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THE ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF DAPAGLIFLOZIN BY USING RP-HPLC METHOD

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INTRODUCTION

Pharmaceutical analysis plays a vital role in the Quality Assurance and Quality control of bulk drugs. Analytical chemistry involves separating, identifying, and determining the relative amounts of components in a sample matrix. Pharmaceutical analysis is a specialized branch of analytical chemistry. Pharmaceutical analysis derives its principles from various branches of sciences like physics, microbiology, nuclear science, and electronics etc. Qualitative analysis reveals the chemical identity of the sample. Quantitative analysis establishes the relative amount of one or more of these species or analytes in numerical terms. Qualitative analysis is required before a quantitative analysis can be undertaken. A separation step is usually a necessary part of both qualitative and quantitative analysis. The results of typical quantitative analysis can be computed from two measurements. One is the mass or volume of sample to be analyzed and second is the measurement of some quantity that is proportional to the amount of analyte in that sample and normally completes the analysis.

Assay of Drugs in Dosage Forms:

Assay is the process of determining the percent amount of the analyte present in the sample by its name. Dosage forms require a variety of tests and standards to assure therapeutic benefit.

According to WHO, a drug may be defined as any substance or product that is used or intended to be used for modifying or exploring physiological systems or pathological states for the benefit of the patient.

Typical Instrumental Techniques:

The methods of estimation of drugs are divided into

- Physical Methods
- Chemical Methods
- Physicochemical Methods

Of them, physical and physicochemical methods are used mostly.

Physical methods of analysis involve the studying of the physical properties of a substance. They include determination of the solubility, transparency or degree of turbidity, color density or specific gravity (for liquids), moisture content, melting, freezing and boiling points.

The chemical methods include the gravimetric and volumetric procedures, which are based on complex formation, acid-base, precipitation and redox reactions. Titrations in non-aqueous media and complexometry have been widely used in pharmaceutical analysis whenever the existing amounts are in milligram level and the interferences are negligible.

Physicochemical methods are used to study the physical phenomenon's that occur as a result of chemical reactions. Among the physicochemical methods are optical refractometry, polarimetry, emission and fluorescent methods of analysis, photometry including photo colorimetry, spectrophotometry, nephelometry and turbidimetry; electrochemical (potentiometry, ampherometry, coulometry, voltametry, polarography) and chromatography (column, paper, thin layer, gas-liquid, high performance liquid chromatography) methods are

generally preferable. Methods involving nuclear reactions such as nuclear magnetic resonance (NMR) and paramagnetic resonance (PMR) are becoming more and more popular.

Advances in both chemistry and technology are making new techniques available and expanding the use of existing ones. Photo acoustic spectroscopy is an example of an emerging analytical technique. A number of existing techniques have been combined to expand the utility of the component methods. Gas chromatography-mass spectrometry (GC-MS), inductively coupled plasma - mass spectrometry (ICP-MS) and gas chromatography-infrared spectroscopy (GC-IR) are examples of successful hyphenated methods.

CHROMATOGRAPHY

3 Chromatography is a separation of mixture into individual components using a stationary phase and a mobile phase. This may be regarded as an analytical technique employed for the purification and separation of organic and inorganic substances. There are various advanced chromatographic techniques, widely used for the estimation of multicomponent drugs in their formulations. The various chromatographic techniques are

- High Performance Liquid Chromatography
- High Performance Thin Layer Chromatography
- Gas Chromatography

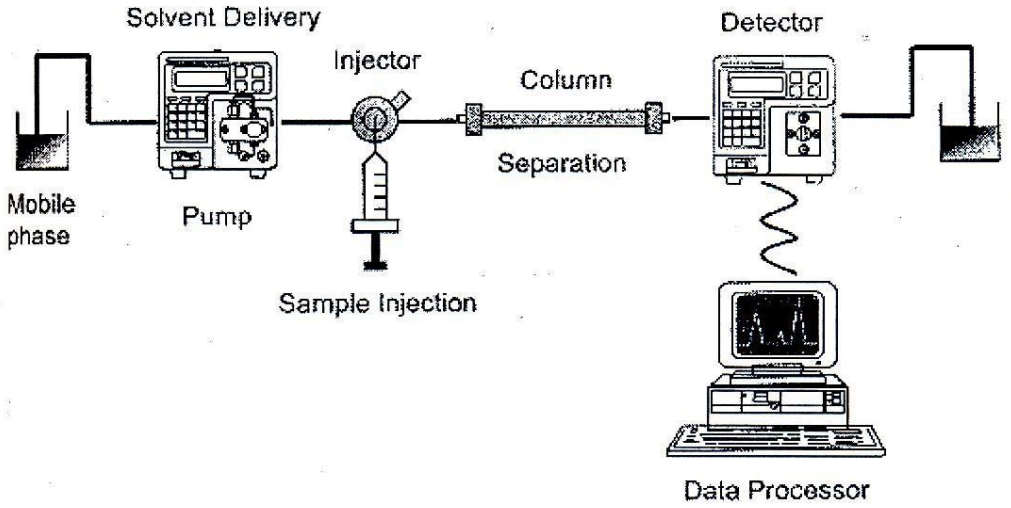
HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC is a type of liquid chromatography that employs a liquid mobile phase and a very finely divided stationary phase. The technique of high performance liquid chromatography is so called because of its improved performance when compared to column chromatography. Advances in column technology, high-pressure pumping system and sensitive detectors have transformed liquid column chromatography into high speed, efficient, accurate and highly resolved method of separation.

The HPLC is the method of choice in the field of analytical chemistry, since this methodis specific, robust, linear, precise and accurate and the limit of detection is low and also it offersthe following advantages.

- 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 viscosity
- Easy sample recovery, handling and maintenance.
- Instrumentation leads itself to automation and quantification (less time and less labour)
- Precise and reproducible
- Integrator itself does calculations.

Fig:-1 HPLC BASIC INSTRUMENTATION



Types of HPLC Techniques

Based on Modes of Chromatography:

- Normal phase chromatography
- Reverse phase chromatography

Based on Principle of Separation:

- Adsorption chromatography
- Ion exchange chromatography

- Size exclusion chromatography
- Affinity chromatography
- Chiral phase chromatography

Base on Elution Technique:

- Isocratic separation
- Gradient separation

Based on the Scale of Operation:

- Analytical HPLC
- Preparative HPLC

Instrumentation 5

The essential parts of the High Performance Liquid Chromatography are:

- Solvent reservoir and treatment system
- Mobile phase
- Pump system
- Sample injection system
- Column
- Detector

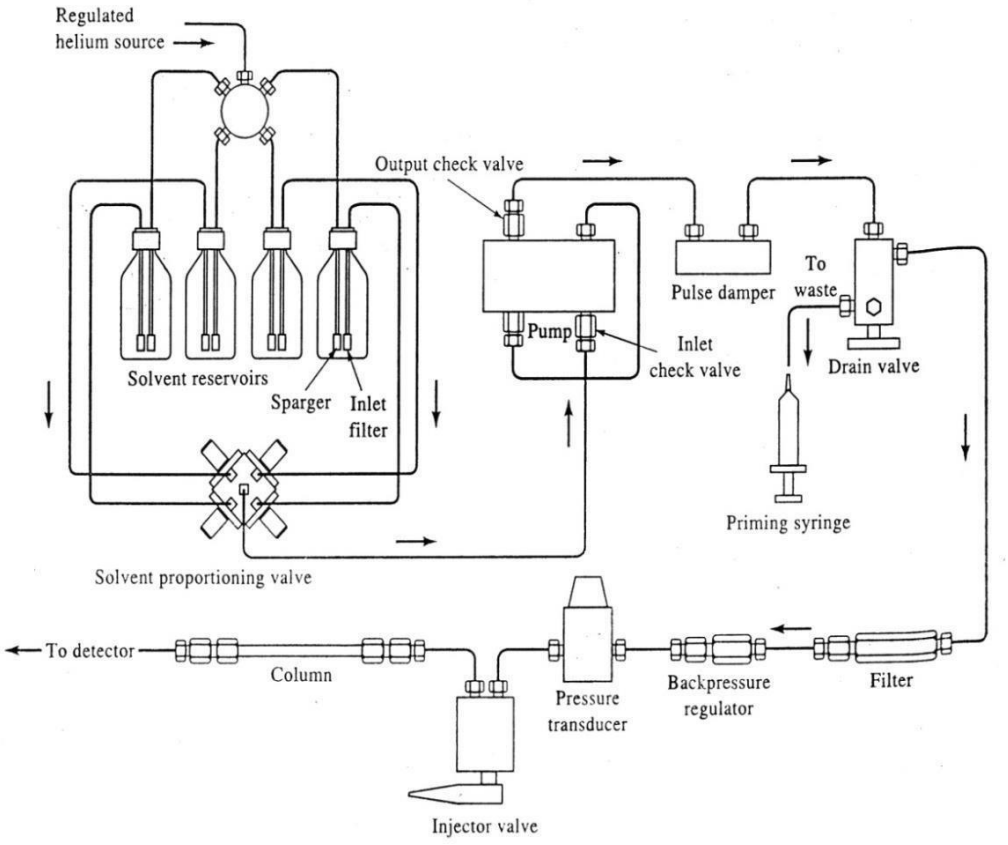


Fig2: Schematic of an apparatus for HPLC

SOLVENT RESERVOIR AND SOLVENT TREATMENT SYSTEMS

A modern HPLC apparatus is equipped with one or more glass or stainless steel reservoirs. The reservoir is often equipped with an online degasser which removes the dissolved gasses usually oxygen and nitrogen, which interfere by forming bubbles. Degasser may consist of vacuum pumping system, distillation system, system devices for heating, and solvent stirrer.

MOBILE PHASE:

Mobile phases used for HPLC typically are mixtures of organic solvents and water or aqueous buffers. Table given below lists the physical properties of organic solvents commonly used for HPLC. Isocratic methods are preferable to gradient methods. Gradient methods will some times be required when the molecules being separated have vastly different partitioning properties. When a gradient elution method is used, care must be taken to ensure that all solvents are miscible.

The following points should also be considered when choosing a mobile phase:

1. It is essential to establish that the drug is stable in the mobile phase for at least the duration ofthe analysis.

2. Excessive salt concentrations should be avoided. High salt concentrations can result inprecipitation, which can damage HPLC equipment.

3. The mobile phase should have a pH 2.5 and pH 7.0 to maximize the lifetime of the column.

4. Reduce cost and toxicity of the mobile phase by using methanol instead of acetonitrile whenpossible.

5. Minimize the absorbance of buffer. Since trifluoroacetic acid, acetic acid or formic acid absorb at shorter wavelengths, they may prevent detection of products with out chromophores above 220 nm. Carboxylic acid modifiers can be frequently replaced by phosphoric acid, whichdoes not absorb above 200 nm.

6. Use volatile mobile phases when possible, to facilitate collection of products and LC-MS analysis. Volatile mobile phases include ammonium acetate, ammonium phosphate, formic acid,acetic acid, and trifluoroacetic acid. Some caution is needed as these buffers absorb below 220 nm.

Table:1 PHYSICAL PROPERTIES OF COMMON HPLC SOLVENTS

Solvent	MW	BP	RI (25°C)	UV[a] Cut-off (nm)	Density g/ml (25°C)	Viscosity CP (25°C)	Dielectric Constant
Acetonitrile	41.0	82	1.342	190	0.787	0.358	38.8
Methanol	32.0	65	1.326	205	0.792	0.584	32.7
THF	72.1	66	1.404	210	0.889	0.51	7.58
Water	18.0	100	1.333	170	0.998	1.00	78.5

a: The wavelength at which the absorbance of 1cm cell is 1.0

Ionizable compounds in some cases can present some problems when analyzed by reverse phase chromatography. Two modifications of the mobile phase can be useful in reverse phase HPLC for ionizable compounds. One is called ion suppression and other ion pairing chromatography. In both techniques, a buffer is used to ensure that the pH of the solution is constant and usually at least 1.5 pH units from a pKa of the drug to ensure that one form predominates. If pH is approximately equal to pKa, peak broadening can occur. In ion suppression chromatography, the pH of the aqueous portion of the mobile phase is adjusted to allow the neutral form of the drug to predominate. This ensures that the drug is persistent in only one form and results in improvement of the peak shape and consistency of retention times. In ion pairing chromatography, the pH of the mobile phase is adjusted so that the drug is completely ionized. If necessary to improve peak shape or lengthen retention time, an alkyl sulfonic acid salt or bulky anion such as trifluoroacetic acid is added to the ion pair to cationic drugs or a quaternary alkyl ammonium salt is added to ion-pair to anionic drugs. Ion pairing chromatography also allows the simultaneous analysis of both neutral and charged compounds **PUMPING SYSTEM**

The function of the pump in HPLC is to pass mobile phase through the column at a controlled flow rate. Features of an ideal pumping system include:

- Generating pressure from 6000 psi to 10000 psi.
- Pulse free output.
- Flow rates ranging from 0.1 to 10 ml/min.
- Flow control and reproducibility of 0.5% relative or better.
- Corrosion resistant components.

There are three types of pumps commonly used:

Reciprocating Pumps

Reciprocating pumps usually consist of a small chamber in which the solvent is pumped by the back and forth motion of a motor driven piston. Two check valves control the flow of solvent. Reciprocating pumps have a disadvantage of producing pulsed flow, which must be damped as its presence is manifested as base line noise on the chromatogram. Advantages of this pump include their small internal volume, high output pressure, ready adaptability to gradient elution, and independent of column backpressure and viscosity of solvent.

Displacement Pumps

Displacement pumps usually consist of large syringe like chambers equipped with aplunger that is activated by a screw driven mechanism powered by stepping motor. Displacement pumps also produce a flow that tends to be independent of viscosity and backpressure. In addition, the output is pulse free. Disadvantages include limited solvent capacity (250 mL) and considerable inconvenience when solvents must be changed.

Pneumatic Pumps

In pneumatic pumps, the mobile phase is contained in a collapsible container housed in a vessel that can be pressurized by a compressor gas. Pumps of this kind are inexpensive and pulse free. They suffer from limited capacity, pressure output, dependence of flow rate on solvent viscosity and column backpressure. In addition, they are not amenable to gradient elution and are limited to pressures less than about 2000 psi.

AIM AND OBJECTIVE

Literature review reveals that there is no analytical method reported for the analysis of Dapagliflozin by 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 estimation of Dapagliflozin in pharmaceutical dosage form.

Present work is aimed to develop a new, simple, fast, rapid, accurate, efficient and

reproducible RP-HPLC method for the analysis of Dapagliflozin. The developed method will be validated according to ICH guidelines.

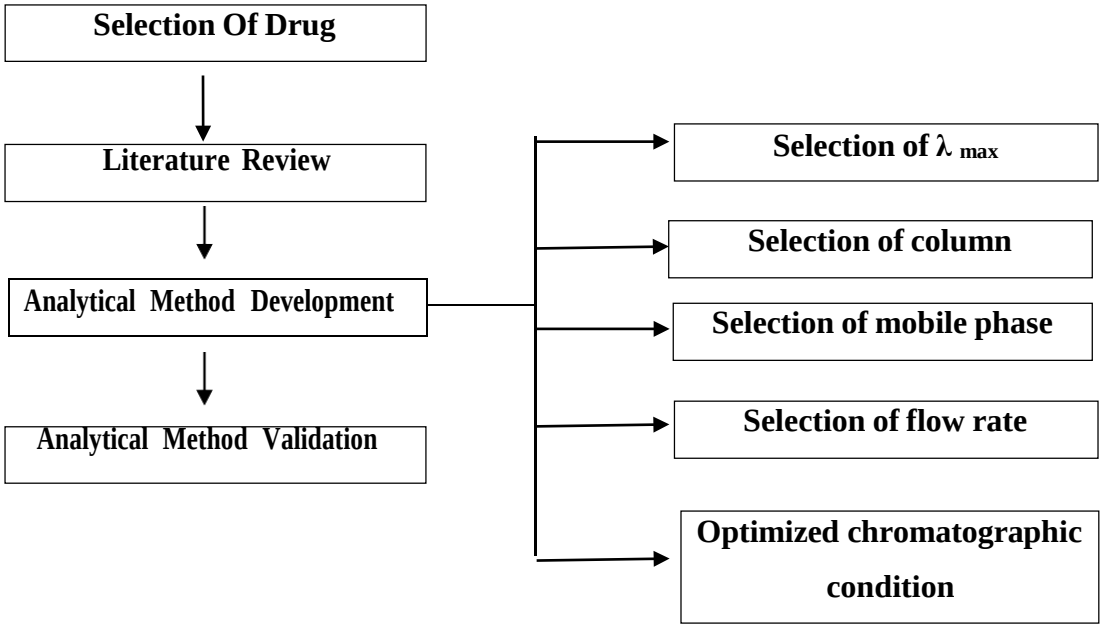
Objective of the work

- The analytical method for the estimation of Dapagliflozin will be developedby RP-HPLC method by optimizing the chromatographic conditions.
- The developed method is validated according to ICH guidelines for variousparameters specified in ICH guidelines, Q2 (R1).

PLAN OF WORK

To develop a new analytical method for the estimation of Dapagliflozin by RP-HPLC.

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



LITERATURE REVIEW

Based On Posaconazole

Halde Supriya et.al., has developed high performance liquid chromatography tandem mas spectrometric method was developed for the estimation of Posaconazole in human urine using Abacavir as an internal standard (IS). Posaconazole and abacavir were extracted from urine by solid phase extraction using Water Oasis HLB cartridges. The samples were chromatographed on Hypurity Advance, 50 x 2.1, 5µ column using a mobile phase consisting 5mM ammonium acetate: Acetonitrile: Methanol: (30:30:40 v/v). The chromatographic separation is achieved in 2.6 min. The method was validated over a concentration range of 0.50µg/mL to 80.00µg/mL. Method was validated for its sensitivity, selectivity, accuracy and precision, matrix effect, recovery and various stabilities. The validated method was used for analysis of urine samples. As per literature approximately 86% of Posaconazole is recovered in the urine unchanged hence urine analysis can serve as a useful tool to monitor patient adherence in HIV treatment.[16]

Deepthi Komaroju et.al., has been developed a simple, precise, accurate and rapid RP-HPLC method with PDA detector and subsequently validated for the simultaneous estimation of Posaconazole and tenofovir disoproxil fumarate in pure and tablet dosage form. The estimation was carried out on a Phenomenax Luna C18 (250mm x 4.6mm i.d; particle size 5µm) column with mixture of methanol: phosphate buffer pH-3 (70:30 v/v) as mobile phase. The flow rate was 1ml/min. UV detection was performed at 258 nm. The method was validated for linearity, accuracy, precision, robustness, LOD, LOQ as per ICH guidelines. The developed and validated method was successfully used for the quantitative analysis of commercial available dosage form. The retention time was 2.605 min and 3.781 min for Posaconazole and tenofovir disoproxil fumarate, respectively. The calibration curve was linear over concentration range of 45-105µg/ml for tenofovir disoproxil fumarate and 30-70µg/ml for Posaconazole. The correlation coefficient (r2) was found to be 0.999.Amount of Posaconazole and tenofovir disoproxil fumarate was found to be 199.4mg/tab and 298.6mg/tab respectively. The %RSD values were less than 2 for method precision. The high percentage of recovery and low percentage coefficient of variance confirm the suitability of the method for the simultaneous estimation of Posaconazole and tenofovir disoproxil fumarate in pure and tablet dosage form.[17]

Nagaraju P.T. et.al., was developed two simple, precise and economical UV methods for the estimation of Posaconazole in bulk and pharmaceutical formulations. Posaconazole has the absorbance maxima at 241.1nm (Method A), and in thefirst order derivative spectra, Showed zero crossing at 241.1nm, with a sharp peak at 232.7nm when n=1 (Method B).Drug followed the Beer's Lamberts range of 5-30 µg/ml for the Method A&B. The limits of detection were found to be 0.0684µg/ml and 0.185 µg/ml for Method A and Method B respectively. The limit of quantification for Method A and Method B were found to be 0.207µg/ml and 0.555µg/ml respectively. Results of analysis were validated statistically and by recovery studies and were found to be satisfactory.[18]

Patel Bhavini N et.al., was developed a simple, precise, rapid and accurate reverse phase HPLC method for the estimation of Posaconazole in capsule dosage form. A Phenomenex (Torrance, CA) C8 column, 250× 4.6 mm id, column, at ambient temperature with mobile phase consisting of 0.03M potassium dihydrogen phosphate (pH 4.86±0.02): Acetonitrile: Methanol (40:20:40 v/v/v) was used. The flow rate was 1 mL/min and the effluent was monitored at 260 nm. The retention time was 2.95 min. The detector response was linear in the concentration of 8-60 µg/mL. The respective linear regression equation being Y=27675x+41556. The limit of detection and limit of quantification was 0.06 and 0.20µg/mL respectively. The percentage assay of Posaconazole was 100.53%. The method was validated by determining its accuracy, precision and system suitability. The results of the study showed that the proposed RP-HPLC method is simple, rapid, precise and accurate, which is useful for the routine determination of Posaconazole in bulk drug and in its pharmaceutical dosage form.[19]

Pradeep kumar* et.al., was developed a rapid, precise, accurate, specific and simple RP-HPLC (reversed phase – high performance liquid chromatography) method for the assay of Posaconazole from tablets. A High performance liquid chromatograph 10AT SHIMADZU- SPD10A, using Phenomenex - Luna RP-18(2),250X4.6mm, 5 µm column, with a mobile phase composition of buffer : acetonitrile [85:15 %(v/v)] was used. The flow rate of 1.0 mL min-1 and the effluent was detected at 280 nm. The retention time of

Posaconazole was 9.341 minutes. Linearity was observed over the concentration range of 20-600µg mL-1. The limit of detection was found to be 5.539µg mL-1 while the quantification limit was 16.786µg mL- 1. The accuracy of the proposed method was determined by recovery studies and was found to be 99.468% to 101.110 %. The commercial tablet formulation was successfully analyzed using the developed method and the proposed method is applicable to stability studies and routine analysis of Posaconazole in bulk and pharmaceutical formulations. The proposed method was validated for various ICH (International Conference on Harmonization) parameters like linearity, limit of detection, limits of quantification, accuracy, precision, range and specificity.[20]

Based On Zidovudime

Palani Venkatesh et.al., was developed Simultaneous quantification of Lamivudine and Zidovudine in tablets by HPTLC method and validated. The chromatograms were developed using a mobile phas eof toluene : ethyl acetate : methanol (4:4:2,v/v/v) on precoated plate of silica gel G Faluminum TLC, plate and quantified by densitometric absorbance mode at 276 nm. The Rf values were 0.4170.03 and 0.6070.04 for Lamivudine and Zidovudine, respectively. The linearity of the method was found to be within the concentration range of 50-250 ng/spot for Lamivudine and for Zidovudine, it was100-500ng/spot. The lower limits of detection and quantification were 2.23ng/spo tand 7.90 ng/spot for Lamivudine and 2.90ng/spot and 8.85ng/spot for Zidovudine. The method was also validated for precision, specificity and recovery.This developed method was used to analyze fixed-dose tablets (Duovir, CiplaLtd) samples of Lamivudine and Zidovudine. & 2011 Xi'anJiaotong University .Production and hosting by Elsevier B.V.All rights reserved.[21]

Yadavalli Rekha1, et.al., has been developed A simple, economic, specific, accurate and precise validated reverse phase liquid chromatographic method for the estimation of Efavirenz, Lamivudine and Zidovudine in Tablet dosage forms. Here in present method, chromatography was carried out using the instrument Waters HPLC 2695 mode with empower software on a Xterra C18(150mm×4.6mm, 5µ) column with mobile phase of 70

volumes of Water (pH was adjusted to 2.1 with o-phosphoric acid) and 30 volumes of Methanol in isocratic mode. The flow rate was 1ml/min, with injection volume 10µl. Detection was done by using PDA detector at 275nm. The retention time was found to be 1.91, 2.90 and 7.52 min The method was validated in terms of linearity, precision, accuracy, LOD, LOQ and robustness in accordance with ICH guidelines. The linearity was found to be in the range of 300-900 µg/ml, 75-225 µg/ml, 150-450 µg/ml for Efavirenz, Lamivudine and Zidovudine with correlation coefficient 0.999. The LOD values were 1.8196, 0.796, 3.166 µg/ml.The LOQ values were 6.065, 2.654 and 10.55 µg/ml respectively. The percentage assay was 99.89, 99.22 and 99.64% for Efavirenz, Lamivudine and Zidovudine. No chromatographic interference from tablet excipients was found. The developed method with good separation could be successfully applied for the determination of Efavirenz, Lamivudine and Zidovudine in its Tablet dosage form.[22]

CH Venkata Reddiah et.al., A novel rapid, was developed sensitive and reproducible mass compatible, ultra performance liquid chromatographic method for quantitative determination of Lamivudine, Zidovudine and Abacavir in active pharmaceutical ingredients and its dosage forms. The synthetic nucleoside reverse transcriptase inhibitor analogues Abacavir, Lamivudine and Zidovudine form one of the fixed dosage combinations used in the effective management of HIV. It belongs to a group of anti-HIV medicines called non-nucleoside reverse transcriptase inhibitors (NNRTIs). The method is applicable to the quantification of related compounds of Abacavir, Lamivudine and Zidovudine form one of the fixed dosage combinations. Chromatographic separation of drugs from the possible impurities and the degradation products was achieved on an Inertsil ODS-3V 250 x 4.6 mm, 5.0µm column; the gradient elution achieved with in 90.0 min. Ammonium dihydrogen phosphate and Diammonium hydrogen phosphate buffers pH 3.9 as mobile phase A and methanol as mobile phase B . The flow rate was 1.0 mL/min, column temperature 50°C and the detection was done at 270 nm. The above developed HPLC method was further subjected to hydrolytic, oxidative, photolytic and thermal stress conditions. The performance of the method was validated according to the present ICH guidelines for specificity, limit of detection, limit of quantification, linearity, accuracy, precision, and ruggedness.[23]

Devyani dube et.al., has been developed a novel, simple, rapid and sensitive spectrophotometric method for simultaneous estimation of Lamivudine and Silymarin. The method employs formation and solving of simultaneous equation using 270.9 nm and 326.4 nm as two analytical wavelengths. Both the drugs obey Beer's Law in the concentration ranges employed for this method. Accuracy and reproducibility of the proposed method was statistically validated by recovery studies. The method is found to be rapid, precise and accurate and can easily be employed in the laboratory for the routine estimation of drugs.[24]

Narendra Devanaboyina*, et.al., has been developed a new simple, rapid, selective, precise and accurate isocratic reverse phase high performance liquid chromatography assay and validated for the estimation of zidovudine in tablet formulation. The separation was achieved by using C-18 column (250x4.6mm, 5µm in particle size) at ambient temperature coupled with a guard column of silica in mobile phase Acetonitrile: Water (90%; 10%) with the pH value adjusted to 4.8 .The flow rate was 1ml/min and the drug was detected by using UV detector at the wavelength 240nm and the run time was 10min. The retention time was found 4.7 minutes. The percentage of RSD for precision and accuracy of the method was found to be less than 2%. The method was validated as per ICH guidelines. The proposed method was found to be accurate, repeatability and consistent. It can be successfully applied for the analysis of the drug in marketed formulation and could be effectively used for the routine analysis of the same drug without any alteration in the chromatographic conditions.[25]

K. Balamuralikrishna, et.al., have been developed Reverse phase high performance liquid chromatography methods for the simultaneous estimation of Efavirenz, Lamivudine and Zidovudine in tablet dosage form. In reverse phase high performance liquid chromatography analysis is carried out using Acetonitrile, Methanol and 0.05M dipotassium hydrogen orthophosphate in the ratio of 40:40:20 v/v/v (pH was adjusted to 4.0 with o-phosphoric acid) as the mobile phase and Luna C18 (4.6 x 250 mm) column as stationary phase with detection wavelength of 259 nm. Linearity was obtained in the

concentration range of 100-200, 15-45 and 40-120 µg/ml for Efavirenz, Lamivudine and Zidovudine, respectively. Reverse phase high performance liquid chromatography method was statistically validated and can be used for analysis of combined dose tablet formulation containing Efavirenz, Lamivudine and Zidovudine.[26]

J. Priyanka et.al., was developed a rapid, sensitive and specific RP-HPLC [1-5] method involving UV detection and validated for determination and quantification of Lamivudine and Zidovudine. Chromatography was carried out on Thermo Hypersil BDS, C18,(150 x 4..6 mm,5□) column using filtered and degassed mixture of Buffer : Methanol :Acetonitrile (70:5:25) as mobile phase at a flow rate of 0.8ml/min and effluent was monitored at 267nm. The method was validated in terms of linearity, precision, accuracy, robustness and specificity, limit of quantification and limit of detection. The assay was linear over the concentration range of Lamivudine and Zidovudine was 37.5µg - 225µg/ml and 75µg to 450µg/ml respectively. Accuracy of the method was determined through recovery studies by adding known quantities of standard drug to the pre analyzed test solution and was found to be 99.50%-100.7% and 99.9%-100.7% within precision RSD of 1.30 and 0.61 for Lamivudine and Zidovudine respectively. The method does require only 10 minutes as run time for analysis which prove the adoptability of the method for the routine quality control of the drug.[27]

Aim and Objectives

The drug analysis plays an significant role in the development of drugs, their manufacture and the therapeutic use. These drugs or formulation may be either in the novel entities in the market or fractional structural modification of the already presented drugs or novel dosage forms or multi component dosage forms. The multi component dosage form proves to be effective due to the combined mode of action on the body. The combined dosage form may show synergetic action in the body so the multi component dosage form is more effective than the single component dosage form.

The extensive literature survey carried out and revealed that very few analytical methods have reported for the simultaneous determination of Posaconazole and Zidovudine drugs individually or in the combination with the other drugs. Hence to develop a simple, rapid, accurate, specific, linear and precise method was developed and validated for the simultaneous estimation of Posaconazole and Zidovudine in combined dosage forms by using RP-HPLC.

The specific aim of the research was

- To develop a method for the simultaneous estimation of Posaconazole and Zidovudine in Pharmaceutical dosage form and validate the proposed methods in accordance with ICH guidelines for the intended analytical application.
- To validate the developed RP-HPLC method by using various validation parameters such as linearity, accuracy, system suitability, precision, robustness, ruggedness according to ICH guidelines.

Plan And Design Of Work

i) Preparation of drug standard and sample

ii) Optimization chromatographic conditions like,

a. Selection of wavelength

b. Selection of initial separation conditions

c. Nature of stationary phase

d. Nature of mobile phase (pH, solvent strength, solvent ratio, flow rate)

iii) Validation of proposed method

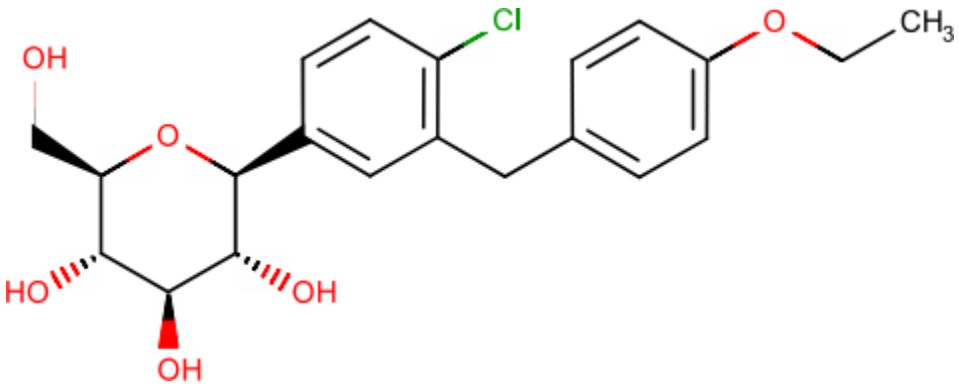
DRUG PROFILE[30]

DAPAGLIFLOZIN

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

Chemical formula : $C_{21}H_{25}ClO_6$

Structure:



Molecular weight : 408.873 g/mol

Cas no : 461432-26-8

Solubility : Dapagliflozin is not a significant P-gp inhibitor. Solubility : Soluble in ethanol (~30**mg**/ml), **DMSO** (82 **mg**/ml at 25 °C), DMF (~30 **mg**/ml), ethanol:PBS(pH7.2)(1:1) (~0.5 **mg**/ml), and methanol.

Melting Point : **65-70**° C

Mechanism of action: A competitive inhibitor of the sodium-glucose transport subtype 2 protein, dapagliflozin blocks glucose reabsorption into the kidney, resulting in the elimination of blood glucose through the urine.

MATERIALS AND METHODSEQUIPMENTS

Table.No.1. List of instruments used

S.No	Instrument name	Model number	Soft ware	Manufacturers Name
1	HPLC-auto sampler –UV detector	Separation module2695, UV.detector2487	Empower-software version-2	Waters
2	U.V double beam spectrometer	UV 3000+	U.V win soft ware	Lab India
3	Digital weighing balance(sensitivity 5mg)	ER 200A	-	Ascoset
4	pH meter	AD 102U	-	ADWA
5	Sonicator	SE60US	-	Enertech

Chemicals used:

Table.No.2 List of chemicals and standards used

S.No	Chemicals	Manufacturer Name	Grade
1.	Water	Merck	HPLC grade
2.	Methanol	Merck	HPLC grade
3.	Acetonitrile	Merck	HPLC grade
4.	Ortho phosphoric acid	Merck	G.R
5.	KH_2PO_4	Merck	G.R

Active pharmaceutical Ingredient (pure drug)

Table.No.3 Active pharmaceutical Ingredient

S.No	Name	Specification
1	Dapagliflozin	Reference Standard

Marketed Formulation

Table.No.4 Marketed Formulation

S.No	Name	Manufacturer
1	Dapagliflozin	--

Solubility

Solubility of drug was observed by dissolving it in different solvents and it was found that drug having good solubility in followings.

Solubility of drugs in different solvents

Table.No.5 Solubility of drugs in different solvents

Solvent	Solubility Dapagliflozin
Water	-
Acetonitrile	+
Methanol	-

METHOD DEVELOPMENT

Selection of mobile phase:

The method development and validation of Dapagliflozin requires greater resolution. Hence different solvent systems were tried.

The trails are using UV 3000+ equipment with PDA detector and isocratic pump. The system controlled by LC solution software.

Selection of flow rate:

The flow rate of Dapagliflozin was tried from 0.8 ml to 1.5ml.

Trial-1

Buffer preparation:

About 7.0g of potassium di hydrogen ortho phosphate was dissolved in 1000ml of HPLC grade water and P^H 2.5 was adjusted with ortho phosphoric acid. It was filtered through 0.45µm nylon membrane filter and degassed with sonicator. It was used as a diluent for the preparation of sample and standard solution.

Preparation of mobile phase:

Mobile phase consist of water: methanol HPLC of P^H 2.5 (30:70) was taken sonicated and degassed for 10 min and filtered through 0.45 µm nylon membrane filter.

Standard Preparation:

Weigh accurately 10mg Dapagliflozin Working Reference Standard and 15mg of Zidovudine Working Reference Standard is taken in to 100ml volumetric flask and then it was dissolved and diluted to volume with mobile phase up to the mark. After that 50ml of the above solution was taken into 100ml standard flask and made up with mobile phase. (Stock solution)

Further pipette 0.5ml of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent.

Chromatographic conditions:

Column	:	Chromosil Column C_{18} (150mm x 4.6mm)5µg.
Mobile phase	:	Water : Methanol P^H 2.5 (30:70 v/v)
Flow rate	:	0.8ml/ min

Detector wavelength	:	254 nm
Injection mode	:	Auto injector (vial)
Injection volume	:	20µl

Trial-2

Buffer preparation:

About 7.0g of potassium di-hydrogen orthophosphate was dissolved in 1000ml of HPLC grade water and P^H 2.5 was adjusted with orthophosphoric acid. It was filtered through 0.45µm nylon membrane filter and degassed with sonicator. It was used as a diluent for the preparation of sample and standard solution.

Preparation of mobile phase:

Mobile phase consist of water: acetonitrile of P^H 2.5 (30:70) was taken sonicated and degassed for 10 min and filtered through 0.45µm nylon membrane filter.

Standard Preparation: Weigh accurately 10mg Dapagliflozin Working Reference Standard taken in to 100ml volumetric flask and then it was dissolved and diluted to volume with mobile phase up to the mark. After that 50ml of the above solution was taken into 100ml standard flask and made up with mobile phase. (Stock solution)

Further pipette 0.5ml of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent.

Chromatographic conditions:

Column	:	Chromosil C_{18} Column (150mm x 4.6mm)5µg.
Mobile phase	:	water: Acetonitrile P^H 2.5 (30:70 v/v)
Flow rate	:	1ml/ min
Detector wavelength	:	254 nm
Injection mode	:	Auto injector (vial)
Injection volume	:	20µl

Trial-3

Buffer preparation:

About 7.0g of potassium dihydrogen orthophosphate was dissolved in 1000ml of HPLC grade water and P^H 2.5 was adjusted with orthophosphoric acid. It was filtered through 0.45µm nylon membrane filter and degassed with sonicator. It was used as a diluent for the preparation of sample and standard solution.

Preparation of mobile phase:

Mobile phase consist of buffer: Methanol of P^H 2.5 (20:80) was taken sonicated and degassed for 10min and filtered through 0.45µm nylon membrane filter.

Standard Preparation:

Weigh accurately 10mg Dapagliflozin Working Reference Standard is taken in to 100ml volumetric flask and then it was dissolved and diluted to volume with mobile phase up to the mark. After that 50ml of the above solution was taken into 100ml standard flask and made up with mobile phase. (Stock solution)

Further pipette 0.5ml of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent.

Chromatographic conditions:

Column	:	Xterra C_{18} Column (150mm x 4.6mm) 5µg.
Mobile phase	:	Phosphate buffer: Methanol P^H 2.5 (45:55 v/v)
Flow rate	:	1ml/ min
Detector wavelength	:	254 nm
Injection mode	:	Auto injector (vial)
Injection volume	:	20µl

Trial-4

Buffer preparation:

About 7.0g of potassium dihydrogen orthophosphate was dissolved in 1000ml of HPLC grade water and P^H 2.5 was adjusted with orthophosphoric acid. It was filtered through 0.45µm nylon membrane filter and degassed with sonicator. It was used as a diluent for the preparation of sample and standard solution.

Preparation of mobile phase:

Mobile phase consist of buffer: Methanol of P^H 2.5 (30:70) was taken sonicated and degassed for 10min and filtered through 0.45µm nylon membrane filter.

Standard Preparation:

Weigh accurately 10mg Dapagliflozin Working Reference Standard is taken in to 100ml volumetric flask and then it was dissolved and diluted to volume with mobile phase up to the mark. After that 50ml of the above solution was taken into 100ml standard flask and made up with mobile phase. (Stock solution)

Further pipette 0.5ml of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent.

Chromatographic conditions:

Column	:	Xterra C_{18} Column (150mm x 4.6mm)5µg.
Mobile phase	:	Phosphate buffer: Methanol P^H 2.5 (30:70 v/v)
Flow rate	:	1ml/ min
Detector wavelength	:	254 nm
Injection mode	:	Auto injector (vial)
Injection volume	:	20µl

Trial-5

Buffer preparation:

About 7.0g of potassium di hydrogen ortho phosphate was dissolved in 1000ml of HPLC grade water and P^H 2.5 was adjusted with ortho phosphoric acid. It was filtered through 0.45 µm nylon membrane filter and degassed with sonicator. It was used as a diluent for the preparation of sample and standard solution.

Preparation of mobile phase:

Mobile phase consist of buffer: Methanol of P^H 2.5 (30:70) was taken sonicated and degassed for 10min and filtered through 0.45µm nylon membrane filter.

Standard Preparation:

Weigh accurately 10mg Dapagliflozin Working Reference Standard is taken in to 100ml volumetric flask and then it was dissolved and diluted to volume with mobile phase up to the mark. After that 50ml of the above solution was taken into 100ml standard flask and made up with mobile phase. (Stock solution)

Further pipette 0.5ml of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent.

Chromatographic conditions:

Column	:	Thermosil C_{18} Column (100mm x 4.6mm) 5µg.
Mobile phase	:	Phosphate buffer: Methanol P^H 2.5 (30:70 v/v)
Flow rate	:	1ml/ min
Detector wavelength	:	254 nm
Injection mode	:	Auto injector (vial)
Injection volume	:	20µl

Trial-6

Buffer preparation:

About 7.0g of potassium dihydrogen orthophosphate was dissolved in 1000ml of HPLC grade water and pH 2.5 was adjusted with orthophosphoric acid. It was filtered through 0.45µm nylon membrane filter and degassed with sonicator. It was used as a diluent for the preparation of sample and standard solution.

Preparation of mobile phase:

Mobile phase consist of buffer: Methanol of P^H 2.5 (35:65) was taken sonicated and degassed for 10min and filtered through 0.45 µm nylon membrane filter.

Standard Preparation:

Weigh accurately 10mg Dapagliflozin Working Reference Standard is taken in to 100ml volumetric flask and then it was dissolved and diluted to volume with mobile phase up to the mark. After that 50ml of the above solution was taken into 100ml standard flask and made up with mobile phase. (Stock solution)

Further pipette 0.5ml of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent.

Chromatographic conditions:

Column	:	Thermosil C_{18} Column (100mm x 4.6mm) 5µg.
Mobile phase	:	Phosphate buffer: Methanol P^H 2.5 (35:65 v/v)
Flow rate	:	1ml/ min
Detector wavelength	:	254 nm
Injection mode	:	Auto injector (vial)
Injection volume	:	20µl

Method Validation

The chromatographic conditions were validated by evaluating linearity, accuracy, method precision, limit of detection (LOD), limit of quantization (LOQ), ruggedness and robustness in accordance with ICH guidelines.

Specificity

Preparation of solutions

a) Placebo interference

Amount of 352.6 mg of the capsule powder was taken in to 100ml standard flask. A volume of 70ml of mobile phase was added and sonicate for 30min.Then the solution was cooled and diluted to volume with mobile phase and filtered through 0.45µm membrane filter. (Stock solution)

Further pipette 0.25ml of Dapagliflozin of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent.

Acceptance criteria

Chromatogram of placebo should not show any peak at the retention time of analyte peak.

Standard preparation

Weigh accurately 10mg Dapagliflozin Working Reference Standard is taken in to 100ml volumetric flask and then it was dissolved and diluted to volume with mobile phase up to the mark. After that 50ml of the above solution was taken into 100ml standard flask and made up with mobile phase. (Stock solution)

Further pipette 0.5ml of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent.

Sample preparation

Amount of 352.6 mg of the tablet powder was taken in to 100ml standard flask. A volume of 70ml of mobile phase was added and sonicate for 30min.Then the solution was cooled and diluted to volume with mobile phase and filtered through 0.45µm membrane filter.

(stock solution)

Further pipette 0.25ml of Dapagliflozin of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent.

Linearity and Range

Preparation of stock solution

Weigh accurately 10mg Dapagliflozin Working Reference Standard is taken in to 100ml volumetric flask and then it was dissolved and diluted to volume with mobile phase up to the mark. After that 50ml of the above solution was taken into 100ml standard flask and made up with mobile phase. (Stock solution)

Dapagliflozin:

Preparation of linearity solution (20%)

ml of stock solution was taken in 10mlof volumetric flask dilute up to the mark with diluent. The solution mixed well and used for chromatographic injection.

Preparation of linearity solution (30%)

ml of stock solution was taken in 10ml of volumetric flask dilute up to the mark with diluent. The solution mixed well and used for chromatographic injection

Preparation of linearity solution (40%)

ml of stock solution was taken in 10ml of volumetric flask dilute up to the mark with diluent. The solution mixed well and used for chromatographic injection.

Preparation of linearity solution (50%)

ml of stock solution was taken in 10ml of volumetric flask dilute up to the mark with diluent. The solution mixed well and used for chromatographic injection.

Preparation of linearity solution (60%)

ml of stock solution was taken in 10ml of volumetric flask dilute up to the mark with diluent The solution mixed well and used for chromatographic injection.

Procedure

Each level of the above solutions was injected into the chromatographic system for five replicate and the peak area was measured. A graph was plotted (peak area versus concentration) and the correlation coefficient (r^2) was calculated.

Accuracy

Accuracy is the measure of exactness of an analytical method, or closeness of agreement between the measured value and the value that is accepted either as a conventional, true value or an accepted reference value. Accuracy is measured as the percentage of analyte recovered by assay, spiking samples in a blind study.

Preparation of stock solution

Weigh accurately 10mg Dapagliflozin Working Reference Standard is taken in to 100ml volumetric flask and then it was dissolved and diluted to volume with mobile phase up to the mark. After that 50ml of the above solution was taken into 100ml standard flask and made up with mobile phase. (Stock solution)

Further pipette 0.5ml of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation Sample solutions

50% Sample preparation

Weigh accurately 5 mg Dapagliflozin Working Reference Standard is taken in to 100ml volumetric flask and then it was dissolved and diluted to volume with mobile phase up to the mark. After that 50ml of the above solution was taken into 100ml standard flask and made up with mobile phase. (Stock solution)

Further pipette 0.5ml of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent. The above solution were inject into the HPLC column same procedure was repeated for three replicate.

100% Sample preparation

Weigh accurately 10mg Dapagliflozin Working Reference Standard is taken in to 100ml volumetric flask and then it was dissolved and diluted to volume with mobile phase up to the mark. After that 50ml of the above solution was taken into 100ml standard flask and made up with mobile phase. (Stock solution)

Further pipette 0.5ml of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluents. The above solution were inject into the HPLC column same procedure was repeated for three replicate.

150% Sample preparation

Weigh accurately 15mg Dapagliflozin Working Reference Standard is taken in to 100ml volumetric flask and then it was dissolved and diluted to volume with mobile phase up to the mark. After that 50ml of the above solution was taken into 100ml standard flask and made up with mobile phase. (Stock solution)

Further pipette 0.5ml of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent. The above solution were inject into the HPLC column same procedure was repeated for three replicate.

Procedure

The standard solution was injected in triplicate for Accuracy -50%, Accuracy -100% and Accuracy -150% solutions.

Calculate the Amount found and Amount added for Dapagliflozin & Zudovudine and calculate the individual recovery and mean recovery values.

$$\text{\% Recovery} = \frac{\text{Sample peak area x weight of standard}}{\text{Standard peak area x weight of sample}} \times 100$$

Precision

Precision was the measure of the degree of repeatability of an analytical method under normal operation and it was normally expressed as the relative standard deviation for a statistically number of samples. Precision should be performed at three different levels: repeatability, intermediate precision and reproducibility.

Standard preparation

Weigh accurately 10mg Dapagliflozin Working Reference Standard is taken in to 100ml volumetric flask and then it was dissolved and diluted to volume with mobile phase up to the mark. After that 50ml of the above solution was taken into 100ml standard flask and made up with mobile phase. (Stock solution)

Further pipette 0.5ml of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent.

Intermediate Precision (Ruggedness)

Ruggedness is the degree of reproducibility of results obtained by the analysis of the same sample under a variety of normal test conditions i.e. different analysts, laboratories, instruments, reagents, assay temperatures, small variations in mobile phase, different days etc. (i.e. from laboratory to laboratory, from analyst to analyst). Acceptance criteria for ruggedness, the % RSD for the area of five standard injections should not be more than 2%.

Standard preparation

Weigh accurately 10mg Dapagliflozin Working Reference Standard is taken in to 100ml volumetric flask and then it was dissolved and diluted to volume with mobile phase up to the mark. After that 50ml of the above solution was taken into 100ml standard flask and made up with mobile phase. (Stock solution)

Further pipette 0.5ml of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure

Five replicate Sample solutions were prepared as per the test method and injected as per the test procedure.

Robustness

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

a) The flow rate was varied at 0.8ml/min to 1.2ml/min.
b) The organic composition in the mobile phase was varied from 65% to75 % standard solution 10µg/ml of prepared and analysed using the varied mobile phase composition along with the actual mobile phase composition in the method.

Limit of detection (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 based on the standard deviation of y-intercepts of regression lines.

Formula:

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

Where

σ - Standard deviation (SD)

S - Slope

Limit of quantification

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:

$$\mathbf{LOD = 10\ X\ \frac{\sigma}{s}}$$

Where

σ - Standard deviation

S - Slope

System Suitability:

- System is suitable for analysis if the relative standard deviation (RSD) of area counts in the six replicate injections for each peak should not be more than 2.0%. The USP plate count of peak should not be less than 2000 theoretical plates for HPLC. The tailing factor for each peak should not be more than 2.0 and the resolution for two peaks should not be less than 2.0. The results obtained indicate the good precision of the developed method.

Amount of Dapagliflozin present in mg per average net content of the using the formula

Assay

Preparation of samples for Assay

Standard preparation:

Weigh accurately 10mg Dapagliflozin Working Reference Standard is taken in to 100ml volumetric flask and then it was dissolved and diluted to volume with mobile phase up to the mark. After that 50ml of the above solution was taken into 100ml standard flask and made up with mobile phase. (Stock solution)

Further pipette 0.5ml of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent.

Calculation

The amount of Dapagliflozin present in the formulation by using the formula given below, and results shown in above table:

$$\%\ \text{Assay} = \frac{\text{AT}}{\text{AS}} \times \frac{\text{WS}}{\text{DS}} \times \frac{\text{DT}}{\text{WT}} \times \frac{\text{P}}{100} \times \frac{\text{AW}}{\text{LC}} \times 100$$

Where,

AS: Average peak area due to standard preparation

AT: Peak area due to assay preparation

WS: Weight of Dapagliflozin in mg

WT: Weight of sample in assay preparation

DT: Dilution of assay preparation

System Suitability Results:

1). Tailing factor Obtained from the standard injection is 1.2

2). Theoretical Plates Obtained from the standard injection is 5404.6

Assay Results:

Calculation: (For Dapagliflozin)

Assay % =

AT WS DT P Avg. Wt

$$- \frac{AT}{AS} \times \frac{DS}{WT} \times \frac{WT}{100} \times \frac{100}{Label\ Claim} \times 100$$

Where:

AT = average area counts of sample preparation.

As= average area counts of standard preparation.

WS = Weight of working standard taken in mg.

P = Percentage purity of working standard

LC = label claim of Dapagliflozin mg/ml.

$$= \frac{2005829}{2008408} \times \frac{15}{10} \times \frac{0.5}{10} \times \frac{100}{694.2} \times \frac{10}{0.25} \times \frac{99.89}{100} \times \frac{694.2}{200} \times 100 = 99.77175$$

P = Percentage purity of working standard

LC = label claim of Zidovudine mg/ml.

$$\frac{1189695}{1185786} \times \frac{15}{10} \times \frac{0.5}{10} \times \frac{100}{694.2} \times \frac{10}{0.25} \times \frac{99.8}{100} \times \frac{694.2}{300} \times 100 = 100.129$$

Discussion:

The amount of Dapagliflozin present in the taken dosage form was found to be 99.77175% and 100.129 % respectively.

TRIALS

Trial-1

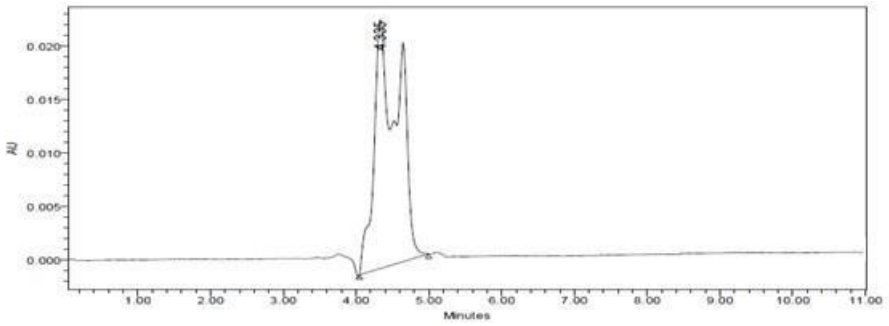


Fig.1: Chromatogram of Dapagliflozin

Table 6: Chromatogram results

S.No	Peak Name	R_t	Area	Height	USP Plate Count	USP Tailing	USP Resolution
1	Dapagliflozin	4.335	536443	23306	1894	1.4	

Discussion: The separation of one analytical peak was not proper, So the mobile phase ratio has been changed for next trial.

Trial-2

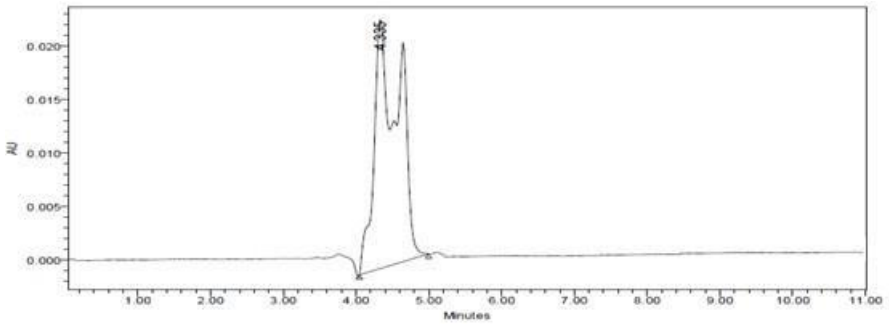


Fig. 2: Chromatogram of Dapagliflozin

Table 7: Chromatogram results

S.No	Peak Name	R_t	Area	Height	USP Plate Count	USP Tailing	USP Resolution
1	Dapagliflozin	4.333	518384	22211	92	1.4	

Discussion: The separation of analytical peak was not proper, So the mobile phase ratio has been changed for next trial.

Trial-3

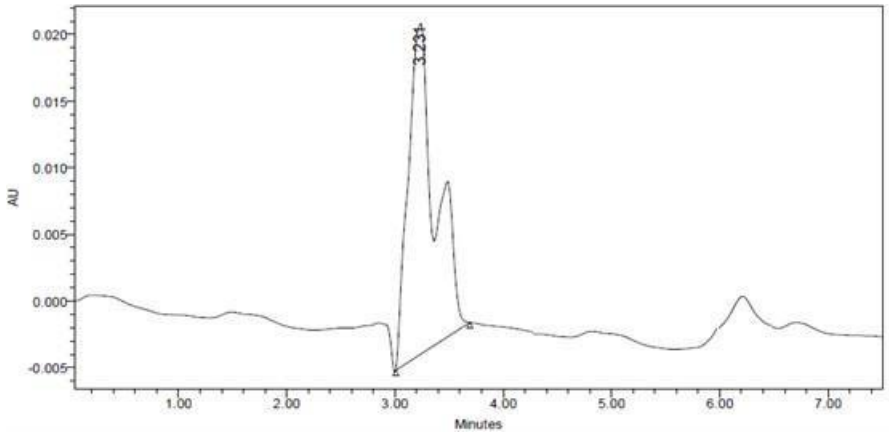


Fig. 3: Chromatogram of Dapagliflozin

Table 8: Chromatogram results

S.No	Peak Name	R_t	Area	Height	USP Plate Count	USP Tailing	USP Resolution
1	Dapagliflozin	3.231	432752	25062	1439	1.4	

Discussion: The separation of analytical peak was not proper, so the mobile phase ratio has been changed for next trial.

Trial-4

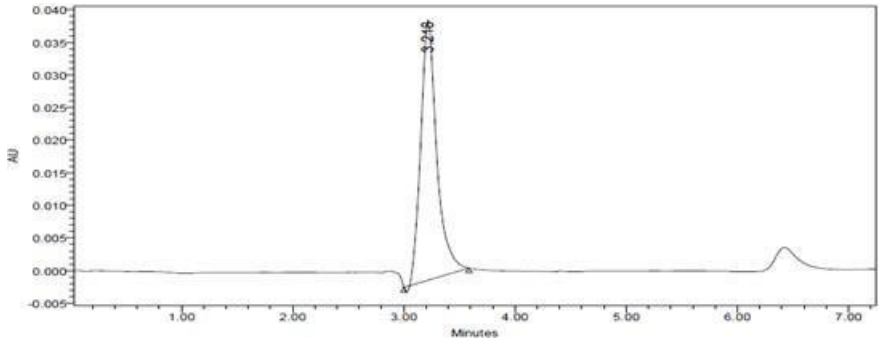


Fig.4: Chromatogram of Dapagliflozin

Table 9: Chromatogram results

S.No	Peak Name	R_t	Area	Height	USP Plate Count	USP Tailing	USP Resolution
1	Dapagliflozin	3.218	400986	39855	2602	1.2	

Discussion: The separation of analytical peak is occurred but fronting occurs in Dapagliflozin peak.

Trial-5

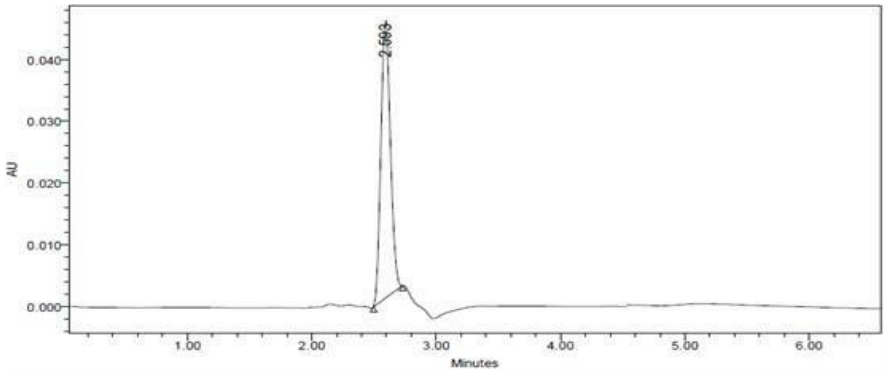


Fig. 5: Chromatogram of Dapagliflozin

Table 10: Chromatogram results

S.No	Peak Name	R_t	Area	Height	USP Plate Count	USP Tailing	USP Resolution
1	Dapagliflozin	2.593	239603	44771	5354	1.1	

Discussion: The separation of analytical peak was good but base line noise is occurred. So the mobile phase ratio has been changed for next trial.

Optimized method

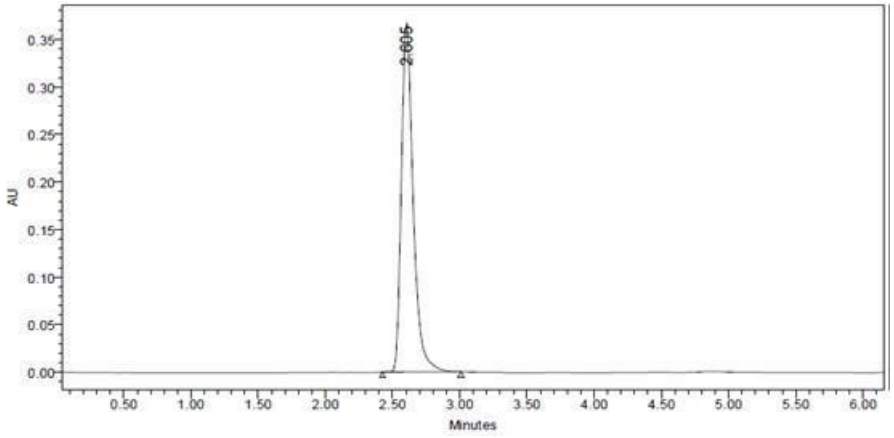


Fig. 6: Chromatogram of Optimized method

Table 11: Chromatogram results

S.No	Peak Name	R_t	Area	Height	USP Plate Count	USP Tailing	USP Resolution
1	Dapagliflozin	2.605	2233704	365596	4456	1.4	

Discussion: The separation of two analytical peaks was good. The plate count also above 2000, tailing factor below 2, and the resolution is above 2. The condition is taken as optimized method.

ASSAY

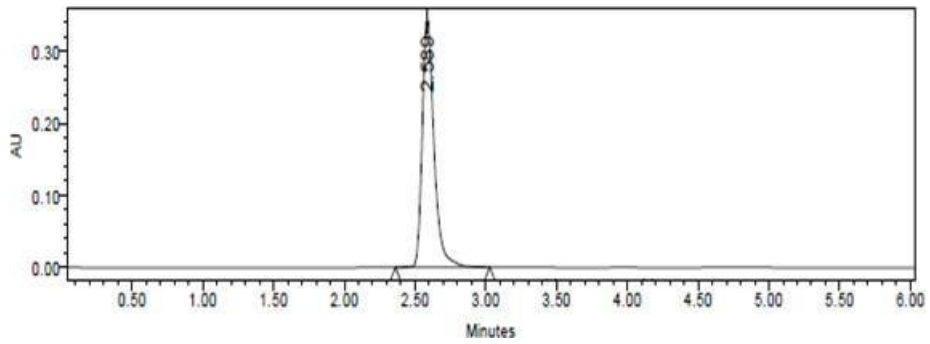


Fig.7: Chromatogram of Assay standard preparation-1

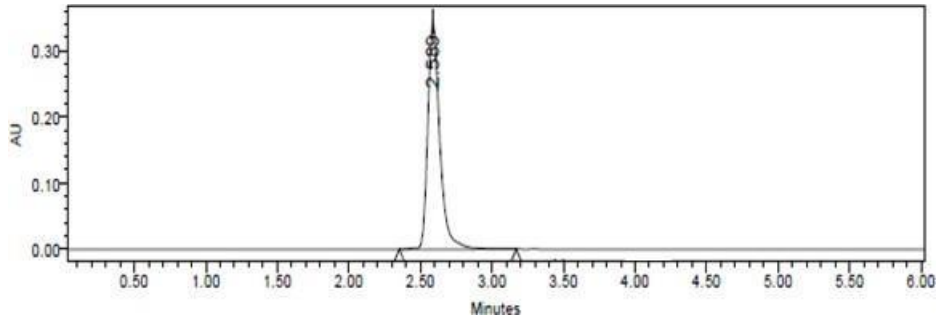


Fig.8: Chromatogram of Assay standard preparation-2

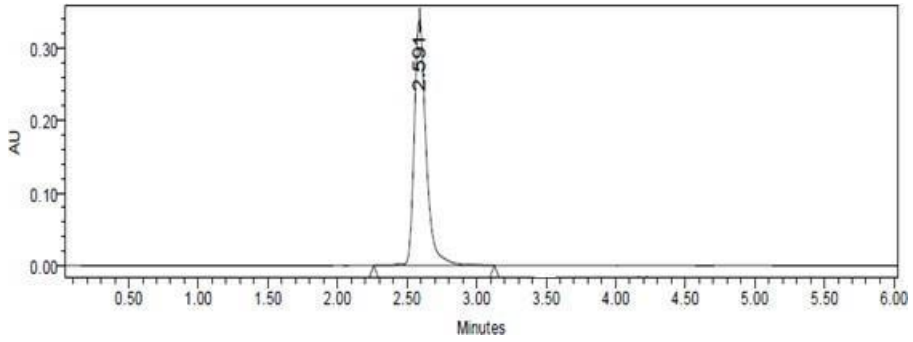


Fig.9: Chromatogram of Assay sample preparation-1

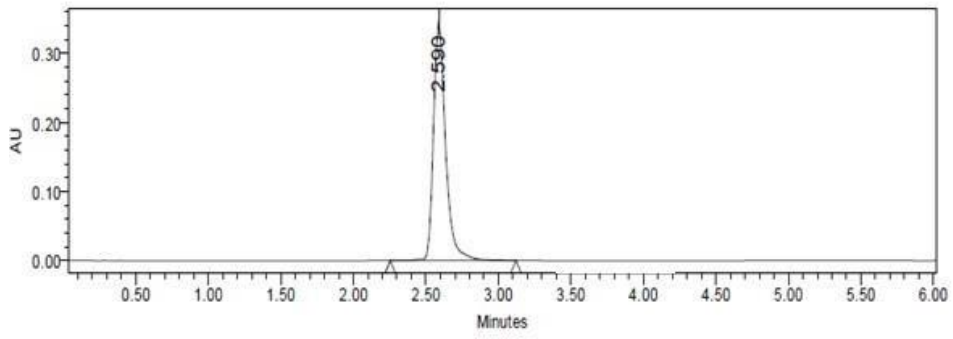


Fig.10: Chromatogram of Assay sample preparation-2

METHOD VALIDATION

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.No.11-13.

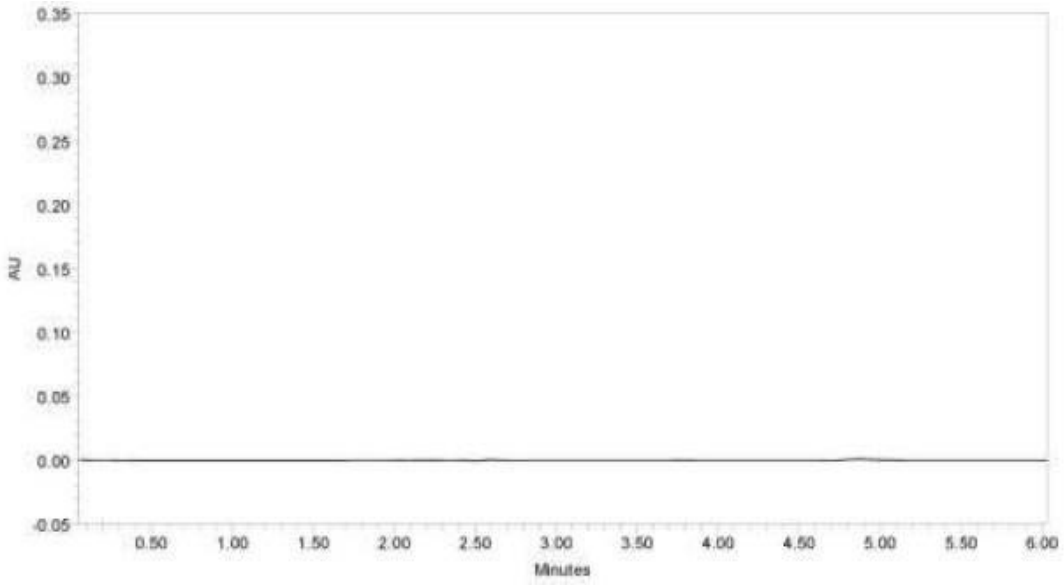


Fig.No.11. Chromatogram showing blank (mobile phase preparation)

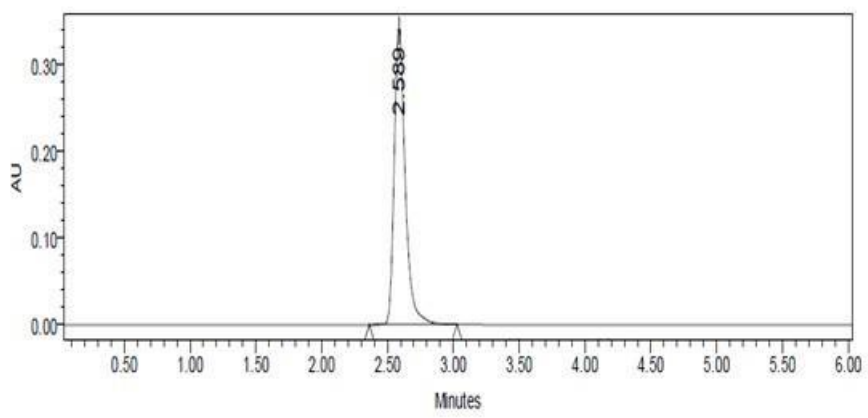


Fig.No.12. Chromatogram showing standard injection

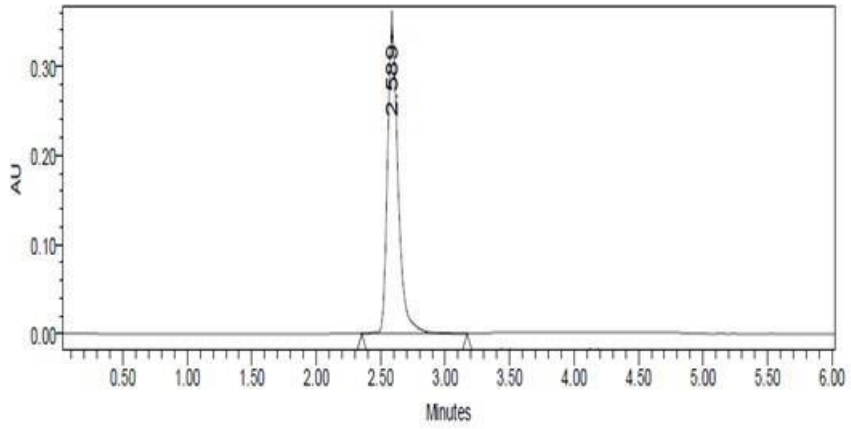


Fig.No.13. Chromatogram showing sample injection

Table 12: Specificity results

S.No	Peak Name	R_t	Area	Height	USP Plate Count	USP Tailing	USP Resolution
1	Dapagliflozin	2.589	2004682	342227	5167	1.3	

Discussion: The specificity test was performed for Dapagliflozin. It was found that there was no interference of impurities in retention time of analytical peak.

Linearity:

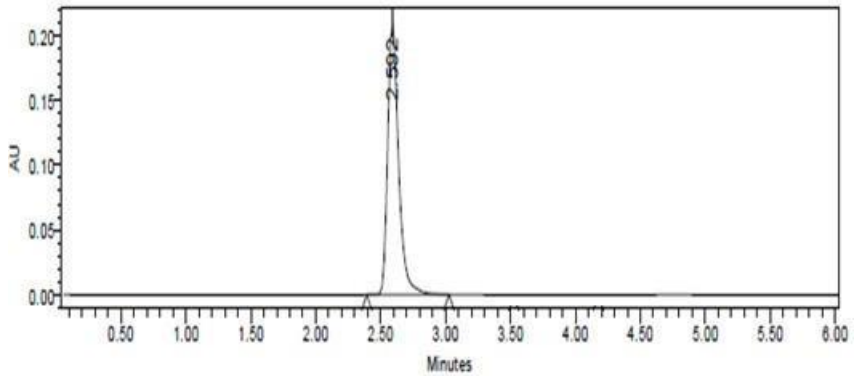


Fig. 14: Chromatogram Of Dapagliflozin Linearity-1

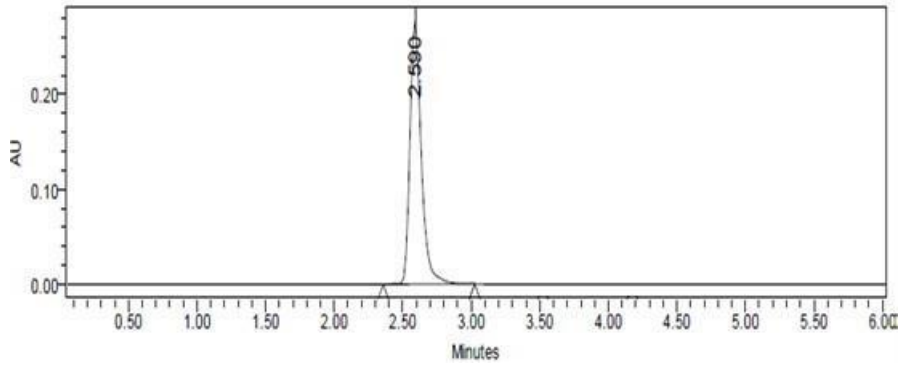


Fig. 15: Chromatogram of Dapagliflozin Linearity-2

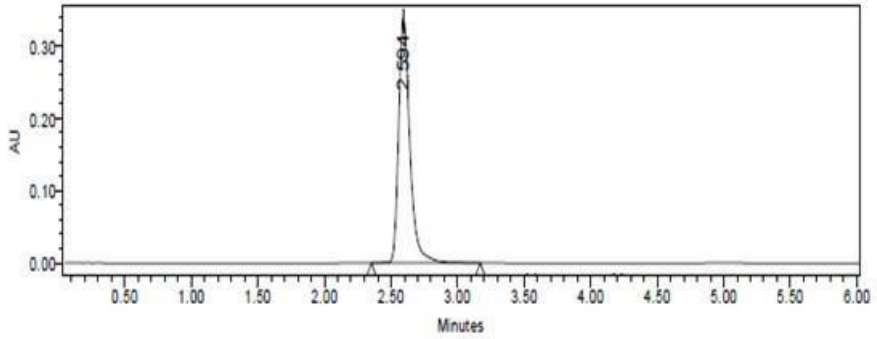


Fig. 16: Chromatogram of Dapagliflozin for Linearity-3

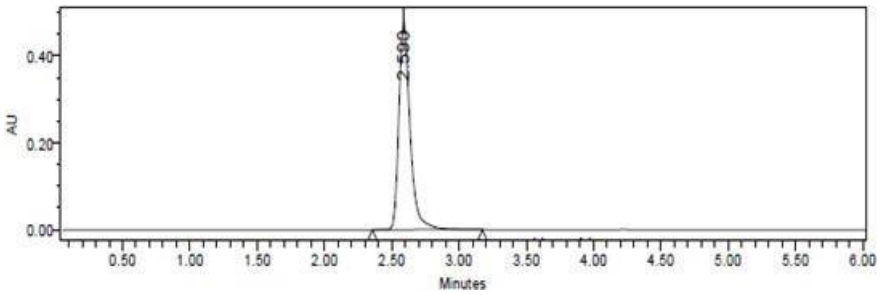


Fig. 17: Chromatogram of Dapagliflozin for Linearity-4

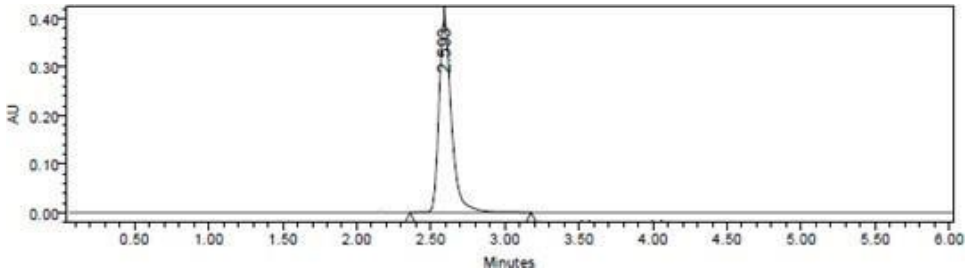


Fig.18: Chromatogram of Dapagliflozin for Linearity-5

Table 13: Linearity of Dapagliflozin

Sample ID	Emitricibine	
	Concentration	Area
20% of operating concentration	20	1224140
40% of operating concentration	30	1595681
60% of operating concentration	**40***	**1992966**
80% of operating concentration	50	2356546
100% of operating concentration	60	2797214
Correlation Coefficient 0.999		

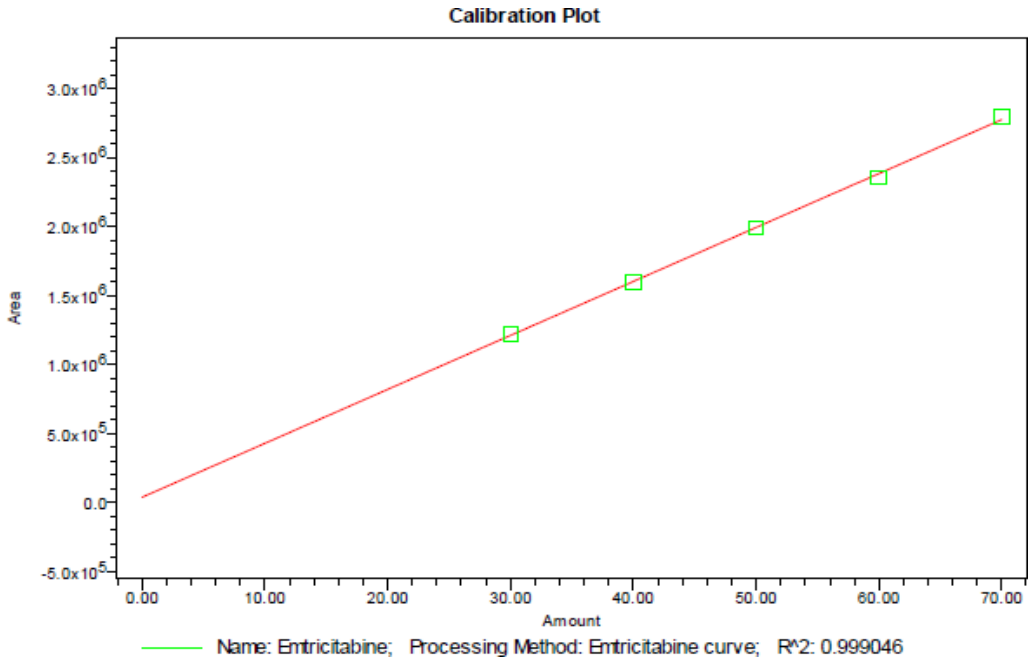


Fig.No.19.Showing calibration graph for Dapagliflozin

Fig.No.20.Showingcalibration graph for zidovudine

Acceptance criteria*:* Correlation Coefficient should be NLT 0.999

Discussion: The relationship between the concentration of Dapagliflozin was linear in the specific range and the correlation coefficient was found to be within limit only. The correlation coefficient of Dapagliflozin was found to be 0.9999 .

Accuracy:

Table-14: Accuracy for Dapagliflozin

Recovery level	Accuracy of Dapagliflozin					Average % Recovery
	Amount taken (mcg/ml)	Area	Average area	Amount recovered (mcg/ml)	Percentage Recovery	
50%	5.05	1011326	1017498.5	101.3927	101.3927	100.599%
	5.05	1015029				
	5.05	1026141				
100%	10	1986534	1987384.8	100.0106	100.0106	
	10	1987425				
	10	1988195				
150%	15	2989367	2992493.4	100.3936	100.3936	
	15	2991556				
	15	2996557				

Accuracy 50%

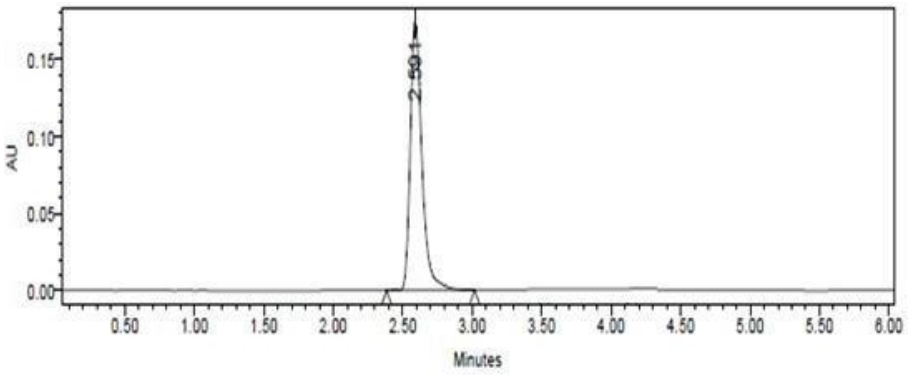


Fig.21: Chromatogram of Dapagliflozin for 50%spiking-1

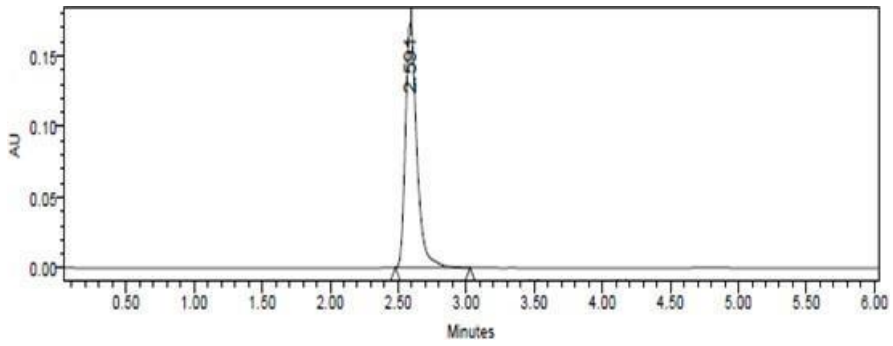


Fig.22: Chromatogram of Dapagliflozin for 50% spiking-2

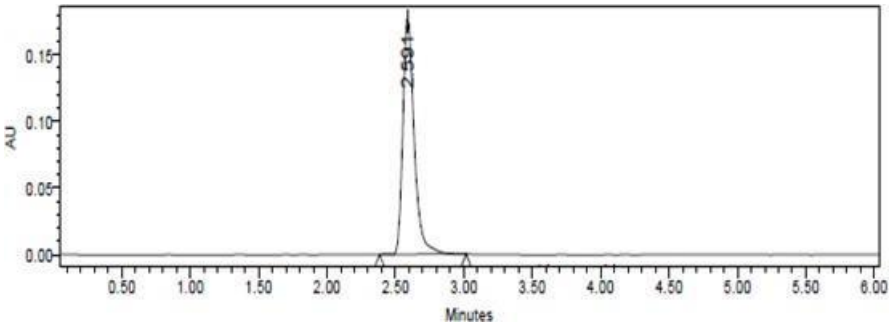


Fig. 23: Chromatogram of Dapagliflozin for 50%spiking-3

Accuracy 100%

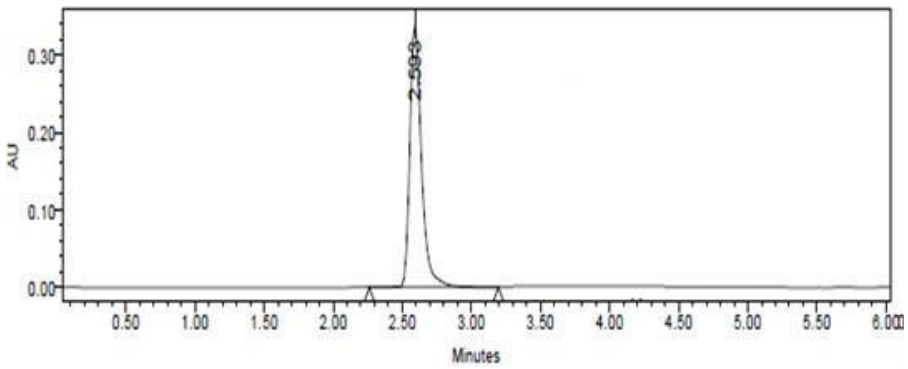


Fig. 24: Chromatogram of Dapagliflozin for 100% spiking-1

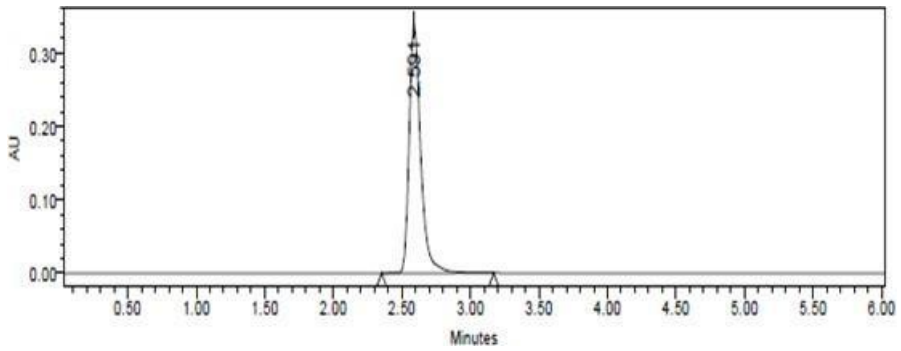


Fig. 25: Chromatogram of Dapagliflozin for 100% spiking-2

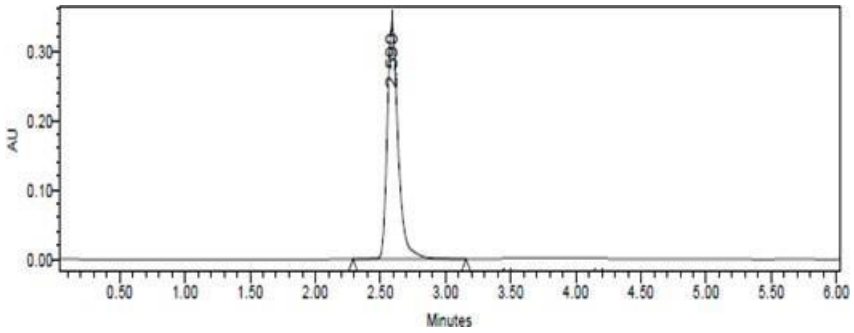


Fig. 26: Chromatogram of Dapagliflozin for 100% spiking-3

Accuracy 150%

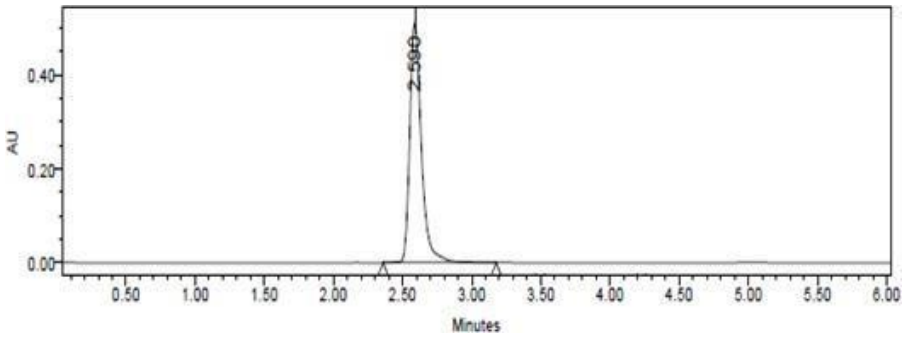


Fig. 27: Chromatogram of Dapagliflozin for 150% spiking-1

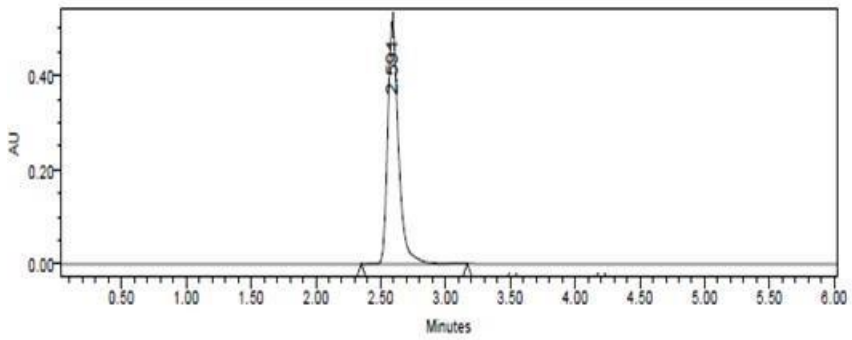


Fig. 28: Chromatogram of Dapagliflozin for 150% spiking-2

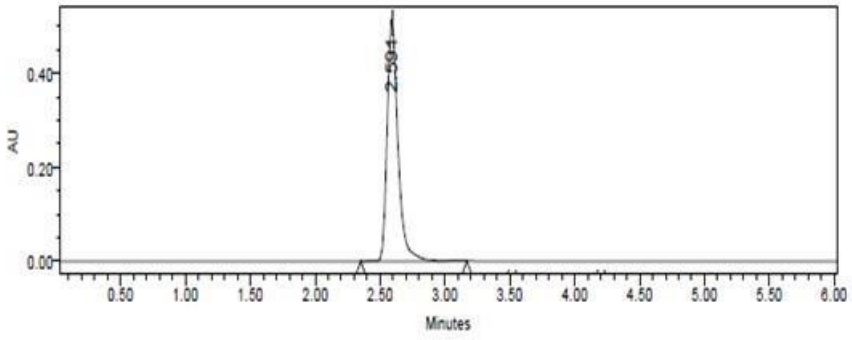


Fig. 29: Chromatogram of Dapagliflozin for 150% spiking-3

Acceptance criteria:

The mean percentage recovery at each spike level should be NLT 98.0% and NMT 102.0%.

Discussion*:*

From the Accuracy table it was found that % Recovery of the drug was found to be in the range of 100.01-101.39 % and 99.66-101.09 % for Dapagliflozin and Zidovudine .This indicates that the method was accurate.

Precision:

- ❖ Repeatability
- ❖ Intermediate Precision

Repeatability

The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

Emitricibine:

Table 16: Method precision Dapagliflozin

S.No	Injection	Peak Name	R_t	Area	Height
1	Injection-1	**Dapagliflozin**	**2.586**	**2010800**	346322
2	Injection-2	**Dapagliflozin**	**2.588**	**2002956**	340800
3	Injection-3	**Dapagliflozin**	**2.590**	**2012800**	**346911**
4	Injection-4	**Dapagliflozin**	**2.590**	**2005243**	**344089**
5	Injection-5	**Dapagliflozin**	**2.591**	**2011092**	**345720**
Average				2008578.1	
Standard Deviation				4237	
%RSD				0.2	

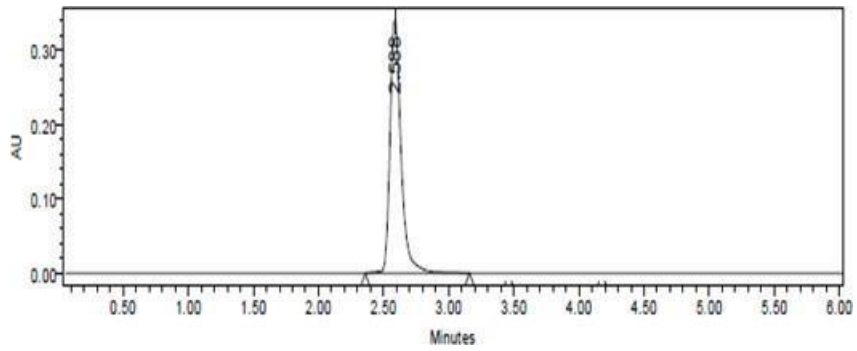


Fig. 30: Chromatogram of Dapagliflozin precision-1

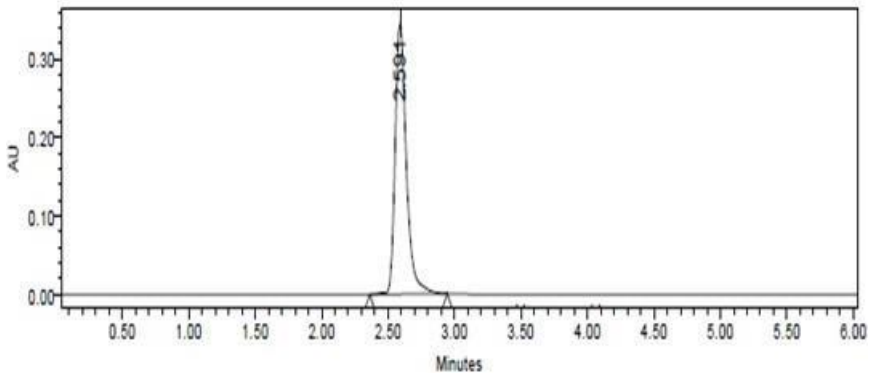


Fig. 31: Chromatogram of Dapagliflozin for precision -2

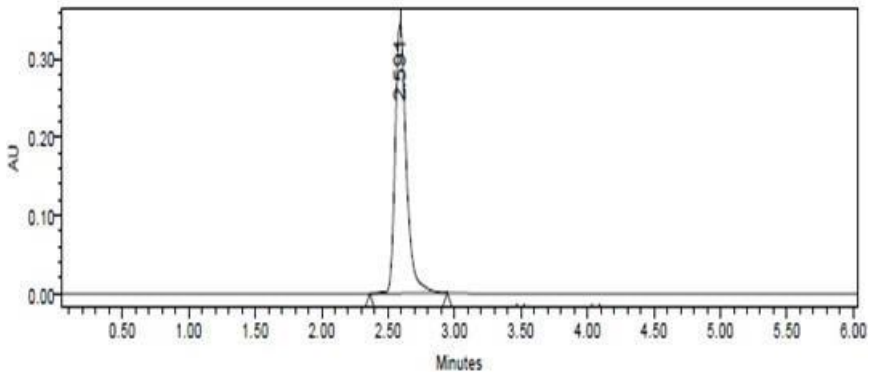


Fig. 32: Chromatogram of Dapagliflozin for precision -3

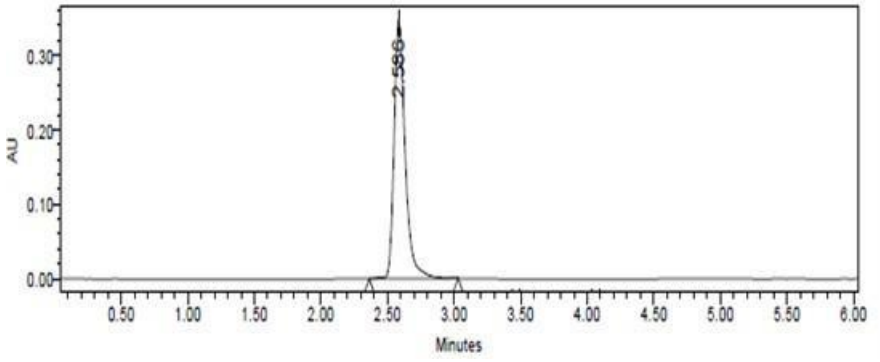


Fig.33: Chromatogram of Dapagliflozin for precision -4

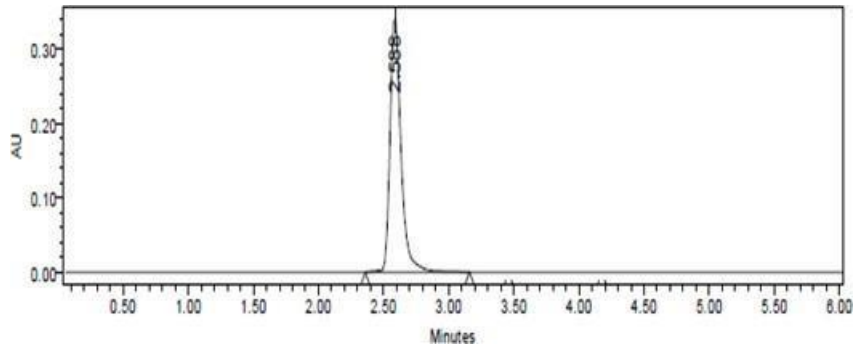


Fig.34: Chromatogram of Dapagliflozin for precision -5

Acceptance Criteria:

% RSD of the sample replicate should not be more than 2.

Discussion: The % RSD value indicates a good degree of precision within the specified range.

Ruggedness

Intermediate Precision/Ruggedness:

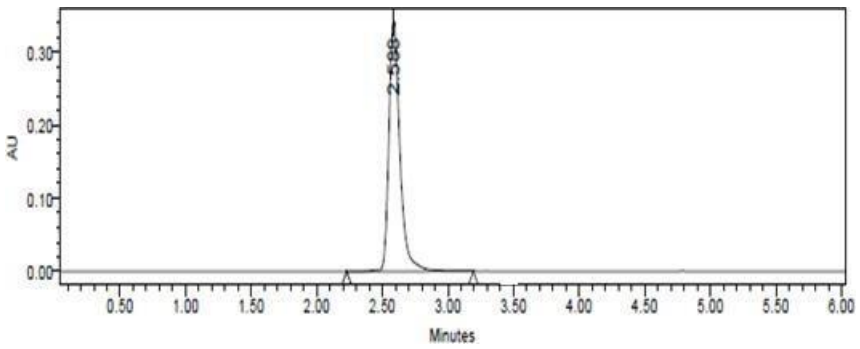


Fig.No.35. Chromatogram showing intermediate precision injection -1

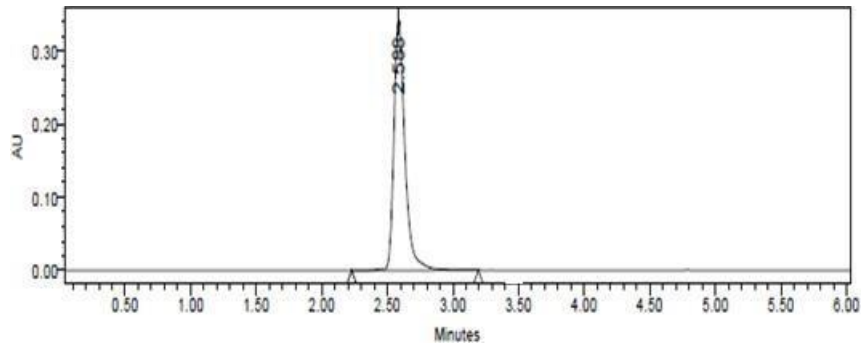


Fig.No.36. Chromatogram showing intermediate precision injection -2

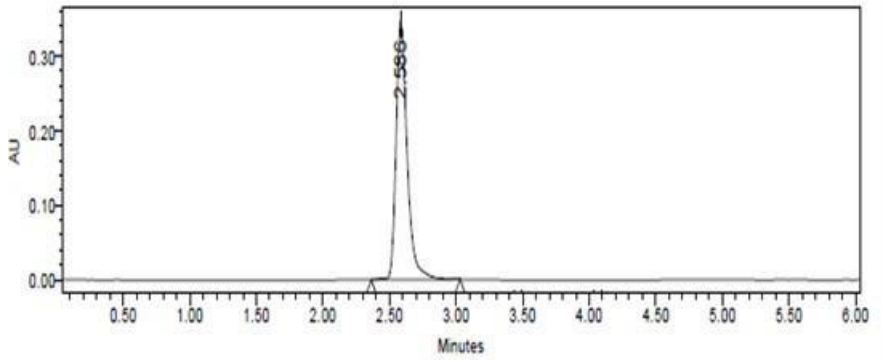


Fig.No.37. Chromatogram showing intermediate precision injection -3

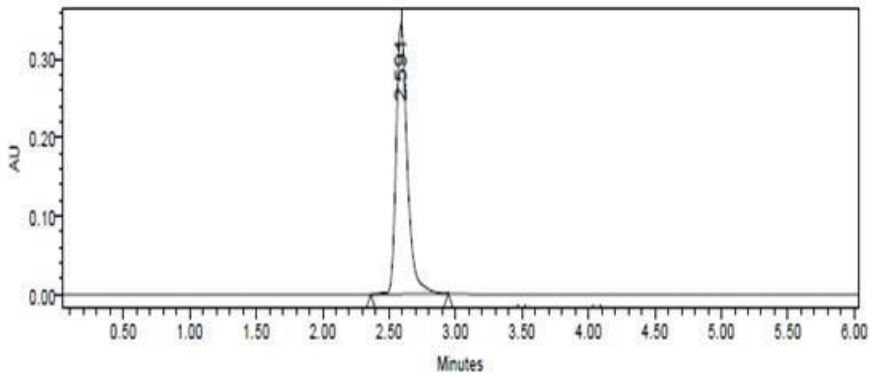


Fig.No.38. Chromatogram showing intermediate precision injection -4

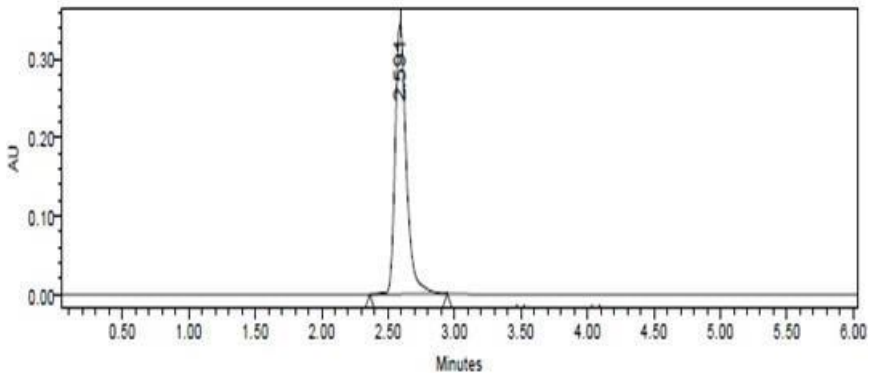


Fig.No.39. Chromatogram showing intermediate precision injection -5

Table 18: The results are summarized Dapagliflozin

Injection	**Area**
Injection-1	2005053
Injection-2	2007362
Injection-3	2007473
Injection-4	2009153
Injection-5	2012800
Average	2008368.1
Standard Deviation	2874.8
%RSD	0.1

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

Robustness:

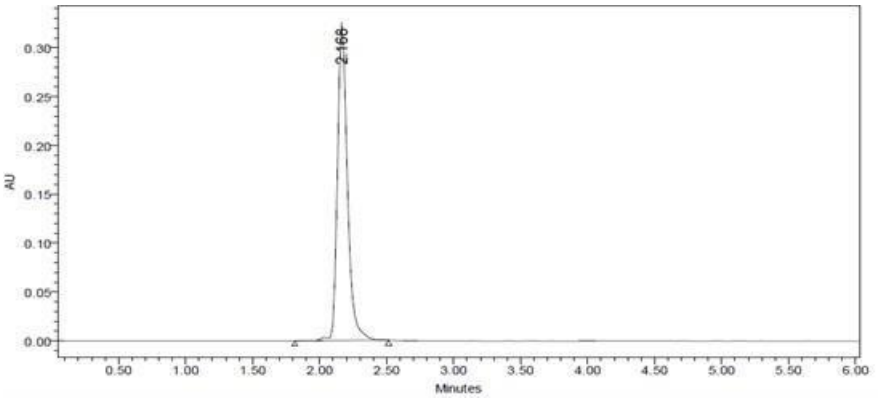


Fig.No.40. Chromatogram showing more flow rate 1.2 ml/min

S.No	Peak Name	R_t	Area	Height	USP Plate Count	USP Tailing	USP Resolution
1	Dapagliflozin	2.168	1676589	321224	4207	1.3	

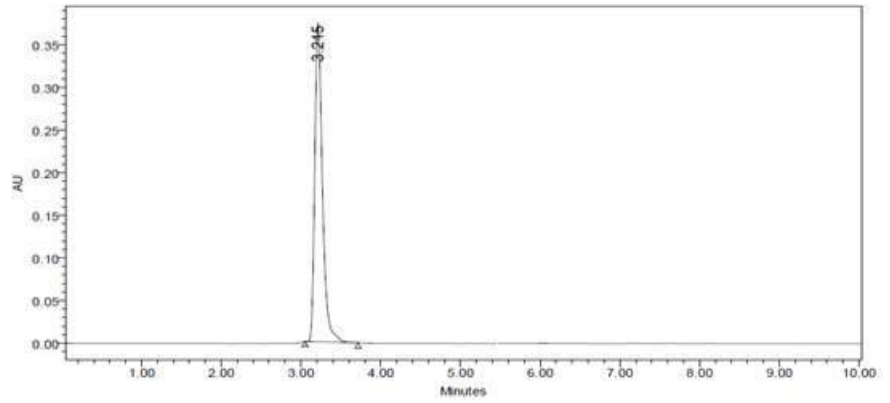


Fig.No.41. Chromatogram showing less flow rate 0.8ml/min

S.No	Peak Name	R_t	Area	Height	USP Plate Count	USP Tailing	USP Resolution
1	Dapagliflozin	3.215	2492492	372153	5752	1.4	

Flow rate results for Dapagliflozin:

Table 20: Flow rate results for Dapagliflozin

S.No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.8	5752	1.4
2	1.0	5026.5	1.3
3	1.2	4476	

Discussion: 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 by change in the flow rate ±0.2ml/min. The method is robust only in less flow condition.

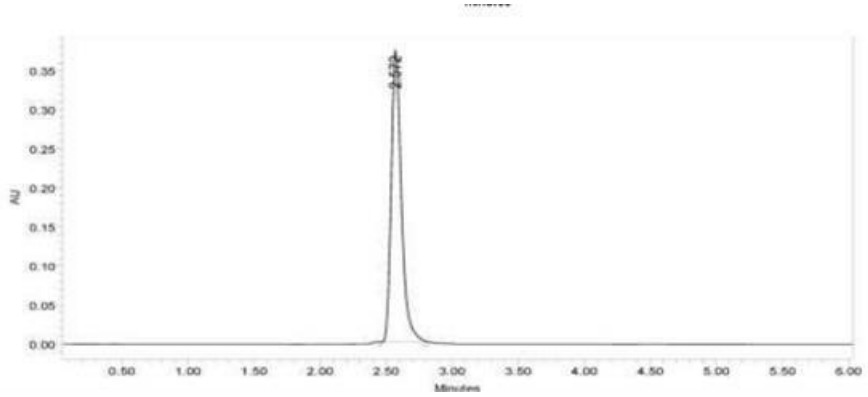


Fig.No.42 Chromatogram showing more organic phase ratio

S.No	Peak Name	R_t	Area	Height	USP Plate Count	USP Tailing	USP Resolution
1	Dapagliflozin	2.572	1979168	371985	4476	1.3	

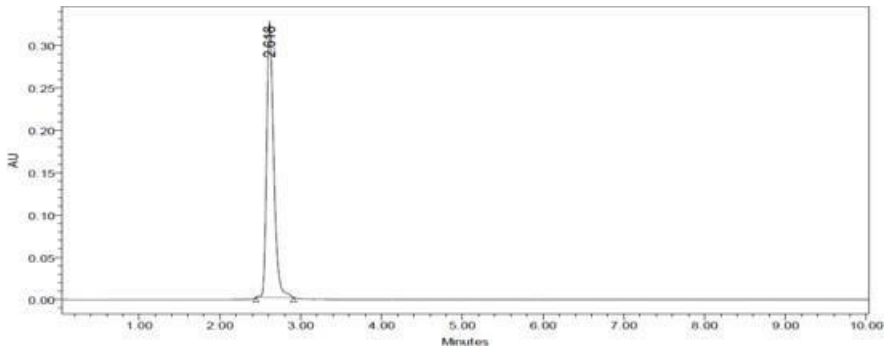


Fig.No.43. Chromatogram showing less organic phase ratio

S.No	Peak Name	R_t	Area	Height	USP Plate Count	USP Tailing	USP Resolution
1	Dapagliflozin	2.618	1951632	324982	4577	1.3	

Table.No.22. Organic phase results for Dapagliflozin

S. No	Change in organic composition in the mobile phase	System suitability results	
		USP Plate Count	USP Tailing
1	5 % less	6498	1.2
2	***Actual**	5026.5	1.3
3	5 % more	6471	1.2

Discussion: On evaluation of the above results, it can be concluded that the variation in±5%. 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 ±5%.

LIMIT OF DETECTION: (for Dapagliflozin)

Table No 24: Results for calibration graph

	Dapagliflozin	
S.No.	Concentration µg/ml	Peak Area
1	30	1224140
2	40	1595681
3	50	1992966
4	60	2356546
5	70	2797214
S.D.	15.81	618048
Slope	39092	

Table .No.25 Showing results for Limit of Detection

Drug name	**Standard deviation(σ)**	**Slope(s)**	**LOD(µg)**
Dapagliflozin	618048	39092	0.001

The LOD was performed for Dapagliflozin was found to be 0.001.

Quantitation limit

Table.No.26. Showing results for Limit of Quantitation

Drug name	**Standard deviation(σ)**	**Slope(s)**	**LOQ(µg)**
Dapagliflozin	618048	39092	0.004

The LOQ was performed for Dapagliflozin was found to be 0.004

System suitability

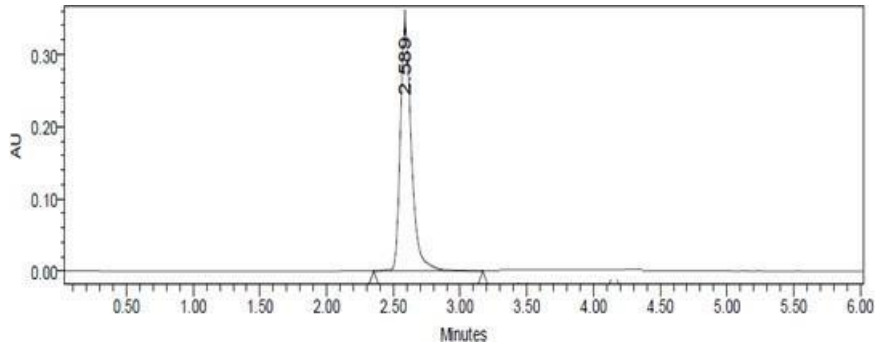


Fig.No.44. Chromatogram showing standard injection

Table.No.27 showing system suitability results

S.No	Peak Name	R_t	Area	Height	USP Plate Count	USP Tailing	USP Resolution
1	Dapagliflozin	2.589	2004682	342227	5167	1.3	

Discussion: The retention time of Dapagliflozin and 3.711mins respectively. The system suitability parameters for Dapagliflozin such as theoretical plates and tailing factor were found to be 5167,1.3 the Resolution were found to be 6.5. The % purity of Dapagliflozin in pharmaceutical dosage form was found to be and 100.59% ,Overall summarized method validation results are tabulated in Table.No.27.

CONCLUSION

In the present study, novel reverse phase High performance liquid chromatography method for simultaneous determination of Dapagliflozin in pharmaceutical dosage form was developed. The developed method was validated for various parameters such as accuracy, precision, ruggedness, linearity, robustness, system suitability, specificity as per ICH guidelines.

A) Method development:

- Trial 6 was optimized for the method development of deliberately changing the chromatographic conditions.
- Column used was Trerosil C_{18} (100 mm x 4.6 mm)5µg.mobile phase composition of buffer: Methanol in the ratio (35:65V/V) and buffer pH 2.5 adjusted with ortho phosphoric acid. The Flow rate set to 0.8ml min^{-1} with UV detection was carried out at 254 nm.

B) Validation Parameters

- The calibration was linear with correlation coefficient 0.999 for Dapagliflozin
- In precision it was found that % RSD is less than 2% which indicates that the Proposed method has good reproducibility.
- The system suitability parameter indicates good resolution of both the peaks > 2.
- From the Accuracy was found that % Recovery of the drug was found to be in the range of % for Dapagliflozin.
- Robustness, When pH was altered RT has no changed significantly, when mobile phase was altered there was no change in the RT significantly.

Table no.28 Overall summarized method validation results

S. No	Parameter	Requirement	Acceptance criteria	
			Dapagliflozin	
1.	**System suitability**	R_t	2.589	
2.		Tailing factor	1.3	NMT 2
3.		Resolution		NLT 2
4.		Plate count	5167	NLT 2000
5.		Assay value	99.77%	100 ± 2.0%
6.	**Accuracy**	% recovery	100.59%	100 ± 2.0%
7.	**Precision**	%RSD	0.2	NMT 2%
8.	**Intermediate precision**	%RSD	0.1	NMT 2%
9.	**Linearity**	Correlation coefficient	0.999	NLT 0.999
10..	**LOD**		0.001	LOQ is three times more than LOQ
11.	**LOQ**		0.004	
12	**Robustness**	More flow	R_t=2.168	Robust even by change in the flow rate ±0.2ml/min
		Less flow	R_t=3.215	
		More organic	R_t=2.572	Robust even by change in the mobile phase ±5%.
		Less organic	R_t=2.618	

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