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METHOD DEVELOPMENT AND METHOD VALIDATION OF EMPAGLIFLOZIN AND METFORMIN BY RP-HPLC

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1. INTRODUCTION

Analytical method development and validation play important roles in the discovery, development, and manufacture of pharmaceuticals. One of the most critical factors in developing pharmaceutical drug substances and drug products today is ensuring that the UV-Visible, HPLC analytical test methods that are used to analyze the products to generate meaningful data.

Analytical Chemistry (B.K. Sharma 2004) is a branch of chemistry that deals with the separation, identification and determination of components in a sample. It is the science of making quantitative measurements, which requires background knowledge of chemical and physical concepts.

Analytical chemistry may be defined as the ***"Science and art of determining the composition of materials in terms of the elements or compounds contained".***

Analytical chemistry is important since the early days of chemistry, providing methods for determining which elements and chemicals are present in the world around us. During this period significant analytical contributions to chemistry include the development of systematic elemental analysis by Justis von Liebig and systematized organic analysis based on the specific reactions of functional groups (J.W. Robinson 2009).

To be effective and efficient, analyzing samples requires expertise in

1. The chemistry that can occur in a sample

2. Analysis and sample handling methods for a wide variety of problems (the tools-of the-trade)
3. Proper data analysis and record keeping.

Pharmaceutical Analysis (www.pubmed.com) plays a major role today, and it can be considered as interdisciplinary subject. Analytical instrumentation plays an important role in the production and evaluation of new products and in the protection of consumers and the environment.

Pharmaceutical Analysis Techniques are applied mainly in two areas

1. **Qualitative**: Qualitative analysis seeks to establish the presence of a given element or compound in a sample.

2. Quantitative: Quantitative analysis seeks to establish the amount of a given element or compound in a sample.

Quantitative chemical analysis is an important tool to assure that the raw material used and the intermediate products meet the required specifications. Drug analysis is the base for the determination of the product.

Every year number of drugs is introduced into the market. Also quality is important in every product or service but it is vital in medicines as it involves life (K.A. Connors 1994).

Need For Drug Analysis

The number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing one (J. Swarbrick 1998).

Very often there is a time delay from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias.

Quality control is a concept, which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different stage of production. The decision to release or reject a product is based on one or more type of control action. Providing simple analytical procedure for complex formulation is a matter of most importance.

In brief, the reasons for the development of newer methods of drug analysis are

- The drug or drug combination may not be official in any pharmacopoeias,

- A proper analytical procedure for the drug may not be available in the literature due to patent regulations,
- Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipients,
- Analytical methods for the quantitation of the drug in biological fluids may not be available,
- Analytical methods for a drug in combination with other drugs may not be available,
- The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable.

Varieties of analytical methods are used for the analysis of drugs in bulk, formulations and biological samples. In pharmaceutical industry, spectrophotometric and chromatographic methods have gained the significance in recent years (J. Mendham 2004).

Importance of Analytical Methods

Drug analysis reveals identification characterization & determination of the drugs in mixtures like dosage forms & biological fluids. The number of drugs introduced in to the market has been increasing at very fast rate. These drugs may be either new entities in the market or partial structural modification of the existing drugs (B.K. Sharma 2004). Newer analytical methods are developed for these drugs or drug combination of the below reasons: -

1. Official pharmacopoeia may not reveal an analytical procedure for the drugs or its combination.
2. Analytical method may not be available for the drug combination due to interference caused by excipients.

1.1. Spectrophotometry

Analysts have developed large number of instrumental techniques and these techniques are extremely sensitive and can yield results rapidly to a high degree of accuracy. Among these instrumental analytical techniques, spectrophotometric technique occupies a unique position, because of its simplicity, sensitivity, accuracy and rapidity. Spectrophotometry is a branch, which embraces the measurement of absorption of radiation energy of definite and narrow wavelength approximating monochromatic radiations by chemical species (G.R. Chatwal 2003).

While relatively simple in concept, determining the reflectance or transmittance involves careful consideration of the geometrical and spectral conditions of the measurement. The national scales for reflectance and transmittance in the ultraviolet, visible and near infrared spectral regions arise from 200 nm to 2500 nm. The availability of spectrophotometer made this technique indispensable to the modern analytical chemists.

Ultraviolet-Visible Absorption Spectrophotometry

This deals with the absorption of electromagnetic radiation in the wavelength region of 160 to 780 nm. UV absorption spectrophotometry deals with absorption of light by a sample in the Ultra Violet (UV) region (190-380 nm) while visible region (380-780 nm) absorption spectrophotometry (colorimetry) deals with absorption of light by a sample in the visible region (380-780 nm).

Origin and Theory of Ultraviolet Spectra

All atoms and molecules are capable of absorbing energy in accordance with certain restriction, these limitations depending upon the structure of the substance. The kind and amount of radiation absorbed by a molecule depend upon the structure of the molecule and its interaction with the radiation. (H.H. Willard 1996).

Ultraviolet absorption spectra arise from transition of electrons or electron with in a molecule or an ion form a lower to a higher electronic energy and the ultraviolet emission spectra arise from the reverse type of transition for radiation to cause electronic excitation it must be in the UV region of the electromagnetic spectrum.

When a molecule absorbs ultraviolet radiation of frequency v sec^{-1}, the electron in the molecules undergoes transition from a lower to a higher energy level or molecular orbital, the energy difference is given by

$$E=h\upsilon \qquad \textbf{Equation 1}$$

Where,

E = Energy of the photon

υ = Frequency of the monochromatic radiation

h = Plank's constant.

Types of Transition in Organic Molecules

Energy absorbed in the ultraviolet region by complex organic molecules causes transition of valance electron in the molecules (L. Pavia, 2011).

These transitions are in the following order,

$$n \rightarrow \pi^* < \pi \rightarrow \pi^* < n \rightarrow \sigma^* < \sigma \rightarrow \sigma^*$$

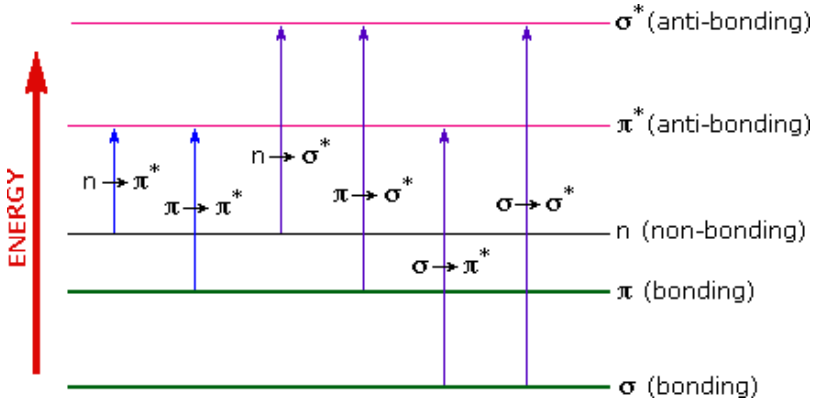


Figure 1: Energy Levels of Electronic Transitions.

Both organic and inorganic molecules may exhibit absorption and emission of UV-VIS radiation. Molecular groups that absorb visible or UV light are called ***chromophores***.

An auxochrome is a substituent that contains unshared (non-bonding) pair of electrons, such as OH, NH and halogens. An auxochrome attached to a chromophore with π electrons shifts the absorption maximum to longer wavelengths.

A shift to longer wavelengths is called a ***bathochromic shift or red shift***. A shift to shorter wavelengths is called ***hypsochromic shift or blue shift***. An increase in the intensity of an absorption band (i.e., an increase in ε_{max}) is called ***hyperchromism*** and a decrease in intensity is called ***hypochromism.***

Absorption Law

The fundamental law that governs the quantitative spectrophotometric analysis is the Beer-Lambert's law which is stated as; (G.R. Chatwal 2003)

"*When a beam of monochromatic light is passed through a transparent cell containing a solution of an absorbing substance, reduction of intensity of the light may occurs; the rate of reduction in intensity with the thickness of the medium is proportional to the intensity of the light and the concentration of the absorbing substances".*

Mathematically Beer-Lamberts law is expressed as;

A = a b c **Equation 2**

Where,

A = absorbance or optical density,

a = absorptivity or extinction coefficient,

b = path length of radiation through sample (cm),

c = concentration of solute in solution.

Light absorption in the UV - Visible region causes the transition of electron from ground state to excited state. The important consequences of rapid relaxation of the excited states are not appreciably distributed by absorption of light energy from any source.

Therefore, the fraction of light absorbed from an incident beam is independent of the intensity which is integrated to obtain Beer's and Lambert's law.

Deviations from Beer-Lambert's Law

As per the Beer's law discussed above, there is a direct proportionality between the absorbance and concentration. A plot of absorbance versus concentration is expected to be a straight line passing through origin. However, this is not always true; there are certain limitations.

The law does not hold for all species under every condition. Many a times instead of a straight line, a curvature in the plot may be observed as shown in **figure 1.2.**

The upward curvature, curve (a), is known as **positive deviation** and the downward curvature, curve(c), as **negative deviation**.

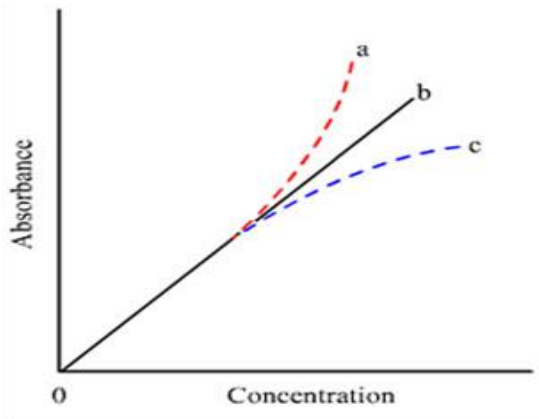


Figure 2: Beer-Lambert's Law Plot; the Curvatures Show Deviations from the Law.

Spectrophotometers

A spectrophotometer can be either *single beam* or *double beam*

1. Single Beam Spectrophotometer

In a single beam instrument all of the light passes through the sample cell. It contains monochromators and phototube as detectors and design to measure % transmittance or absorbance.

This was the earliest design, but is still in common use in both teaching and industrial labs.

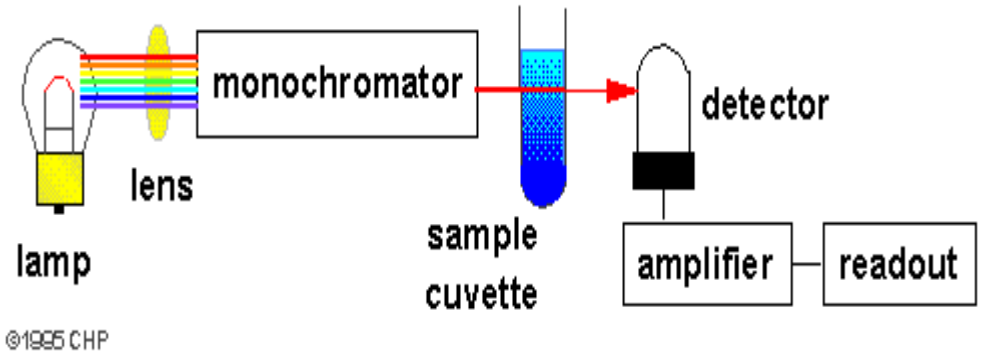


Figure 3: Schematic Diagram of Single Beam Spectrophotometer.

2. Double Beam Spectrophotometer

Two sources are required to scan the entire UV-VIS band:

- Deuterium lamp
- Hydrogen lamp

In a double-beam instrument, the light is split into two beams before it reaches the sample. One beam is used as the reference; the other beam passes through the sample.

The reference beam intensity is taken as 100% Transmission (or 0 Absorbance), and the measurement displayed is the ratio of the two beam intensities.

Double beam instruments became quite popular in the early days of spectrophotometry due to the instability of light sources, detectors, and the associated electronics (Y.R. Sharma 2011).

The figure below illustrates the general configuration of a double beam spectrophotometer.

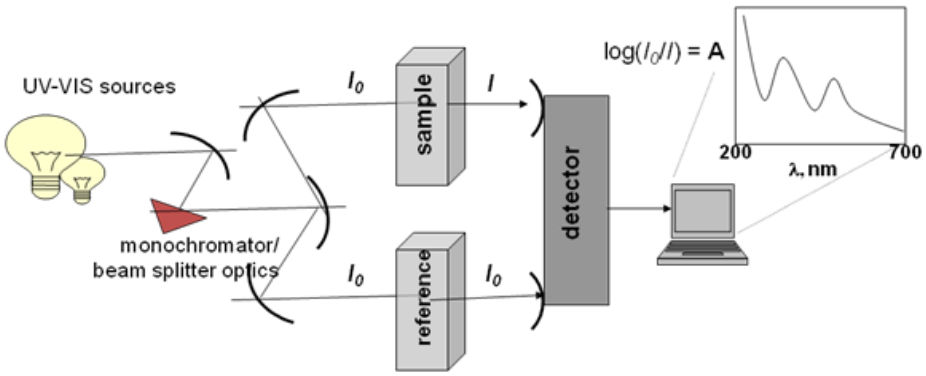


Figure 4: Schematic Diagram of Double Beam Spectrophotometer.

Terms used in absorption spectroscopy

1. Transmittance (T)

It is the ratio of intensity of transmitted light to that of incident light.

$$T = I_t / I_o \qquad \text{Equation 3}$$

2. Absorbance (A)

It is the negative logarithm of transmittance to the base 10.

$$A = -\log_{10} T$$

$$= -\log_{10} I_0/I_t \qquad \text{Equation 4}$$

3. Molar Absorptivity (ε)

When concentration c in equation A= abc is expressed in mole/lit and cell length in ‘cm then Absorptivity is called as molar absorptivity.

$$\varepsilon = A/bc \qquad \text{Equation 5}$$

Spectrophotometric Assay Methods

Single Component Analysis

When the absorption of each of a series of solutions of the same substance are measured at the same wavelength, temperature and solvent conditions, a graph of absorbance measured can be plotted against its concentration. If the graph is a straight line passing through the origin, then it is said to obey Beer’s law over that concentration range. The slope of the line is equal to “ab”, where “b” is the internal path length of the sample cell in cms and “a” is the absorptivity calculated which is constant.

The concentration of a component in a sample which contains other absorbing substances

may be determined by a simple spectrophotometric measurement of absorbance as described above, provided that the other components have a sufficiently small or negligible absorbance at the wavelength of measurement.

Once this is determined, the analysis of known samples of this substance can be easily done under the same experimental conditions. The absorbance is measured and from the Beer's plot, the unknown concentration can be calculated (A.H. Beckett 2002).

Multicomponent Analysis

Now a day's multicomponent formulation are finding their place in clinical therapy. Hence there is a need to develop new methods to analyze the drugs simultaneously. A brief review of the methods used for multicomponent analysis is discussed. The absorbance of a solution is the sum of absorbances of the individual components, or the measured absorbance is the difference between the total absorbance of the solution in the sample cell and that of the solution in the reference (blank) cell.

Following are the spectrophotometric methods

1. Assay Using Absorbance Corrected for Interference/ Corrective Absorbance
2. Simultaneous Equation Method
3. Absorbance Ratio Method
4. Geometric Correction Method
5. Orthogonal Polynomial Method
6. Difference Spectrophotometry
7. Derivative Spectrophotometry
8. Dual Wavelength method
9. Ratio Derivative method
10. Area under Curve Method

Applications

UV/Vis spectrophotometry is routinely used in analytical chemistry for the quantitative determination of different analytes, such as transition metal ions, highly conjugated organic compounds, and biological macromolecules.

1. Solutions of transition metal ions can be colored (i.e. absorb visible light) because d electrons within the metal atoms can be excited from one electronic state to another. The color of metal ion solutions is strongly affected by the presence of other species, such as

certain anions or ligands. For instance, the color of a dilute solution of copper sulphate is a very light blue; adding ammonia intensifies the color and changes the wavelength of maximum absorption (λ_{max}).

2. Organic compounds, especially those with a high degree of conjugation, also absorb light in the UV or visible regions of the electromagnetic spectrum. The solvents for these determinations are often water for water soluble compounds, or ethanol for organic-soluble compounds. Organic solvents may have significant UV absorption; not all solvents are suitable for use in UV spectroscopy. Ethanol absorbs very weakly at most wavelengths. Solvent polarity and pH can affect the absorption spectrum of an organic compound.
3. The Beer-Lambert law states that the absorbance of a solution is directly proportional to the concentration of the absorbing species in the solution and the path length. Thus, for a fixed path length, UV/Vis spectroscopy can be used to determine the concentration of the absorber in a solution. It is necessary to know how quickly the absorbance changes with concentration. This can be taken from references (tables of molar extinction coefficients), or more accurately, determined from a calibration curve.
4. A UV/Vis spectrophotometer may be used as a detector for HPLC. The presence of an analyte gives a response assumed to be proportional to the concentration. For accurate results, the instrument's response to the analyte in the unknown should be compared with the response to a standard; this is very similar to the use of calibration curves. The response (e.g., peak height) for a particular concentration is known as the response factor.

1.2. Chromatography

Russian botanist Michael Tswett invented chromatography as a separation technique. He described in detail the separation of pigments, the colored substances by filtration through column, followed by developments with pure solvents.

Recently, the IUPAC has defined chromatography as; (D.A. Skoog 2005)

"Methods used primarily for the separation of the components of a sample, in which the components are distributed between two phases, one of which is stationary while other moves. The stationary phase may be a solid or a liquid supported on a solid or a gel, and may be packed in a column, spread as a layer or distributed as a film. The mobile phase may be gaseous or liquid".

Chromatography is Mainly Divided into Two Categories

1. Adsorption Chromatography

Separation is mainly due to the interaction between solute and surface on the adsorbent. In this, stationary phase is solid and mobile phase is liquid.

e.g: TLC, HPTLC, and GC

2. Partition Chromatography

Separation is based on the partition between two phases. In this mode, both stationary phase and mobile phase are liquids

e.g: HPLC, GLC, and PC.

Partition chromatography can be divided into ***normal phase and reverse- phase chromatography***.

In ***Normal Phase Chromatography***, the stationary bed is strongly polar in nature (e.g., Silica gel), and the mobile phase is nonpolar (such as n-hexane or tetrahydrofuran). Polar samples are thus retained on the polar surface of the column packing longer than less polar materials.

In ***Reverse Phase Chromatography***, the stationary bed is non-polar (hydrophobic) in nature, while the mobile phase is polar liquid, such as mixtures of water and methanol or acetonitrile. Here the more nonpolar the material is, the longer it will be retained.

High Performance Liquid Chromatography (HPLC)

The typical HPLC separation is based on the selective distribution of analytes between a liquid mobile phase and an immiscible stationary phase. The sample is first introduced by means of an injection port into the mobile phase stream that is delivered by a high-pressure pump (A.H. Beckett 2002).

Next, the components of this sample mixture are separated on the column, a process monitored with a flow-through detector as the isolated components emerge from the column.

The HPLC is classified into two modes depending on the relative polarity of the two phases *viz*. **normal and reverse- phase chromatography.**

There are two elution types in HPLC they are **isocratic** and **gradient**. In **isocratic** elution composition of solvent is pumped through the column during complete analysis. In **gradient**

system eluent composition and strength is steadily changed during the run (G. G. Alfonso 2006).

Instrumentation and Principle of HPLC

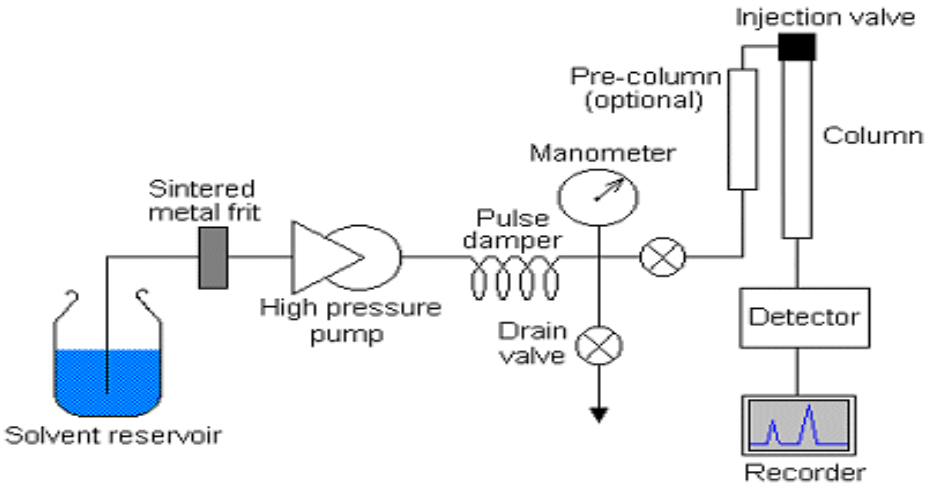


Figure 5: Schematic Diagram of Instrumentation of HPLC.

The Various Components of HPLC Equipment are

1. Reservoir - that holds the mobile phase.
2. Pumps - A & B to pump the mobile phase.
3. Sample injection - to inject the sample.
4. Columns - that contains the stationary phase.
5. Detectors - to detect the results.
6. Recorder – to display the results.

Quantification

Quantitative analysis using chromatography is based on calibration curves obtained from each of the substances analyzed. Calibration is needed in all those cases in which a signal related to mass or concentration of a component in mixture, is obtained. Chromatographic test methods use either external or internal standards for quantification (A.V. Kasture 2002).

1. External Standard Method

An external standard method is used when the standard is analyzed on a separated chromatogram from the sample. Quantification is based on a comparison of the peak area / height (HPLC or GC) or spot intensity (TLC) of the sample to that of a reference standard of the analyte of interest.

The external standard method is more appropriate for samples as follows:

- Samples with a single target concentration and narrow concentration range, e.g., acceptance and release tests. Simple sample preparation procedure.
- Increased baseline time for detection of potential extraneous peaks, e.g., impurities test.

2. Internal Standard Method

With an internal standard method, compound of known purity that does not cause interference in the analysis is added to the sample mixture. Quantification is based on the response ratio of compound of interest to the internal standard vs. the response ratio of a similar preparation of the reference standard (HPLC or GC). This technique is rarely used for TLC methods.

3. Standard Addition Method

When matrix interactions are found to be important, a standard addition method may prove useful. In this method, a known quantity of standard was added to unknown compound. But it is not much accurate. Although CDER does not specify whether the method must use an internal or external standard for quantification, it is commonly observed that HPLC methods for release and stability. The working concentration is the target concentration of the compound of interest as described in the method (P.D. Sethi 2001).

System Suitability Specifications

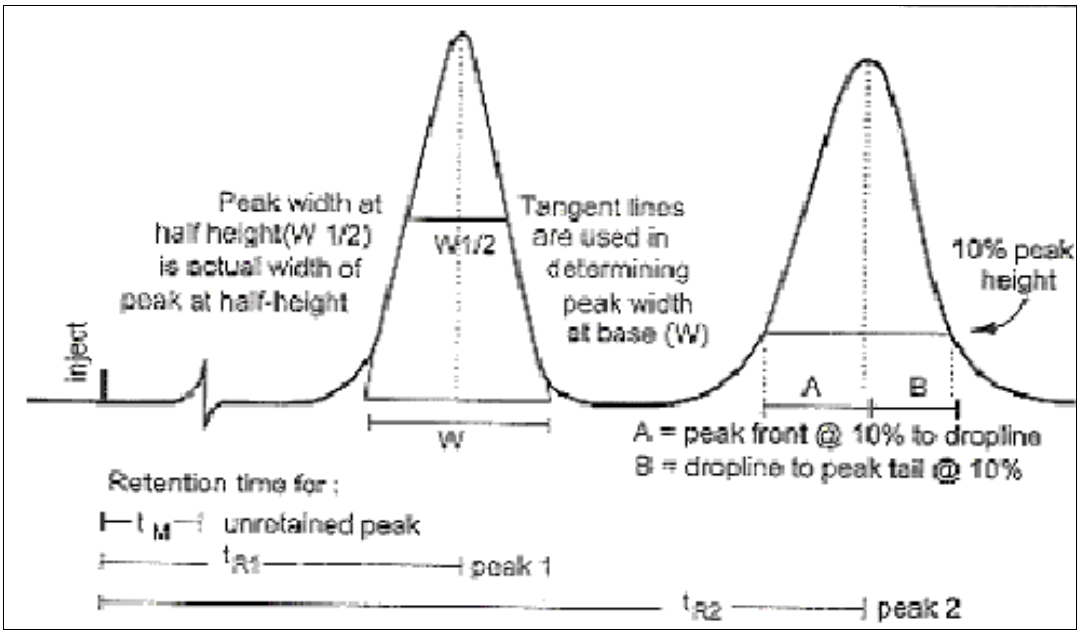


Figure 6: Typical Chromatogram with Examples of Retention Measurements.

Where,

W_x = Width of the peak determined at either 5% or 10% above baseline

f = Distance between peak maximum and peak front at W_x

t_o = Elution time of void volume or non-retained components

t_r = Retention time of the analyte, R

t_w = Peak width measured at baseline of the extrapolated straight sides to baseline.

$k' = (t_R - t_o) / t_o$.

a) Capacity Factor (k')

The capacity factor is a measure of location of the peak of interest with respect to the void volume, i.e., elution time of non-retained components.

$$k' = (t_R - t_o) / t_o \qquad \text{Equation 6}$$

b) Resolution (R_S)

R_S is the measure of how well two peaks are separated for reliable quantification, well-separated peaks are essential for quantification. The closest potential eluting peak to the analyte should be selected R_S is minimally influenced by the ratio of two compounds being measured.

$$R_S = (t_{R2} - t_{R1}) / 0.5(w_1 + w_2) \qquad \text{Equation 7}$$

c) Tailing Factor (T)

The accuracy of quantification decreases with increase in peak tailing as the integrator encounters difficulty in determining where and when the peak ends. Hence, the calculation of the area under the peak differs with tailing. If the integrator is unable to determine exactly when an up slope or down slope occurs, accuracy drops.

$$T = W_x / 2f \qquad \text{Equation 8}$$

d) Theoretical Plate Number (N)

Theoretical plate number is a measure of column efficiency, i.e., the number of peaks located per unit run time of the chromatogram.

$$N = 16 (t_R/w)^2 = L / H \qquad \text{Equation 9}$$

N is fairly constant for each peak on a chromatogram with a fixed set of operating conditions. H or HETP, the height equivalent of a theoretical plate, measures the column efficiency per unit length (L) of the column.

Parameters, which can affect N or H, include peak position, particle size in column, flow-rate of mobile phase, column temperature, viscosity of mobile phase, and molecular weight of the analyte.

e) Dead Volume

Dead Volume means any empty space or unoccupied volume, the presence of which can lead to disastrous losses in efficiency. There will be dead volume in the column itself, which will be the space that is not occupied by the stationary phase.

The other sources of dead volume are the injection unit, the tubing and fittings at each end of the column, and the detector cell.

f) Retention Time (R_t)

Retention time is the difference in time between the point of injection and appearance of peak maxima. Retention time is the time required for 50% of a component to be eluted from a column. Retention time is measured in minutes of seconds. Retention time is also proportional to the distance moved on a chart paper, which can be measured in cm or mm.

g) Retention Volume (V_r)

Retention volume is the mobile required to elute 50% of the component from the column. It is the product of retention time and flow rate (Phyllis 1998).

Retention Volume = Retention time x flow rates Equation 10

Table 1: System Suitability Parameters and Recommendations.

Parameter	Recommendation
Capacity Factor (k')	The peak should be well-resolved from other peaks and the void volume, generally k'>2.0
Repeatability	RSD $\leq$ 1% for N $\geq$ 5 is desirable.
Relative Retention	Not essential as long as the resolution is stated.
Resolution (R_s)	R_s of > 2 between the peak of interest and the closest eluting potential interferent (impurity, excipient, degradation product, internal standard, etc.
Tailing Factor (T)	T of $\leq$ 2
Theoretical Plates (N)	N > 2000

Applications of HPLC

1) Preparative HPLC

It refers to the process of isolation and purification of compounds. Importance is the degree of solute purity and the throughput, which is the amount of compound, produced per unit time.

2) Analytical HPLC

Here the focus is to obtain information about the sample compound, which includes relative comparison, quantification and resolution of a compound.

3) Identification

For this purpose a clean peak of known sample assay has to be observed from the chromatogram. Selection of column mobile phase and flow rate matter to certain level in this process by comparing with reference compound does identification and it can be assured by combining two or more detection methods (L.R. Synder 1979).

4) Chemical Separation

This can be accomplished using HPLC by utilizing the fact that, certain compounds have different migration rates given at a particular column and mobile phase. The extent or degree of separation is mostly determined by the choice of stationary phase and mobile phase.

5) Quantification

It is used to estimate the concentration (potency) of API as well as dosage formulation by using the known reference standards Quantification of known and unknown areas with respect to the principal peak by various methods like:

- Area normalization method.
- Internal standard method
- External standard method.

1.3. Analytical Method Development

Methods are developed for new products when no official methods are available. Alternate methods for existing products are developed to reduce the cost and time for better precision and ruggedness. Trial runs are conducted, method is optimized and validated. When alternate method proposed is intended to replace the existing procedure comparative laboratory data including merit / demerits are made available (M.E. Schartz 2004).

Steps of Method Development

Documentation starts at the very beginning of the development process, a system for full documentation of the development studies must be established. All data relating to these studies must be recorded in laboratory notebook or an electronic database.

1. Analyte Standard Characterization

a) All known information about the analyte and its structure is collected i.e., physical and chemical properties.
b) The standard analyte (≈100% purity) is obtained. Necessary arrangement is made for the proper storage (refrigerator, desiccators and freezer).
c) When multiple components are to be analyzed in the sample matrix, the number of components is noted, data is assembled and the availability of standards for each one is determined.
d) Only those methods (Spectroscopic, MS, GC, HPLC etc.,) that are compatible with sample stability are considered.

2. Method Requirements

The goals or requirements of the analytical method that need to be developed are considered and the analytical figures of merit are defined. The required detection limits, selectivity, linearity, range, accuracy and precision are defined.

3. Literature Search and Prior Methodology

The literature for all types of information related to the analyte is surveyed. For synthesis, physical and chemical properties, solubility and relevant analytical methods, books, periodicals, chemical manufacturers and regulatory agency compendia such as USP / NF, AOAC and ASTM publications are reviewed. Chemical Abstracts Service (CAS) automated computerized literature searches are convenient.

4. Choosing a Method

a) Using the information in the literatures and prints, methodology is adapted. The methods are modified wherever necessary. Sometimes it is necessary to acquire additional instrumentation to reproduce, modify, improve or validate existing methods for in-house analytes and samples.
b) If there are no prior methods for the analyte in the literature, from analogy, the compounds that are similar in structure and chemical properties are investigated and are

worked out. There is usually one compound for which analytical method already exist that is similar to the analyte of interest.

5. Instrumental Setup and Initial Studies

The required instrumentation is setup. Installation, operational and performance qualification of instrumentation using laboratory standard operating procedures (SOP's) are verified.

Always new consumables (e.g. solvents, filters and gases) are used, for example, method development is never started, on a HPLC column that has been used earlier.

The analyte standard in a suitable injection / introduction solution and in known concentrations and solvents are prepared. It is important to start with an authentic, known standard rather than with a complex sample matrix.

If the sample is extremely close to the standard (e.g., bulk drug), then it is possible to start work with the actual sample.

6. Optimization

During optimization one parameter is changed at a time, and set of conditions are isolated, rather than using a trial and error approach. Work has been done from an organized methodical plan, and every step is documented (in a lab notebook) in case of dead ends.

7. ***Documentation of Analytical Figures of Merit***

The originally determined analytical figures of merit limit of quantitation (LOQ), Limit of detection (LOD), linearity, time per analysis, cost, sample preparation etc., are documented.

8. Evaluation of Method Development with Actual Samples

The sample solution should lead to unequivocal, absolute identification of the analyte peak of interest apart from all other matrix components.

9. Determination of Percent Recovery of Actual Sample and Demonstration of Quantitative Sample Analysis

Percent recovery of spiked, authentic standard analyte into a sample matrix that is shown to contain no analyte is determined. Reproducibility of recovery (average +/- standard deviation) from sample to sample and whether recovery has been optimized has been shown.

It is not necessary to obtain 100% recovery as long as the results are reproducible and known with a high degree of certainty.

The validity of analytical method can be verified only by laboratory studies. Therefore documentation of the successful completion of such studies is a basic requirement for determining whether a method is suitable for its intended applications.

1.4. Analytical Method Validation

Method validation can be defined as (International Conference of Harmonization) "Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics" (ICH Q2R1, 2005).

Method validation is an integral part of the method development; it is the process by which a method is tested by the developer or user for reliability, accuracy and preciseness of its intended purpose and demonstrating that analytical procedures are suitable for their intended use that they support the identity, quality, purity, and potency of the drug substances and drug products.

Data thus generated become part of the methods validation package submitted to Center for Drug Evaluation and Research (CDER). Simply, method validation is the process of proving that an analytical method is acceptable for its intended purpose.

Methods should be reproducible when used by other analysts, on other equivalent equipment, on other days or locations, and throughout the life of the drug product. Data that are generated for acceptance, release, stability, or pharmacokinetic will only be trustworthy if the methods used to generate the data are reliable.

The process of validation and method design also should be clearly in the development cycle before important data are generated. Validation should be on going in the form of re-validation with method changes.

Advantages of Analytical Method Validation

The biggest advantage of method validation is that it builds a degree of confidence, not only for the developer but also to the user. Although the validation exercise may appear costly and

time consuming, it results inexpensive, eliminates frustrating repetitions and leads to better time management in the end.

Minor changes in the conditions such as reagent supplier or grade, analytical setup are unavoidable due to obvious reasons but the method validation absorbs the shock of such conditions and pays for more than invested on the process.

Guidelines for Analytical Method validation

For pharmaceutical method guidelines are prescribed by

- United states Pharmacopoeia (USP) (USP NF, 2004)
- Food and Drug Administration (FDA)
- World Health Organization (WHO)
- International Conference on Harmonization (ICH)

These Guidelines provide a framework for performing validation. In general, methods for routine analysis, standardization or regulatory submission must include studies on specificity, linearity, accuracy, precision, range detection limit, quantitation limit and robustness.

Validation Parameters

These parameters are termed "analytical performance characteristics" or sometimes "analytical figures of merit." The various validation parameters are: (ICH Q2B, 1996)

1) Accuracy,
2) Precision (Repeatability and Reproducibility),
3) Linearity and Range,
4) Limit of detection (LOD) / Limit of quantitation (LOQ),
5) Selectivity / Specificity,
6) Robustness / Ruggedness,
7) Stability and System suitability studies.

(1) Accuracy

The accuracy of an analytical method may be defined as a closeness of the test results obtained by the method to the true value. It is the measure of the exactness of the analytical method developed. Accuracy may often express as percent recovery by the assay of a known amount of analyte added.

Accuracy may be determined by applying the method to samples or mixtures of excipients to which known amount of analyte have been added both above and below the normal levels expected in the samples. Accuracy is then calculated from the test results as the percentage of the analyte recovered by the assay.

Dosage form assays commonly provide accuracy within 3-5% of the true value. The ICH documents recommend that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels, covering the specified range (i.e. three concentrations and three replicated of each concentration).

(2) Precision

The precision of an analytical method is the degree of agreement among individual test results, when the method is applied repeatedly to multiple samplings of homogenous samples. This is usually expressed as the standard deviation or the relative standard deviation (coefficient of variation). Precision is a measure of the degree of reproducibility or of the repeatability of the analytical method under normal operating circumstances.

Repeatability involves analysis of replicates by the analyst using the same equipment and method and conducting the precision study over short period of time while reproducibility involves precision study at Different Occasions, Different Laboratories, Different Batch of Reagent, Different Analysts and Different Equipments.

Determination of Repeatability: - Repeatability can be defined as "the precision of the procedure when repeated by same analyst under the same operating conditions (same reagents, equipments, settings and laboratory) over a short interval of time".

It is normally expected that at least six replicates be carried out and a table showing each individual result provided from which the mean, standard deviation and co-efficient of variation should be calculated for set of n value. The RSD values are important for showing degree of variation expected when the analytical procedure is repeated several time in a standard situation. (RSD below 1% for built drugs, RSD below 2% for assays in finished product).

The ICH documents recommend that repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure (i.e. three concentrations

and three replicates of each concentration or using a minimum of six determinations at 100 % of the test concentration).

Determination of Reproducibility: - Reproducibility means the precision of the procedure when it is carried out under different conditions usually in different laboratories on separate, putatively identical samples taken from the same homogenous batch of material. Comparisons of results obtained by different analysts, by the use of different equipments, or by carrying out the analysis at different times can also provide valuable information.

(3) Linearity and Range

The linearity of an analytical method is its ability to elicit test results that are directly (or by a well-defined mathematical transformation) proportional to the analyte concentration in samples within a given range. Linearity usually expressed in terms of the variance around the slope of regression line calculated according to an established mathematical relationship from test results obtained by the analysis of samples with varying concentrations of analyte.

The linear range of detectability that obeyed Beer's law is dependent on the compound analysed and the detector used. The working sample concentration and samples tested for accuracy should be in the linear range. The claim that the method is linear is to be justified with additional mention of zero intercept by processing data by linear least square regression. Data is processed by linear least square regression declaring the regression co-efficient and b of the linear equation $y = mx + c$ together with the correlation coefficient of determination r^2. For the method to be linear the r^2 value should be close to 1.

The range of an analytical method is the interval between the upper and lower levels of the analyte (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written.

(4) Limit of Detection and Limit of Quantitation

Limit of Detection: - The limit of detection is the parameter of limit tests. It is the lowest level of analyte that can be detected, but not necessarily determined in a quantitative fashion, using a specific method under the required experimental conditions. The limit test thus merely substantiates that the analyte concentration is above or below a certain level.

The determination of the limit of detection of instrumental procedures is carried out by determining the signal-to-noise ratio by comparing test results from the samples with known

concentration of analyte with those of blank samples and establishing the minimum level at which the analyte can be reliably detected.

A signal-to-noise ratio of 2:1 or 3:1 is generally accepted. The signal-to-noise ratio is determined by dividing the base peak by the standard deviation of all data points below a set threshold. Limit of detection is calculated by taking the concentration of the peak of interest divided by three times the signal-to-noise ratio. For spectroscopic techniques or other methods that rely upon a calibration curve for quantitative measurements, the IUPAC approach employs the standard deviation of the intercept (σ) which may be related to LOD and the slope of the calibration curve, S, by

$$LOD = \frac{3.3\sigma}{S} \qquad \textbf{Equation 11}$$

Limit of Quantitation: - Limit of quantitation is a parameter of quantitative assays for low levels of compounds in sample matrices such as impurities in bulk drugs and degradation products in finished pharmaceuticals. The limit of quantitation is the lowest concentration of analyte in a sample that may be determined with acceptable accuracy and precision when the required procedure is applied.

It is measured by analyzing samples containing known quantities of the analyte and determining the lowest level at which acceptable degrees of accuracy and precision are attainable. Where the final assessment is based on an instrumental reading, the magnitude of background response by analyzing a number of blank samples and calculating the standard deviation of this response. The standard deviation multiplied by a factor (usually 10) provides an estimate of the limit of quantitation. In many cases, the limit of quantitation is approximately thrice the limit of detection.

$$LOQ = \frac{10\sigma}{S} \qquad \textbf{Equation 12}$$

(5) Selectivity and Specificity

The selectivity of an analytical method is its ability to measure accurately and specifically the analyte of interest in the presence of components that may be expected to be present in the sample matrix.

If an analytical procedure is able to separate and resolve the various components of a mixture and detect the analyte qualitatively the method is called selective. On the other hand, if the

method determines or measures quantitatively the component of interest in the sample matrix without separation, it is said to be specific.

Hence one basic difference in the selectivity and specificity is that, while the former is restricted to qualitative detection of the components of a sample, the latter means quantitative measurement of one or more analyte.

(6) Robustness and Ruggedness

Robustness: - The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variation in method parameters and provides an indication of its reliability during normal usage.

Ruggedness:- The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions such as different laboratories, different analysts, using operational and environmental conditions that may differ but are still within the specified parameters of the assay.

(7) Stability and System Suitability Tests

Stability of the sample, standard and reagents is required for a reasonable time to generate reproducible and reliable results. For example, 24 h stability is desired for solutions and reagents that need to be prepared for each analysis.

System suitability test provide the added assurance that on a specific occasion the method is giving, accurate and precise results. System suitability test are run every time a method is used either before or during analysis. The results of each system suitability test are compared with defined acceptance criteria and if they pass, the method is deemed satisfactory on that occasion.

Table 2: Characteristics to be Validated in HPLC.

Characteristics	Acceptance Criteria
Accuracy/trueness	Recovery 98-102% (individual)
Precision	RSD < 2%
Repeatability	RSD < 2%
Intermediate Precision	RSD < 2%
Specificity / Selectivity	No interference
Detection Limit	S/N > 2 or 3
Quantitation Limit	S/N > 10
Linearity	Correlation coefficient $r^2 > 0.999$
Range	80 –120 %

1.5. Statistical Parameters

1. Linear Regression

Once linear relationship has been shown to have a high probability by the value of the correlation coefficient 'r' then the best straight line through the data points has to be estimated. This can be often done by visual inspection of graph but in many cases it is far more sensible to evaluate the best straight line by linear regression. (J. Mendham 2004)

The equation of straight line is

$$Y = mx + c \qquad \textbf{Equation 13}$$

Where, y the dependent variable is plotted as result changing x the independent variable.

To obtain regression line 'y ' on 'x ' the slope 'm' of the line intercept 'c' on the y axis are given by the following formula.

$$m = \frac{N\sum xy - (\sum x)(\sum y)}{N\sum x^2 - (\sum x)^2} \qquad \textbf{Equation 14}$$

$$c = \frac{(\sum y)(\sum x^2) - (\sum x)(\sum xy)}{N\sum x^2 - (\sum x)^2} \qquad \textbf{Equation 15}$$

2. Correlation Coefficient

The correlation co-efficient is used as a measure of the correlation between two variables. When variables x and y are correlated rather than being functionally related. The person correlation co efficient is one of the most convenient to calculate. This is given by

$$r = \frac{n\sum x_1y_1 - \sum x_1y_1}{\{[n\sum x_1^2 - (\sum x_1^2)][n\sum y_1^2 - (\sum y_1^2)]\}^{1/2}} \qquad \textbf{Equation 16}$$

Where n is the number of data points

The maximum, value of r is 1. When this occurs, there is exact correlation between the two variables. When r is zero, there is complete independence of the variables. The minimum value of r is -1. A negative correlation co-efficient indicates that the assumed dependence is opposite to what exists and therefore a positive co-efficient for the revered relation.

The fit must be quite poor before r become smaller than about 0.98 and is really very poor when less than 0.9.

3. Standard Deviation

It is commonly used in statistics as a measure of precision and is more meaning full than is the average deviation. It may be thought of as a root mean square deviation of values from their average and is expressed mathematically as

$$S = \sum(x - \bar{x})^2 / n - 1 \qquad \textbf{Equation 17}$$

Where,

S = Standard deviation

If n is large (50 or more) then of course it of immaterial whether the term in the denomination is n - 1 or n.

Σ = Sum

$\bar{x}$ = Mean or arithmetic average.

$x - \bar{x}$ = Deviation of a value from the mean

N = Number of observations

4. Percentage Relative Standard Deviation (% RSD)

It is also known as co efficient of variation CV. It is defined as the standard deviation (S.D) expressed as the percentage of mean.

$$\%\ RSD = \frac{SD}{\bar{x}} * 100 \qquad \textbf{Equation 18}$$

Where,

SD = Standard deviation

The variance is defined as S^2 and is more important in statistics than S itself. However the latter is much more commonly used with chemical data.

5. Standard Error Of Mean (S.E)

The standard error of mean can be defined as the value obtained by the division of standard deviation by square root of number of observations. It is mathematically expressed as,

$$S.E = \frac{SD}{\sqrt{n}} \qquad \textbf{Equation 19}$$

Where,

SD = Standard deviation.

n = number of observations.

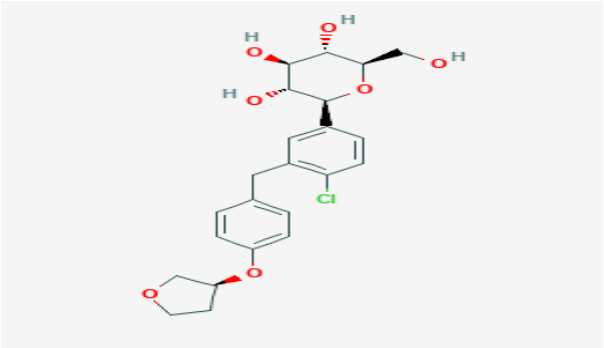
2. DRUG PROFILE

2.1. Drug Profile of Empagliflozin

Description

Empagliflozin is an inhibitor of sodium-glucose co-transporter-2 (SGLT2), the transporters primarily responsible for the reabsorption of glucose in the kidney. It is used clinically as an adjunct to diet and exercise, often in combination with other drug therapies, for the management of type 2 diabetes mellitus.

Structure



Weight

Average: 450.91

Monoisotopic: 450.1445309

Chemical Formula

$C_{23}H_{27}ClO_7$

Synonyms

- Empagliflozin
- Empagliflozina
- Empagliflozine
- Empagliflozinum

PHARMACOLOGY

Indication

Empagliflozin is indicated as an adjunct to diet and exercise to improve glycemic control in adult patients with type 2 diabetes. It is also indicated to reduce the risk of cardiovascular death in adult patients with both type 2 diabetes mellitus and established cardiovascular disease.

Empagliflozin is also available as a combination product with either metformin and Linagliptin as an adjunct to diet and exercise in the management of type 2 diabetes mellitus in adults.

Associated Conditions

- Cardiovascular Mortality
- Type 2 Diabetes Mellitus

Pharmacodynamics

Empagliflozin lowers blood glucose levels by preventing glucose reabsorption in the kidneys, thereby increasing the amount of glucose excreted in the urine. It has a relatively long duration of action requiring only once-daily dosing. Patients should be monitored closely for signs and symptoms of ketoacidosis regardless of blood glucose level as empagliflozin may precipitate diabetic ketoacidosis in the absence of hyperglycemia.

Mechanism of action

The vast majority of glucose filtered through the glomerulus is reabsorbed within the proximal tubule, primarily via SGLT2 (sodium-glucose linked co-transporter-2) which is responsible for ~90% of the total glucose reabsorption within the kidneys. Na^+/K^+-ATPase on the basolateral membrane of proximal tubular cells utilize ATP to actively pump Na+ ions into the interstitium surrounding the tubule, establishing a Na^+ gradient within the tubular cell.

Absorption

Following oral administration, peak plasma concentrations are reached in approximately 1.5 hours (T_{max}). At steady-state, plasma AUC and C_{max} were 1870 nmol·h/L and 259 nmol/L, respectively, following therapy with empagliflozin 10mg daily and 4740 nmol·h/L and 687 nmol/L, respectively, following therapy with empagliflozin 25mg daily. Administration with food does not significantly affect the absorption of empagliflozin.

Volume of distribution

The estimated apparent steady-state volume of distribution is 73.8 L.

Protein binding

Empagliflozin is approximately 86.2% protein-bound in plasma.

Metabolism

Empagliflozin undergoes minimal metabolism. It is primarily metabolized via glucuronidation by 5'-diphospho-glucuronosyltransferases 2B7, 1A3, 1A8, and 1A9 to yield three glucuronide metabolites: 2-O-, 3-O-, and 6-O-glucuronide. No metabolite represented more than 10% of total drug-related material.

Route of elimination

After oral administration of radiolabeled empagliflozin approximately 41.2% of the administered dose was found eliminated in feces and 54.4% eliminated in urine. The majority of radioactivity in the feces was due to unchanged parent drug while approximately half of the radioactivity in urine was due to unchanged parent drug.

Half-life

The apparent terminal elimination half-life was found to be 12.4 h based on population pharmacokinetic analysis.

Clearance

Apparent oral clearance was found to be 10.6 L/h based on a population pharmacokinetic analysis.

Toxicity

Experience with empagliflozin overdose is limited - employ standard symptomatic and supportive measures, as well as gastric decontamination when appropriate. The use of hemodialysis in empagliflozin overdose has not been studied but is unlikely to be of benefit given the drug's relatively high protein-binding.

Affected organisms

- Humans and other mammals

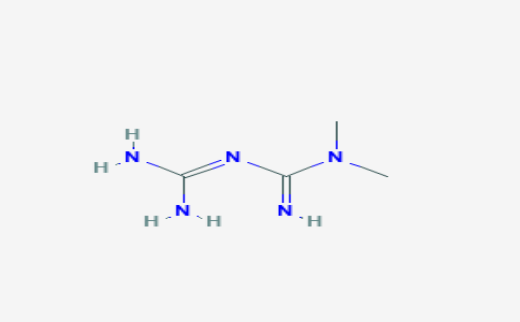
2.2. Drug Profile of Metformin

Description

Metformin is an antihyperglycemic agent of the *biguanide* class, used for the management of type II diabetes. Currently, metformin is the first drug of choice for the management of type II diabetes and is prescribed to at least 120 million people worldwide.

Metformin is considered an antihyperglycemic drug because it lowers blood glucose concentrations in type II diabetes without causing hypoglycemia. Metformin is commonly described as an *insulin sensitizer* leading to a decrease in insulin resistance and a clinically significant reduction of plasma fasting insulin levels. Another well-known benefit of this drug is modest weight loss. Metformin is the drug of choice for obese type II diabetes patients.

Structure



Weight

Average: 129.1636

Monoisotopic: 129.101445377

Chemical Formula

$C_4H_{11}N_5$

Synonyms

- Dimethylbiguanide
- Metformin
- Metformina
- Metformine
- Metforminum

PHARMACOLOGY

Indication

Metformin tablet

Metformin is indicated as an adjunct to diet and exercise to increase glycemic control in *adults and pediatric patients* 10 years of age and older diagnosed with type 2 diabetes mellitus.

Metformin extended-release tablet (XR)

The extended-release form is indicated as an adjunct to diet and exercise to improve glycemic control in only *adults* with type 2 diabetes mellitus. Safety in children has not been determined to this date.

An extended-release combination product containing empagliflozin, linagliptin, and metformin was approved by the FDA in January 2020 for the improvement of glycemic control in adults with type 2 diabetes mellitus when used adjunctively with diet and exercise.

Associated Therapies

- Glycemic Control

Pharmacodynamics

General effects

Insulin is an important hormone that regulates blood glucose levels. Type II diabetes is characterized by a decrease in sensitivity to insulin, resulting in eventual elevations in blood glucose when the pancreas can no longer compensate. In patients diagnosed with type 2 diabetes, insulin no longer exerts adequate effects on tissues and cells (called insulin resistance) and insulin deficiency may also be present.

Effect on fasting plasma glucose (FPG) and Glycosylated hemoglobin (HbA1c)

HbA1c is an important periodic measure of glycemic control that is used to monitor diabetic patients. Fasting plasma glucose is also a useful and important measure of glycemic control. In a 29-week clinical trial of subjects diagnosed with type II diabetes, metformin decreased the fasting plasma glucose levels by an average of 59 mg/dL from baseline, compared to an average increase of 6.3 mg/dL from baseline in subjects taking a placebo Glycosylated hemoglobin (HbA1c) was decreased by about 1.4% in subjects receiving metformin and increased by 0.4% in subjects receiving placebo only.

Mechanism of action

Metformin's mechanisms of action are unique from other classes of oral antihyperglycemic drugs. Metformin decreases blood glucose levels by decreasing hepatic glucose production (gluconeogenesis), decreasing the intestinal absorption of glucose, and increasing insulin sensitivity by increasing peripheral glucose uptake and utilization. It is well established that metformin inhibits mitochondrial complex I activity, and it has since been generally

postulated that its potent antidiabetic effects occur through this mechanism. The above processes lead to a decrease in blood glucose, managing type II diabetes and exerting positive effects on glycemic control.

Absorption

Regular tablet absorption

The absolute bioavailability of a metformin 500 mg tablet administered in the fasting state is about 50%-60%. Single-dose clinical studies using oral doses of metformin 500 to 1500 mg and 850 to 2550 mg show that there is a lack of dose proportionality with an increase in metformin dose, attributed to decreased absorption rather than changes in elimination.

Extended-release tablet absorption

After a single oral dose of metformin extended-release, Cmax is reached with a median value of 7 hours and a range of between 4 and 8 hours. Peak plasma levels are measured to be about 20% lower compared to the same dose of regular metformin, however, the extent of absorption of both forms (as measured by area under the curve - AUC), are similar.

Effect of food

Food reduces the absorption of metformin, as demonstrated by about a 40% lower mean peak plasma concentration (Cmax), a 25% lower area under the plasma concentration versus time curve (AUC), and a 35-minute increase in time to peak plasma concentration (Tmax) after ingestion of an 850 mg tablet of metformin taken with food, compared to the same dose administered during fasting.

Though the extent of metformin absorption (measured by the area under the curve - AUC) from the metformin extended-release tablet is increased by about 50% when given with food, no effect of food on Cmax and Tmax of metformin is observed. High and low-fat meals exert similar effects on the pharmacokinetics of extended-release metformin.

Volume of distribution

The apparent volume of distribution (V/F) of metformin after one oral dose of metformin 850 mg averaged at 654 ± 358 L.

Protein binding

Metformin is negligibly bound to plasma proteins, in contrast to sulfonylureas, which are more than 90% protein bound.

Metabolism

Intravenous studies using a single dose of metformin in normal subjects show that metformin is excreted as unchanged drug in the urine and does not undergo hepatic metabolism (no metabolites have been identified in humans) or biliary excretion.

Route of elimination

This drug is substantially excreted by the kidney.

Renal clearance of metformin is about 3.5 times higher than creatinine clearance, which shows that renal tubular secretion is the major route of metformin elimination. After oral administration, about 90% of absorbed metformin is eliminated by the kidneys within the first 24 hours post-ingestion.

Half-life

Approximately 6.2 hours in the plasma and in the blood, the elimination half-life is approximately 17.6 hours, suggesting that the erythrocyte mass may be a compartment of distribution.

Clearance

Renal clearance is about 3.5 times greater than creatinine clearance, which indicates that tubular secretion is the major route of metformin elimination. Following oral administration, approximately 90% of the absorbed drug is eliminated via the renal route within the first 24 hours.

Toxicity

Metformin (hydrochloride) toxicity data

Oral LD50 (rat): 1 g/kg; Intraperitoneal LD50 (rat): 500 mg/kg; Subcutaneous LD50 (rat): 300 mg/kg; Oral LD50 (mouse): 1450 mg/kg; Intraperitoneal LD50 (mouse): 420 mg/kg; Subcutaneous LD50 (mouse): 225 mg/kg.

A note on lactic acidosis

Metformin decreases liver uptake of lactate, thereby increasing lactate blood levels which may increase the risk of lactic acidosis. There have been reported postmarketing cases of metformin-associated lactic acidosis, including some fatal cases. Such cases had a subtle onset and were accompanied by nonspecific symptoms including malaise, myalgias,

abdominal pain, respiratory distress, or increased somnolence. In certain cases, hypotension and resistant bradyarrhythmias have occurred with severe lactic acidosis.

A note on renal function

In patients with decreased renal function, the plasma and blood half-life of metformin is prolonged and the renal clearance is decreased.

A note on hypoglycaemia

When used alone, metformin does not cause hypoglycemia, however, it may potentiate the hypoglycemic effects of sulfonylureas and insulin when they are used together.

Use in pregnancy

Available data from post-marketing studies have not indicated a clear association of metformin with major birth defects, miscarriage, or adverse maternal or fetal outcomes when metformin was ingested during pregnancy. Despite this, the abovementioned studies cannot definitively establish the absence of any metformin-associated risk due to methodological limitations, including small sample size and inconsistent study groups.

Use in nursing

A limited number of published studies indicate that metformin is present in human milk. There is insufficient information to confirm the effects of metformin on the nursing infant and no available data on the effects of metformin on the production of milk. The developmental and health benefits of breastfeeding should be considered as well as the mother's clinical need for metformin and any possible adverse effects on the nursing child.

Affected organisms

- Humans and other mammals

3. LITERATURE REVIEW

1. J.Archana *et al* (2019) reported Two simple spectrophotometric methods have been developed for the simultaneous estimation of Empagliflozin and Metformin hydrochloride from the tablet dosage form. Method-I simultaneous equation method involves the measurement of absorbances at two wavelengths 224 nm (λ_{max} of Empagliflozin) and 233nm (λ_{max} of Metformin hydrochloride) using Methanol and Water as diluent. Method – II Absorption ratio method involves the measurement of absorbances at two wavelengths 233 nm (λ_{max} of Metformin Hydrochloride) and 266 nm

(λ_{max} of Isobestic point). The linearity lies between 0.1-25 µg/ml for Empagliflozin and 0.5-25 µg/ml Metformin hydrochloride in both methods. The accuracy and precision of the methods were determined and validated statistically. The two methods exhibited good reproducibility and recovery with a relative standard deviation of <2%. Both the methods were found to be rapid, specific, precise, accurate, and reproducible and can be successfully applied for the routine analysis of Empagliflozin and Metformin hydrochloride in a combined dosage form.

2. S. Baokar *et* al (2020) reported that, Worldwide the R & D divisions of Pharma industry are actively involved in the development of new therapeutic agents. These agents may be either new entities or partial structural modification of the existing one. The recent FDA statistics represent that the average number of drug filings are increasing every year in the thrust areas like anti-cancer agents, anti-diabetic, antibiotics, cardio-vascular drugs, respiratory drugs etc. Sodium glucose co-transporter-2(SGLT-2) inhibitors, dipeptidyl peptidase-4 (DPP-4) inhibitors and biguanides are effective oral anti-diabetic agents used in treatment of type 2 Diabetes Mellitus. Therefore, the necessity to explore and compare the existing analytical and bioanalytical assays used for determination of such drugs either single or in combination is crucial. Many methods were reported in the literature for the bio-analysis and analysis of four novel gliptins combinations, empagliflozin-linagliptin, empagliflozin-metformin HCl, linagliptin-metformin HCl, empagliflozin-linagliptin-metformin HCl combination with application on Glyxambi®, Synjardy®, Jentadueto®, Trijardy® XR tablets respectively. Furthermore, this review offered an overview of different methods used for determination of every drug alone as empagliflozin from SGLT-2 inhibitors, linagliptin from DPP-4 inhibitors and metformin from biguanides in a tabulated comparative way. Moreover, the current review emphasizes the most common stability indicating assays to be of interest to the analysts in the area of drug control. This review helps in understanding the further need for the development of analytical methods for the estimation of such drugs.

3. Deepak patil *et al* (2017) reported an accurate, precise and reproducible high performance liquid chromatographic method was developed for quantitative estimation of Metformin and teneligliptin simultaneously in tablet dosage forms. Younglin (S.K.) gradient System UV Detector and C8 (Agilent) column with 250mm x4.6 mm i.d. and 5µm particle size. Methanol : water 0.05 % OPA (50:50) was used as the mobile phase for the method. The

detection wavelength was 235 nm and flow rate was 0.7 ml/min. In the developed method, the retention time of Metformin and Teneligliptin were found to be 2.1 min and 7.6 min. The developed method was validated according to the ICH guidelines. The linearity, precision, range, robustness was within the limits as specified by the ICH guidelines. Hence the method was found to be simple, accurate, precise, economic and reproducible. So the proposed methods can be used for the routine quality control analysis of Metformin and Teneligliptin simultaneously in tablet dosage forms.

4. A.Geetha susmitha *et al* (2019)reported the objective of this study was to develop and validate a stability-indicating reverse-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of the metformin and empagliflozin in tablet dosage forms. Methods: The chromatographic conditions were optimized and it was run through Std. BDS (250 mm × 4.6 mm, 5 m) column with mobile phase consisting of 0.1% orthophosphoric acid buffer: acetonitrile in the ratio of 50:50. The flow rate was 1 ml/min and optimized wavelength was 210 nm. Temperature was maintained at 30°C. Results: The retention times of metformin and empagliflozin were found to be 2.588 min and 3.679 min and percentage relative standard deviation (RSD) of the metformin and empagliflozin was found to be 0.59 and 1.2, respectively. Percentage recovery was in the range of 100.01–100.65% for metformin and empagliflozin, respectively. Conclusion: A sensitive, rapid, and specific method has been developed for the simultaneous estimation of metformin and empagliflozin using RP-HPLC in tablet dosage form.

5. Ramesh jayaprakash *et al* (2017) reported that the present study was aimed to develop a rapid, accurate, linear, sensitive and validate stability-indicating high performance liquid chromatographic [RP-HPLC] method for determination of vildagliptin and metformin in pharmaceutical dosage form. Methods: The chromatographic separation was performed on kromasil-C18 column [4.5 x 250 mm; 5 µm] using a mobile phase consisting of 0.05 mmol potassium dihydrogen phosphate buffer: acetonitrile [80:20 v/v], [pH adjusted to 3.5 using orthophosphoric acid]. The flow rate is 0.9 ml/min and the detection was carried out at 263 nm. Results: The chromatographic condition, the peak retention time of metformin and vildagliptin were found to be 2.215 min and 2.600 min respectively. Stress testing was performed in accordance with an international conference on harmonization [ICH] Q1A R2 guidelines. The method was validated as per ICH Q2 R1 guidelines. The calibration curve was found to be linear in the concentration range of 5-17.5 µg/ml and

50-175 µg/ml for vildagliptin and metformin. The limit of detection and quantification was found to be 0.0182 µg/ml and 0.0553 µg/ml for vildagliptin and 0.4451 µg/ml and 1.3490 µg/ml for metformin respectively. Conclusion: A new sensitive, simple and stability indicating reverse-phase high-performance liquid chromatography [RP-HPLC] method has been developed and validated for the determination of vildagliptin and metformin. The proposed method can be used for routine determination of vildagliptin and metformin.

6. Shyamala M *et al* (2016) reported the present work describes the development and following validation of a stability indicating reverse phase HPLC (RP-HPLC) method for the analysis of Empagliflozin in its API. The proposed method utilizes Hypersil BDS column Mobile phase 0.1% OPA: Acetonitrile in the ratio of 70:30 and flow rate was maintained at 1ml/min, detection wave length was 233nm, column temperature was set to 30°C. The developed method was successfully validated for different validation parameters as per ICH guidelines. The stability of the drug was determined by studying the degradation of the drug under acidic, alkaline, peroxide, neutral, heat and UV conditions.

7. Srikanth M V *et al* (2019) reported that this study presents a rapid, sensitive and accurate high performance liquid chromatographic (HPLC) method for determina-tion of propranolol HCl in human plasma. Human plasma samples were subjected to protein precipitation by methanol and protein free plasma samples were directly injected into HPLC C18 column. Mobile phase consisting of a mixture of acetonitrile: pH 4.5 phosphate buffer (35:65) was delivered at a flow rate of 1 ml/min. A good separation of propranolol HCl and diltiazem HCl as internal standard was achieved with retention times of 6.6 min and 9.9 min respectively. Linearity was obtained for propranolol (conc. range 20-280 ng/ml) and followed regression equation, Y= 0.0162 X – 0.0515. Peak areas were reproducible as indicated by low coefficient of variation (< 2.13%). Introduction Propranolol, a nonselective β-adrenergic receptor blocking agent possessing no other autonomic nervous system activity, is used for the treatment of hypertension. It is highly lipophilic and almost completely absorbed after oral administration 1-4. Among various methods developed for propranolol from biological matrices (serum, plasma, blood or urine), most of the methods 5-8 require relatively large plasma volumes (> 1 ml), multiple steps of sample preparation and laborious time consuming extraction procedures. This

study, approved from an independent Institutional Ethics Committee of Andhra University, Visakhapatnam, India, proposed a HPLC method, where human plasma (HP) samples were subjected to protein precipitation and protein free plasma samples were directly injected into HPLC column. Proposed method is simplified one-step sample preparation and can be adapted for routine pharmacokinetic studies of propranolol in clinical situations or small animal studies using a small amount (< 0.3 ml) of plasma.

8. Kavitha K Y *et al* (2019) reported a simple, RP-HPLC method was established for determining linagliptin and metformin in pharmaceutical formulations. Linagliptin, metformin and their degradation products were separated using C8 column with Acetonitrile: Water: Methanol (25: 50: 25 (v/v/v) to pH 4. 1 with 0. 1% orthophosphoric acid as the mobile phase. Detection was performed at 243 nm using a diode array detector. The method was validated using ICH guidelines and was linear in the range 5-30µg/ and 10-100 µg /ml for linagliptin and metformin respectivily. Good separation of both the analytes and their degradation products was achieved using this method. The developed method can be applied successfully for the determination of linagliptin and metformin.

9. Murthy TGK *et al* (2019) reported a simple, precise, rapid, selective, and economic reversed phase high-performance liquid chromatography (RPHPLC) method has been established for simultaneous analysis of Metformin hydrochloride and Rosuvastatin in bulk powder and In-House Formulation on a Phenomenex C18 (250×4.6 mm i.d) chromatographic column equilibrated with mobile phase containing Acetonitrile/0.02 M Sodium dihydrogen o-phosphate. Experimental conditions such as pH of mobile phase, ratio of organic phase, flow rate, wavelength, etc. were critically studied and the optimum conditions were selected. Efficient chromatographic separation was achieved with mobile phase containing combination of Phosphate buffer pH 2.8 and Acetonitrile in ratio of 65:35(v/v) adjusted to pH 3.8 at flow rate of 1.0 ml/min and eluents were monitored at 252 nm. 20 µl of sample was injected into chromatographic system and the total run time was 10 min. The retention time for Metformin and Rosuvastatin were 2.147 min and 3.80 min respectively. The method was linear in the range of 5µg mL-1to 30µg mL-1and 0.4 µg mL-1to2.4µg mL-1for Metformin and Rosuvastatin respectively. The proposed method was successfully applied to the analysis of Metformin and Rosuvastatin in bulk and in-house formulation without interference from other additives. The developed

method was validated according to ICH guidelines. Linearity, regression value, recovery and % RSD of intra and interday precision values were found within the limits and the method was found to be satisfactory. This validated HPLC procedure is economic, sensitive, user-friendly and less time consuming than other chromatographic procedures.

10. Mohammad Y *et al* (2015) reported a simple and precise stability indicating reversed-phase high-performance liquid chromatography method was developed and validated for the simultaneous determination of metformin (MET) hydrochloride and dapagliflozin (DAP) in bulk and pharmaceutical dosage form. Methods: Chromatography was carried out on hypersil BDS C18(250 mm × 4.6 mm, 5 µ particle size) column containing mobile phase of buffer (0.1% orthophosphoric acid) adjusted to pH 6.8 with triethylamine:acetonitrile in the ratio of 50:50%/v/v at a flow rate of 1 ml/minutes. The analyte was monitored using photodiode array detector at 240 nm. Results: The retention time was found to be 2.791 minutes and 3.789 minutes for MET hydrochloride and DAP respectively. The proposed method was found to be having linearity in the concentration range of 85-510 µg/ml for MET (r2=0.99995) and 0.5-3.0 µg/ml for DAP (r2=0.99978), respectively. The mean % recoveries obtained were found to be 99.66-100.23% for MET and 99.61-100.38% for DAP respectively. Stress testing which covered acid, base, peroxide, photolytic and thermal degradation was performed on under test to prove the specificity of the method and the degradation was achieved. The developed method has been statistically validated according to ICH guidelines. Conclusion: Thus, the proposed method can be successfully applied for the stability indicating the simultaneous determination of MET hydrochlorideand DAP in bulk and combined tablet dosage form and in the routine quality control analysis. © 2015, Asian Journal of Pharmaceutical and Clinical Research.

11. Vishnu shinde *et al* (2016) reported a simple, rapid, sensitive, precise and specific U V spectrophotometric and High-performance thin layer chromatographic (HPTLC) methods for the determination of Teneligliptin Hydrobromide both in bulk drug and pharmaceutical dosage form were developed and validated. In UV spectrophotometric method, the solutions of Teneligliptin HBr were prepared in water. The standard solution of Teneligliptin HBr showed maximum absorption at wavelength 243.5 nm. The drug obeyed Beer–Lambert's law in the concentration range of 10– 90 µg/mL with coefficient of correlation (r2) of 0.999. For HPTLC method, the method employed aluminium plates

precoated with silica gel G60 F254 as the stationary phase. The solvent system consisted of toluene: chloroform: ethanol: diethyl amine in the proportion of 4:4:1:1, v/v/v/v. This solvent system was found to give compact spots for Teneligliptin HBr with Rf value 0.16 ± 0.01. Densitometric analysis of Teneligliptin HBr was carried out in the absorbance mode at 254 nm. Linear regression analysis showed good linearity (r2 =0.998) with respect to peak area in the concentration range of 100–600 ng/spot. The developed methods were validated as per the ICH guidelines. Statistical analysis proved that the methods are repeatable and specific for the estimation of the said drug. These methods can be adopted in routine assay analysis of Teneligliptin HBr in bulk or tablet dosage form.

12. Manojkumar K Munde *et al* (2020) reported SGLT-2 is the newly developed class of antidiabetic medicine also called as gliflozins. Empagliflozin, dapagliflozin and canagliflozin are the SGLT-2 class inhibitors for the treatment of type II diabetes mellitus. SGLT-2 inhibitors shows the 82% of plasma protein binding, 36.8% of partitioning of red blood cells, 78% of bioavailability, 5.6 to 13.1 hrs half life in oral route of administration. In this review we complied analytical methods for the development and determination of the SGLT-2 inhibitors. Table no. 1, 2, 3 shows the analytical method development and validation of empagliflozin dapagliflozin and canagliflozin alone and with its combination by the HPLC method respectively also table no. 4 shows the various formulations available in SGLT-2 Inhibitors.

13. Mohammad rizhk *et al* (2020) reported the thermal behavior of anti-diabetic drugs Linagliptin (LNG) and Empagliflozin (EMP) in their pure form was examined using several thermal strategies. The thermo gravimetry (TGA), derivative thermo gravimetry (DrTGA), differential thermal analysis (DTA) and differential scanning calorimetry (DSC) were used to study the thermal decomposition and purity of both drugs. Thermodynamic parameters such as; activation energy (E*), frequency factor (A), reaction order (n), enthalpy (ΔH*), entropy (ΔS*) and Gibbs free energy change of the decomposition (ΔG*) were calculated using different kinetic models. The data exhibit that the thermal degradation of the studied drugs followed first order kinetic behavior. The results of experimental purity items obtained from the thermal analysis technique for the cited drugs in their drug substances such as; purity, melting point, loss on drying, ash content and infra-red spectrum were in agreement with those obtained from the reported

method, where no significant difference was observed. Thermal analysis justifies its application in quality control of pharmaceutical compounds due to its simplicity, sensitivity, speed and low operational costs.

14. Godasu SK *et al* (2017) reported a new method was established for simultaneous estimation of Metformin and Empagliflozin by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Metformin and Empagliflozin by using Symmetry C18 column (4.6×150mm) 5µ, flow rate was 1ml/min, mobile phase ratio was (70:30 v/v) methanol: phosphate buffer (KH_2PO_4 and K_2HPO_4) phosphate pH 3 (pH was adjusted with orthophosphoricacid), detection wavelength used was Waters HPLC Auto Sampler, Separation module 2695, photo diode array detector 996, Empower-software version-2. The retention times were found to be 2.403 mins and 3.907 mins. The % purity of Metformin and Empagliflozin was found to be 99.87% and 100.27% respectively. The analytical method was validated according to ICH guidelines (ICH, Q2 (R1). The linearity study of Metformin and Empagliflozin was found in concentration range of 50µg-250µg and 5µg-25µg and correlation coefficient (r^2) was found to be 0.999 and 0.999, % recovery was found to be 99.56% and 99.48%, %RSD for repeatability was 0.3and 0.3, % RSD for intermediate precision was 1.3 and 0.4respectively. LOD value was 2.17 and 0.0372 and LOQ value was 6.60 and 0.1125 respectively.

4. AIM AND OBJECTIVE

Quest for new drug discovery is an ongoing R&D work. The assurance of quality is achieved through analysis of the drug product. Now-a-days the pharmaceutical dosage forms are widely presented with multiple active components i.e. in combined dosage forms. This has opened new task for analyst to develop simultaneous estimation of different drugs in such combined dosage forms.

Since only few methods have been reported in the literature for the quantitative estimation of Empaglifozin and Metformin, there is scope for investigation of new analytical methods for Empaglifozin and Metformin in bulk and pharmaceutical dosage forms and many of the previously published methods are not directly applicable for this issue and need more investigation for method development and validation.

A. Consequently focus of the present study was aimed to develop the following analytical method for Empagliflozin and Metformin with high sensitivity, accuracy and precision.

- To develop a HPLC method for simultaneous determination of Empagliflozin and Metformin.
- The new method shall be validated for analytical parameters such as accuracy, precision, specificity, robustness, ruggedness, linearity and range as per the guidelines of ICH.
- The validated analytical method shall then be applied for the simultaneous estimation of Empagliflozin and Metformin in dosage forms.

5. ANALYTICAL METHOD DEVELOPMENT AND VALIDATION

5.1. Simultaneous Estimation of Empaglifozin and Metformin by RP-HPLC Method

A. Selection of Sampling Wavelength for Analysis and Preparation of Standard Calibration Curves.

1. Selection of Mobile Phase

The standard solutions containing Empaglifozin and Metformin were injected into the HPLC system and run in different solvent systems. By studying literature survey, different mobile phases in different proportions and different pH were tried in order to find the best conditions for the separation.

It was found that methanol and water gives satisfactory results as compared to other mobile phases. This mobile phase system was tried with different proportions and using different flow rates. Finally, the optimal composition of the mobile phase was determined to be Methanol: Water (50:50).

2. Preparation of Mobile Phase

Mobile phase was prepared by mixing methanol and water in the ratio of 50:50 and was initially filtered through 0.45µm millipore membrane filter and sonicated for 15 min before use.

3. Preparation of Standard Stock Solution

The separate stock solutions of EMP and MET were prepared by accurately weighing 10 mg each into a separate 10 mL volumetric flasks A and B and made up to the volume with mobile phase to get 1000µg/mL respectively. From the above standard stock solutions 1mL from volumetric flask A and 0.5 mL from volumetric flask B was transferred to a 10 mL

volumetric flask and made up to the volume with same mobile phase to get 100µg/mL and 50µg/mL of EMP and MET respectively (Working stock solution).

4. Selection of Analytical Wavelength

By appropriate dilution of each standard stock solution with mobile phase, various concentrations of Empaglifozin and Metformin were prepared separately. Each solution was scanned using double beam UV visible spectrophotometer between the range of 200 nm to 400 nm and their spectra was overlaid. From the overlain spectra shown in figure 13 of Empaglifozin and Metformin, 277 nm was selected as analytical wavelength for Multicomponent analysis using HPLC method.

5. Optimized Chromatographic Conditions

Mobile phase consisting of Methanol: Water (50:50 v/v) was used in isocratic mode. The mobile phase was initially filtered through 0.45µm millipore membrane filter and sonicated for 15 min before use. The flow rate was maintained at 1 mL/min and the injection volume was 20µL. UV detection was performed at 277 nm and the separation was achieved at ambient temperature.

6. Selection of Analytical Concentration Range and Construction of Calibration Curve for Empaglifozin and Metformin

Appropriate aliquots ranging from 0.5 mL to 2.5 mL were pipetted out from the working stock solution (100 µg/mL of Empaglifozin and 50 µg/mL of Metformin) in to a series of 10 mL volumetric flasks. The volume was made up to the mark with the mobile phase to get a set of solutions having the concentration range, ranging from 5-25 µg/mL of Empaglifozin and 2.5-12.5 µg/mL of Metformin respectively. Chromatograms representing linearity was shown in figure 16 to 20. Triplicate dilutions of each of the above mentioned concentrations were prepared separately and from these triplicate solutions, 20 µL of each concentration of the drug were injected into the HPLC system three times separately and their chromatograms were recorded under the same chromatographic conditions as described above.

Peak areas were recorded for all the peaks and a standard calibration curve of area against concentration was plotted as concentration of the drug Vs peak area (figure 21 and 22). The results were shown in table 24. Both the drugs follow the Beer‘s Lambert‘s law in the concentration range of 5-25 µg/mL of Empaglifozin and 2.5-12.5 µg/mL of Metformin.

The linearity of calibration curves and adherence of the system to Beer‘s Lambert‘s law was validated by high value of correlation coefficient and less than 2% percent relative standard deviation (%RSD) for the intercept value which were shown in table 25.

B. Analysis of Tablet Formulation.

The tablets (JARDIANCE-MET) were initially powdered and an amount equivalent to 100 mg of Empaglifozin and 50 mg of Metformin was accurately weighed into a 100 mL volumetric flask, mixed with 50 mL of mobile phase. The solution was made up to the volume with mobile phase and sonicated for 5 minutes. The solution was then filtered through 0.45µm millipore membrane filter. Final stock containing 20µg/mL and 10µg/mL of Empaglifozin and Metformin respectively was prepared by subsequent dilution with the same mobile phase. 20µL of sample solution was injected into chromatographic system and the peak responses were measured. The solution was injected three times into the column. The amount present in each tablet was calculated by comparing the areas of test with that of the standard. A typical chromatogram of test solution containing 20 µg/ml of Empaglifozin and 10 µg/ml of Metformin was shown in figure 23. The results were shown in table 26.

C. Method Validation

The method was validated according to ICH Q2 B guidelines for validation of analytical procedures in order to determine system suitability, linearity, sensitivity, precision, accuracy and robustness for the analytes (ICH Q2B, 1996).

1. System Suitability

The system suitability parameters were evaluated from standard chromatograms by calculating the % RSD from six replicate injections for Empaglifozin and Metformin retention times and peak areas.

System suitability was carried out by injecting 100% concentration (sample having 25µg/mL of Empaglifozin and 12.5µg/mL of Metformin) into the HPLC system. This was repeated for six times under similar condition. The tailing factor (T) and no. of theoretical plates (N) obtained were shown in figure 24 and the results were given in table 27 and 28.

2. Accuracy

To confirm the accuracy of the proposed method, recovery experiments were performed by standard addition technique. In this method a known quantity of pure drug was added at three

different levels i.e. 80 %, 100% and 120% to pre-analyzed sample solutions and calculated the recovery of Empaglifozin and Metformin for each concentration. Chromatograms showing different levels of recovery were shown in figure 25 to 27. The results of recovery studies by proposed method were validated by statistical evaluation and were given in table 29 and 30.

3. Linearity and Range

The linearity of analytical method is its ability to elicit test results that are directly proportional to the concentration of analyte in sample within a given range. Linearity of the method was determined by means of calibration curve using different concentration of the drugs. Linearity was evaluated by visual inspection of a calibration curve shown in figure 21 and 22. The linearity of the method was determined in concentration range of 5-25µg/mL for Empaglifozin and 2.5-12.5µg/mL for Metformin. Each solution was injected in triplicate. Chromatograms representing linearity were shown in figure 16 to 20. The slope, intercept was reported as required by ICH which were given in table 24 and 25.

4. Precision

The precision of an analytical method was studied by performing intraday and inter day precision.

Intraday Precision

Variation of results within the same day was analyzed. Intraday precision was determined by analyzing a set of six combined standard solutions of Empaglifozin (25 µg/mL) and Metformin (12.5 µg/mL) in linearity range as 100% concentration at three different time intervals on same day. Chromatogram representing intraday precision was shown in figure 28 and the results were given in table 31.

Inter day Precision

Variation of results between the days was analyzed. Inter day precision was determined by analyzing a set of six combined standard solutions of Empaglifozin (25 µg/mL) and Metformin (12.5 µg/mL) in linearity range as 100% concentration on three consecutive days. Chromatogram representing interday precision was shown in figure 29 and the results were given in table 31.

5. Specificity and Selectivity

The specificity of the RP-HPLC method was determined by complete separation of Empaglifozin and Metformin with parameters like retention time (Rt), resolution (Rs) and tailing factor (T_f). Here tailing factor for peaks of Empaglifozin and Metformin was less than 2% and resolution was also more than 2%. The average retention time and standard deviation for Empaglifozin and Metformin were found to be satisfactory for six determinations of sample solution containing 25µg/mL of Empaglifozin and 10µg/mL of Metformin respectively. The peaks obtained for Empaglifozin and Metformin were sharp and have clear baseline separation as none of the excipients interfered with the analytes of interest. The chromatogram to represent specificity was shown in figure 30 and the results were given in table 32.

6. Robustness

The evaluation of robustness should be considered during the development phase and depends upon the type of procedure under study. It should show the reliability of analysis with respect to deliberate variations in method parameters like different column temperate, different analytical wavelength, different flow rate. The solution containing 25 µg/mL of Empagliflozin and 12.5 µg/mL of Metformin was injected into sample injector of HPLC three times under different parameters like deliberate variations in flow rate (±0.1mL/min) and detection wavelength (± 2 nm).

Chromatogram representing robustness was shown in figure 31 and 32 for change in flow rate and the results were given in table 33. Chromatogram representing robustness was shown in figure 33 and 34 for change in detection wavelength and the results were given in table 34.

7. Ruggedness

The evaluation of ruggedness should be considered during the development phase and depends upon the type of procedure under study. It should show the reliability of analysis with respect to deliberate variations in method parameters like different instruments, analysts, laboratories, reagents, days etc. The solution containing 25 µg/mL of Empaglifozin and 12.5 µg/mL of Metformin was injected into HPLC three times under different parameters like different analysts. Chromatogram representing ruggedness was shown in figure 35 and 36 for change in analysts and the results were given in table 35.

8. LOD and LOQ

The LOD and LOQ values were determined by the formulae LOD = 3.3 σ/S and LOQ = 10 σ/S (Where, σ is the standard deviation of the responses and S is mean of the slopes of the calibration curves). The results were given in table 25.

5.2. RESULTS

6.1. Simultaneous Estimation of Empaglifozin and Metformin by RP-HPLC Method

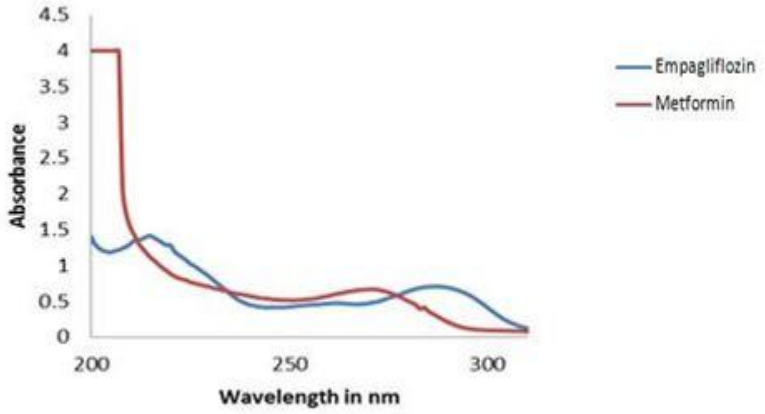


Figure 13: Overlain Spectra of Empaglifozin and Metformin.

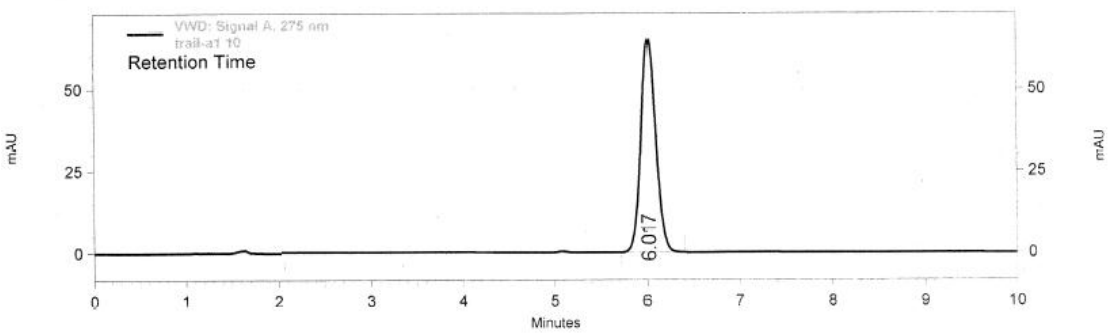


Figure 14: RP-HPLC Chromatogram of Empagliflozin.

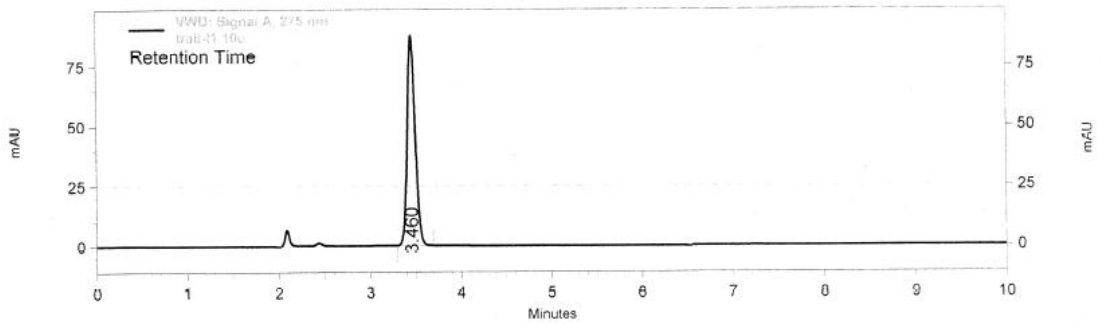


Figure 15: RP-HPLC Chromatogram of Metformin.

Linearity

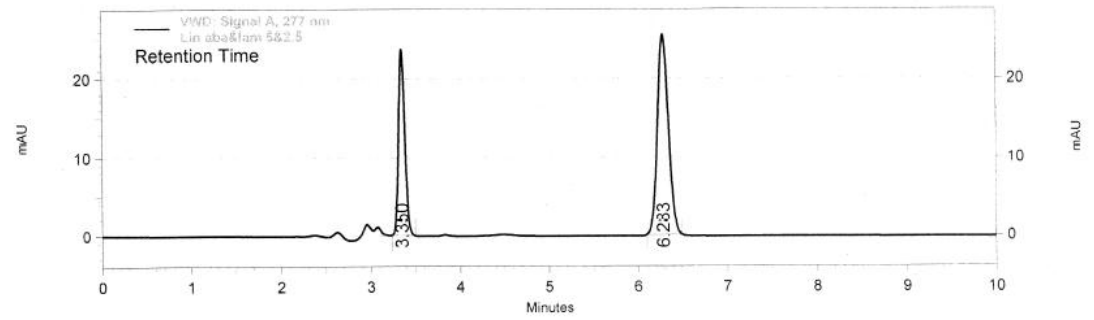


Figure 16: RP-HPLC Chromatogram of Linearity-1.

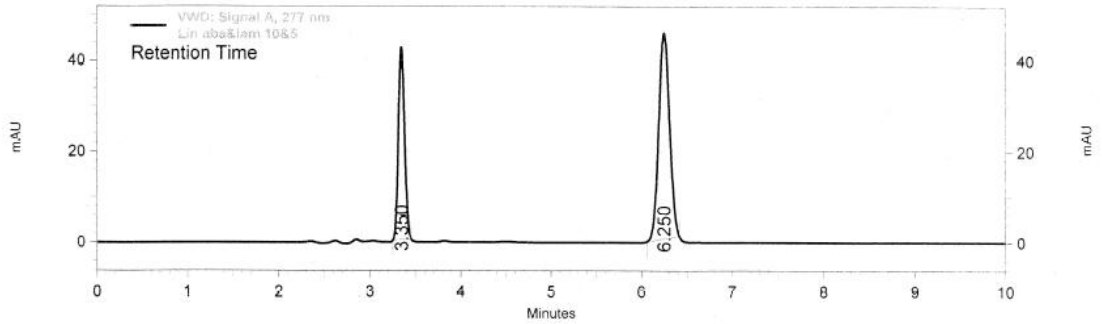


Figure 17: RP-HPLC Chromatogram of Linearity-2.

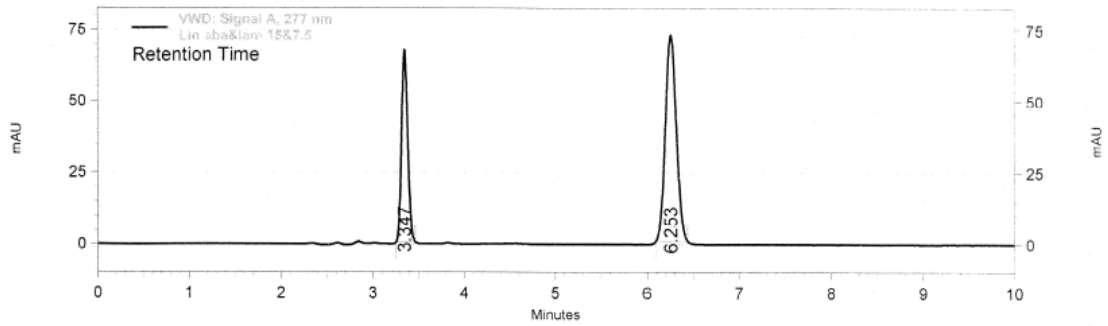


Figure 18: RP-HPLC Chromatogram of Linearity-3.

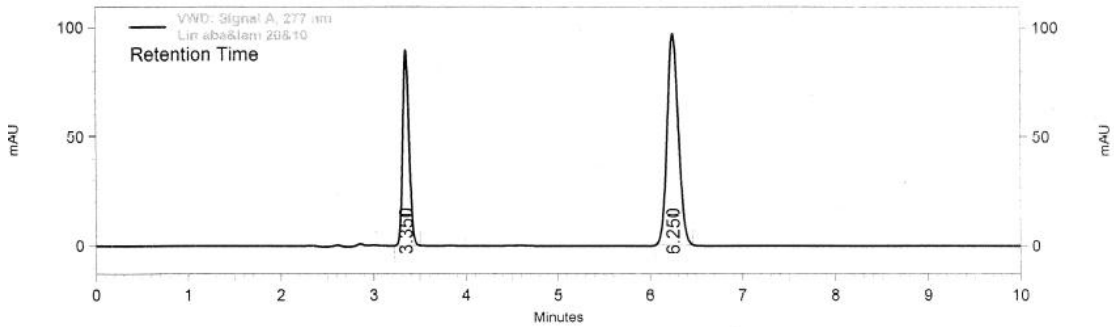


Figure 19: RP-HPLC Chromatogram of Linearity-4

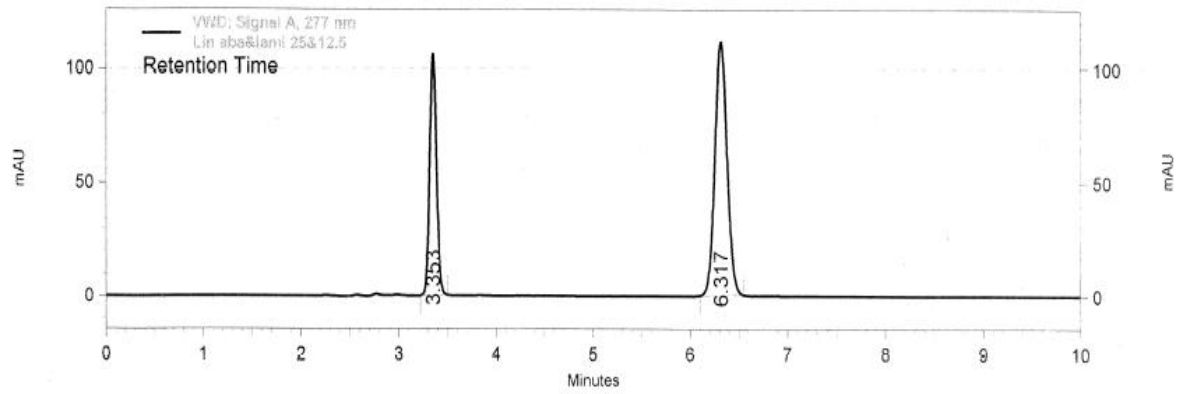


Figure 20: RP-HPLC Chromatogram of Linearity-5.

Table 24: Linearity Data of Empagliflozin and Metformin at 277 nm by RP−HPLC Method.

S. No	Empagliflozin			Metformin		
	Conc. (µg/mL)	R_t (min)	Peak Area	Conc. (µg/mL)	R_t (min)	Peak Area
0	0	0	0	0	0	0
1	5	6.2	3644264	2.5	3.3	1941262
2	10	6.2	6570423	5	3.3	3498562
3	15	6.2	10025283	7.5	3.3	5228345
4	20	6.2	13025471	10	3.3	7072569
5	25	6.2	16246592	12.5	3.3	8734279

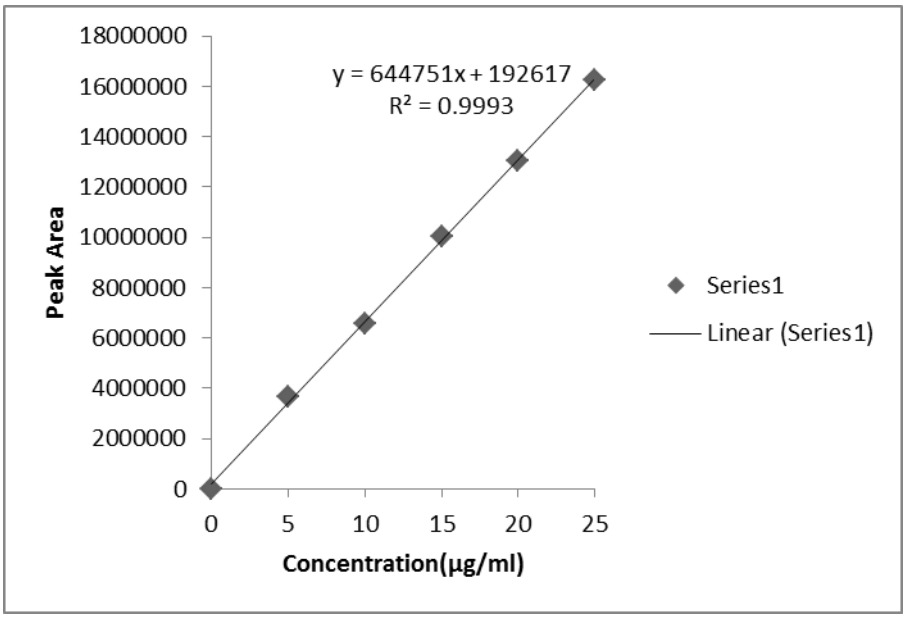


Figure 21: Calibration Curve of Empagliflozin at 277 nm by RP−HPLC Method.

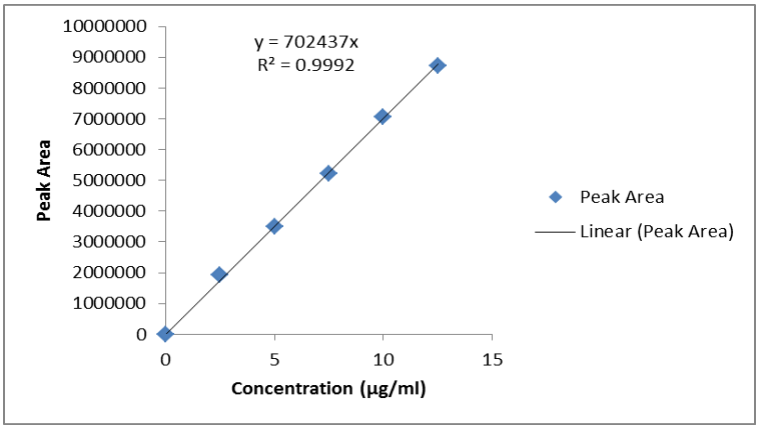


Figure 22: Calibration Curve of Metformin at 277 nm by RP−HPLC Method.

Table 25: Statistical Data of Empagliflozin and Metformin at 277 nm by RP−HPLC Method.

Parameter	Empagliflozin	Metformin
Linearity Range (µg/mL)	5-25	2.5-12.5
Regression Equation	Y=644751x+192617	Y=694801x+69996
Slope (m)	644751	694801
Intercept (c)	192617	69996
Regression Coefficient (r^2)	0.9993	0.9997
Limit of Detection (µg/mL)	0.16	0.33
Limit of Quantitation (µg/mL)	0.49	1.01

Assay

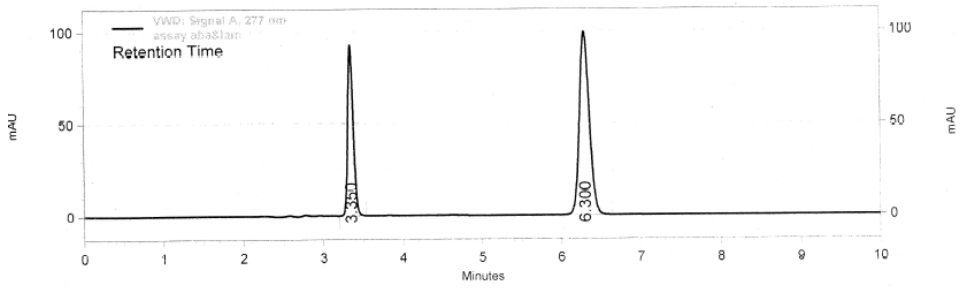


Figure 23: RP-HPLC Chromatogram of Test Formulation.

Table 26: Assay of Empagliflozin and Metformin in Tablet Formulation.

S. No	Amount Present in (mg/tab)		Amount Obtained in (mg/tab)		Label Claim %w/w	
	EMP	MET	EMP	MET	EMP	MET
1	600	300	595.86	303.8	99.31	101.1
2	600	300	601.28	299.98	100.21	99.99
3	600	300	599.85	301.56	99.97	100.52
Mean			598.99	301.78	99.83	100.53
SD			2.8085	1.9194	0.4660	0.5551
%RSD			0.468	0.636	0.466	0.552

Table 27: System Suitability Data of Empagliflozin and Metformin.

S. No	Empagliflozin			Metformin		
	Conc. (µg/mL)	R_t (min)	Peak Area	Conc. (µg/mL)	R_t (min)	Peak Area
1	25	6.40	8795252	12.5	3.37	16254852
2	25	6.39	8734279	12.5	3.36	16411866
3	25	6.40	8768665	12.5	3.37	16411866
4	25	6.38	8662457	12.5	3.36	16215954
5	25	6.43	8754215	12.5	3.39	16452585
6	25	6.40	8698524	12.5	3.37	16326898
Mean		6.4	8735565		3.37	95520.862
SD		0.016	48424.04		0.010	16345670
% RSD		0.261	0.554		0.325	0.584

Table 28: Summary of System Suitability Parameters.

Parameters	Empagliflozin	Metformin
Retention Time (min)	6.4	3.37
Resolution (R_s)	3.0	
Tailing Factor (T)	1.2	1.4
Theoretical Plates (N)	11456	10366

Accuracy

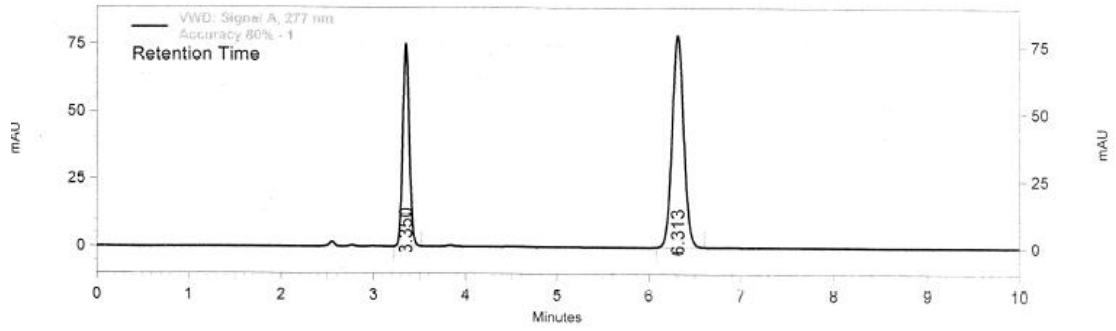


Figure 25: RP-HPLC Chromatogram of 80% Recovery Level.

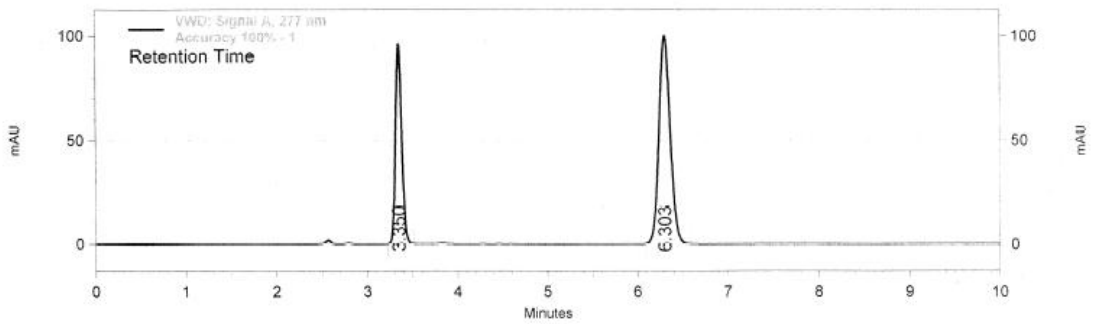


Figure 26: RP-HPLC Chromatogram of 100% Recovery Level.

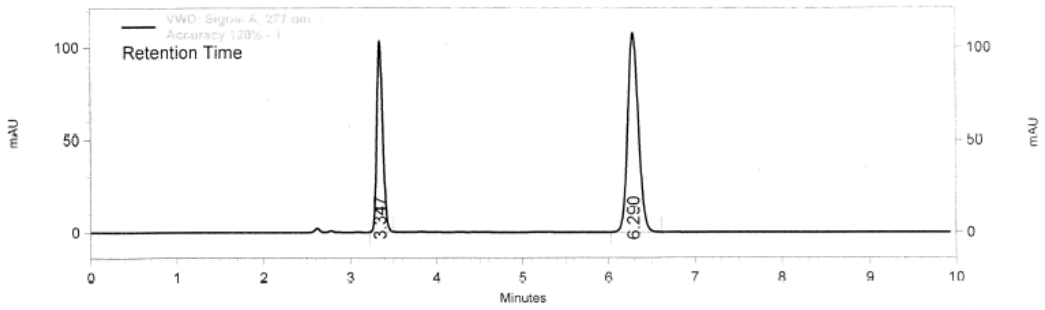


Figure 27: RP-HPLC Chromatogram of 120% Recovery Level.

Table 29: Determination of Accuracy for Empagliflozin and Metformin by RP-HPLC Method.

S. No	Recovery Level	Empagliflozin				Metformin			
		Amount Added (µg/mL)		Amount Found (mg)	% Recovery	Amount Added (µg/mL)		Amount Found (mg)	% Recovery (w/w)
		Std.	Test			Std.	Test		
1	80%	8	10	595.9	99.9	4	5	294	98.0
2		8	10	590.0	98.4	4	5	294.6	98.2
3		8	10	598.2	99.7	4	5	297.8	99.2
4	100%	10	10	599.5	99.9	5	5	304.8	101.6
5		10	10	592.4	98.7	5	5	303.0	101.0
6		10	10	598.4	99.7	5	5	303.8	101.2
7	120%	12	10	604.2	100.7	6	5	300.6	100.2
8		12	10	597.8	99.6	6	5	295.9	98.6
9		12	10	603.2	100.5	6	5	299.8	99.9

Table 30: Statistical Validation Data for % Recovery Determinations.

Level of % Recovery	Empagliflozin			Metformin		
	Mean	SD	%RSD	Mean	SD	%RSD
80	99.3	0.814	0.819	98.4	0.642	0.652
100	99.4	0.642	0.646	101.2	0.305	0.301
120	100.2	0.585	0.584	99.5	0.850	0.854

Precision

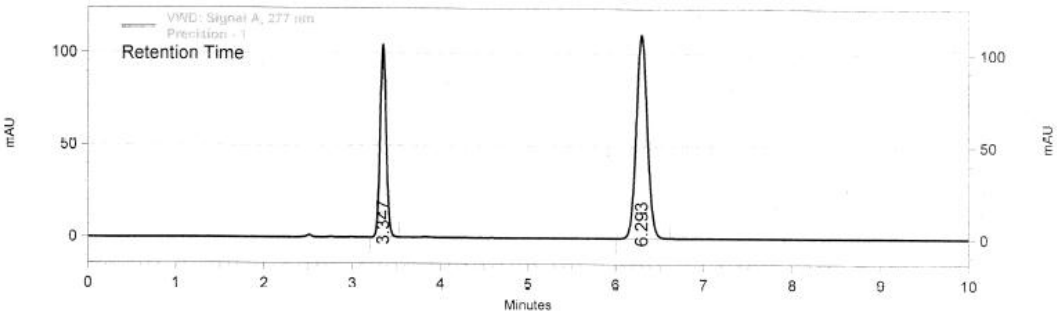


Figure 28: RP-HPLC Chromatogram to Show Intraday Precision of Empagliflozin and Metformin.

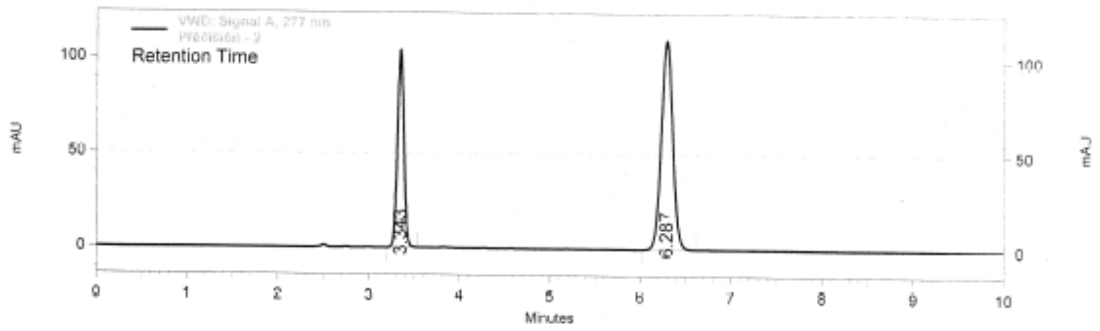


Figure 29: RP-HPLC Chromatogram to Show Inter day Precision of Empagliflozin and Metformin.

Table 31: Determination of Precision for Empagliflozin and Metformin by RP-HPLC.

S. No	Empagliflozin(25µg/mL)		Metformin (12.5µg/mL)	
	Peak Areas			
	Intra day	Inter day	Intra day	Inter day
1	16147878	15036416	8701823	8605248
2	16041854	15294672	8589857	8794482
3	16411866	15629990	8584943	8734279
4	16246592	15311383	8571319	8662145
5	16072779	15388356	8568665	8798391
6	16215954	15333986	8554585	8768665
Mean	16189487	15332467	8595199	8727202
SD	134581.9	190349.7	53714.44	78062.7
% RSD	0.831	1.241	0.624	0.894

Specificity

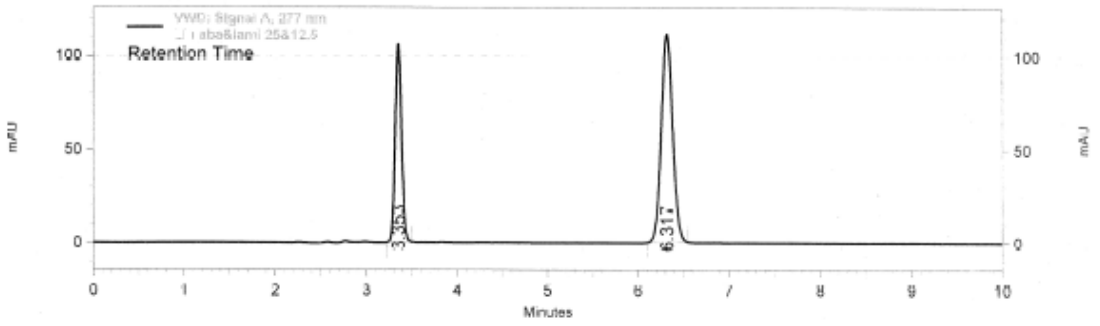


Figure 30: RP-HPLC Chromatogram to Show Specificity of Sample Solution.

Table 32: Specificity Parameters of Empagliflozin and Metformin by RP-HPLC.

Parameters	Empagliflozin	Metformin
Retention Time (min)	6.31	3.35
Resolution (R_s)	3.0	
Tailing Factor (T)	1.17	1.35
Theoretical Plates (N)	11750	10587

Robustness

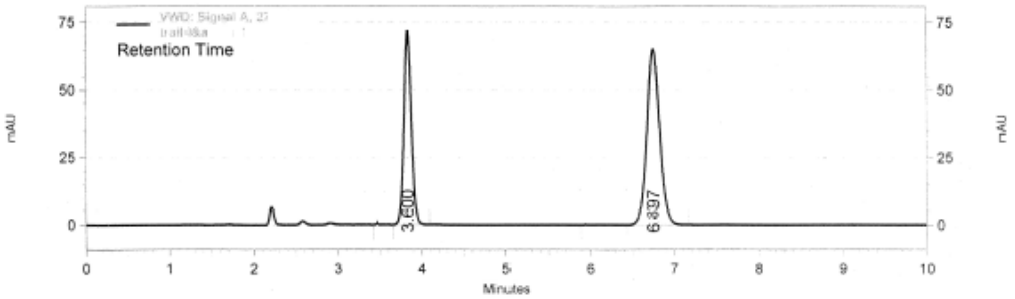


Figure 31: RP-HPLC Chromatogram of Robustness Study for Empagliflozin and Metformin at Flow rate 0.9 mL/min.

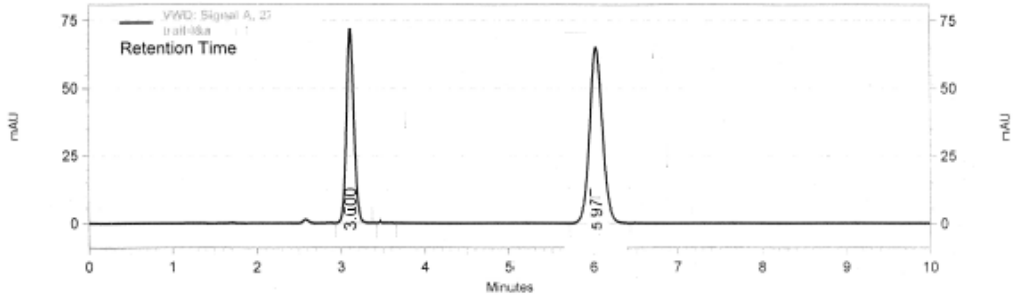


Figure 32: RP-HPLC Chromatogram of Robustness Study for Empagliflozin and Metformin at Flow rate 1.0 mL/min.

Table 33: Robustness Data with Change in Flow Rate.

S. No	Flow Rate (±0.1mL)	Empagliflozin		Metformin	
		R_t (min)	Peak Area	R_t (min)	Peak Area
1	0.9	3.8	15036416	6.9	8185320
2	0.9	3.7	14965852	7.01	8201356
3	0.9	3.8	15162532	6.8	8126548
Mean		3.7	15054933	6.90	8171075
SD		0.057	99638.97	0.105	39385.99
% RSD		1.532	0.661	1.521	0.482
1	1.1	2.9	14652068	5.8	8835268
2	1.1	3.0	14756825	5.9	8725698
3	1.1	2.9	14865922	5.9	8854256
Mean		2.93	14758272	5.8	8805074
SD		0.057	106934.3	0.057	69394.15
% RSD		1.968	0.724	0.984	0.788

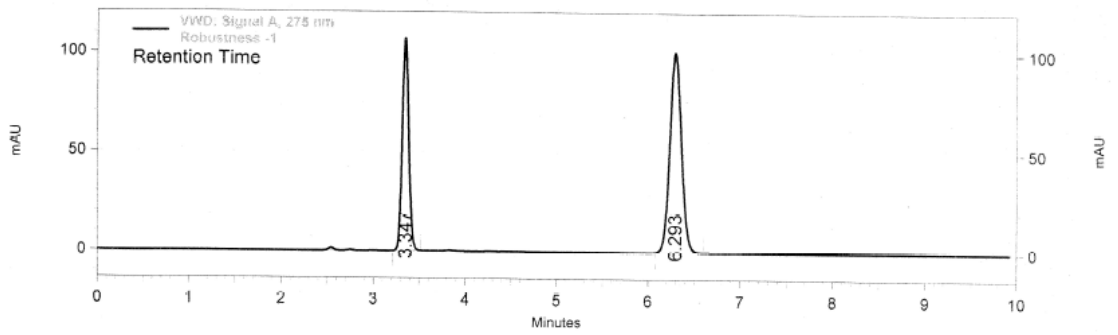


Figure 33: RP-HPLC Chromatogram of Robustness Study for Empagliflozin and Metformin at Detection Wavelength 275nm.

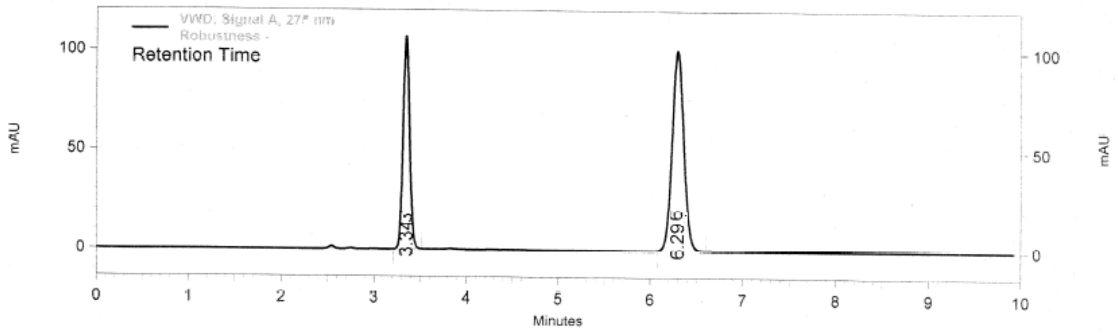


Figure 34: RP-HPLC Chromatogram of Robustness Study for Empagliflozin and Metformin at Detection Wavelength 279nm.

Table 34: Robustness Data with Change in Detection Wavelength.

S. No	Detection wavelength (±0.1nm)	Empagliflozin		Metformin	
		R_t (min)	Peak Area	R_t (min)	Peak Area
1	275	6.29	14434097	3.34	8828159
2	275	6.30	14358174	3.35	8795685
3	275	6.34	14478514	3.39	8752352
Mean		6.31	14423595	3.36	8792065
SD		0.026	60853.5	0.026	38032.9
% RSD		0.419	0.421	0.787	0.432
1	279	6.29	15988374	3.34	8898391
2	279	6.32	16072779	3.32	8794482
3	279	6.31	16215954	3.40	8862145
Mean		6.30	16092369	3.35	8851673
SD		0.015	115047.8	0.041	52740.14
% RSD		0.242	0.71	1.24	0.595

Ruggedness

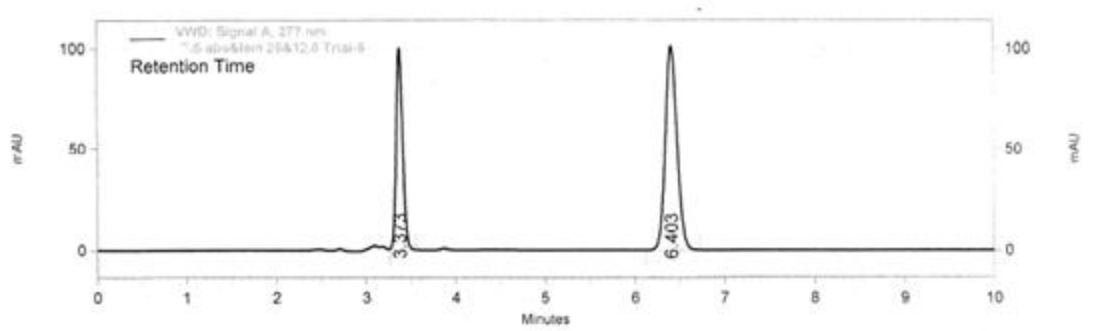


Figure 35: RP-HPLC Chromatogram of Ruggedness Study for Empagliflozin and Metformin by Analyst-I.

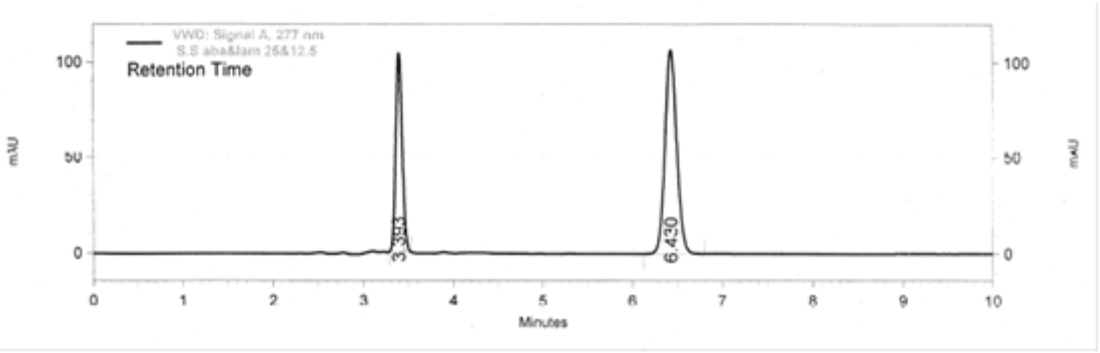


Figure 36: RP-HPLC Chromatogram of Ruggedness Study for Empagliflozin and Metformin by Analyst-II.

Table 35: Ruggedness Data of Empagliflozin and Metformin.

S. No.	Condition	Empagliflozin		Metformin	
		Rt	Peak Area	Rt	Peak Area
1	Analyst-1	6.40	15629990	3.37	8605248
2		6.39	15162532	3.37	8794482
3		6.38	15388356	3.34	8735241
Mean		6.39	15393626	3.36	8711657
SD		0.01	233773.6	0.017	96796.34
%RSD		0.156	1.518	0.515	1.111113
4	Analyst-2	6.43	15253688	3.39	8571319
5		6.41	15162532	3.40	8662145
6		6.40	15036416	3.38	8568665
Mean		6.413333	15150879	3.39	8600710
SD		0.01527	109103.8	0.01	53221.11
%RSD		0.238	0.720	0.294	0.618

6. SUMMARY AND CONCLUSION

6.1. Simultaneous Estimation of Empagliflozin and Metformin by RP-HPLC Method

In HPLC method, the conditions were optimized to obtain an adequate separation of eluted compounds. Initially, various mobile phase compositions were tried to separate title ingredients

The objective of this study was to develop a rapid and sensitive RP-HPLC method for the analysis of Empagliflozin and Metformin in bulk drug and pharmaceutical dosage form by using the most commonly employed C-18 column with UV-detection.

Initially, various mobile phase compositions were tried to elute the drug. Mobile phase ratio and flow rate were selected based on peak parameters (height, capacity, theoretical plates, tailing or symmetry factor), run time and resolution. The system with Methanol: Water (50: 50 v/v) and 1 mL / min flow rate was selected.

The optimum wavelength selected was 277 nm from the overlain spectra obtained which was shown in figure 13 at which better detector response for the drug was obtained. The retention time for Empagliflozin and Metformin was found to be 6.4 min and 3.37 min respectively which were shown in figure 14 and 15. The linearity was observed in concentration range of 5-25 µg/ mL and 2.5-12.5 µg/ mL for Empagliflozin and Metformin respectively. Calibration curves of the respective drugs were shown in figure 21 and 22. Summary of validation parameters were given in table 25.

System suitability was assessed by injecting 5 replicate injections of 100% test concentration. Number of theoretical plates was more than 2000 for both the drugs and tailing factor was less than 1.5 for both Empagliflozin and Metformin was reported. A Resolution of greater than 2 was observed. The relative retention times of six replicate injections and system suitability parameters were given in table 27 and 28.

The low % RSD values (≤ 2) indicated that the method was precise and accurate. The mean recoveries were found in the range of 99.3 – 100.2% w/w.

Specificity of the chromatographic method was tested by injecting sample concentration prepared from marketed formulation. The response was compared with that obtained from the standard drug. The chromatogram confirms the presence of Empagliflozin and Metformin at

6.3min and 3.3min respectively without any interference. Thus the developed method was specific to Empagliflozin and Metformin and the parameters were given in table 32.

The robustness of an analytical method was determined by analysis of aliquots from homogenous lots by differing physical parameters such as change in flow rate to 1.0 ± 0.1 mL and changing detection wavelength 277nm ± 2nm. The obtained values were given in table 33 and 34. These values with low % RSD (<2) indicated that the method was quite robust.

Ruggedness of the proposed method was determined by analysis of aliquots from homogeneous slot by different analysts, using similar operational and environmental conditions; the % RSD reported was found to be less than 2 and these values were listed in table 35.

The proposed method was validated in accordance with ICH parameters and was applied for analysis of the same in marketed formulations. The content of each component in the formulation was estimated by comparing the peak area of the test sample with that of the peak area of the standard and the results were given in table 26 which were found to be 99.83% w/w for Empagliflozin and 100.53% w/w for Metformin respectively. High % recovery and low % RSD suggested that the method can be applicable for the routine analysis of commercial formulations.

Hence, the developed HPLC method can be adopted for the routine analysis of Empagliflozin and Metformin in pharmaceutical formulations.

Drug combinations are commonly used clinically and analyst is required to develop suitable methods of their analysis. For routine analytical purposes it is always of interest to establish methods capable of analyzing a large number of samples in a short time period with good accuracy and precision. The commonly used tests of pharmaceutical analysis generally entail compendia testing method development, setting specifications, and method validation.

Analytical testing is one of the more interesting ways for scientists to take part in quality process by providing actual data on the identity, content and purity of the drug products. New methods are now being developed with a great deal of consideration to worldwide harmonization. As a result, new products can be assured to have comparable quality and can be brought to international markets faster.

A liquid chromatographic technique coupled with spectrophotometric analysis is a versatile tool that can generate extensive analytical data that is highly useful in the routine drug analysis. For routine analytical purposes it is always of interest to establish methods capable of analyzing a large number of samples in a short time period with good accuracy and precision.

In the present work, an attempt was made to provide a newer, simple, accurate and low cost spectrophotometric and HPLC methods for the effective quantitative determination of Empagliflozin and Metformin as an active pharmaceutical ingredients as well as in pharmaceutical preparations in their single and combined dosage forms, without the interferences of other constituent in combined formulations. Hence it is planned to develop both HPLC and Spectrophotometric methods.

The results were summarized as follows

6.2. Simultaneous Estimation of Empagliflozin and Metformin by RP-HPLC Method

Table 38: Summarized Results of RP-HPLC Method.

Parameter	Results	
	Empagliflozin	**Metformin**
Detection Wavelength	277	
R_t (min)	3.37	6.40
Beer's Law Range (µg/mL)	5-25	2.5-12.5
Regression Equation	Y= 644751x+192617	Y= 694801x+69996
Correlation Coefficient (r^2)	0.9993	0.9997
% Recovery (w/w)	99.3-100.2%	98.4-101.2%
LOD (µg/mL)	0.16	0.33
LOQ (µg/mL)	0.49	1.01
Assay (% purity) w/w	99.83%	100.53%
Precision		
Intraday Precision	0.83	0.62
Inter day Precision	1.24	0.89
Robustness		
Flow Rate 0.9 mL/min	1.53	1.52
Flow Rate 1.1 mL/min	1.96	0.98
Detection Wavelength 275 nm	0.42	0.43
Detection Wavelength 279 nm	0.71	0.59
Ruggedness		
Analyst 1	1.51	1.11
Analyst 2	0.72	0.61

6.3. CONCLUSION

Development of methods to achieve the final goal of ensuring the quantity of drug substances and drug products is not a trivial undertaking. The capabilities of the methods developed were complementary to each other. Hence they can be regarded as simple, specific and sensitive methods for the estimation of Empagliflozin and Metformin in single and combined pharmaceutical dosage forms. The developed UV Spectrophotometric methods and RP-HPLC methods were validated according to ICH guidelines and were found to be applicable for the routine analysis of Empagliflozin and Metformin in their single and combined dosage forms.

The proposed UV methods were simple, sensitive and reliable with good precision and accuracy. This method was specific while estimating the commercial formulations without interference of excipients and other additives. Hence, this method can be used for the estimation of Empagliflozin and Metformin in bulk samples and their pharmaceutical formulations individually and in combination by simultaneous equation method.

The developed and validated RP-HPLC method was found to be economical due to the use of higher percentage of water as a solvent in mobile phase. The low solvent consumption (1mL/min), along with short analytical run time of less than 10.0 minutes lead to an environmental friendly chromatographic procedure that allows the analysis of a large number of samples in a short period of time. This method has been found to be better than previously reported methods, due to its wider range of linearity, use of readily available mobile phase, lack of extraction procedures. Hence above method can be used in quality control for routine analysis of finished products of Empagliflozin and Metformin simultaneously without any interference.

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