for the determination of Alogliptin (ALO) and Pioglitazone (PIO). The mobile phase used was mixture of Phosphate buffer- pH-3.6 and acetonitrile in the ratio of 35:65, by Manzoor et al (2013). The detection wavelength was 268.0 nm. The retention time of ALO and PIO was found to be 3.113 and 5.234 respectively. The linearity of the proposed method was in the range of 2.5-15 µg/ml (r = 0.9998) for ALO and 03-18 µg/ml (r = 0.9999) for PIO respectively. [49]

Simple, sensitive, rapid and accurate UV spectroscopic methods have been developed for the simultaneous estimation of alogliptin and pioglitazone in bulk and pharmaceutical dosage forms by Kashyap et al (2014). First order derivative and dual wavelength methods were developed and validated using methanol as solvent. Both methods were found to be linear in the concentration range of 5-30 µg/ml. The first order derivative spectra of each solution were obtained. ZCP of Alogliptin was found to be 275.60 nm and ZCP of pioglitazone was found to be 268.20 nm. The zero crossing point (ZCP) of alogliptin at which pioglitazone is measured and ZCP of pioglitazone at which alogliptin is measured. In dual wavelength method, spectra two wavelengths 270.20 nm and 265 were selected as λ_1 and λ_2 for the estimation of alogliptin. Pioglitazone shows the same absorbance at these wavelengths. Similarly, wavelengths 280 nm and 271 nm were selected as λ_3 and λ_4 for estimation of Pioglitazone. Alogliptin shows the same absorbance at these wavelengths. [50]

MET+PIO

It has been reported that a simple, sensitive and rapid reverse phase high performance liquid chromatographic method can be used for the estimation of Metformin HCl (MET) and Pioglitazone (PIO) in pure and in pharmaceutical dosage forms by Lakshmi et al (2009). A Gemini C_{18} column (150x4.6mm, 5µ) was used

with a mobile phase containing a mixture of acetonitrile and ammonium acetate buffer (pH-3) in the ratio of 42:58. The flow rate was 0.3 ml/min and effluents were monitored at 255 nm. MET eluted at 5.17 min and PIO at 8.1 min. Calibration curve was plotted with a range from 0.5-50 µg/ml for MET and 0.3-30 µg/ml for PIO. **[51]**

A simple, selective, rapid, precise and economical reverse phase HPLC method for the simultaneous estimation of metformin and pioglitazone from pharmaceutical dosage forms was published by Alexander et al (2010). The column used for the study was phenomenex C_{18} (25 cm x 4.6 mm i.d., 5 µ) with a mobile phase consisting of acetonitrile: phosphate buffer (adjusted to pH 5.0 using orthophosphoric acid) (50:50 v/v) at a flow rate of 1 ml/min. The wavelength of detection was 258 nm. The internal standard used was Etoricoxib **[52]**

It has been reported that a reversed-phase high-performance liquid chromatography method can be used for the simultaneous estimation of metformin hydrochloride (MET), pioglitazone hydrochloride (PIO), and glimepiride (GLP) present in multicomponent dosage forms by Jain et al (2008). Chromatography is carried out isocratically at 25°C ± 0.5°C on an Inertsil-ODS-3 (C-18) Column (250 × 4.60 mm, 5 µm) and the mobile phase composed of methanol-phosphate buffer (pH 4.3) in the ratio of 75:25 v/v at a flow rate of 1 mL/min. Detection is carried out using a UV-PDA detector at 258 nm. The retention times for MET, PIO, and GLP are 2.66 + 0.5 min, 7.12 + 0.5 min, and 10.17 + 0.5 min, respectively. The linearity range and percentage recoveries for MET, PIO, and GLP are 10-5000, 10-150, and 1-10 µg/mL and 100.4%, 100.06%, and 100.2%, respectively. **[53]**

There was a simple, precise, and accurate reported HPLC method for simultaneous estimation of metformin hydrochloride (MET), pioglitazone hydrochloride (PIO), and glimepiride (GLIMP) by Pandit et al (2012). Chromatographic separation of the drugs were performed on a Phenomenex-ODS-3 (C-18) column (250 × 4.60 mm, 5 µm) column and the mobile phase used was methanol:acetonitrile:15 mM potassium dihydrogen phosphate (pH 4) in the proportion of 40:35:25 (v/v) at a flow rate of 1 ml/min. Detection was carried out using a UV-SPD-10AVP detector at 240 nm. The retention time for MET, PIO, and GLIMP were 2.85 ± 0.03 min, 4.52 ± 0.03 min, and 7.08 ± 0.02min, respectively. Parameters such as linearity (0.2–50 µg/ ml for MET,

0.2–30 μg/ml for PIO, and GLIMP, respectively), precision, accuracy (99.66 ± 0.14 for MET, 98.46 ± 0.40 for PIO, and 98.62 ± 0.39 for GLIMP), specificity and robustness were calculated in accordance with ICH guidelines. The method was proved to be simple, precise, accurate, rapid and cost effective. [54]

MET+STG

A simple and precise liquid chromatographic method has been developed and validated for the determination of either sitagliptin (STG), vildagliptin (VLG) or saxagliptin HCL (SXG) and metformin HCL (MET) in the presence of metformin degradation product, 1-cyanoguanidine (CGN) El-Bagary et al (2013). The chromatography was performed on a SymmetryÂ® cyanide column (150 mm Ã— 4.6 mm, 5 Î¼m). The mode of elution was Isocratic and the mobile phase used was potassium dihydrogen phosphate buffer (pH = 4.6) - acetonitrile (30:70, v:v) at a flow rate of 1 mL/min with UV detection at 210 nm. The LC method was used for the simultaneous estimation of STG, VLG, and SXG and MET in the ranges of 5-200, 5-200, 0.5-80.0 and 20-800 Î¼g/mL, respectively. The results were statistically compared with the reference method for each drug using one-way analysis of variance (ANOVA). The developed method was proved to be specific and accurate hence can be satisfactorily applied to the analysis of the pharmaceutical formulations. **[55]**

A research paper was published on simple, rapid and validated liquid chromatographic method for the simultaneous determination of three novel Gliptins namely Vildagliptin (VLD), Sitagliptin (STG) and Linagliptin (LIN) in their binary mixture with Metformin (MET) by Attimarad et al (2014). The separation was performed on fast monolithic column using isocratic mode and the mobile phase consisted of a mixture of sodium dihydrogen phosphate, sodium dedosyl sulphate and acetonitrile. The flow rate was 2.5 mL/min and UV detection for MET, VLD and SIT was performed at 208 nm, whereas, that of MET and LIN was at 228 nm. All three tablet formulations were assayed with accuracy and precision and without interference from excipients. The method is also stability indicating with respect to linagliptin. **[56]**

MET+OTHERS

There was a simple, economic and sensitive RP-HPLC method for the simultaneous estimation of metformin and saxagliptin in tablets. Cumar et al (2012). The method was carried out on C-18 column using phosphate buffer (pH 5), acetonitrile and methanol in the ratio 75:15:10 as a mobile phase at a flow rate of 1.5 ml/min. The wavelength of detection was 225 nm. The retention time of metformin and saxagliptin was found to be 5.65 and 6.20 respectively. The developed method is found to be sensitive and rapid which can be used for estimation of metformin and saxagliptin pharmaceutical dosage forms. **[57]**

It was reported that a simple, rapid, and precise RP-HPLC method for simulataneous estimation of metformin hydrochloride, pioglitazone hydrochloride and Glibenclamide in a tablet dosage form has been developed and validated by Doredla et al (2012). C18 column with 60:40 (v/v) acetonitrile-0.5 Mm dihydrogen phosphate buffer (pH adjusted to 3.0±0.1 with 5% orthophosporic acid) as mobile phase was used for the study. Flow rate employed was of 1.2 ml/min. UV detection was performed at 230 nm. Calibration plots were found to be linear over the concentration ranges 200-1000 µg/ml. **[58]**

A stability indicating high performance thin layer chromatography (HPTLC) method was developed and validated for determination of two anti-diabetic drugs, nateglinide and metformin hydrochloride in co-formulations by Thomas et al (2011).The solvent system used for the study was chloroform: ethyl acetate: acetic acid (4:6:0.1 v/v/v). Wavelength 216 nm was used for direct evaluation of the chromatograms in the reflectance/absorbance mode. The method had an accuracy of 99.72% and100.08% for nateglinide and metformin hydrochloride respectively. Nateglinide and metformin hydrochloride were subjected to acid, base, oxidation, wet, heat and photo-degradation studies. The degradation products obtained were well resolved from the pure drugs and different Rf values were significantly different. The method can be used for stability-indicating analysis as the method could effectively separate the drugs from its degradation products **[59]**

An article was published on development and validation of a simple HPLC method for the simultaneous determination of metformin (MTF) and gliclazide (GCZ) in the presence of glibenclamide, in human plasma, for the clinical monitoring of MTF and GCZ after oral administration or for bioequivalence studies. Ranetti et al (2007). Ion-pair separation followed by UV detection performed on deproteinised plasma samples was chosen for the determination of metformin and gliclazide. The HPLC method was performed on a Zorbax Eclipse XDB-C_{18} 150x4.6 mm i.d. (5µm) column and analytical guard column 12.5x4.6 mm (5µm), with a gradient elution (1 mL/min) at 40°C column temperature. The mobile phase used was acetonitrile: methanol (1:1v/v) and sodium dodecylsulphate 5mM, pH=3.5 with H_3PO_4 85% and gradient elution. The eluent was monitored at 236 nm. The calibration curve was linear within the range of 0.05-5.00 µg/mL (r^2=0.99, n=6). **[60]**

Studies have stated that a simple, specific, accurate and isocratic reversed phase liquid chromatographic method has been developed and subsequently validated for the determination of metformin hydrochloride, rosiglitazone and pioglitazone hydrochloride by Havaldar et al (2010). Separation was achieved with a Zorbax C_8 column of 150×4.6 mm i.d. with 5 µm particle size and ammonium dihydrogen phosphate buffer adjusted to pH 3.0 using diluted ortho phosphoric acid and acetonitrile (65:35 v/v) as eluent at a constant flow rate of 0.7 ml per min. UV detection was performed at 215 nm. The retention time of metformin hydrochloride, rosiglitazone and pioglitazone hydrochloride were about 1.9, 3.4 and 6.7 min, respectively. **[61]**

A rapid, precise, sensitive, economical, and validated analytical method for simultaneous separation and quantification of three anti-diabetic drugs, viz., glibenclamide (GLB), gliclazide (GLC), and metformin hydrochloride (MHC) using ultra fast liquid chromatography (UFLC) has been developed by Bandarkara et al (2010).The column used for the separation of three drugs was XR-ODS C18 (30^0C) with a mobile phase comprised of acetonitrile-water-trifluoroaceticacid-triethylamine (54:46:0.1:0.1v/v) in isocratic elution mode at a flow rate of 0.38 $mLmin^{-1}$ and detected at 230 nm. MHC (Rt¼0.98 min), GLC (Rt¼4.10 min), and GL B (Rt¼6.40 min) separated with good resolution in a single chromatographic run of 7.5 min.

Linear relationship (r2>0.999) was observed between the peak area and concentration for all the three compounds within the range of 5–50 ng/mL. [62]

An article was published on high-throughput assay for the simultaneous analysis of metformin and sitagliptin from mouse and human dried blood spot samples using laser diode thermal desorption interfaced with atmospheric pressure chemical ionization tandem mass spectrometry (LDTD–APCI–MS/MS) for use in a pharmaceutical discovery environment as an alternative to traditional plasma analysis by Swales et al (2011). Analytes were extracted from dried blood spots using a simple punch disc and solvent extract procedure. Analyte stability was determined in dried blood spots on FTA cards and as extracts of dried blood spots. The method was subsequently used to determine the oral pharmacokinetics of metformin and sitagliptin after dosing to male mice. Intra-assay and inter-assay accuracy and precision across the analytes and species deviated by less than 30% at all calibration levels and less than 20% at all quality control levels. The method was successfully applied to spiked mouse and human dried blood spot samples. [63]

Two simple, precise and economical UV methods have been developed for the simultaneous estimation of Sitagliptin phosphate and Metformin hydrochloride in bulk and pharmaceutical dosage form by Loni et al (2012). Method A is absorbance maxima method, which is based on measurement of absorption at 266 nm for sitagliptin phosphate and 232 nm for metformin hydrochloride. Method B is area under curve (AUC), in the wavelength range of 244-279 nm for sitagliptin phosphate and 222-240 nm for metformin hydrochloride. Linearity for detector response was observed in the concentration range of 25-225 μg/ml for sitagliptin phosphate and 2-12 μg/ml for metformin hydrochloride. The accuracy of the methods was assessed by recovery studies and was found to be 99.64 % and 98.98% for Sitagliptin phosphate and metformin hydrochloride respectively. [64]

There was a simple, accurate, economical and reproducible UV spectrophotometric method for the simultaneous estimation of a two component drug mixture of pioglitazone and glimipiride in the combined tablet dosage form by Kottu et al (2012). Simultaneous method that involves maximum absorbance of pioglitazone and glimipiride at 279 nm and 238 nm respectively was developed. [65]

There was a reported liquid chromatographic method for the simultaneous determination of glipizide, rosiglitazone, pioglitazone, glibenclamide and glimepiride in pharmaceutical dosage forms and human plasma, Lakshmi et al (2011).The mobile phase employed in the method was a mixture of 0.05% Triethylamine (pH-3.5, adjusted with *ortho* phosphoric acid), acetonitrile and methanol in the ratio of 5:15:30 and column used was phenomenex C_{18} column. The preparation of plasma samples and mixtures of formulations were prepared by extraction with acetonitrile. From the results of method validation parameters it was found that the developed method can be used for quality control of drugs and can also be applied to pharmacokinetic studies. **[66]**

Three spectrophotometric methods and one HPLC method for analysis of anti-diabetic drugs in tablets were reported by Onal (2009). The two developed spectrophotometric methods explains the reaction of rosiglitazone with bromocresol green and 2, 3-dichloro-5, 6-dicyano-1, 4-benzoquinone. The third method was a zero-crossing first-derivative spectrophotometric which is used for simultaneous estimation of rosiglitazone and metformin in tablet dosage form. The last method developed was a stability-indicating HPLC method for the determination of rosiglitazone. **[67]**

Two antidiabetic drugs, metformin and glyburide, in pharmaceutical tablet formulations were investigated by using HPTLC, Ghasssempour et al. (2006). Silica gel 60 F254 was used as stationary phase and mobile phase used was water/methanol/ammonium sulfate (2/1/0.5 w/v). The detection and quantification was carried out at 237 nm. Stability study also has been performed for samples and standard **[68]**

A study was published on high-performance thin-layer chromatography (HPTLC) method for the simultaneous determination of pioglitazone, metformin, and glimepiride in multicomponent pharmaceutical preparations by Kala et al (2011). The chromatographic separation was done on silica gel 60 F_{254} HPTLC plates. The mobile phase used for the analysis was acetonitrile, methanol, propyl alcohol, and ammonium acetate solutions (7:2:1:1 *v/v*). Detection and quantification was carried out at 240 nm. **[69]**

There was a reported HPLC method for the simultaneous estimation of metformin in combination with gliclazide and glipizide in multicomponent dosage form. Vasudevan et al (2001). The column and mobile phase used was Inertsil C_{18} column and Acetonitrile-water containing camphor sulphonic acid (pH 7 using 0.1 N NaOH; 75 mM) respectively. The detection was done at 225 nm. **[70]**

A research paper was published on capillary zone electrophoresis for the simultaneous estimation of rosiglitazone and metformin in pharmaceutical dosage form, Yardimci et al (2005).The column used for analysis was fused-silica capillary and the mobile phase employed was 25 mM acetate buffer at pH 4.0. Detection was performed at wavelength of 203 nm. **[71]**

There was a reverse phase high performance liquid chromatographic method developed for the simultaneous estimation of metformin, pioglitazone and glibenclamide in tablet dosage form and the internal standard used was gliclazide by Tengli et al (2013). A phenomenex luna CN (100R 250×4.60 (mm) column was used for the separation of components. Mobile phase was consisted acetonitrile, water and buffer (0.5% potassium dihydrogen phosphate) (60:20:20). The pH of the mobile phase was adjusted to 2.5 using orthophosphoic acid. The detection wavelength using UV detector was at 230 nm. This developed method can be used for of these antidiabetic drugs in combined dosage forms. **[72]**

A research paper was published on high-performance liquid chromatographic (HPLC) method for the simultaneous determination of diltiazem, metformin, pioglitazone, and rosiglitazone hydrochloride in pharmaceutical formulations, raw materials, and human serum by Sultana et al. (2011). Acetonitrile-methanol-water (30:20:50, v/v, pH 2.59 ± 0.02) was used as the mobile phase for the developed method. The column used for the separation was Hiber, 250-4.6 RP-18 column, and wavelength of detection was 230 nm. Statistical analysis was also performed for the validated parameters of developed method which proves the reliability of this method. **[73]**

There was a reported method for estimation of pioglitazone hydrochloride (PIO) and metformin hydrochloride (MET) in their combined dosage forms using

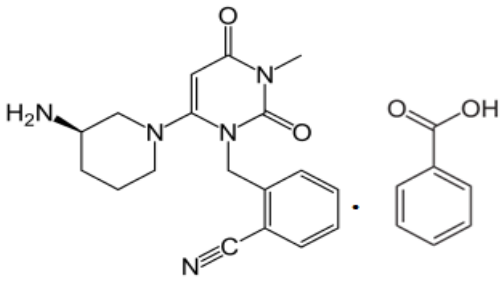
second-derivative spectrophotometry and reversed-phase liquid chromatography (LC) by Shankar et al (2005). Second-derivative responses at 257.25 nm for MET and 227.55 nm for PIO were used for the proposed study. A mixture of methanol and acetonitrile (30 + 70) was used as solvent for the method. A Hypersil ODS-C18 column which has a particle size of 5 micron was used for analysis. The mobile phase used for the method was acetonitrile-water-acetic acid (75 + 25 + 0.3), and pH of the mobile phase was adjusted to 5.5 by using liquor ammonia. Detection wavelength was 230 nm. **[74]**

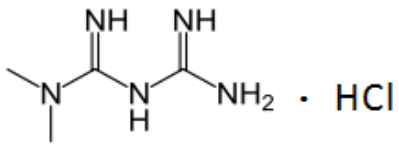
SITAGLIPTIN

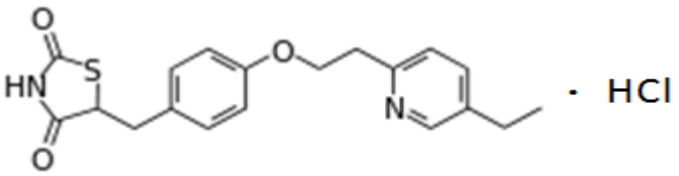
There was a high performance liquid chromatography (RP-HPLC) method for the quantitative determination of Sitagliptin in human plasma, Kashid et al (2012). In this work first the drug was spiked in the plasma and then the drug was extracted by using mobile phase by precipitation method. An INTERSIL C18 column (150 mm × 4.6 mm, 5μm) was used for analysis into which the extracted analyte was injected. The column effluents were detected at 267 nm. The mobile phase used for the present study was acetonitrile: methanol: buffer (2:3:5 v/v). *O*-phosphoric acid was used to adjust the mobile phase pH .The developed method found to have good specificity for sitagliptin. The linearity range was from 25-125 μg/mL (r2>0.9994). 25 μg/mL was the lower limit of quantification (LLOQ). This developed and validated RP-HPLC method is appropriate for determining the concentration of sitagliptin in human plasma. **[75]**

A simple, sensitive and reproducible spectrophotometric method was developed for the determination of sitagliptin phosphate in bulk and in pharmaceutical formulations by Sekaran et al (2010). The developed method explains, primary amino group of sitagliptin phosphate condenses with acetyl acetone and formaldehyde which produces a product which is yellow in color and analyzed spectrophotometrically at 430 nm. All the variables were studied to optimize the reaction conditions. There were no interferences from excipients. **[76]**

Drug Profile: [77-86]

Drug 1	**Alogliptin benzoate**
Chemical Structure	
Molecular Formula	$C_{18}H_{21}N_5O_2 \cdot C_7H_6O_2$
Molecular Weight	461.51
Chemical Name	2-({6-[(3*R*)-3-aminopiperidin-1-yl]-3-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl}methyl)benzonitrile monobenzoate
Description	Alogliptin benzoate is a white to off-white, crystalline powder
Solubility	It is soluble in dimethyl sulfoxide; sparingly soluble in water and methanol; slightly soluble in ethanol and very slightly soluble in octanol and isopropyl acetate.
Melting Range	185-188˚C
Pka value	9.47
Dose	6.25/12.5/25 mg
Category	Antidiabetic

Drug 2	**Metformin hydrochloride**
Chemical Structure	
Molecular Formula	$C_4H_{11}N_5$•HCl
Molecular Weight	165.63
Chemical Name	*N*, *N*-Dimethylimidodicarbonimidic diamide hydrochloride
Description	White to off-white crystalline powder
Solubility	Freely soluble in water, methanol and practically insoluble in acetone, ether and chloroform.
Melting Range	223-226˚C
Pka value	12.4
Dose	500/1000 mg
Category	Antidiabetic

Drug 3	**Pioglitazone hydrochloride**
Chemical Structure	
Molecular Formula	$C_{19}H_{20}N_2O_3S$•HCl
Molecular Weight	392.90
Chemical Name	(*RS*)-5-(4-[2-(5-ethylpyridin-2-yl) ethoxy] benzyl) thiazolidine-2, 4-Dione monohydrochloride
Description	Odorless white crystalline powder
Solubility	It is soluble in N, N dimethylformamide and methanol; slightly soluble in anhydrous ethanol; very slightly soluble in acetone and acetonitrile; practically insoluble in water and ether
Melting Range	183-184˚C
Pka value	6.66
Dose	15/30/45 mg
Category	Antidiabetic

3. AIM AND OBJECTIVES OF THE STUDY

Literature survey reveals various analytical methods for the estimation of alogliptin benzoate alone and with other drugs in combinations like metformin hydrochloride and pioglitazone hydrochloride by UV Spectroscopy, HPLC and other analytical methods.

However, no HPTLC method has been reported for alogliptin benzoate in combination with metformin hydrochloride and pioglitazone hydrochloride. Still there was need for more sensitive methods for the determination of ALO in combination with MET and PIO in tablet dosage form by UV Spectroscopy, HPLC along with HPTLC, which can cover up the deficiencies of existing methods.

More over it was observed that most of the reported methods did not describe about procurement of tablet formulation and remaining methods did not use tablet formulation for assay. All these observations were taken in to consideration and it was thought to prepare tablet formulation in laboratory using all the excipients as per the marketed formulation.

So objectives of the study are to develop and validate newer analytical methods as per ICH guidelines as follows:

Formulation 1: (Alogliptin benzoate and metformin hydrochloride)

Quantitative simultaneous determination of alogliptin benzoate and metformin hydrochloride in bulk and combined tablet formulation by

a) UV Spectroscopy
    i. Simultaneous equation method
    ii. Absorbance ratio method
    iii. Derivative spectroscopic method
b) RP-HPLC
c) HPTLC

Formulation 2: (Alogliptin benzoate and pioglitazone hydrochloride)

Simultaneous determination of alogliptin benzoate and pioglitazone hydrochloride in combined tablet formulation by

a) UV Spectroscopy
    i. Simultaneous equation method
    ii. Absorbance ratio method
b) RP-HPLC
c) HPTLC

4. MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 Pure drugs (Reference substance):

4.1.1.1 Alogliptin benzoate: Alogliptin benzoate reference standard was purchased from Swapnroop Drugs Pvt. Ltd., Aurangabad, India.

4.1.1.2 Metformin hydrochloride: Metformin hydrochloride pure drug was received as gift sample from IPCA laboratories, Mumbai, Maharashtra, India.

4.1.1.3 Pioglitazone hydrochloride: Pioglitazone hydrochloride reference standard was received as gift sample from Cadila Healthcare, Ahmedabad, India.

4.1.2 Formulations (Samples): (Tablet formulations made in laboratory which contains most of the ingredients used in the branded formulations, i.e. KAZANO and OSENI Tablets)

4.1.2.1 Formulation 1: Laboratory made tablet formulation

Alogliptin benzoate and Metformin hydrochloride tablet (Alogliptin benzoate 17 mg equivalent to 12.5 mg of alogliptin and 500 mg of metformin hydrochloride)

Table No. 4.1: Formulation 1

Sr. No.	Ingredients used	Quantity (mg)
1	Alogliptin benzoate	17
2	Metformin hydrochloride	500
4	Microcrystalline cellulose	50
5	Povidone	20
6	Crospovidone	12
7	Magnesium stearate	6
8	Distilled water	q.s
Total weight (mg)		**605**

4.1.2.1.1 Wet granulation method

Two different batches of tablets were prepared using wet granulation technique. The composition of single tablet per batch is given in **Table No.4.1**. Exactly weighed quantities of Alogliptin benzoate, metformin hydrochloride and MCC were passed through #22 mesh and blended. Povidone was dissolved in sufficient quantity of water and added to the dry mix and kneaded to form granules. The wet mass was passed through #22 mesh and granules were dried in hot air oven at 60^0c. The mixture was passed through #44 mesh. Superdisintegrant crospovidone and lubricant magnesium stearate was weighed and sifted through #60 mesh and added to the above mixture and blended for 1 minute. The final blend was mixed thoroughly for 2-3 minutes in polybag; tablets were compressed in 12 mm round concave shaped punches.

4.1.2.1.2 Organoleptic properties

Organoleptic properties of tablet formulation like colour, odor, and physical properties were characterized and recorded using descriptive terminology and the results are shown in **Table.No.4.2.**

Table No: 4.2. Organoleptic Chracteristics

Properties	Results
Colour	Colourless
Odor	Odourless
Shape	Round

4.1.2.1.3 Hardness test

Tablets require a certain amount of strength, or hardness and resistance to friability, to withstand mechanical shocks of handling in manufacture, packaging and shipping. Hardness of all batches were determined using Monsanto hardness tester. The test was carried out in triplicate for all batches as per USP XXIV monograph for uncoated tablets and the results are shown in **Table No.4.3.**

Table No: 4.3 Hardness Test

Tablet No.	1	2	3	4	5	Average	SD
Hardness (kg/cm^2)	6.5	7	6.9	7.1	7.2	6.9	0.2702

Hardness of the tablets was found to be 6.9±0.2702 which was found to be within the limits.

4.1.2.1.4 Friability test

Friability testing was done by Roche Friabilator with readings in triplicate. Tablets of all batches were used to evaluate friability as per USP XXIV monograph. Ten tablets were weighed and placed in friabilator and rotated at 25 rpm for 4 minutes. Then the tablets were taken out, dusted and reweighed. The percentage friability of the tablets was calculated by the formula: Conventional compressed tablets that lose less than 0.5%-1% of their weight are considered acceptable.

Percentage Friability = [(Initial Weight – Final Weight)/ Initial Weight] × 100

Initial Weight: **6070.89**

Final Weight: **6062.77**

% Friability: **0.14%**

4.1.2.1.5 Weight variation test

The weight of the tablet being made is routinely measured to help ensure that a tablet contains the proper amount of drug. The USP weight variation test is run by weighing 20 tablets individually, calculating the average weight, and comparing the individual tablet weights to the average. The tablet meet the USP test if no more than 2 tablets are outside the percentage limit and if no tablet differs by more than two times the percentage limit. The weight variation tolerances for uncoated tablets differ depending on average tablet weight. Twenty tablets were selected at random and weighed individually. The weights of individual tablets were noted. Average weight was calculated and the individual weights were compared with the average weight. The weight of not more than two tablets must not deviate from the average weight by more than 5%.The results of weight variation test is shown in **Table No. 4.5**

Table No. 4.4: Weight variation tolerances for uncoated tablets

Average weight of tablets (mg)	Maximum percentage differences allowed
130 or less	10
130-324	7.5
More than 324	5

Table No. 4.5: Weight variation test

1	2	3	4	5	6	7	8	9	10
605.47	610.38	607.69	612.32	606.81	609.66	611.62	603.1	601.62	605.21
11	12	13	14	15	16	17	18	19	20
608.85	606.78	612.32	613.98	615.67	610.45	600.67	599.62	600.34	605.32

Total weight of 20 Tablets: **12137.78**

Average weight of 20 tablets: **606.35**

No. of tablets differing by more than double the limit: **NIL**

No. of tablets differing by double the prescribed limit: **NIL**

4.1.2.1.6 Disintegration

Disintegration test was carried out by using Disintegration test apparatus. One tablet is placed in each tube, and the basket rack was positioned in a 1-litre beaker of water, at 37°C ±2°C. A standard motor-driven device is used to move the basket assembly containing the tablets up and down through a distance of 5 to 6 cm at a frequency of 28 to 32 cycles per minutes. The time taken for the tablet to disintegrate completely was noted.

Uncoated tablets have disintegration time standards as low as 5 minutes, but the majority of the tablets have maximum disintegration time of 30 min. [87-89]

Medium: Distilled water.

Volume: 1 liter

No. of tablets used: 6

Disintegration time: **26 minutes**

4.1.2.2 Formulation 2: Laboratory made formulation

Alogliptin benzoate and Pioglitazone hydrochloride tablet (Alogliptin benzoate 17/34 mg is equivalent to 12.5/25 mg of alogliptin and Pioglitazone hydrochloride 49.6 mg is equivalent to 45 mg of pioglitazone).[81-83]

Table No. 4.6: Formulation 2

Sr. No.	Ingredients used	Quantity (mg)
1	Alogliptin benzoate	17
2	Pioglitazone hydrochloride	45
3	Mannitol	10
4	Microcrystalline cellulose	20
5	Hydroxypropyl cellulose	6
6	Croscarmellose sodium	4
7	Magnesium stearate	2
8	Lactose monohydrate	100
9	Distilled water	q.s
Total weight (mg)		**204**

4.1.2.2.1 Wet granulation method

Tablet formulation of Alogliptin and Pioglitazone was made using wet granulation technique. Two different batches of tablets were prepared using wet granulation technique. The composition of single tablet per batch is given in **Table No.3.6**. Exactly weighed quantities of Alogliptin benzoate, Pioglitazone hydrochloride, mannitol and micro crystalline cellulose were passed through #22 mesh and blended. Weighed amount of hydroxypropyl cellulose was dissolved in minimum quantity of water and added to the dry mix and kneaded to form granules. The wet mass was passed through #22 mesh and granules were dried in hot air oven at 60^0c. The mixture was passed through #44 mesh. Lubricant magnesium stearate was weighed and sifted through #60 mesh and added to the above mixture. Disintegrant

croscarmellose sodium and lactose also was added and blended for 1 minute. The final blend was mixed thoroughly for 2-3 minutes in polybag; tablets were compressed in 6 mm round concave shaped punches.

4.1.2.2.2 Organoleptic properties

Organoleptic properties of tablet formulation like colour, odor, and physical properties were characterized and recorded using descriptive terminology and the results are shown in **Table.4.7**

Table No. 4.7: Organoleptic Chracteristics

Properties	Results
Colour	Colourless
Odor	Odourless
Shape	Round

4.1.2.2.3 Hardness test

Tablets require a certain amount of strength, or hardness and resistance to friability, to withstand mechanical shocks of handling in manufacture, packaging and shipping. Hardness of all batches was determined using Monsanto hardness tester. The test was carried out in triplicate for all batches as per USP XXIV monograph for uncoated tablets and the results are shown in **Table No.4.8**

Hardness of the tablets was found to be 5.98±0.3271 which was found to be within limits.

Table No. 4.8: Hardness Test

Tablet No.	1	2	3	4	5	Average	SD
Hardness (kg/cm^2)	5.5	6.3	6.1	5.8	6.2	5.98	0.3271

4.1.2.2.4 Friability test

Friability testing was done by Roche friabilator with readings in triplicate. Tablets of all batches were used to evaluate friability as per USP XXIV monograph.

Ten tablets were weighed and placed in friabilator and rotated at 25 rpm for 4 minutes. Then the tablets were taken out, dusted and reweighed. The percentage friability of the tablets was calculated by the formula: Conventional compressed tablets that lose less than 0.5%-1% of their weight are considered acceptable.

Percentage Friability = [(Initial Weight – Final Weight)/ Initial Weight] × 100
Initial Weight: 2094.54
Final Weight: 2085.88
% Friability: 0.413%

4.1.2.2.5 Weight variation test

The weight of the tablet being made is routinely measured to help ensure that a tablet contains the proper amount of drug. The USP weight variation test is run by weighing 20 tablets individually, calculating the average weight, and comparing the individual tablet weights to the average. The tablet meet the USP test if no more than 2 tablets are outside the percentage limit and if no tablet differs by more than two times the percentage limit. The weight variation tolerances for uncoated tablets differ depending on average tablet weight **(Table No. 4.9).** Twenty tablets of each formulation were selected at random and weighed individually. The weights of individual tablets were noted. Average weight was calculated and the individual weights were compared with the average weight. The weight of not more than two tablets must not deviate from the average weight by more than 5%. **(Table No. 4.10)**

Table No. 4.9: Weight variation tolerances for uncoated tablets

Average weight of tablets(mg)	Maximum percentage differences allowed
130 or less	10
130-324	7.5
More than 324	5

Table No. 4.10: Weight variation test

1	2	3	4	5	6	7	8	9	10
210.67	220.44	205.34	206.89	210.87	212.45	205.23	212.49	209.37	202.29
11	12	13	14	15	16	17	18	19	20
200.87	205.54	202.19	208.37	209.84	203.52	205.67	201.32	210.66	212.84

Total weight of 20 Tablets: 4150.8

Average weight of 20 tablets: 207.415

No. of tablets differing by more than double the limit: **NIL**

No. of tablets differing by double the prescribed limit: **NIL**

4.1.2.2.6 Disintegration Test

Disintegration test was carried out by using Disintegration test apparatus. One tablet is placed in each tube, and the basket rack was positioned in a 1-litre beaker of water, at 37°C ±2°C. A standard motor-driven device is used to move the basket assembly containing the tablets up and down through a distance of 5 to 6cm at a frequency of 28 to 32 cycles per minutes. The time taken for the tablet to disintegrate completely was noted.

Uncoated tablets have disintegration time standards as low as 5 minutes, but the majority of the tablets have maximum disintegration time of 30 min. **[87-89]**

Medium: Distilled water.

Volume: 1 liter

No. of tablets used: 6

Disintegration time: **15 minutes**

4.1.3 Solvents & Chemicals

1. Methanol: AR grade (Loba Chemie Pvt. Ltd., Mumbai, India.) & HPLC Grade (Merck Specialities Private Limited, Mumbai, India)
2. Ethanol: AR grade, SDFCL, Mumbai, India.
3. Acetonitrile: AR grade (Loba Chemie Pvt. Ltd., Mumbai, India.) & HPLC Grade (Merck Specialities Private Limited, Mumbai, India)
4. Water: HPLC grade, Millipore Direct Q3, Millipore India, Bangalore, India
5. Chloroform: AR Grade, SDFCL, Mumbai, India.
6. Ethyl acetate: AR Grade, SDFCL, Mumbai, India.
7. Toluene: AR Grade, SDFCL, Mumbai, India.
8. Ammonia solution: AR Grade, SDFCL, Mumbai, India.
9. Glacial acetic acid: AR Grade, SDFCL, Mumbai, India.
10. Diethylether: AR Grade, SDFCL, Mumbai, India.
11. Acetone: AR Grade, SDFCL, Mumbai, India.
12. Triethylamine: HPLC Grade, Loba Chemie Pvt. Ltd., Mumbai, India.
13. Orthophosphoric acid: HPLC Grade, Loba Chemie Pvt. Ltd., Mumbai, India.
14. Benzoic acid: AR Grade, SDFCL, Mumbai, India.
15. Microcrystalline cellulose: AR Grade, SDFCL, Mumbai, India.
16. Povidone: AR Grade, SDFCL, Mumbai, India.
17. Cros povidone: AR Grade, SDFCL, Mumbai, India.
18. Magnesium stearate: AR Grade, SDFCL, Mumbai, India.
19. Mannitol: AR Grade, SDFCL, Mumbai, India.
20. Hydroxypropyl cellulose: AR Grade, SDFCL, Mumbai, India.
21. Croscarmellose sodium: AR Grade, SDFCL, Mumbai, India.
22. Lactose monohydrate: AR Grade, SDFCL, Mumbai, India.

4.1.4 Instruments and equipments

4.1.4.1 UV-Visible Spectrophotometer

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

4.1.4.2 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)

4.1.4.3 RP-HPLC

Liquid Chromatography	:	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

4.1.4.4 Digital balance

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

4.1.4.5 Water purifier (HPLC grade water)

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

4.1.4.6 Digital pH meter

Model	:	S901
Manufacturer	:	Systonic, Delhi, India

4.1.4.7 TOSHCON Ultrasonic cleaner (Sonicator)

Model	:	SW 4

Manufacturer	:	Toshniwal Instruments Mfg. Pvt. Ltd., Ajmer, India.

4.1.4.8 Tablet punching machine

Manufacturer	:	Create (12 Station tablet punching machine), Ahmadabad, India.

4.2 Methods

Formulation 1:

Method 1

4.2.1 "Development and validation of simultaneous equation method for the simultaneous determination of alogliptin benzoate and metformin hydrochloride in tablets"

4.2.1.1 Selection of solvent:

By checking solubility in different solvents, methanol was selected as solvent for further studies**.**

4.2.1.2 Preparation of standard solution

Stock solution of ALO and MET were prepared by weighing accurately 13.60 mg of alogliptin benzoate (13.60 mg of alogliptin benzoate is equivalent to 10 mg of alogliptin) and 10 mg of metformin hydrochloride 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.

4.2.1.3 Selection of wavelength

Standard stock solutions of ALO and MET were further diluted separately with methanol to get the drug solutions containing 6 µg/ml of ALO and MET, 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.

4.2.1.4 Preparation of calibration curve

Different concentrations of ALO and MET (0.5-18 µg/ml) were prepared from respective stock solutions. The absorbances were noted at 224 and 237 nm for both the drugs. Calibration curves were plotted for both the drugs (ALO and MET) at 224

and 237 nm. Both the drugs should show optimum absorbance and linearity at each of the wavelength.

4.2.1.5 Determination of absorptivity values

The absorptivity values were calculated for ALO and of MET at both the wavelengths by using the following formula and tabulated.

Absorptivity = Absorbance/Concentration (gm/100 ml)

4.2.1.6 Preparation of sample solution

Twenty laboratory made tablets (12.5 mg of ALO and 500 mg of MET) were accurately weighed and average weight was calculated. All the tablets were crushed to fine powder and quantity equivalent to 5 mg of ALO and 200 mg of MET 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 and filtered through whatman filter paper. From this, 1 ml solution was transferred to 10 ml volumetric flask and 3.9 mg equivalent of pure ALO was also added and volume was made up to the mark with methanol to maintain the same concentration for both the drugs. (400 µg/ml of ALO&MET). From this 2.5 ml was pipetted out into a 10 ml volumetric flask and volume was made up with methanol to get 100 µg/ml of ALO and MET. Suitable aliquots were prepared to get desired concentrations (ALO 6 µg/ml and MET 6 µg/ml). Absorbance was measured and percentage assay was calculated solving simultaneous equation method.

4.2.1.7 Analysis of formulation

After scanning the sample solution (Formulation) between 200 to 400 nm, absorbances were noted at 224 and 237 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 C_x and C_y are the concentrations of ALO and MET, ax_1 and ax_2 are absorptivities of ALO at 224 nm and 237 nm, respectively. ay_1 and ay_2 are absorptivities of MET at 224 nm and 237 nm, respectively. A_1 and A_2 are the absorbances of mixture at 224 and 237 nm

4.2.1.8 Validation of the method

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

4.2.1.9 Specificity

To check the interference between tablet excipients used in the 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.

4.2.1.10 Linearity and range

Linearity and range of the method was checked by analyzing all the standard solutions separately, containing both the drugs ALO and MET (0.5, 3, 6, 9, 12, 15 and 18 µg/ml) and absorbances were measured at 224 nm and 237 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. Absorptivity values of individual solutions were calculated and average absorptivity values at specific wavelength of particular drug was used for calculating the concentration of drugs.

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

4.2.1.11 Precision

The precision of the method was checked by taking standard solutions of ALO and MET (4 and 6 µg/ml) and carrying out repeatability of measurement, intraday and interday precision.

4.2.1.11.1 Repeatability of measurement

To check the repeatability of the measurement standard solution of both the drugs (ALO: 4 & 6; MET: 4 & 6 µg/ml) were subjected to six times analysis and %RSD was calculated.

4.2.1.11.2 Intraday precision

Intraday precision was carried out by repeated measurements of the absorbance of standard solutions in triplicate at two different concentration levels (ALO: 4 & 6; MET: 4 & 6 µg/ml) for three times on the same day within the linearity range and % RSD was calculated.

4.2.1.11.3 Interday precision

Interday precision was studied by comparing the results on three different days analyzing three replicate measurements at two different concentration levels (ALO: 4 & 6; MET: 4 & 6 µg/ml) within the linearity range. Percentage RSD was calculated.

4.2.1.12 Accuracy

Recovery studies were carried out by measuring the absorbance of the added standard drug to pre-analyzed sample solution (Formulation, ALO: 2, 4 and 6 µg/ml; MET: 2, 4 and 6 µg/ml where ALO pure standard drug was added to make the same concentration of both the analytes) at 50, 100 and 150% levels at 224 nm and 237 nm

to check the accuracy of the method. The resulting solutions were reanalyzed and % recovery was calculated.

The result of the accuracy study was assessed based on the percentage of standard ALO and MET recovered from the formulation by using following formula.

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

4.2.1.13 LOD and LOQ

The limit of detection and limit of quantification were calculated to determine sensitivity of the method 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.

4.2.1.14 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 (±1 nm) in the wave length of measurement at 224 nm and 237 nm and % RSD was calculated.

4.2.1.15 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.

4.2.1.16 Analysis of formulation using developed method

Sample solutions were prepared and diluted with methanol by extracting the formulation as described in sample preparation to get desired concentration. Absorbance was measured and percentage assay was calculated by solving simultaneous equation method.

Method 2

4.2.2 "Development and validation of absorbance ratio (Q analysis) method for the simultaneous determination of alogliptin benzoate and metformin hydrochloride in tablets"

4.2.2.1 Selection of solvent:

By checking solubility in different solvents methanol was selected as solvent for further studies**.**

4.2.2.2 Preparation of standard solution:

Stock solution of ALO and MET were prepared by weighing accurately 13.60 mg of alogliptin benzoate (13.60 mg of alogliptin benzoate is equivalent to 10 mg of alogliptin) and 10 mg of metformin hydrochloride 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.

4.2.2.3 Selection of wavelength

Standard stock solution of ALO and MET were further diluted separately with methanol to get the drug solutions containing 6 µg/ml. 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.

4.2.2.4 Preparation of calibration curve

Different concentrations of ALO and MET (0.5-18 µg/ml) were prepared separately from respective stock solutions. The absorbances were noted at 224 and 251 nm for both the drugs. Calibration curves were plotted for both the drugs (ALO and MET) at 224 and 251 nm. Each of the drugs showed optimum absorbance and linearity at each of the wavelength.

4.2.2.5 Determination of absorptivity value

The absorptivity values were calculated for ALO and MET at both the wavelengths by using the following formula and tabulated.

Absorptivity = Absorbance/Concentration (gm/100 ml)

4.2.2.6 Preparation of sample solution:

Twenty laboratory made tablets (12.5 mg of ALO and 500 mg of MET) were accurately weighed and average weight was calculated. All the tablets were crushed to fine powder and quantity equivalent to 5 mg of ALO and 200 mg of MET 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 and filtered through whatman filter paper. From this 1 ml solution was transferred to 10 ml volumetric flask and 3.9 mg equivalent of ALO pure drug was also added and volume was made up to the mark with methanol to maintain the same concentration for both the drugs. (400 µg/ml of ALO and MET.) Suitable aliquots were prepared to get desired concentrations (ALO 6 µg/ml and MET 6 µg/ml). Absorbances were measured and percentage assay was calculated using absorbance ratio method.

4.2.2.7 Analysis of formulation

After scanning the sample solution (Formulation) between 200 to 400 nm, absorbances were noted at 224 and 251 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 ALO at 224 nm and 251 nm, respectively. ay_1 and ay_2 are absorptivities of MET at 224 nm and 251 nm, respectively.

A1 and A2 are the absorbances of mixture at 224 nm and 251 nm. Cx and Cy are the concentrations of ALO and MET, respectively in sample solution.

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

Absorbance was measured and percentage assay was calculated solving absorbance ratio equation method

4.2.2.8 Validation of the method:

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

4.2.2.9 Specificity

To check the interference between tablet excipients used in the 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.

4.2.2.10 Linearity and range

Linearity and range of the method was checked by analyzing all the standard solutions separately, containing ALO and MET (0.5, 3, 6, 9, 12, 15 and 18 µg/ml) and absorbances were measured at 224 nm and 251 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. Absorptivity values of individual solutions were calculated and average absorptivity values at specific wavelength of particular drug was used for calculating the concentration of drugs.

4.2.2.11 Precision

The precision of the method was checked by carrying out repeatability of measurement, intraday and interday precision.

4.2.2.11.1 Repeatability of measurement

To check the repeatability of the measurement standard solution of both the drugs (ALO: 4 & 6; MET: 4 & 6 µg/ml) were subjected to six times analysis and %RSD was calculated.

4.2.2.11.2 Intraday precision

Intraday precision was carried out by repeated measurements of the absorbance of standard solutions in triplicate at two different concentration levels (ALO: 4 & 6; MET: 4 & 6 µg/ml) for three times on the same day within the linearity range and % RSD was calculated.

4.2.2.11.3 Interday precision

Interday precision was studied by comparing the results on three different days analyzing three replicate measurements at two different concentration levels (ALO: 4 & 6; MET: 4 & 6 µg/ml) within the linearity range and percentage RSD was calculated.

4.2.2.12 Accuracy

Recovery studies were carried out by measuring the absorbance of the added standard drug to pre-analyzed sample solution (Formulation, ALO: 2, 4 and 6 µg/ml; MET: 2, 4 and 6 µg/ml) where pure ALO standard drug was added to make the same concentration of both the analytes) at 50, 100 and 150% at 224 nm and 251 nm to check the accuracy of the method. The resulting solutions were reanalyzed and % recovery was calculated. The result of the accuracy study was assessed based on the percentage of standard ALO and MET recovered from the formulation by using following formula

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

4.2.2.13 LOD and LOQ

The limit of detection and limit of quantification were calculated to determine sensitivity of the method 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.

4.2.2.14 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 (±1 nm) in the wave length of measurement at 224 nm and 251 nm and % RSD was calculated.

4.2.2.15 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.

4.2.2.16 Analysis of formulation using developed method

Sample solutions were prepared and diluted with methanol by extracting the formulation as described in sample preparation to get desired concentration. Absorbance was measured and percentage assay was calculated by using absorbance ratio method.

Method 3

4.2.3 "Development and validation of first-derivative (Zero crossing) spectroscopic method for the simultaneous determination of alogliptin benzoate and metformin hydrochloride in tablets"

Derivative UV spectroscopy has been widely used as a tool for quantitative analysis and for 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.

4.2.3.1 Selection of solvent

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

4.2.3.2 Preparation of standard solution

Stock solution of ALO and MET were prepared by weighing accurately 13.60 mg of alogliptin benzoate (13.60 mg of alogliptin benzoate is equivalent to 10 mg of alogliptin) and 10 mg of metformin hydrochloride 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.

4.2.3.3 Selection of wavelength

Standard stock solutions of ALO and MET were further diluted separately with methanol to get the drug solutions containing 6 µg/ml of ALO and MET. Both the solutions were scanned in the UV region (200-400 nm) and spectra were recorded. The spectra of ALO and MET 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 were showed typical zero-crossing points at 247.3 nm for ALO and 237 nm for MET. From the overlain spectra, 237 nm and 247.3 nm were selected for further studies.

4.2.3.4 Preparation of sample solution:

Twenty laboratory made tablets (12.5 mg of ALO and 500 mg of MET) were accurately weighed and average weight was calculated. All the tablets were crushed to fine powder and quantity equivalent to 5 mg of ALO and 200 mg of MET 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 and filtered through whatman filter paper. From this 1 ml solution was transferred to 10 ml volumetric flask and 3.9 mg equivalent of ALO pure drug was also added and volume was made up to the mark with methanol to maintain the same concentration for both the drugs (400 µg/ml of ALO and MET). Suitable aliquots were prepared to get desired concentrations (ALO 6 µg/ml and MET 6 µg/ml). Absorbance was measured and percentage assay was calculated using absorbance ratio method.

4.2.3.5 Validation of the method

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

4.2.3.6 Specificity

To check the interference between tablet excipients used in the 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.

4.2.3.7 Linearity and range

Standard stock solutions of ALO and MET were further diluted separately with methanol to get a series of drug solutions containing 0.5-18 µg/ml for ALO and MET. All the solutions were scanned in the UV region (200-400 nm) and spectra were recorded and converted into their 1st derivative spectra. Linearity and range of the method was checked by measuring 1stderivative signal and plotting calibration curve for both the drugs separately, containing ALO and MET (0.5, 3, 6, 9, 12, 15 and 18 µg/ml) at 237 nm and 247.3 nm, respectively. Calibration graphs were plotted using 1st derivative 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.

4.2.3.8 Precision

The precision of the method was checked by carrying out repeatability of measurement, intraday and interday precision.

4.2.3.8.1 Repeatability of measurement

To check the repeatability of the measurement standard solution of both the drugs (ALO: 4 & 6; MET: 4 & 6 µg/ml) were subjected to six times analysis and %RSD was calculated.

4.2.3.8.2 Intraday precision

Intraday precision was carried out by repeated measurements of the absorbance of standard solutions in triplicate at two different concentration levels (ALO: 4 & 6; MET: 4 & 6 µg/ml) for three times on the same day within the linearity range and % RSD was calculated.

4.2.3.8.3 Interday precision

Interday precision was studied by comparing the results on three different days analyzing three replicate measurements at two different concentration levels (ALO: 4 & 6; MET: 4 & 6 µg/ml) within the linearity range. Percentage RSD was calculated.

4.2.3.9 Accuracy

Recovery studies were carried out by measuring the absorbance of the added standard drug to pre-analyzed sample solution (Formulation, ALO: 2, 4 and 6 µg/ml; MET: 2, 4 and 6 µg/ml) where ALO pure standard drug was added to make the same concentration of both the analytes) at 50, 100 and 150% at 237 nm and 247.3 nm to check the accuracy of the method. The resulting solutions were reanalyzed and % recovery was calculated. The result of the recovery study was assessed based on the percentage of standard ALO and MET recovered from the formulation by using following formula

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

4.2.3.10 LOD and LOQ

The limit of detection and limit of quantification were calculated to determine sensitivity of the method 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.

4.2.3.11 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 247.3 nm.

4.2.3.12 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.

4.2.3.13 Analysis of formulation using developed method

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 amplitudes were measured. The concentration of drugs in sample/formulation was determined by using the regression equation and percentage assay was calculated**.**

Method 4

4.2.4 "Development and validation of RP-HPLC method for the simultaneous determination of alogliptin benzoate and metformin hydrochloride in tablets"

4.2.4.1 Selection of mode of chromatographic method:

Based on the literature survey RP-HPLC mode was selected

4.2.4.2 Selection of column:

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

4.2.4.3 Selection of wavelength:

Overlain UV spectra of both the drugs were taken in RP-HPLC system and 236 nm was selected as the wavelength for study.

4.2.4.4 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.

4.2.4.5 Optimization of separation conditions:

Strength of buffer, mobile phase composition, pH, flow rate, detection wave length etc. were tried.

4.2.4.6 Preparation of standard solution:

Mixed standard stock solution of ALO and MET were prepared by weighing accurately 13.60 mg of alogliptin benzoate (13.60 mg of alogliptin benzoate is equivalent to 10 mg of alogliptin) and 10 mg of metformin hydrochloride 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 drugs 1000 µg/ml. Further dilutions were made to get the desired concentration with mobile phase.

4.2.4.7 Preparation of sample solution

Twenty laboratory made tablets (12.5 mg of ALO and 500 mg of MET) were accurately weighed and average weight was calculated. All the tablets were crushed to fine powder and quantity equivalent to 5 mg of ALO and 200 mg of MET 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 and filtered through whatman filter paper. From this 1 ml solution was transferred to 10 ml volumetric flask and 3.9 mg equivalent of pure ALO was also added and volume was made up to the mark with methanol to maintain the same concentration for both the drugs. (400 µg/ml ALO&MET). From this 2.5 ml was pipetted out into a 10 ml volumetric flask and volume was made up with methanol to get 100 µg/ml of ALO and MET. Suitable aliquots were prepared to get desired concentrations (ALO 10 µg/ml and MET 10 µg/ml).

4.2.4.8 Validation of chromatographic method:

Developed method was validated according to ICH guidelines using following parameters

4.2.4.9 Specificity

To check the interference between tablet excipients used in the 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.

4.2.4.10 Linearity and range

Linearity of the method was checked by analyzing mixed standard solutions containing ALO and MET (0.5, 1, 10, 20, 30, 40 and 50 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 Vs concentration. Results were subjected to regression analysis by the least square method to calculate the values of slope, intercept and correlation coefficient.

4.2.4.11 Precision

The precision of the method was checked by carrying out repeatability, intraday and interday precision.

4.2.4.11.1 Repeatability of measurement

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

4.2.4.11.2 Intraday precision

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

4.2.4.11.3 Interday precision

Interday precision was studied by comparing the results on three different days analyzing three replicate injections at two different concentration levels (ALO: 5 & 10; MET: 5 & 10 µg/ml) within the linearity range and % RSD was calculated.

4.2.4.12 Accuracy

Recovery studies were carried out by the addition of standard drug to pre-analyzed sample solution (Formulation, ALO: 5, 10 and 15 µg/ml; MET: 5, 10 and 15 µg/ml) where pure ALO standard drug was added to make the same concentration of both the analytes) at 50, 100 and 150% levels to check the accuracy of the method. The resulting solutions were reanalyzed and % recovery was calculated. The result of the accuracy study was assessed based on the percentage of standard ALO and MET recovered from the formulation by using following formula

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

4.2.4.13 LOD and LOQ

The limit of detection and limit of quantification of ALO and MET 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.

4.2.4.14 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 flow rate (± 0.1 ml/min), buffer pH (± 0.2 units) and buffer strength (± 0.1%).

4.2.4.15 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.

4.2.4.16 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, no of theoretical plates, peak, asymmetry factor, resolution, tailing factor)

4.2.4.17 Analysis of formulation by developed method:

Sample solutions were prepared and diluted with mobile phase by extracting the formulation as described in sample preparation to get desired concentration. Peak area was measured and percentage assay was calculated.

Method 5

4.2.5 "Development and validation of HPTLC method for the simultaneous determination of alogliptin benzoate and metformin hydrochloride in tablets"

4.2.5.1 Selection of mode of chromatographic method:

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

4.2.5.2 Selection of solvent:

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

4.2.5.3 Selection of wavelength

UV spectra of drugs on pre-coated plate were recorded and 237 nm was selected as wavelength of detection.

4.2.5.4 Development of optimum mobile phase

A solvent system that gave dense compact spots, good separation between ALO, MET and BA (Benzoic acid: Which is separating from alogliptin benzoate) and separation from solvent front and application position was selected. Initially different solvent systems were tried and optimized mobile phase was selected.

4.2.5.5 Optimization of separation conditions

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

4.2.5.6 Preparation of standard solution

Stock solution containing 1000 µg/ml of ALO and MET was prepared by weighing 13.60 mg (13.60 mg of alogliptin benzoate is equivalent to 10 mg of alogliptin) of alogliptin benzoate and 10 mg of metformin hydrochloride in to a 10 ml

volumetric flask using methanol as solvent and sonicated. Working standard solution (ALO and MET 100 µg/ml) was prepared by mixing 1 ml of each stock solution and volume was made up to 10 ml with methanol.

4.2.5.7 Preparation of sample solution

Twenty laboratory made tablets (12.5 mg of ALO and 500 mg of MET) were accurately weighed and average weight was calculated. All the tablets were crushed to fine powder and quantity equivalent to 5 mg of ALO and 200 mg of MET were weighed and transferred to a 50 ml volumetric flask. Flasks were vortexed after adding 30 ml of methanol, shaken for 10 minutes and volume was made up to the mark with methanol and filtered through whatman filter paper. From this 1 ml solution was transferred to 10 ml volumetric flask and 3.9 mg equivalent of pure ALO was also added and volume was made up to the mark with methanol to maintain the same concentration for both the drugs (400 µg/ml ALO&MET). From this 2.5 ml was pipetted out into a 10 ml volumetric flask and volume was made up with methanol to get 100 µg/ml of ALO and MET. Suitable aliquots were prepared to get desired concentrations (ALO 500 ng/band and MET 500 ng/band).

4.2.5.8 Validation of chromatographic method

Developed method was validated according to ICH guidelines using following parameters

4.2.5.9 Specificity

Specificity of the method was checked by analyzing chromatographic peaks of drugs for peak purity.

4.2.5.10 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 (ALO and MET 50-1000 ng/band) of mixed working standard solution (ALO and MET 100 µg/ml). The developed plate was analyzed and chromatograms were recorded. Calibration graphs were plotted using peak area versus

ng/band. Results were subjected to regression analysis by the least squares method to calculate values of slope, intercept and correlation coefficient**.**

4.2.5.11 Precision

The precision of the method was checked by carrying out repeatability, intraday and interday precision.

4.2.5.11.1 Repeatability of measurement

To check the repeatability of the method a standard mixed solution (ALO: 200 & 400 ng/band; MET: 200 & 400 ng/band) was spotted on the TLC plate six times, chromatograms were recorded and %RSD was calculated.

4.2.5.11.2 Intraday precision

Intraday precision studies were performed by spotting two different aliquots (ALO: 200 & 400 ng/band; MET: 200 & 400 ng/band) of the mixed standard solution in triplicate on the same day and %RSD was calculated.

4.2.5.11.3 Interday precision

Interday precision studies were performed by spotting two different aliquots (ALO: 200 & 400 ng/band; MET: 200 & 400 ng/band) of the mixed standard solution in triplicate on three different days within the linearity range and % RSD was calculated.

4.2.5.12 Accuracy

Recovery studies were carried out by the addition of standard drug to pre-analyzed sample solution (Formulation, ALO: 200,300 and 400 ng/band; MET: 200, 300 and 400 ng/band) at 50, 100 and 150% levels to check the accuracy of the method. The resulting solutions were reanalyzed and % recovery was calculated. The result of the recovery study was assessed based on the percentage of standard ALO and MET recovered from the formulation by using following formula

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

4.2.5.13 LOD and LOQ

The limit of detection and limit of quantification of ALO and MET 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.

4.2.5.14 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, chamber saturation time, time from spotting to chromatography, time from chromatography to scanning, development distance, volume of mobile phase, etc.

4.2.5.15 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.

4.2.5.16 Analysis of formulation

Sample solutions were prepared and diluted with methanol by extracting the formulation as described in sample preparation to get desired concentration. Peak area was measured and percentage assay was calculated.

Formulation 2:

Method 6

4.2.6 "Development and validation of simultaneous equation method for the simultaneous determination of alogliptin benzoate and pioglitazone hydrochloride in tablets"

4.2.6.1 Selection of solvent

By checking solubility in different solvents methanol was selected as solvent for further studies**.**

4.2.6.2 Preparation of standard solution

Stock solution of ALO and PIO were prepared by weighing accurately 6.80 mg of ALO (6.80 mg of alogliptin benzoate is equivalent to 5 mg of alogliptin) and 18 mg of PIO (19.84 mg of pioglitazone hydrochloride is equivalent to 18 mg of pioglitazone) standard drugs which were then transferred to a 10 ml volumetric flask separately and diluted to 10 ml with methanol to get the concentration of the drugs 500 and 1800 µg/ml, respectively. Further dilutions were made to get the desired concentration with methanol.

4.2.6.3 Selection of wavelength

Standard stock solutions of ALO and PIO were further diluted separately with methanol to get the drug solutions containing 6 µg/ml of ALO and 21.6 µg/ml of PIO, 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.

4.2.6.4 Preparation of calibration curve

Different concentrations of ALO (0.5-12 µg/ml) and PIO (1.8-43.2 µg/ml) were prepared from respective stock solutions. The absorbances were noted at 224 and 268 nm for both the drugs. Calibration curves were plotted for both the drugs

(ALO and PIO) at 224 and 268 nm. Each of the drugs should show optimum absorbance and linearity at each of the wavelength.

4.2.6.5 Determination of absorptivity value

The absorptivity values were calculated for ALO and of PIO at both the wavelengths by using the following formula.

Absorptivity = Absorbance/Concentration (gm/100 ml)

4.2.6.6 Preparation of sample solution

Twenty tablets (12.5 mg of ALO and 45 mg of PIO) were accurately weighed and crushed to fine powder. Quantity of fine powder equivalent to 5 mg of ALO & 18 mg of PIO was transferred to a 50 ml volumetric flask (100 µg/ml ALO & 360 µg/ml PIO). 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. From this 1 ml solution was transferred to 10 ml volumetric flask (10 µg/ml ALO & 36 µg/ml PIO). Suitable aliquots were prepared to get desired concentrations (ALO 6 µg/ml and PIO 21.6 µg/ml).

4.2.6.7 Analysis of formulation

After scanning the sample solution (Formulation) between 200 to 400 nm, absorbances were noted at 224 and 268 nm. The concentrations of drugs in sample/formulation were determined by the simultaneous equation method using the following formula

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

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

Where C_x and C_y are the concentrations of ALO and PIO, ax_1 and ax_2 are absorptivities of ALO at 224 nm and 268 nm, respectively. ay_1 and ay_2 are absorptivities of PIO at

224 nm and 268 nm, respectively. A_1 and A_2 are absorbances of mixture at 224 and 268 nm.

4.2.6.8 Validation of the method

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

4.2.6.9 Specificity

To check the interference between tablet excipients used in the 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.

4.2.6.10 Linearity and range

Linearity and range of the method was checked by analyzing all the standard solutions separately, containing ALO (0.5, 2, 4, 6, 8, 10 and 12 µg/ml) and PIO (1.8, 7.2, 14.4, 21.6, 28.8, 36 and 43.2 µg/ml) in methanol and absorbances were measured at 224 nm and 268 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. Absorptivity values of individual solutions were calculated and average absorptivity values at specific wavelength of particular drug was used for calculating the concentration of drugs.

4.2.6.11 Precision

The precision of the method was checked by carrying out repeatability of measurement, intraday and interday precision.

4.2.6.11.1 Repeatability of measurement

To check the repeatability of the measurement a standard solution (ALO: 4 & 14.4 µg/ml; PIO: 6 & 21.6 µg/ml) of both the drugs were subjected to six times analysis and %RSD was calculated.

4.2.6.11.2 Intraday precision

Intraday precision was carried out by repeated measurement of the absorbance of standard solutions (ALO: 4 & 14.4 µg/ml; PIO: 6 & 21.6 µg/ml) in triplicate at two different concentration levels for three times on the same day within the linearity range.

4.2.6.11.3 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 (ALO: 4 & 14.4 µg/ml; PIO: 6 & 21.6 µg/ml) within the linearity range. Percentage RSD was calculated.

4.2.6.12 Accuracy

Recovery studies were carried out by measuring the absorbance of the added standard drug to pre-analyzed sample solution (Formulation, ALO: 1, 2 and 4 µg/ml; MET: 3.6, 7.2 and 14.4 µg/ml) at three different levels: 50, 100 and 150% at 224 nm and 268 nm to check the accuracy of the method. The resulting solutions were reanalyzed and % recovery was calculated. The result of the accuracy study was assessed based on the percentage of standard ALO and PIO recovered from the formulation by using following formula.

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

4.2.6.13 LOD and LOQ

The limit of detection and limit of quantification of ALO and PIO 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.

4.2.6.14 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 (±1 nm) in the wave length of measurement at 224 nm and 268 nm**.**

4.2.6.15 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.

4.2.6.16 Analysis of formulation by developed method:

Sample solutions were prepared and diluted with methanol by extracting the formulation as described in sample preparation to get desired concentration. Absorbance was measured and percentage assay was calculated by solving simultaneous equation method.

Method 7

4.2.7 "Development and validation of absorbance ratio (Q analysis) method for the simultaneous determination of alogliptin benzoate and pioglitazone hydrochloride in tablets"

4.2.7.1 Selection of solvent:

By checking solubility in different solvents methanol was selected as solvent for further studies**.**

4.2.7.2 Preparation of standard solution

Stock solution of ALO and PIO were prepared by weighing accurately 6.80 mg of ALO (6.80 mg of alogliptin benzoate is equivalent to 5 mg of alogliptin) and 18 mg of PIO (19.84 mg of pioglitazone hydrochloride is equivalent to 18 mg of pioglitazone) standard drugs which were then transferred to a 10 ml volumetric flask separately and diluted to 10 ml with methanol to get the concentration of the drugs 500 and 1800 µg/ml, respectively. Further dilutions were made to get the desired concentration with methanol.

4.2.7.3 Selection of wavelength

Standard stock solutions of ALO and PIO were further diluted separately with methanol to get the drug solutions containing 6 µg/ml of ALO and 21.6 µg/ml of PIO, 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, 224 nm (λ_{max} of ALO) and 279 nm (isobestic point) were selected for further studies.

4.2.7.4 Preparation of calibration curve

Different concentrations of ALO (0.5-12 µg/ml) and PIO (1.8-43.2 µg/ml) were prepared from respective stock solutions. The absorbances were noted at 224 and 279 nm for both the drugs. Calibration curves were plotted for both the drugs

(ALO and PIO) at 224 and 279 nm. Each of the drugs showed optimum absorbance and linearity at each of the wavelength.

4.2.7.5 Determination of absorptivity value

The absorptivity values were calculated for ALO and of PIO at both the wavelengths by using the following formula.

Absorptivity = Absorbance/Concentration (gm/100 ml)

4.2.7.6 Preparation of sample solution

Twenty tablets (12.5 mg ALO and 45 mg of PIO) were accurately weighed and crushed to fine powder. Quantity of fine powder equivalent to 5 mg of ALO & 18 mg of PIO was transferred to a 50 ml volumetric flask (100 µg/ml ALO & 360 µg/ml PIO). 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. From this 1 ml solution was transferred to 10 ml volumetric flask (10 µg/ml ALO & 36 µg/ml PIO). Suitable aliquots were prepared to get desired concentrations (ALO 6 µg/ml and PIO 21.6 µg/ml).

4.2.7.7 Analysis of formulation

After scanning the sample solution (Formulation) between 200 to 400 nm, absorbances were noted at 224 and 279 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 ALO at 224 nm and 279 nm, respectively. ay_1 and ay_2 are absorptivities of PIO at 224 nm and 279 nm, respectively.

A1 and A2 are the absorbances of mixture at 224 nm and 279 nm. Cx and Cy are the concentrations of ALO and PIO, respectively in sample solution.

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

4.2.7.8 Validation of the method

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

4.2.7.9 Specificity

To check the interference between tablet excipients used in the 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.

4.2.7.10 Linearity and range

Linearity and range of the method was checked by analyzing all the standard solutions separately, containing ALO (0.5, 2, 4, 6, 8, 10 and 12 µg/ml) and PIO (1.8, 7.2, 14.4, 21.6, 28.8, 36 and 43.2 µg/ml), in methanol and absorbance was noted at 224 nm and 279 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**.** Absorptivity values of individual solutions were calculated and average absorptivity values at specific wavelength of particular drug was used for calculating the concentration of drugs.

4.2.7.11 Precision

The precision of the method was checked by carrying out repeatability of measurement, intraday and interday precision.

4.2.7.11.1 Repeatability of measurement

To check the repeatability of the measurement a standard solution of both the drugs (ALO: 4 & 14.4 µg/ml; PIO: 6 & 21.6 µg/ml) were subjected to six time analysis and %RSD was calculated.

4.2.7.11.2 Intraday precision

Intraday precision was carried out by repeated measurement of the absorbance of standard solutions in triplicate at two different concentration levels (ALO: 4 & 14.4 µg/ml; PIO: 6 & 21.6 µg/ml) for three times on the same day within the linearity range.

4.2.7.11.3 Interday precision

Interday precision was studied by comparing the results on three different days taking three replicate measurement at two different concentration levels (ALO: 4 & 14.4 µg/ml; PIO: 6 & 21.6 µg/ml) within the linearity range and %RSD was calculated. Percentage RSD was calculated.

4.2.7.12 Accuracy

Recovery studies were carried out by measuring the absorbance of the added standard drug to pre-analyzed sample solution (Formulation, ALO: 1, 2 and 4 µg/ml; PIO: 3.6, 7.2 and 14.4 µg/ml) at 50, 100 and 150% at 224 nm and 279 nm to check the accuracy of the method. The resulting solutions were reanalyzed and % recovery was calculated**.** The result of the accuracy study was assessed based on the percentage of standard ALO and PIO recovered from the formulation by using following formula

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

4.2.7.13 LOD and LOQ

The limit of detection and limit of quantification of ALO and PIO 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,

4.2.7.14 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 (±1 nm) in the wave length of measurement at 224 nm and 279 nm.

4.2.7.15 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.

4.2.7.16 Analysis of formulation using developed method:

Sample solutions were prepared and diluted with methanol by extracting the formulation as described in sample preparation to get desired concentration. Absorbance was measured and percentage assay was calculated using absorbance ratio method.

Method 8

4.2.8 "Development and validation of RP-HPLC method for the simultaneous determination of alogliptin benzoate and pioglitazone hydrochloride in tablets"

4.2.8.1 Selection of mode of chromatographic method:

Based on the literature survey RP-HPLC mode was selected

4.2.8.2 Selection of column:

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

4.2.8.3 Selection of wavelength:

Overlain UV spectra of both the drugs were taken in RP-HPLC system and 267 nm was selected as the wavelength for study.

4.2.8.4 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.

4.2.8.5 Optimization of separation conditions:

Strength of buffer, mobile phase composition, pH, flow rate, detection wave length etc. were tried.

4.2.8.6 Preparation of standard solution:

Mixed standard stock solution of ALO and PIO were prepared by weighing accurately 13.60 mg of alogliptin benzoate (13.60 mg of alogliptin benzoate is equivalent to 10 mg of alogliptin) 19.84 mg of pioglitazone hydrochloride (19.84 mg of pioglitazone hydrochloride is equivalent to 18 mg of pioglitazone) standard drug which was then transferred to a 10 ml volumetric flask and diluted to 10 ml with mobile phase to get the concentration of the drugs 1000 µg/ml and 1800 µg/ml, respectively. Further dilutions were made to get the desired concentration with mobile phase.

4.2.8.7 Preparation of sample solution

Twenty tablets (25 mg ALO and 45 mg of PIO) were accurately weighed and crushed to fine powder. Quantity of fine powder equivalent to 5 mg of ALO & 18 mg of PIO was transferred to a 50 ml volumetric flask (100 µg/ml ALO & 360 µg/ml PIO). 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. Suitable aliquots were prepared to get desired concentrations.

4.2.8.8 Validation of chromatographic method

Developed method was validated according to ICH guidelines using following parameters

4.2.8.9 Specificity

To check the interference between tablet excipients used in the 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.

4.2.8.10 Linearity and range

Linearity of the method was checked by analyzing mixed standard solutions containing ALO (0.5, 1, 10, 20, 30, 40 and 50 µg/ml) and PIO (0.9, 1.8, 18, 36, 54, 72 and 90 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 values of slope, intercept and correlation coefficient**.**

4.2.8.11 Precision

The precision of the method was checked by carrying out repeatability, intraday and interday precision.

4.2.8.11.1 Repeatability of measurement

To check the repeatability of the method, a standard mixed solution (ALO: 5µg/ml &10 µg/ml; PIO: 9 µg/ml &18 µg/ml) was injected 6 times and %RSD was calculated.

4.2.8.11.2 Intraday precision

Intraday precision was carried out by analyzing six replicate injections at two different concentration levels (ALO: 5 µg/ml & 10 µg/ml; PIO: 9 µg/ml &18 µg/ml) on the same day within the linearity range and % RSD was calculated.

4.2.8.11.3 Interday precision

Interday precision was studied by comparing the results on three different days analyzing six replicate injections at two different concentration levels (ALO: 5 µg/ml & 10 µg/ml; PIO: 9 µg/ml &18 µg/ml)within the linearity range and % RSD was calculated.

4.2.8.12 Accuracy

Recovery studies were carried out by the addition of standard drug to pre-analyzed sample solution (Formulation, ALO: 5, 10 and 15 µg/ml; PIO: 9, 18 and 27 µg/ml) at 50, 100 and 150% to check the accuracy of the method. The resulting solutions were reanalyzed and % recovery was calculated**.** The result of the accuracy study was assessed based on the percentage of standard ALO and PIO recovered from the formulation by using following formula:

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

4.2.8.13 LOD and LOQ

The limit of detection and limit of quantification of ALO and PIO 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.

4.2.8.14 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 flow rate (± 0.1 ml/min), buffer pH (± 0.2 units) and buffer strength (± 0.1%).

4.2.8.15 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.

4.2.8.16 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, no of theoretical plates, peak asymmetry factor, resolution, tailing factor)

4.2.8.17 Analysis of formulation using developed method

Sample solutions were prepared and diluted with mobile phase by extracting the formulation as described in sample preparation to get desired concentration. The solutions were injected and chromatograms were recorded. Based on the peak area of analytes, percentage assay of the formulation was calculated.

Method 9

4.2.9 "Development and validation of HPTLC method for the simultaneous determination of alogliptin benzoate and pioglitazone hydrochloride in tablets"

4.2.9.1 Selection of mode of chromatographic method:

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

4.2.9.2 Selection of solvent:

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

4.2.9.3 Selection of wavelength

UV spectra of drugs on pre-coated plate were recorded and 274 nm was selected as wavelength of detection.

4.2.9.4 Development of optimum mobile phase

A solvent system that gave dense compact spots, good separation between ALO, PIO and BA (Benzoic acid: Which is separating from alogliptin benzoate) and separation from solvent front and application position was selected. Initially different solvent systems were tried and optimized mobile phase was selected.

4.2.9.5 Optimization of separation conditions

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

4.2.9.6 Preparation of standard solution

Mixed standard stock solution of ALO and PIO were prepared by weighing accurately 13.60 mg of alogliptin benzoate (13.60 mg of alogliptin benzoate is equivalent to 10 mg of alogliptin) and 18 mg of pioglitazone hydrochloride standard

drug which was then transferred to a 10 ml volumetric flask and diluted to 10 ml with methanol to get the concentration of the drugs 1000 µg/ml and 1800 µg/ml, respectively. Further dilutions were made to get the desired concentration with methanol.

4.2.9.7 Preparation of sample solution

Twenty tablets (25 mg of ALO and 45 mg of PIO) were accurately weighed and crushed to fine powder. Quantity of fine powder equivalent to 5 mg of ALO & 18 mg of PIO was transferred to a 50 ml volumetric flask (100 µg/ml ALO & 360 µg/ml PIO). 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. Suitable aliquots were prepared to get desired concentrations.

4.2.9.8 Validation of chromatographic method

Developed method was validated according to ICH guidelines using following parameters

4.2.9.9 Specificity

Specificity of the method was checked by analyzing chromatographic peaks of drugs for peak purity.

4.2.9.10 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 (50-1000 ng/band of ALO and 90-1800 ng/band of PIO) of mixed working standard solution (ALO 100 µg/ml and PIO 180 µg/ml). The developed plate was analyzed and chromatograms were recorded. Calibration graph was plotted using peak area versus ng/band. Results were subjected to regression analysis by the least squares method to calculate values of slope, intercept and correlation coefficient.

4.2.9.11 Precision

The precision of the method was checked by carrying out repeatability, intraday and interday precision.

4.2.9.11.1 Repeatability of measurement

To check the repeatability of the method a standard mixed solution (ALO: 200 ng/band & 400 ng/band; PIO: 360 ng/band & 720 ng/band) was spotted on the TLC plate six times and %RSD was calculated.

4.2.9.11.2 Intraday precision

Intraday precision studies were performed by spotting two different aliquots of the mixed standard solution (ALO: 200 ng/band & 400 ng/band; PIO: 360 ng/band &720 ng/band) in triplicate on the same day within the linearity range and % RSD was calculated.

4.2.9.11.3 Interday precision

Interday precision studies were performed by spotting two different aliquots of the mixed standard solution (ALO: 200 ng/band & 400 ng/band; PIO: 360 ng/band &720 ng/band) in triplicate on three different days within the linearity range and % RSD was calculated.

4.2.9.12 Accuracy

Recovery studies were carried out by the addition of standard drug to pre-analyzed sample solution ((Formulation, ALO: 200, 300 and 400 ng/band; PIO: 360, 540, and 720 ng/band) at three different levels: 50, 100 and 150% to check the accuracy of the method. The resulting solutions were reanalyzed and % recovery was calculated. The result of the accuracy study was assessed based on the percentage of standard ALO and PIO recovered from the formulation by using following formula

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

4.2.9.13 LOD and LOQ

The limit of detection and limit of quantification of ALO and PIO 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.

4.2.9.14 Robustness

The robustness of an analytical procedure is refers to its ability to remain unaffected by small and deliberate variations in the method parameters. The method should be robust enough with respect to all critical parameters so as to allow routine laboratory use. Robustness of the method was checked on the basis of slight alteration in the mobile phase composition, chamber saturation time, time from spotting to chromatography, time from chromatography to scanning, development distance, volume of mobile phase, etc.

4.2.9.15 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.

4.2.9.16 Analysis of formulation

Sample solutions were prepared and diluted with methanol by extracting the formulation as described in sample preparation to get desired concentration. Peak area was measured and percentage assay was calculated**.**

5. RESULTS AND DISCUSSION

Method 1

5.1 "Development and validation of simultaneous equation method for the simultaneous determination of alogliptin benzoate and metformin hydrochloride in tablets"

Estimation of ALO and MET was achieved by simultaneous equation method using Shimadzu UV 1800 (UV Pro), double beam UV-Visible spectrophotometer.

5.1.1 Selection of solvent

By checking solubility in different solvents methanol was selected as solvent for further studies, which showed higher absorbance and distinct λ_{max} for both the drugs**.**

5.1.2 Selection of wavelength

Standard solutions containing 6 µg/ml of ALO and MET were scanned in the UV region (200-400 nm) and spectra were recorded using methanol as blank. Their overlain spectra are shown in **Figure No. 5.1.1**

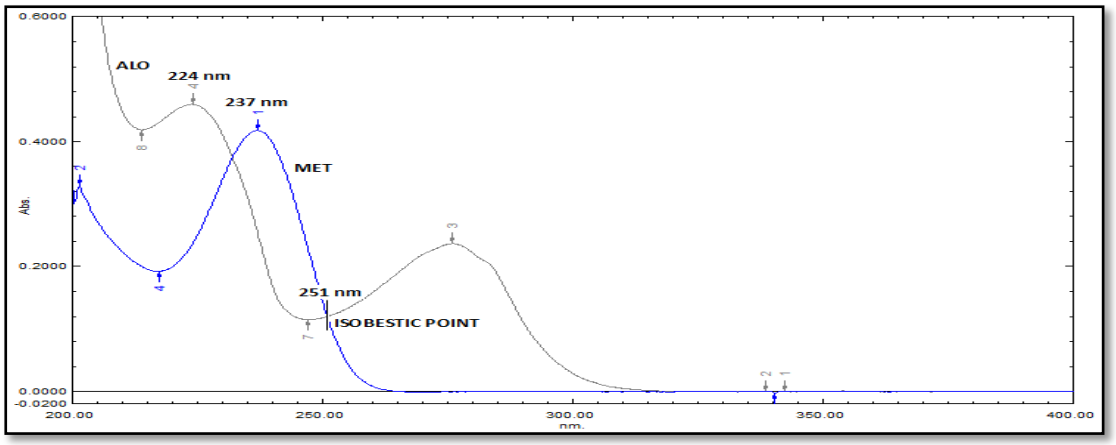


Figure No. 5.1.1: Overlain UV spectra of ALO and MET (6 µg/ml)

From the overlain spectra, different wavelengths were tried for the method. After comparing all wavelengths, 224 nm and 237 nm were selected which showed good linearity over the given concentration range.

5.1.3 Determination of absorptivity values

The developed method was found to be linear in the concentration range of 0.5-18 µg/ml for both the drugs. Absorbances were measured at 224 nm and 237 nm for both the drugs and absorptivity values were calculated which is shown in **Table No.5.1.1 & 5.1.2**

Table No. 5.1.1: Absorbances and absorptivities of ALO at selected wavelength

Conc. (µg/ml)	ALO					
	224 nm			237 nm		
	Abs.*	Absorptivity	Avg. Absorptivity	Abs.*	Absorptivity	Avg. Absorptivity
0.5	0.0386	772.0000	771.9972 (ax_1)	0.0215	429.6667	430.5562 (ax_2)
3	0.2315	771.6111		0.1284	428.1111	
6	0.4565	760.8611		0.2533	422.1667	
9	0.6866	762.8889		0.3819	424.3148	
12	0.9398	783.1250		0.5291	440.9028	
15	1.1682	778.8000		0.6608	440.5000	
18	1.3945	774.6944		0.7708	428.2315	

**average of six determinations*

Table No. 5.1.2: Absorbances and absorptivities of MET at selected wavelength

Conc. (µg/ml)	MET					
	224 nm			237 nm		
	Abs.*	Absorptivity	Avg. Absorptivity	Abs.*	Absorptivity	Avg. Absorptivity
0.5	0.0196	391.6667	396.854 (ay_1)	0.0342	684.3333	698.0052 (ay_2)
3	0.1183	394.1667		0.2116	705.2778	
6	0.2357	392.8333		0.4195	699.1111	
9	0.3619	402.1111		0.6320	702.1852	
12	0.4829	402.4306		0.8410	700.8194	
15	0.5930	395.3000		1.0492	699.4667	
18	0.7190	399.4444		1.2507	694.8426	

**average of six determinations*

5.1.4 Validation of the method

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

5.1.5 Specificity

Overlain spectra of placebo and drug solution indicate that there was no interference between excipients and standard drugs (**Figure No. 5.1.2.).**

5.1.6 Linearity

From the linearity study, ALO and MET were found to be linear in the concentration range of 0.5-18 µg/ml. The overlain spectra of ALO and MET are shown in **Figure No. 5.1.3-5.1.5.**

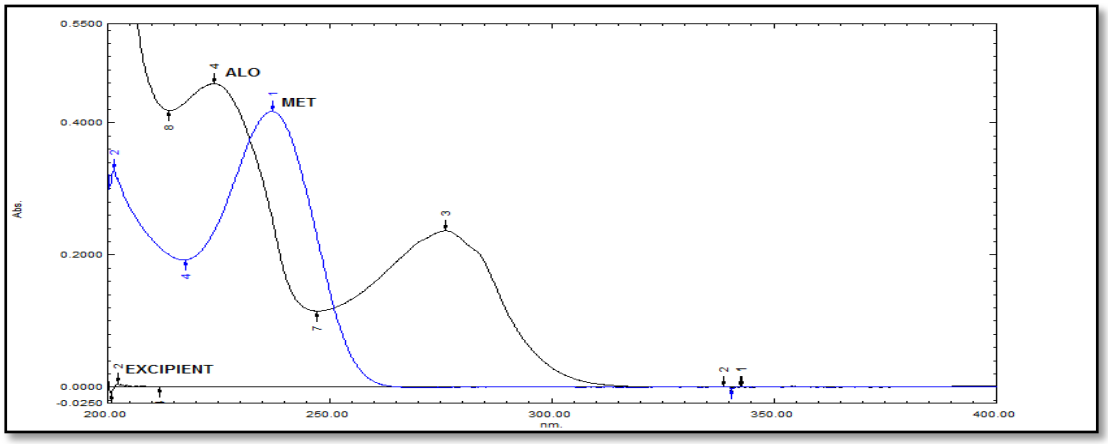


Figure No. 5.1.2: Overlain UV Spectra of ALO and MET(6 µg/ml) and formulation excipient

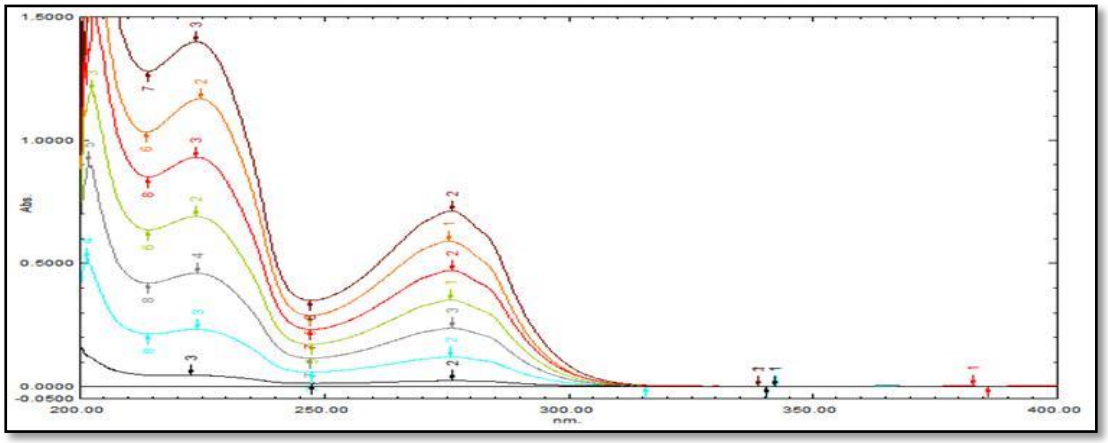


Figure No. 5.1.3: Overlain UV spectra of ALO (0.5-18 µg/ml)

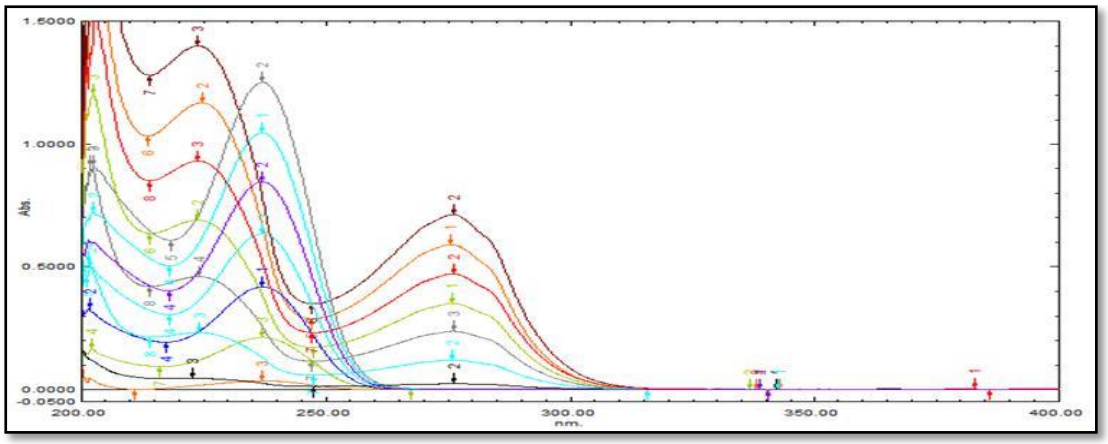


Figure No. 5.1.4: Overlain UV spectra of MET (0.5-18 µg/ml)

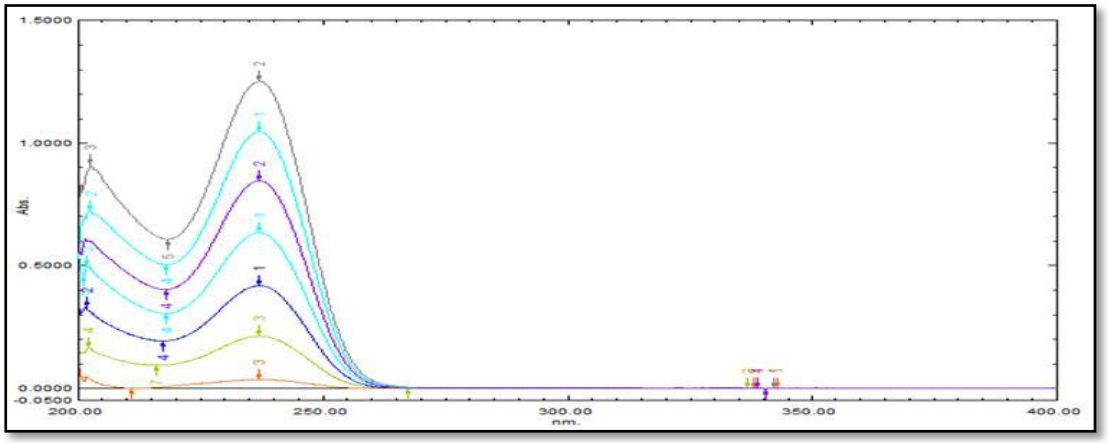


Figure No. 5.1.5: Overlain UV spectra of ALO & MET(0.5-18 µg/ml)

Calibration graphs were plotted using absorbances of standard drug solutions versus concentration **(Figure No. 5.1.6 - 5.1.9).** Results were subjected to regression analysis by the least squares method to calculate the values of slope, intercept and correlation coefficient at 224 and 237 nm and are given in **Table No.5.1.8**

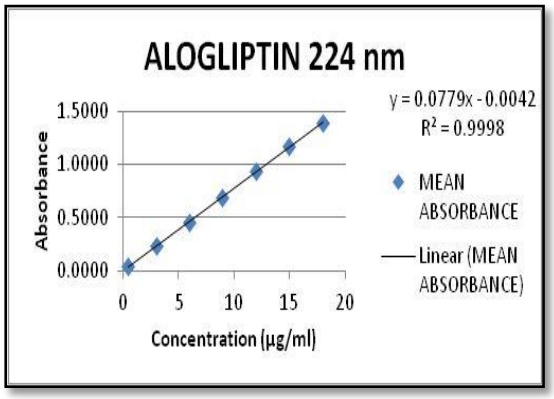


Figure No. 5.1.6: Calibration graph of ALO at (0.5-18 µg/ml) 224 nm

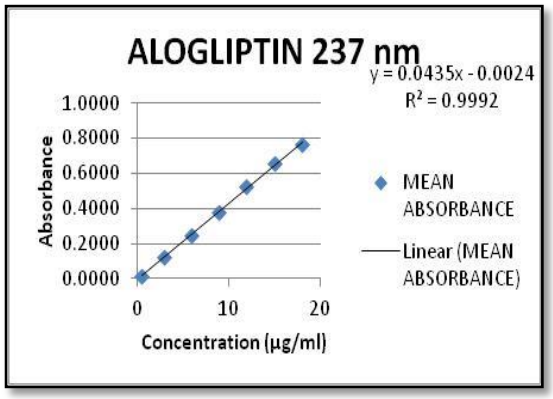


Figure No. 5.1.7: Calibration graph of ALO at (0.5-18 µg/ml) 237 nm

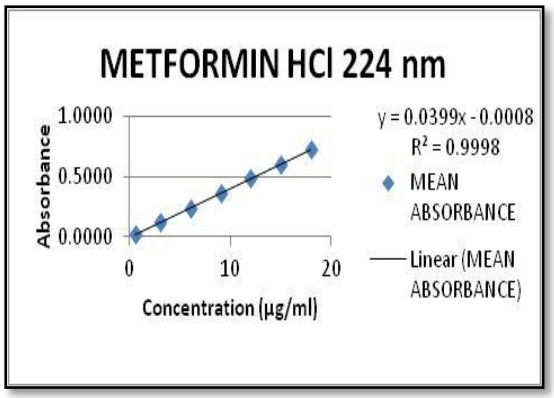


Figure No. 5.1.8: Calibration graph of MET at (0.5-18 µg/ml) 224 nm

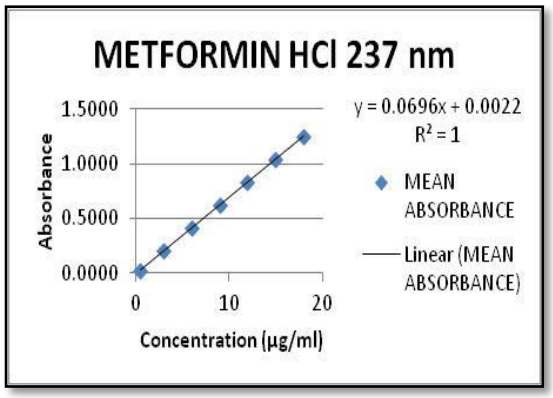


Figure No. 5.1.9: Calibration graph of MET at (0.5-18µg/ml) 237 nm

5.1.7 Precision

Precision studies were performed and % RSD was calculated which is in accordance with ICH guideline acceptable limits (<2), which indicates that the method has good precision **(Table No. 5.1.3, 5.1.4 & 5.1.5.).**

Table No. 5.1.3: Results of repeatability of measurement

Repeatability					
Sr. No.	**Conc.**	**Absorbance**			
		ALO		**MET**	
		224 nm	**237 nm**	**224 nm**	**237 nm**
1	ALO & MET (4 µg/ml)	0.3025	0.1715	0.1576	0.2812
2		0.3038	0.1698	0.1581	0.2841
3		0.3016	0.1725	0.1565	0.2823
4		0.2989	0.1734	0.1546	0.2814
5		0.2983	0.1746	0.1584	0.2863
6		0.2999	0.1695	0.1546	0.2815
Mean±SD*		**0.3008±0.0022**	**0.1719±0.0020**	**0.1566±0.007**	**0.2828±0.0020**
% RSD		**0.7159**	**1.1702**	**1.0870**	**0.7143**
1	ALO & MET (6 µg/ml)	0.4512	0.2490	0.2356	0.4113
2		0.4528	0.2564	0.2375	0.4132
3		0.4581	0.2536	0.2361	0.4113
4		0.4535	0.2541	0.2357	0.4165
5		0.4524	0.2538	0.2361	0.4131
6		0.4535	0.2547	0.2365	0.4175
Mean±SD*		**0.4536±0.0024**	**0.2536±0.0025**	**0.2363±0.0007**	**0.4138±0.0026**
% RSD		**0.5227**	**0.9739**	**0.2930**	**0.6332**
Mean % RSD		**0.6193**	**1.0721**	**0.6900**	**0.6737**

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

Table No. 5.1.4: Results of intra-day precision

Intra-day precision					
Sr. No.	**Conc.**	**Absorbance**			
		ALO		**MET**	
		224 nm	**237 nm**	**224 nm**	**237 nm**
1	ALO & MET (4µg/ml)	0.3056	0.1724	0.1569	0.2821
2		0.3046	0.1713	0.1567	0.2815
3		0.3064	0.1740	0.1564	0.2845
4		0.3069	0.1735	0.1574	0.2863
5		0.3046	0.1751	0.1568	0.2812
6		0.3010	0.1723	0.1578	0.2865
7		0.2945	0.1741	0.1523	0.2813
8		0.2996	0.1732	0.1556	0.2821
9		0.2989	0.1723	0.1545	0.2831
Mean±SD*		**0.3025±0.0042**	**0.1731±0.0012**	**0.1560±0.0017**	**0.2832±0.0021**
% RSD		**1.3861**	**0.6767**	**1.0956**	**0.7399**
1	ALO & MET (6µg/ml)	0.4625	0.2543	0.2368	0.4189
2		0.4634	0.2568	0.2397	0.4163
3		0.4618	0.2546	0.2367	0.4155
4		0.4638	0.2531	0.2398	0.4165
5		0.4619	0.2587	0.2334	0.4165
6		0.4639	0.2498	0.2357	0.4198
7		0.4787	0.2541	0.2361	0.4231
8		0.4631	0.2529	0.2389	0.4233
9		0.4647	0.2547	0.2365	0.4213
Mean±SD*		**0.4649±0.0053**	**0.2543±0.0025**	**0.2371±0.0021**	**0.4190±0.0030**
% RSD		**1.1346**	**0.9784**	**0.8765**	**0.7223**
Mean % RSD		**1.2603**	**0.8275**	**0.9860**	**0.7311**

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

Table No.5.1.5: Results of Inter-day precision

Inter-day precision						
Sr. No.		**Conc.**	**Absorbance**			
			ALO		**MET**	
			224 nm	**237 nm**	**224 nm**	**237 nm**
1	1	ALO & MET (4 µg/ml)	0.2969	0.1689	0.1578	0.2815
2			0.2987	0.1724	0.1564	0.2824
3			0.2998	0.1736	0.1574	0.2865
4	2		0.3016	0.1711	0.1498	0.2874
5			0.3012	0.1721	0.1534	0.2863
6			0.3124	0.1734	0.1543	0.2823
7	3		0.3038	0.1715	0.1576	0.2814
8			0.3045	0.1734	0.1573	0.2875
9			0.3125	0.1713	0.1556	0.2896
Mean±SD*			**0.3035±0.0056**	**0.1720±0.0015**	**0.1555±0.0026**	**0.2850±0.0031**
% RSD			**1.8438**	**0.8674**	**1.6969**	**1.0841**
1	1	ALO & MET (6 µg/ml)	0.4687	0.2498	0.2374	0.4215
2			0.4545	0.2541	0.2365	0.4212
3			0.4568	0.2629	0.2357	0.4256
4	2		0.4563	0.2513	0.2361	0.4213
5			0.4575	0.2534	0.2365	0.4311
6			0.4678	0.2541	0.2324	0.4298
7	3		0.4614	0.2604	0.2453	0.4189
8			0.4558	0.2535	0.2389	0.4213
9			0.4569	0.2537	0.2374	0.4245
Mean±SD*			**0.4595±0.0053**	**0.2548±0.0042**	**0.2374±0.0035**	**0.4239±0.0042**
% RSD			**1.1518**	**1.6421**	**1.4583**	**0.9920**
Mean % RSD			**1.4978**	**1.2548**	**1.5776**	**1.0381**

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

5.1.8 Accuracy

The method showed good accuracy as the results of recovery studies ranged from 98-101% for both the drugs **(Table No. 5.1.6).** The results of recovery study indicate that there is no interference from excipients used in the tablet formulaion.

Table No. 5.1.6: Results of recovery studies

Accuracy (% Recovery)				
ALO				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	2	1	1.0190	101.8992
50	4	2	1.9894	99.4724
50	6	3	2.9512	98.3720
100	2	2	1.9974	99.8689
100	4	4	4.0275	100.6871
100	6	6	6.0406	100.6772
150	2	3	2.9736	99.1215
150	4	6	6.0280	100.4673
150	6	9	9.0568	100.6315
Mean±SD*				**100.1330±1.0436**
% RSD				**1.0422**
MET				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	2	1	1.0045	100.4496
50	4	2	2.0303	101.5168
50	6	3	3.0429	101.4315
100	2	2	2.0142	100.7080
100	4	4	3.9924	99.8093
100	6	6	6.0111	100.1855
150	2	3	3.0380	101.2674
150	4	6	6.0248	100.4134
150	6	9	8.9959	99.9540
Mean±SD*				**100.6373±0.6372**
% RSD				**0.6332**

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

5.1.9 LOD and LOQ

The developed method found to be sensitive as the values of LOD and LOQ were found to be very low which is shown in **Table No. 5.1.8.**

5.1.10 Robustness

Robustness study was carried out for proposed method, but no significant changes (% RSD<2) found in absorption (**Table No. 5.1.7).**Thus the method was found to be robust.

Table No. 5.1.7: Results of robustness study

Parameter	**Drugs**			
Wavelengths	**ALO**		**MET**	
(224 & 237 ±1 nm)	**Assay (%)***	**% RSD**	**Assay (%)***	**% RSD**
223 & 236 nm	99.2155	1.1531	101.5432	1.6687
224& 237 nm	100.2554		100.0223	
225 & 238nm	101.5255		98.2125	

** (n= 3) number of determination*

5.1.11 Stability of the solution

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

Table No. 5.1.8: Summary of validation parameters for the proposed method

Parameters	ALO		MET	
Detection wavelengths(nm)	224	237	224	237
Linearity range (µg/ml)	0.5-18			
Correlation coefficient	0.9998	0.9992	0.9998	1
Regression equation	y = 0.0779x - 0.0042	y = 0.0435x - 0.0024	y = 0.0399x - 0.0008	y = 0.0696x + 0.0022
Precision (%RSD) Intra-day (n=3) Inter-day (n=3) Repeatability of measurement (n=6)	 1.2603 1.4978 0.6193	 0.8275 1.2548 1.0721	 0.9860 1.5776 0.6900	 0.7311 1.0381 0.6737
Accuracy* % Recovery (n=3) %RSD (n=3)	 100.1330±1.0436 1.0422		 100.6373±0.6372 0.6332	
Specificity	No interference			
LOD (µg/ml)	0.0695	0.0933	0.1095	0.0885
LOQ (µg/ml)	0.2107	0.2826	0.3317	0.2682

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

5.1.12 Analysis of formulation

The proposed method was successfully used for the quantitative determination of ALO and MET in tablet formulation (12.5 mg of ALO and 500 mg of MET). Six replicate determinations were carried out and average experimental values were found to be 98.89 and 99.69 %w/w for ALO and MET, respectively and hence the developed method can be used for the simultaneous estimation of drugs in tablet dosage form.[90] **Table No. 5.1.9.** Overlain spectra of standard drugs and formulation are shown in **Figure No. 5.1.10.**

Table No. 5.1.9: Results of formulation analysis

Drugs	Amount (mg/tablet)		% Drug found*	% RSD
	Labelled	Found		
ALO	12.5	12.36	98.8995±1.5902	1.6079
MET	500	498.47	99.6935±1.3084	1.3124

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

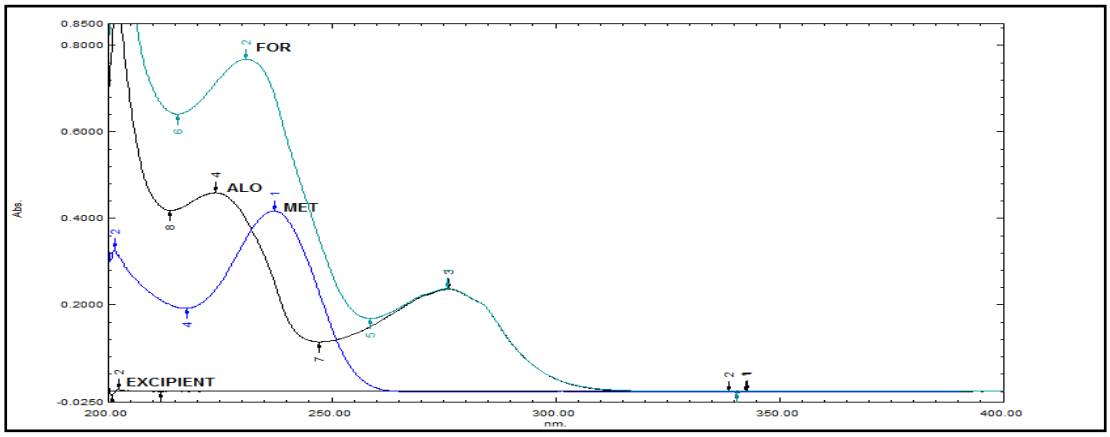


Figure No. 5.1.10: Overlain spectra of standard ALO , MET (6 µg/ml) & formulation(6 µg/ml)

Method 2

5.2 "Development and validation of absorbance ratio (Q analysis) method for the simultaneous determination of alogliptin benzoate and metformin hydrochloride in tablets"

Estimation of ALO and MET was achieved by absorbance ratio method using Shimadzu UV 1800 (UV Pro), double beam UV-Visible spectrophotometer.

5.2.1 Selection of solvent

By checking solubility in different solvents methanol was selected as solvent for further studies

5.2.2 Selection of wavelength

Standard stock solutions containing 6 µg/ml of ALO and MET were scanned in the UV region (200-400 nm) and spectra were recorded using methanol as blank. Their overlain spectra are shown in **Figure No. 5.2.1**

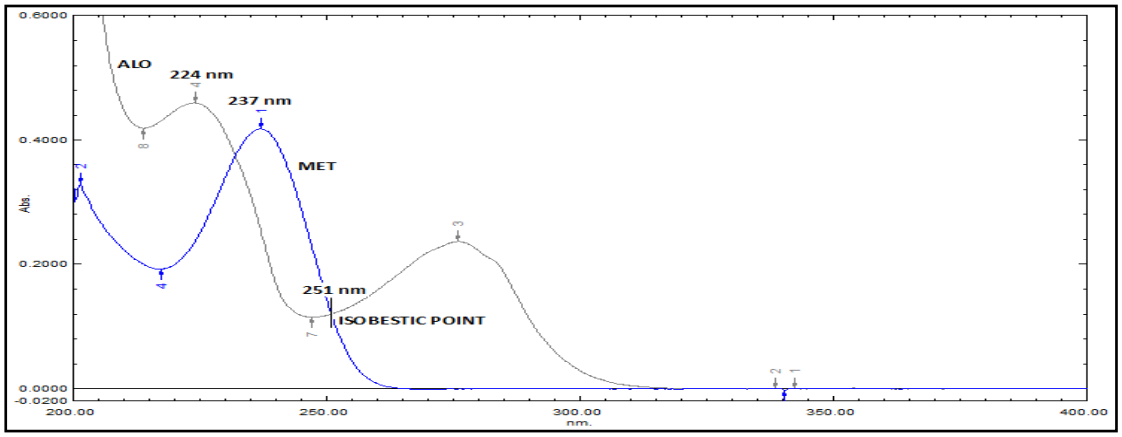


Figure No. 5.2.1: Overlain UV spectra of ALO and MET (6 µg/ml)

From the overlain spectra, different wavelengths were tried for the method. After comparing all wavelengths 224 nm and 251 nm were selected which showed good linearity over the given concentration range.

5.2.3 Determination of absorptivity values

The developed method was found to be linear in the concentration range of 0.5-18 µg/ml for both the drugs. Absorbances were measured at 224 nm and 251 nm for both the drugs and absorptivity values were calculated which is shown in **Table No. 5.2.1 & 5.2.2**

Table No. 5.2.1: Absorbances and absorptivities of ALO at selected wavelength

Conc. (µg/ml)	ALO					
	224 nm			251 nm		
	Abs.*	Absorptivity	Avg. Absorptivity	Abs.*	Absorptivity	Avg. Absorptivity
0.5	0.0386	772.0000	771.9972 (ax_1)	0.0103	206.0000	202.6708 (ax_2)
3	0.2315	771.6111		0.0609	202.8333	
6	0.4565	760.8611		0.1193	198.7500	
9	0.6866	762.8889		0.1787	198.5370	
12	0.9398	783.1250		0.2468	205.6806	
15	1.1682	778.8000		0.3046	203.0333	
18	1.3945	774.6944		0.3670	203.8611	

**average of six determinations*

Table No. 5.2.2: Absorbances and absorptivities of MET at selected wavelength

Conc. (µg/ml)	MET					
	224 nm			251nm		
	Abs.*	Absorptivity	Avg. Absorptivity	Abs.*	Absorptivity	Avg. Absorptivity
0.5	0.0196	391.6667	396.854 (ay_1)	0.0103	206.0000	202.6708 (ay_2)
3	0.1183	394.1667		0.0609	202.8333	
6	0.2357	392.8333		0.1193	198.7500	
9	0.3619	402.1111		0.1787	198.5370	
12	0.4829	402.4306		0.2468	205.6806	
15	0.5930	395.3000		0.3046	203.0333	
18	0.7190	399.4444		0.3670	203.8611	

**average of six determinations*

5.2.4 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 Specificity

Overlain spectra of placebo and drug solution indicate that there was no interference between excipients and standard drugs **(Figure No. 5.2.2.).**

5.2.6 Linearity

From the linearity study, ALO and MET were found to be linear in the concentration range of 0.5-18 µg/ml. Overlain spectra of drug solution and formulation excipients prove that there was no interference between standard drugs and excipients. The overlain spectra of ALO and MET are shown in **Figure No. 5.2.3-5.2.5**

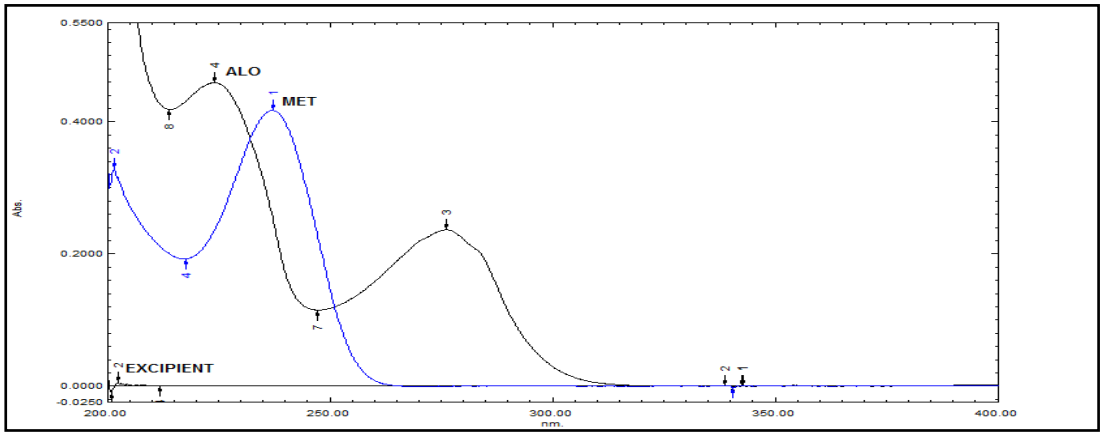


Figure No. 5.2.2: Overlain UV Spectra of ALO, MET(6 µg/ml) and formulation excipient

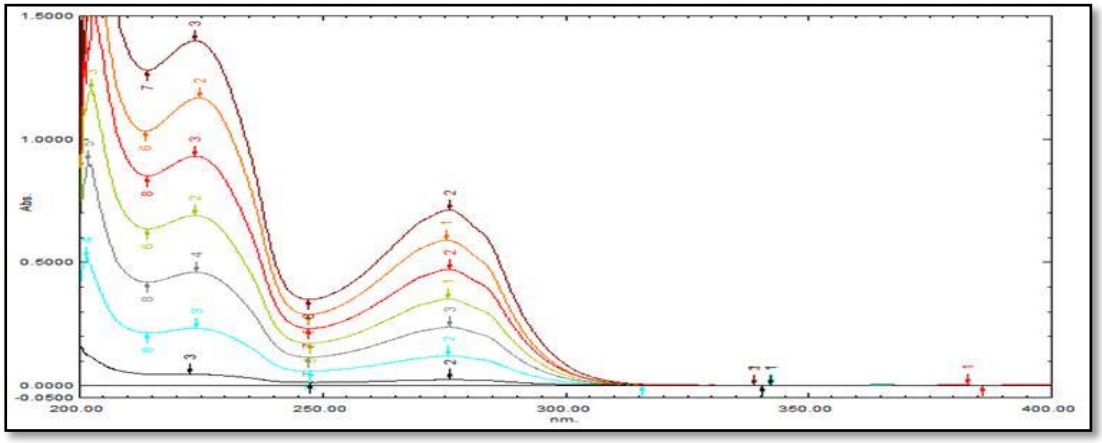


Figure No. 5.2.3: Overlain UV spectra of ALO (0.5-18 µg/ml)

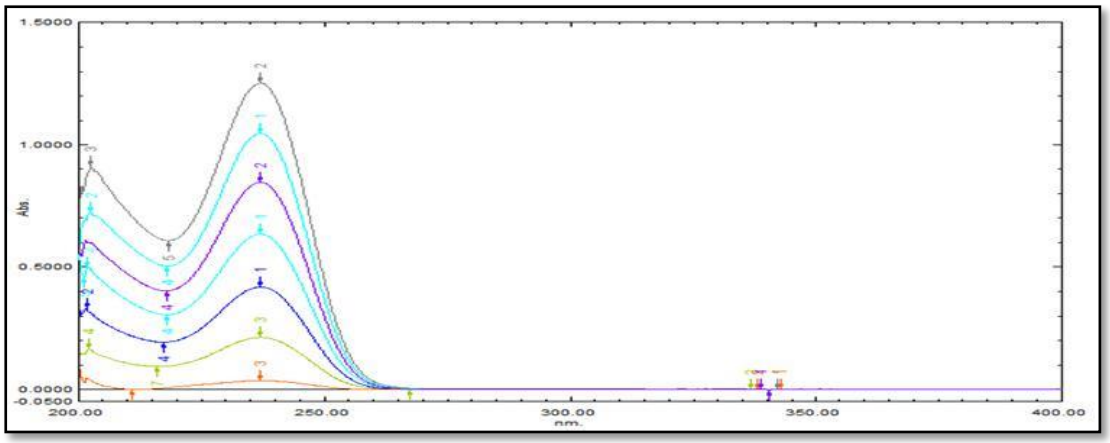


Figure No. 5.2.4: Overlain UV spectra of MET (0.5-18 µg/ml)

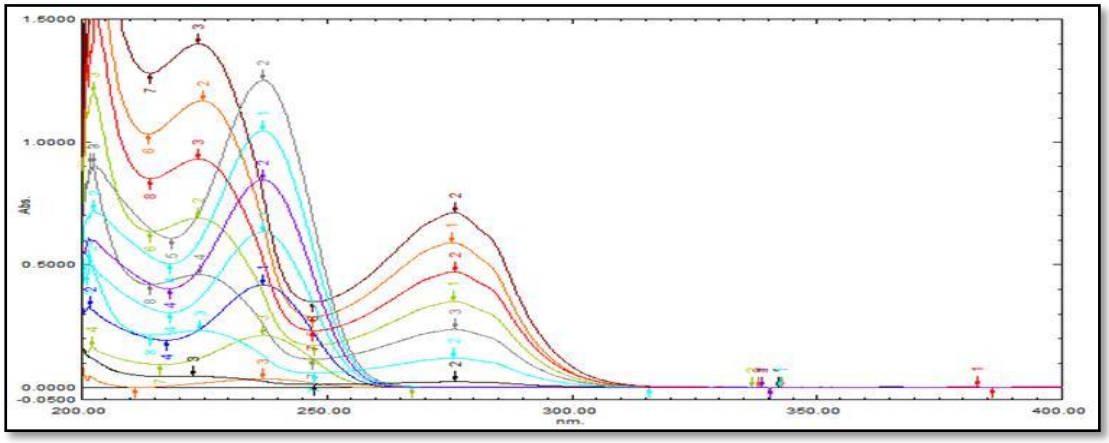


Figure No. 5.2.5: Overlain UV spectra of ALO & MET(0.5-18 µg/ml)

Calibration graphs were plotted using absorbances of standard drug solutions versus concentration **(Figure No. 5.2.6-5.2.9).** Results were subjected to regression analysis by the least squares method to calculate the values of slope, intercept and correlation coefficient at 224 and 251 nm and are given in **Table No. 5.2.8.**

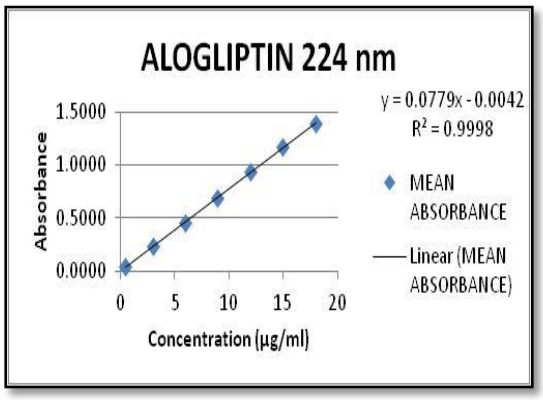


Figure No. 5.2.6: Calibration graph of ALO at 224 nm

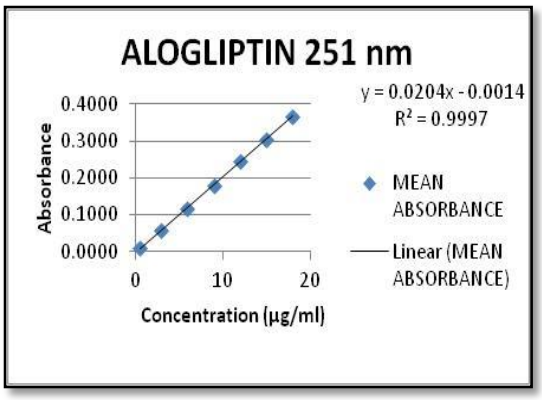


Figure No. 5.2.7: Calibration graph of ALO at 251nm

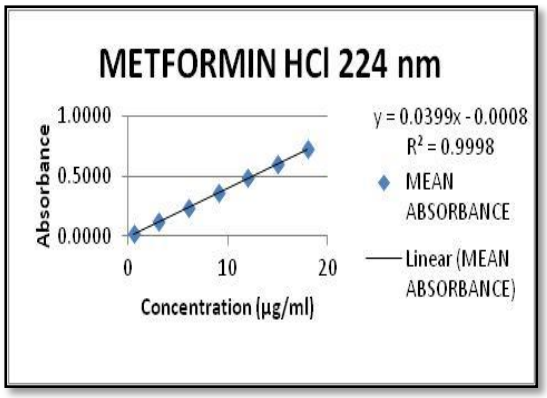


Figure No. 5.2.8: Calibration graph of MET at 224 nm

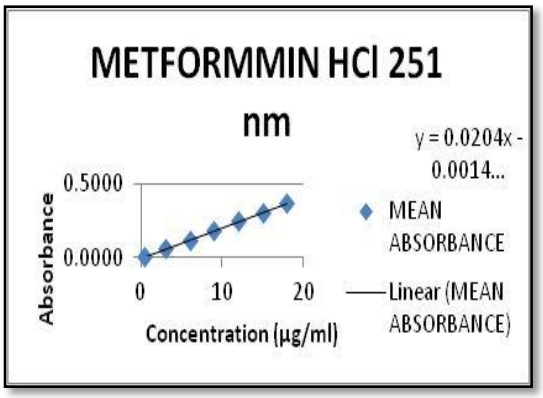


Figure No. 5.2.9: Calibration graph of MET at 251nm

5.2.7 Precision

Precision studies were performed and % RSD was calculated which is in accordance with ICH guideline acceptable limits (<2), which indicates that the method has good precision (**Table No. 5.2.3, 5.2.4 & 5.2.5.).**

Table No. 5.2.3: Results of repeatability of measurement

Repeatability					
Sr. No.	**Conc.**	**Absorbance**			
		ALO		**MET**	
		224 nm	**251 nm**	**224 nm**	**251 nm**
1	ALO & MET (4µg/ml)	0.3025	0.0796	0.1576	0.0796
2		0.3038	0.0788	0.1581	0.0788
3		0.3016	0.0781	0.1565	0.0781
4		0.2989	0.0789	0.1546	0.0789
5		0.2983	0.0769	0.1584	0.0769
6		0.2999	0.0792	0.1546	0.0792
Mean±SD*		**0.3008±0.0022**	**0.0786±0.0010**	**0.1566±0.007**	**0.0786±0.0010**
% RSD		**0.7159**	**1.2243**	**1.0870**	**1.2243**
1	ALO & MET (6 µg/ml)	0.4512	0.1165	0.2356	0.1165
2		0.4528	0.1178	0.2375	0.1178
3		0.4581	0.1191	0.2361	0.1191
4		0.4535	0.1182	0.2357	0.1182
5		0.4524	0.1164	0.2361	0.1164
6		0.4535	0.1173	0.2365	0.1173
Mean±SD*		**0.4536±0.0024**	**0.1176±0.0010**	**0.2363±0.0007**	**0.1176±0.0010**
% RSD		**0.5227**	**0.8820**	**0.2930**	**0.8820**
Mean % RSD		**0.6193**	**1.0532**	**0.6900**	**1.0532**

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

Table No. 5.2.4: Results of intra-day precision

Intra-day precision					
Sr. No.	**Conc.**	**Absorbance**			
		ALO		**MET**	
		224 nm	**251 nm**	**224 nm**	**251 nm**
1	ALO & MET (4µg/ml)	0.3056	0.0786	0.1569	0.0786
2		0.3046	0.0775	0.1567	0.0775
3		0.3064	0.0768	0.1564	0.0768
4		0.3069	0.0782	0.1574	0.0782
5		0.3046	0.0791	0.1568	0.0791
6		0.3010	0.0789	0.1578	0.0789
7		0.2945	0.0769	0.1523	0.0769
8		0.2996	0.0792	0.1556	0.0792
9		0.2989	0.0786	0.1545	0.0786
Mean±SD*		**0.3025±0.0042**	**0.0782±0.0009**	**0.1560±0.0017**	**0.0782±0.0009**
% RSD		**1.3861**	**1.1755**	**1.0956**	**1.1755**
1	ALO & MET (6 µg/ml)	0.4625	0.1161	0.2368	0.1161
2		0.4634	0.1169	0.2397	0.1169
3		0.4618	0.1164	0.2367	0.1164
4		0.4638	0.1138	0.2398	0.1138
5		0.4619	0.1154	0.2334	0.1154
6		0.4639	0.1137	0.2357	0.1137
7		0.4787	0.1146	0.2361	0.1146
8		0.4631	0.1154	0.2389	0.1154
9		0.4647	0.1163	0.2365	0.1163
Mean±SD*		**0.4649±0.0053**	**0.1154±0.0012**	**0.2371±0.0021**	**0.1154±0.0012**
% RSD		**1.1346**	**0.9994**	**0.8765**	**0.9994**
Mean % RSD		**1.2603**	**1.0874**	**0.9860**	**1.0874**

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

Table No. 5.2.5: Results of inter- day precision

Inter-day precision						
Sr. No.		**Conc.**	**Absorbance**			
			ALO		**MET**	
			224 nm	**251nm**	**224 nm**	**251 nm**
1	1	ALO & MET (4µg/ml)	0.2969	0.0782	0.1578	0.0782
2			0.2987	0.0789	0.1564	0.0789
3			0.2998	0.0769	0.1574	0.0769
4	2		0.3016	0.0792	0.1498	0.0792
5			0.3012	0.0786	0.1534	0.0786
6			0.3124	0.0763	0.1543	0.0763
7	3		0.3038	0.0768	0.1576	0.0768
8			0.3045	0.0792	0.1573	0.0792
9			0.3125	0.0756	0.1556	0.0756
Mean±SD*			**0.3035±0.0056**	**0.0777±0.0014**	**0.1555±0.0026**	**0.0777±0.0014**
% RSD			**1.8438**	**1.7496**	**1.6969**	**1.7496**
1	1	ALO & MET (6µg/ml)	0.4687	0.1186	0.2374	0.1186
2			0.4545	0.1154	0.2365	0.1154
3			0.4568	0.1141	0.2357	0.1141
4	2		0.4563	0.1134	0.2361	0.1134
5			0.4575	0.1157	0.2365	0.1157
6			0.4678	0.1168	0.2324	0.1168
7	3		0.4614	0.1178	0.2453	0.1178
8			0.4558	0.1185	0.2389	0.1185
9			0.4569	0.1156	0.2374	0.1156
Mean±SD*			**0.4595±0.0053**	**0.1162±0.0019**	**0.2374±0.0035**	**0.1162±0.0019**
% RSD			**1.1518**	**1.5945**	**1.4583**	**1.5945**
Mean % RSD			**1.4978**	**1.6721**	**1.5776**	**1.6721**

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

5.2.8 Accuracy

The method showed good accuracy as the results of recovery studies ranged from **98-102%** for both the drugs **(Table No. 5.2.6).**

Table No. 5.2.6: Results of recovery studies

Accuracy (% Recovery)				
ALO				
Recovery level (%)	**Initial con. of formulation (µg/ml)**	**Standard added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	2	1	1.0156	101.5570
50	4	2	2.0390	101.9512
50	6	3	3.0494	101.6458
100	2	2	2.0393	101.9654
100	4	4	4.0393	100.9825
100	6	6	6.1310	101.5400
150	2	3	2.9876	99.5867
150	4	6	5.9876	99.7654
150	6	9	8.8976	98.7644
Avg.				**100.8620**
SD				**1.1834**
% RSD				**1.1733**
MET				
Recovery level (%)	**Initial con. of formulation (µg/ml)**	**Standard added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	2	1	1.0238	102.3781
50	4	2	2.0347	101.7371
50	6	3	3.0687	102.2892
100	2	2	2.0082	100.4113
100	4	4	4.0854	102.1343
100	6	6	6.0017	100.0284
150	2	3	2.8775	99.8976
150	4	6	5.9876	99.7685
150	6	9	8.9876	98.6789
Avg.				**100.8137**
SD				**1.3458**
% RSD				**1.3349**

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

5.2.9 LOD and LOQ

The developed method found to be sensitive as the values of LOD and LOQ were found to be very low which is shown in **Table No. 5.2.8.**

5.2.10 Robustness

Robustness study was carried out for proposed method, but no significant changes (% RSD<2) found in absorption. (**Table No. 5.2.7).**Thus the method was found to have good robustness.

Table No. 5.2.7: Results of robustness study

Parameter	**Drugs**			
Wavelengths	**ALO**		**MET**	
(224 & 251 ±1 nm)	**Assay (%)***	**% RSD**	**Assay (%)***	**% RSD**
223 & 250 nm	100.1275	0.9432	100.6523	1.6361
224& 251 nm	101.2354		101.3234	
225 & 252 nm	99.3543		98.2125	

** (n= 3) number of determination*

5.2.11 Stability of the solution

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

Table No. 5.2.8: Summary of validation parameters for the proposed method

Parameters	ALO		MET	
Detection wavelengths(nm)	224	251	224	251
Linearity range (µg/ml)	0.5-18			
Correlation coefficient	0.9998	0.9997	0.9998	0.9997
Regression equation	y = 0.0779x - 0.0042	y = 0.0204x - 0.0014	y = 0.0399x - 0.0008	y = 0.0204x - 0.0014
Precision (%RSD) Intra-day (n=3) Inter-day (n=3) Repeatability of measurement (n=6)	 1.2603 1.4978 0.6193	 1.0874 1.6721 1.0532	 0.9860 1.5776 0.6900	 1.0874 1.6721 1.0532
Accuracy* % Recovery (n=3) %RSD (n=3)	 100.8620±1.1834 1.7334		 100.8137±1.3458 1.3349	
Specificity	No interference			
LOD (µg/ml)	0.0695	0.1412	0.1045	0.1412
LOQ (µg/ml)	0.2107	0.4279	0.3166	0.4279

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

5.2.12 Analysis of formulation

The proposed method was successfully used for the quantitative determination of ALO and MET in tablet formulation (12.5 mg of ALO and 500 mg of MET). Six replicate determinations were carried out and average experimental values were found to be 100.0827 and 98.7660 %w/w for ALO and MET, respectively and hence the developed method can be used for the simultaneous estimation of drugs in tablet dosage form. **Table No. 5.2.9.** Overlain spectra of standard drugs and formulation are shown in **Figure No. 5.2.10.**

Table No. 5.2.9: Results of formulation analysis

Drugs	Amount (mg/tablet)		% Drug found*	% RSD
	Labelled	Found		
ALO	12.5	12.51	100.0827±1.1262	1.1253
MET	500	493.83	98.7660±1.3702	1.3873

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

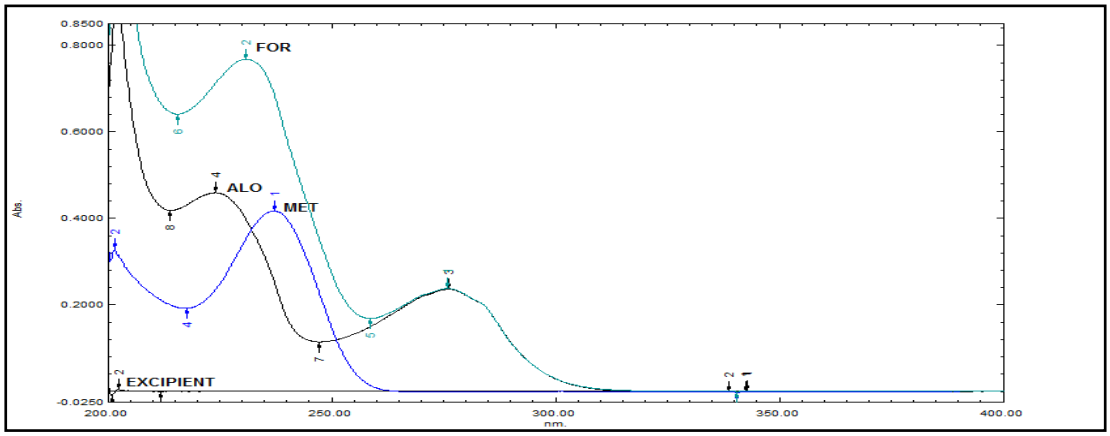


Figure No. 5.2.10: Overlain spectra of standard ALO , MET (6 µg/ml) & formulation(6 µg/ml)

Method 3

5.3 "Development and validation of first-derivative spectroscopic method for the simultaneous determination of alogliptin benzoate and metformin hydrochloride in tablets"

Estimation of ALO and MET was achieved by first-derivative spectroscopic method using Shimadzu UV 1800 (UV Pro), double beam UV-Visible spectrophotometer.

5.3.1 Selection of solvent

The UV spectra of ALO and MET were recorded individually in methanol. All the spectra were processed to obtain their derivative spectra. In methanol derivative spectra of ALO and MET showed favorable zero crossing points and good linearity was observed. Hence methanol was selected as solvent for further studies.

5.3.2 Selection of wavelength

Standard solutions of ALO and MET (6 µg/ml) were scanned in the UV region (200-400 nm) and spectra 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. [91, 92] First derivative spectra were showed typical zero-crossing points at 247.3 nm for ALO and 237 nm for MET. From the overlain spectra, 237 nm and 247.3 nm were selected for further studies are shown in **Figure No. 5.3.1**

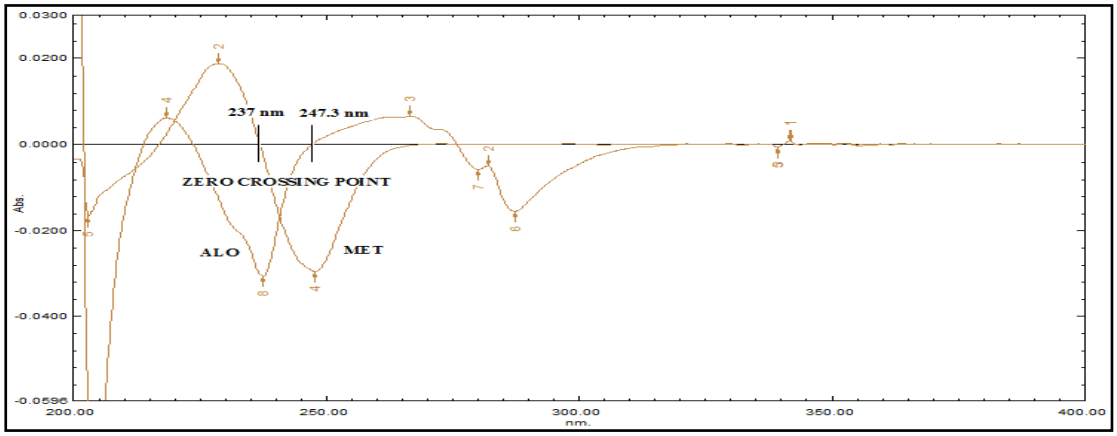


Figure No. 5.3.1: Overlain 1st derivative UV spectra of ALO, MET (6 µg/ml)

At 237 nm, MET showed zero absorbance but ALO had considerable absorbance. Similarly at 247.3 nm, ALO showed zero absorbance but MET had considerable absorbance (**Table No. 5.3.1).**

Table No. 5.3.1: Selection of zero crossing points for ALO & MET

Drugs	Zero crossing point (nm)
ALO	237
MET	247.3

5.3.3 Preparation of calibration curve

A calibration curve was plotted for both ALO and MET in the range of 0.5-18 µg/ml, respectively (**Table No. 5.3.2).** The slope, intercept and correlation coefficient values are shown in **Table No.5.3.8, Figure No.5.3.6&5.3.7**

Table No. 5.3.2: Linearity data of 1st derivative UV spectroscopic method

Sr. No.	ALO at 237 nm			MET at 247.3 nm		
	Conc. (µg/ml)	First derivative signal*	% RSD	Conc. (µg/ml)	First derivative signal *	% RSD
1	0.5	0.0026	1.9610	0.5	0.0027	1.9365
2	3	0.0152	1.1769	3	0.0154	1.7720
3	6	0.0305	1.0864	6	0.0294	1.7409
4	9	0.0453	1.0315	9	0.0446	0.9912
5	12	0.0608	0.7625	12	0.0598	0.6993
6	15	0.0777	0.7747	15	0.0737	1.2297
7	18	0.0928	0.6742	18	0.0879	0.9407

**average of six determinations*

5.3.4 Validation of the method:

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

5.3.5 Specificity

Overlain spectra of placebo and drug solution indicate that there was no interference between excipients and standard drugs (**Figure No. 5.3.2).**

5.3.6 Linearity and range

From the linearity study, ALO and MET were found to be linear in the concentration range of 0.5-18 µg/ml. The overlain spectra of ALO and MET are shown in **Figure No. 5.3.3-5.3.5**

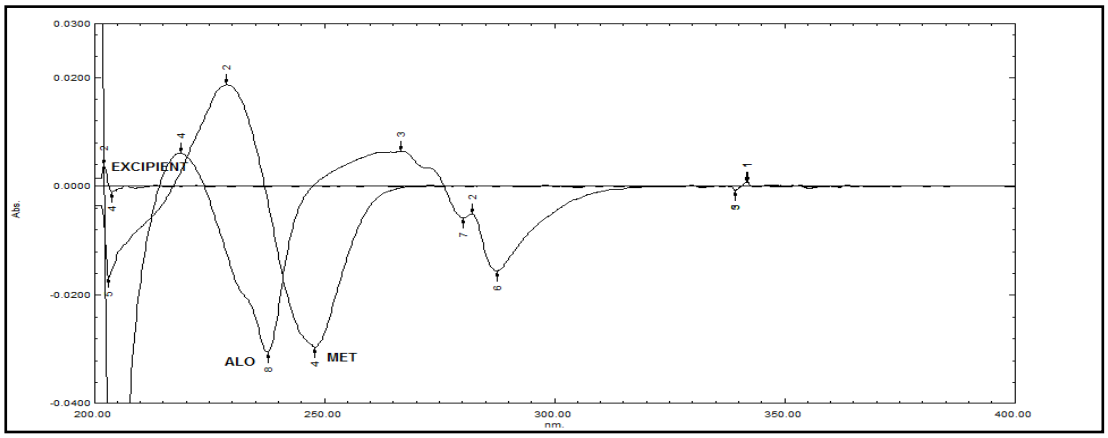


Figure No. 5.3.2: Overlain first derivative UV Spectra of ALO and MET(6 µg/ml) and formulation excipient

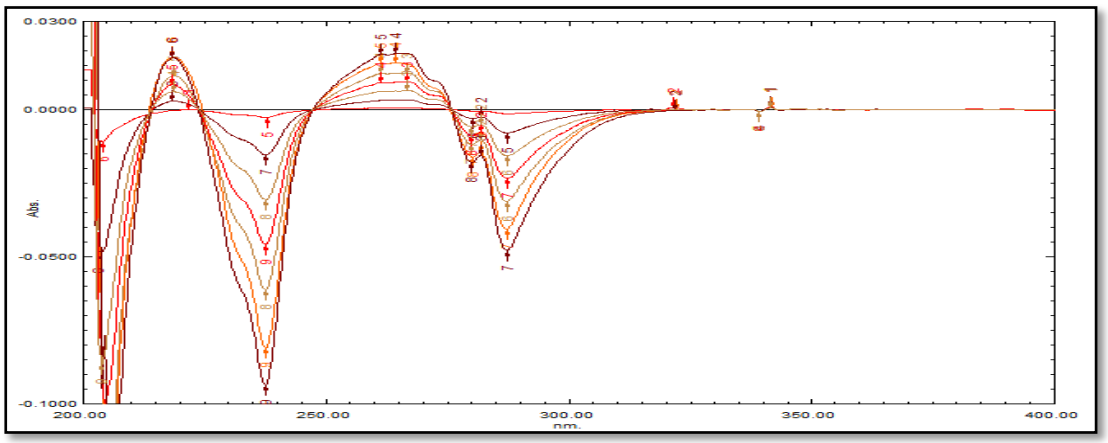


Figure No. 5.3.3: Overlain first derivative UV spectra of ALO (0.5-18 µg/ml)

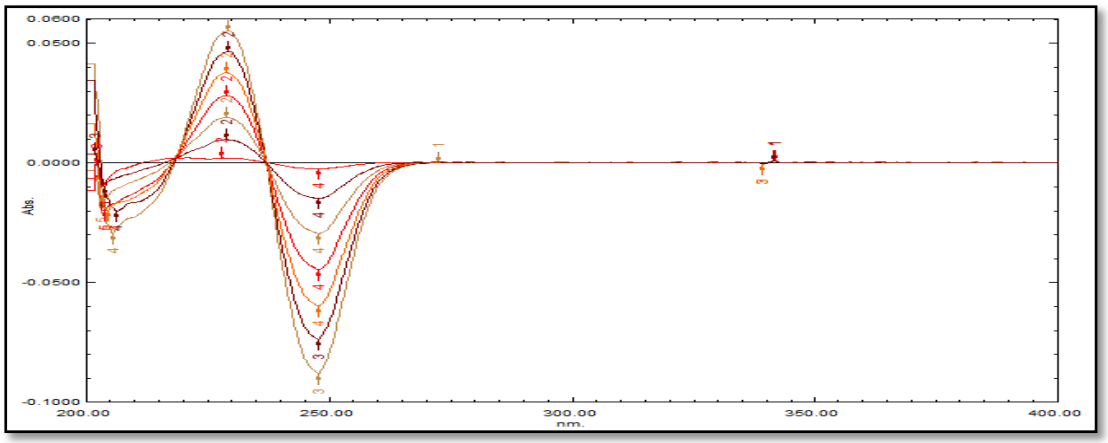


Figure No. 5.3.4: Overlain first derivative UV spectra of MET (0.5-18 µg/ml)

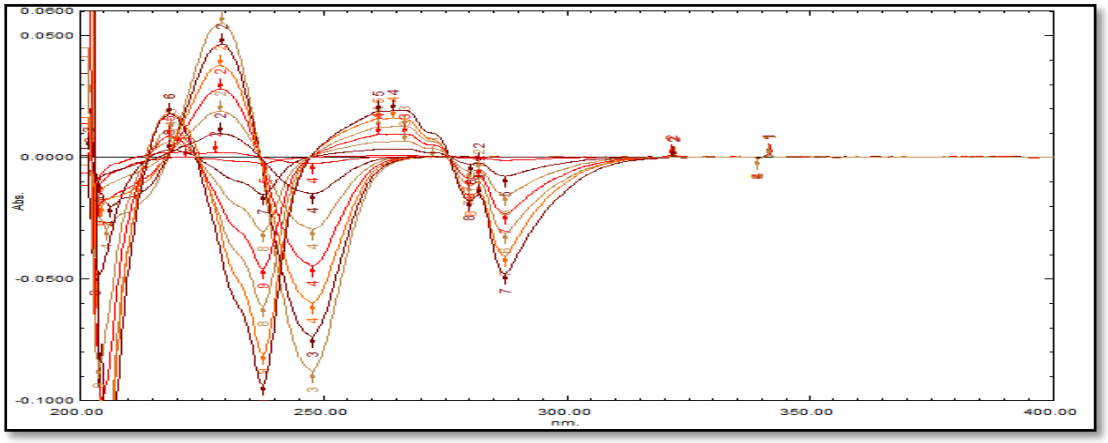


Figure No. 5.3.5: Overlain first derivative UV spectra of ALO & MET (0.5-18 µg/ml)

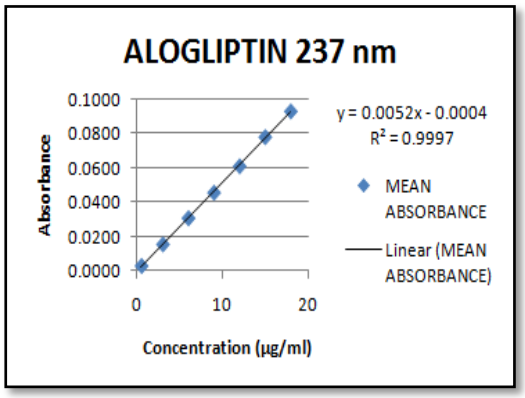


Fig. No.5.3.6: Calibration graph of ALO at 237 nm

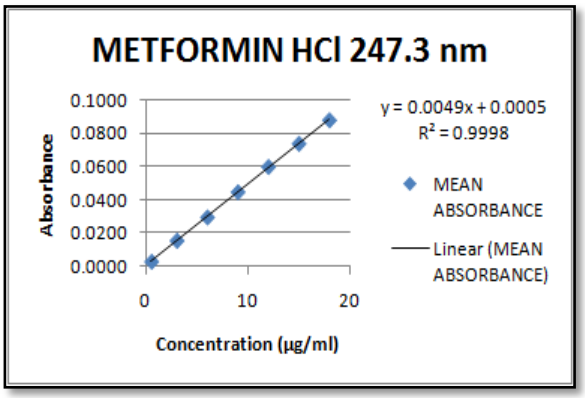


Figure No. 5.3.7: Calibration graph of MET at 247.3 nm

5.3.7 Precision

Precision studies were performed and % RSD was calculated which is in accordance with ICH guideline acceptable limits, which indicates good repeatability and low intra and inter-day variability (**Table No. 5.3.3-5.3.5.).**

Table No. 5.3.3: Results of repeatability of measurement

Repeatability			
Sr. No.	**Conc.**	**1st derivative signal**	
		ALO	**MET**
		237 nm	**247.3 nm**
1	ALO & MET (4 µg/ml)	0.0206	0.0192
2		0.0205	0.0195
3		0.0208	0.0193
4		0.0208	0.0195
5		0.0205	0.0195
6		0.0208	0.0197
Mean±SD*		**0.0207±0.0002**	**0.0195±0.0002**
% RSD		**0.7285**	**0.9052**
1	ALO & MET (6 µg/ml)	0.0305	0.0291
2		0.0302	0.0287
3		0.0306	0.0288
4		0.0308	0.0292
5		0.0301	0.0285
6		0.0304	0.0288
Mean±SD*		**0.0304±0.0003**	**0.0289±0.0003**
% RSD		**0.8484**	**0.8972**
Mean % RSD		**0.7884**	**0.9012**

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

Table No. 5.3.4: Results of intra-day precision

Intraday precision			
Sr. No.	**Conc.**	**1st derivative signal**	
		ALO	**MET**
		237 nm	**247.3 nm**
1	ALO & MET (4 µg/ml)	0.0204	0.0198
2		0.0205	0.0195
3		0.0208	0.0196
4		0.0207	0.0192
5		0.0206	0.0196
6		0.0209	0.0195
7		0.0211	0.0193
8		0.0204	0.0197
9		0.0206	0.0194
Mean±SD*		**0.0207±0.0002**	**0.0195±0.0002**
% RSD		**1.1348**	**0.9740**
1	ALO & MET (6 µg/ml)	0.0305	0.0291
2		0.0302	0.0295
3		0.0308	0.0294
4		0.0311	0.0292
5		0.0312	0.0296
6		0.0301	0.0299
7		0.0304	0.0297
8		0.0307	0.0293
9		0.0312	0.0291
Mean±SD*		**0.0307±0.0004**	**0.0294±0.0003**
% RSD		**1.3675**	**0.9428**
Mean % RSD		**1.2511**	**0.9584**

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

Table No. 5.3.5: Results of inter-day precision

Inter-day precision				
Sr. No.		**Conc.**	**1st derivative signal**	
			ALO	**MET**
			237 nm	**247.3 nm**
1	Day 1	ALO & MET (4 µg/ml)	0.0205	0.0199
2			0.0206	0.0192
3			0.0208	0.0195
4	Day 2		0.0204	0.0189
5			0.0207	0.0194
6			0.0206	0.0196
7	Day 3		0.0207	0.0197
8			0.0212	0.0196
9			0.0215	0.0195
Mean±SD*			**0.0208±0.0004**	**0.0195±0.0003**
% RSD			**1.6913**	**1.4919**
1	Day 1	ALO & MET (6 µg/ml)	0.0301	0.0288
2			0.0304	0.0296
3			0.0307	0.0299
4	Day 2		0.0315	0.0297
5			0.0311	0.0291
6			0.0312	0.0293
7	Day 3		0.0308	0.0294
8			0.0316	0.0294
9			0.0315	0.0285
Mean±SD*			**0.0310±0.0005**	**0.0293±0.0004**
% RSD			**1.6956**	**1.5071**
Mean % RSD			**1.6935**	**1.4995**

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

5.3.8 Accuracy

The method showed good accuracy as the results of recovery studies ranged from 98-101% for both the drugs **(Table No. 5.3.6).**

Table No. 5.3.6: Results of recovery studies

Accuracy (% Recovery)				
ALO				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	2	1	0.9808	98.0769
50	4	2	1.9615	98.0769
50	6	3	2.9808	99.3590
100	2	2	1.9808	99.0385
100	4	4	4.0192	100.4808
100	6	6	5.8077	96.7949
150	2	3	2.9615	98.7179
150	4	6	5.9615	99.3590
150	6	9	8.8846	98.7179
Mean±SD				**98.7358±1.0338**
% RSD				**1.0470**
MET				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	2	1	0.9796	97.9592
50	4	2	1.9796	98.9796
50	6	3	3.0204	100.6803
100	2	2	2.0204	101.0204
100	4	4	4.0204	100.5102
100	6	6	6.0816	101.3605
150	2	3	2.9592	98.6395
150	4	6	6.0204	100.3401
150	6	9	9.1837	102.0408
Mean±SD				**100.17±1.3552**
% RSD				**1.3529**

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

5.3.9 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. 5.3.8.**

5.3.10 Robustness

The proposed method was checked for robustness study, but no significant changes (% RSD<2) found in absorption (**Table No. 5.3.7).** Thus the method was found to be robust.

Table No. 5.3.7: Results of robustness study

Parameter		**Drugs**			
Wavelengths (237 & 247.3 ±1 nm)		**ALO**		**MET**	
ALO	**MET**	**Assay (%)***	**% RSD**	**Assay (%)***	**% RSD**
236	246.3	96.7654	1.4967	98.3651	1.6710
237	247.3	97.7647		101.6554	
238	248.3	99.6554		100.5478	

** (n= 3) number of determination*

5.3.11 Stability of the solution

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

Table No.5.3.8: Summary of validation parameters for the proposed method

Parameters	**ALO**	**MET**
Detection wavelengths (nm)	237	247.3
Linearity range (µg/ml)	0.5-18	
Correlation coefficient	0.9997	0.9998
Regression equation	y = 0.0052x - 0.0004	y = 0.0049x + 0.0005
Precision (%RSD) Intra-day (n=3) Inter-day (n=3) Repeatability of measurement (n=6)	 1.2511 1.6935 0.7884	 0.9584 1.4995 0.9012
Accuracy* % Recovery (n=3) %RSD (n=3)	 98.7358±1.0338 1.0470	 100.1701±1.3552 1.3529
Specificity	No interference	
LOD (µg/ml)	0.0858	0.0908
LOQ (µg/ml)	0.2601	0.2752

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

5.3.12 Analysis of formulation

The proposed method was successfully used for the quantitative determination of ALO and MET in tablet formulation (12.5 mg ALO and 500 mg of MET). Six

replicate determinations were carried out and average experimental values were found to be 100.1883 and 98.1409 %w/w for ALO and MET, respectively and hence the developed can be used for the simultaneous estimation of drugs in tablet dosage form (**Table No. 5.3.9.).** Overlain spectra of standard drugs and formulation are shown in **Figure No. 5.3.8**.

Table No. 5.3.9: Results of formulation analysis

Drugs	Amount (mg/tablet)		% Drug found*	% RSD
	Labelled	Found		
ALO	12.5	12.52	100.1883±1.5413	1.5384
MET	500	490.70	98.1409±1.2661	1.2901

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

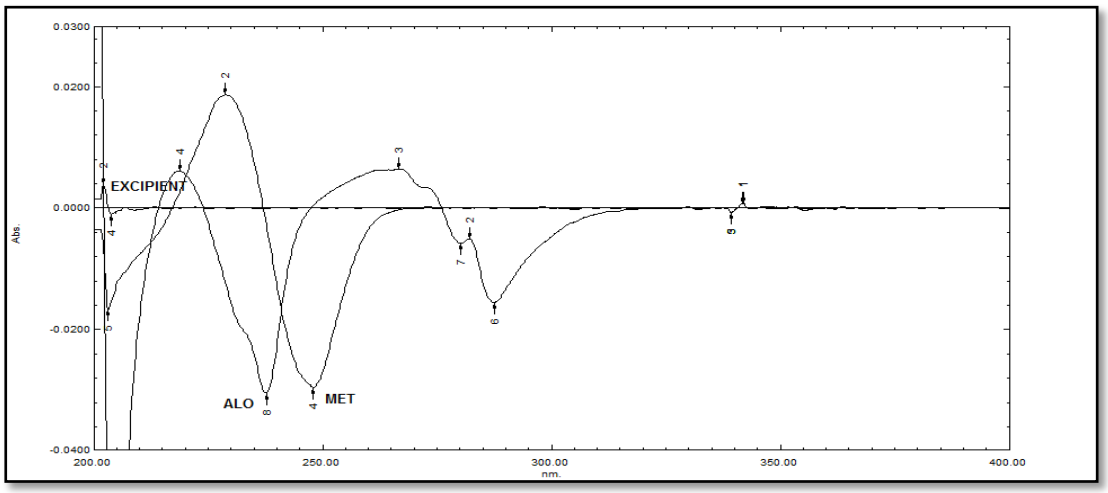


Figure No. 5.3.8: Overlain spectra of standard ALO , MET (6 µg/ml) & formulation(6 µg/ml)

Method 4

5.4 "Development and validation of RP-HPLC method for the simultaneous determination of alogliptin benzoate and metformin hydrochloride in tablets"

5.4.1 Selection of mode of chromatographic method

For most regular samples, reversed phase chromatography is the first choice for analysis. RPC is most convenient and rugged and produces more satisfactory results compared to other form of liquid chromatography. Substances under investigation are polar in nature and hence, reverse phase chromatographic method was selected.

5.4.2 Selection of column:

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

5.4.3 Selection of wavelength

UV spectra of both the drugs were taken in RP-HPLC system and 236 nm was selected as wavelength (**Figure No. 5.4.1**).

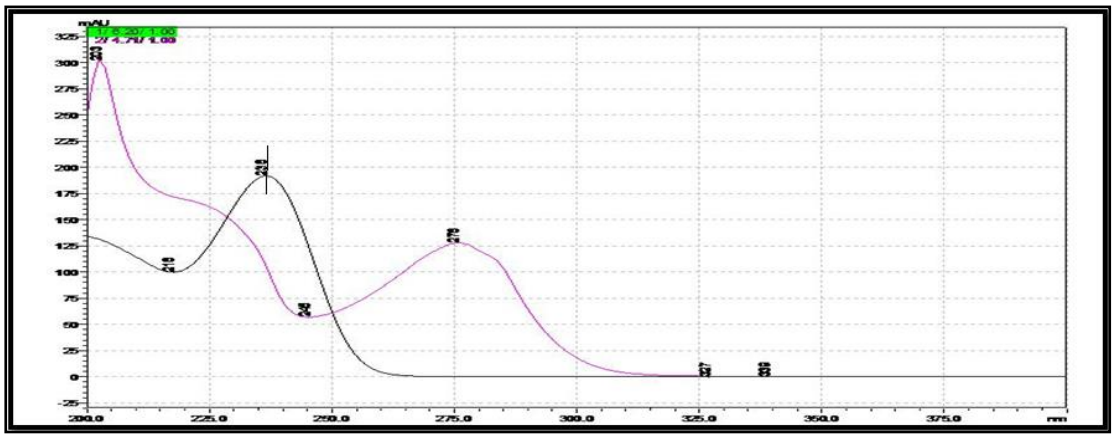


Figure No. 5.4.1: Overlain UV standard spectra of ALO & MET at 236 nm

5.4.4 Trials for selection of mobile phase

Different trials were performed based on the literature survey and suitable mobile phase was selected for further studies **Table No. 5.4.1 & Figure No. 5.4.2-5.4.13.**

Table No. 5.4.1: Selection of mobile phase

Sr. No.	Mobile Phase	Observation	Remarks	Fig. No.
1	Sodium phosphate (pH 3): Acetonitrile (60:40 %v/v)	Broad peak with tailing	Not satisfactory	5.4.2
2	Acetonitrile: water: 0.01% ortho phosphoric acid (45:55:0.01%v/v)	No separation of peaks and splitting	Not satisfactory	5.4.3
3	20 mM Phosphate buffer (pH 4.6): Methanol (50:50 %v/v)	Peaks were merged with no separation	Not satisfactory	5.4.4
4	20 mM Phosphate buffer (pH 4.6): Methanol (25:75 %v/v)	Peaks were merged and fronting	Not satisfactory	5.4.5
5	20 mM Phosphate buffer (pH 4.6): Acetonitrile (50:50 %v/v)	Peaks were merged together	Not satisfactory	5.4.6
6	20 mM Phosphate buffer (pH 4.6): Acetonitrile (20:80 %v/v)	No resolution with bad peak shape	Not satisfactory	5.4.7
7	0.2% TEA (pH 3): Acetonitrile (50:50 %v/v)	No resolution with bad peak shape	Not satisfactory	5.4.8
8	0.1% TEA (pH 3): Methanol (50:50 %v/v)	Showed improvement in peak separation	Not satisfactory	5.4.9
9	0.2% TEA (pH 3): Methanol (30:70 %v/v)	Better separation with tailing	Not satisfactory	5.4.10
10	0.2% TEA (pH 5): Methanol (15:85 %v/v)	Two peaks merged together	Not satisfactory	5.4.11
11	0.2% TEA (pH 5): Methanol (5:95 %v/v)	Good separation with tailing	Not satisfactory	5.4.12
12	0.2% triethylamine (pH 5.5 was adjusted with orthophosphoric acid) and methanol (2:98%v/v)	**Optimum peak parameters**	**Satisfactory**	5.4.13

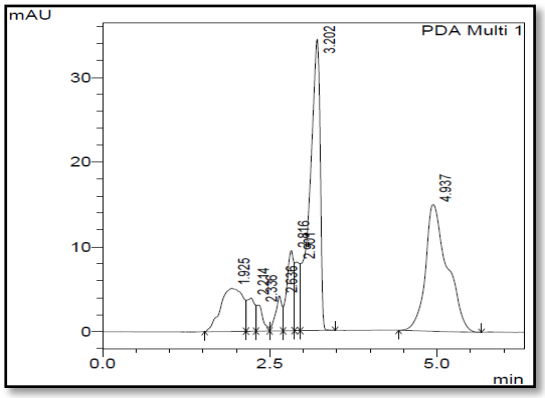


Figure No. 5.4.2: Sodium phosphate (pH 3): Acetonitrile (60:40 %v/v)

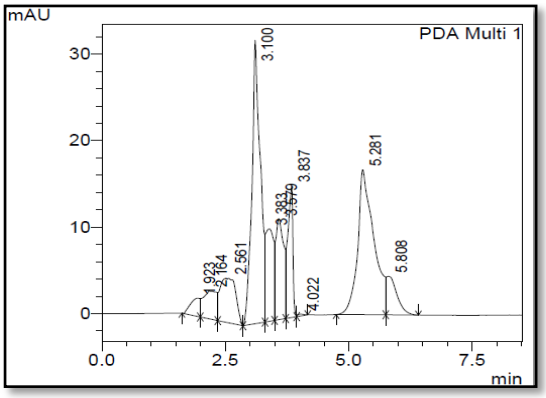


Figure No. 5.4.3: Acetonitrile: water: 0.01% ortho phosphoric acid (45:55:0.01%v/v)

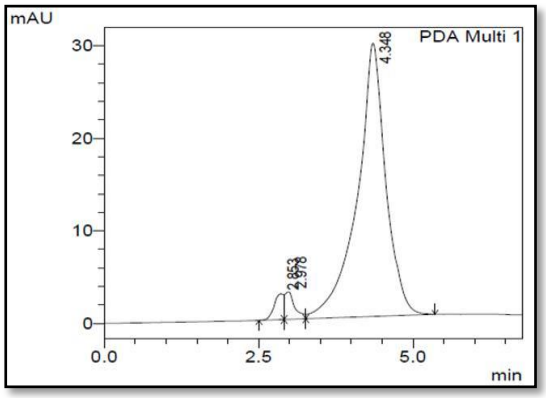


Figure No. 5.4.4: 20 mM Phosphate buffer (pH 4.6): Methanol (50:50 %v/v

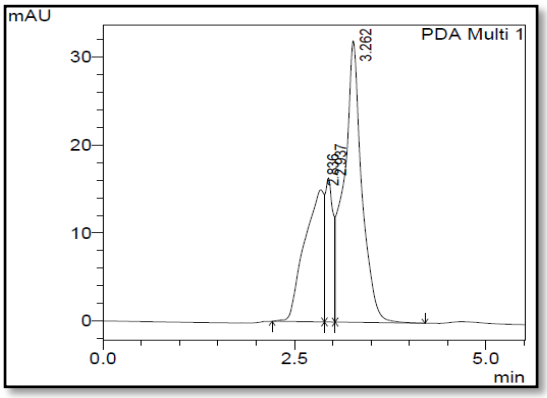


Figure No. 5.4.5: 20 mM Phosphate buffer (pH 4.6): Methanol (25:75 %v/v)

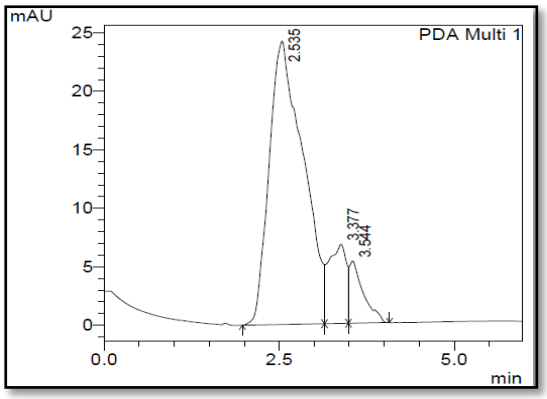


Figure No. 5.4.6: 20 mM Phosphate buffer (pH 4.6): Acetonitrile (50:50 %v/v)

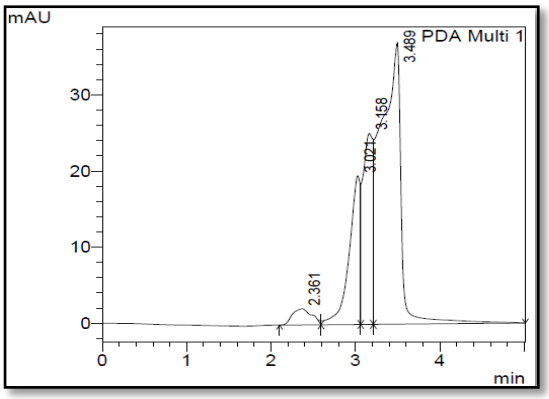


Figure No. 5.4.7: 20 mM Phosphate buffer (pH 4.6): Acetonitrile (20:80 %v/v)

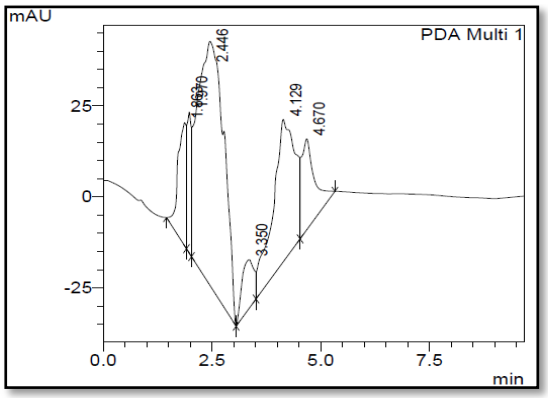


Figure No. 5.4.8: 0.2% TEA (pH 3): Acetonitrile (50:50 %v/v)

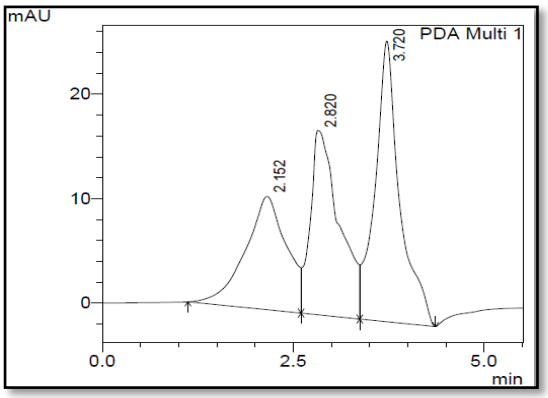


Figure No. 5.4.9: 0.1% TEA (pH 3): Methanol (50:50 %v/v)

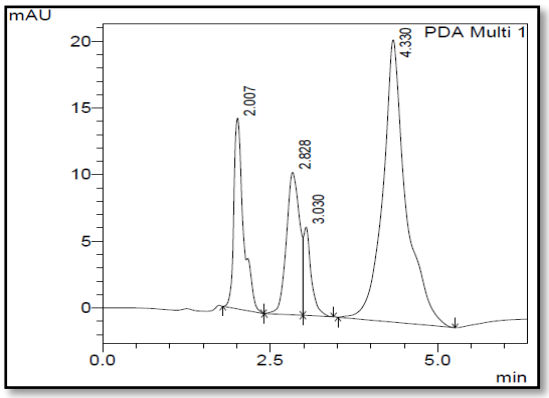


Figure No. 5.4.10: 0.2% TEA (pH 3): Methanol (30:70 %v/v)

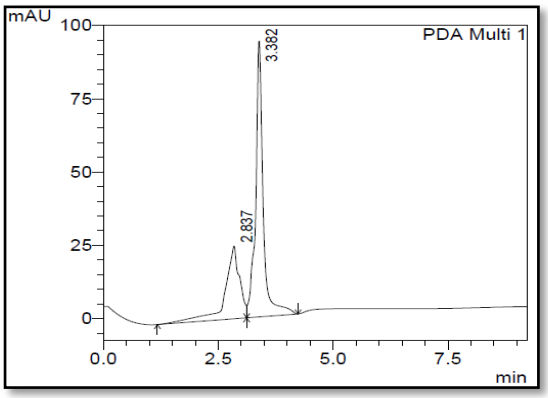


Figure No. 5.4.11: : 0.2% TEA (pH 5): Methanol (15:85 %v/v)

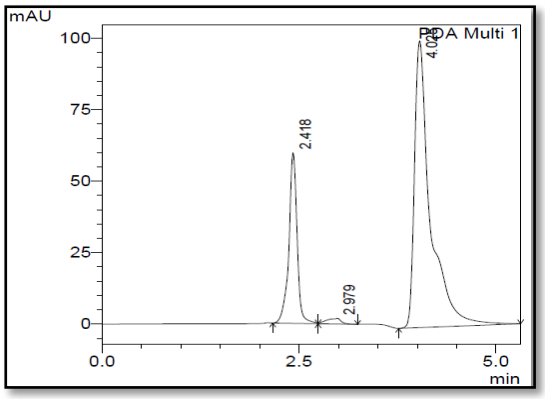


Figure No. 5.4.12: 0.2% TEA (pH 5): Methanol (5:95 %v/v)

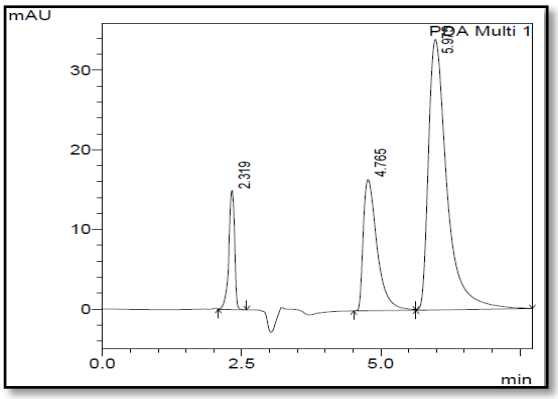


Figure No. 5.4.13: : 0.2% triethylamine (pH 5.5 was adjusted with orthophosphoric acid) and methanol (2:98 %v/v)

5.4.5 Optimization of separation condition

Initially various solvents like methanol, acetonitrile and buffers were tried as mobile phase for separation of ALO, MET & BA. But desired separation could not be achieved. Based on the literature review and preliminary studies, methanol and tri ethyl amine was selected as mobile phase components. Different chromatographic conditions like detection wave length, strength of buffer (0.1-0.3), flow rate (0.8-1.2), mobile phase composition, pH (3-7), etc. were varied to get optimum chromatographic conditions. Enable C18 column with a mobile phase 0.2% triethylamine (pH 5.5 was adjusted with orthophosphoric acid) and methanol (2:98 %v/v) at a flow rate of 1 ml/min was employed for the separation of components and PDA detection at 236 nm was employed. [92-96]

5.4.5 Fixed chromatographic condition

Stationary phase	:	Enable C_{18} column (250x 4.6 mm, 5 µm, 120 Å)
Mobile phase	:	0.2%v/v triethylamine (pH 5.5 with orthophosphoric acid) and methanol
Solvent ratio	:	2: 98%v/v
pH	:	5.5
Detection wavelength	:	236 nm

Flow rate	:	1 ml/ minute
Operating pressure	:	85 kgf
Temperature	:	Room temperature

The retention time of ALO, MET & BA were found to be 4.7296±0.0267, 6.1089±0.0301 min & 2.28 ± 0.0324 respectively, are shown in **Figure No. 5.4.14**

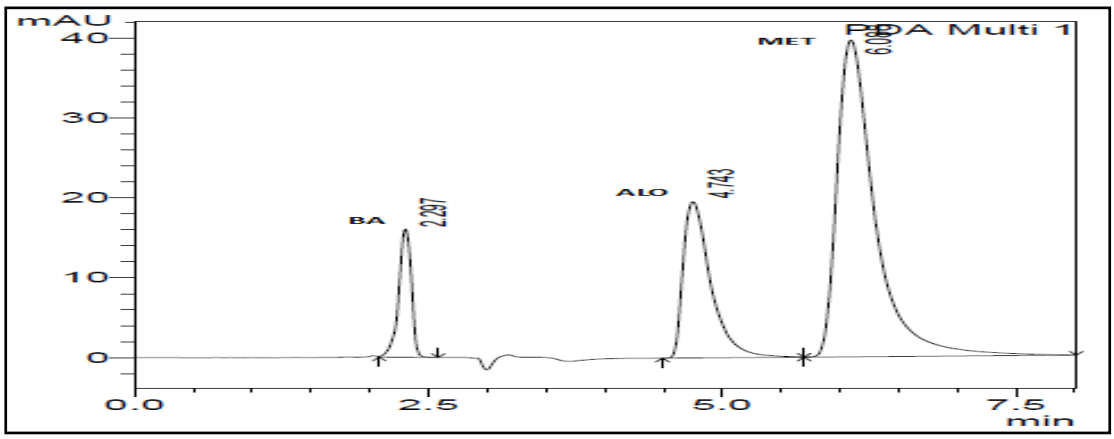


Figure No. 5.4.14: RP-HPLC chromatogram of ALO and MET (10 µg/ml)

5.4.6 Validation of chromatographic method

Developed method was validated according to ICH guidelines using following parameters

5.4.7 Specificity

The method was found to be specific as no interfering peaks were found within the stipulated run time **(Figure No. 5.4.17)**

5.4.8 Linearity and range

Calibration graphs were plotted using peak areas of standard drugs versus concentration for establishing linearity and range of the method **(Figure No. 5.4.15-5.4.16).** ALO and MET were found to be linear in the concentration range of 0.50-50 µg/ml **(Table No. 5.4.2, Figure No. 5.4.18-5.4.27.).** Results were subjected to regression analysis by the least squares method to calculate values of slope, intercept and correlation coefficient are shown in **Table No. 5.4.9**

Table No 5.4.2: Linearity data of ALO & MET

Sr. No.	ALO			MET		
	Conc. (µg/ml)	Peak Area*	% RSD	Conc. (µg/ml)	Peak Area*	% RSD
1	0.5	17204.33	1.2911	0.5	43744.50	1.2932
2	1	35063.83	1.7159	1	85039.50	0.7244
3	10	324059.33	1.0709	10	887975.00	0.9274
4	20	650996.67	0.6738	20	1804660.33	0.4192
5	30	972146.33	0.9494	30	2875761.33	0.7704
6	40	1314659.83	1.0144	40	3651080.33	0.6546
7	50	1634921.67	0.7977	50	4556899.17	0.1675

*(n=6) Avg. of six determination

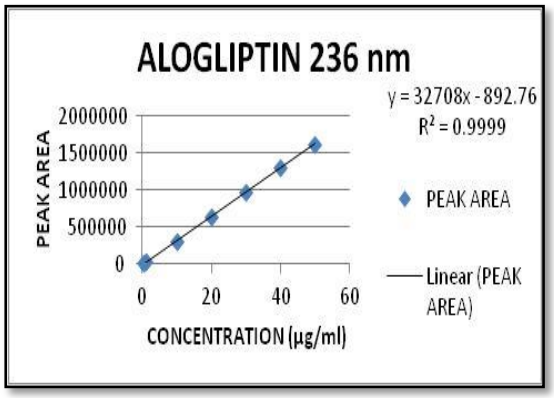


Figure No. 5.4.15: Calibration graph of ALO at 236 nm

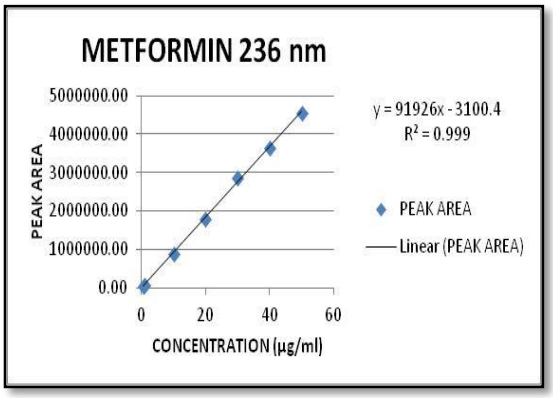


Figure No. 5.4.16: Calibration graph of MET at 236 nm

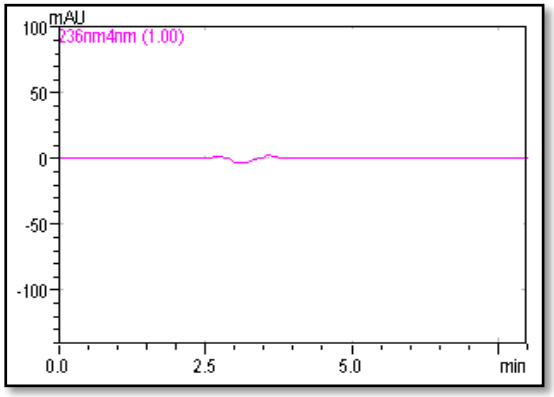


Figure No. 5.4.17: Chromatogram of blank (methanol)

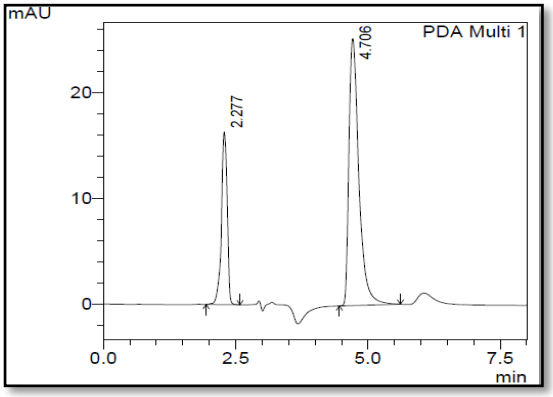


Figure No. 5.4.18: Chromatogram of ALO (10 µg/ml)

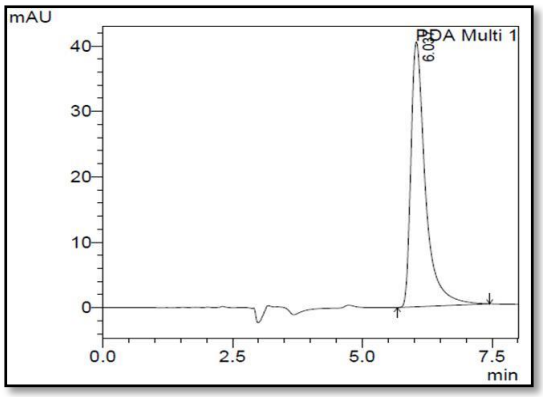


Figure No. 5.4.19: Chromatogram of MET (10 µg/ml)

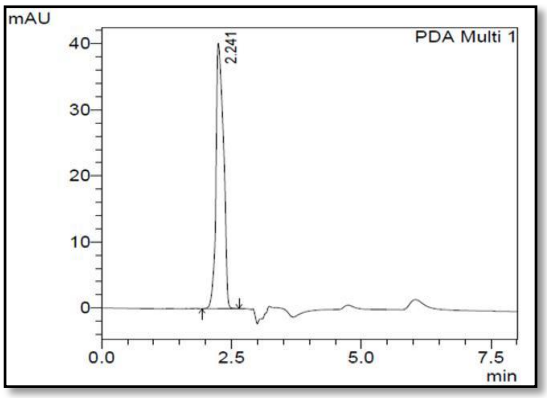


Figure No. 5.4.20: Chromatogram of BA (10 µg/ml)

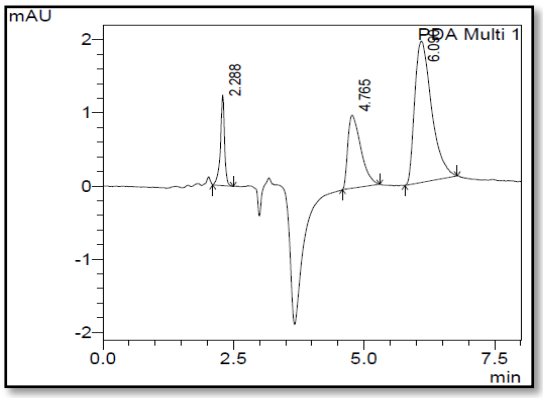


Figure No. 5.4.21: Chromatogram of ALO&MET (0.5 µg/ml)

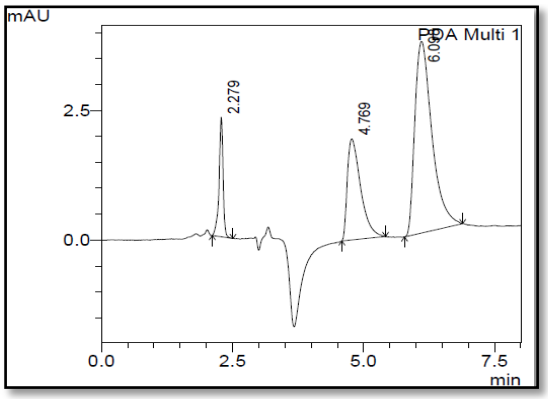


Figure No. 5.4.22: Chromatogram of ALO&MET (1 µg/ml)

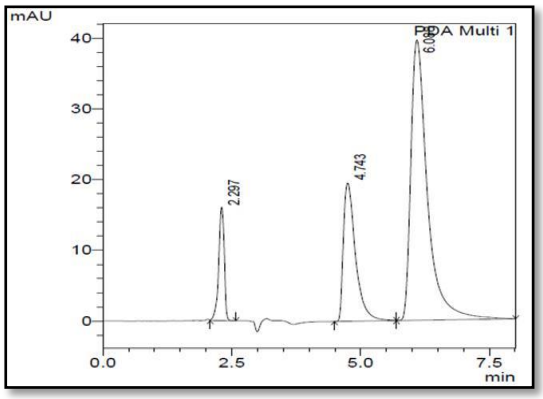


Figure No. 5.4.23: Chromatogram of ALO&MET (10 µg/ml)

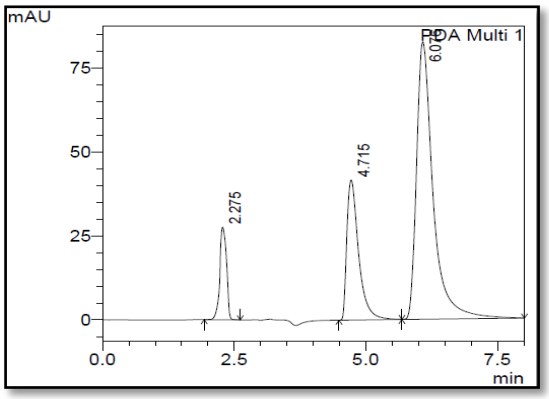


Figure No. 5.4.24: Chromatogram of ALO&MET (20 µg/ml)

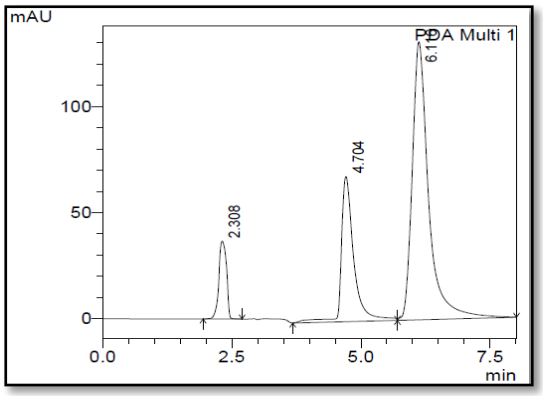


Figure No. 5.4.25: Chromatogram of ALO&MET (30 µg/ml)

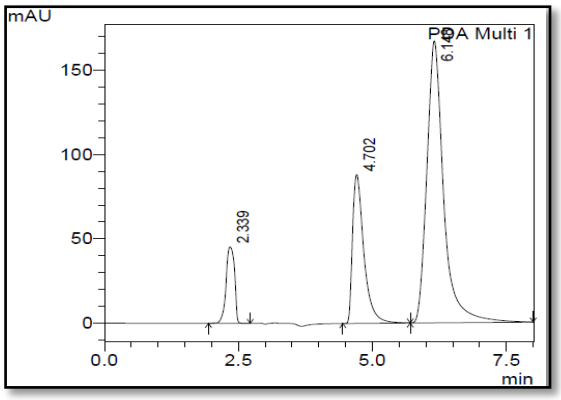


Figure No. 5.4.26: Chromatogram of ALO&MET (40 µg/ml)

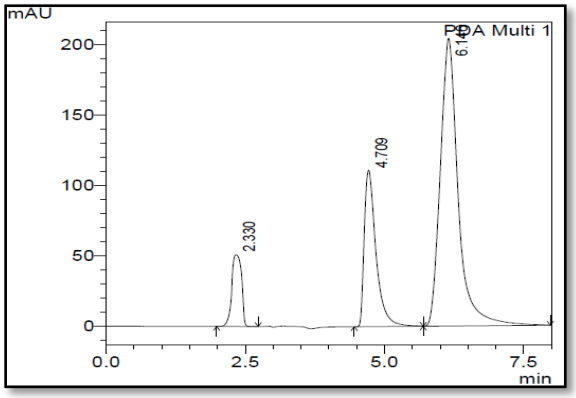


Figure No. 5.4.27: Chromatogram of ALO&MET (50 µg/ml)

5.4.9 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 indicates good repeatability and low inter-day variability (**Table No. 5.4.3, 5.4.4 & 5.4.5.).**

Table No. 5.4.3: Results of repeatability of measurement

Repeatability								
Sr. No.	ALO				MET			
	Conc. (µg/ml)	Peak area	Mean ± SD	% RSD	Conc. (µg/ml)	Peak area	Mean ± SD	% RSD
1	5	166324	165696.83± 1471.0347	0.8878	5	448756	444878.67 ± 2614.5644	0.5877
2		164256				445465		
3		163658				446878		
4		165843				443454		
5		166545				442156		
6		167555				442563		
1	10	333225	335046.67± 1575.0800	0.4701	10	887878	887867.33 ± 2698.1444	0.3039
2		335454				891655		
3		333469				890125		
4		336598				887545		
5		336989				885456		
6		334545				884545		
Mean % RSD				**0.6789**				**0.4458**

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

Table No. 5.4.4: Results of intra-day precision

Intra-day precision								
Sr. No.	ALO				MET			
	Conc. (µg/ml)	Peak area	Mean ± SD	% RSD	Conc. (µg/ml)	Peak area	Mean ± SD	% RSD
1	5	165254	165036.4 ± 1613.621	0.9777	5	442655	448027.7 ± 6161.283	1.3752
2		163455				446555		
3		162566				443655		
4		164365				455557		
5		166445				455785		
6		165456				456587		
7		167985				441255		
8		165548				444655		
9		164254				445545		
1	10	336544	335741.8 ± 2745.497	0.8177	10	884544	867601 ± 11573.98	1.3340
2		335697				875454		
3		336544				868545		
4		333456				871545		
5		336889				876245		
6		338645				865855		
7		332569				846255		
8		331545				865321		
9		339787				854645		
Mean % RSD				0.8977				1.3546

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

Table No. 5.4.5: Results of inter-day precision

Inter-day precision									
Sr. No.		**ALO**				**MET**			
		Conc. (µg/ml)	**Peak area**	**Mean ± SD**	**% RSD**	**Conc. (µg/ml)**	**Peak area**	**Mean ± SD**	**% RSD**
1	1	5	167155	164997.13 ± 1950.206	1.1820	5	445454	451616.33 ± 7523.439	1.6659
2			164544				443556		
3			165365				443548		
4	2		163546				446787		
5			162565				457845		
6			165455				456985		
7	3		168325				465455		
8			162565				453132		
9			165454				451785		
1	1	10	342124	338934.17 ± 5184.488	1.5296	10	884588	869474.98 ± 11939.02	1.3731
2			338455				884654		
3			335465				874566		
4	2		345654				876225		
5			334678				865423		
6			336458				865425		
7	3		334784				846985		
8			334544				862154		
9			348245				865254		
Mean % RSD					1.3558				1.5195

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

5.4.10 Accuracy

The results of recovery studies ranged from 98-100% for both the drugs showing the accuracy of the method (**Table No. 5.4.6**). The results of recovery studies indicate that there were no interferences from tablet excipients.

Table No. 5.4.6: Results of recovery studies

Accuracy (% Recovery)				
ALO				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	5	2.5	2.4493	97.9727
50	10	5	5.0016	100.0319
50	15	7.5	7.2819	97.0919
100	5	5	5.0453	100.9057
100	10	10	10.0123	100.1230
100	15	15	15.1760	101.1733
150	5	7.5	7.5427	100.5688
150	10	15	15.2404	101.6025
150	15	22.5	22.9429	101.9686
Mean± SD				**100.1598±1.6324**
% RSD				**1.6298**
MET				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	5	2.5	2.4566	98.2632
50	10	5	4.9456	98.9116
50	15	7.5	7.4001	98.6684
100	5	5	4.9576	99.1525
100	10	10	10.1407	101.4071
100	15	15	14.9421	99.6139
150	5	7.5	7.4777	99.7022
150	10	15	14.8294	98.8625
150	15	22.5	21.9031	97.3471
Mean± SD				**99.1032±1.1182**
% RSD				**1.1283**

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

5.4.11 LOD and LOQ

The values of LOD and LOQ were found to be 0.1253 and 0.3797 µg/ml for ALO, 0.1092 and 0.3308 µg/ml for MET, respectively **(Table No. 5.4.8).**

5.4.12 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. 5.4.7).**

Table No. 5.4.7: Results of robustness study

Sr. No.	Modification	ALO		MET	
		R_t	Peak area	R_t	Peak area
1	Strength of buffer (0.2 ± 0.1%v/v)	4.686	653478	6.234	1804567
		4.823	657869	6.123	1812345
		4.765	650987	6.276	1809876
% RSD* (<2)		**1.4453**	**0.5327**	**1.2728**	**0.2197**
2	Effect of pH (5.5 ± 0.2 unit)	4.723	643569	6.123	1804567
		4.749	654356	6.234	1814569
		4.867	657896	6.194	1809876
% RSD* (<2)		**1.6056**	**1.1447**	**0.9091**	**0.2765**
3	Effect of flow rate (1± 0.1ml/min)	4.8275	659865	6.234	1814567
		4.678	654556	6.134	1829876
		4.768	652869	6.247	1809876
% RSD* (<2)		**1.5820**	**0.5567**	**0.9965**	**0.5753**

**%RSD of three observation*

5.4.13 Stability of the solution

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

Table No. 5.4.8: Summary of validation parameters for the proposed RP-HPLC method

Parameters	**ALO**	**MET**
Linearity range (µg/ml)	0.50-50	
Correlation coefficient	0.9999	0.9997
Regression equation	y = 32708x - 892.76	y = 91926x - 3100.4
Precision (%RSD) Intra-day (n=3) Inter-day (n=3) Repeatability of injection (n=06)	 0.8977 1.3558 0.6789	 1.3546 1.5195 0.4458
Accuracy % Recovery (n=3) %RSD (n=3)	 100.1598±1.6324 1.6298	 99.1032±1.1182 1.1283
Specificity	No interference	
LOD (µg/ml)	0.1253	0.1092
LOQ (µg/ml)	0.3797	0.3308

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

5.4.14 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 the proposed method is suitable for the analysis intended, **Table No. 5.4.9**

Table No. 5.4.9: Result of system suitability studies

Parameters	**Values**		**Acceptance criteria**
	ALO*	**MET***	
Retention time (R_t) %RSD	4.72±0.0267	6.10±0.0301	%RSD ≤ 2
Peak area reproducibility %RSD	164662±641.49 0.3896	446581±1468.36 0.3288	%RSD<2
Theoretical plates (N)	2656	2258.43	N>2000
Tailing factor (T)	1.87	1.75	T<2
Resolution (R_S)	7.63	3.006	R_S>2

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

5.4.15 Analysis of formulation

The proposed method was successfully used for the quantitative determination of ALO and MET in tablet formulation (12.5 mg ALO and 500 mg of MET). Six replicate determinations were carried out and average experimental values were found to be 99.0123 and 99.1963 %w/w for ALO and MET, respectively and hence the developed method can be used for the simultaneous estimation of drugs in tablet dosage form. **Table No. 5.4.9.** Overlain chromatogram of standard drugs and formulation are shown in **Figure No. 5.4.28.**

Table No. 5.4.10: Results of formulation analysis

Drugs	Amount (mg/tablet)		% Drug found*	% RSD
	Labelled	Found		
ALO	12.5	12.38	99.0123±0.9374	0.9468
MET	500	495.98	99.1963±0.7314	0.7373

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

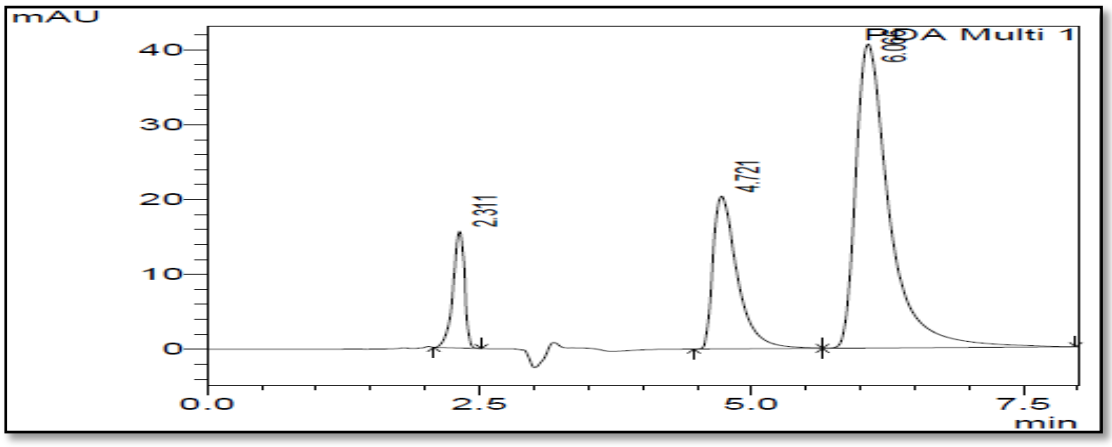


Figure No. 5.4.28: Chromatogram of formulation of ALO & MET (10 µg/ml)

Method 5

5.5 "Development and validation of HPTLC method for the simultaneous determination of alogliptin benzoate and metformin hydrochloride in tablets"

A simple, rapid, precise, specific and accurate reverse phase high performance thin layer chromatography (HPTLC) method was developed and validated for simultaneous estimation of Alogliptin and Metformin in their tablet dosage form. HPTLC method has several advantages like less cost per analysis, lower analysis time, simple sample preparation, mobile phases with pH 8 and above can be employed, no need of sample pretreatment, suspensions or turbid samples can be directly applied etc over other chromatographic techniques.

5.5.1 Selection of mode of chromatographic method

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

5.5.2 Selection of solvent

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

5.5.3 Selection of wavelength

UV spectra of both the drugs on pre-coated plate were recorded and 237 nm was selected as wavelength of detection. **Figure No. 5.5.1**

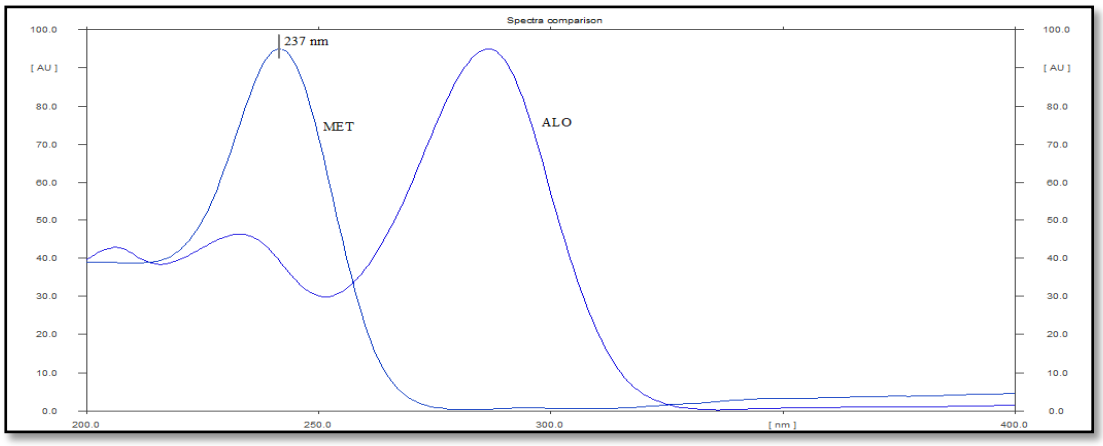


Figure No. 5.5.1: Overlain UV Spectra of ALO & MET in HPTLC

Table No. 5.5.1: Selection of mobile phase

Sr.No	Mobile Phase	Observation	Result
1	Toluene:Methanol:Ammonia (7:3:0.1v/v/v)	MET did not run from initial spot	Not satisfactory
2	Chloroform:Methanol:Ammonia (10:1:0.1v/v/v)	MET did not run from initial spot	Not satisfactory
3	Toluene:Ethyl acetate:Methanol (7:2:1v/v/v)	BA did not separate from ALO	Not satisfactory
4	Chloroform:Toluene:Methanol: Ammonia (8:2:1:0.12v/v/v/v)	MET did not run from initial spot and ALO showed tailing	Not satisfactory
5	Ethylacetate:Chloroform: Methanol:IPA:Ammonium acetate: Glacial acetic acid (3:2:3:1:1:0.12v/v/v/v/v/v)	ALO&BA showed good separation but MET showed tailing	Not satisfactory
6	Ethylacetate:Methanol:IPA:Ammonium acetate: Glacial acetic acid(3:2:3:1:0.12v/v/v/v/v)	MET showed tailing	Not satisfactory
7	Ethylacetate:Methanol:IPA:Ammoniumacetate: Glacial acetic acid:Ammonia (3:2:3:1:0.12:0.12v/v/v/v/v/v)	MET showed tailing	Not satisfactory
8	Ethyl Acetate: Methanol: Isopropyl Alcohol: Ammonium acetate: Ammonia (4:4:1:1:0.12v/v/v/v/v)	Rf value of ALO was high and BA moved with solvent front	Not satisfactory
9	Ethyl Acetate: Methanol: Isopropyl Alcohol: Ammoniumacetate: Ammonia (4:3:1:1:0.12v/v/v/v/v)	Rf value of ALO decreased but BA moved with solvent front	Not satisfactory
10	Ethyl acetate: methanol: isopropyl alcohol: ammoniumacetate : ammonia (4:2:1:1:0.12v/v/v/v/v)	**Good separation with symmetrical peaks**	**Satisfactory**

5.5.4 Development of optimum mobile phase

A solvent system that gave dense compact spots, good separation between ALO, MET and BA (Benzoic acid: Which is separating from alogliptin benzoate) and separation from solvent front and application position was selected. Initially a fixed concentration of drug solution was spotted and then developed using solvents such as methanol, acetonitrile, chloroform, ethyl acetate, toluene, hexane etc. and also mobile phases obtained from literature review was tried. After various trials, optimum mobile phase was selected. **[97-102]**

5.5.5 Optimization of separation conditions:

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

5.5.6 Fixed chromatographic condition:

Stationary Phase	:	Pre-coated silica gel $60F_{254}$ on aluminium sheets
Mobile phase	:	Ethyl acetate: methanol: isopropyl alcohol: ammonium acetate, ammonia (4:2:1:1:0.12 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	:	237 nm

All the components were scanned at 237 nm and reproducible Rf values were found to be 0.393± 0.0052**,** 0.663±0.0041, and 0.97± 0.0031for MET, ALO and BA, respectively, are shown in **Figure No. 5.5.2.**

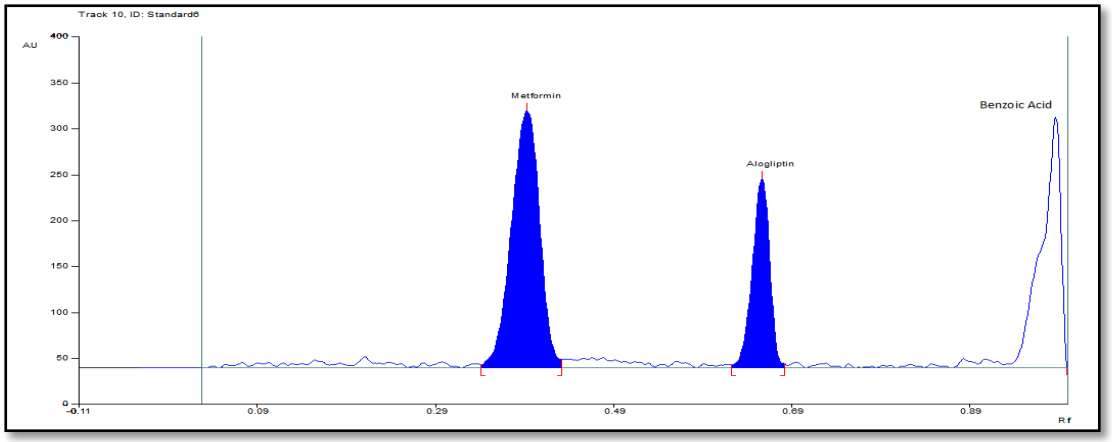


Figure No. 5.5.2: Standard chromatogram of ALO and MET (800 ng/band)

5.5.7 Validation of chromatographic method:

Developed method was validated according to ICH guidelines using following parameters

5.5.8 Specificity

The peak purity of both the drugs ALO and MET 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 ALO {correlation r(s, m) = 0.9999, r (m, e) = 0.9999} and MET {correlation r(s, m) = 0.9999, r(m, e) = 0.9996}. It can be concluded that no impurities or degradation products migrated with the peaks obtained from standard solutions of the drugs.

5.5.9 Linearity and range

Calibration curve was plotted using peak area versus ng/band. ALO and MET were found to be linear in the concentration range of 50-1000 ng/band (**Figure No. 5.5.6-5.5.16)**. Results were subjected to regression analysis by the least squares method to calculate values of slope, intercept and correlation coefficient are shown in **Table No: 5.5.2.**

Table No. 5.5.2: Linearity data of ALO & MET

Sr. No.	ALO			MET	
	Amount / Band (ng/band)	Peak Area*	Rf Value	Peak Area*	Rf Value
1	50	347.12	0.67	727.33	0.39
2	100	553.22	0.67	1430.97	0.39
3	200	1020.43	0.66	2407.15	0.4
4	400	1888.55	0.66	4105.52	0.39
5	600	2693.35	0.66	5871.73	0.4
6	800	3501.88	0.66	7861.92	0.39
7	1000	4366.52	0.66	9714.23	0.39

*(n=6) Avg. of six determination

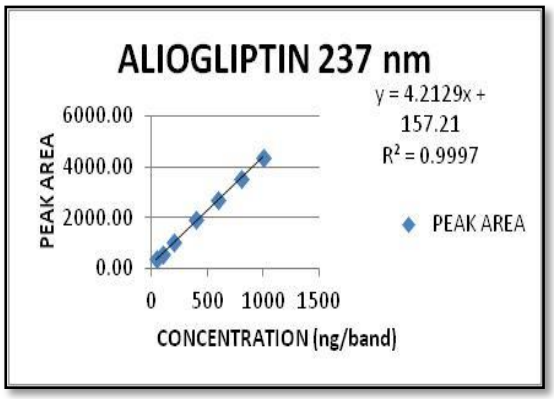


Fig. No. 5.5.3: Calibration curve of ALO at 237 nm

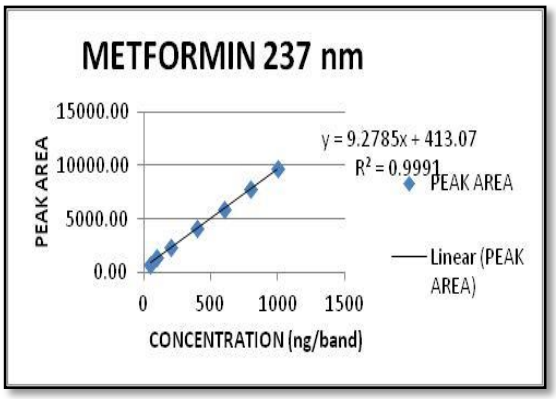


Fig. No. 5.5.4:Calibration curve of MET at 237 nm

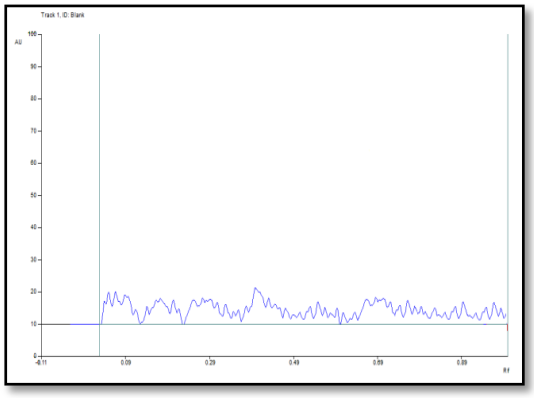


Fig. No. 5.5.5: Chromatogram of blank (methanol)

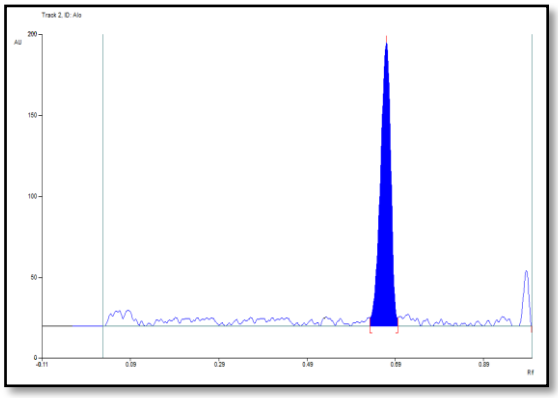


Fig. No. 5.5.6: Chromatogram of Alogliptin

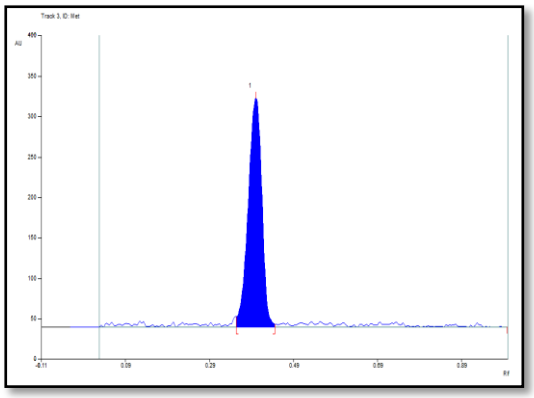


Fig. No. 5.5.7: Chromatogram of Metformin

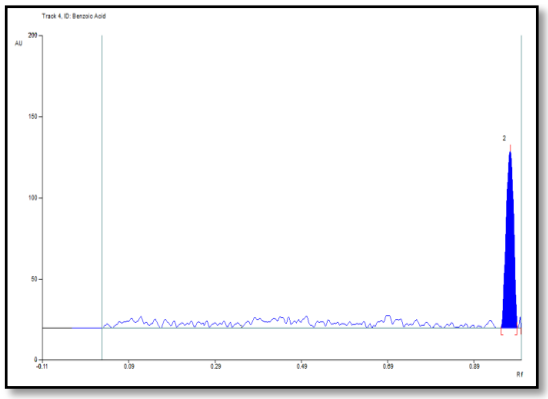


Fig. No. 5.5.8: Chromatogram of Benzoic acid

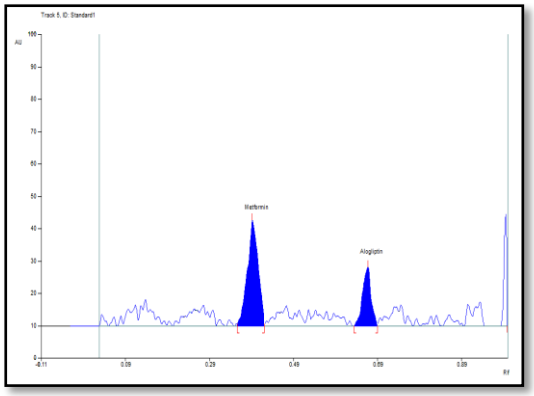


Fig. No. 5.5.9: Chromatogram of ALO & MET (50ng/band)

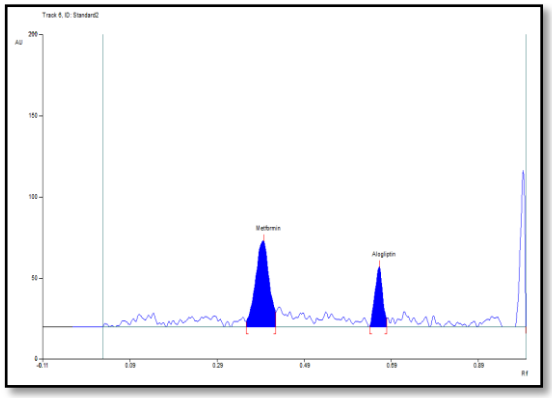


Fig. No. 5.5.10: Chromatogram of ALO & MET100 (ng/band)

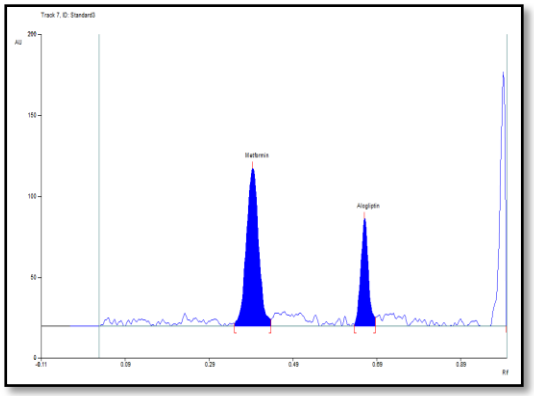


Fig. No. 5.5.11: Chromatogram of ALO & MET (200ng/band)

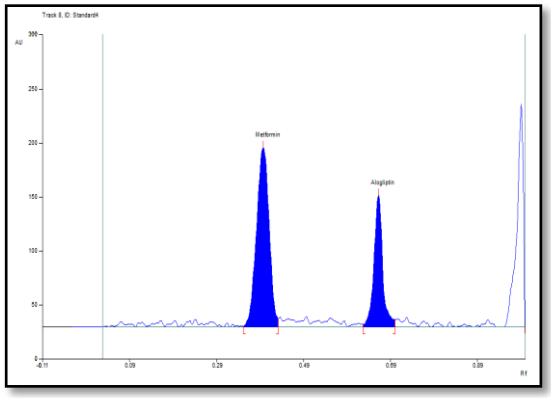


Fig. No. 5.5.12: Chromatogram of ALO & MET (400ng/band)

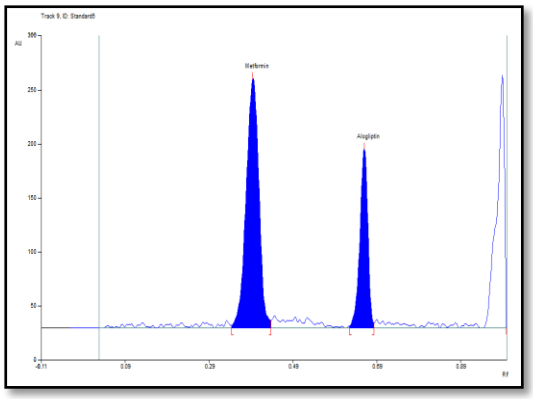


Fig. No. 5.5.13: Chromatogram of ALO & MET (600 ng/band)

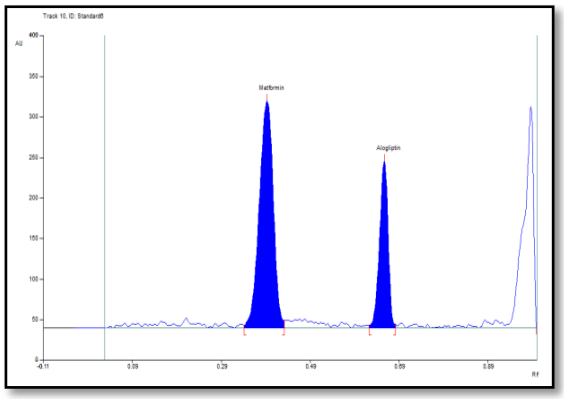


Fig. No. 5.5.14: Chromatogram of ALO & MET (800 ng/band)

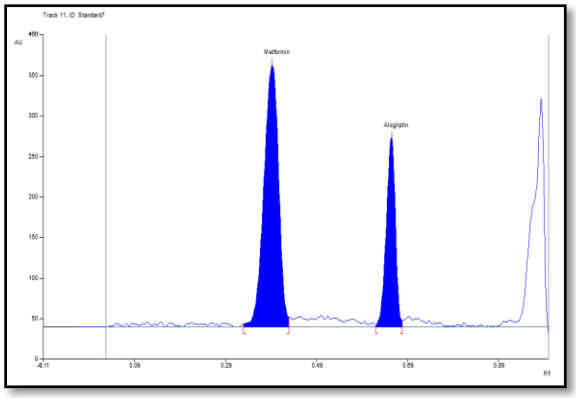


Fig. No. 5.5.15: Chromatogram of ALO & MET (1000 ng/band)

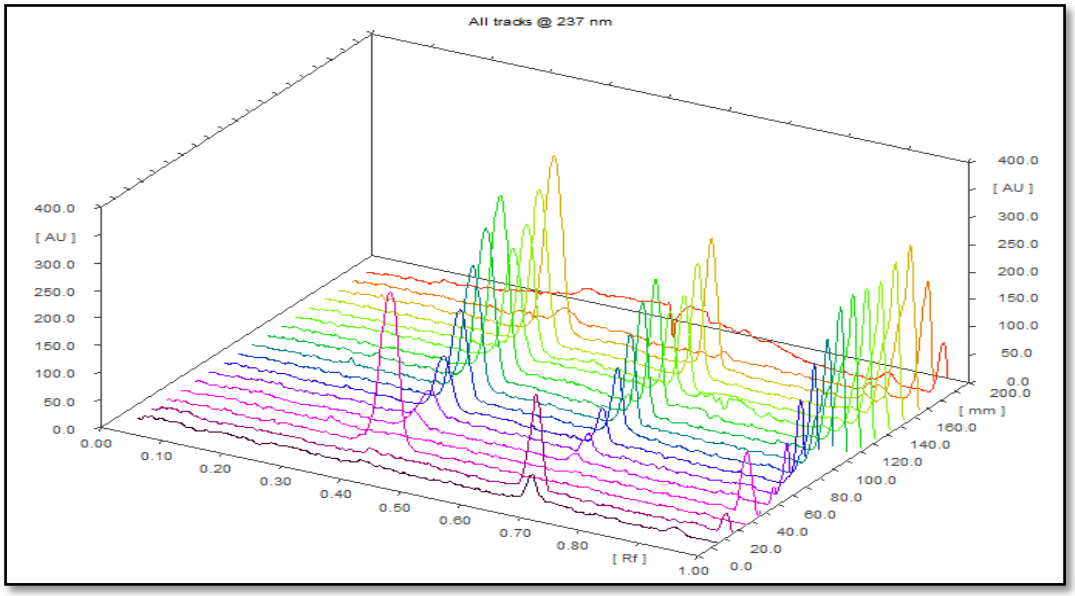


Fig. No. 5.5.16: Overlain 3D Chromatogram of ALO&PIO at 237 nm

5.5.10 Precision

The precision of the method was checked by carrying out repeatability, intraday and interday precision. Results of precision studies expressed in %RSD follows ICH guideline acceptable limits, (RSD<2) which indicates good repeatability and low inter-day variability. **Table No. 5.5.3, 5.5.4 & 5.5.5**

Table No. 5.5.3: Results of repeatability of measurement

Repeatability								
Sr. No.	**ALO**				**MET**			
	Conc. (ng/band)	**Peak area**	**Mean ± SD**	**% RSD**	**Conc. (ng/band)**	**Peak area**	**Mean ± SD**	**% RSD**
1	200	1025.1	1033.78 ± 8.1116	0.7846	200	2418.4	2414.40 ± 16.3498	0.6772
2		1035.6				2435.5		
3		1039.7				2415.3		
4		1046.5				2418.2		
5		1028.5				2415.3		
6		1028.9				2384.7		
1	400	1894.2	1875.45 ± 13.8055	0.7361	400	4016.3	4005.70 ± 31.0820	0.7759
2		1876.2				3958.3		
3		1885.3				4042.2		
4		1869.5				4022.7		
5		1854.3				4016.2		
6		1874.8				3978.5		
Mean % RSD				**0.7604**				**0.7266**

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

Table No. 5.5.4: Result of intra-day precision

Intra-day precision								
Sr. No.	**ALO**				**MET**			
	Conc. (ng/band)	**Peak area**	**Mean ± SD**	**% RSD**	**Conc. (ng/band)**	**Peak area**	**Mean ± SD**	**% RSD**
1	200	1048.7	1035.011 ± 10.2394	0.9893	200	2395.7	2409.489 ± 25.6731	1.0655
2		1026.5				2385.3		
3		1045.4				2457.4		
4		1031.8				2418.5		
5		1047.6				2384.2		
6		1029.9				2379.2		
7		1039.2				2431.0		
8		1024.1				2419.4		
9		1023.1				2415.2		
1	400	1865.3	1865.278 ± 15.7625	0.8450	400	4018.5	4086.344 ± 46.9959	1.1501
2		1847.9				4102.2		
3		1862.2				4125.3		
4		1854.4				4138.2		
5		1874.1				4119.2		
6		1895.3				41238		
7		1878.5				4039.0		
8		1865.5				4023.7		
9		1846.7				4089.2		
Mean % RSD				**0.9172**				**1.1078**

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

Table No. 5.5.5: Results of inter-day precision

Inter-day precision									
Sr. No.		**ALO**				**MET**			
		Conc. (µg/ml)	**Peak area**	**Mean ± SD**	**% RSD**	**Conc. (µg/ml)**	**Peak area**	**Mean ± SD**	**% RSD**
1	1	200	1028.3	1045.068 ± 13.9508	1.3349	200	2415.2	2421.444 ± 26.0981	1.0778
2			1049.2				2453		
3			1071.3				2417.5		
4	2		1044.2				2387		
5			1036				2413.3		
6			1051.3				2387		
7	3		1045.01				2451.00		
8			1026				2415.2		
9			1054.3				2453.8		
1	1	400	1846	1857.833 ± 22.372	1.2042	400	4124.2	4144.467 ± 69.0941	1.6671
2			1896				4039.3		
3			1875.3				4051.8		
4	2		1854.6				4111		
5			1884.2				4217.8		
6			1826.7				4141.3		
7	3		1846				4215.80		
8			1846.2				4215		
9			1845.5				4184		
Mean % RSD					**1.2696**				**1.3725**

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

5.5.11 Accuracy

The results of recovery studies ranged from 98-101% for both the drugs showing the accuracy of the method. **(Table No. 5.5.6)**

Table No. 5.5.6: Results of recovery studies

Accuracy (% Recovery)				
ALO				
Recovery level (%)	**Initial conc. of formulation (ng/band)**	**Standard added (ng/band)**	**Recovered (ng/band)**	**% Recovered**
50	200	100	98.8417	98.8417
50	300	150	151.4206	100.9471
50	400	200	201.0325	100.5163
100	200	200	201.1465	100.5732
100	300	300	304.2370	101.4123
100	400	400	395.3642	98.8411
150	200	300	293.2683	97.7561
150	300	450	452.7807	100.6179
150	400	600	604.4838	100.7473
Mean± SD				**100.0281±1.2319**
% RSD				**1.2315**
MET				
Recovery level (%)	**Initial conc. of formulation (ng/band)**	**Standard added (ng/band)**	**Recovered (ng/band)**	**% Recovered**
50	200	100	98.3963	98.3963
50	300	150	145.5892	97.0594
50	400	200	198.7207	99.3603
100	200	200	199.4924	99.7462
100	300	300	303.8078	101.2693
100	400	400	401.0422	100.2606
150	200	300	296.8917	98.9639
150	300	450	456.0771	101.3505
150	400	600	602.8033	100.4672
Mean± SD				**99.6526±1.3875**
% RSD				**1.3923**

5.5.12 LOD and LOQ

The values of LOD and LOQ were found to be 6.9223 and 20.9768 ng/band for ALO, 7.2778 and 22.0540 ng/band for MET, respectively **(Table No. 5.5.8)**.

5.5.13 Robustness

The proposed method was checked through all parameters described earlier under robustness studies, but no significant changes found in retention time, peak area or symmetry of the peaks and % RSD was found to be less than 2 showing the method was robust (**Table No. 5.5.7).**

Table No. 5.5.7: Results of robustness study

Sr. No.	Modification	ALO*		MET*	
		R_f value	**Peak area**	**R_f value**	**Peak area**
1	M/P Composition (± 0.1 ml)	0.6670 ± 0.0053	1886.8000 ± 10.6517	0.4000 ± 0.0059	4139.9467 ± 60.6535
% RSD		**0.7933**	**0.5645**	**1.4788**	**1.4651**
2	Volume of M/P (± 5 ml)	0.6707 ± 0.0074	1828.8467 ± 25.3818	0.3980 ± 0.0056	4166.6700 ± 20.2044
% RSD		**1.0991**	**1.3879**	**1.3955**	**0.4849**
3	Chamber saturation time (20 ± 5 min)	0.6707 ± 0.0067	1824.5067 ± 21.5071	0.3994 ± 0.0025	4137.8567 ± 45.2416
% RSD		**0.9928**	**1.1788**	**0.6235**	**1.0934**
4	Development distance (80 ± 5 mm)	0.6877 ± 0.0100	1826.2900 ± 24.4327	0.4025 ± 0.0056	4084.4867 ± 53.5471
% RSD		**1.4566**	**1.3378**	**1.3806**	**1.3116**
5	Time from spotting to chromatography (15± 10 min)	0.6780 ± 0.0100	1887.1000 ± 11.7439	0.3997 ± 0.0051	4106.7900 ± 14.4544
% RSD		**1.4749**	**0.6223**	**1.2857**	**0.3520**
6	Time from chromatography to scanning(15± 10 min)	0.6700 ± 0.0070	1815.8767 ± 13.7670	0.4066 ± 0.0059	4100.5267 ± 66.7944
% RSD		**1.0448**	**0.7581**	**1.4418**	**1.6289**

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

5.5.14 Stability of the solution

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

Table No. 5.5.8: Summary of validation parameters for the proposed HPTLC method

Parameters	**ALO**	**MET**
Linearity range (ng/band)	50-1000	
Correlation coefficient	0.9997	0.9991
Regression equation	y = 4.2129x + 157.21	y = 9.2785x + 413.07
Precision (%RSD)		
Intra-day (n=3)	0.9172	1.1078
Inter-day (n=3)	1.2696	1.3725
Repeatability of injection (n=6)	0.7604	0.7266
Accuracy		
% Recovery (n=3)	100.0281±1.2319	99.6526±1.3875
%RSD (n=3)	1.2315	1.3923
Specificity	No interference	
LOD (ng/band)	6.9223	7.2778
LOQ (ng/band)	20.9768	22.0540

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

5.5.15 Analysis of formulation

The proposed method was successfully used for the quantitative determination of ALO and MET in tablet formulation (12.5 mg ALO and 500 mg of MET). Six replicate determinations were carried out and average experimental values were found to be 99.4565 and 99.6673 %w/w for ALO and MET, respectively and hence the developed method can be used for the simultaneous estimation of drugs in tablet dosage form (**Table No. 5.5.9.)** Overlain chromatogram of standard drugs and formulation are shown in **Fig. No. 5.5.17**

Table No. 5.5.9: Result of formulation analysis

Drugs	Amount (mg/tablet)		% Drug found*	% RSD
	Labelled	Found		
ALO	12.5	12.43	99.4565±1.2097	1.2163
MET	500	498.34	99.6673±0.9757	0.9790

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

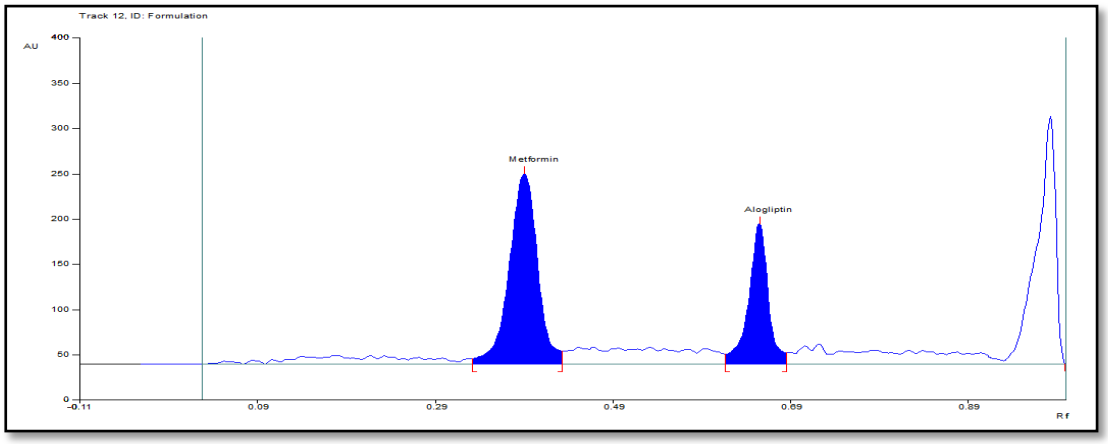


Figure No. 5.5.17: Chromatogram of formulation of ALO and MET (500 ng/band)

Formulation 2:

Method 6

5.6 "Development and validation of simultaneous equation method for the simultaneous determination of alogliptin benzoate and pioglitazone hydrochloride in tablets"

Estimation of ALO and PIO was achieved by simultaneous equation method using Shimadzu UV 1800 (UV Pro), double beam UV-Visible Spectrophotometer.

5.6.1 Selection of solvent:

By checking solubility in different solvents, methanol was selected as solvent for further studies

5.6.2 Selection of wavelength

Standard stock solutions of ALO and PIO containing 6 µg/ml of ALO and 21.6 µg/ml of PIO were scanned in the UV region (200-400 nm) and spectra were recorded using methanol as blank. Their overlain spectra are shown in **Figure No. 5.6.1**

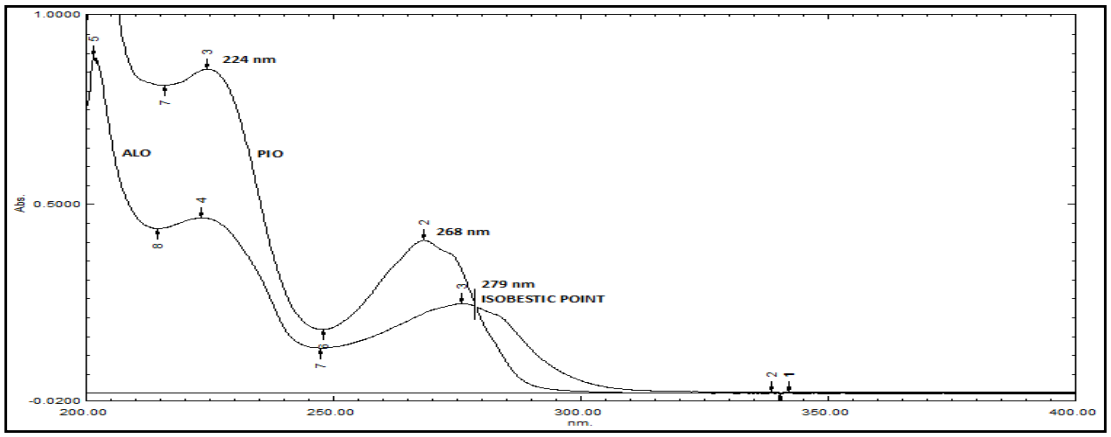


Figure No. 5.6.1: Overlain UV spectra of ALO (6 µg/ml) and PIO (21.6 µg/ml)

From the overlain spectra, different wavelengths were tried for the method. After comparing all wavelengths 224 nm and 268 nm were selected which showed good linearity over the given concentration range.

5.6.3 Determination of absorptivity values

The developed method was found to be linear in the concentration range of 0.5-12 µg/ml for ALO and 1.8-43.2 µg/ml for PIO. Absorbances were measured at 224 nm and 268 nm for both the drugs and absorptivity values were calculated which is shown in **Table No. 5.6.1 & 5.6.2**

Table No. 5.6.1: Absorbances and absorptivities of ALO at selected wavelength

Conc. (µg/ml)	ALO					
	224 nm			268 nm		
	Abs.	Absorptivity	Avg. Absorptivity	Abs.	Absorptivity	Avg. Absorptivity
0.5	0.0390	780.1333	777.0109 (ax_1)	0.0178	355.6667	350.6115 (ax_2)
2	0.1556	778.1667		0.0697	348.6667	
4	0.3123	780.6250		0.1370	342.4167	
6	0.4572	761.9444		0.2118	353.0278	
8	0.6208	775.9375		0.2780	347.4583	
10	0.7745	774.5333		0.3534	353.4333	
12	0.9453	787.7361		0.4243	353.6111	

*average of six determination

Table No. 5.6.2: Absorbances and absorptivities of PIO at selected wavelength

Conc. (µg/ml)	PIO					
	224 nm			268 nm		
	Abs.	Absorptivity	Avg. Absorptivity	Abs.	Absorptivity	Avg. Absorptivity
1.8	0.0711	395.0926	391.1810 (ay_1)	0.0262	145.7407	148.6599 (ay_2)
7.2	0.2744	381.0417		0.1088	151.1111	
14.4	0.5542	384.8727		0.2157	149.7685	
21.6	0.8534	395.0772		0.3207	148.4645	
28.8	1.1380	395.1389		0.4251	147.5926	
36	1.4354	398.7222		0.5361	148.9259	
43.2	1.6776	388.3218		0.6438	149.0162	

*average of six determination

5.6.4 Validation of the method:

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

5.6.5 Specificity

In order to determine the specificity of the method, a mixture of tablet excipients was prepared and analyzed to check whether there is any interference because of excipients. Overlain spectra of drug solution and formulation excipients proved that there was no interference between standard drugs and excipients (**Figure No. 5.6.2.)**

5.6.6 Linearity

From the linearity study, ALO and PIO were found to be linear in the concentration range of 0.5-12 µg/ml & 1.8-43.2 µg/ml respectively. The overlain spectra of ALO and PIO are shown in **Figure No. 5.6.3-5.6.5**

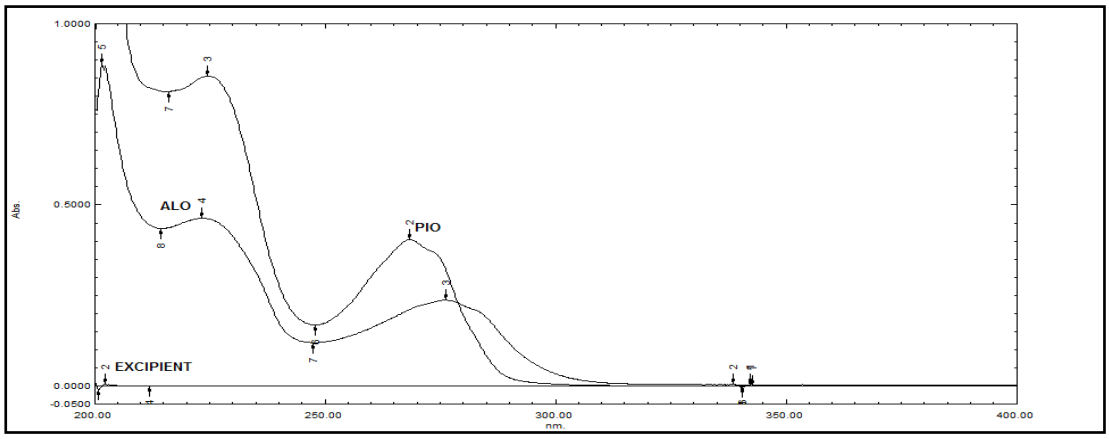


Figure No. 5.6.2: Overlain UV Spectra of ALO (6 µg/ml), PIO (21.6 µg/ml) and formulation excipient

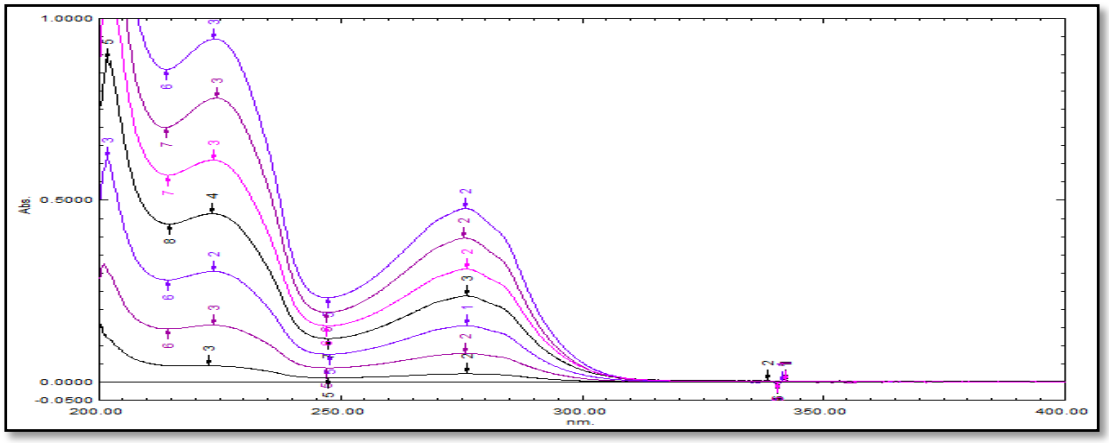


Fig. No. 5.6.3: Overlain UV spectra of ALO (0.5- 12 µg/ml)

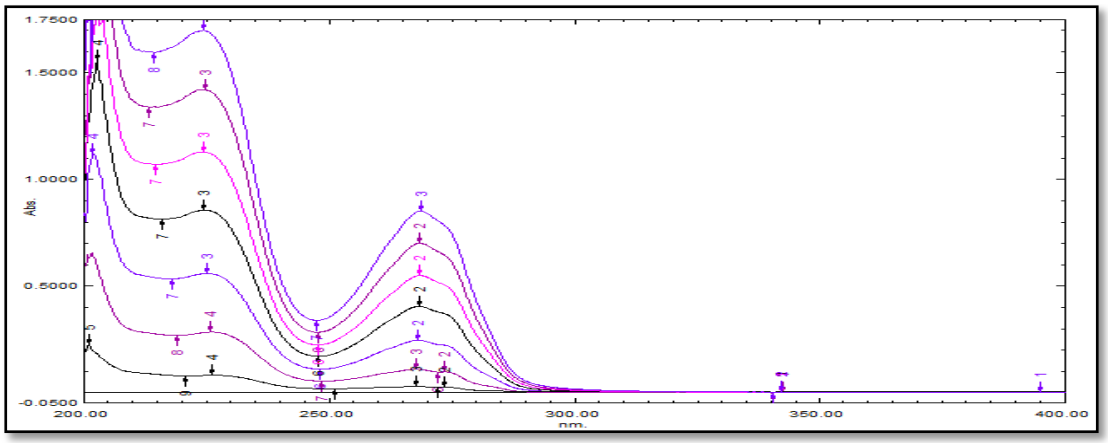


Figure No. 5.6.4: Overlain UV spectra of PIO (1.8-43.2 µg/ml)

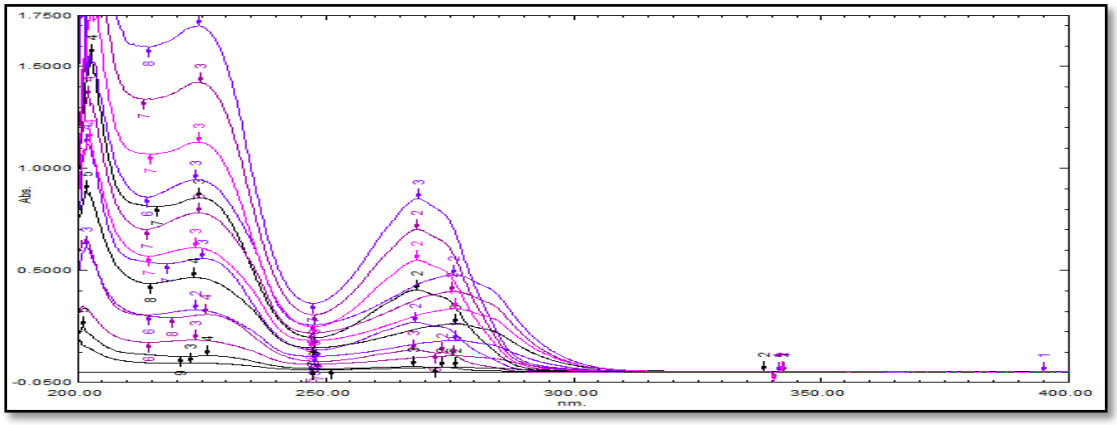


Figure No. 5.6.5:Overlain UV spectra of ALO (0.5- 12 µg/ml) & PIO (1.8-43.2 µg/ml)

Calibration graphs were plotted using absorbances of standard drug solutions versus concentration **(Figure No. 5.6.6-5.6.9).** Results were subjected to regression analysis by the least squares method to calculate the values of slope, intercept and correlation coefficient at 224 and 268 nm and are given in **Table No. 5.6.8.**

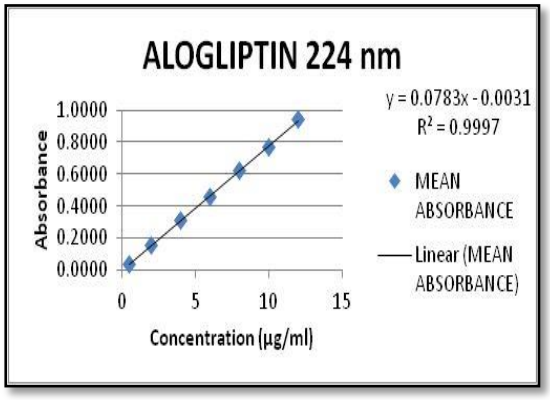


Figure No. 5.6.6: Calibration graph of ALO at 224 nm

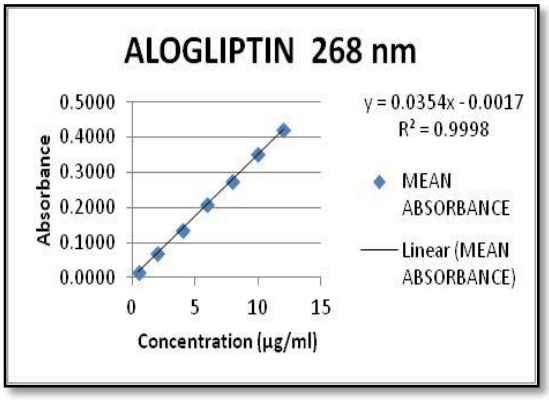


Figure No. 5.6.7: Calibration graph of ALO at 268 nm

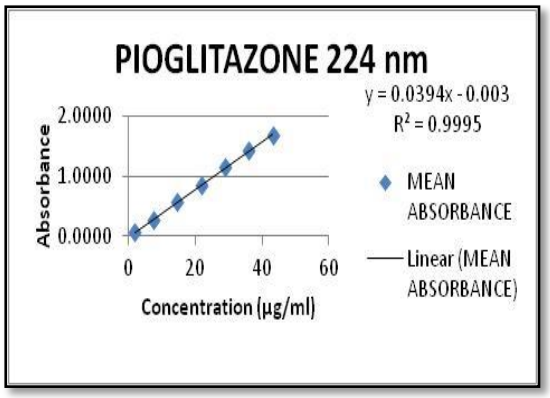


Figure No. 5.6.8: Calibration graph of PIO at 224 nm

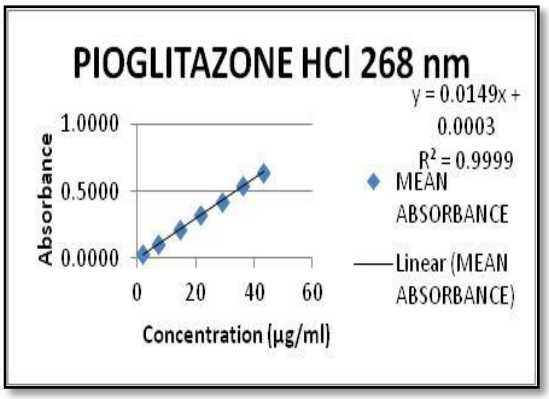


Figure No. 5.6.9: Calibration graph of PIO at 268 nm

5.6.7 Precision

Precision studies were performed and % RSD was calculated which is in accordance with ICH guideline acceptable limits (<2), which indicates that the method has good precision. **Table No. 5.6.3, 5.6.4 & 5.6.5.**

Table No. 5.6.3: Results of repeatability of measurement

Repeatability					
Sr. No.	**Conc.**	**Absorbance**			
		ALO		**PIO**	
		224 nm	**268 nm**	**224 nm**	**268 nm**
1	ALO 4 µg/ml & PIO 14.4 µg/ml	0.3125	0.1377	0.5563	0.2138
2		0.3151	0.1378	0.5534	0.2142
3		0.3128	0.1366	0.5543	0.2135
4		0.3127	0.1385	0.5581	0.2142
5		0.3126	0.1365	0.5538	0.2125
6		0.3128	0.1375	0.5546	0.2135
Mean±SD*		**0.3131±0.0010**	**0.1374±0.0008**	**0.5551±0.0018**	**0.2136±0.0006**
% RSD		**0.3177**	**0.5554**	**0.3213**	**0.2952**
1	ALO 6 µg/ml & PIO 21.6 µg/ml	0.4654	0.2145	0.8654	0.3208
2		0.4646	0.2134	0.8548	0.3215
3		0.4658	0.2142	0.8631	0.3218
4		0.4638	0.2096	0.855	0.3186
5		0.4644	0.2145	0.8654	0.3196
6		0.4589	0.2112	0.8546	0.3218
Mean±SD*		**0.4638±0.0025**	**0.2129±0.0020**	**0.8597±0.0055**	**0.3207±0.0013**
% RSD		**0.5417**	**0.9589**	**0.6342**	**0.4111**
Mean % RSD		**0.4297**	**0.7572**	**0.4778**	**0.3531**

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

Table No. 5.6.4: Results of intra-day precision

Intra-day precision					
Sr. No.	**Conc.**	**Absorbance**			
		ALO		**PIO**	
		224 nm	**268 nm**	**224 nm**	**268 nm**
1	ALO 4 µg/ml & PIO 14.4µg/ml	0.3125	0.1354	0.5634	0.2143
2		0.3258	0.1365	0.5628	0.2138
3		0.3148	0.1377	0.5628	0.2136
4		0.3128	0.1378	0.5563	0.2158
5		0.3152	0.1366	0.5534	0.2154
6		0.3118	0.1348	0.5543	0.2163
7		0.3154	0.1349	0.5684	0.2150
8		0.3149	0.1354	0.5661	0.2196
9		0.3129	0.1342	0.5648	0.2185
Mean±SD*		**0.3151±0.0042**	**0.1359±0.0013**	**0.5614±0.0054**	**0.2158±0.0021**
% RSD		**1.3393**	**0.9485**	**0.9572**	**0.9534**
1	ALO 6µg/ml & PIO 21.6µg/ml	0.4658	0.2156	0.8554	0.3212
2		0.4644	0.2135	0.855	0.3169
3		0.4589	0.2175	0.8654	0.3154
4		0.4578	0.2163	0.8546	0.3128
5		0.4655	0.2187	0.8564	0.3128
6		0.4628	0.2096	0.8547	0.3125
7		0.4638	0.2145	0.8564	0.3156
8		0.4618	0.2112	0.8551	0.315
9		0.4589	0.2157	0.8624	0.3139
Mean±SD*		**0.4622±0.0030**	**0.2147±0.0029**	**0.8573±0.0039**	**0.3151±0.0027**
% RSD		**0.6532**	**1.3571**	**0.4537**	**0.8662**
Mean % RSD		**0.9962**	**1.1528**	**0.7054**	**0.9098**

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

Table No. 5.6.5: Results of inter-day precision

Inter-day precision						
Sr. No.		**Conc.**	**Absorbance**			
			ALO		**PIO**	
			224 nm	**268 nm**	**224 nm**	**268 nm**
1	1	ALO 4 µg/ml & PIO 14.4µg/ ml	0.3158	0.1346	0.5634	0.2142
2			0.3125	0.1347	0.5614	0.2136
3			0.3265	0.1352	0.5562	0.2158
4	2		0.3175	0.1349	0.5478	0.2164
5			0.3162	0.1377	0.5638	0.2176
6			0.3228	0.1378	0.5563	0.2163
7	3		0.3122	0.1366	0.5534	0.2154
8			0.3118	0.1342	0.5543	0.2177
9			0.3158	0.1325	0.5684	0.2139
Mean±SD*			**0.3168±0.0050**	**0.1354±0.0017**	**0.5583±0.0064**	**0.2157±0.0015**
% RSD			**1.5688**	**1.2727**	**1.1443**	**0.7040**
1	1	ALO 6µg/ml & PIO 21.6µg/ ml	0.4684	0.3214	0.8646	0.3251
2			0.4781	0.3164	0.8741	0.3214
3			0.4635	0.3128	0.8562	0.3164
4	2		0.4638	0.3255	0.865	0.3128
5			0.4635	0.3264	0.8654	0.3255
6			0.4695	0.3215	0.8546	0.3264
7	3		0.4744	0.3257	0.8547	0.3215
8			0.4589	0.3261	0.8546	0.3257
9			0.4678	0.3251	0.8621	0.3261
Mean±SD*			**0.4675±0.0060**	**0.3149±0.0042**	**0.8613±0.0068**	**0.3223±0.0048**
% RSD			**1.2755**	**1.6671**	**0.7845**	1.5016
Mean % RSD			**1.4221**	**1.6199**	**0.9644**	1.1028

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

5.6.8 Accuracy

The method showed good accuracy as the results of recovery studies ranged from **98-102%** for both the drugs (**Table No. 5.6.6).**

Table No. 5.6.6: Results of recovery studies

Accuracy (% Recovery)				
ALO				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	1	0.5	0.4945	98.9002
50	2	1	0.9915	99.1534
50	4	2	2.0011	100.0571
100	1	1	0.9754	97.5437
100	2	2	1.9870	99.3512
100	4	4	3.9740	99.3512
150	1	1.5	1.4925	99.5015
150	2	3	2.9420	98.0675
150	4	6	5.8953	98.2543
Mean±SD*				**98.9089±0.8001**
% RSD				**0.8089**
PIO				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	3.6	1.8	1.8284	**101.5782**
50	7.2	3.6	3.6082	**100.2281**
50	14.4	7.2	7.3959	**102.7214**
100	3.6	3.6	3.5618	**98.93994**
100	7.2	7.2	7.3032	**101.4332**
100	14.4	14.4	14.7141	**102.1812**
150	3.6	5.4	5.3671	**99.39026**
150	7.2	10.8	10.9299	**101.2024**
150	14.4	21.6	21.7682	**100.7788**
Mean±SD*				**100.939±1.2434**
% RSD				**0.5837**

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

5.6.9 LOD and LOQ

The developed method was found to be sensitive as the values of LOD and LOQ were found to be very low which is shown in **Table No. 5.6.8.**

5.6.10 Robustness

Robustness study was carried out for proposed method, but no significant changes (% RSD<2) found in absorption (**Table No. 5.6.7).**

Table No. 5.6.7: Results of robustness study

Parameter	**Drugs**			
Wavelengths (224 & 268 ±1 nm)	**ALO**		**PIO**	
	Assay (%)*	**% RSD**	**Assay (%)***	**% RSD**
223 & 267 nm	99.4537	0.9877	100.5432	0.8378
224& 268 nm	100.3256		101.5498	
225 & 269nm	101.4325		99.8745	

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

5.6.11 Stability of the solution

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

Table No. 5.6.8: Summary of validation parameters for the proposed method

Parameters	**ALO**		**PIO**	
Detection wavelengths (nm)	224	268	224	268
Linearity range (µg/ml)	0.5-12		1.8-43.2	
Correlation coefficient	0.9997	0.9998	0.9995	0.9999
Regression equation	y = 0.0783x - 0.0031	y = 0.0354x - 0.0017	y = 0.0394x - 0.003	y = 0.0149x + 0.0003
Precision (%RSD) Intra-day (n=3) Inter-day (n=3) Repeatability of measurement (n=6)	 0.9962 1.4221 0.4297	 1.1528 1.6199 0.7572	 0.7054 0.9644 0.4778	 0.9098 1.1028 0.3531
Accuracy % Recovery (n=3) %RSD (n=3)	 98.9089±0.8001 0.8089		 100.9393±1.2434 0.5837	
Specificity	No interference			
LOD (µg/ml)	0.0539	0.0556	0.0702	0.2138
LOQ (µg/ml)	0.1634	0.1686	0.2127	0.6480

**mean±SD, n=number of determination*

5.6.12 Analysis of formulation

The proposed method was successfully used for the quantitative determination of ALO& PIO in tablet formulation (12.5 mg ALO and 45 mg of PIO). Six replicate determinations were carried out and average experimental values were found to be 97.6596 and 97.3869 %w/w for ALO and PIO, respectively and hence the developed can be used for the simultaneous estimation of drugs in tablet dosage form (**Table No. 5.6.9.)** Overlain spectra of standard drugs and formulation are showed in **Figure No. 5.6.10**.

Table No. 5.6.9: Results of formulation analysis

Drugs	Amount (mg/tablet)		% Drug found*	% RSD
	Labelled	Found		
ALO	12.5	12.21	97.6596±0.7456	0.7635
PIO	45	43.82	97.3869±1.0077	1.0347

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

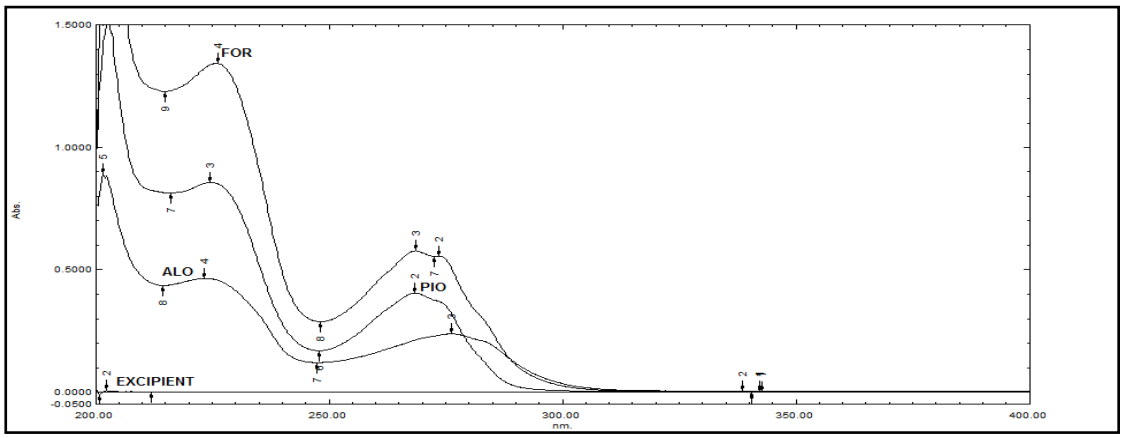


Figure No. 5.6.10: Overlain UV spectra of standard ALO (6 µg/ml), PIO (21.6 µg/ml) & formulation (6& 21.6µg/ml)

Method 7

5.7 "Development and validation of absorbance ratio (Q analysis) method for the simultaneous determination of alogliptin benzoate and pioglitazone hydrochloride in tablets"

Estimation of ALO and PIO was achieved by absorbance ratio method using Shimadzu UV 1800 (UV Pro), double beam UV-Visible spectrophotometer.

5.7.1 Selection of solvent:

By checking solubility in different solvents methanol was selected as solvent for further studies

5.7.2 Selection of wavelength

Standard stock solutions of ALO and PIO containing 6 µg/ml of ALO and 21.6 µg/ml of PIO were scanned in the UV region (200-400 nm) and spectra were recorded using methanol as blank. Their overlain spectra are shown in **Figure No. 5.7.1**

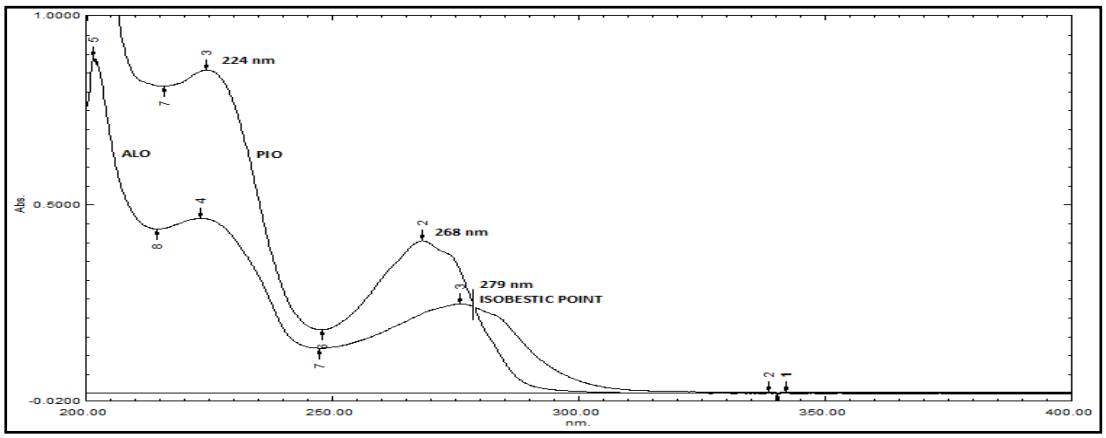


Figure No. 5.7.1: Overlain UV spectra of ALO (6 µg/ml) and PIO (21.6 µg/ml)

From the overlain spectra, different wavelengths were tried for the method. After comparing all wavelengths, 224 nm and 279 nm were selected which showed good linearity over the given concentration range.

5.7.3 Determination of absorptivity values

The developed method was found to be linear in the concentration range of 0.5-12 µg/ml for ALO and 1.8-43.2 µg/ml for PIO, respectively. Absorbances were measured at 224 nm and 279 nm for both the drugs and absorptivity values were calculated which is shown in **Table No. 5.7.1 & 5.7.2**

Table No. 5.7.1: Absorbances and absorptivities of ALO at selected wavelength

Conc. (µg/ml)	ALO					
	224 nm			279 nm		
	Abs.	Absorptivity	Avg. Absorptivity	Abs.	Absorptivity	Avg. Absorptivity
0.5	0.0390	780.1333	777.0109 (ax_1)	0.0192	383.0000	378.007 (ax_2)
2	0.1556	778.1667		0.0747	373.5000	
4	0.3123	780.6250		0.1515	378.6667	
6	0.4572	761.9444		0.2268	378.0556	
8	0.6208	775.9375		0.3015	376.8333	
10	0.7745	774.5333		0.3751	375.0500	
12	0.9453	787.7361		0.4571	380.9444	

*average of six determination

Table No. 5.7.2: Absorbances and absorptivities PIO of at selected wavelength

Conc. (µg/ml)	PIO					
	224 nm			279 nm		
	Abs.	Absorptivity	Avg. Absorptivity	Abs.	Absorptivity	Avg. Absorptivity
1.8	0.0711	395.0926	391.1810 (ay_1)	0.0192	106.3889	105.0020 (ay_2)
7.2	0.2744	381.0417		0.0747	103.7500	
14.4	0.5542	384.8727		0.1515	105.1852	
21.6	0.8534	395.0772		0.2268	105.0154	
28.8	1.1380	395.1389		0.3015	104.6759	
36	1.4354	398.7222		0.3751	104.1806	
43.2	1.6776	388.3218		0.4571	105.8179	

*average of six determination

5.7.4 Validation of the method:

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

5.7.5 Specificity

In order to determine the specificity of the method, a mixture of tablet excipients was prepared and analyzed to check whether there is any interference because of excipients. Overlain spectra of drug solution and formulation excipients prove that there was no interference between standard drugs and excipients. **Figure No. 5.7.2.**

5.7.6 Linearity

From the linearity study, ALO and PIO were found to be linear in the concentration range of 0.5-12 µg/ml & 1.8-43.2 µg/ml respectively. The overlain spectra of ALO and PIO are shown in **Figure No. 5.7.3-5.7.5**

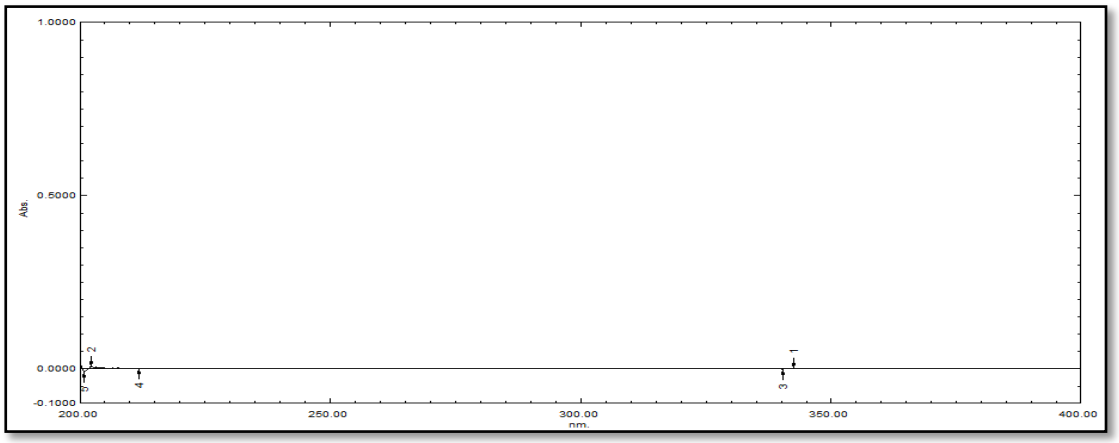


Fig. No. 5.7.2: Spectra of formulation excipient

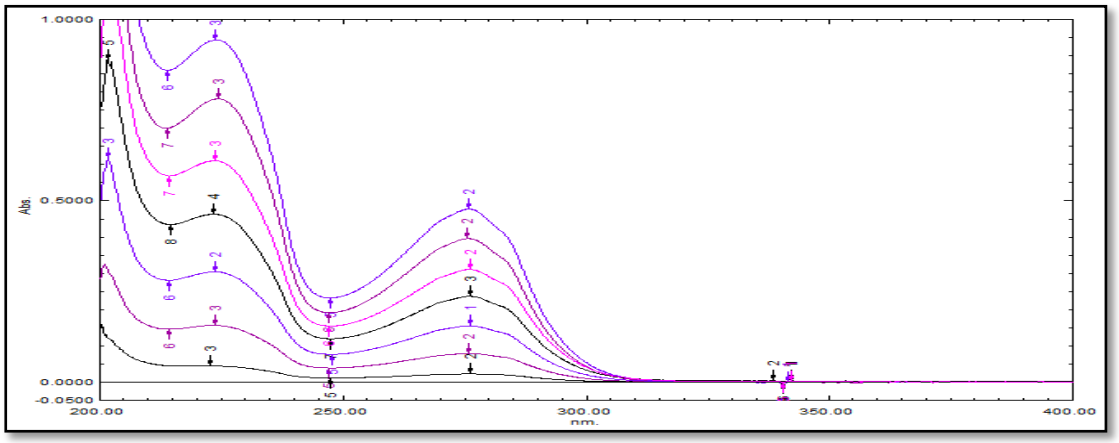


Fig. No. 5.7.3: Overlain UV spectra of ALO (0.5- 12 µg/ml)

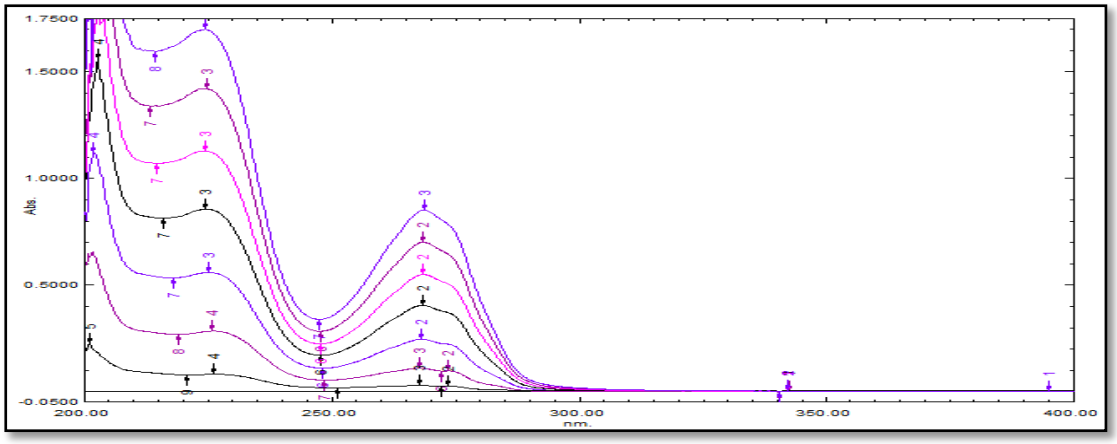


Figure No. 5.7.4: Overlain UV spectra of PIO (1.8-43.2 µg/ml)

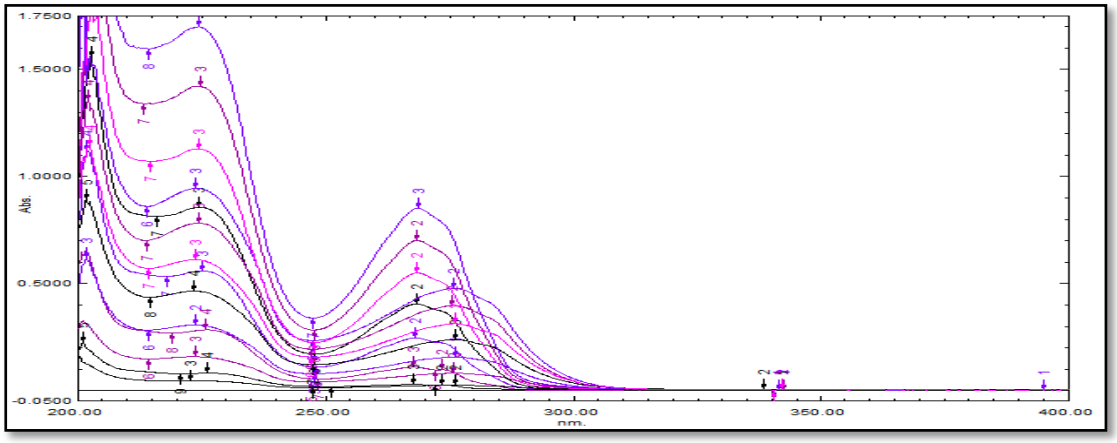


Figure No. 5.7.5:Overlain UV spectra of ALO (0.5- 12 µg/ml) & PIO(1.8-43.2 µg/ml)

Calibration graphs were plotted using absorbances of standard drug solutions versus concentration **(Figure No. 5.7.6-5.7.9).** Results were subjected to regression analysis by the least squares method to calculate the values of slope, intercept and correlation coefficient at 224 and 279 nm and are given in **Table No. 5.7.8**

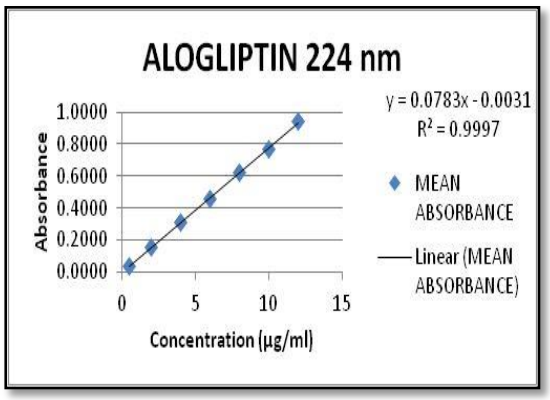


Figure No. 5.7.6: Calibration graph of ALO at 224 nm

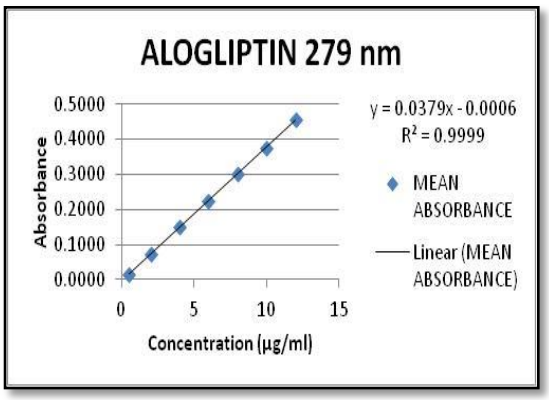


Figure No. 5.7.7: Calibration graph of ALO at 279 nm

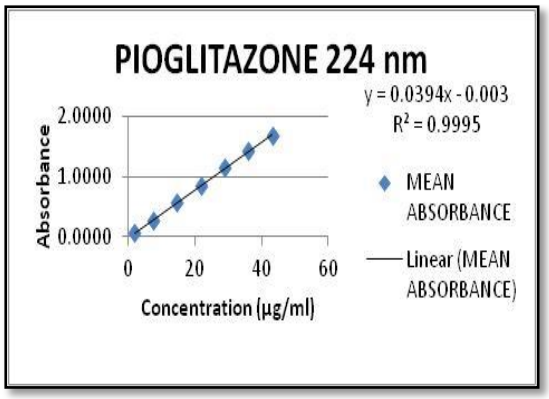


Figure No. 5.7.8: Calibration graph of PIO at 224 nm

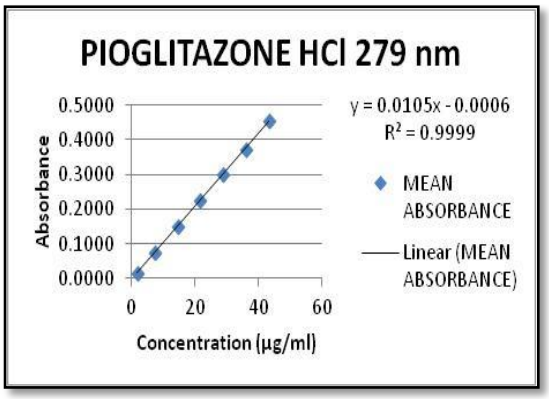


Figure No. 5.7.9: Calibration graph of PIO at 279 nm

5.7.7 Precision

Precision studies were performed and % RSD was calculated which is in accordance with ICH guideline acceptable limits, which indicates good repeatability and low inter-day variability (Table **No. 5.7.3, 5.7.4 & 5.7.5.).**

Table No. 5.7.3: Results of repeatability of measurement

Repeatability					
Sr. No.	**Conc.**	**Absorbance**			
		ALO		**PIO**	
		224 nm	**279 nm**	**224 nm**	**279 nm**
1	ALO 4 µg/ml & PIO 14.4 µg/ml	0.3125	0.1543	0.5563	0.1498
2		0.3151	0.1512	0.5534	0.1537
3		0.3128	0.1512	0.5543	0.1534
4		0.3127	0.1508	0.5581	0.1543
5		0.3126	0.1515	0.5538	0.1512
6		0.3128	0.1498	0.5546	0.1512
Mean±SD*		**0.3131±0.0010**	**0.1515±0.0015**	**0.5551±0.0018**	**0.1523±0.0018**
% RSD		**0.3177**	**0.9966**	**0.3213**	**1.1687**
1	ALO 6 µg/ml & PIO 21.6 µg/ml	0.4654	0.2271	0.8654	0.2265
2		0.4646	0.2268	0.8548	0.2269
3		0.4658	0.2278	0.8631	0.2275
4		0.4638	0.2265	0.855	0.2276
5		0.4644	0.2259	0.8654	0.2273
6		0.4589	0.2268	0.8546	0.2278
Mean±SD*		**0.4638±0.0025**	**0.2268±0.0006**	**0.8597±0.0055**	**0.2273±0.0005**
% RSD		**0.5417**	**0.2780**	**0.6342**	**0.2132**
Mean % RSD		**0.4297**	**0.6373**	**0.4778**	**0.6909**

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

Table No. 5.7.4: Results of intra-day precision

Intra-day precision					
Sr. No.	**Conc.**	**Absorbance**			
		ALO		**PIO**	
		224 nm	**279 nm**	**224 nm**	**279 nm**
1	ALO 4 µg/ml & PIO 14.4µg/ml	0.3125	0.1498	0.5634	0.1543
2		0.3258	0.1543	0.5628	0.1512
3		0.3148	0.1512	0.5628	0.1512
4		0.3128	0.1543	0.5563	0.1532
5		0.3152	0.1549	0.5534	0.1549
6		0.3118	0.1569	0.5543	0.1524
7		0.3154	0.1543	0.5684	0.1522
8		0.3149	0.1546	0.5661	0.1508
9		0.3129	0.1544	0.5648	0.1515
Mean±SD*		**0.3151±0.0042**	**0.1539±0.0021**	**0.5614±0.0054**	**0.1524±0.0014**
% RSD		**1.3393**	**1.3653**	**0.9572**	**0.9505**
1	ALO 6µg/ml & PIO 21.6µg/ml	0.4658	0.2273	0.8554	0.2273
2		0.4644	0.2269	0.855	0.2265
3		0.4589	0.2276	0.8654	0.2274
4		0.4578	0.2269	0.8546	0.2259
5		0.4655	0.2276	0.8564	0.2265
6		0.4628	0.2278	0.8547	0.2267
7		0.4638	0.2265	0.8564	0.2278
8		0.4618	0.2259	0.8551	0.2265
9		**0.4589**	**0.2265**	**0.8624**	**0.2259**
Mean±SD*		**0.4622±0.0030**	**0.2270±0.0006**	**0.8573±0.0039**	**0.2267±0.0007**
% RSD		**0.6532**	**0.2777**	**0.4537**	**0.2899**
Mean % RSD		**0.9962**	**0.8215**	**0.7054**	**0.6202**

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

Table No. 5.7.5: Results of inter-day precision

Inter-day precision						
Sr. No.		**Conc.**	**Absorbance**			
			ALO		**PIO**	
			224 nm	**279 nm**	**224 nm**	**279 nm**
1	1	ALO 4 µg/ml & PIO 14.4µg/ml	0.3158	0.1523	0.5634	0.1543
2			0.3125	0.1522	0.5614	0.1512
3			0.3265	0.1549	0.5562	0.1512
4	2		0.3175	0.1539	0.5478	0.1498
5			0.3162	0.1549	0.5638	0.1537
6			0.3228	0.1532	0.5563	0.1534
7	3		0.3122	0.1498	0.5534	0.1512
8			0.3118	0.1543	0.5543	0.1512
9			0.3158	0.1512	0.5684	0.1532
Mean±SD*			**0.3168±0.0050**	**0.1530±0.0017**	**0.5583±0.0064**	**0.1521±0.0015**
% RSD			**1.5688**	**1.1408**	**1.1443**	**1.0082**
1	1	ALO 6µg/ml & PIO 21.6µg/ml	0.4684	0.2265	0.8646	0.2277
2			0.4781	0.2267	0.8741	0.2263
3			0.4635	0.2276	0.8562	0.2255
4	2		0.4638	0.2274	0.8652	0.2273
5			0.4635	0.2273	0.8654	0.2265
6			0.4695	0.2273	0.8546	0.2274
7	3		0.4744	0.2269	0.8547	0.2274
8			0.4589	0.2278	0.8546	0.2266
9			0.4678	0.2264	0.8621	0.2269
Mean±SD*			**0.4675±0.0060**	**0.2271±0.0005**	**0.8613±0.0068**	**0.2268±0.0007**
% RSD			**1.2755**	**0.2180**	**0.7845**	**0.3055**
Mean % RSD			**1.4221**	**0.6794**	**0.9644**	**0.6568**

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

5.7.8 Accuracy

The method showed good accuracy as the results of recovery studies ranged from **98-102%** for both the drugs **(Table No. 5.7.6)**

Table No. 5.7.6: Results of recovery studies

Accuracy (% Recovery)				
ALO				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	1	0.5	0.5031	100.6180
50	2	1	0.9789	97.8872
50	4	2	1.9401	97.0059
100	1	1	0.9712	97.1181
100	2	2	1.9825	99.1263
100	4	4	4.0189	100.4726
150	1	1.5	1.5167	101.1101
150	2	3	2.9731	99.1045
150	4	6	6.1208	102.0141
Mean±SD*				**99.3841±1.7951**
% RSD				**1.8062**
PIO				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	1.8	0.9	0.9078	100.8625
50	3.6	1.8	1.7709	98.3811
50	7.2	3.6	3.6247	100.6859
100	1.8	1.8	1.7975	99.8595
100	3.6	3.6	3.6482	101.3381
100	7.2	7.2	7.1267	98.9821
150	1.8	2.7	2.7626	102.3178
150	3.6	5.4	5.4025	100.0471
150	7.2	10.8	10.9786	101.6533
Mean±SD*				**100.4586±1.2697**
% RSD				**1.2639**

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

5.7.9 LOD and LOQ

The developed method found to be sensitive as the values of LOD and LOQ were found to be very low which is shown in **Table No. 5.7.8.**

5.7.10 Robustness

Robustness study was carried out for proposed method, but no significant changes (% RSD<2) found in absorption which shows the method is robust (**Table No. 5.7.7).**

Table No. 5.7.7: Results of robustness study

Parameter	**Drugs**			
Wavelengths	**ALO**		**PIO**	
(224 & 279 ±1 nm)	**Assay (%)***	**% RSD**	**Assay (%)***	**% RSD**
223 & 278 nm	99.5432	0.8462	100.5432	1.6147
224& 279 nm	101.2354		101.3467	
225 & 280 nm	100.2657		98.2349	

5.7.11 Stability of the solution

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

Table No. 5.7.8: Summary of validation parameters for the proposed method

Parameters	**ALO**		**PIO**	
Detection wavelengths (nm)	224	279	224	279
Linearity range (µg/ml)	0.5-12		1.8-43.2	
Correlation coefficient	0.9997	0.9999	0.9995	0.9999
Regression equation	y = 0.0783x - 0.0031	y = 0.0379x - 0.0006	y = 0.0394x - 0.003	y = 0.0105x - 0.0006
Precision (%RSD) Intra-day (n=3) Inter-day (n=3) Repeatability of measurement (n=6)	 0.9962 1.4221 0.4297	 0.8215 0.6794 0.6373	 0.7054 0.9644 0.4778	 0.6202 0.6568 0.6909
Accuracy % Recovery (n=3) %RSD (n=3)	 99.38±1.7951 1.8062		 100.46±1.2697 1.2639	
Specificity	No interference			
LOD (µg/ml)	0.0539	0.0590	0.0702	0.2125
LOQ (µg/ml)	0.1634	0.1788	0.2127	0.6440

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

5.7.12 Analysis of formulation

The proposed method was successfully used for the quantitative determination of ALO& PIO and in tablet formulation (12.5 mg ALO and 45 mg of PIO). Six replicate determinations were carried out and average experimental values were found to be 99.8070 and 99.4534 %w/w for ALO and PIO, respectively and hence the developed can be used for the simultaneous estimation of drugs in tablet dosage form. **Table No. 5.7.9.** Overlain spectra of standard drugs and formulation are shown in **Figure No. 5.7.10**.

Table No. 5.7.9: Results of formulation analysis

Drugs	Amount (mg/tablet)		% Drug found*	% RSD
	Labelled	Found		
ALO	12.5	12.48	99.8070±1.2544	1.2568
PIO	45	44.75	99.4534±1.5852	1.5939

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

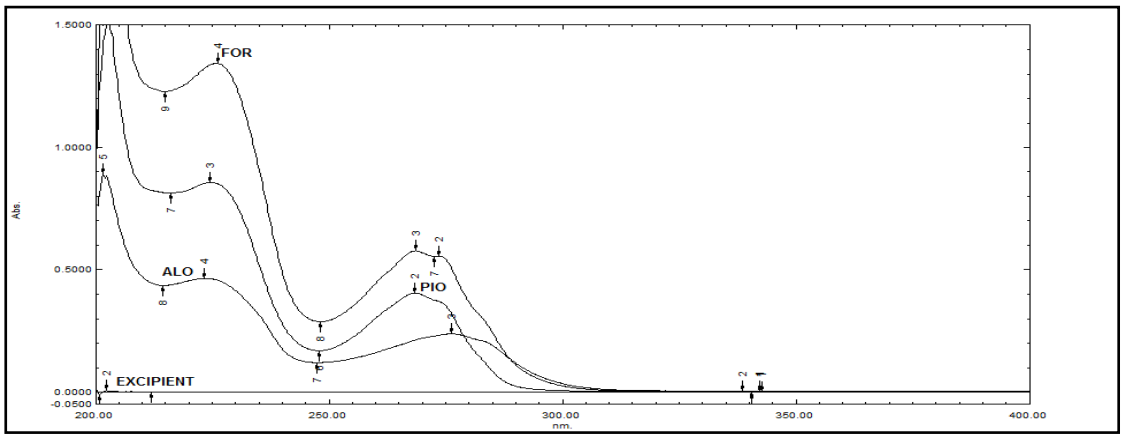


Figure No. 5.7.10: Overlain UV spectra of standard ALO (6 µg/ml), PIO (21.6 µg/ml) & formulation (6 & 21.6 µg/ml)

Method 8

5.8. "Development and validation of RP-HPLC method for the simultaneous determination of alogliptin benzoate and pioglitazone hydrochloride in tablets"

5.8.1 Selection of mode of chromatographic method:

For most regular samples, reversed phase chromatography is the first choice for analysis. RPC is most convenient and rugged and produces more satisfactory results compared to other form of liquid chromatography. Substances under investigation are polar in nature and hence reverse phase chromatographic method was selected.

5.8.2 Selection of wavelength

UV spectra of both the drugs were taken in RP-HPLC system and 267 nm was selected as wavelength (**Figure No. 5.8.1).**

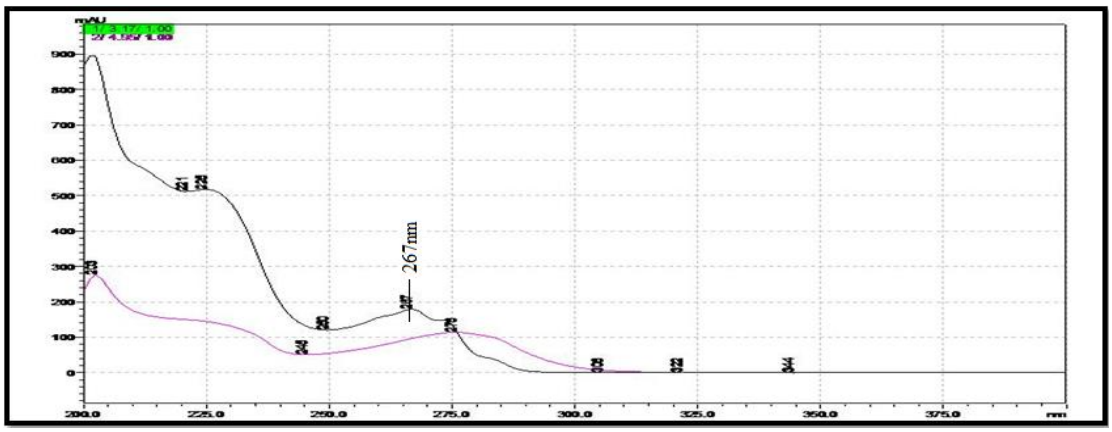


Figure No. 5.8.1: Overlain standard spectra of ALO & PIO at 267 nm

5.8.3 Selection of mobile phase:

Various initial trials were performed to establish basic analytical requirements of the method like type of organic solvent, type of buffer, pH etc. Trials were also done based on the literature survey and suitable mobile phase was selected for further studies **(Table No. 5.8.1 & Figure No. 5.8.2-5.8.13).**

Table No. 5.8.1: Selection of mobile phase

Sr. No.	Mobile Phase	Observation	Remarks	Fig. No.
1	Sodium phosphate (pH 3): Acetronitrile (60:40%v/v)	Alogliptin gave very bad peak	Not satisfactory	Fig. 5.8.2
2	Acetonitrile: water: 0.01% ortho phosphoricacid (45:55:0.01%v/v/v)	No separation between ALO &BA	Not satisfactory	Fig. 5.8.3
3	20 mM Phosphate buffer (pH 4.6): Methanol (50:50% v/v)	Alogliptin peak showed tailing	Not satisfactory	Fig. 5.8.4
4	20 mM Phosphate buffer (pH 4.6): Methanol (25:75 %v/v)	Alogliptin peak showed tailing	Not satisfactory	Fig. 5.8.5
5	20 mM Phosphate buffer (pH 4.6): Acetonitrile (20:80 %v/v)	Alogliptin peak showed splitting	Not satisfactory	Fig. 5.8.6
6	0.2% TEA (pH 3): Acetonitrile (50:50 %v/v)	Alogliptin gave very bad peak	Not satisfactory	Fig. 5.8.7
7	0.1% TEA (pH 3): Methanol (50:50%v/v)	Showed improvement in peak separation	Not satisfactory	Fig. 5.8.8
8	0.2% TEA (pH 3): Methanol (30:70 %v/v)	Good separation of peaks with tailing	Not satisfactory	Fig. 5.8.9
9	0.2% TEA (pH 5): Methanol (10:90 %v/v)	Good separation of peaks with tailing	Not satisfactory	Fig. 5.8.10
10	0.2% TEA (pH 5): Methanol (5:95 %v/v)	Peak of pioglitazone was not symmmetrical	Not satisfactory	Fig. 5.8.11
11	0.2% TEA (pH 5.5): Methanol (5:95 %v/v)	Peak of pioglitazone was not symmmetrical	Not satisfactory	Fig. 5.8.12
12	0.2% triethylamine (pH 5.5 was adjusted with orthophosphoric acid) :methanol (2:98%v/v)	Optimum peak parameters	Satisfactory	Fig. 5.8.13

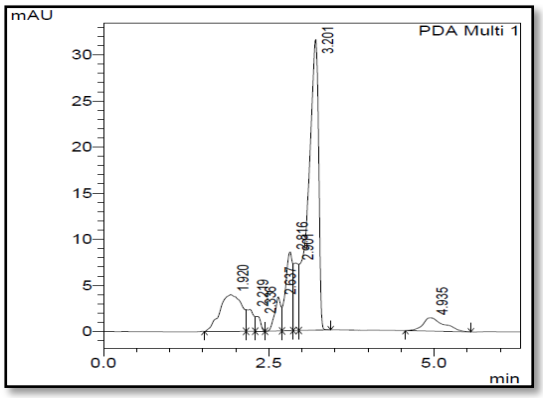


Figure No. 5.8.2: Sodium phosphate (pH 3): Acetonitrile (60:40 %v/v)

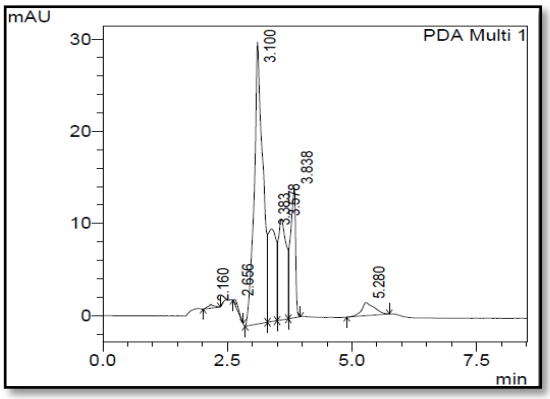


Figure No. 5.8.3: Acetonitrile: water: 0.01% ortho phosphoric acid (45:55:0.01%v/v)

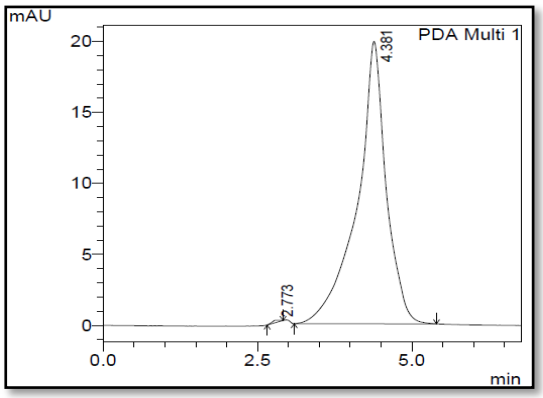


Figure No. 5.8.4: 20 mM Phosphate buffer (pH 4.6): Methanol (50:50 %v/v)

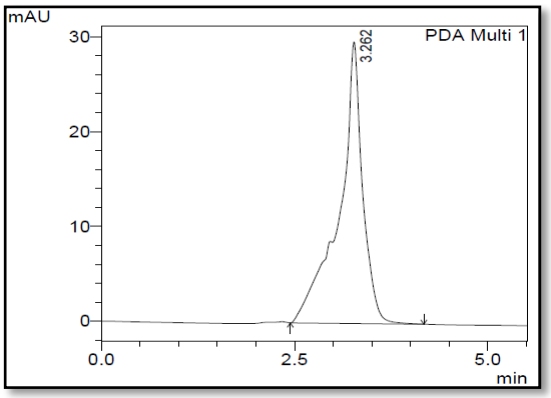


Figure No. 5.8.5: 20 mM Phosphate buffer (pH 4.6): Methanol (25:75 %v/v)

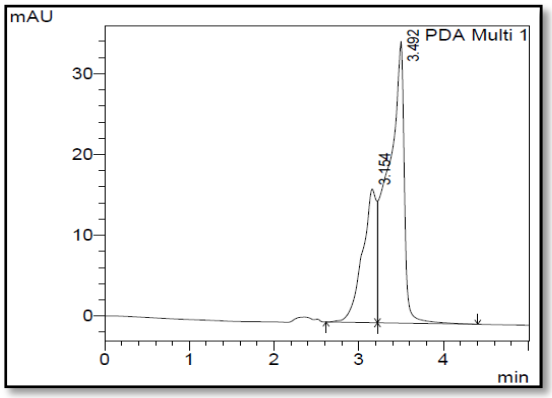


Fig. No. 5.8.6: 20 mM Phosphate buffer (pH 4.6): Acetonitrile (20:80 %v/v)

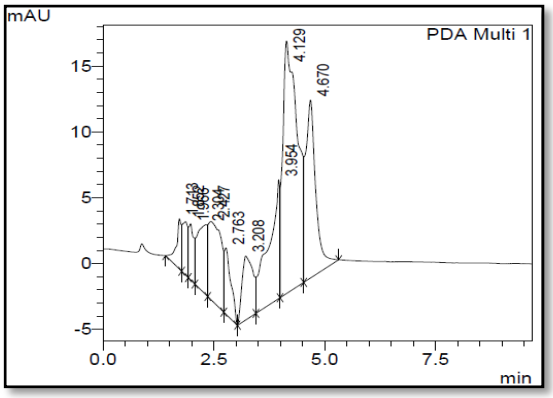


Fig. No. 5. 8.7: 0.2% TEA (pH 3): Acetonitrile (50:50 %v/v)

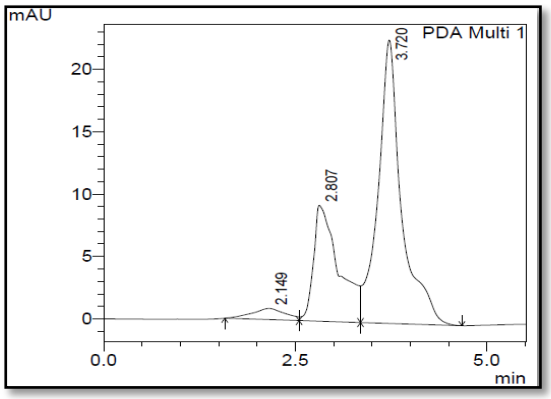


Fig. No. 5.8.8: 0.1% TEA (pH 3): Methanol (50:50 %v/v)

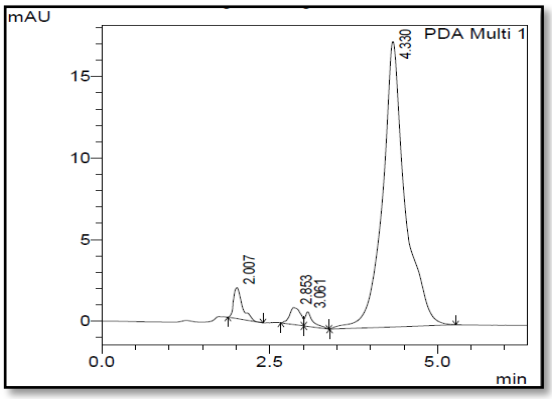


Fig. No. 5.8.9: 0.2% TEA (pH 3): Methanol (30:70 %v/v)

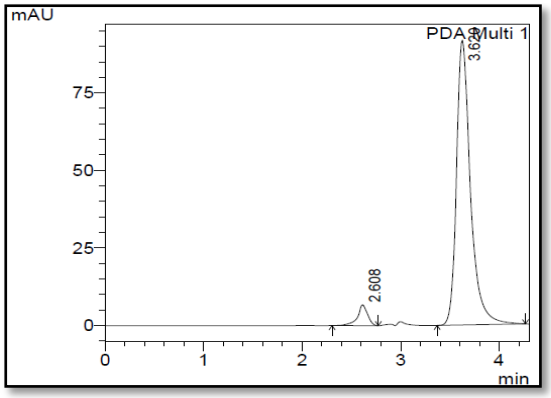


Figure No. 5.8.10: 0.2% TEA (pH 5): Methanol (10:90 %v/v)

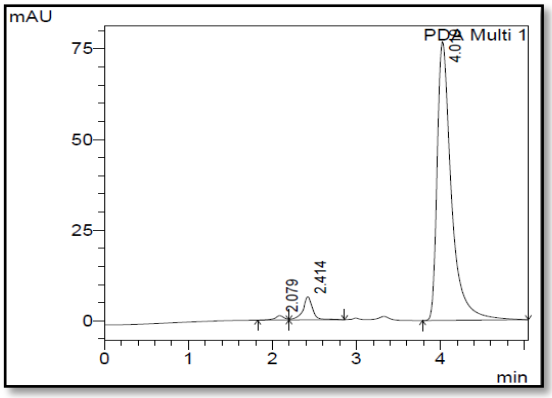


Figure No. 5.8.11:0.2% TEA (pH 5):Methanol (5:95 %v/v)

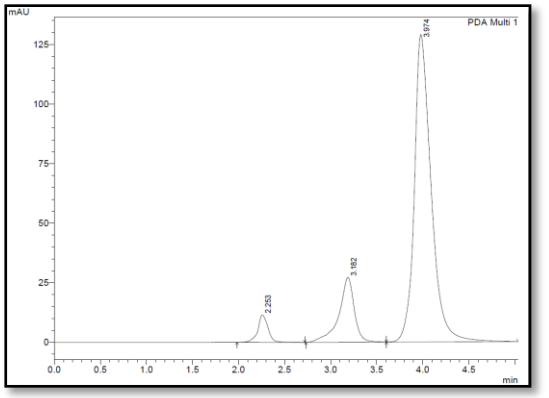


Figure No. 5.8.12: 0.2% TEA (pH 5.5): Methanol (5:95 %v/v)

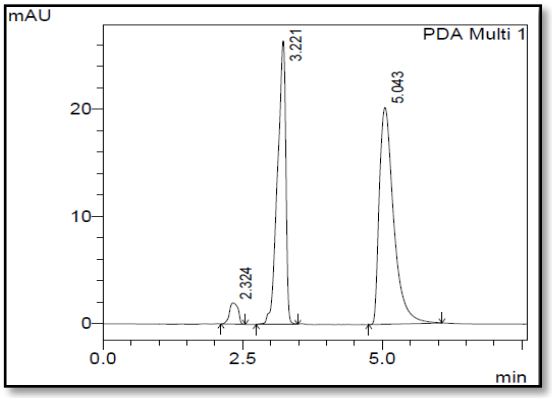


Figure No. 5.8.13: 0.2% TEA (pH 5.5 was adjusted with orthophosphoric acid) and methanol (2:98%v/v)

Initially various solvents like methanol, acetonitrile and buffers were tried as mobile phase for separation of ALO, PIO & BA. But desired separation could not be achieved. Different chromatographic conditions like detection wave length, strength of buffer, flow rate, mobile phase composition, pH, etc. were varied to get optimum chromatographic conditions. Enable C_{18} column with a mobile phase 0.2% triethylamine (pH 5.5 was adjusted with orthophosphoric acid) and methanol (2:98 %v/v) at a flow rate of 1 ml/min was employed for the separation of components and PDA detection at 267 nm was employed.

5.8.4 Fixed chromatographic condition

Stationary phase	:	Enable C_{18} column (250x 4.6 mm, 5 µm, 120 Å)
Mobile phase	:	0.2%v/v triethylamine (pH 5.5 with orthophosphoric acid) and methanol
Solvent ratio	:	2: 98%v/v
pH	:	5.5
Detection wavelength	:	267 nm
Flow rate	:	1 ml/ minute
Operating pressure	:	88 kgf
Temperature	:	Room temperature

The retention time of ALO, PIO&BA were found to be 4.9291±0.0337, 3.1759±0.0058 min& 2.248±0.0024 respectively, are shown in **Figure No. 5.8.14**

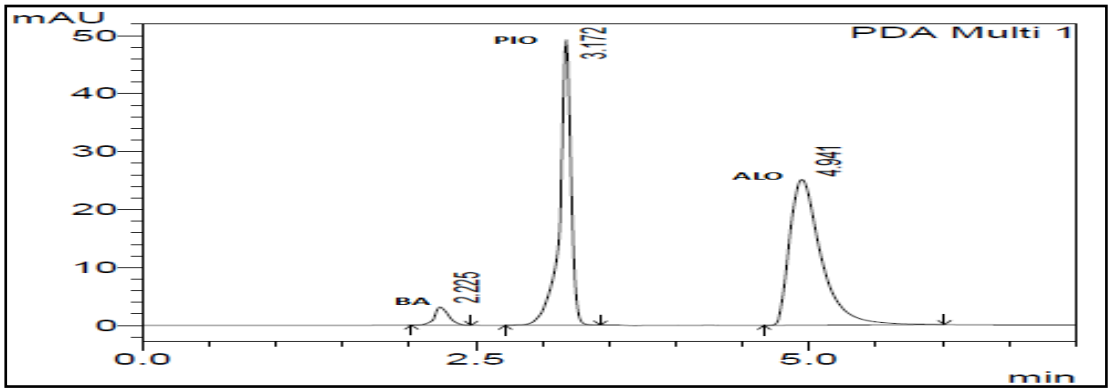


Figure No. 5.8.14: RP-HPLC chromatogram of ALO (10 µg/ml) and PIO (18 µg/ml)

5.8.5 Validation of chromatographic method

Developed method was validated according to ICH guidelines using following parameters

5.8.6 Specificity

The method was found to be specific as no interfering peaks were found within the stipulated run time **(Figure No. 5.8.17).**

5.8.7 Linearity and range

Calibration graphs were plotted using peak areas of standard drugs versus concentration for establishing linearity and range of the method. ALO and PIO were found to be linear in the concentration range 0.50-50 µg/ml and 0.90-90 µg/ml, respectively **(Figure No. 5.8.18-5.8.28 & Table. No. 5.8.2).** Results were subjected to regression analysis by the least squares method to calculate values of slope, intercept and correlation coefficient are shown in **Figure No. 5.8.15-5.8.16.**

Table 5.8.2: Linearity data of ALO & PIO

Sr. No.	ALO			PIO		
	Conc. (µg/ml)	Peak Area*	% RSD	Conc. (µg/ml)	Peak Area*	% RSD
1	0.5	21981.17	1.1687	0.9	14904.00	1.1774
2	1	41857.00	0.6290	1.8	29859.33	0.9378
3	10	416797.33	1.2471	18	295112.50	0.7215
4	20	847656.00	0.7571	36	586018.67	0.8316
5	30	1246609.67	0.7699	54	873878.67	0.6841
6	40	1652797.17	0.5551	72	1143306.50	1.0644
7	50	2024580.33	1.1083	90	1418226.00	0.7760

*(n=6) Avg. of six determination

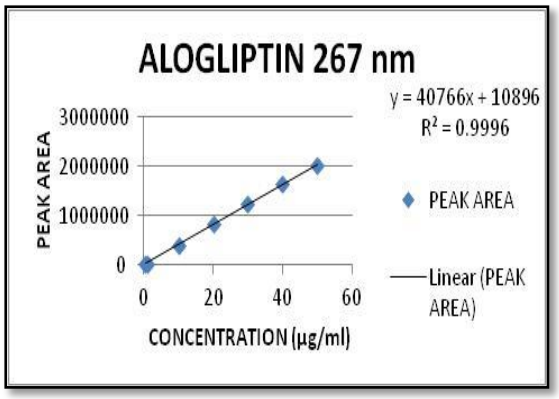


Figure No. 5.8.15: Calibration graph of ALO at 267 nm

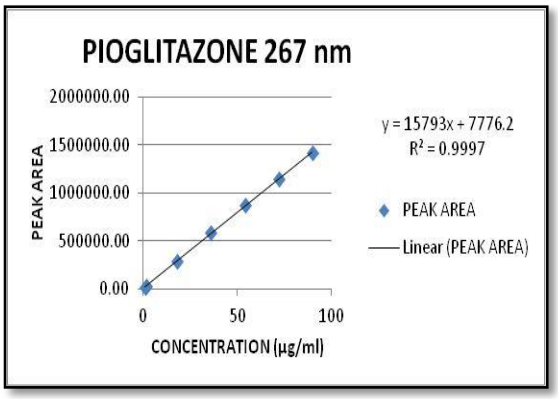


Figure No. 5.8.16: Calibration graph of PIO at 267 nm

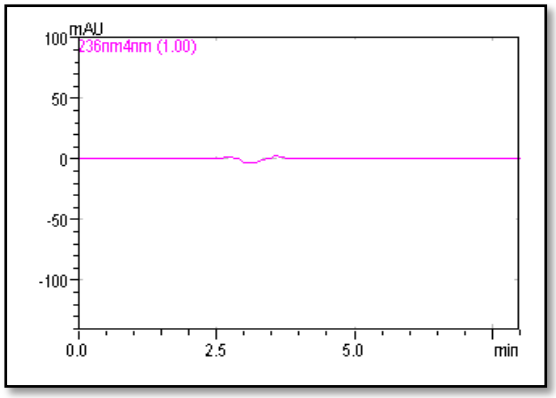


Fig. No. 5.8.17: Chromatogram of blank(methanol)

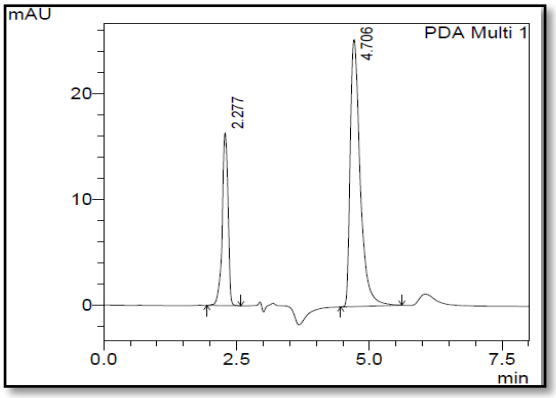


Fig. No. 5.8.18: Chromatogram of ALO (10 µg/ml)

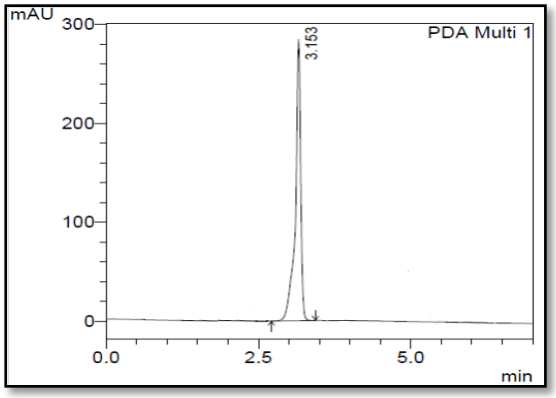


Fig. No. 5.8.19: Chromatogram of PIO (18 µg/ml)

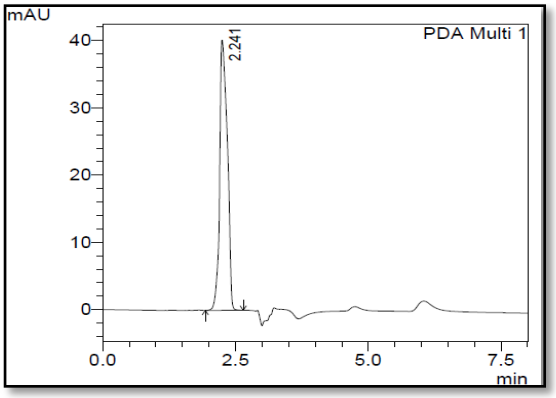


Fig. No. 5.8.20: Chromatogram of BA (10 µg/ml)

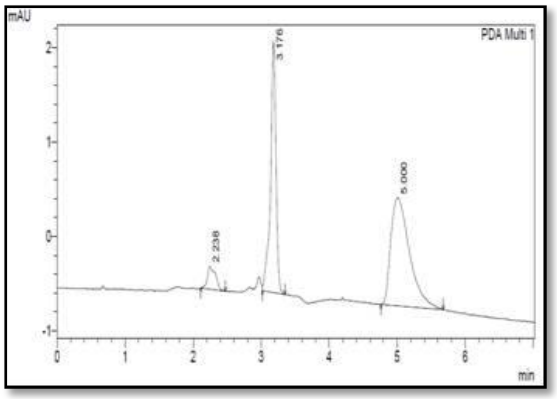


Fig. No. 5.8.21: Chromatogram of ALO (0.5 µg/ml) & PIO (0.9 µg/ml)

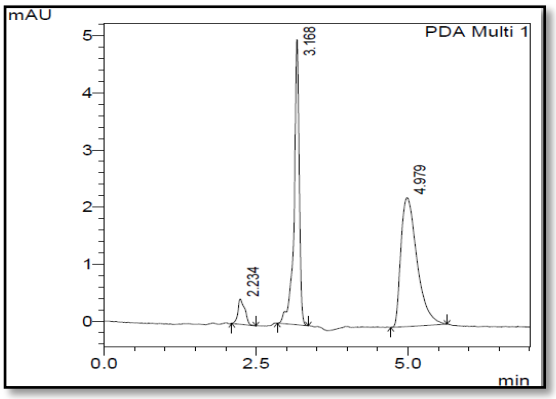


Fig. No. 5. 8.22: Chromatogram of ALO (1 µg/ml) & PIO (1.8 µg/ml)

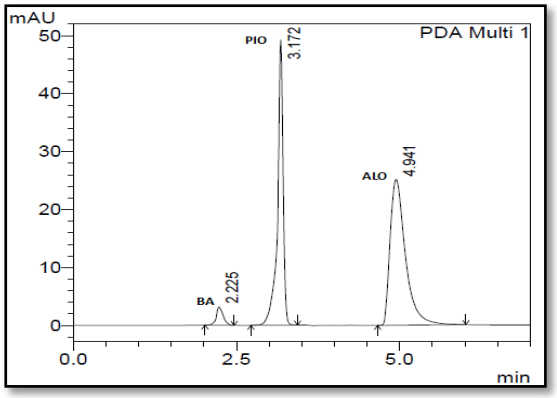


Fig. No. 5. 8.23: Chromatogram of ALO (10 µg/ml) & PIO (18 µg/ml)

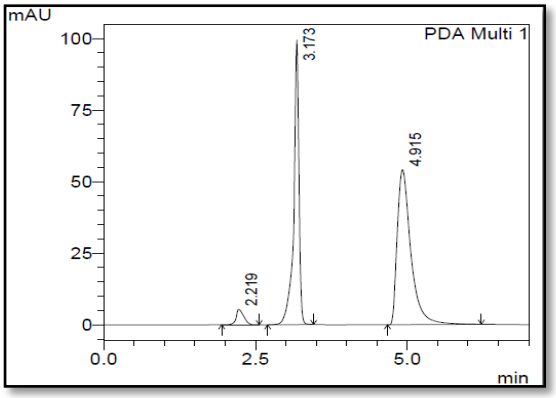


Fig. No. 5. 8.24: Chromatogram of ALO (20µg/ml) & PIO (36 µg/ml)

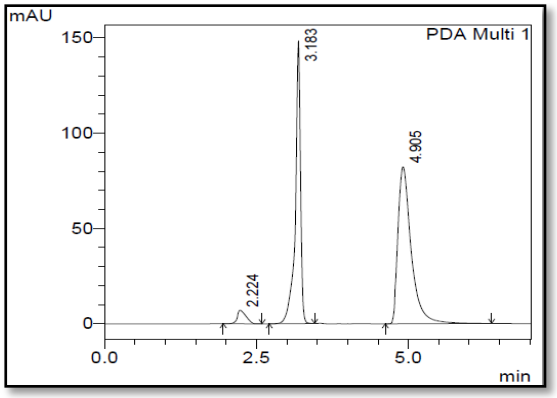


Fig. No. 5.8.25: Chromatogram of ALO (30 µg/ml) & PIO (54 µg/ml)

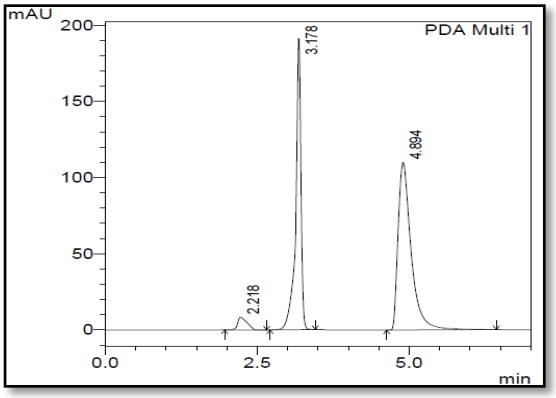


Fig. No. 5.8.26: Chromatogram of ALO (40 µg/ml) & PIO (72 µg/ml)

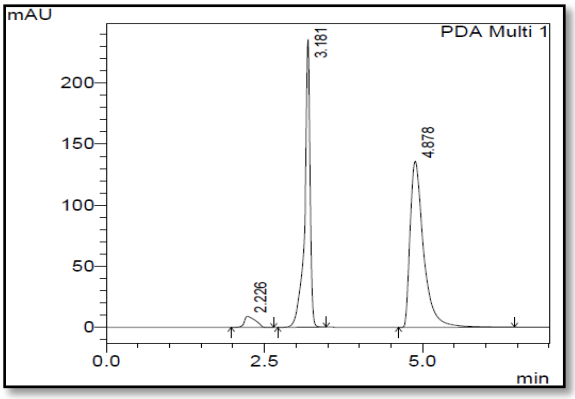


Fig. No. 5. 8.27: Chromatogram of ALO (50 µg/ml) & PIO (90µg/ml)

5.8.4 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, which indicates good repeatability and low inter-day variability (**Table No. 5.8.3, 5.8.4 & 5.8.5.).**

Table No. 5.8.3: Results of repeatability of measurement

Repeatability								
	ALO				PIO			
Sr. No.	Conc. (µg/ml)	Peak area	Mean ± SD	% RSD	Conc. (µg/ml)	Peak area	Mean ± SD	% RSD
1	ALO 5 µg/ml	216878	216372.67 ± 1111.7253	0.5138	PIO 9 µg/ml	150454	150669.67 ± 1255.3319	0.8332
2		214658				148698		
3		215455				151246		
4		217545				152464		
5		216455				151012		
6		217245				150144		
1	ALO 10 µg/ml	418645	417319.33 ± 1222.7616	0.2930	PIO 18 µg/ml	294787	295469.50 ± 2239.4238	0.7579
2		418755				296788		
3		415687				295467		
4		416532				297845		
5		417545				296485		
6		416752				291445		
Mean % RSD				**0.4034**				**0.7955**

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

Table No. 5.8.4: Results of intra-day precision

Intra-day precision								
Sr. No.	**ALO**				**PIO**			
	Conc. (µg/ml)	**Peak area**	**Mean ± SD**	**% RSD**	**Conc. (µg/ml)**	**Peak area**	**Mean ± SD**	**% RSD**
1	ALO 5 µg/ml	215465	215308.3 ± 1077.545	0.5005	PIO 9µg/ml	149688	147848.3± 2116.124	1.4313
2		216455				148697		
3		213455				147658		
4		215655				145789		
5		216445				148545		
6		214556				151545		
7		214545				148145		
8		216544				145445		
9		214655				145123		
1	ALO 10 µg/ml	416544	416396.6± 1730.105	0.4155	PIO 18 µg/ml	293478	295045.4± 1389.939	0.4711
2		414555				295458		
3		417544				293644		
4		418755				296785		
5		418455				294565		
6		416555				296785		
7		415252				294785		
8		413554				293455		
9		416355				296454		
Mean % RSD				**0.4580**				**0.9512**

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

Table No. 5.8.5: Results of inter-day precision

Inter-day precision									
Sr. No.		**ALO**				**PIO**			
		Conc. (µg/ml)	**Peak area**	**Mean ± SD**	**% RSD**	**Conc. (µg/ml)**	**Peak area**	**Mean ± SD**	**% RSD**
1	1	ALO 5 µg/ml	216544	215676.1 ± 1654.742	0.7672	PIO 9 µg/ml	150144	150844.8 ± 1705.172	1.1304
2			214544				149658		
3			216325				148655		
4	2		213556				152455		
5			217544				153455		
6			216555				152455		
7	3		214574				151655		
8			213545				150122		
9			217898				149004		
1	1	ALO 10 µg/ml	418255	415645.4 ± 1859.621	0.4474	PIO 18 µg/ml	296455	296189.3 ± 3328.818	1.1239
2			417874				298788		
3			415327				301244		
4	2		416547				295458		
5			412487				293455		
6			413879				296458		
7	3		416347				297845		
8			414468				296544		
9			415625				289457		
Mean % RSD					**0.6073**				**1.1271**

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

5.8.5 Accuracy

The results of recovery studies ranged from 98-100% for both the drugs showing the accuracy of the method **(Table No. 5.8.6).** The results also reveal that there are no interference from tablet excipients.

Table No. 5.8.6: Results of recovery studies

Accuracy (% Recovery)				
ALO				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	5	2.5	2.5061	100.2424
50	10	5	5.0778	101.5557
50	15	7.5	7.6829	102.4390
100	5	5	4.9261	98.5228
100	10	10	9.7361	97.3605
100	15	15	15.0199	100.1328
150	5	7.5	7.6008	101.3446
150	10	15	14.7917	98.6114
150	15	22.5	22.8730	101.6576
Mean± SD*				**100.2074±1.7208**
% RSD				**1.7173**
PIO				
Recovery level (%)	**Initial conc. of formulation (µg/ml)**	**Standard added (µg/ml)**	**Recovered (µg/ml)**	**% Recovered**
50	9	4.5	4.4540	98.9775
50	18	9	9.0956	101.0622
50	27	13.5	13.4744	99.8107
100	9	9	8.8240	98.0447
100	18	18	18.2806	101.5590
100	27	27	26.5824	98.4533
150	9	13.5	13.5896	100.6638
150	18	27	27.3101	101.1486
150	27	40.5	40.0883	98.9833
Mean± SD*				**99.8559±1.2972**
% RSD				**1.0461**

5.8.6 LOD and LOQ

The values of LOD and LOQ were found to be 0.1410 and 0.4274 µg/ml for ALO, 0.2439 and 0.7392 µg/ml for MET, respectively **(Table No. 5.8.8)**.

5.8.7 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. 5.8.7).**

Table No. 5.8.7: Results robustness study

Sr. No.	Modification	ALO		PIO	
		R_t	Peak area	R_t	Peak area
1	Strength of buffer (0.2 ± 0.1%v/v)	4.986	846789	3.1345	584678
		4.878	867899	3.1346	576896
		4.865	856789	3.1456	587654
% RSD* (<2)		**1.3530**	**1.2320**	**0.2033**	**0.9527**
2	Effect of pH (5.5 ± 0.2 unit)	4.879	846789	3.123	587654
		4.976	835679	3.234	578976
		4.867	847896	3.194	576896
% RSD* (<2)		**1.2180**	**0.8011**	**1.7658**	**0.9818**
3	Effect of flow rate (1 ± 0.1ml/min)	4.9768	846789	3.234	578976
		4.8768	857898	3.134	587654
		4.9877	845678	3.147	587645
% RSD* (<2)		**1.2356**	**0.7949**	**1.7143**	**0.8564**

**%RSD of three observation*

5.8.8 Stability of the solution

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

Table No. 5.8.8: Summary of validation parameters for the proposed RP-HPLC method

Parameters	ALO	PIO
Linearity range (µg/ml)	0.50-50	0.90-90
Correlation coefficient	0.9996	0.9997
Regression equation	y = 40766x + 10896	y = 15793x + 7776.2
Precision (%RSD) Intra-day (n=3) Inter-day (n=3) Repeatability of injection (n=06)	 0.4580 0.6073 0.4034	 0.9512 1.1271 0.7955
Accuracy* % Recovery (n=3) %RSD (n=3)	 100.2074±1.7208 1.7173	 99.8559±1.2972 1.3725
Specificity	No interference	
LOD (µg/ml)	0.1410	0.2439
LOQ (µg/ml)	0.4274	0.7392

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

5.8.9 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 the proposed method is suitable for the analysis intended. **Table No. 5.8.9.**

Table No. 5.8.9: Results of system suitability studies

Parameters	Values		Acceptance criteria
	ALO*	PIO*	
Retention time (R_t) %RSD	4.92±0.0337	3.17±0.0058	RSD ≤ 2 %
Peak area reproducibility %RSD	209280±1182.35 0.5650	81995.1±495.14 0.6039	<2
Theoretical plates (N)	2654	6663	N>2000
Tailing factor (T)	1.77	0.76	T<2
Resolution (R_S)	6.53	4.37	R_s>2

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

5.8.10 Analysis of formulation

The proposed method was successfully used for the quantitative determination of ALO and PIO in tablet formulation (25 mg ALO and 45 mg of PIO). Six replicate determinations were carried out and average experimental values were found to be 99.6987 and 98.6219 %w/w for ALO and PIO, respectively hence the developed

method can be used for the simultaneous estimation of drugs in tablet dosage form. **Table No. 5.8.10.** Chromatogram of standard drugs and formulation are shown in **Figure No. 5.8.28**.

Table No. 5.8.10: Results of formulation analysis

Drugs	Amount (mg/tablet)		% Drug found*	% RSD
	Labelled	Found		
ALO	25	24.92	99.6987±0.8946	0.8973
	45	44.38	98.6219±0.6561	0.6653

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

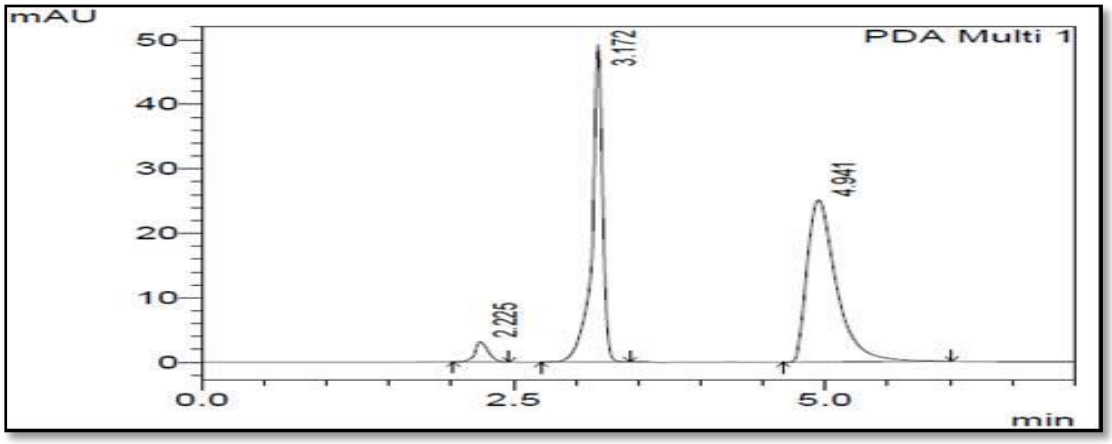


Figure No. 5.8.28: Chromatogram of formulation ALO (50 µg/ml) & PIO (90 µg/ml)

Method 9

5.9 "Development and validation of HPTLC method for the simultaneous determination of alogliptin benzoate and pioglitazone hydrochloride in tablets"

5.9.1 Selection of mode of chromatographic method:

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

5.9.2 Selection of solvent

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

5.9.3 Selection of wavelength

UV spectra of both the drugs on pre-coated plate were recorded and 274 nm was selected as wavelength of detection. **Figure No. 5.9.1**

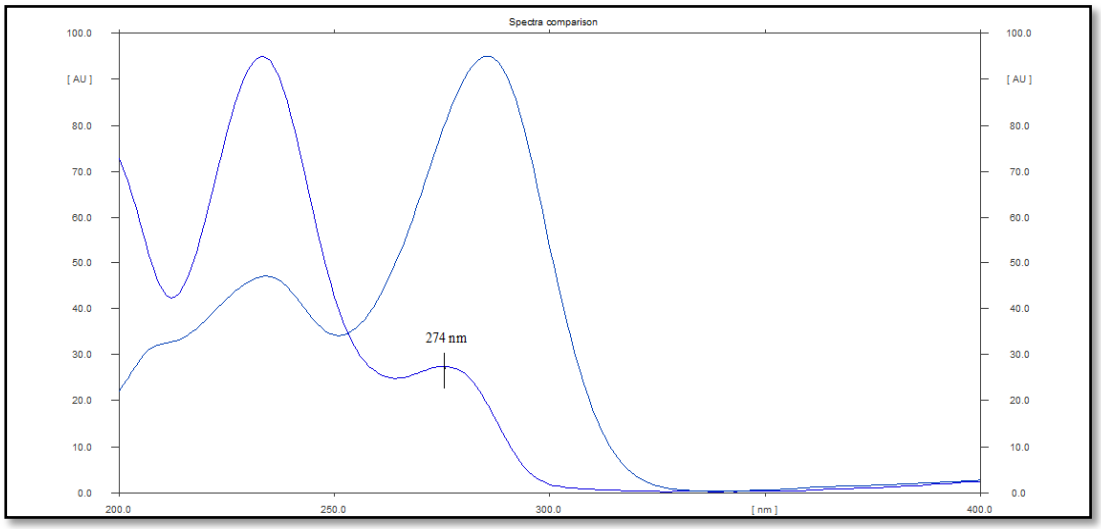


Figure No. 5.9.1: Overlain UV Spectra of ALO &PIO in HPTLC at 274 nm

5.9.4 Development of optimum mobile phase

A solvent system that gave dense compact spots, good separation between ALO, PIO and BA (Benzoic acid: Which is separating from alogliptin benzoate) and

separation from solvent front and application position was selected. Initially different solvent systems were tried and optimum mobile phase was selected. **Table No. 5.9.1.**

Table No. 5.9.1: Selection of solvent system

Sr. No.	Mobile Phase	Observation	Remarks
1	Toluene:Methanol:Ammonia (7:3:0.1/v/v/v)	ALO gave compact spot but MET did not run from initial spot	Not satisfactory
2	Chloroform:Methanol:Toluene (6:3:4v/v/v)	PIO gave good spot but ALO gave only one spot with tailing	Not satisfactory
3	Chloroform:Methanol:Toluene (8:3:4v/v/v)	ALO&BA did not separate	Not satisfactory
4	Chloroform:Methanol (10:1v/v)	ALO&BA separated with tailing.PIO moved with solvent front	Not satisfactory
5	Chloroform:Methanol: GAA (10:1:0.02 v/v)	ALO&BA separated with tailing.	Not satisfactory
6	Chloroform: Methanol: Ammonia: (10:1:0.06 v/v/v/v)	ALO&BA separated with good peak.PIO moved with solvent front	Not satisfactory
7	Chloroform:Methanol:Ammonia:GAA (10:1:0.06:0.02 v/v/v/v)	PIO moved with solvent front	Not satisfactory
8	Chloroform:Methanol:Ammonia:GAA (8:2:0.06:0.02 v/v/v/v)	Rf value of PIO was high	Not satisfactory
9	Chloroform: Methanol: Ammonia: Glacial Acetic Acid (8.5:1.5:0.06:0.02 v/v/v/v)	Rf value PIO was high	Not satisfactory
10	Chloroform: Methanol: Ammonia: Glacial Acetic Acid (8:1:0.06:0.02 v/v/v/v)	Good separation with symmetrical peaks	Satisfactory

5.9.5 Optimization of separation conditions

A solvent system that gave dense compact spots, good separation between ALO, PIO and BA (Benzoic acid: Which is separating from alogliptin benzoate) and separation from solvent front and application position was selected. Initially a fixed concentration of drug solution was spotted and then developed using solvents such as methanol, acetonitrile, chloroform, ethyl acetate, toluene, hexane etc. and also mobile

phases obtained from literature review was tried. After various trials optimum mobile phase was selected.

5.9.6 Fixed chromatographic condition

Fixed chromatographic condition

Stationary Phase	:	Pre-coated silica gel $60F_{254}$ on aluminium sheets
Mobile phase	:	Chloroform: methanol: ammonia: glacial acetic acid (8:1:0.06:0.02 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	:	274 nm

Components were scanned at 274 nm and reproducible R_f values were found to be 0.196±0.0046, 0.843±0.0052 and 0.578±0.0038 for ALO, PIO and BA, respectively, are shown in **Figure No. 5.9.2**

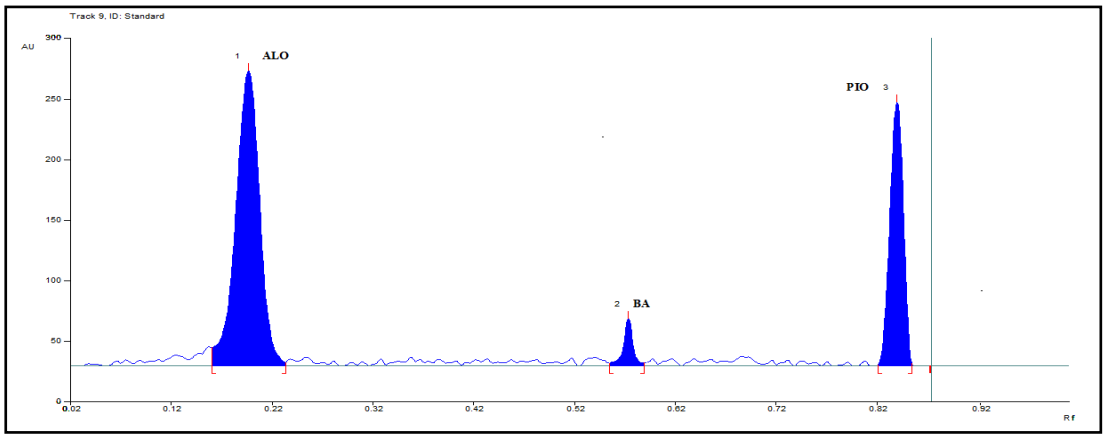


Figure No. 5.9.2: Standard chromatogram of ALO (600 ng/band) and PIO (1080 ng/band)

5.9.7 Validation of chromatographic method

Developed method was validated according to ICH guidelines using following parameters

5.9.8 Specificity

The peak purity of both the drugs ALO and MET 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 ALO {correlation r(s, m) = 0.9999, r (m, e) = 0.9998} and MET {correlation r(s, m) = 0.9999, r(m, e) = 0.9995}. It can be concluded that no impurities or degradation products migrated with the peaks obtained from standard solutions of the drugs.

5.9.9 Linearity and range

Calibration curve was plotted using peak area versus ng/band. **(Figure No. 5.9.3-5.9.4).**ALO and PIO were found to be linear in the concentration range of 50-1000 ng/band of ALO and 90-1800 ng/band of PIO **(Figure No. 5.9.6-5.9.16 & Table No. 5.9.2).** Results were subjected to regression analysis by the least squares method to calculate values of slope, intercept and correlation coefficient are shown in **Table No. 5.9.8.**

Table No: 5.9.2: Linearity data of ALO & PIO

Sr. No.	ALOGLIPTIN			PIOGLITAZONE		
	Amount / Band (ng/band)	Peak Area*	Rf Value	Amount / Band (ng/band)	Peak Area*	Rf Value
1	50	779.9	0.193	90	216.25	0.84
2	100	1214.35	0.194	180	553.33	0.84
3	200	2317.32	0.2	360	1122.32	0.84
4	400	4247.10	0.192	720	2080.27	0.84
5	600	6541.62	0.2	1080	3076.90	0.85
6	800	8634.95	0.2	1440	4086.53	0.85
7	1000	10501.22	0.19	1800	4993.35	0.84

*(n=6) Avg. of six determination

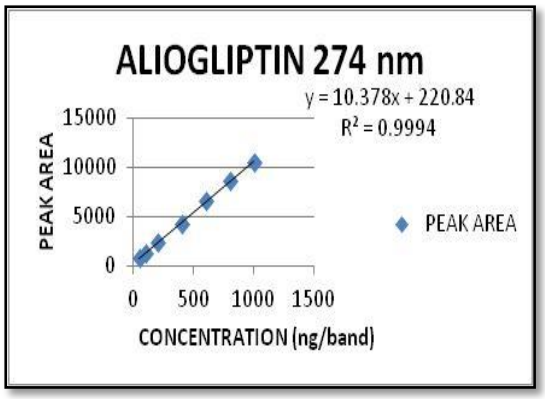


Fig. No. 5.9.3.: Calibration curve of ALO at 274 nm

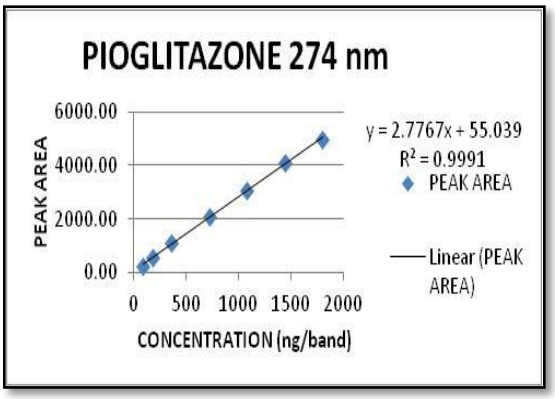


Fig. No. 5.9.4: Calibration curve of PIO at 274 nm

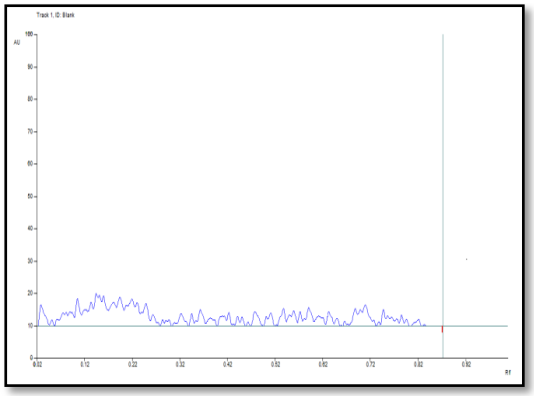


Fig. No. 5.9.5: Chromatogram of blank (methanol)

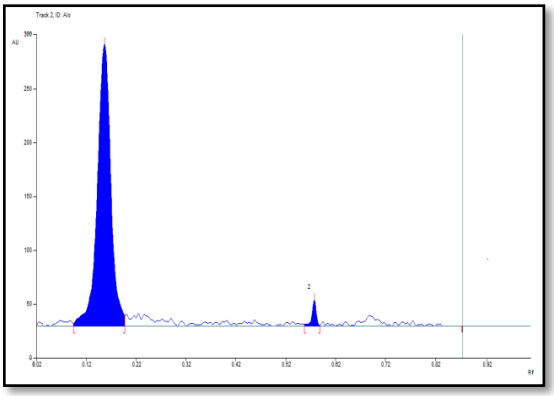


Fig. No. 5.9.6: Chromatogram of ALO

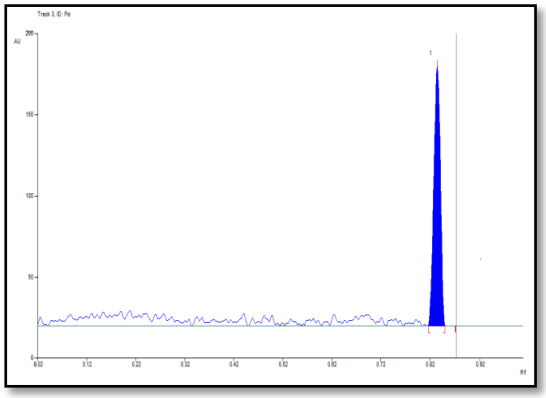


Fig. No. 5.9.7: Chromatogram of PIO

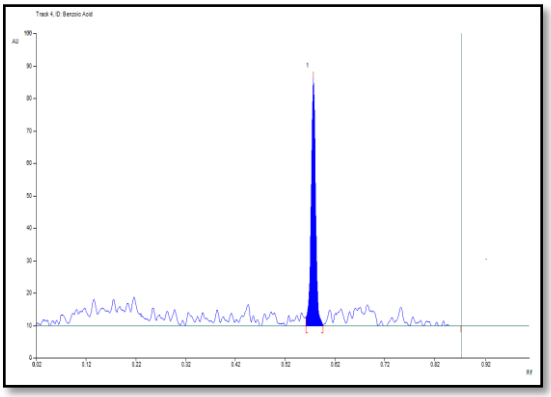


Fig. No. 5.9.8: Chromatogram of Benzoic acid

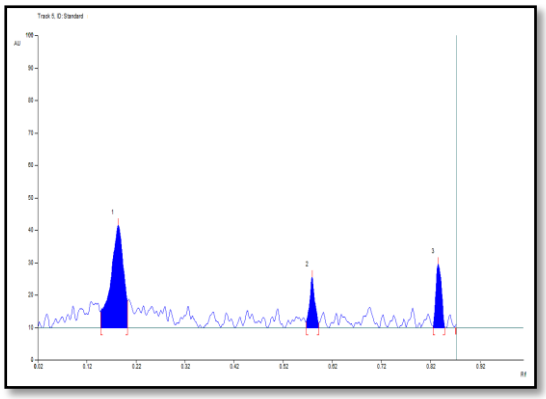


Fig. No. 5.9.9: Chromatogram of ALO (50 ng/band) & PIO(90 ng/band)

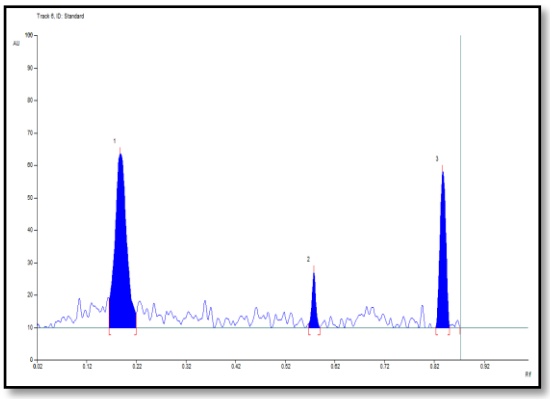


Fig. No. 5.9.10: Chromatogram of ALO (100 ng/band) & PIO (180 ng/band)

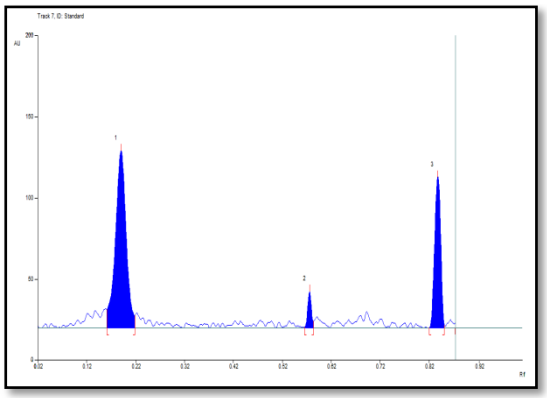


Fig. No. 5.9.11: Chromatogram of ALO (200 ng/band) & PIO (360 ng/band)

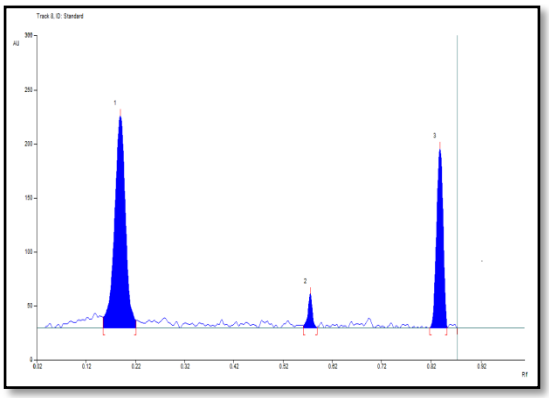


Fig. No. 5.9.12: Chromatogram of ALO (400 ng/band) & PIO (720 ng/band)

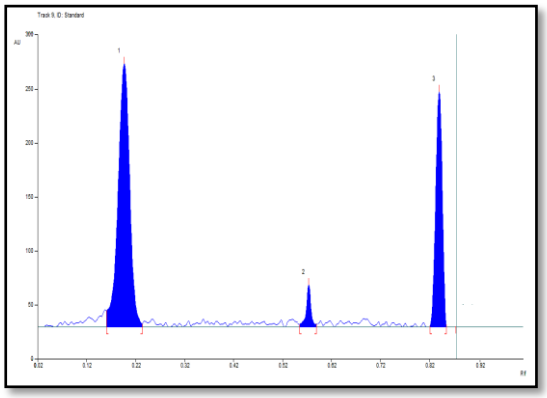


Fig. No. 5. 9.13: Chromatogram of ALO(600 ng/band) & PIO(1080 ng/band)

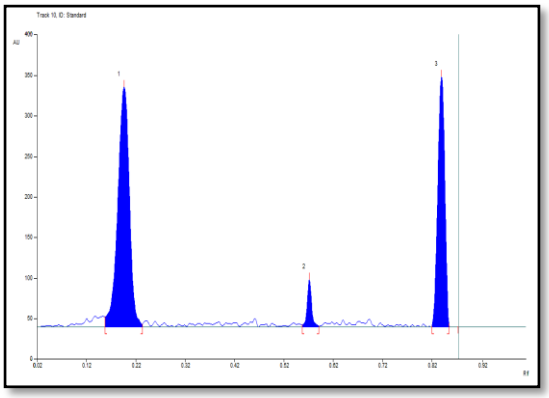


Fig. No. 5.9.14: Chromatogram of ALO (800 ng/band) & PIO (1440 ng/band)

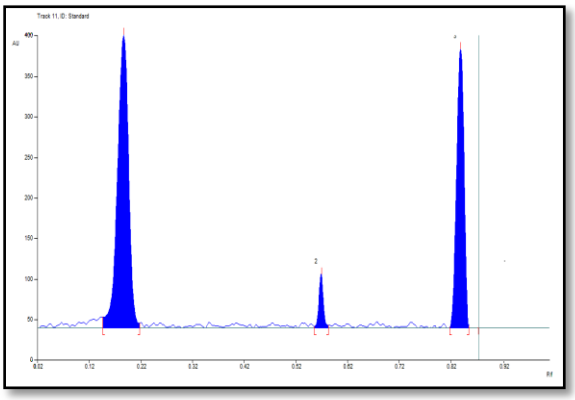


Fig. No. 5.9.15: Chromatogram of ALO (1000 ng/band)& PIO (1800 ng/band)

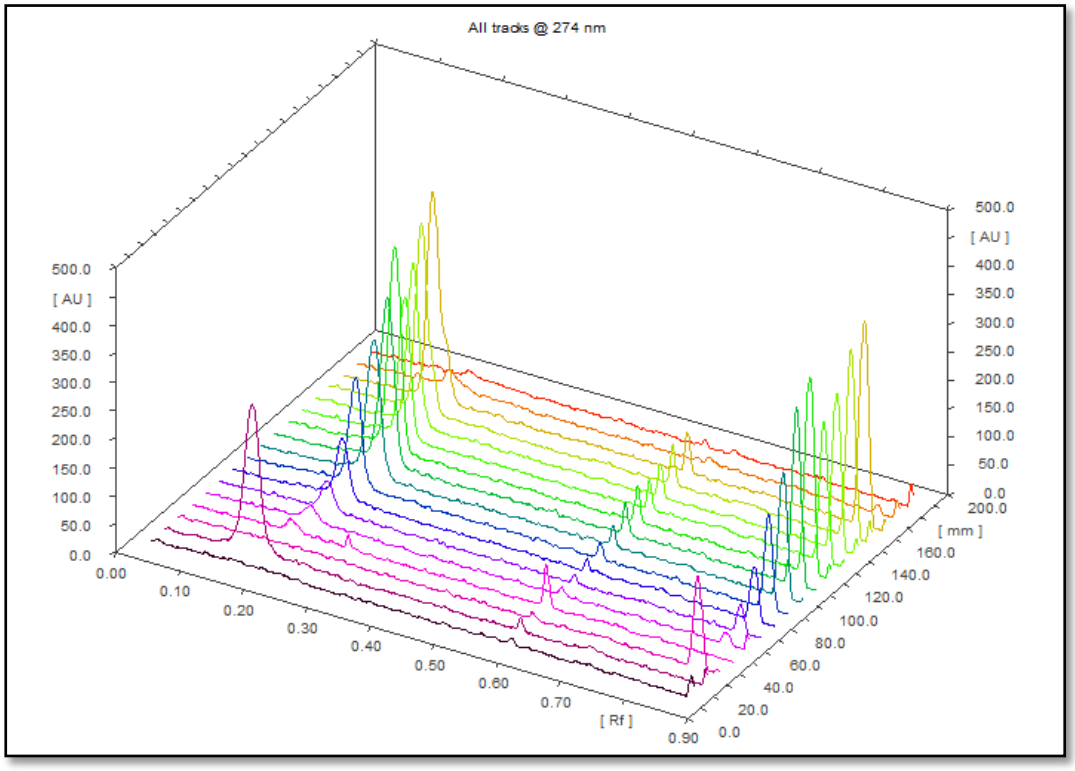


Fig. No. 5.9.16: Overlain 3D Chromatogram of ALO & PIO at 274 nm

5.9.10 Precision

The precision of the method was checked by carrying out repeatability, intraday and interday precision. Results of precision studies expressed in %RSD follows ICH guideline acceptable limits, (<2) which indicates good repeatability and low inter-day variability. **Table No. 5.9.3, 5.9.4 & 5.9.5.**

Table No. 5.9.3: Results of repeatability of measurement

Repeatability								
Sr. No.	**ALO**				**PIO**			
	Conc. (ng/band)	**Peak area**	**Mean ± SD**	**% RSD**	**Conc. (ng/band)**	**Peak area**	**Mean ± SD**	**% RSD**
1	200 ng/band	2293.2	2316.78± 24.2129	1.0451	360 ng/band	1121.3	1120.78 ± 12.9339	1.1540
2		2342				1102.2		
3		2314.1				1134.9		
4		2289.4				1113.7		
5		2348				1116.8		
6		2314				1135.8		
1	400 ng/band	4312.2	4261.83 42.0196	0.9860	720 ng/band	2002.2	2033.05 ± 20.9874	1.0323
2		4274.8				2048.8		
3		4251.8				2043		
4		4272.6				2013.5		
5		4185.9				2055.8		
6		4273.7				2035		
Mean % RSD				**1.0155**				**1.0932**

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

Table No. 5.9.4: Results of intra-day precision

Intra-day precision								
Sr. No.	**ALO**				**PIO**			
	Conc. (µg/ml)	**Peak area**	**Mean ± SD**	**% RSD**	**Conc. (µg/ml)**	**Peak area**	**Mean ± SD**	**% RSD**
1	200 ng/band	2257	2285.222 ± 31.9622	1.3986	360 ng/band	1156.7	1135.178 ± 12.3534	1.0882
2		2276.4				1143		
3		2282.2				1131.3		
4		2334.7				1122.2		
5		2329				1137		
6		2301.4				1146		
7		2267.1				1116.8		
8		2237				1135.8		
9		2282.2				1127.8		
1	400 ng/band	4287.5	4274.133 ± 39.8846	0.9332	720 ng/band	2034.3	2062.422 ± 24.1200	1.1695
2		4331.7				2063		
3		4287				2036.8		
4		4297				2072.1		
5		4251.8				2113.5		
6		4272.6				2055.8		
7		4185.9				2075		
8		4265.8				2065.9		
9		4287.9				2045.4		
Mean % RSD				**1.1659**				**1.1289**

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

Table No. 5.9.5: Results of inter-day precision

Inter-day precision									
Sr. No.		**ALO**				**PIO**			
		Conc.	**Peak area**	**Mean ± SD**	**% RSD**	**Conc.**	**Peak area**	**Mean ± SD**	**% RSD**
1	1	200 ng/band	2342.2	2346.5 ± 22.1157	0.9425	360 ng/band	1139.9	1130.378± 14.7139	1.3017
2			2376.3				1145.5		
3			2329				1128		
4	2		2356.7				1137		
5			2379				1121.3		
6			2334.7				1102.2		
7	3		2329				1116.8		
8			2356.6				1135.8		
9			2315				1146.9		
1	1	400 ng/band	4372.2	4292.456 ± 55.6483	1.2964	720 ng/band	2034.4	2063.256± 21.6521	1.0494
2			4357				2057.8		
3			4298.8				2064.7		
4	2		4287				2054.5		
5			4319.8				2058.8		
6			4251.8				2113.5		
7	3		4272.6				2055.8		
8			4185.9				2075		
9			4287				2054.8		
Mean % RSD					**1.1195**				**1.1755**

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

5.9.11 Accuracy

The results of recovery studies ranged from 98-100% for both the drugs showing the accuracy of the method. **(Table No. 5.9.6)**

Table No. 5.9.6: Results of recovery studies

Accuracy (% Recovery)				
ALO				
Recovery level (%)	**Initial conc. of formulation (ng/band)**	**Standard added (ng/band)**	**Recovered (ng/band)**	**% Recovered**
50	200	100	101.0561	101.0561
50	300	150	148.3966	98.9311
50	400	200	205.3055	102.6527
100	200	200	201.0368	100.5184
100	300	300	305.6042	101.8681
100	400	400	403.8890	100.9722
150	200	300	302.2991	100.7664
150	300	450	446.6814	99.2625
150	400	600	593.8485	98.9748
Mean± SD				**100.5558±1.2942**
% RSD				**1.2871**
PIO				
Recovery level (%)	**Initial conc. of formulation (ng/band)**	**Standard added (ng/band)**	**Recovered (ng/band)**	**% Recovered**
50	360	180	178.6109	99.2283
50	540	270	270.5525	100.2046
50	720	360	357.0198	99.1722
100	360	360	361.6700	100.4639
100	540	540	544.0066	100.7420
100	720	720	710.7491	98.7152
150	360	540	547.8982	101.4626
150	540	810	789.5858	97.4797
150	720	1080	1096.1706	101.4973
Mean± SD				**99.8851±1.3395**
% RSD				**1.3410**

5.9.12 LOD and LOQ

The values of LOD and LOQ were found to be 7.0498 and 21.3630 ng/band for ALO, 12.4164 and 37.6253 ng/band for PIO, respectively **(Table No. 5.9.8)**.

5.9.13 Robustness

The proposed method was checked through all parameters described earlier under robustness studies, but no significant changes found in retention time, peak area or symmetry of the peaks. (**Table No. 5.9.7)**

Table No. 5.9.7: Results of robustness study

Sr. No.	Modification	ALO*		PIO*	
		R_f value	**Peak area**	**R_f value**	**Peak area**
1	M/P composition (± 0.1 ml)	0.1963 ± 0.0032	4284.6267 ± 40.9352	0.8459 ± 0.0055	2112.7000 ± 32.6675
% RSD		**1.6373**	**0.9554**	**0.6540**	**1.5462**
2	Volume of M/P (± 5%)	0.1997 ± 0.0015	4253.9233 ± 11.6501	0.8444 ± 0.0073	2100.2233 ± 28.4421
% RSD		**0.7650**	**0.2739**	**0.8678**	**1.3542**
3	Chamber saturation time (20 ± 5 min)	0.1973 ± 0.0031	4303.8167 ± 74.3316	0.8431 ± 0.0106	2039.0867 ± 29.1867
% RSD		**1.5482**	**1.7271**	**1.2591**	**1.4314**
4	Development distance (80 ± 5 cm)	0.1993 ± 0.0012	4295.3000 ± 26.0688	0.8463 ± 0.0052	2094.5967 ± 14.0016
% RSD		**0.5793**	**0.6069**	**0.6129**	**0.6685**
5	Time from spotting to chromatography (15± 10 min)	0.1967 ± 0.0023	4341.1867 ± 46.179	0.8471 ± 0.0062	2101.0067 ± 21.2359
% RSD		**1.1743**	**1.0633**	**0.7369**	**1.0108**
6	Time from chromatography to scanning (15± 10 min)	0.1983 ± 0.0015	4277.3667 ± 42.0493	0.8500 ± 0.0066	2049.3767 ± 36.4661
% RSD		**0.7702**	**0.9831**	**0.7767**	**1.7794**

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

5.9.14 Stability of the solution

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

Table No. 5.9.8: Summary of validation parameters for the proposed HPTLC method

Parameters	ALO	PIO
Linearity range (ng/band)	50-1000	90-1800
Correlation coefficient	0.9994	0.9991
Regression Equation	y = 10.378x + 220.84	y = 2.7767x + 55.039
Precision (%RSD)		
Intra-day (n=3)	1.1659	1.1289
Inter-day (n=3)	1.1195	1.1755
Repeatability of injection (n=6)	1.0155	1.0932
Accuracy		
% Recovery (n=6)	100.5558±1.2942	99.8851±1.3395
%RSD (n=6)	1.2871	1.3410
Specificity	No interference	
LOD (ng/band)	7.0498	12.4164
LOQ (ng/band)	21.3630	37.6253

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

5.9.15 Analysis of formulation

The proposed method was successfully used for the quantitative determination of ALO and PIO in tablet formulation (25 mg ALO and 45 mg of PIO). Six replicate determinations were carried out and average experimental values were found to be 100.63 and 100.84 %w/w for ALO and PIO, respectively hence the developed can be used for the simultaneous estimation of drugs in tablet dosage form (**Table No. 5.9.9.).** Overlain spectra of standard drugs and formulation are shown in **Figure No. 5.9.17.**

Table No. 5.9.9: Results of formulation analysis

Drugs	Amount (mg/tablet)		% Drug found*	% RSD
	Labelled	Found		
ALO	25	24.99	99.9890±0.9493	0.9494
PIO	45	44.47	98.8227±1.1623	1.1761

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

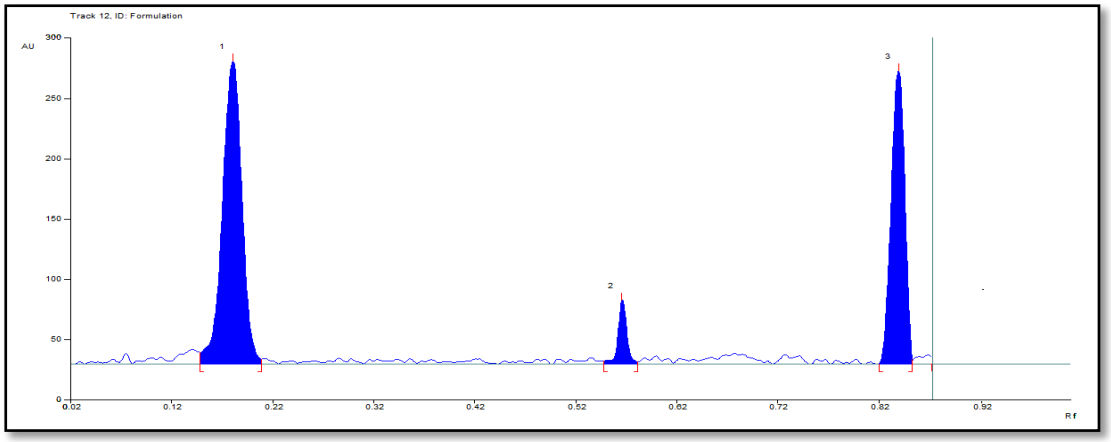


Figure No. 5.9.17: Chromatogram of formulation of ALO (500 ng/band) & PIO (900 ng/band)

5.10 Statistical analysis

Five different methods namely RP-HPLC, HPTLC, simultaneous equation, abosorbance ratio and first derivative spectroscopic methods were developed and validated for two 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 test.

Formulation 1: Results of ANOVA are presented in Table No 5.10.1. The results of assay reveal that there was no significant difference between all the five methods.

Table No. 5.10.1: Results of statistical comparison using one way ANOVA & Bonferroni multiple comparison test for formulation 1 (ALO & MET tablet)

Drugs	RP-HPLC	HPTLC	Simultaneous Equation Method	Absorbance Ratio Method	First Derivative Method
ALO	99.0123 ± 0.9374	99.4565 ± 1.2097	98.8995 ± 1.5902	100.0827 ± 1.1262	100.1883 ± 1.5413
MET	99.1963 ± 0.7314	99.6673 ± 0.9757	99.6935 ± 1.3084	98.7660 ± 1.3702	98.1409 ± 1.2661
All values are expressed in Mean±SD (n=6)					

Formulation 2: Results of ANOVA are presented in Table No. 5.10.2. The results reveal that there was no statistical significant difference between RP-HPLC and HPTLC methods for the determination of ALO. There was significant ($p<0.05$) difference between simultaneous equation and HPTLC method, where assay of ALO by HPTLC method was found to be superior as compared to simultaneous equation method. Moreover, simultaneous equation method was significantly better than absorbance ratio method for the determination of ALO or PIO. In addition, there was no significant difference between all other comparisons except above mentioned methods for formulation 2.

Table No. 5.10.2 : Results of statistical comparison using one way ANOVA & Bonferroni multiple comparison test for formulation 2 (ALO & PIO tablet)

Drugs	RP-HPLC	HPTLC	Simultaneous Equation Method	Absorbance Ratio Method
ALO	99.6987 ± 0.8946	99.9890* ± 0.9493	97.6596# ± 0.7456	99.8070 ± 1.2544
PIO	98.6219 ± 0.6561	98.8227 ± 1.1623	97.3869$ ± 1.0077	99.4534 ± 1.5852

All values are expressed in Mean±SD (n=6), where *$p<0.05$, compared to aimultaneous equation method; #$p<0.05$, compared to absorbance ratio method; $$p<0.05$, compared to absorbance ratio method

6. Conclusion

Precise, accurate, specific, sensitive & cost effective methods compared to existing methods for the determination of ALO in combination with MET and PIO in tablet dosage form by UV Spectroscopy, HPLC along with HPTLC were developed and validated.

Most of the reported methods did not describe about procurement of tablet formulation and remaining methods did not use tablet formulation for assay. All these observations were taken in to consideration and it was thought to prepare tablet formulation for the analysis in laboratory using all the excipients as per the marketed formulation.

The results of the analysis of pharmaceutical dosage forms by the proposed methods are highly reproducible, reliable, and are in good agreement with the label claims of the drug. The excipients usually present in the pharmaceutical formulations of the assayed samples did not interfere with the drugs. Hence, all the developed methods can be used successfully for routine analysis of tablet dosage form.

Statistical analysis was performed to assess the effect of all the developed methods based on assay results obtained. The statistical significance between all the methods were tested using one way ANOVA followed by Bonferroni multiple comparison test. Results of ANOVA revealed that there is no statistical significant difference between all the five methods for formulation 1 and where assay of ALO by HPTLC method was found to be statistically superior than simultaneous equation method. Moreover, simultaneous equation method was significantly better than absorbance ratio method for the determination of ALO or PIO. In addition, there was no significant difference between all other comparisons except above mentioned methods for formulation 2.

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