PREAMBLE
1. Introduction:

1.1 Analytical Chemistry:

A branch of science which deals with the quantitative measurements of analyte in a given sample. In practice, quantifying an analyte in a sample is a complex practice. The branch also studies the separation mechanism, qualification and identification of the chemical species in its natural or complex synthetic form. Qualitative analysis identifies the component present in that the simple or complex matrix, whereas Quantitative analysis calculate the purity of the component in the substance

1.1.1 Classifications of Analysis:

Qualitative analysis: It deals with the identification/characterization of elements, ions or compounds present in the sample.

Quantitative analysis: Quantitative analysis deals with the determination of how much quantity and hence the purity of one or more constituents is present.

Further, the analytical method is classified into two, Classical method and Instrumental method.

Classical method: This method is also known as wet chemistry method. The principles of classical methods are based on the separations of chemical species on the basis of precipitation, extraction and distillation. Classical method further divided into Qualitative analysis and Quantitative analysis.

Quantitative analysis determines only presence or the absence of various components in the present sample. It does not measure the amount present in it. Whereas, Quantitative analysis determines the sample qualitatively as well as measures the quantity of various components present in the given sample.

Examples

- Chemical test: Identification of Lead or heavy metals by lead-acetate test.
- Flame test: Identification of sodium ion by flame test. It gives yellow flame.
Gravimetric analysis: Precipitation silver as silver chloride using silver nitrate as precipitating agent or Barium as Barium chloride.

Volumetric analysis- It can be further classified as

- Acid-Base titration: Hydrochloric acid v/s sodium hydroxide as base.
- Redox titration- Cerelium v/s iron where one get reduced and other oxidized simultaneously during titration and further determination potentiometricaly using quinoline as an indicator.

Instrumental method: In this, the method utilizes an instrument for the detection and quantification of present various analyte in the given sample. The various physical properties used in the instrumental methods are Light absorption spectroscopy, Fluorescence spectroscopy, Conductivity, refractive index. The separation of various components is done by using Chromatography, Electrophoresis, Spectrophotometer and Field Flow Fractionation methods.

1.1.2 Modern analytical chemistry

a) Spectroscopy: In this, the presence of analyte can be measure through the molecular interaction of analyte molecule with the various electromagnetic radiations. Various types of spectroscopy instruments are AAS, UV-Spectroscopy, X-ray fluorescence spectroscopy, IR-Spectroscopy, Raman spectroscopy, NMR Spectroscopy.

b) Mass Spectrometry: In this analytical tool, the analyte is measured according to the mass of the molecule to the charge ratio. Generally it is used to calculate molecular mass of the fine particle and for the determination of composition of a given sample.

c) Electrochemical analysis: In this method, the potential (volts) across the membrane is calculated with the help of an electrochemical cell. These methods further categorized into three main which are potentiometry, coulometry and voltammetry.

d) Hyphenated techniques: Combinations of classical method with the instrumental method results into some new hyphenated techniques. Presently several hyphenated techniques
are used to Qualify and quantify the complex sample material. Example. Protein separation, Drug-protein binding, Identification of molecular weight of unknown impurity in the degraded sample. The examples of some popular new hybrid techniques used are GC-MS, GC-IR, and LC-MS, LC-NMR.

The most commonly used hyphenated technique is combination of Liquid chromatography with various detectors method such as UV-visible Spectrophotometer, Infrared spectrophotometer, Mass spectrophotometer, Refractive index detector, Fluorescence Detector and Photo-diode array detector. These techniques uses various available instruments individual or in combination to detect and separate various chemicals and analyte in the solution or in suspension form. The applications of Hyphenated techniques are in a very wide range of sample as well as in the various branches of chemistry and biochemistry.

1.1.3 Application of analytical chemistry:

- In Pharmaceuticals and Clinical research, it serves the purpose of diagnosis of disease and to check the progress in Recovery. It also used to establish the purity of the medicinal product.

- In industry, it is used in raw materials testing and finished products testing which ensures the quality of the product. It is also used to find out the critical attributes in the raw material which can affect the finished product in terms of quality. Many products, such as fuel, paints, pharmaceuticals etc. are analyzed by analytical procedure developed by chemist before the product being sold to consumer.

- It is also used in Environmental monitoring of waste which can affect the biodiversity adversely. Analytical chemistry is used to detect the poisonous substance. It is also used to find out various attributes which can affect the human life adversely.

- Analytical chemistry also used in the determining the nutritional value of food by the chemical analysis for major components such as protein and carbohydrates and trace component such as vitamin and minerals. The calories can also be calculated by chemical analysis

1.1.4 Selection of analytical method

Important factors which must be taken into account while selecting an analytical method.
1. The size of sample available and proportion of the constituents to be determined.
2. The purpose for which analytical data is required,
3. The nature of the information is available, Speed and Cost.
1.2 Definition of Chromatography

Chromatography is defined as the quantitative and qualitative estimation of various components on the basis of their various physical and chemical properties. The Substance to be analyzed is dissolved in the specific solvent and passed through the stationary phase. The speed of travel of analyte depends upon the interaction of analyte with the stationary phase. This causes them to separate from each other. This separation of constituents is based on various Physico-chemical properties of the constituents. The separation of analyte depends upon the partition co-efficient between the mobile phase and the stationary phase. The difference in the partition co-efficient of analyte results into different retention.

The IUPAC system definition for chromatography is “A method which is used to separate the various components in the given sample. The separation is achieved due to distribution of various components into two different phases. One is stationary phase which is generally solid or gel in nature, another phase is mobile phase. The mobile phase can be liquid or gaseous in nature.”

Chromatograph can be considered to be the most commonly and precisely used analytical technique in the current pharmaceutical industry scenario. No other separation method is as powerful and generally applicable as is chromatography. An understanding of the parameters which govern chromatographic performance has given rise to improvements in chromatography systems, so the ability to achieve high-resolution separation is continuously increasing. The system suitability tests are now routinely included in chromatographic software packages so that the chromatographic performance of a system can be monitored routinely.

1.2.1 Type of Chromatography

**Column chromatography:** The separation of components in a mixture depends upon the transfer of analyte between the Mobile phase and the stationary phase. The most attractive and commonly used stationary phase can be silica gel column, a paper, silica gel plates.
a) **Planar chromatography:** This separation technique uses a planar surface that can be paper of glass plate coated with silica. The retention of analyte depends upon the affinity of analyte and stationary phase with each other. Compound which is having high affinity towards the stationary phase retains for the longer time compared to the compound having low affinity towards the stationary phase.

b) **Gas chromatography:** In this chromatography the mobile phase is in the form of gas and the stationary phase is solid. Partition equilibrium of analyte with that of stationary phase is determines the retention of analyte in gas chromatography.

c) **Liquid chromatography:** In this the mobile phase is in the liquid state. Whereas, stationary phase is made up of solid particles. In High pressure liquid chromatography, the sample is forced through the stationary phase i.e. packed in either a stainless steel cylinder or a plastic cylinder or a glass cylinder. The most commonly used cylinders are of stainless steel. The sample is injected into the system under high pressure. The typical stationary phase having particles of either natural silica or hybrid silica fuzzed with various other functional groups. The general characteristics of silica particles are spherically shaped, a porous in nature, etc.

d) **Supercritical fluid chromatography (SFC):** SFC is similar to that of liquid chromatography. The difference lies in mobile phase. The mobile phase used in SFC is carbon dioxide, nitrogen, oxygen, nitrous oxide, ammonia or rarely water can also be used. The SFC can be very useful in the quantification, purification and separation of low molecular weight compounds. It is also used in the quantification of thermally labile molecules. The most useful and effective application of SFC is Chiral separation. Above the critical temperature and pressure the form of substance changes. Supercritical carbon dioxide readily dissolves saturated hydrocarbons containing up to 30 carbon atoms. It can also dissolve many polymers and aromatic compounds. The Stationary Phase in supercritical fluid chromatography is generally Octasilane. The Supercritical CO$_2$ has a property equivalent to non-polar solvent.
e) **Ion exchange chromatography**: It is also referred as Ion Chromatography. The separation of molecules depends upon the charge present on it. In Ion exchange chromatography, stationary phase is either charged with anions or cations exchange resins. Sometimes amino acids, peptides and proteins are also used as a stationary phase.

f) **Size-exclusion chromatography**: It is also termed as Size exclusion Chromatography. In this the separation of molecules depends upon the molecular weight. It is also known as Gel Permeation Chromatography.

g) **Chiral chromatography**: Chiral chromatography is used where the separation is needed between the stereoisomers. The enantiomers cannot be separated by any other techniques, as both the chemical as well as physical properties are same to each other. The only difference between the two molecules is in the three dimensional structure. These structures are generally mirror images of each other. The Prerequisites for the chiral separation is to have either one of it to be chiral in nature. i.e. Either mobile phase or stationary phase. The availability of chiral stationary phase is abundant.

    It involves Qualitative and Quantitative separation. Also identifies and determines the various compounds present in the complex sample matrix.

    This technique is also used in assessing the quality of various pharmaceuticals. Quality of various pharmaceuticals can be maintained by using varies analytical techniques and some critical components of Quality Assurance/Quality Control.
1.2.2 High performance liquid Chromatography and its principals

HPLC uses high pressure system to quantify and qualify various components in the complex sample matrix. In the beginning, instruments have the capability of generating the pressure of 500 psi (35 bar). The early 1970’s new HPLC system were developed which can sustain up to the pressure of 6000 psi (400 bars). These developed HPLC systems were also attached with improved detectors and columns.

HPLC then nearly became the common in the analytical laboratories in 1970’s. The modern HPLC was the newer version of older HPLC’s with having more advancements than that of the older version. The name further modified to High Performance Liquid Chromatography.

Where now a day’s these two terminologies are interchangeable. HPLC now became the most powerful tools in analytical chemistry. In the HPLC, The analyte is passed through the Silica/Polymer based column under high pressure. The separation of analyte from the complex sample matrix depends upon the interaction with the stationary phase.

Every analyte has its own characteristic retention on the stationary phase. This retention depends upon the Stationary phase and its chemistry. Generally, the mobile phase used in the HPLC is a mixture of aqueous as well as organic liquids. The most commonly used organic liquids are Acetonitrile, methanol etc. The aqueous phase generally contain buffer such as TEA, TFA, KH$_2$PO$_4$, Acetate buffers and Ion pairing reagents.

The elution in HPLC is based upon the composition of mobile phase. If the composition of mobile phase is constant it is termed as Isocratic HPLC. If the mobile phase composition changes with the time then it is termed as Gradient Elution. Isocratic separation is useful when only single molecule is to be separated. Multiple molecules cannot be separated using Isocratic Separation. Gradient elution is useful when multiple components are to be separated from its complex matrix.
1.2.3 Types of HPLC:
HPLC can be classified according to the stationary phase and the separation process.

I] Adsorption chromatography: In this the analyte interacts with the stationary phase and the separation is based on the adsorption and desorption of analyte with the stationary phase.

II] Ion exchange chromatography: In this chromatography, the stationary phase is hypothetically charged with the anions or cation species, which further interacts with the analyte and results in the retention of compound.

III] Size Exclusion Chromatography: In this chromatography, the separation of analyte is based on the molecular size of the analyte. Bigger the molecule lesser will be the retention, smaller the size larger will be the retention. The alternate name for this technique is gel filtration or gel permeation chromatography.

IV] Ion-pair chromatography: This method is generally used for the ionic samples. On reversed phase, the samples which are completely ionized cannot be retained using normal C18 column. Additional additives are required to retain such ionic species. Additives are Ion pairing reagents. The reagents give stationary phase a temporary charge which makes ionic species to retain. The retention of these species is due to ionic interaction between the ionized functional group of analyte and the temporary ionized stationary phase. The stronger is the attraction longer will be retention. Weaker the attraction shorter will be retention of ionized analyte.
• Normal Phase Chromatography:

In this, the stationary phase is polar in nature (i.e. Silica) and the mobile phase is non-polar (ex. n-Hexane, Heptane). The analyte which are polar in nature interacts with the stationary phase and gets retained on the stationary phase. The care must be taken during the preparation of mobile phase, as the normal phase system is very sensitive to the water.

Figure 1.3 Retention mechanisms for various types of Chromatography
- **Reversed Phase Chromatography:**

  In this the stationary phase is non polar (C8, C18) in nature whereas; mobile phase is comparatively polar (ex. Mixture of water, Acetonitrile, Methanol) in nature. Here more non-polar the material, longer it will be retained.

**1.2.4 Mechanism of retention in Reverse Phase Chromatography:**

When a solute is dissolved in water, the strong attractive force between water molecules become distorted or disrupted. These attractive forces arises from the three dimensional network of inter-molecular H-bonds. In RP-HPLC, the driving force for retention is not the favorable interaction of solute with stationary phase but the partition co-efficient of analyte between the aqueous phase of mobile phase and the non-polar stationary phase. The hydrophobic retention involves mainly non-polar substances or non-polar portion of the molecule. Better resolution of sample, for a given stationary phase is achieved when the aqueous portion is higher than that of organic.
1.2.5 Mechanism of retention in Normal Phase Chromatography:

The normal phase consists of polar stationary phase and non-polar mobile phase. The stationary phase polarity can be increased or decreased by covering bare silica gel with ligand of various functional groups.

There are four main parameters to be considered during the selection of detection wavelength in normal phase, UV cutoff, Solvent strength, Localization and the Basicity.

The Competition of solvent and the analyte for the adsorption on the adsorptive surface of the stationary phase is the important factor in the normal phase selectivity. The compounds that are polar in nature have the highest affinity towards the polar stationary phase than that of organic solvents. The interaction between the stationary phase is week for the weekly polar solvents. The solvents which are highly polar interacts with the stationary phase very freely of solvents that are weekly polar. Selectivity can be changed by the use of basic solvents such as methyl-tertiary butyl ether or non-basic solvents such as acetonitrile.
Figure 1.5 The relationship between polarity and elution time for Normal phase and Reversed phase Chromatography

1.2.6 Separation of Molecules with Different Functional Groups.

The adsorption of analyte increases in the following order: saturated hydrocarbons, olefins, aromatics, organic halogen compounds, Sulphides, Nitro-compounds, Ethers, Esters, Aldehydes, Ketones, Alcohols, Amines, amides and carboxylic acids. The analyte having different functional groups has the retention based on most polar group. For Reversed phase, stationary phase having C18 chain gives excellent separation. Whereas in Normal phase, Silica gives the excellent separation of compound having different functional groups.

1.3 Instrumentation

The main components of HPLC are shown in the figure.3. HPLC contains solvent storage system, an Injector (loop containing 20-200µL), Pump (flow rate from 0.2-10.0 Milliliter/Minute, Back Pressure regulator (100-5500psi), Column (50mm to 300mm) with or without column oven, sample storage and a Detector (US, DAD, IR, RI, etc.). The whole system is attached to the data collection system (personal computer)
Figure 1.6: Schematic diagram of HPLC system
I. High Pressure Pumps

As a consequence of the large back pressure encountered, due to the small particle size of packing used in HPLC columns, pumps must be employed to achieve acceptable eluent flow rates. HPLC pumping system delivers required amount of mobile phase from the reservoir to the high pressure system. The system maximum pressure can be up to 5000 psi. Some modern ultra-pressure system can withstand the back pressure up to 14000-15000 psi. The primary requisite of pump is to be corrosion free and it should deliver pulse free flow across the stationary phase. The pulse type flow can results into some detector noise. There are two types of pumps that are generally used in HPLC: syringe pumps and reciprocating pumps;

a. **Syringe pump:**
Figure 1.7: Syringe pump

b. Reciprocating pump:
Figure 1.8: Reciprocating pump
Figure 9: Various components of HPLC System
II. Sample Injection System

After dissolving the component in the suitable diluent were injected, either manually by syringes or automatically by auto samplers. The modern system contains an auto sampler which is programmed to inject specific quantity of sample into the system. The older HPLC system was having Rheodyne injector, where the sample is to be injected manually.
III. Column

The chromatographic columns dimension varies from 2 cm to 30 cm in length and 2.1 mm-4.6 mm internal diameter. The columns are constructed of stainless steel to cope with the high back pressure and are glass lined to prevent metal catalysis of solvent-solute reactions at the

Figure 1.10: Rheodyne injector
high column pressures experienced. The HPLC is conveniently divided into two, on the basis of stationary phase and the mobile phase.

HPLC system is classified on the basis of stationary phase and mobile phase. When the stationary phase is polar and mobile phase is non-polar then it is termed as Normal Phase. When the stationary phase is non-polar a mobile phase is polar then it is termed as Reversed phase.

In the modern era, stationary phase consist of organic phase/ functional groups chemically bounded to the silica. The polarity of the stationary phase depends upon the functional group attached to it. The most commonly used functional groups are Nitrile, Phenyl, Cyano, Amino hexyl-phenyl, etc.
IV. Detectors:

There are many detectors that can be linked with the traditional HPLC system. The most common detectors are Ultra-Violet detector, Diode Array Detector and IR detector. Most of the regulator methods comprises of UV detector for assay and dissolution. For the qualification of related substances Diode array detector is preferred. In HPLC, a detector is in a series connection to the column. The sample which exit from the column flows through the detector cell. The detector contains light source, monochromatic and a lens.
V. Data Collection Device

A modern collector receives and stores detector output and printout chromatogram complete with the peak height, peak area.

Figure 1.12: Photo diode array detector
1.4 Diabetes:

It is a simply a metabolic disorder in which a person can have a high blood glucose level. The prominent reason for such a disorder is either a body cannot produce sufficient insulin or body doesn’t have the required insulin in the body. The main reason for such condition is abnormal functioning of the cells that are responsible for the production of insulin. The general indication of diabetes is Urination (frequent), increase in thrust and increase in hunger. If these symptoms are seen in the patient then the person can have diabetes. There are typically three types of diabetes in human being.

• **Type-1:** Type-1 diabetes results due to loss of insulin producing beta cells. This can be further classified as an immune-mediated nature. The immune-mediated nature of the beta cell losses its T-cell mediated autoimmune attack

• **Type-2:** Type-2 diabetes is most commonly found diabetes in the world. The reason for the Type-2 diabetes is resistance to the decreased level of insulin secretion. However so specific data is available on this.

• **Gestational diabetes:** Gestational Diabetes is similar to the Type-2 diabetes. In several aspects it resembles with type-2 diabetes. In gestational diabetes the cells secretes inadequate insulin and looses responsiveness. The occurrence of gestational diabetes is about 2% - 5% of pregnancies and can disappear after pregnancy.

  Gestation diabetes can be fully treatable by careful medical supervision. The medical supervision is needed throughout the complete course of pregnancy

  On an average 20%-50% of woman gets affected by gestational diabetes can result into Type-2 diabetes in the later stage of life (after 40 years).

  Further the anti-diabetes drugs can be classified according to the pharmacological classification. These classes are as follows:
1. **Biagunides**: Metformin, Phenformin and buformin.
2. **Alpha-glucose inhibitors**: Miglitol, Acarbose and Viglibose
3. **Insuline therapy**
4. **Meglitinides**: Repaglinide, Nateglinide
5. **Sulfonylureas**: Tolbutamide, Tolazamide, Glipizide, Glibenclamide, Glimepiride and Gliclazide.
6. **Dipeptidyl peptidase-4 inhibitors**: Vildagliptin, Sitagliptin and Saxagliptin.
7. **Thiazolidinediones**: Rosiglitazone, Pioglitazone and Trioglitazone.

1.5 **Objective of the study**

The Quality, safety and efficacy of drug are the most important factors since it’s directly applied to the human being. Hence, to control purity of drug substance and impurities present in it throughout shelf life (during transportation, handling and storage) is a major concern.

The objective of the given study is to develop a Stability indicating analytical method to estimate the drug content in the various formulations. This will help to assess the drug substance in various formulations and will make dosage form assessment easy during its stability period. Also, the present work could be extending to identification of impurities observed during the study.

There are some research papers, which are on different technologies. These technologies cannot be utilized due to its availability and operative cost. So, objective of this work is to develop an analytical method for the estimation of some anti-diabetic drugs which can use commercially. This method will be on simple Reversed Phase High Pressure Liquid Chromatography. This will make scientist as well as industrial community to make a use of research work for the formulation of good quality of pharmaceutical dosage forms.

The objective of the present study was to develop and validate the “**Analytical methods for anti-diabetic drugs**” with better selectivity and this method applies in industry to assure the good quality of drugs.

Generally, dosage form used to treat diabetes used as in single as well as in combination. An analytical method has to be developed for the quantitative estimation of various anti-diabetic
drugs. The methods will be help to determine the assay of more than three drugs from pharmaceutical products.

Also, the developed analytical method should be linear, precise, accurate and robust that will help scientist for regular analysis in pharmaceutical organization by Quality control department.

The validation of these analytical methods was performed according to the ICH guidelines.

1.6 Scope of Thesis:

Scope of this thesis relates to the stability indicating method development and its validation for some anti-diabetics drugs. The analytical method development will be done systematically. The validation of the analytical method will be performed according to the ICH guidelines and USP general chapter 1225.

Base of my experience will contribute to develop accurate method with better selectivity. It will help to scientists in industries in quality control and institutes also. This method not only use in domestic purpose but will be used in regulatory market also.
1.7 Hypothesis:

Still there are many method are available in the various literature having old classical method for the estimation of various components of the drugs. Those methods lacks in the accuracy and reproducibility. As well HPLC assay methods are available for single diabetic dosage form and combined of 2 molecules dosage forms. Very less assay method available for combined of 3 molecules dosage forms. No method available for combined of <3 molecules dosage forms.

Isocratic and gradient methods has been evolved as primary techniques in the pharmaceutical analysis. The detector of choice varies from PDA to the variable wavelength detector. The use of DAD detector is to determine the peak purity and the % degradation in the stress sample. It can also be used to demonstrate the specificity of stability indicating power of the given analytical method.

Once the specificity is demonstrated the PAD/DAD can be replace by variable wavelength detector. New developed instrumental methods by HPLC will help to pharmaceutical and API industry to improve the quality of drugs as well as reliability of analytical method.

The following are the instruments used to conduct the degradation behavior of drugs.
- HPLC with Diode Array Detector and UV- detector
- UV-Visible Spectrophotometer.

The method will be developed for following drugs.

a) Biagunides: Metformin
b) Dipeptidyl peptidase-4 inhibitors: Vildagliptin, Sitagliptin
c) Thiazolidinediones: Rosiglitazone, Pioglitazone.

a) Method I

HPLC method development will be carried by clubbing the three molecules simultaneously by single method like Metformin Hydrochloride, Rosiglitazone Maleate and Sitagliptin Phosphate.
b) Method II
HPLC method development will be carried by clubbing the two molecules simultaneously by single method like Vildagliptin and Pioglitazone Hydrochloride

Step 4 - Application

After finalizing the method, it will be apply for available commercial pharmaceutical drug products and calculate the % assay. It will be helpful to determine the assay from drug products with accuracy. We will collect the market samples (Single dosage form or combined dosage form) from medical store and perform the analysis and calculate the potency of drugs in the percentage. Results will be recorded and reported.

1.8 Utility (usefulness) of the study:

The conclusion on the developed method can be drawn after looking on the various parameters involves in the study. An analytical method is said to be useful when all the parameters involves in the study are tested to its extreme. The parameters which are observed in this study were Stationary phase, Mobile phase composition, Buffer used in the mobile phase, Column temperature, Sample temperature, Injection volume, sample filtration and flow rate. The final method parameters were decided on the basis of Chromatographic behavior of various peak of interest. The developed method then validated according to the ICH guidelines. The validated method is documented in the various journals.

So, developed method is very useful for commercial practice. The method can be used by quality control chemist in industry for regular analysis. Also, they can give release for product on base of developed methods.

1.9 Limitations (restriction) of the study

The method development takes more time for research than classical method, but in present scenario regulatory market requires instrumental method. Now a day the HPLC method is compulsory for regulatory as well as domestic market for analysis a pharmaceuticals drugs.
The time requirement for regular analysis is more than that of classical method. It is more cost effective than classical method. But the cost of modern technique is very high. But due to requirement of regulatory authorities, the instrumental method has to be used to for the development and commercial purpose to maintain quality of drugs.

Also in ensuring the quality of the drugs and drug product, the instrumental method is more specific, selective, accurate and precise than classical method.