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NEW ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF METFORMIN AND ENPAGLIFLOZIN BY USING REVERESE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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INTRODUCTION

1. Importance of drug analysis

'Health is wealth'. It is vital fact that a healthy body is desire of every human being. Good health is first condition to enjoy the life and all other things which mankind is having. Nowadays peoples are more concentrating towards health. Even governmental bodies of different countries and World health organization (WHO) are also focusing for health of human being. Health care is prevention, treatment and management of illness and preservation of mental and physical well being. Health care embraces all the goods and services designed to promote health including preventive, curative and palliative in interventions. The Health care industry is considered an industry or profession which includes people's exercise of skill or judgment or providing of a service related to the prevention or improvement of the health of the individuals or the treatment or care of individuals who are injured, sick, disabled or infirm. The delivery of modern health care depends on an Interdisciplinary Team.

The medical model of health focuses on the eradication of illness through diagnosis and effective treatment. A traditional view is that improvement in health results from advancements in medical science. Advancements in medical science bring varieties of medicines. Medicines are key part of the health care system. The numerous medicines are introducing into the world-market and also, that is increasing every year. These medicines are being either new entities or partial structural modification of the existing one. So, to evaluate quality and efficacy of these medicines is also important factor. Right from the beginning of discovery of any medicine quality and efficacy of the same are checked by quantification means. Quality and efficacy are checked by either observing effect of drug on various animal models or analytical means. The option of animal modelsis not practically suitable for every batch of medicine as it's require long time, high cost and more man-power. Later option of analytical way is more suitable, highly precise, safeand selective.

The analytical way deals with quality standards which are assigned for products tohave desirable efficacy of the medicines. Sample representing any batch are analyzed for these standards and it is assumed that drug/medicine which is having such standards are having desire effect on use. 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.

Due to rapid growth of pharmaceutical industry during last several years, number of pharmaceutical formulations are enter as a part of health care system and thus, there has been rapid progress in the field of pharmaceutical analysis. Developing analytical method for newly introduced pharmaceutical formulation is a matter of most importance because drug or drug combination may not be official in any pharmacopoeias and thus, noanalytical method for quantification is available. To check the quality standards of the medicine various analytical methods are used. Modern analytical techniques are playing key role in assessing chemical quality standards of medicine. Thus analytical techniques are required for fixing standards of medicines and its regular checking. Out of all analytical techniques, the technique which is widely used to check the quality of drug is known as 'CHROMATOGRAPHY'.

2. History of chromatography and HPLC

In 1903 a Russian botanist Mikhail Tswett produced a colorful separation of plantpigments through calcium carbonate column. Chromatography word came from Greek language chroma = color and graphein = to write i.e. color writing or chromatography[1, 2].

Prior to the 1970's, few reliable chromatographic methods were commercially available to the laboratory scientist. During 1970's, most chemical separations were carried out using a variety of techniques including open-column chromatography, paper chromatography, and thin-layer chromatography. However, these chromatographic techniques were inadequate for quantification of compounds and resolution between similar compounds. During this time, pressure liquid chromatography began to be used to decrease flow through time, thus reducing purification times of compounds being isolated by column chromatography. However, flow rates were inconsistent, and the question of whether it was better to have constant flow rate or constant pressure was debated[3]. High pressure liquid chromatography was developed in the mid-1970's and quickly improved with the development of column packing materials and the additional convenience of online detectors. In the late 1970's, new methods including reverse phase liquid chromatography allowed for improved separation between very similar compounds.

By the 1980's HPLC was commonly used for the separation of chemical compounds. New techniques improved separation, identification, purification and quantification far above the previous techniques. Computers and automation added to the convenience of HPLC. Improvements in type of columns and thus reproducibility were

made as such terms as micro-column, affinity columns, and Fast HPLC began to immerge.

By the 2000 very fast development was undertaken in the area of column material with small particle size technology and other specialized columns. The dimensions of the General Introduction typical HPLC column are 100-300 mm in length with an internal diameter between 3-5 mm. The usual diameter of micro-columns, or capillary columns, ranges from 3 μm to 200 μm[4]. In this decade sub 2 micron particle size technology (column material packed with silica particles of < 2μm size) with modified or improved HPLC instrumentation becomes a popular with different instrument brand name like UPLC (Ultra Performance Liquid Chromatography) of Waters and RRLC (Rapid Resolution Liquid Chromatography) of Agilent.

3. Modern High Performance Liquid Chromatography (HPLC)

The highly sophisticated reliable and fast liquid chromatographic (LC) separation techniques are become a requirement in many industries like pharmaceuticals, agrochemicals, dyes, petrochemicals, natural products and others. Early LC used gravity fed open tubular columns with particles 100s of microns in size; the human eye was used for a detector and separations often took hours (days?) to develop.

Isocratic and Gradient LC System Operation

Two basic elution modes are used in HPLC.

The first is called isocratic elution. In this mode, the mobile phase, either a pure solvent or a mixture, *remains the same throughout the run.*

The second type is called gradient elution, wherein, as its name implies, *the mobile phase composition changes during the separation.* This mode is useful for samples that contain compounds that span a wide range of chromatographic polarity. As the separation proceeds, the elution strength of the mobile phase is increased to elute the more strongly retained sample components.

In the simplest case, there are two bottles of solvents and two pumps. The speed of each pump is managed by the gradient controller to deliver more or less of each solvent over the course of the separation. The two streams are combined in the mixer to create the actual mobile phase composition that is delivered to the column over time. At the beginning, the mobile phase contains a higher proportion of the weaker solvent [Solvent A]. Over time, the proportion of the stronger solvent [Solvent B] is increased, according to a predetermined timetable. Note that in Figure 2, the mixer is downstream of the pumps; thus the gradient is created under *high pressure*. Other HPLC systems are designed to mix multiple streams of solvents under *low pressure*, ahead of a single pump. A gradient proportioning valve selects from the four solvent bottles, changing the strength of the mobile phase over time.

Today's HPLC requires very special apparatus which includes the following.

1. Extremely precise gradient mixers.
2. HPLC high pressure pumps with very constant flow.
3. Unique high accuracy, low dispersion, HPLC sample valves.
4. Very high efficiency HPLC columns with inert packing materials.
5. High sensitivity low dispersion HPLC detectors.

6. High speed data acquisition systems.

7. Low dispersion connecting tubes for valve to column and column to detector.

HPLC Gradient mixtures

HPLC gradient mixers must provide a very precise control of solvent composition to maintain a reproducible gradient profile. This can be complicated in HPLC by the small elution volumes required by many systems. It is much more difficult to produce a constant gradient when mixing small volumes then when mixing large volumes. For low pressure systems this requires great precision in the operation of the miniature mixing General Introduction valves used and low dispersion flows throughout the mixer. For multi-pump high pressure systems it requires a very precise control of the flow rate while making very small changes of the flow rate.

HPLC Pumps

Because of the small particles used in modern HPLC, modern LC pumps need to operate reliably and precisely at pressures of 10,000 psi or at least 6,000 psi. To operate at these pressures and remain sensibly inert to the wide variety of solvents used HPLC pumps usually have sapphire pistons, stainless steel cylinders and return valves fitted with sapphire balls and stainless steel seats. For analytical purposes HPLC pumps should have flow rates that range from 0 to 10 ml/min., but for preparative HPLC, flow rates in excess of 100 ml/min may be required. It is extremely difficult to provide a very constant flow rate at very low flow rates. If 1% is considered acceptable then for 1ml/min a flow variation of less than 10µl/min is required. This level of constancy is required because most HPLC detectors are flow sensitive and errors in quantization will result from change in flow rate.

HPLC Sample Valves

Since sample valves come between the pump and the column it follows that HPLC sample valves must also tolerate pressures up to 10,000 psi. For analytical HPLC, the sample volume should be selectable from sub micro liter to a few micro liters, whereas in preparative HPLC the sample volume may be even greater than 10 ml. To maintain system efficiency the sample valve must be designed to have very low dispersion characteristics, this is true not only for flow dispersion but also for the less obvious problems of dispersion caused by sample adsorption/desorption on valve surfaces and diffusion of sample into and out of the mating surfaces between valve moving parts. It goes without saying that the valves must deliver a very constant sample size but this is usually attained by the use of a constant size sample loop.

HPLC Columns

HPLC columns are packed with very fine particles (usually a few microns in diameter). The very fine particles are required to attain the low dispersion that give the high plate counts expected of modern HPLC. Plate counts in excess of 25,000 plates per column are possible with modern columns, however, these very high efficiencies are very rarely found with real samples because of the dispersion associated with injection valves, detectors, data acquisition systems and the dispersion due to the higher molecular weight of real samples as opposed to the common test samples. Packing these small particles into the column is a difficult technical problem but even with good packing a great amount of care must be given to the column end fittings and the inlet and outlet connection to keep dispersion to a minimum. The main consideration with HPLC is the much wider variety of solvents and packing materials that can be utilized because of the much lower

quantities of both which are required. In particular very expensive optically pure compounds can be used to make Chiral HPLC stationary phases and may even be used as (disposable) HPLC solvents.

HPLC Detectors [5-10]

UV/Vis spectrophotometers, including diode array detectors, are the most commonly employed detectors. Fluorescence spectrophotometers, differential refractometers, electrochemical detectors, mass spectrometers, light scattering detectors, radioactivity detectors or other special detectors may also be used.

Detector consists of a flow-through cell mounted at the end of the column. A beam of UV radiation passes through the flow cell and into the detector. As compounds elute from the column, they pass through the cell and absorb the radiation, resulting in measurable energy level changes. Fixed (mercury lamp), variable (deuterium or highpressure xenon lamp), and multi-wavelength detectors are widely available. Modern variable wavelength detectors can be programmed to change wavelength while an analysis is in progress. Multi-wavelength detectors measure absorbance at two or more wavelengths simultaneously. In diode array multi-wavelength detectors, continuous radiation is passed through the sample cell, and then resolved into its constituent wavelengths, which are individually detected by the photodiode array. These detectors acquire absorbance data over the entire UV-visible range, thus providing the analyst with chromatograms at multiple, selectable wavelengths, spectra of the eluting peaks and also peak purity.

Differential refractometer detectors measure the difference between the refractive index of the mobile phase alone and that of the mobile phase containing chromatographic compounds as it emerges from the column. Refractive index detectors are used to detect non-UV absorbing compounds.

Fluorometric detectors are sensitive to compounds that are inherently fluorescent or that can be converted to fluorescent derivatives either by chemical transformation of the compound or by coupling with fluorescent reagents at specific functional groups.

Potentiometric, voltametric, or polarographic electrochemical detectors are useful for the quantitation of species that can be oxidized or reduced at a working electrode. These detectors are selective, sensitive, and reliable, but require conducting mobile phases free of dissolved oxygen and reducible metal ions. Electrochemical detectors with carbon-paste electrodes may be used advantageously to measure nanogram quantities of easily oxidized compounds, notably phenols and catechols.

In order to give an accurate chromatographic profile the detector sampling (cell) volume must be a small fraction of the solute elution volume. If the detector volume were larger than the elution volume then you would have peaks that appeared with flat tops as the whole peak would be resident in the detector at the same time. This means that as column volumes decrease and system efficiencies increase the volume of the detector cell volume must also decrease. It is odds for the requirement for detector to maintain high sensitivity as this is usually dependant on having a larger cell volume. Again, this requires the very careful design of modern detectors. Many types of detectors can use with HPLC system like UV-Visible or PDA (Photo Diode Array), RI (Refractive Index),

Fluorescence, ECD (Electro Chemical Detector), ELSD (Evaporative Light Scattering detector) and many others hyphenated techniques like MS, MS/MS and NMR as well as evaporative IR.

HPLC Data acquisition

In HPLC data acquisition system the higher sampling rate needed for the rapidly eluting narrow peaks of the HPLC chromatogram. Although the theoretical number of samples needed for good quantization are actually quite small, for real systems a hundred samples or more per peak is recommended; thus, for a 4 sec wide peak, a rate of 25 samples per second may be required. The same data analysis and reporting software can be used as in ordinary LC.

Conclusion

HPLC is probably the most universal type of analytical procedure; its application areas include quality control, process control, forensic analysis, environmental monitoring and clinical testing. In addition HPLC also ranks as one of the most sensitive analytical procedures and is unique in that it easily copes with multi-component mixtures. It has achieved this position as a result of the constant evolution of the equipment used in LC to provide higher and higher efficiencies at faster and faster analysis times with a constant incorporation of new highly selective column packings.

Introduction to HPLC Methods of Analysis for Drugs [11-13]

Most of the drugs in single/multi component dosage forms can be analyzed by HPLC method because of the several advantages like rapidity, specificity, accuracy, precision

and ease of automation in this method. HPLC method eliminates tedious extraction and isolation procedures. Some of the advantages are:

- Speed (analysis can be accomplished in 20 minutes or less).
- Greater sensitivity (various detectors can be employed).
- Improved resolution (wide variety of stationary phases).
- Reusable columns (expensive columns but can be used for many analysis).
- Ideal for the substances of low volatility.
- Easy sample recovery, handling and maintenance.
- Instrumentation tends itself to automation and quantitation (less time and less labour).
- Precise and reproducible.
- Calculations are done by integrator itself.
- Suitable for preparative liquid chromatography on a much larger scale.

There are different modes of separation in HPLC. They are normal phase mode, reversed phase mode, reverse phase ion pair chromatography, affinity chromatography and size exclusion chromatography (gel permeation and gel filtration chromatography).

In the normal phase mode, the stationary phase is polar and the mobile phase is non-polar in nature. In this technique, non-polar compounds travel faster and are eluted first. This is because of the lower affinity between the non-polar compounds and the stationary phase. Polar compounds are retained for longer times because of their higher affinity with the stationary phase. These compounds, therefore take more times to elute.

Normal phase mode of separation is therefore, not generally used for pharmaceutical applications because most of the drug molecules are polar in nature and hence takes longer time to elute.

Reversed phase mode is the most popular mode for analytical and preparative separations of compound of interest in chemical, biological, pharmaceutical, food and biomedical sciences. In this mode, the stationary phase is non-polar hydrophobic packing with octyl or octa decyl functional group bonded to silica gel and the mobile phase is polar solvent. An aqueous mobile phase allows the use of secondary solute chemical equilibrium (such as ionization control, ion suppression, ion pairing and complexation) to control retention and selectivity. The polar compound gets eluted first in this mode and nonpolar compounds are retained for longer time. As most of the drugs and pharmaceuticals are polar in nature, they are not retained for longer times and hence elute faster. The different columns used are Octa Decyl Silane (ODS) or C18, C8, C4, etc. (in the order of increasing polarity of the stationary phase).

In ion exchange chromatography, the stationary phase contains ionic groups like $NR3^{+}$ or $SO3^{-2}$, which interact with the ionic groups of the sample molecules. This is suitable for the separation of charged molecules only. Changing the pH and salt concentration can modulate the retention.

Ion pair chromatography may be used for the separation of ionic compounds and this method can also substitute for ion exchange chromatography. Strong acidic and basic compounds may be separated by reversed phase mode by forming ion pairs (coulumbic association species formed between two ions of opposite electric charge) with suitable

counter ions. This technique is referred to as reversed phase ion pair chromatography or soap chromatography.

Affinity chromatography uses highly specific biochemical interactions for separation. The stationary phase contains specific groups of molecules which can absorb the sample if certain steric and charge related conditions are satisfied. This technique can be used to isolate proteins, enzymes as well as antibodies from complex mixtures.

Size exclusion chromatography separates molecules according to their molecular mass. Largest molecules are eluted first and the smallest molecules last. This method is generally used when a mixture contains compounds with a molecular mass difference of at least 10%. This mode can be further subdivided into gel permeation chromatography (with organic solvents) and gel filtration chromatography (with aqueous solvents).

Method Development and Design of Separation Method

Methods for analyzing drugs in single or multi component dosage forms can be developed, provided one has knowledge about the nature of the sample, namely, its molecular weight, polarity, ionic character and the solubility parameter. An exact recipe for HPLC, however, cannot be provided because method development involves considerable trial and error procedures. The most difficult problem usually is where to start, what type of column is worth trying with what kind of mobile phase. In general one begins with reversed phase chromatography, when the compounds are hydrophilic in nature with many polar groups and are water soluble.

The organic phase concentration required for the mobile phase can be estimated by gradient elution method. For aqueous sample mixtures, the best way to start is with gradient reversed phase chromatography. Gradient can be started with 5-10 % organic phase in the mobile phase and the organic phase concentration (methanol or acetonitrile) can be increased up to 100 % within 30-45 min. Separation can then be optimized by changing the initial mobile phase composition and the slope of the gradient according to the chromatogram obtained from the preliminary run. The initial mobile phase composition can be estimated on the basis of where the compounds of interest were eluted, namely at what mobile phase composition.

Changing the polarity of mobile phase can alter elution of drug molecules. The elution strength of a mobile phase depends upon its polarity, the stronger the polarity, higher is the elution. Ionic samples (acidic or basic) can be separated, if they are present in undissociated form. Dissociation of ionic samples may be suppressed by the proper selection of pH.

The pH of the mobile phase has to be selected in such a way that the compounds are not ionized. If the retention times are too short, the decrease of the organic phase concentration in the mobile phase can be in steps of 5%. If the retention times are too long, an increase of the organic phase concentration is needed.

In UV detection, good analytical results are obtained only when the wavelength is selected carefully. This requires knowledge of the UV spectra of the individual components present in the sample. If analyte standards are available, their UV spectra can be measured prior to HPLC method development.

The molar absorbance at the detection wavelength is also an important parameter. When peaks are not detected in the chromatograms, it is possible that the sample quantity is not enough for the detection. An injection of volume of 20 µL from a solution of 1mg/mL concentration normally provides good signals for UV active compounds around 220 nm. Even if the compounds exhibit higher λmax, they absorb strongly at lower wavelength.

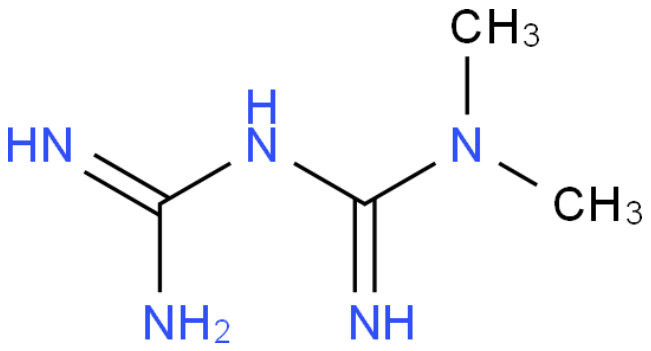
It is not always necessary to detect compounds at their maximum absorbance. It is, however, advantageous to avoid the detection at the sloppy part of the UV spectrum for precise quantitation. When acceptable peaks are detected on the chromatogram, the investigation of the peak shapes can help further method development.

The addition of peak modifiers to the mobile phase can affect the separation of ionic samples. For examples, the retention of the basic compounds can be influenced by the addition of small amounts of triethylamine (a peak modifier) to the mobile phase. Similarly for acidic compounds small amounts of acids such as acetic acid can be used. This can lead to useful changes in selectivity.

When tailing or fronting is observed, it means that the mobile phase is not totally compatible with the solutes. In most case the pH is not properly selected and hence partial dissociation or protonation takes place. When the peak shape does not improve by lower (1-2) or higher (8-9) pH, then ion-pair chromatography can be used. For acidic compounds, cationic ion pair molecules at higher pH and for basic compounds, anionic ion-pair molecules at lower pH can be used. For amphoteric solutes or a mixture of acidic and basic compounds, ion-pair chromatography is the method of choice.

DRUG PROFILE

METFORMIN



IUPAC Name : 1-carbamimidamido-N,N-dimethylmethanimidamide

Chemical formula : C4H11N5

Molecular weight : 129.16364

pKa : 12.4

Description : white to off-white crystalline compound

Solubility : freely soluble in water,
Insoluble in acetone, ether, chloroform.

Melting point : 215-218°C

Category : antidiabetic drug, Antihypoglycemic agent.

Mechanism of action: Metformin decreases blood glucose levels by decreasing hepatic glucose production, decreasing intestinal absorption of glucose, and improving insulin sensitivity by increasing peripheral glucose uptake and utilization. These effects are mediated by the initial activation by metformin of AMP-activated protein kinase (AMPK), a liver enzyme that plays an important role in insulin signaling, whole body energy balance, and the metabolism of glucose and fats. Activation of AMPK is required for metformin's inhibitory effect on the production of glucose by liver cells. Increased peripheral utilization of glucose may be due to improved insulin binding to insulin receptors. Metformin administration also increases AMPK activity in skeletal muscle. AMPK is known to cause GLUT4

deployment to the plasma membrane, resulting in insulin-independent glucose uptake. The rare side effect, lactic acidosis, is thought to be caused by decreased liver uptake of serum lactate, one of the substrates of gluconeogenesis. In those with healthy renal function, the slight excess is simply cleared. However, those with severe renal impairment may accumulate clinically significant serum lactic acid levels. Other conditions that may precipitate lactic acidosis include severe hepatic disease and acute/decompensated heart failure.

Generic Name: Metformin

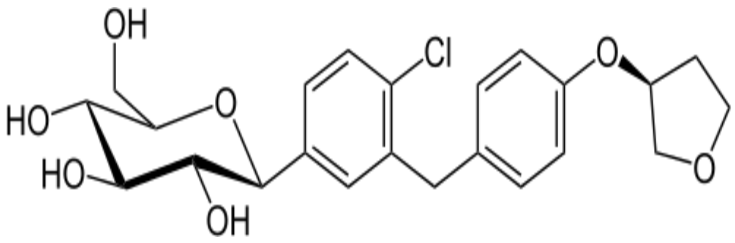
***Brand Name**: Glucophage, Glucophage XR, Glumetza, Fortamet, Riomet*

EMPAGLIFLOZINE

IUPAC Name **:** (2S,3R,4R,5S,6R)-2-[4-chloro-3-({4-[(3S)-oxolan-3-yloxy]phenyl}methyl)phenyl]-6-(hydroxymethyl)oxane-3,4,5-triol

Chemical formula **:** $C_{23}H_{27}ClO_7$

Structure **:**



Molecular weight **:** 450.91 g/mol

Cas no **:** 864070-44-0

Solubility **:** Soluble in water

Melting Point **:** 127-130 °C

Pka : 12.57

Mechanism of action : Empagliflozin is a sodium glucose co-transporter-2 (SGLT-2) inhibitor. SGLT2 co-transporters are responsible for reabsorption of glucose from the glomerular filtrate in the kidney. The glucuretic effect resulting from SGLT2 inhibition reduces renal absorption and lowers the renal threshold for glucose, resulting in increased glucose excretion. Additionally, it contributes to reduced hyperglycaemia, assists weight loss, and reduces blood pressure.

REVIEW OF LITERATURE

- **Liandong Hu et.al.,** A selective, rapid and sensitive high-performance liquid chromatography (HPLC) method has been developed for the simultaneous determination of oxycodone and related substances in oxycodone and acetaminophen capsule. An isocratic elution was performed to determine content of oxycodone on a Welchrom C18 column (5μm, 250 ×4.6 mm) for the separation, with 7mM potassium dihydrogen phosphate aqueous solution (containing 0.1% phosphoric acid, 0.1% n-nonylamine, with potassium hydroxide solution (1 → 2), the pH was adjusted to 4.9 ± 0.1) and methanol (9:1) as the mobile phase at the flow rate of 1 mL/min.The detection wavelength for oxycodone was 214 nm. The accuracy of this method, measured by the recovery of oxycodone was above 99% at three spiking levels. The linear regression analysis data for the calibration.plots showed good linear relationship at the concentration range. Gradient elution was used in order to assay the contents of related substances at the detection wavelength of 230nm. All the parameters of recovery study were within the limits. Both of the two methods showed good linearity for oxycodone and related substances, respectively. The main component oxycodone was well separated from other ingredients and degradation products. Both of the two methods were capcable to confirm the contents of corresponding substances. This method is fast, simple, and can be used for determination of oxycodone and related substances in this oxycodone and acetaminophen preparation.[32]

AIM AND OBJECTIVE

Literature review reveals that there is no analytical method reported for the analysis of Metformin and Empagliflozin by simultaneous estimation by RP-HPLC. Spectrophotometer, HPLC and HPTLC are the reported analytical methods for compounds either individually or in combination with other dosage form. Hence, it was felt that, there is a need of new analytical method development for the simultaneous estimation of Metformin and Empagliflozin in pharmaceutical dosage form.

Present work is aimed to develop a new, simple, fast, rapid, accurate, efficient and reproducible RP-HPLC method for the simultaneous analysis of Metformin and Empagliflozin. The developed method will be validated according to ICH guidelines.

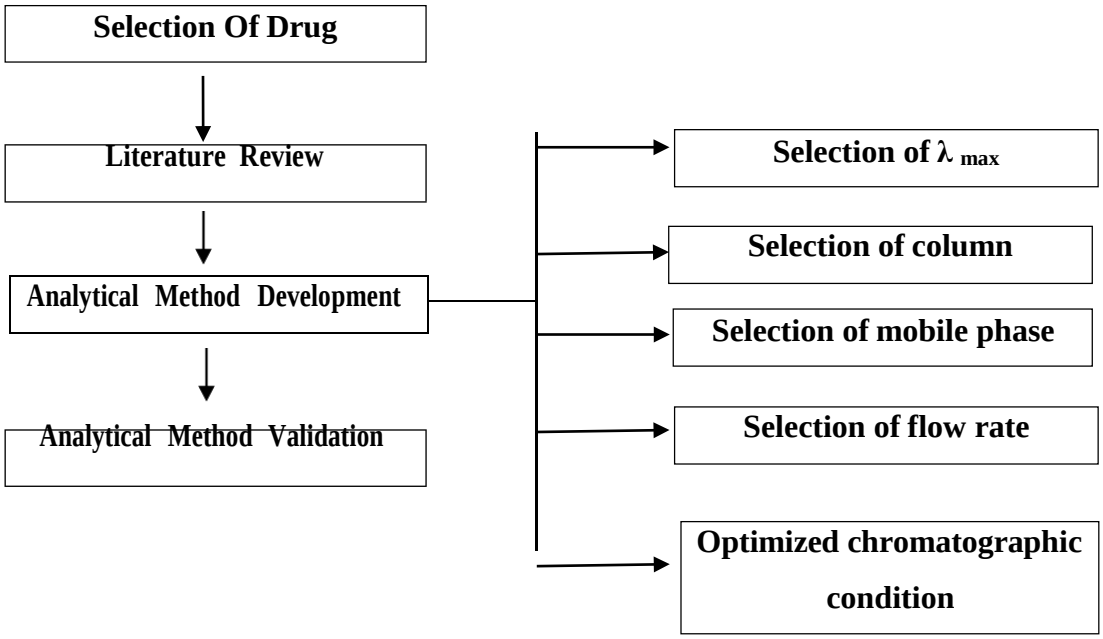
Objective of the work

- The analytical method for the simultaneous estimation of Metformin and Empagliflozin will be developed by RP-HPLC method by optimizing the chromatographic conditions.
- The developed method is validated according to ICH guidelines for various parameters specified in ICH guidelines, Q2 (R1).

PLAN OF WORK

To develop a new analytical method for the simultaneous estimation of Metformin and Empagliflozin by RP-HPLC.

The dissertation work has been carried out in the following steps:



MATERIALS AND METHODS

MATERIALS:

The list of instruments used in the course of experimental work is as follows:

Table 1: List of Instruments

S.No.	Instrument	Model No.	Software	Manufacturer's name
1	HPLC Alliance	Waters 2695	Empower	Waters
2	UV double beam	UV 3000	UV Win 5	Lab India
3	Digital weighing	BSA224SC	-	Satorius
4	pH meter	AD102U	-	Lab India
5	Ultra sonicator	SE60US	-	-
6	Suction pump	VE115N	-	-

The experimental work involves several chemicals. Chemicals used presently are listed below:

Table:2 List of Chemicals

S.No.	Chemical	Manufactur	Grade
1	Water	Me	HPLC Grade
2	Methanol	Me	HPLC Grade
3	Acetonitrile	Me	HPLC Grade
4	Potassium dihydrogen orthophosphate	Me rck	A.R
5	Metformin and Empagliflozin	-	-
6	Eurepa mf tablets	Local	-

METHOD DEVELOPMENT:

Method development for simultaneous estimation of Metformin and Empagliflozin in Pharmaceutical dosage forms includes the following steps:

1. Selection of detection wavelength (λ_{max})
2. Selection of column
3. Selection of mobile phase
4. Selection of flow rate
5. Preparations and procedures

1. Selection of Detection wavelength:

10 mg of Metformin and Empagliflozin was dissolved in mobile phase. The solution was scanned from 200-400 nm the spectrum was obtained. The overlay spectrum was used for selection of wavelength for Metformin and Empagliflozin. The isobestic point was taken as detection wavelength. The overlay spectrums are shown in Figs. 4.1, 4.2, & 4.3.

2. Selection of column:

Column is selected based on solubility, polarity and chemical differences among

Analytes [Column: Inertsil C18 (4.6 x 250mm, 5µm, Make: Waters)]

3. Selection of mobile phase:

Phosphate buffer (0.05M) pH 4.6: ACN (30:70%v/v) has been selected as mobile phase. Buffer pH should be between 2 to 8. If the buffer pH is below 2 siloxane linkages are cleaved. If the buffer pH is above 8 dissolution of silica takes place. pH controls the elution properties by controlling the ionization characteristics. It also decreases the retention and improves separation. Good Response, Area, Tailing factor, Resolution will be achieve.

4. Selection of flow rate:

Flow rate selected was 1ml/min

Flow rate is selected based on

1. Retention time
2. Column back pressure
3. Peak symmetry
4. Separation of impurities

5. Preparations and procedures:

Preparation of Phosphate buffer :(PH: 4.6):

Weighed 6.8 grams of KH_2PO_4 was taken into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC water, adjusted the pH to 4.6 with ortho phosphoric acid.

Preparation of mobile phase:

A mixture of pH 4.6 Phosphate buffer 300 mL (30%), 700 mL of ACN (70%) are taken and degassed in ultrasonic water bath for 5 minutes. Then this solution is filtered through 0.45 µ filter under vacuum filtration.

Diluant Preparation:

Mobile phase is used as Diluant**.**

Preparation of the individual Empagliflozin standard preparation:

10mg of Empagliflozin working standard was accurately weighed and transferred into a 10ml clean dry volumetric flask and about 2ml of DMF is added. Then it is sonicated to dissolve it completely and made volume upto the mark with the diluant. (Stock solution). Further 10.0 ml from the above stock solution is pipette into a 100 ml volumetric flask and was diluted upto the mark with diluant.

Preparation of the individual Metformin standard preparation:

10mg of Metformin working standard was accurately weighed and transferred into a 10ml clean dry volumetric flask and about 2ml of DMF is added. Then it is sonicated to dissolve it completely and made volume upto the mark with the diluant. (Stock solution). Further 10.0 ml from the above stock solution is pipette into a 100 ml volumetric flask and was diluted upto the mark with diluant.

Preparation of Sample Solution :(Tablet)

Accurately 10 tablets are weighed and crushed in mortar and pestle and weight equivalent to 10 mg of Metformin and Empagliflozin (marketed formulation) sample into a 10mL clean dry volumetric flask and about 7mL of Diluents is added and sonicated to dissolve it completely and made volume upto the mark with the same solvent. (Stock solution) Further 3 ml of above stock solution was pipetted into a10ml volumetric flask and diluted upto the mark with diluant.

Procedure:

20µL of the standard, sample are injected into the chromatographic system and the areas for Metformin and Empagliflozin peaks are measured and the %Assay are calculated by using the formulae.

System Suitability:

Tailing factor for the peaks due to Metformin and Empagliflozin in Standard solution should not be more than 2.0.

Theoretical plates for the Metformin and Empagliflozin peaks in Standard solution should not be less than 2000

Assay calculation:

$$Assay\ \% = \frac{sample\ area}{Standard\ area} \times \frac{dilution\ sample}{dilution\ of\ standard} \times \frac{P}{100} \times \frac{Avg.wt}{Lc} \times 100$$

Where,

P = Percentage purity of working standard

Lc = LABEL CLAIM OF drug in mg/ml.

ANALYTICAL METHOD VALIDATION

Accuracy:

Preparation of standard solution (Empagliflozin and Metformin):

Accurately weighed 10 mg of Metformin and 10mg of Empagliflozin working standard were transferred into a 10mL and 100ml of clean dry volumetric flasks.

About 7mL and 70ml of Diluents are added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution) Further 3ml and 0.3ml of the above stock solution was pipetted into a 10ml volumetric flask and diluted upto the mark with diluents.

Preparation of Sample solutions:

For preparation of 50% solution (With respect to target Assay concentration):

Accurately 5mg of Metformin and 5mg of Empagliflozin working standard were weighed and transferred into a 10mL and 100ml of clean dry volumetric flask and about 7mL of Diluents was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock Solution). Further 3ml and 0.3ml of the above Metformin a n d Empagliflozin stock solution were pipetted into a 10ml volumetric flask and diluted up to the mark with diluant.

***For preparation of 100% solution (With respect to target Assay concentration)*:**

Accurately 10mg of Metformin and 10mg of Empagliflozin working standard were weighed and transferred into a 10mL and 100ml of clean dry volumetric flask and about 7mL of Diluents was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock Solution). Further 3ml and 0.3ml of the above Metformin and Empagliflozin stock solution were pipetted into a 10ml volumetric flask and diluted up to the mark with diluant.

For preparation of 150% solution (With respect to target Assay concentration):

Accurately 15mg of Metformin and 15mg of Empagliflozin working standard were weighed and transferred into a 10mL and 100ml of clean dry volumetric flask and about 7mL of Diluents was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock Solution). Further 3ml and 0.3ml of the above Metformin a nd Empagliflozin stock solution were pipetted into a 10ml volumetric flask and diluted up to the mark with diluant.

Procedure:

The standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions were injected. The Amount found and Amount added for Metformin & Empagliflozin and

the individual recovery and mean recovery values were calculated.

Acceptance criteria

- Correlation coefficient should be not less than 0.999.

Precision

A) Repeatability:

Preparation of standard stock solution:

Accurately 10 mg of Metformin and 10mg of Empagliflozin working standard were weighed and transferred into a 10mL and 100ml of clean dry volumetric flasks and about 7mL and 70ml of Diluant was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution) Further it was pipette (3ml and 0.3ml) into a 10ml volumetric flask and diluted up to the mark with diluents.

Procedure:

The standard solution was injected for five times and the areas for all five injections in HPLC were measured. The %RSD for the area of five replicate injections was found to be within the specified limits. The chromatograms are shown in Figs. 4.27 –4.31 and results are tabulated in Tables 4.21&4.22

Acceptance criteria

- The % RSD for the area of five standard injections results should not be more than 2.

B) Intermediate Precision (Ruggedness):

To evaluate the intermediate precision (also known as ruggedness) of the method, precision was performed on different days by using different make column of

same dimensions.

Preparation of standard stock solution:

Accurately 10 mg of Metformin and 10mg of Empagliflozin working standard were weighed and transferred into a 10mL and 100ml of clean dry volumetric flasks and about 7mL and 70ml of Diluant was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution) Further this Stock was pipette (3ml and 0.3ml) into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure

The standard solution was injected for five times and the area for all five injections measured in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits. The chromatograms are shown in Fig. 4.32-4.36 and results are tabulated in Table. 4.23, 4.24

Acceptance criteria

- The % RSD for the area of five sample injections results should not be more than 2%.

Specificity

The system suitability for specificity was carried out to determine whether there is any interference of any impurities in retention time of analytical peak. The specificity was performed by injecting blank. The chromatograms are shown in Fig. 4.37, 4.38.

LOD:

LOD's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) at levels approximating the LOD according to the formula. The standard deviation of the response can be determined ba sed on the standard deviation of y-intercepts of regression lines.

Formula:

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

Where

σ - Standard deviation (SD)

S – Slope

LOQ:

LOQ's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) according to the formula. Again, the standard deviation of the response can be determined based on the standard deviation of y-intercepts of regression lines.

Formula:

LOQ = 10 σ /Slope

Where

σ - Standard deviation

S – Slope

Linearity

Preparation of stock solution*:*

Accurately 10 tablets were weighed & crushed in mortar and pestle and weight equivalent to 10 mg of Metformin and Empagliflozin (marketed formulation) sample were transferred into a 10mL clean dry volumetric flask and about 7mL of Diluant was added and sonicated to dissolve it completely and made volume up to the mark with the same

solvent. (Stock solution)

Preparation of Level – I (20ppm of Metformin &10pm of Empagliflozin):

1ml of stock solution has taken in 10ml of volumetric flask and diluted up to the mark with diluant.

Preparation of Level – II (40ppm of Metformin &20ppm of Empagliflozin):

2ml of stock solution has taken in 10ml of volumetric flask and diluted up to the mark with diluant.

Preparation of Level – III (60ppm of Metformin &30ppm of Empagliflozin):

3ml of stock solution has taken in 10ml of volumetric flask and diluted up to the mark with diluant.

Preparation of Level – IV (80ppm of Metformin &40ppm of Empagliflozin):

4ml of stock solution has taken in 10ml of volumetric flask and diluted up to the mark with diluant.

Preparation of Level – V (100ppm of Metformin &50ppm of Empagliflozin)

5ml of stock solution has taken in 10ml of volumetric flask and diluted up to the mark with diluant.

Procedure:

Each level was injected into the chromatographic system and the peak area was measured. A graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) was plotted and the correlation coefficient was calculated. The chromatograms are shown in Fig. 4.39 – 4.43 and results are tabulated in Table. 4.26,

4.27. Calibration graph for Metformin and Empagliflozinare shown in Fig. 4.44, 4.45

Acceptance criteria

- Correlation coefficient should be not less than 0.999.

Range:

Based on precision, linearity and accuracy data it can be concluded that the assay method is precise, linear and accurate in the range of 1µg-5µg and 100µg- 500µg of Metformin and Empagliflozinrespectively.

Robustness:

As part of the robustness, deliberate change in the flow rate, mobile phase composition was made to evaluate the impact on the method.

a) The flow rate was varied at 0.8ml/min to 1.2 ml/min. Standard solution 3ppm of Empagliflozin and 300ppm of Metformin was prepared and analyzed using the varied flow rates along with method flow rate. The chromatograms are shown in Fig. 4.46, 4.47 and results are tabulated in Table.4.30, 4.31

b) The organic composition in the mobile phase was varied from 65% to75 % standard solution 3 µg/ml of Empagliflozin and 300 µg/ml of Metformin were prepared and analyzed using the varied mobile phase composition along with the actual mobile phase composition in the method. The chromatograms are shown in Fig.4.48, 4.49 and results are tabulated in Table.4.32, 4.33

System suitability:

5 mg of Empagliflozin and 500 mg of Metformin working standard was accuratel y weighed and transferred into a 100ml clean dry volumetric flask and add about 20ml of diluant and sonicated to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further 10 ml of Metformin and Empagliflozin was pipetted out from the above stock solution into a 100ml volumetric flask and was diluted up to the mark with diluant.

METHOD DEVELOPMENT

WAVELENGTH DETECTION:

The detection wavelength was selected by dissolving the drug in mobile phase to get a concentration of 10µg/ml for individual and mixed standards. The resulting solution was scanned in U.V range from 200-400nm. The overlay spectrum of Metformin and Empagliflozin.

was obtained and the isobestic point of Metformin and Empagliflozin showed absorbance's maxima at 260 nm. The spectrums are shown

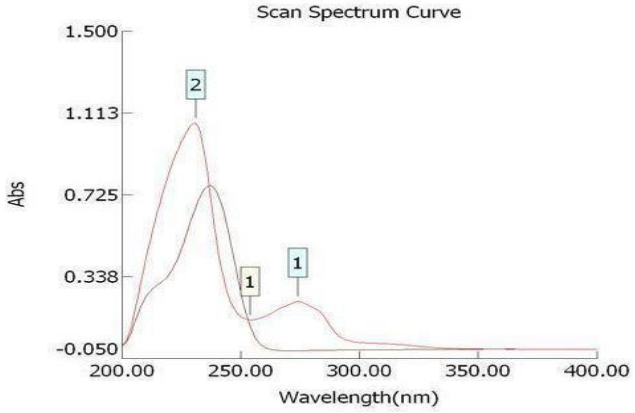


Fig. 1 : Overlay spectrum of Metformin and Empagliflozin

The UV spectra of individual drugs are as follows:

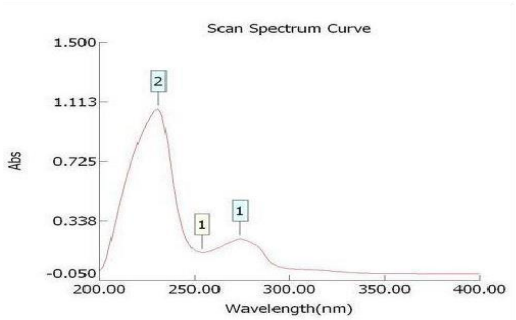


Fig. 2 Spectrum of Metformin

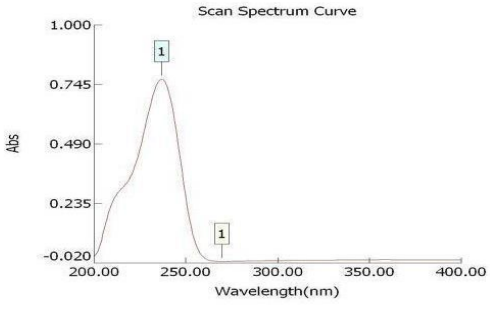


Fig. 3 Spectrum of Empagliflozin

METHOD DEVELOPMENT:

The chromatographic method development for the simultaneous estimation of Metformin and Empagliflozin were optimized by several trials for various parameters as different column, flow rate and mobile phase, finally the optimized chromatographic method was selected for the separation and quantification of Metformin and Empagliflozin API and pharmaceutical dosage form by RP-HPLC method.

Trial-1:

Chromatographic conditions.

Column : Agilent C18 (4.6*150mm) 5µm

Mobile phase ratio : Water: Methanol (40:60%v/v)

Detection wavelength : 255nm

Flow rate : 1ml/min

Injection volume : 10µl Column temperature : Ambient Auto sampler temperature : Ambient

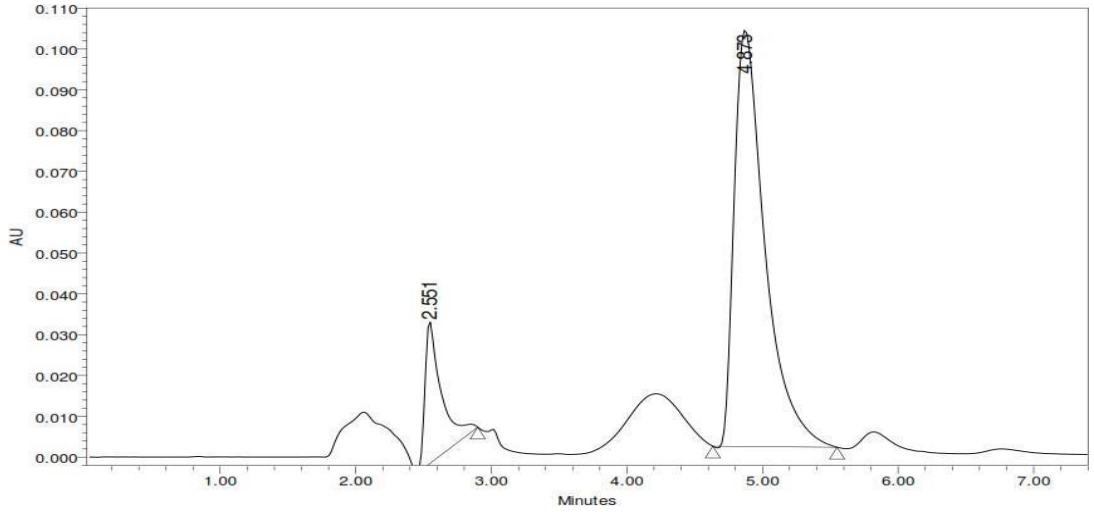


Fig. 4 Chromatogram of Trial-1

Table 3 Details of Trial-1

S.No	Peak name	R_t	Area	Height	USP Plate	USP Tailin	USP Resolutio
1	Metformin	2.551	867192	460798	745	2.19	
2	Empagliflozin	4.879	228369	179357	1911	2.79	1.45

Observation: Empagliflozin and Metformin w e r e separated and two individual peaks are displayed. But they are not clear.

Trial-2:

Chromatographic conditions:

Column	:	Thermosil C18 (4.6*150mm) 5µm
Mobile phase ratio	:	Water: Methanol (40:60%v/v)
Detection wavelength	:	255nm
Flow rate	:	1ml/min
Injection volume	:	10µl
Column temperature	:	40^{o}
Auto sampler temperature	:	Ambient

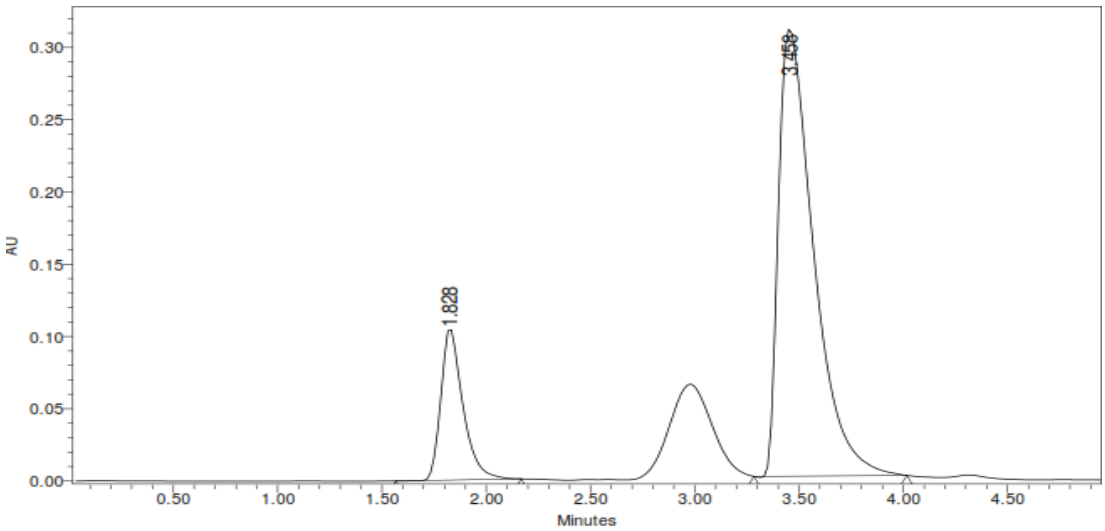


Fig 5 Chromatogram of Trial-2

Table 4 Details of Trial-2

S.No	Peak name	Rt	Area	Height	USP Plate count	USP Tailin	USP Resolutio
1	Metformin	1.8	7913799	394185	722	2.21	
2	Empagliflozin	3.4	1853381	162758	2614	2.85	1.52

Observation:

Peaks symmetry is being improved when compared to the previous trial. Further trials are conducted for better resolution.

Trial-3:

Chromatographic conditions:

Column Agilent C18 5µm (4.6*250mm)

Mobile phase ratio : Phosphate buffer (0.05m) pH 5.0: Methanol(50:50%v/v)

Detection wavelength : 255nm

Flow rate : 1ml/min

Injection volume : 10µl

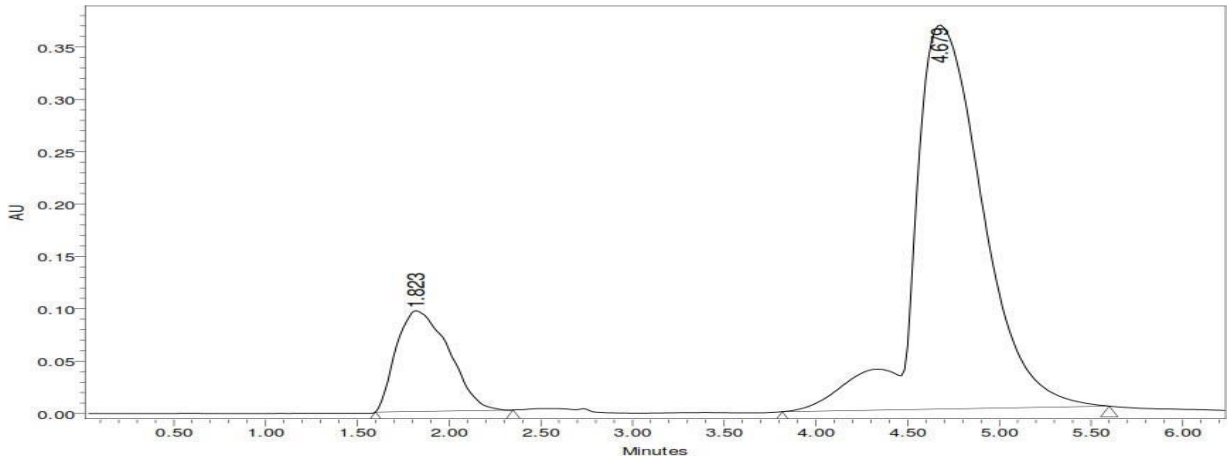


Fig 6 Chromatogram of Trial-3

Table 5 Details of Trail-3

S.No	Peak name	Rt	Area	Height	USP Plate count	USP Tailing	USP Resolution
1	Metformin	1.823	9849287	482363	198	1.97	
2	Empagliflozin	4.679	3272312	356630	5036	1.15	4.23

Observation:

There is noticeable improvement in resolution. But peak symmetry is not achieved.

Trial-4:

Chromatographic conditions:

Column : Inertsil ODS C18 5µm (4.6*250mm)

Mobile phase ratio : Phosphate buffer (0.05M) pH 4.6: MeOH

Detection wavelength : 255nm

Flow rate : 1ml/min

Injection volume : 20µl

Auto sampler temperature : Ambient

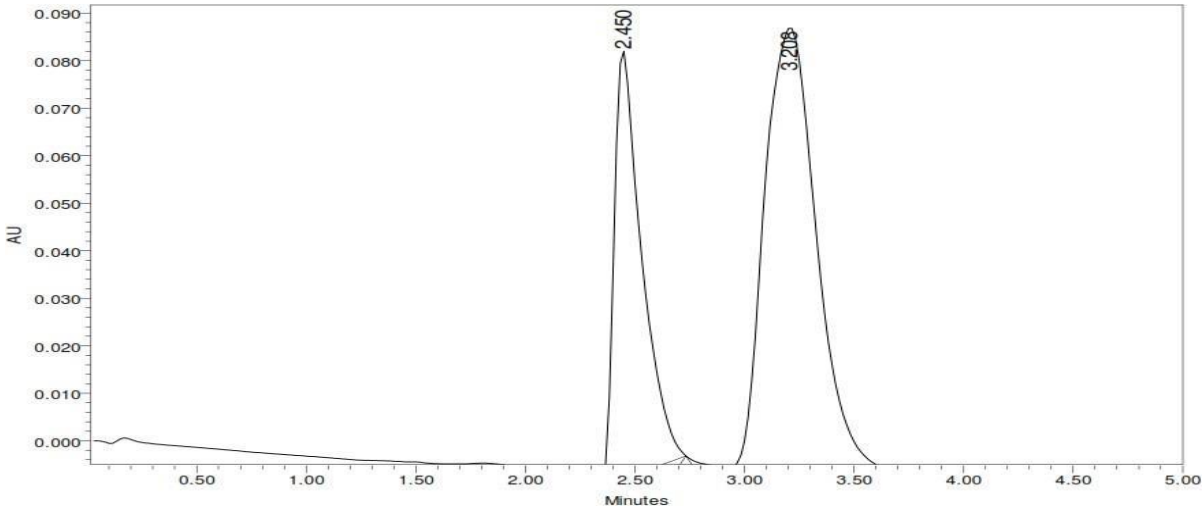


Fig 7 Chromatogram of Trial-4

Table 6 Details of Trial-4

S.No	Peak name	Rt	Area	Height	Plate count	Tailing	Resolution
1	Metformin	3.191	11286305	813690	1587	1.46	
2	Empagliflozinm	3.945	3443649	160557	616	1.80	1.46

Observation:

The tailing factor is within the limit. But the other parameters are not within the limit

Trial-5:

Chromatographic conditions:

Column : Inertsil C18 5µm (4.6*250mm)

Mobile phase ratio : Phosphate buffer (0.05M) pH 4.6: ACN (30:70%v/v)

Detection wavelength : 255nm

Flow rate : 1ml/min

Injection volume : 20µl Column temperature: Ambient

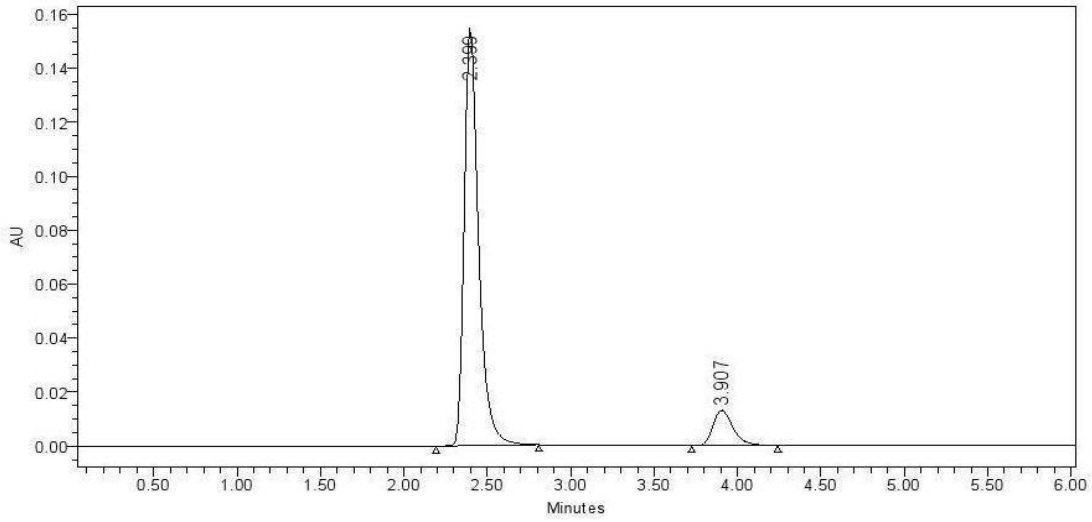


Fig. 8 Chromatogram of Trail-5

Table 7 Details of Trail-5

S.No	Peak name	Rt	Area	Height	USP Plate count	USP Tailin	USP Resolutio
1	Metformin	2.399	946124	155429	5105	1.3	8.1
2	Empagliflozin	3.907	111541	13239	3788	1.4	

Observation:

The chromatogram is perfect with clear separation of components. The peak symmetry and system suitability parameters are within the limits. Hence this method is chosen as optimized one

ASSAY CALCULATIONS FOR METFORMIN AND EMPAGLIFLOZIN

The assay study was performed fo r the Metformin and Empagliflozin. Each three injections of sample and standard was inject into chromatographic system. The chromatograms are shown in Fig. No.4.12 – 4.17 and results are tabulated in Tables 4.9 – 4.12

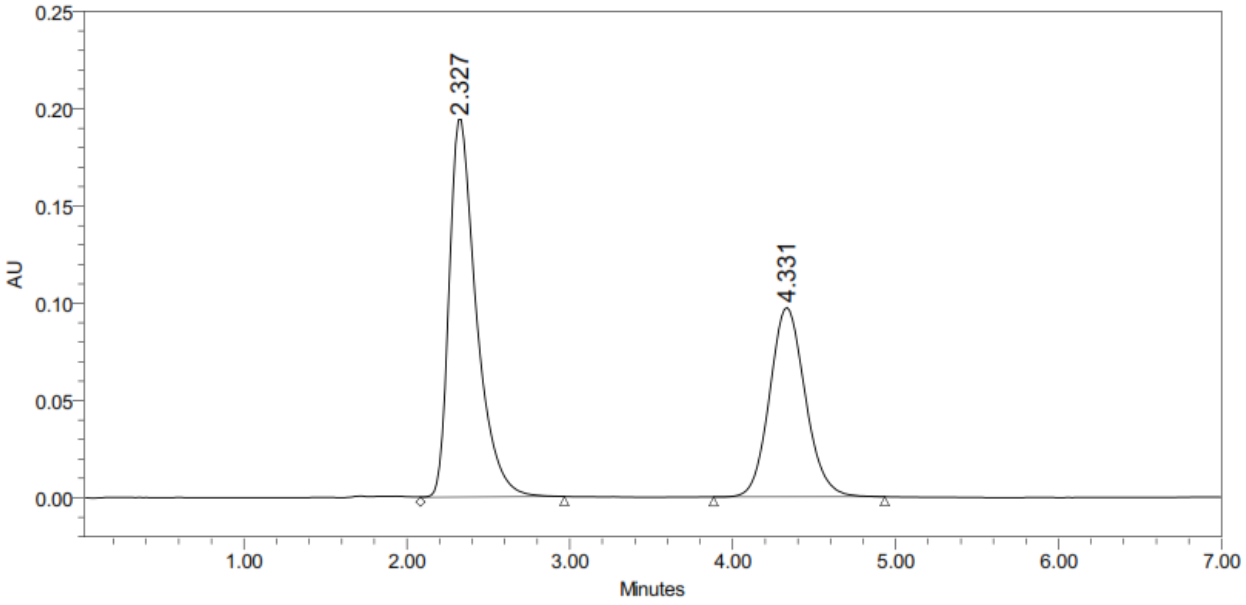


Fig. 9 Chromatogram showing sample injection-1

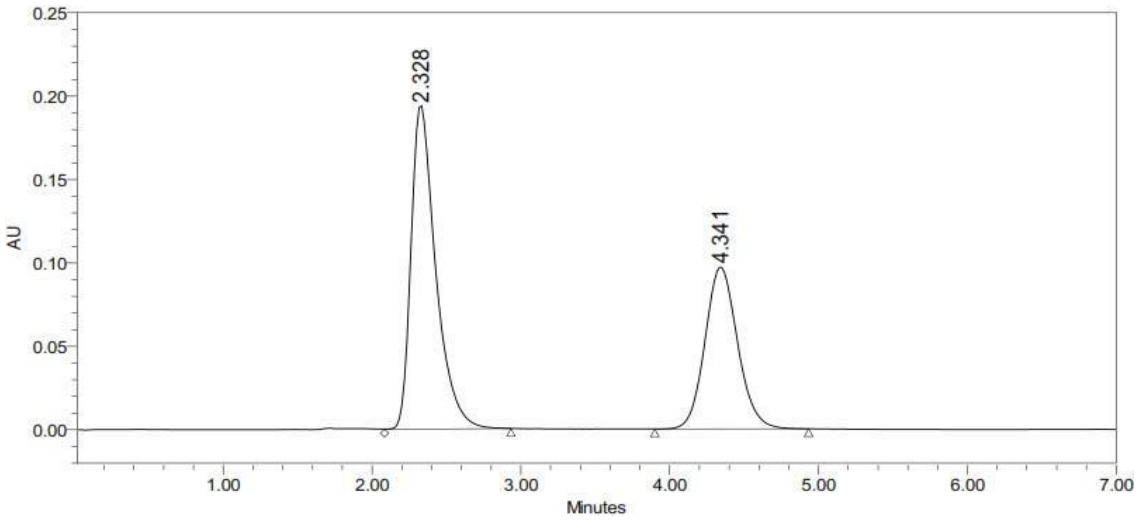


Fig. 10 Chromatogram showing sample injection-2

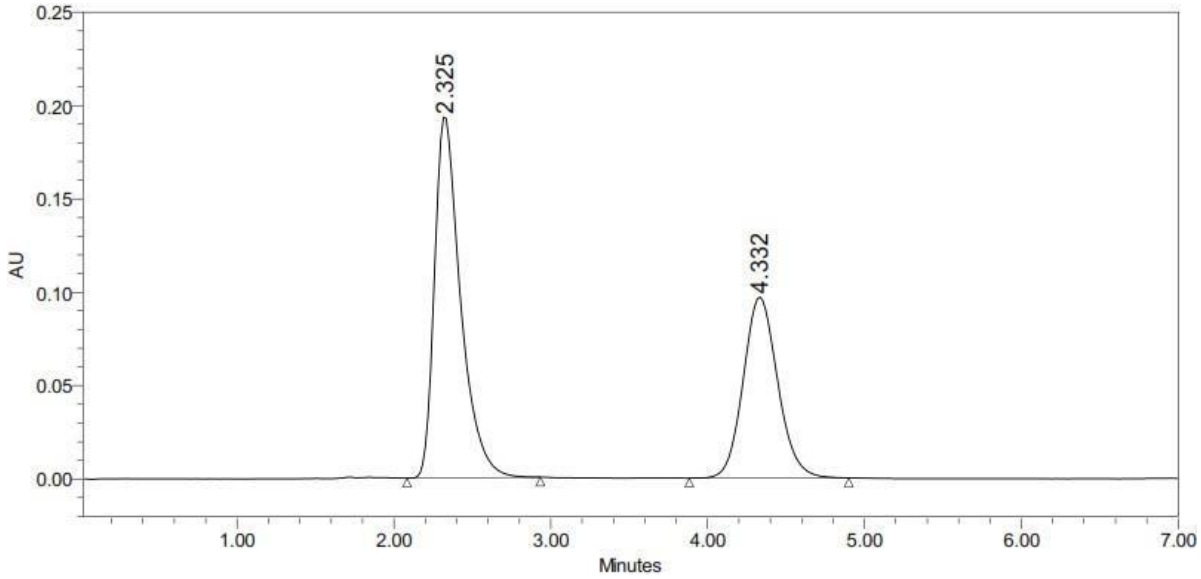


Fig. 11 Chromatogram showing sample injection-3

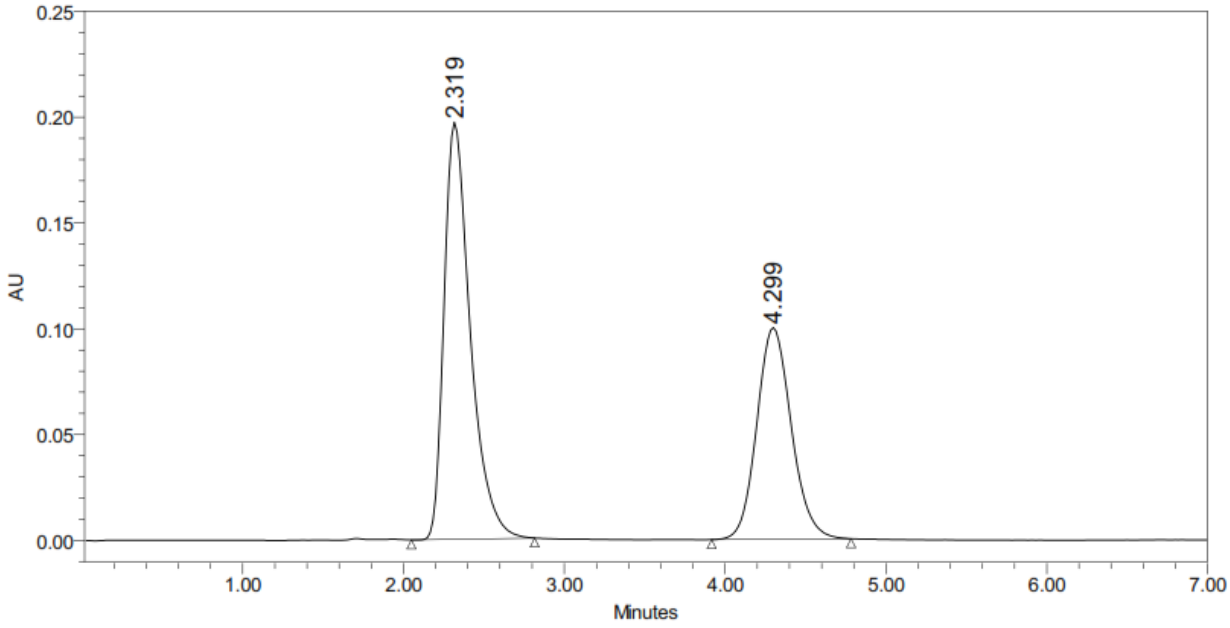


Fig. 12 Chromatogram showing standard injection-1

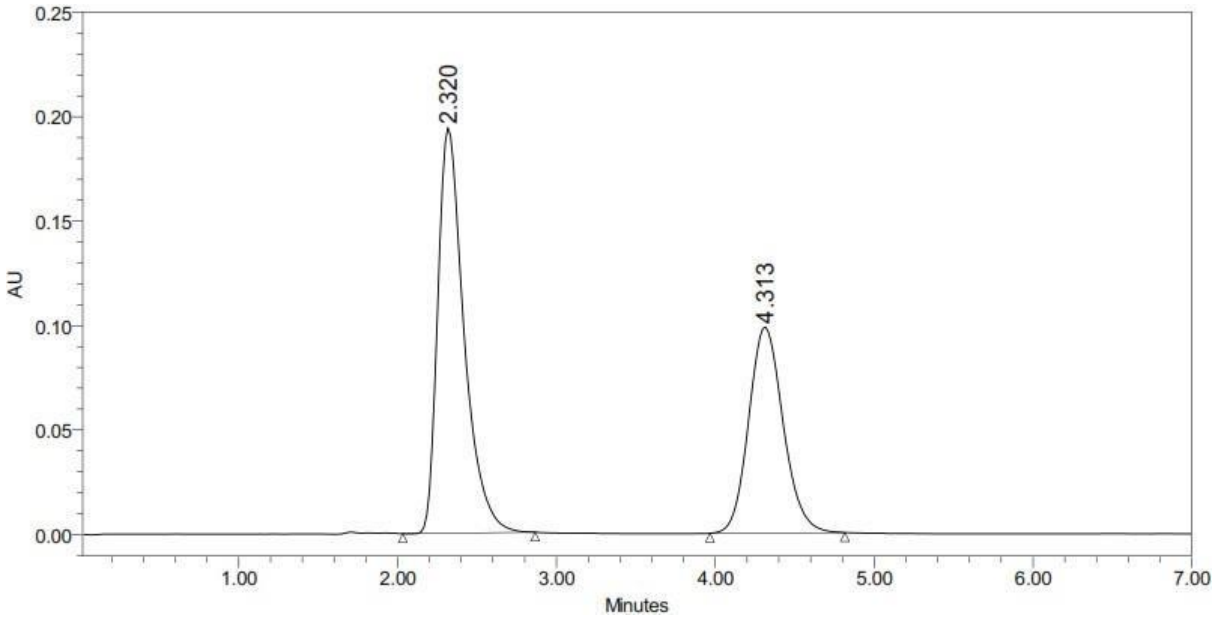


Fig. 13 Chromatogram showing standard injection-2

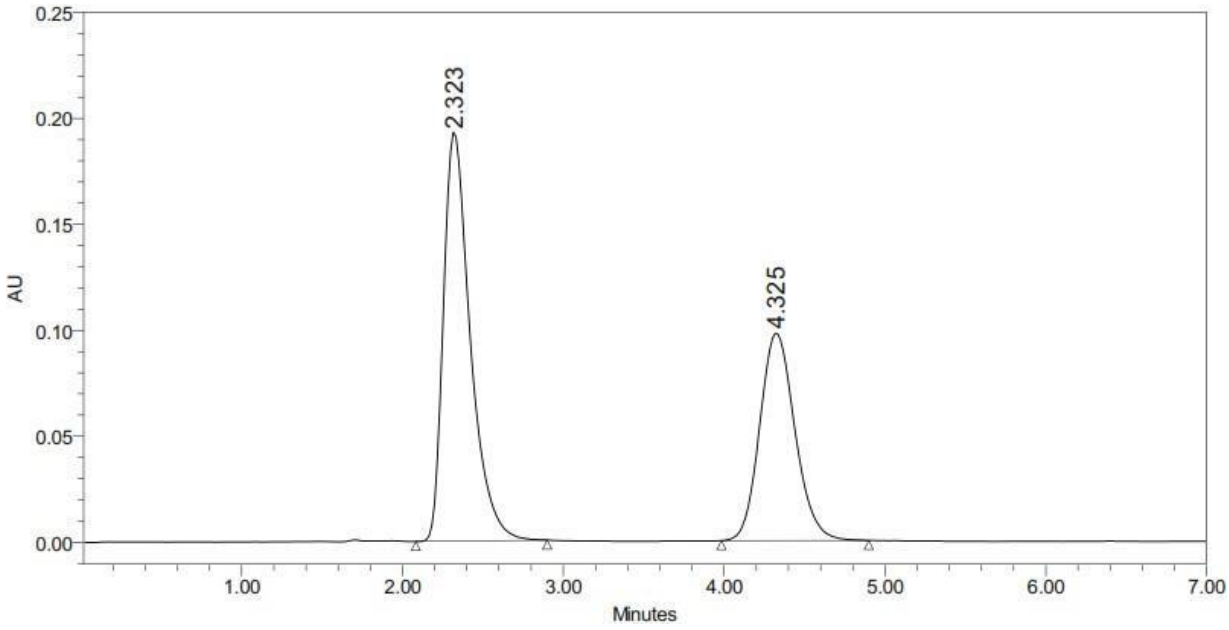


Fig. 14 Chromatogram showing standard injection-3

Calculations:

Empagliflozin*:*

Wt of 10 tablets : 668 g

Avgas wt : 0.668 g

$$Assay\ \% = \frac{sample\ area}{Standard\ area} \times \frac{dilution\ sample}{dilution\ of\ standard} \times \frac{P}{100} \times \frac{Avg.wt}{Lc} \times 100$$

$$= \frac{776673.9 \times 10 \times 0.5 \times 100 \times 10 \times 99.8 \times 0.668}{771716.1 \times 10 \times 10 \times 458 \times 0.33 \times 100 \times 500} \times 100$$

= 101.4

Metformin*:*

Wt of 10 tablets458 g.

Avgas wt :0.458 g.

Assay% =100.7%

- Tailing factor Obtained from the standard injection of Metformin and Empagliflozin are 1.3 & 1.4
- Theoretical Plates Obtained from the standard injection of Metformin and Empagliflozin are 5117.5 & 3877.3

Observation:

The system suitability parameters for Metformin and Empagliflozinsuch as theoretical plates and tailing factor were found to be 5117.5, 1.3 and 3877.3, 1.4. Resolution was8.1. The % purity of Metformin and Empagliflozinin pharmaceutical dosage form was found to be 100.7% and 101.4% respectively.

VALIDATION RESULTS

1. Accuracy:

The accuracy study was performed for 50%, 100% and 150 % for Metformin and Empagliflozin. Each level was injected in triplicate into chromatographic system. The area of each level was used for calculation of % recovery. Chromatograms are shown in Figs. 4.18-4.26 and results are tabulated in Tables 4.13-4.20

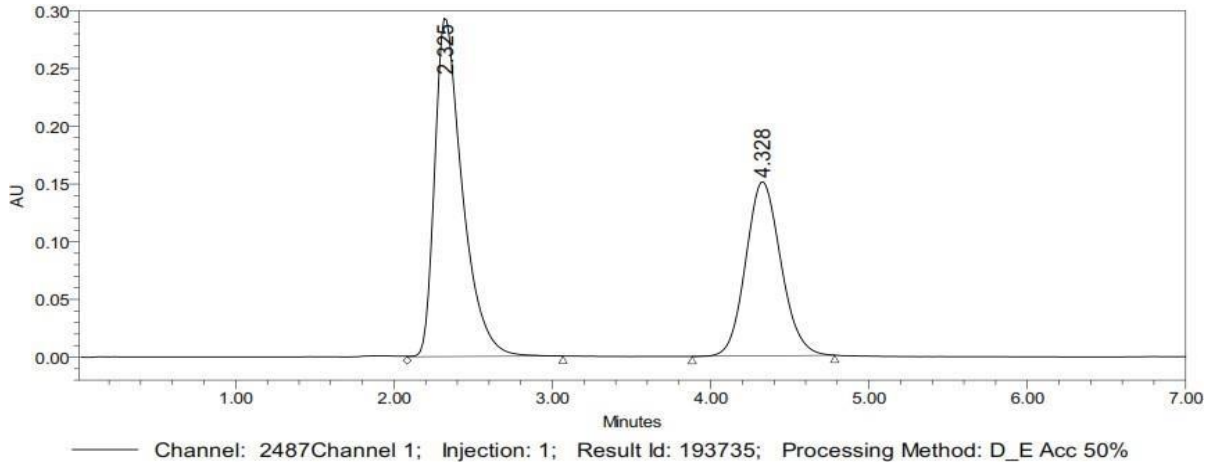


Fig. 15 Chromatogram showing accuracy 50%injection-1

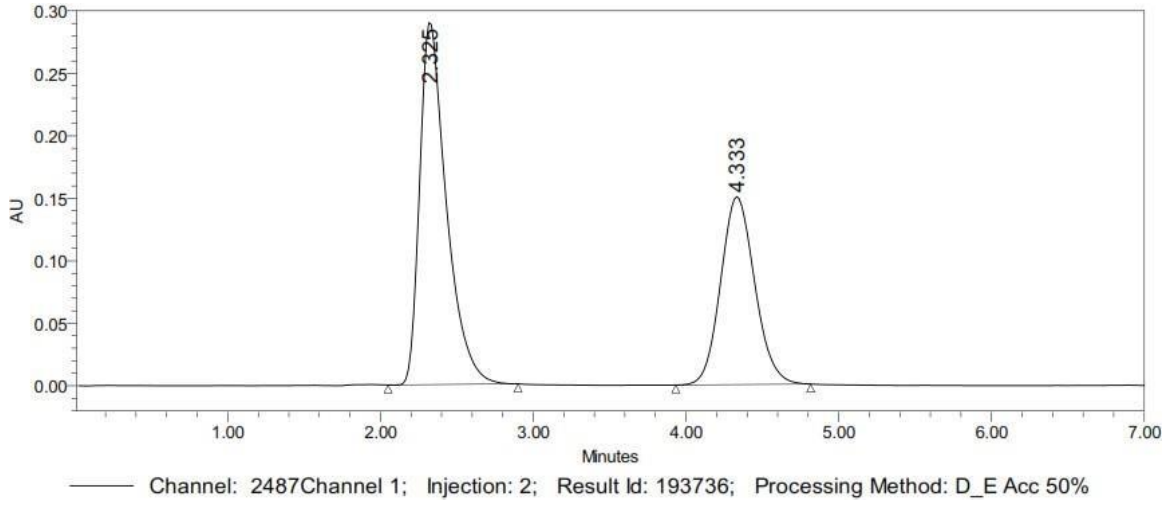


Fig. 16 Chromatogram showing accuracy 50%injection-2

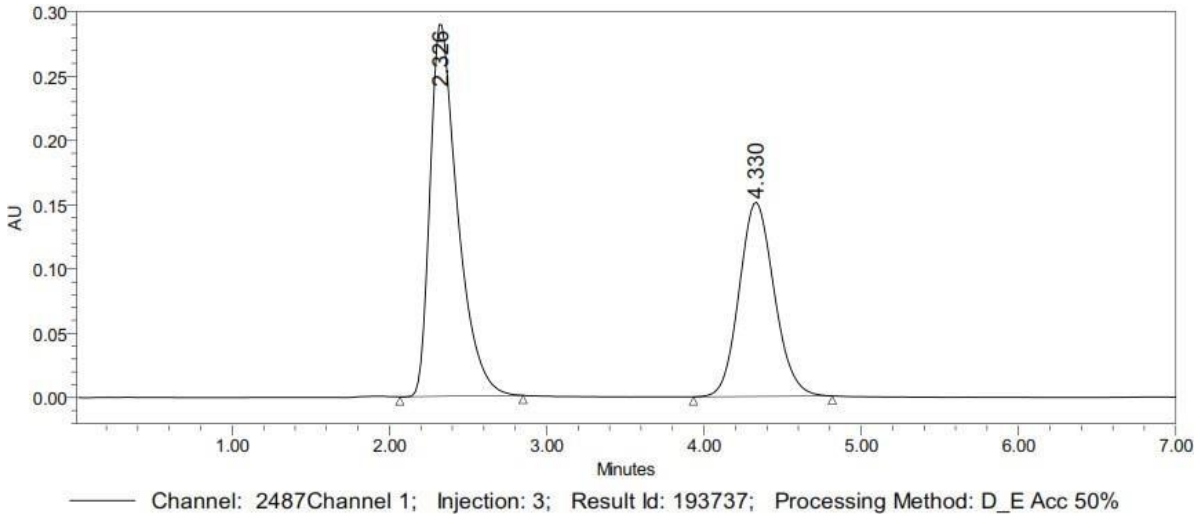


Fig. 17 Chromatogram showing accuracy 50%injection-3

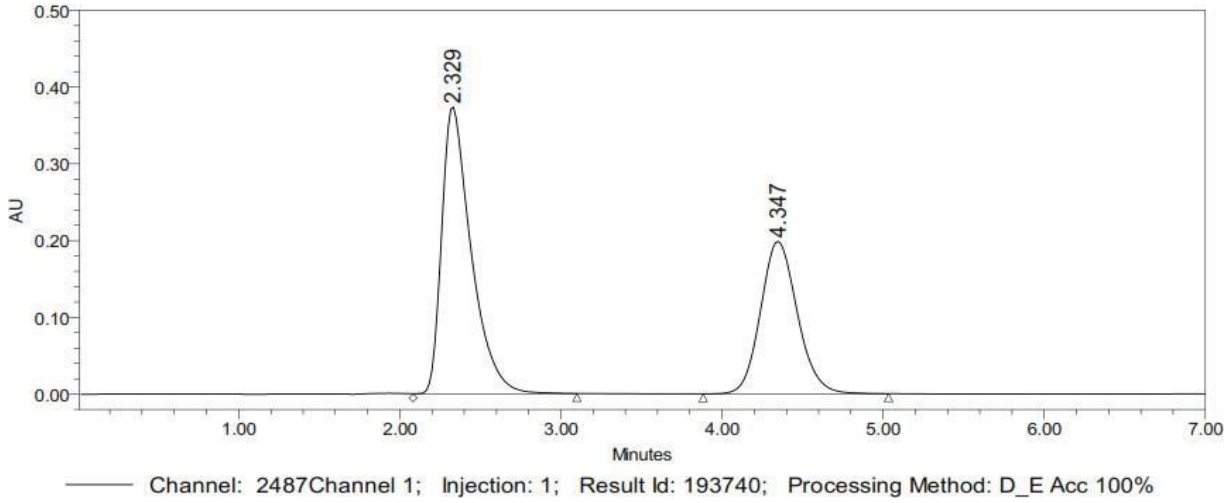


Fig 19 Chromatogram showing accuracy 100%injection-1

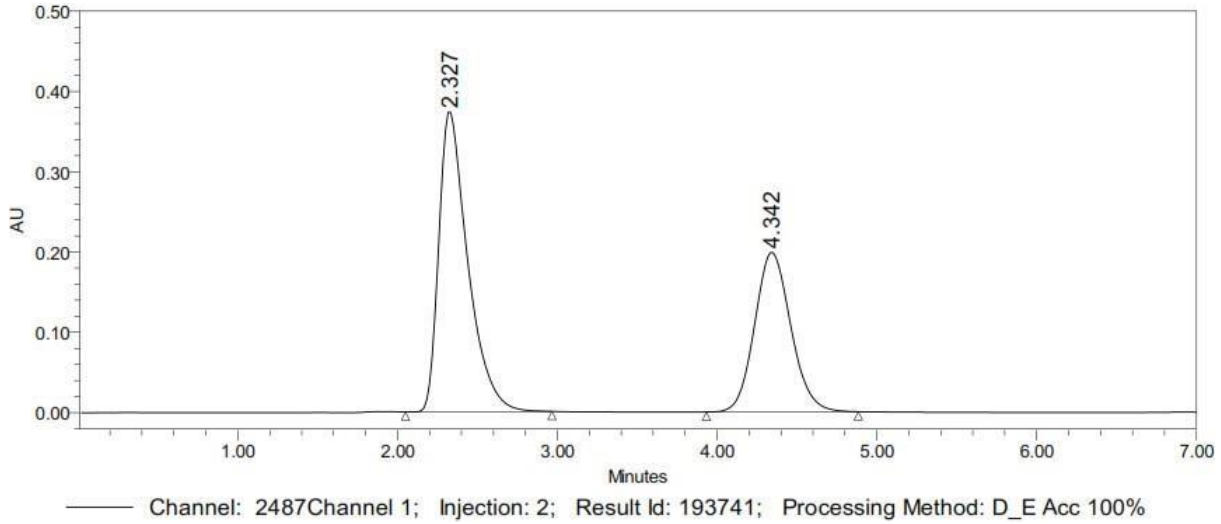


Fig 20 Chromatogram showing accuracy 100%injection-2

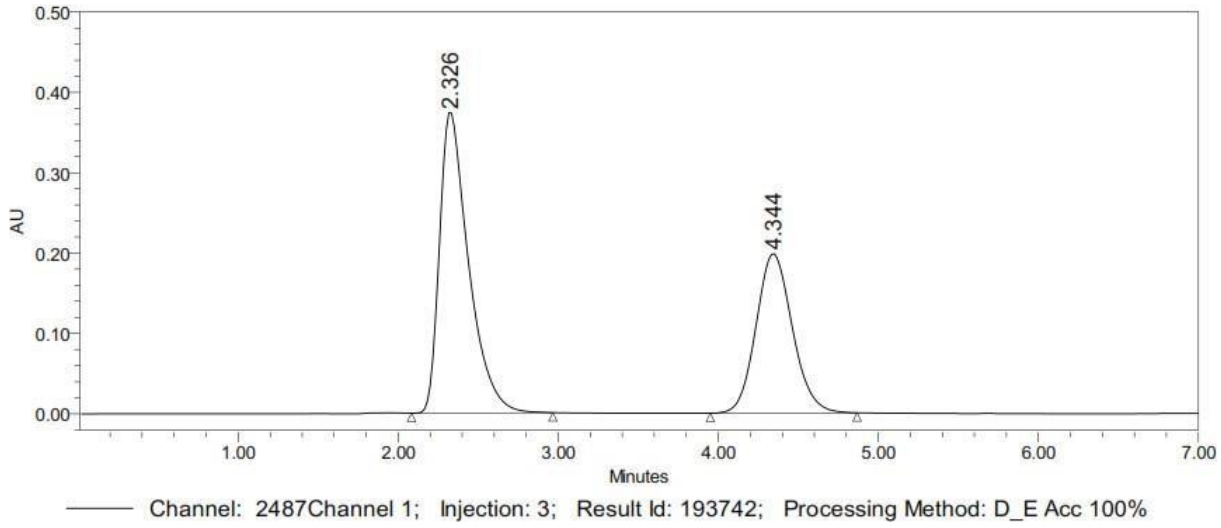


Fig 21Chromatogram showing accuracy 100%injection-3

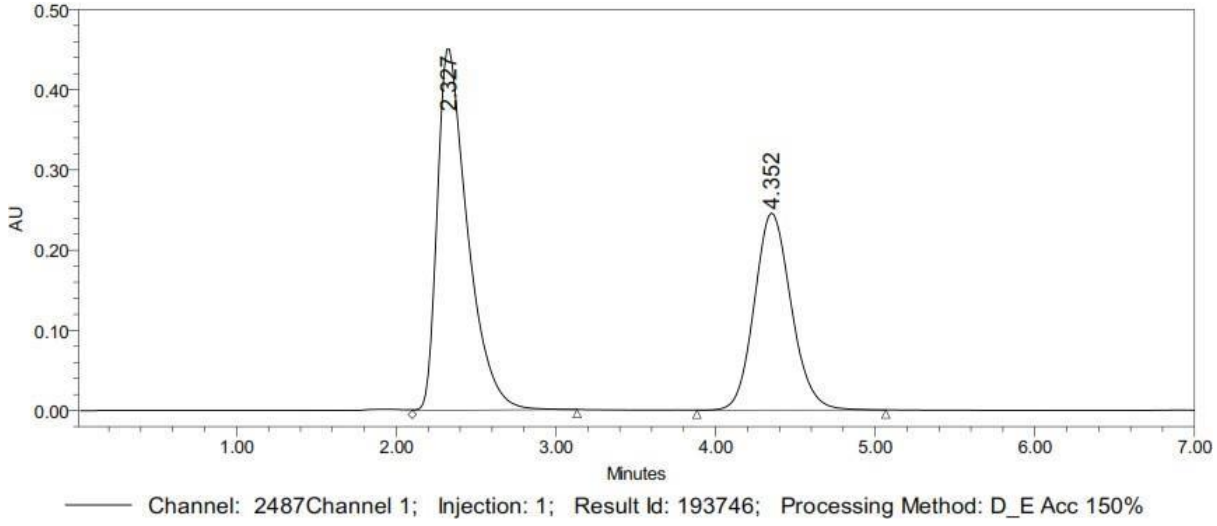


Fig 22 Chromatogram showing accuracy 150%injection-1

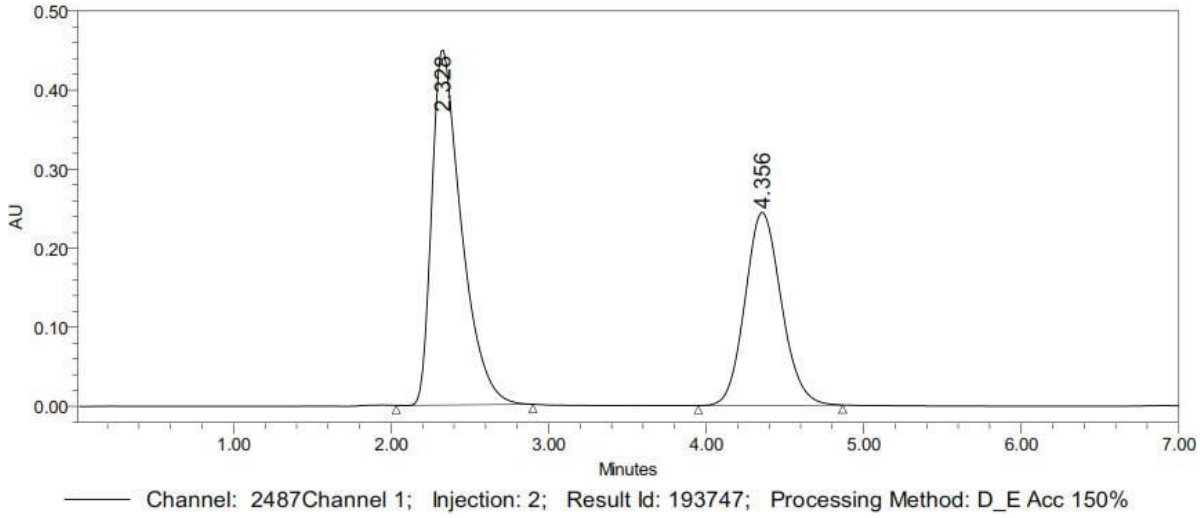


Fig 23 Chromatogram showing accuracy 150%injection-2

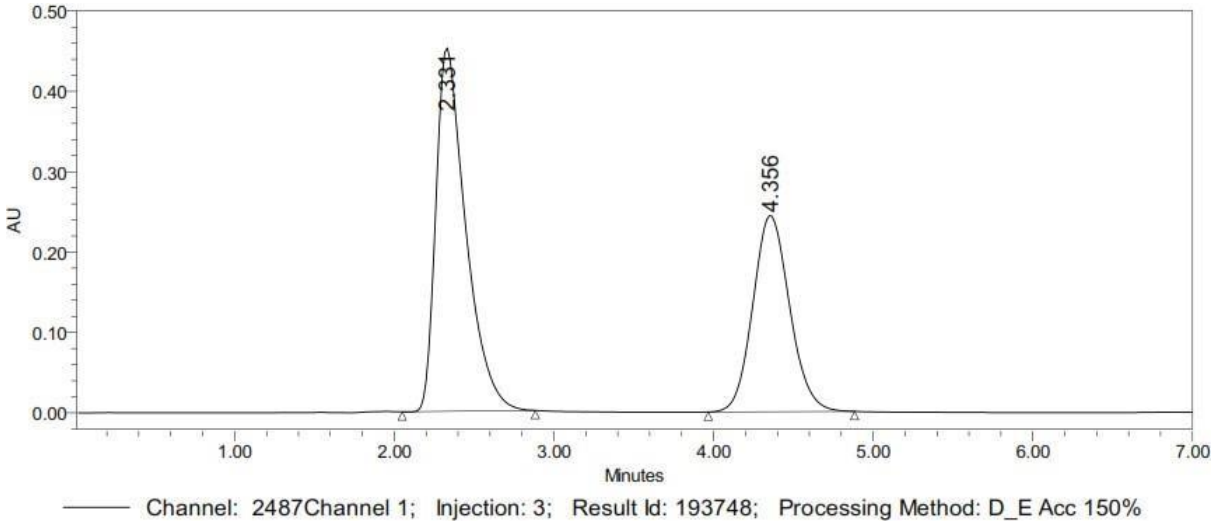


Fig 24 Chromatogram showing accuracy 150%injection-3

The accuracy results for Empagliflozin

Table 8 Accuracy results of Empagliflozin

%Concentration (at specification Level)	Area	Amount added(mg)	Amount found(mg)	% Recovey	Mean Recovery
50%	2332744	5	5.10	101.8%	100.5%
100%	3132697	10	9.99	99.9%	
150%	3918997	15	14.9	99.1%	

Acceptance Criteria:

- The % Recovery for each level should be between 98.0 to 102.0%.

The accuracy results for Metformin

Table 9 Accuracy results of Metformin

%Concentration(at specification level)	Area	Amount Added(mg)	Amount Found(mg)	% Recovery	Mean Recovery
50%	353867	5	5.0	101.3%	100.0%
100%	4735088	10	9.94	99.4%	
150%	5911798	15	14.8	99.2%	

Acceptace Criteria;

% Recovery was with In the limit of 95-105%.

2. Precision

i) Repeatability

ii) Intermediate precision (Ruggedness)

Repeatability

The precision study was performed for five injections of Metformin and Empagliflozin. Each standard injection was injected in to chromatographic system. The area of each Standard injection was used for calculation of % RSD. The chromatograms are shown in Fig.4.27 – 4.31 and results are tabulated in Table 4.21&4.22.

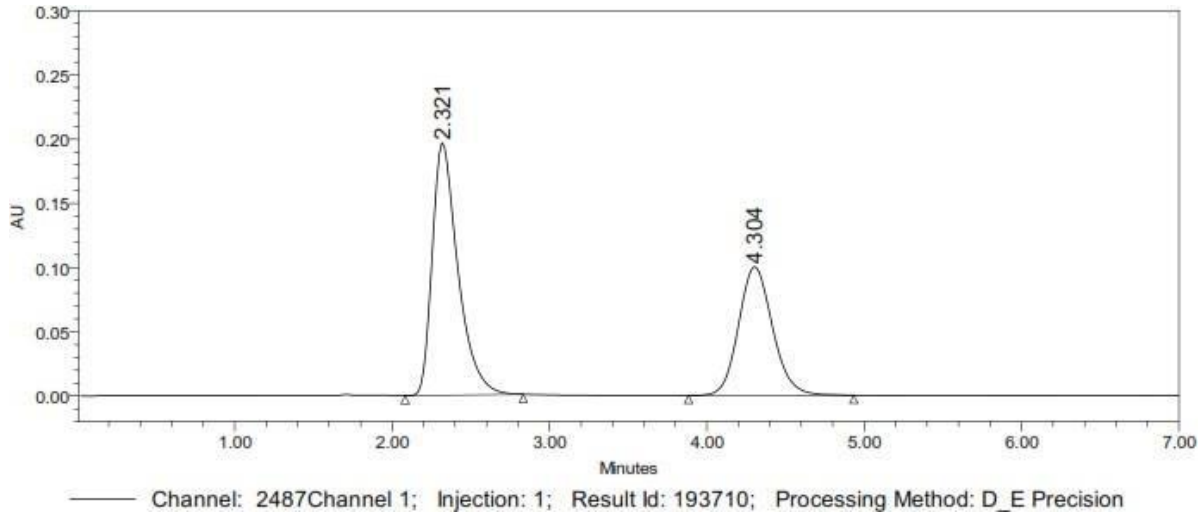


Fig 25 Chromatogram of Standard Inj-1

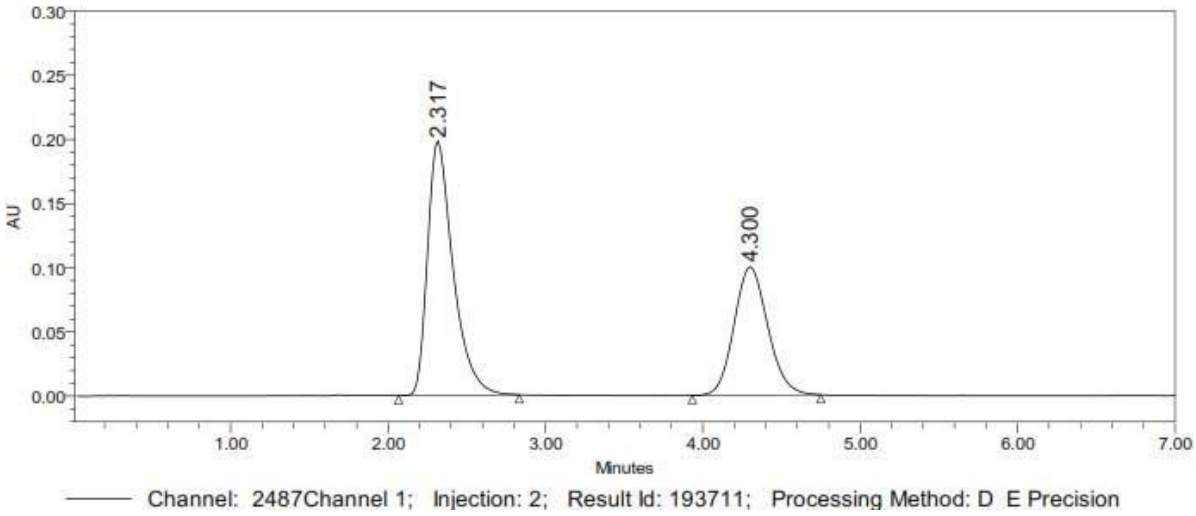


Fig 26 Chromatogram of Standard Inj-2

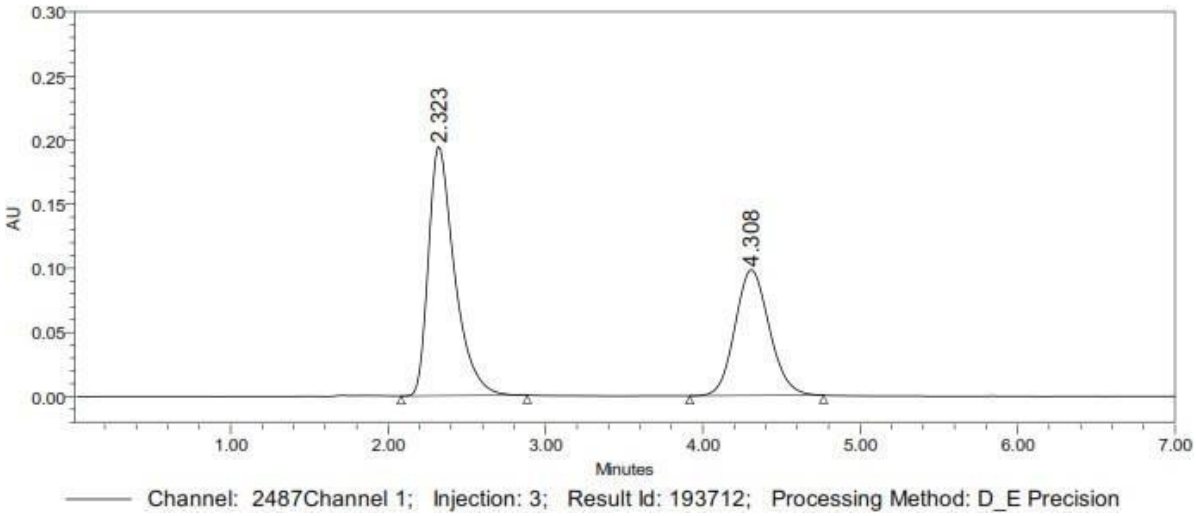


Fig 27 Chromatogram of Standard Inj-3

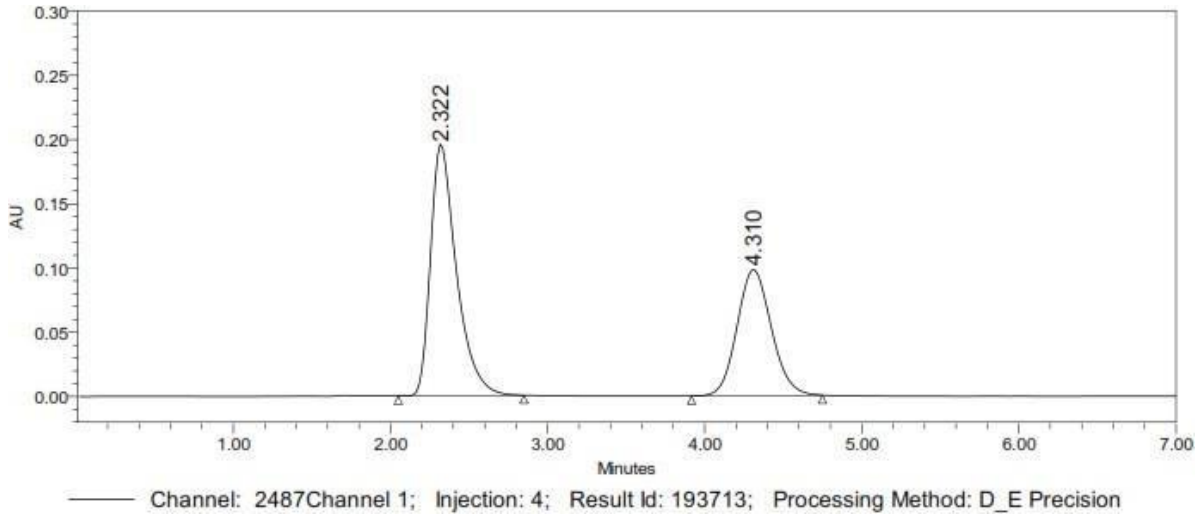


Fig 28 Chromatogram of Standard Inj-4

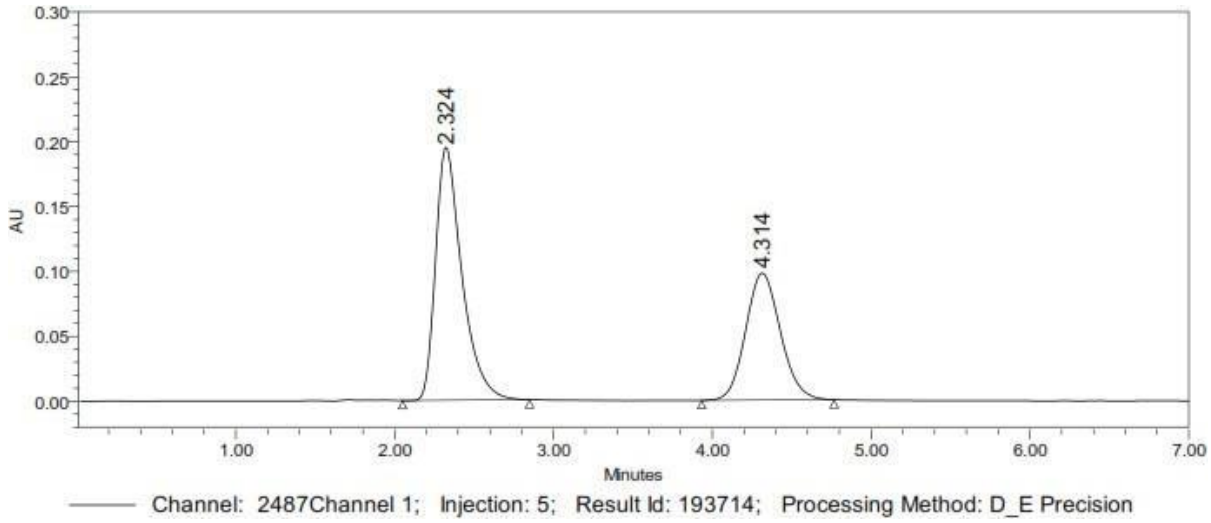


Fig 29 Chromatogram of Standard Inj-5

The results are summarized Metformin

Table 10 Repeatability results of Metformin & Empagliflozin.

Injection	Area
Injection-1	1501417
Injection-2	1486940
Injection-3	1490656
Injection-4	1487329
Injection-5	1490384
Average	1491345
Standard Deviation	5881.4
%RSD	0.39

Injection	Area
Injection-1	2235319
Injection-2	2240678
Injection-3	2249490
Injection-4	2245822
Injection-5	2251694
Average	2244601
Standard Deviation	6656.8
%RSD	0.32

Acceptance Criteria:

 The % RSD for the area of five standard injections results should not be more than 2%The Method precision study was performed for the %RSD of Metformin and Diazepam was found to be 0.3 and 0.3 (NMT 2).

Intermediate precision/Ruggedness

The intermediate precision study was performed for five injections of Metformin and Empagliflozin. Each standard injection was injected into chromatographic system. The area of each standard injection was used for calculation of % RSD. The chromatograms are shown in Fig.4.32-4.36 and results are tabulated in Table 4.23,4.24.

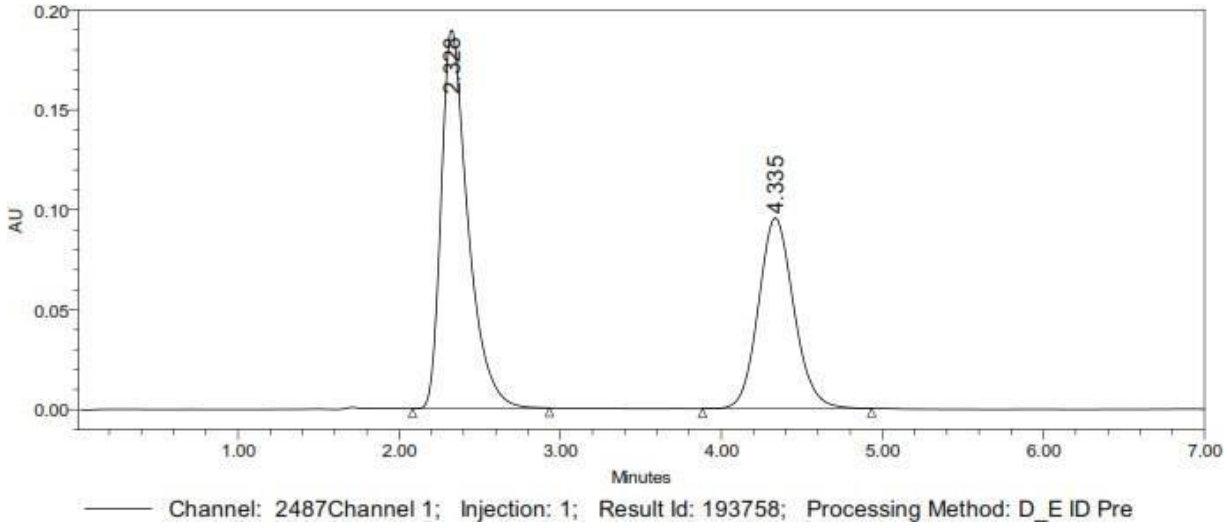


Fig 30 Chromatogram of Standard Inj-1(ID Precision)

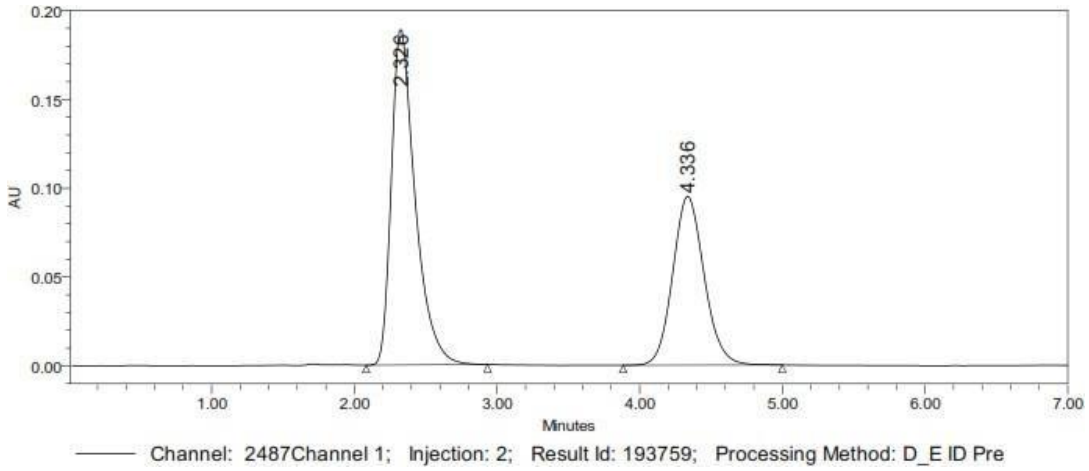


Fig 31 Chromatogram of Standard Inj-2(ID Precision)

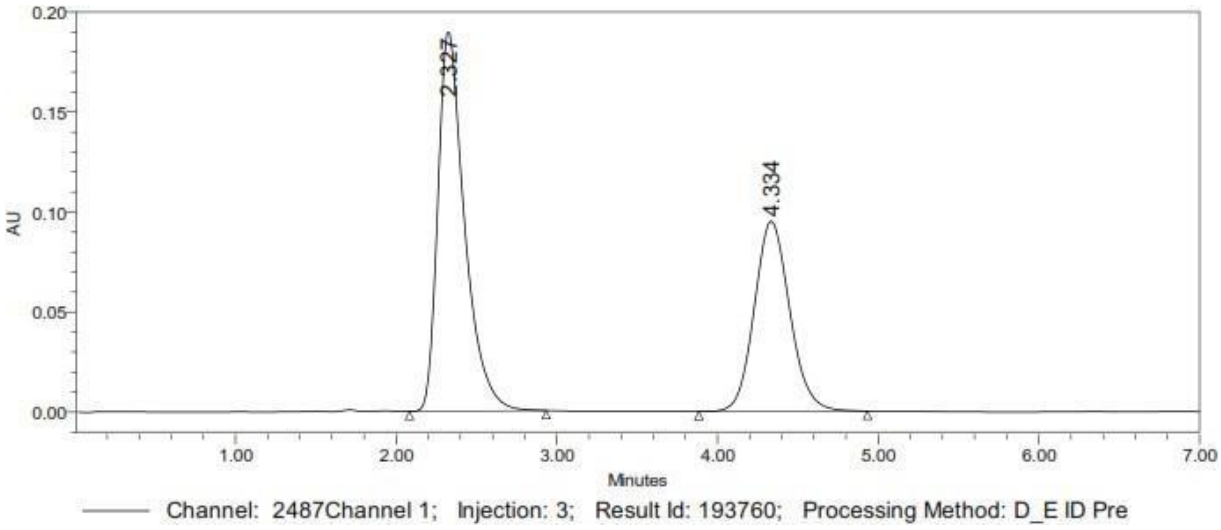


Fig 32 Chromatogram of Standard Inj-3(ID Precision)

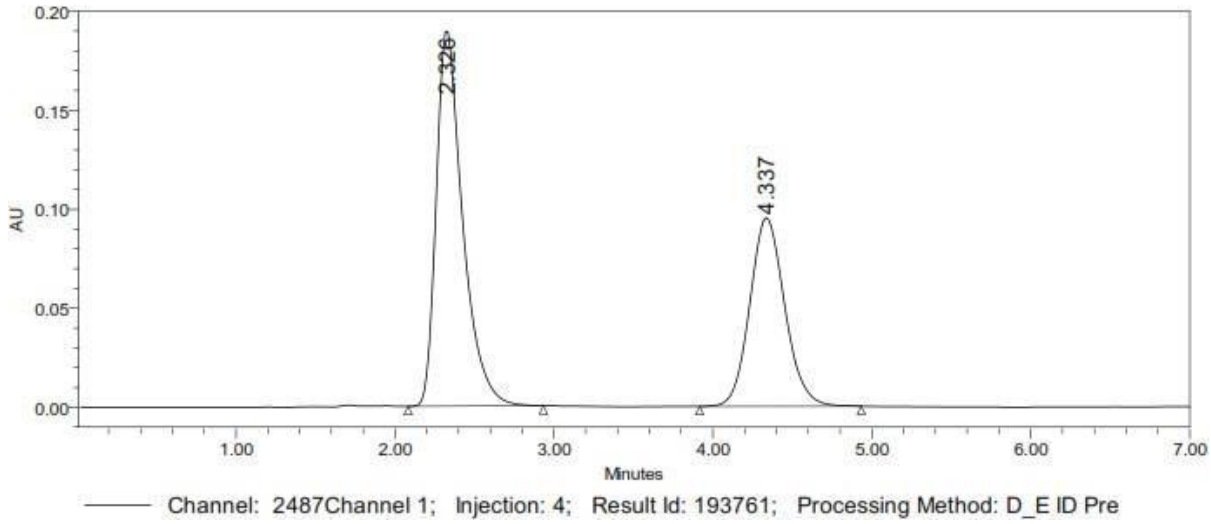


Fig 33 Chromatogram of Standard Inj-4(ID Precision)

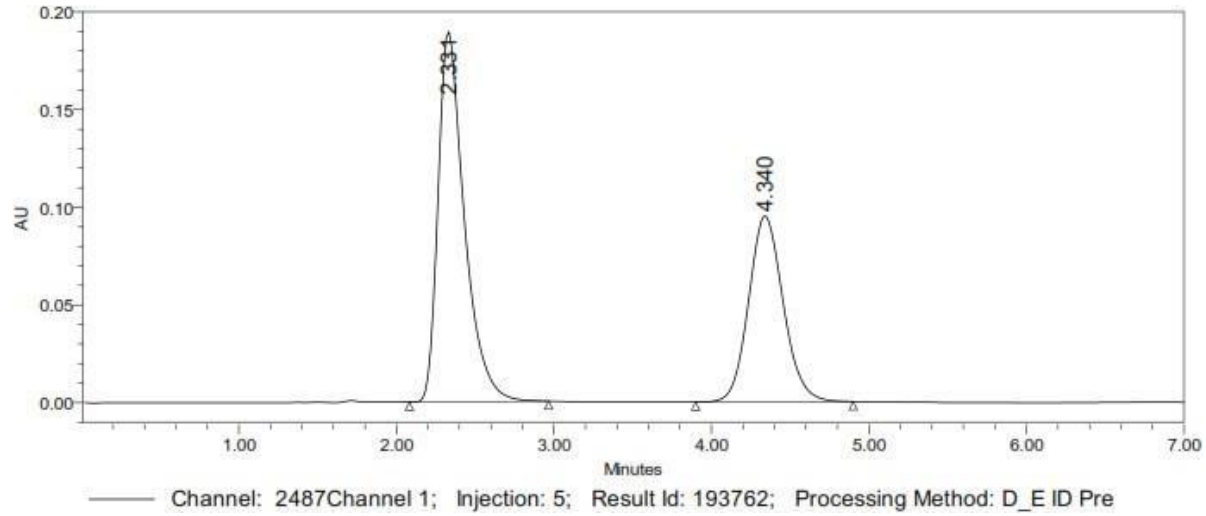


Fig 34 Chromatogram of Standard Inj-5(ID Precision)

The results are summarized Empagliflozin

Table 11 Ruggedness results of Empagliflozin& Metformin

Injection	Area

Injection-1	2194758
Injection-2	2195700
Injection-3	2196191
Injection-4	2195326
Injection-5	2200951
Average	2196585
Standard Deviation	2496.0
%RSD	0.11

Table12 :

Injection	**Area**
Injection-1	1456296
Injection-2	1457422
Injection-3	1456513
Injection-4	1454579
Injection-5	1451483
Average	1455259
Standard Deviation	2347.6
%RSD	0.16

Acceptance Criteria:

The % RSD for the area of five standard injections results should not be more than 2%.

The intermediate precision was performed for %RSD of Metformin and Empagliflozin was found to be 0.1 and 0.1 respectively (NMT 2).

3. Specificity:

The system suitability for specificity was carried out to determine whether there is any interference of any impurities in retention time of analytical peak. The study was performed by injecting blank. The chromatograms are shown in Fig.4.37, 4.38.

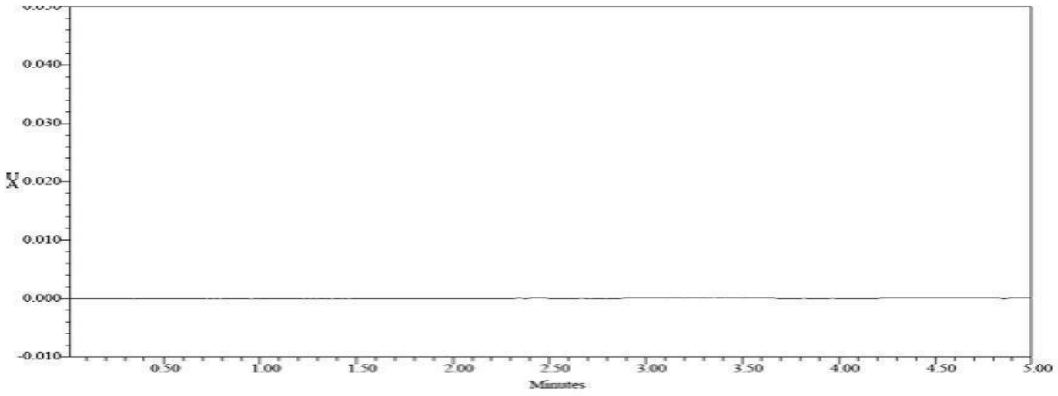


Fig. 35 Chromatogram of blank Inj

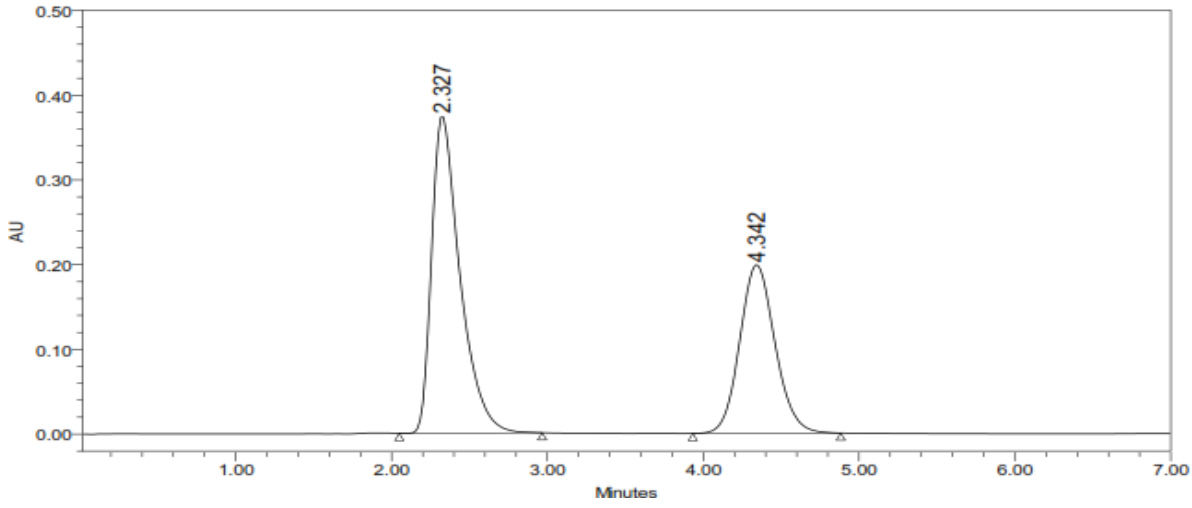


Fig. 36 Chromatogram of Standard Injection

Table13:

S.No	Peak name	Rt	Area	Height	USP Plate count	USP Tailing	USP Resolution
1	Metformin	2.237	7913799	394185	2632	1.8	
2	Empagliflozin	4.342	1853381	162758	2614	1.6	5.23

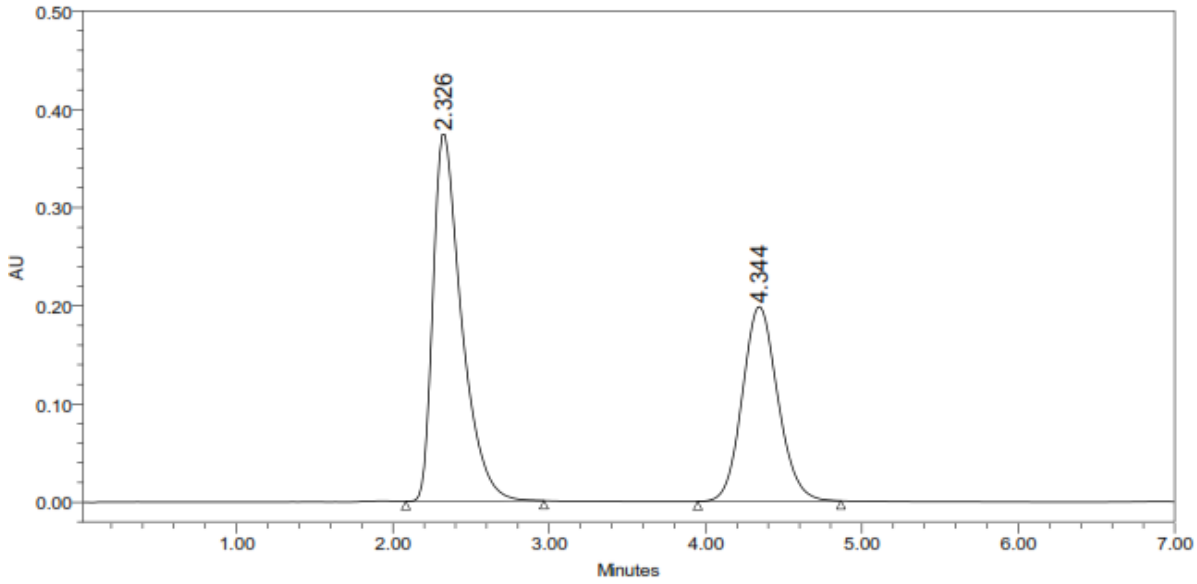


Fig:37

Table14:

S.No	Peak name	Rt	Area	Height	USP Plate count	USP Tailin	USP Resolutio
1	Metformin	2.326	4726354	376488	2455	1.60	
2	Empagliflozin	4.344	3122571	198418	2614	1.11	5.52

The specificity test was performed for Metformin and Empagliflozin. It was

found that there was no interference of impurities in retention time of analytical peak.

4. Detection of limit:

LOD's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) at levels approximating the LOD according to the formula. The standard deviation of the response can be determined based on the standard deviation of y-intercepts of regression lines.

Formula:

$$\mathbf{LOD} = 3.3 \text{ X } \frac{\sigma}{S}$$

Where

σ - Standard deviation (SD)

S – Slope

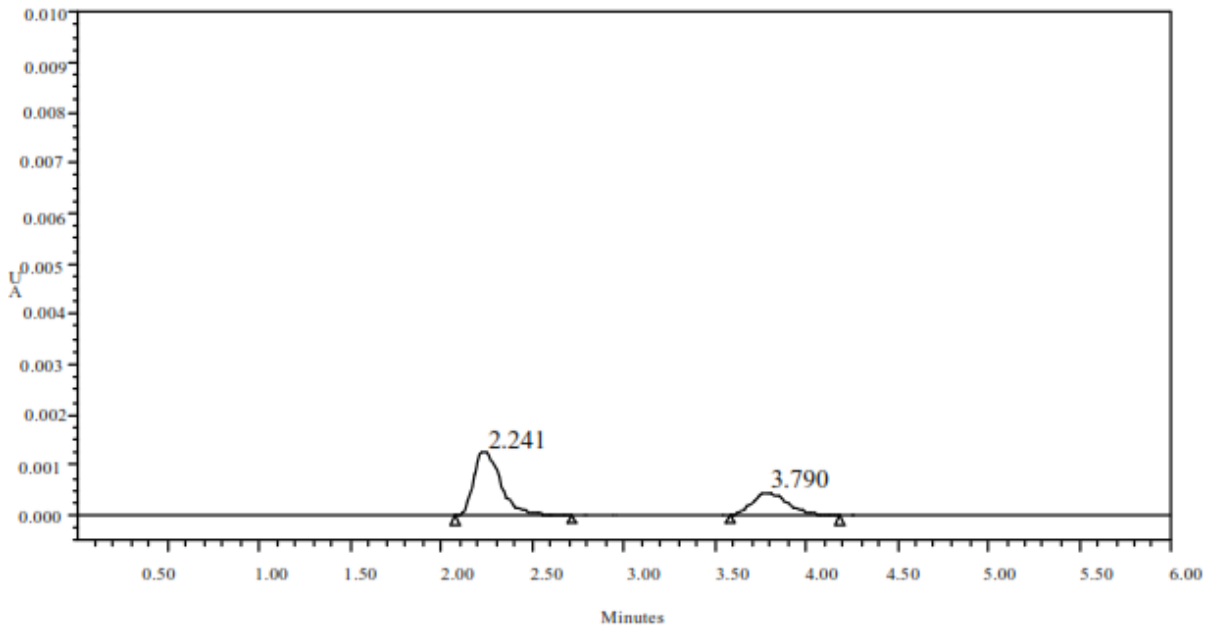


Fig:38

Empagliflozin.

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank : 41µV Signal

Obtained from LOD solution : 125 µV

S/N = 125/41= 3.04

Acceptance Criteria:

S/N Ratio value shall be 3 for LOD solution

Metformin

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank : 41 µV Signal

Obtained from LOD solution : 121 µV

S/N = 121/41 = 2.95

Acceptance Criteria:

S/N Ratio value shall be 3 for LOD solution.

The LOD was performed for Metformin and Empagliflozin was found to be 2.95and 3.04 respectively.

5. Qantitation Limit:

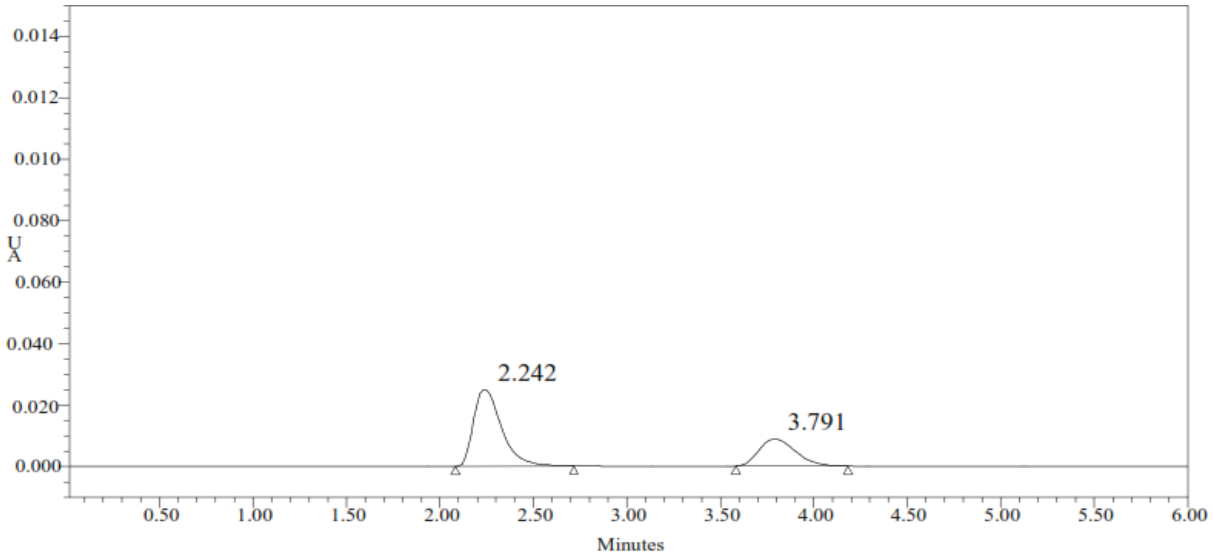


Fig:39

Empagliflozin

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank : 41 µV Signal

Obtained from LOQ solution : 412µV

S/N = 412/41 = 10.0

Acceptance Criteria:

S/N Ratio value shall be 10 for LOQ solution.

Metformin

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank : 41 µV

Signal Obtained from LOQ solution : 405µV

S/N = 405/41 = 9.87

Acceptance criteria

S/N Ratio value shall be 10 for LOQ solution.

The LOQ was performed for Metformin and Empagliflozin was found to be 9.87and 10 respectively**.**

6. Linearity:

The linearity study was performed for the concentration of 100ppm to 500ppm and1ppm to 5ppm level. Each level was injected into chromatographic system. The area of each level was used for calculation of correlation coefficient. The chromatograms are shown in Fig.4.39 – 4.43 and results are tabulated in Table. 4.26, 4.27 Calibration graph for Metformin and Empagliflozin are shown in Fig. 4.44, 4.4

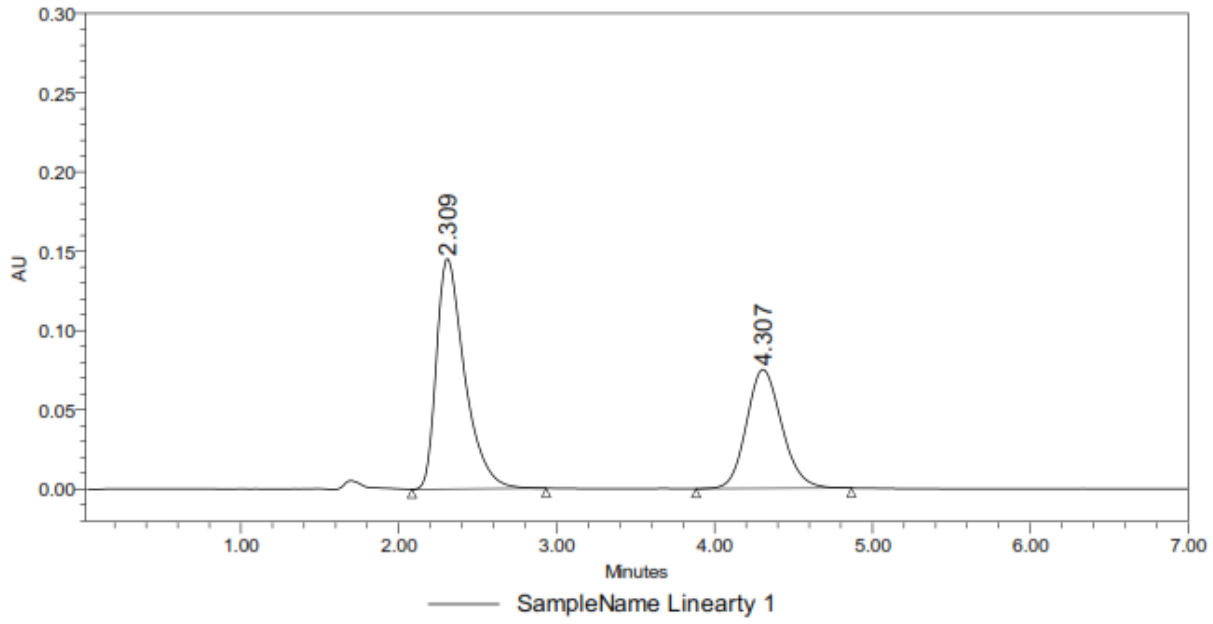


Fig:40 Chromatogram for Inj Level-1

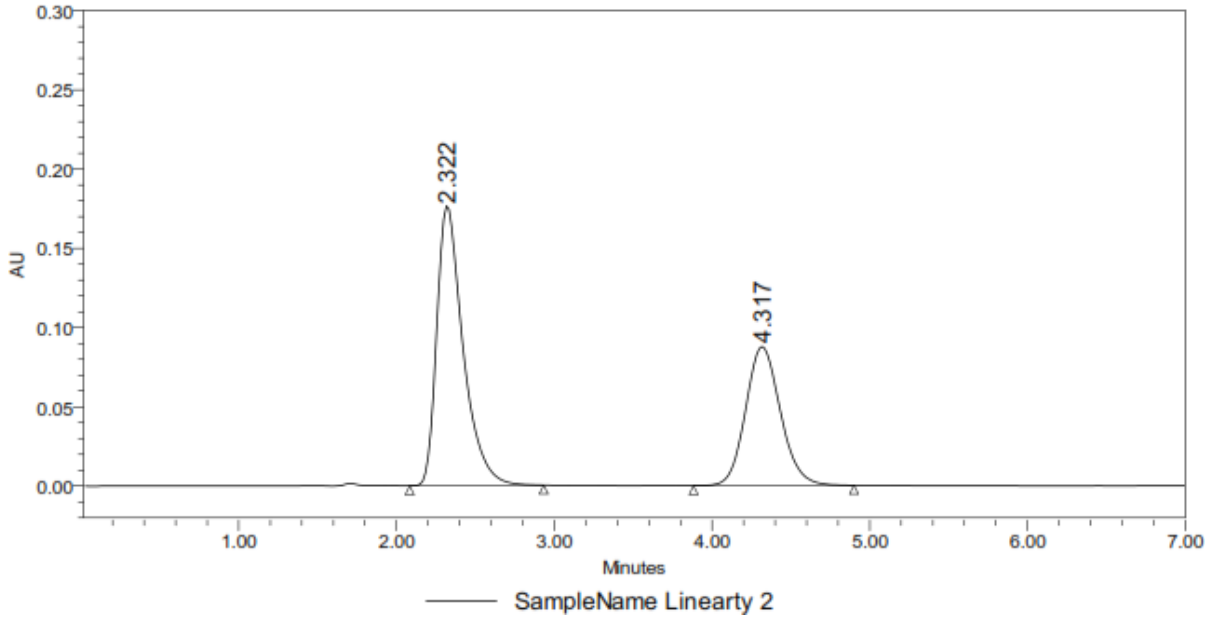


Fig. 41 Chromatogram for Inj Level-II

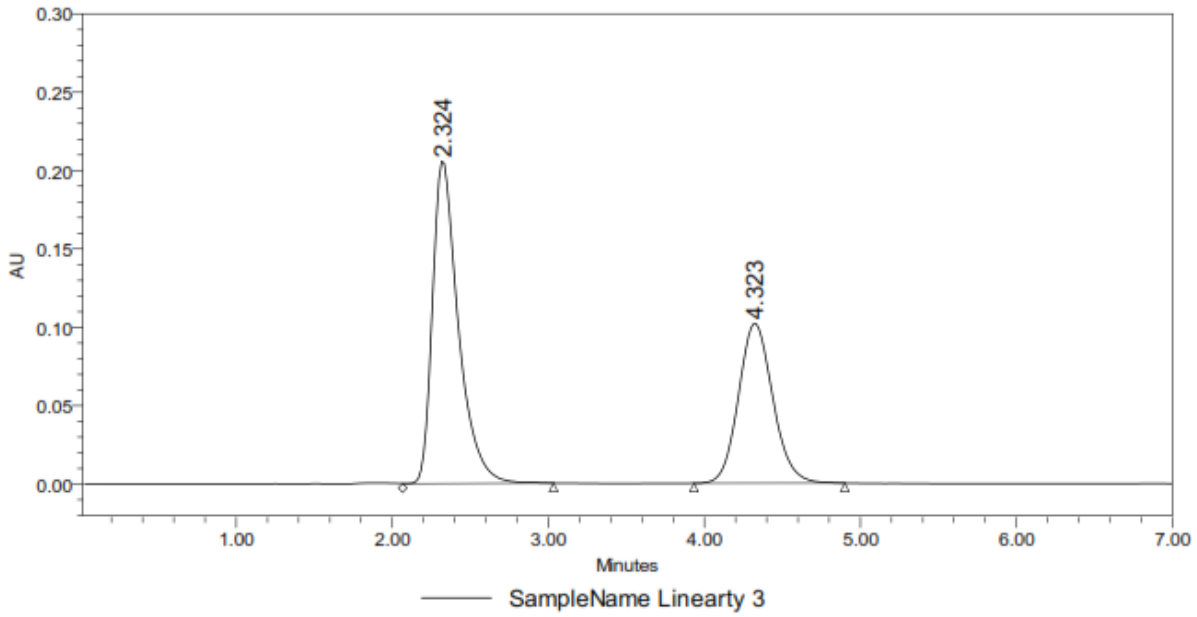


Fig 42 Chromatogram for Inj Level-III

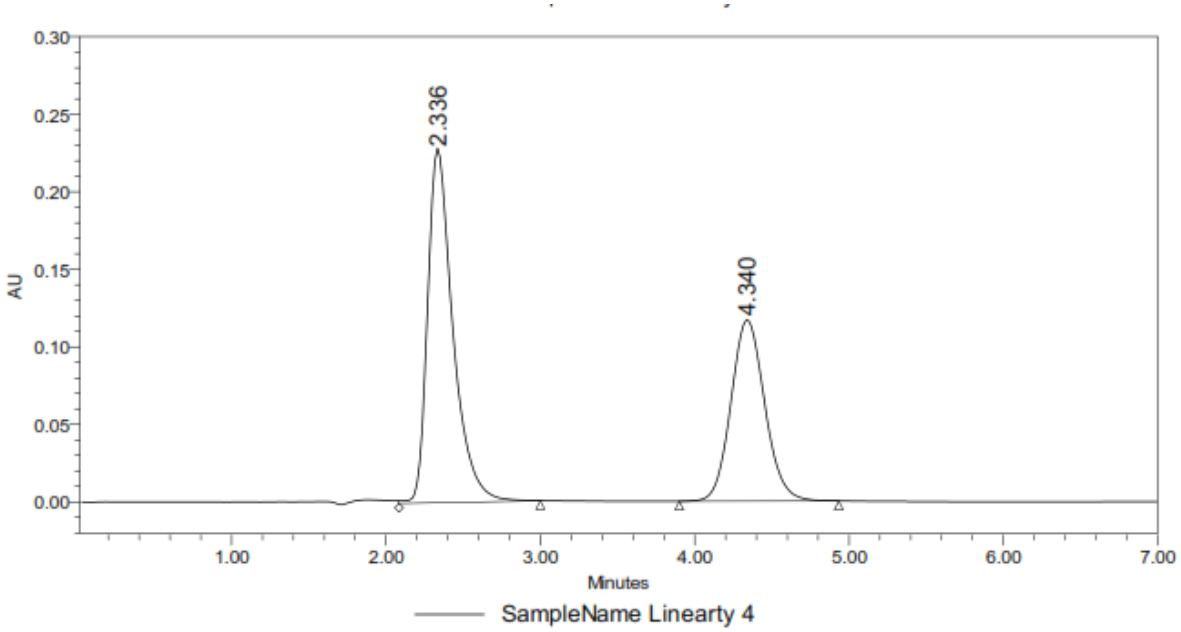


Fig 43 Chromatogram for Inj Level-IV

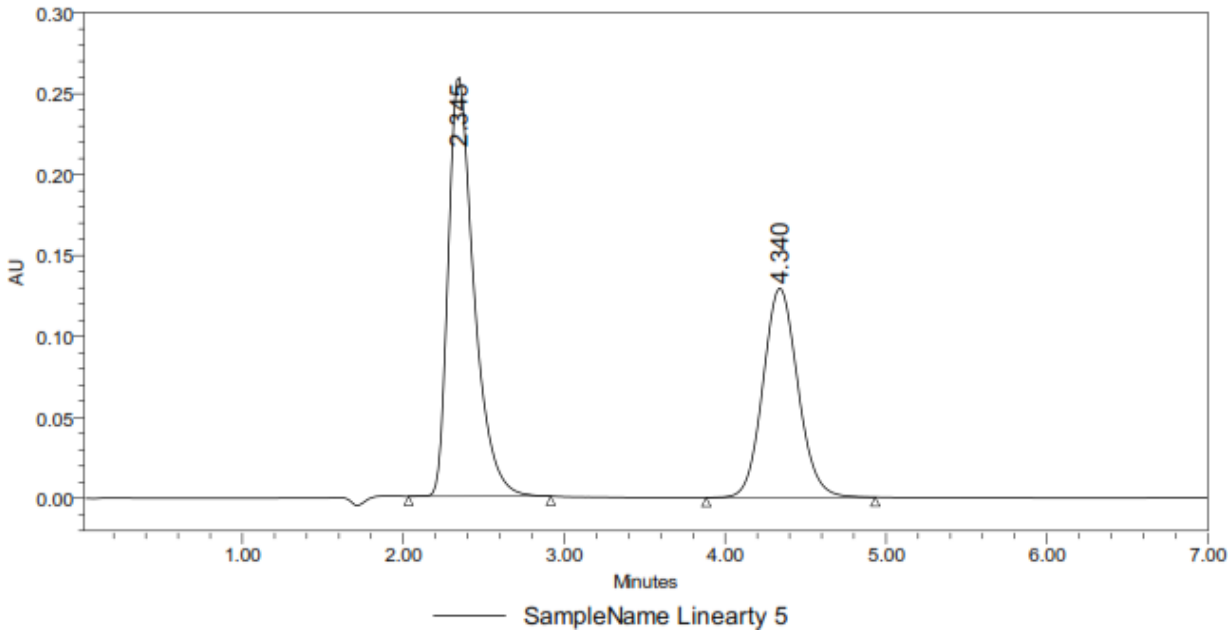


Fig 44 Chromatogram for Inj Level-V

Plotting of calibration graphs:

The resultant areas of linearity peaks are plotted against Concentration

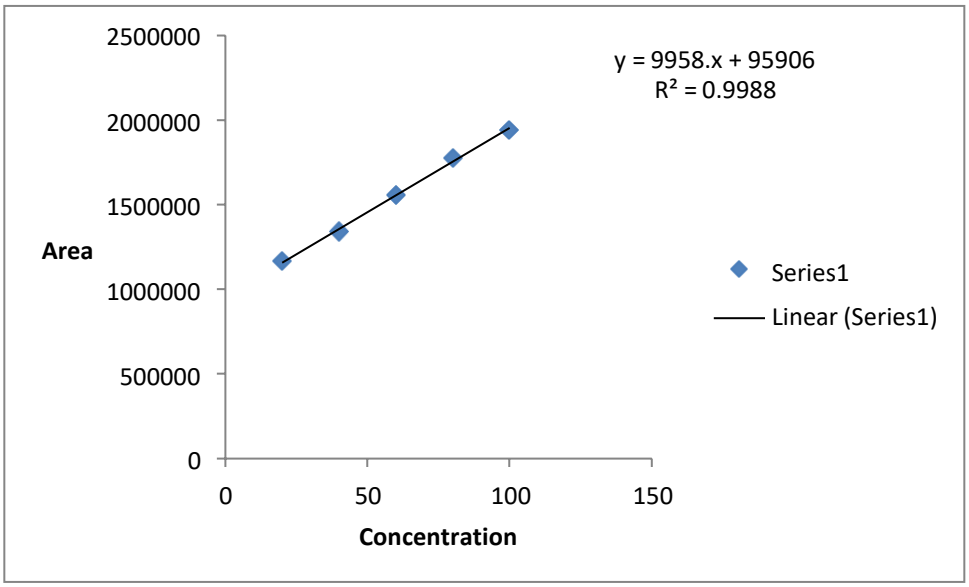


Fig. 45 Calibration curve of Empagliflozin

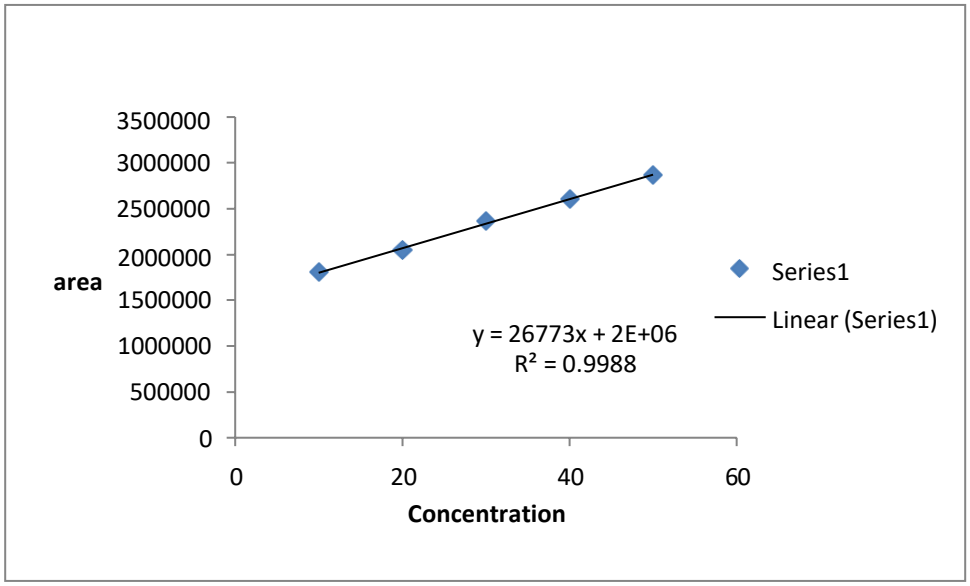


Fig 46 Calibration curve of Metformin

7. Range

The linearity study was performed for concentration range of 10µg - 50µg and 20µg-100µg of Metformin and Empagliflozin and the correlation coefficient was found to be 0.9988 and 0.999. (NLT 0.9988).

8. Robustness

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

A) Flow Rate:

The robustness was performed for the flow rate variations from 0.8 ml/min to 2ml/min. Standard solution 60 µg/ml of Empagliflozin & 30µg/ml of Metformin was prepared and analyzed using the varied Mobile phase composition along with the actual mobile phase composition in the method.

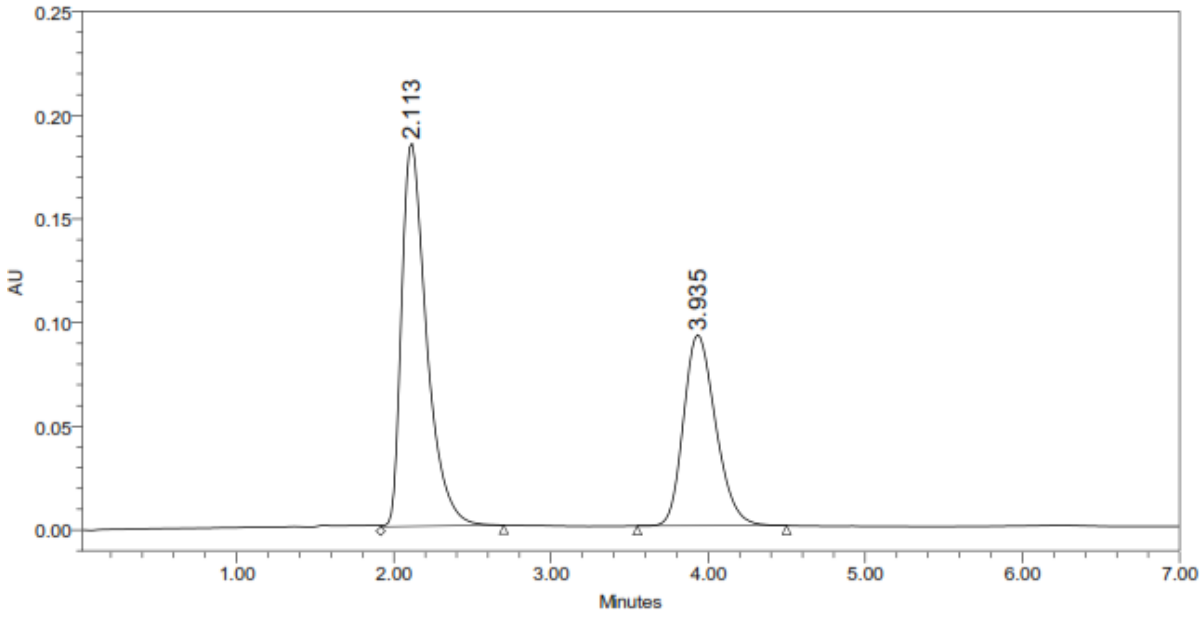


Fig 47 Chromatogram for Robustness more flow

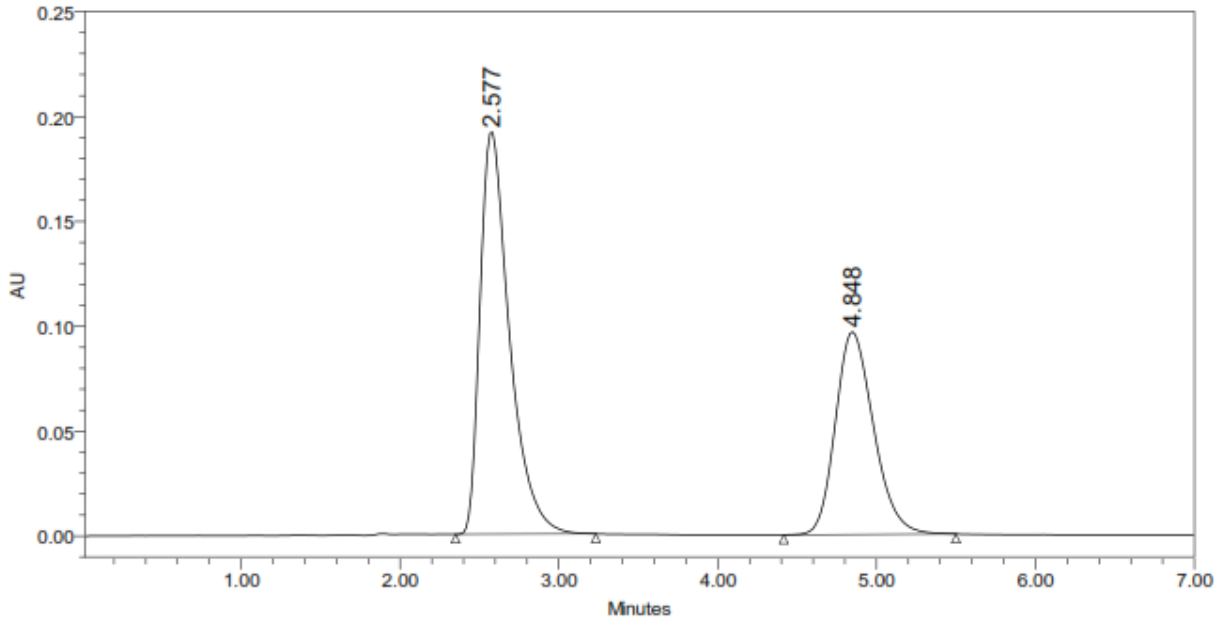


Fig. 48 Chromatogram for Robustness less flow

The results are summarized

On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even b y change in the flow rate ±0.2ml/min.

System suitability results for Empagliflozin*:*

Table 15 System suitability results For Empagliflozin (Flow rate)

***** Results for actual flow (1.0 ml/min) have been considered from Assay standard.

S.No	Flow Rate(ml/min)	System suitability results	
		USP Plate count	USP Tailing
1	0.8	1748.5	1.22
2	1.0	1548.2	1.2
3	1.2	1948.0	1.2

System suitability results for Metformin:

Table 16 System suitability results for Metformin (Flow rate)

S.No	Flow Rate(ml/min)	System suitability results	
		USP Plate count	USP Tailing
1	0.8	883.3	1.56
2	1.0	1234.0	1.1
3	1.2	969.2	1.6

***** Results for actual flow (1.0ml/min) have been considered from Assay standard

B) Mobile Phase:

The Organic composition in the Mobile phase was varied from 70% to 60%. Standard solution 300 µg/ml of Empagliflozin & 3µg/ml of Metformin was prepared and analyzed using the varied Mobile phase composition along with the actual mobile phase composition in the method.

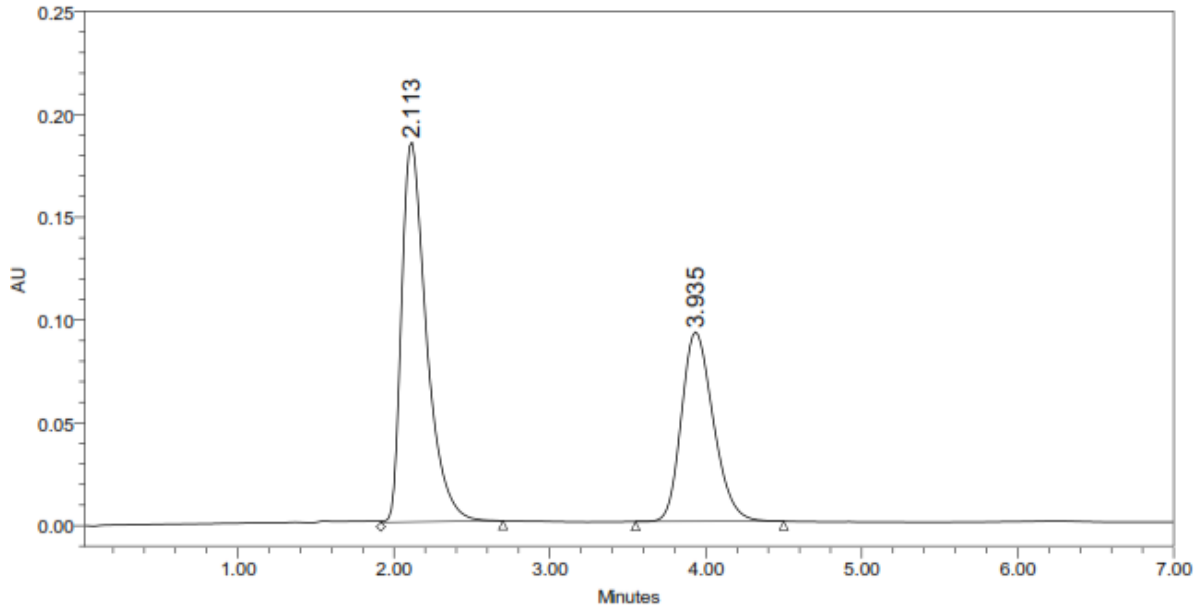


Fig. 49 Chromatogram for Robustness more organic

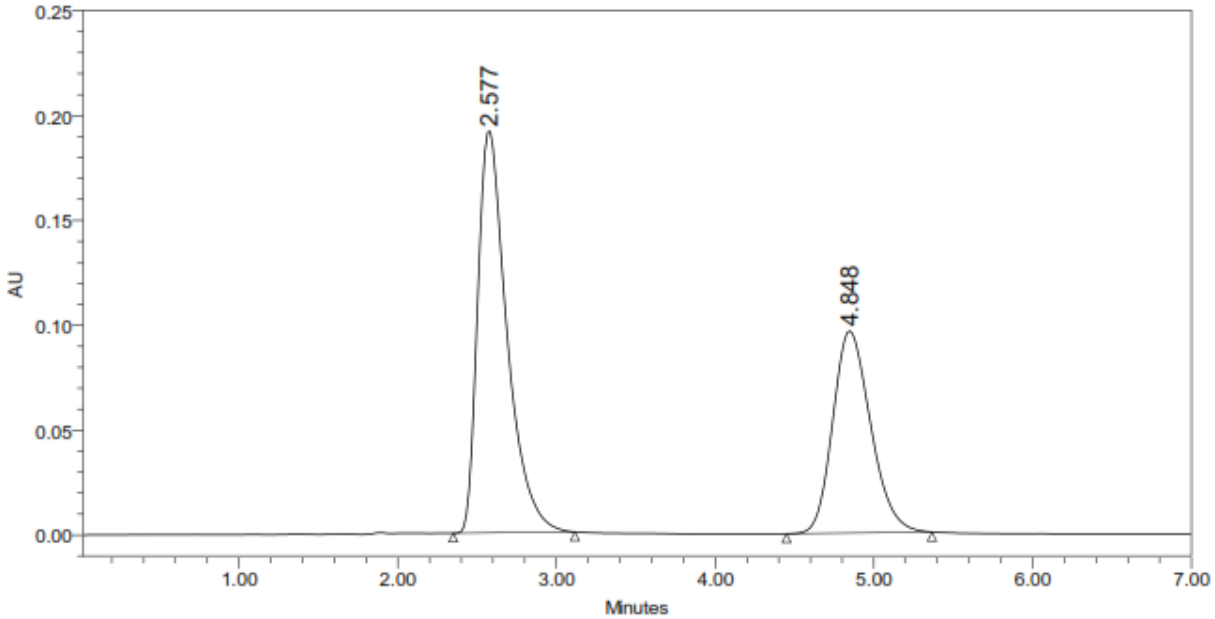


Fig 50 Chromatogram for Robustness less organic

The results are summarized. On evaluation of the above results, it can be concluded that the variation in 10% Organic composition in the mobile phase affected the method significantly. Hence it

indicates that the method is robust even by change in the Mobile phase ±10

System suitability results for Empagliflozin:

Table 17 System suitability results for Empagliflozin (Mobile phase)

S.No	Changein Organic Composition in the Mobile Phase	System suitability results	
		USP Plate count	USP Tailing
1	10% Less	1748.5	1.22
2	Actual	1548.2	1.2
3	10% More	1948.0	1.2

***** Results for actual Mobile phase composition (45:55Buffer: ACN) have been considered from Accuracy standard

System suitability results for Metformin:

Table 18 System suitability results for Metformin (Mobile phase)

S.No	Changein Organic Composition in the Mobile Phase	System suitability results	
		USP Plate count	USP Tailing
1	10% Less	883.3	1.56
2	Actual	1234.0	1.1
3	10% More	969.2	1.6

***** Results for actual Mobile phase composition (55:45Buffer: ACN) have been considered from Accuracy standard.

SUMMARY AND CONCLUSION

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 Xterra C18 5µm (4.6*250mm) column, flow rate was 1ml/min, mobile phase ratio was Phosphate buffer (0.05M) pH 4.6: ACN (55:45%v/v) (pH was adjusted with orthophosphoric acid), detection wave length was 255nm. The instrument used was WATERS HPLC Auto Sampler, Separation module 2695, PDA Detector 996, Empower-software version-2.

The retention times were found to be 2.399mins and 3.907mins. The % purity of Metformin and Empagliflozin was found to be 100.7% and 101.4% respectively. The system suitability parameters for Metformin and Empagliflozin such as theoretical plates and tailing factor were found to be 1.3, 5117.5and 1.4, 3877.3 the resolution was found to be 8.0.

The analytical method was validated according to ICH guidelines (ICH, Q2 (R1)). The linearity study for Metformin and Empagliflozin was found in concentration range of 1µg-5µg and 100µg-500µg and correlation coefficient (r2) was found to be 0.999 and 0.999, % mean recovery was found to be 100% and 100.5%, %RSD for repeatability and 0.4, % RSD for intermediate precision was 0.5 and 0.1 respectively. The precision study was precise, robust, and repeatable. LOD value was 2.95 and 3.04, and LOQ value was 9.87 and 10 respectively. Hence the suggested RP-HPLC method can be used for routine analysis of Metformin and Empagliflozin in API and Pharmaceutical dosage form.

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