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Research

Development And Validation Of Analytical Method For Simultaneous Estimation Of Empagliflozin And Metformin By Rp-Hplc

Abhishek Chaturvedi[1], Pankaj Chasta[2], Tanya Sharma[3]*

[1]*Research Scholar, Mewar University, Chittorgarh, Rajasthan, India*
[2]*Associate Professor, Mewar University, Chittorgarh, Rajasthan, India*
[3]*Assistant Professor, Mewar University, Chittorgarh, Rajasthan, India*

*Author for Correspondence: Tanya Sharma
Email: 28taney@gmail.com

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Abstract

The objective of this study was to develop and validate a reliable, efficient, and robust RP-HPLC method for the simultaneous estimation of Empagliflozin and Metformin in pharmaceutical dosage forms. A high-performance liquid chromatography (HPLC) method was developed using a mobile phase consisting of methanol and acetonitrile (75:25 v/v), with an X-Bridge C18 column (4.6 × 150 mm, 5.0 µm) for the separation of both drugs. The chromatographic separation was performed at a flow rate of 1 mL/min and the detection was carried out at 260 nm. The column temperature was maintained at 35ºC, and the injection volume was 10 µL. The total run time was 14 minutes, allowing for efficient separation of Empagliflozin and Metformin within a short analysis time. The method was validated in accordance with ICH guidelines, covering parameters such as accuracy, precision, linearity, specificity, and robustness. The results demonstrated that the method is accurate, precise, and reproducible, with good resolution between the two drugs. The method exhibited excellent linearity with correlation coefficients greater than 0.999 for both Empagliflozin and Metformin. The limit of detection (LOD) and limit of quantification (LOQ) were found to be suitable for routine pharmaceutical analysis. This validated RP-HPLC method can be effectively employed for the quality control of Empagliflozin and Metformin combination products in the pharmaceutical industry, ensuring the integrity and safety of the drug formulation.

Keywords: RP-HPLC, Empagliflozin & Metformin, X-Bridge C18 column, simultaneous estimation, validation.

INTRODUCTION

High Performance Liquid Chromatography (HPLC) was derived from the classical column chromatography and, is one of the most important tools of analytical chemistry today.[1] In the modern pharmaceutical industry, high performance liquid chromatography (HPLC) is the major and integral analytical tool applied in all stages of drug discovery, development, andproduction.[2] HPLC is the method of choice for checking peak purity of new chemical entities, monitoring reaction changes is in synthetic procedures or scale up, evaluating new formulations and carrying out quality control/ assurance of the final drug products.[3] The Goal of HPLC method is to try & separate, quantify the main drug, any reaction impurities, all available synthetic intermediates and any degradants.[4] High Performance Liquid Chromatography is now one of the most powerful tools inanalyticalchemistry.Ithastheabilitytoseparate,identify,andquantifythecompoundsthatare present in any sample that can be dissolved in a liquid. HPLC is the most accurate analytical methods widely used for the quantitative as well as qualitative analysis of drug product and used for determining drug product stability. 5HPLCprinciple is the solution of sample is injected in to a column of porous material (stationary phase) and liquid phase (mobile phase) is pumped at higher pressure through the column. The principle of separation followed is the adsorption of solute on stationary phase based on its affinity towards stationary phase. (Figure-1)

The technique of HPLC has following features.

- High resolution
- Small diameter, Stainless steel, Glass column
- Rapid analysis
- Relatively higher mobile phase pressure
- Controlled flow rate of mobile phase

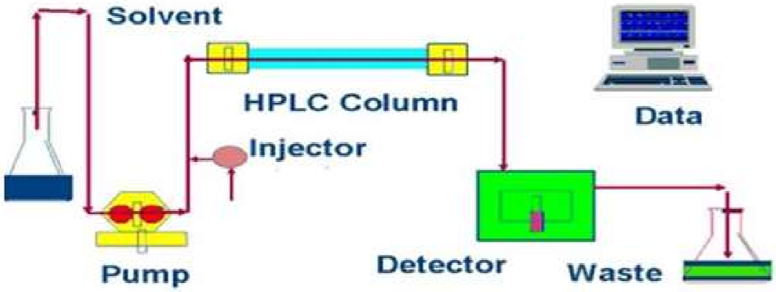

Fig 1: Flow Diagram of HPLC

HPLC system

Typical HPLC system consists of the following main components:

Solvent Reservoirs:

Storage of sufficient amount of HPLC solvents for continuous operation of the system. Could be equipped with an online degassing system and special filters to isolate the solvent from the influence of the environment.

Pump:

This provides the constant and continuous flow of the mobile phase through the system; most modern pumps allow controlled mixing of different solvents from different reservoirs.

Injector:

This allows an introduction (injection) of the analytes mixture into the stream of the mobile phase before it enters the column; most modern injectors are auto samplers, which allow programmed injections of different volumes of samples that are withdrawn from the vials in the auto sampler tray.

Column:

This is the heart of HPLC system; it actually produces a separation of the analytes in the mixture. A column is the place where the mobile phase is in contact with the stationary phase, forming an interface with enormous surface. Most of the chromatography development in recent years went toward the design of many different ways to enhance this interfacial contact.

Detector:

This is a device for continuous registration of specific physical (sometimes chemical) properties of the column effluent. The most common detector used in pharmaceutical analysis is UV (ultraviolet), which allows monitoring and continuous registration of the UV absorbance at a selected wavelength or over a span of wavelengths (diode array detection). Appearance of the analyte in the detector flow-cell causes the change of the absorbance. If the analyte absorbs greater than the background (mobile phase), a positive signal is obtained.

Method optimization

Most of the optimization of HPLC method development has been focused on the optimization of HPLC conditions.[14] The mobile phase and stationary phase compositions need to be considered. Optimization of mobile phase parameters is always considered first as this is much easier and convenient than stationary phase optimization. To minimize the number of trial chromatograms involved, only the parameters that are likely to have a significant effect on selectivity in the optimization must be examined. Primary control variables in the optimization of liquid chromatography (LC) methods are the different components of the mobile phase determining acidity, solvent, gradient, flow rate, temperature, sample amounts, injection volume, and diluents solvent type. This is used to find the desired balance between resolution and analysis time after satisfactory selectivity has been achieved. The parameters involved include column dimensions, column-packing particle size and flowrate. These parameters maybe changed without affecting capacity factor or selectivity.

Method Validation

Validation of an analytical method is the process by which it is established by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical application. Validation is required for any new or amended method to ensure that it is capable of giving reproducible and reliable results, when used by different operators employing the same equipment in the same or different laboratories. The type of validation program required depends entirely on the particular method and its proposed applications.13Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice. Use of equipment that is within specification, working correctly and adequately calibrated is fundamental to the method validation process. Analytical methods need to be validated or revalidated.

- Before their introduction into routine use;
- Whenever the conditions change for which the method has been validated
- Whenever the method is changed Typical parameters recommended by FDA, USP and ICH.

Materials methods

Instruments used

Table 1: Instruments used

S.No	Instruments And Glass wares	Model
1	HPLC	WATERS Alliance 2695 separation module, Software: Empower 2, 996 PDA detector.
2	pH meter	Lab India
3	Weighing machine	Sartorius
4	Volumetric flasks	Borosil
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil
7	Digital ultra sonicator	Labman

Chemicals used

Table 2: chemicals used

S.No	Chemical	Brand names
1	Empagliflozin	Procured from Sun pharma, provided by Sura labs
2	Metformin	Procured from Sun pharma, provided by Sura labs
3	Water and Methanol for HPLC	LICHROSOLV (MERCK)
4	Acetonitrile for HPLC	Merck

HPLC method development

Trails

Preparation of standard solution:

Accurately weigh and transfer 10 mg of Empagliflozin and Metformin working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.

Further pipette 2.25ml of the above Empagliflozinand 0.45ml of the Metformin stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure

Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

Mobile Phase Optimization:

Initially the mobile phase tried was Methanol: Water, Acetonitrile: Water with varying proportions. Finally, the mobile phase was optimized to Methanol and water in proportion 75:25 v/v respectively.

Optimization of Column:

The method was performed with various columns like C18 column, Phenomenex Luna C18, Xterra. X- bridge C18 (4.6 x 150mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

Validation

Preparation of mobile phase

Preparation of mobile phase

Accurately measured 750ml (75%) of Methanol and 250ml of Water (25%) were mixed and degassed in a digital ultrasonicater for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:

The Mobile phase was used as the diluent.

Validation parameters

System suitability

Accurately weigh and transfer 10 mg of Empagliflozin and Metformin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 2.25ml of the above Empagliflozin and 0.45ml of the Metformin stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure:

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.

Specificity study of drug

Preparation of Standard Solution

Accurately weigh and transfer 10 mg of Empagliflozin and Metformin working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 2.25ml of the above Empagliflozin and 0.45ml of the Metforminstock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Preparation of Sample Solution

Take average weight of the Tablet and crush in a mortar by using pestle and weight 10 mg equivalent weight of Empagliflozin and Metformin sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette 2.25ml of Empagliflozin and Metformin above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure

Inject the three replicate injections of standard and sample solutions and calculate the assay by using formula:

%ASSAY =

$$\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

Preparation of drugsolutions for linearity

Accurately weigh and transfer 10 mg of Empagliflozin and Metformin working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (75ppm of Empagliflozinand 15ppm of Metformin):
Pipette out 0.75ml of the Empagliflozin and 0.15ml of the Metformin from the above stock solutions in to a 10ml of volumetric flask and dilute the solution. Performes sonication for 10minutes.

Preparation of Level – II (150ppm of Empagliflozinand 30ppm of Metformin):
Pipette out 1.5ml of the Empagliflozin and 0.3ml of the Metformin from the above stock solutions in to a 10ml of volumetric flask and dilute the solution. Performes sonication for 10minutes.

Preparation of Level – III (225ppm of Empagliflozinand 45ppm of Metformin):
Pipette out 2.25ml of the Empagliflozin and 0.45ml of the Metformin from the above stock solutions in to a 10ml of volumetric flask and dilute the solution. Performes sonication for 10 minutes.

Preparation of Level – IV (300ppm of Empagliflozinand 60ppm of Metformin):
Pipette out 3.0ml of the Empagliflozin and 0.6ml of the Metforminfrom the above stock solutions in to a 10ml of volumetric flask and dilute the solution. Performes sonication for 10minutes.

Preparation of Level – V (375ppm of Empagliflozinand 75ppm of Metformin):
Pipette out 3.75ml of the Empagliflozin and 0.75ml of the Metformin from the above stock solutions in to a 10ml of volumetric flask and dilute the solution. Performs sonication for 10 minutes.

Procedure
Inject each level into the chromatographic system and measure the peak area.
Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

Precision
Repeatability
Preparation of Empagliflozinand Metformin Product Solution for Precision
Accurately weigh and transfer 10 mg of Empagliflozin and Metformin working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 2.25ml of the above Empagliflozin and 0.45ml of the Metformin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.
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.

Intermediate precision
To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days by maintaining same conditions.
Procedure

Day 1
The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Day 2
The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Accuracy
For preparation of 50% Standard stock solution:
Accurately weigh and transfer 10 mg of Empagliflozin and Metformin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 1.12ml of the above Empagliflozin and 0.225ml of the Metformin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

For preparation of 100% Standard stock solution

Accurately weigh and transfer 10 mg of Empagliflozin and Metformin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 2.25ml of the above Empagliflozin and 0.45ml of the Metformin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

For preparation of 150% Standard stock solution

Accurately weigh and transfer 10 mg of Empagliflozin and Metformin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 3.37ml of the above Empagliflozin and 0.675ml of the Metformin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Procedure

Inject the Three replicate injections of individual concentrations (50%, 100%, 150%) were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for Empagliflozin and Metformin and calculate the individual recovery and mean recovery values.

Robustness

The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results.

For preparation of Standard solution:

Accurately weigh and transfer 10 mg of Empagliflozin and Metformin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 2.25ml of the above Empagliflozin and 0.45ml of the Metformin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Effect of Variation of flow conditions

The sample was analyzed at 0.9 ml/min and 1.1 ml/min instead of 1ml/min, remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded

Effect of Variation of mobile phase organic composition

The sample was analyzed by variation of mobile phase i.e. Methanol: Water was taken in the ratio and 60:40, 70:30 instead of 75:25, remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded.

Optimized Chromatogram (Standard)

Mobile phase : Methanol: ACN (75:25v/v)
Column : X- bridge C18 (4.6×150mm, 5.0 µm)
Flow rate : 1 ml/min
Wavelength : 260 nm
Column temp : 35ºC
Injection Volume : 10 µl
Run time : 14 minutes

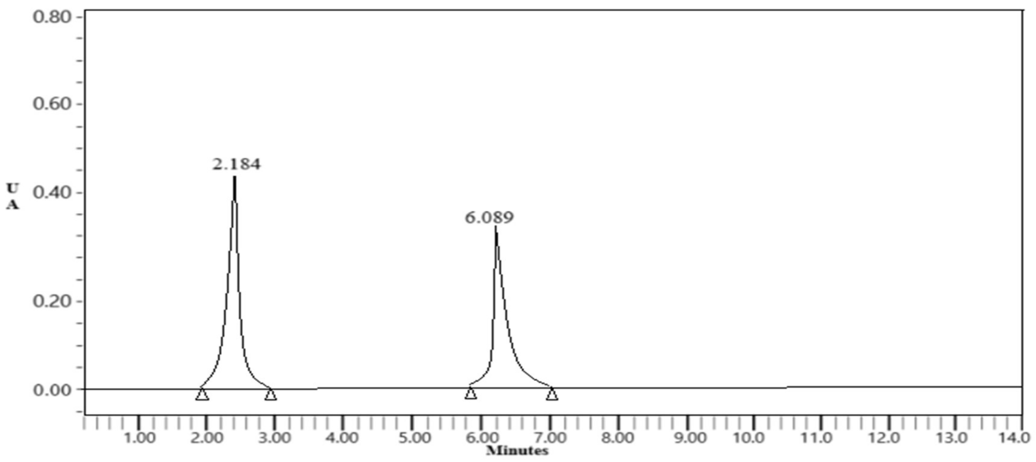

Fig 2: Optimized Chromatogram

Table 3: Peak Results for Optimized Chromatogram

S. No.	Peak name	R_t	Area	Height	USP Resolution	USP Tailing	USP plate count
1	Empagliflozin	2.184	3425413	567933		1.0	5565.5
2	Metformin	6.089	1629854	517733	2.5	1.1	5355.2

From the above chromatogram it was observed that the Empagliflozin and Metformin peaks are well separated and they shows proper retention time, resolution, peak tail and plate count. So it's optimized trial.

Results for Robustness

Empagliflozin

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Flow rate of 1.0 mL/min	3425413	2.185	5568.2	1.0
Flow rate of 0.9 mL/min	3425282	2.184	5922.2	1.2
Flow rate of 1.1 mL/min	3517879	2.190	5868.8	1.2
Less aqueous phase	3175485	2.191	5836.2	1.2
More aqueous phase	3365431	2.189	5282.6	1.1

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

Metformin

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Flow rate of 1.0 mL/min	2029854	6.068	5359.2	1.1
Flow rate of 0.9 mL/min	1738319	6.089	5999.1	1.2
Flow rate of 1.1 mL/min	1638304	6.092	5989.2	1.1
Less aqueous phase	1973724	6.090	5387.2	1.1
More aqueous phase	2102838	6.091	5938.1	1.1

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

DISCUSSIONS

Trails

Trail 1

Mobilephase :Methanol:Orthophosphoricacid (80:20%v/v)

Column :ODSC18(4.6×150mm, 5µmparticlesize)Make;waters
Flow rate :0.6ml/min
Wavelength :260nm
Column temp :28ºC Injection
Volume :10 µl
Runtime :10minutes

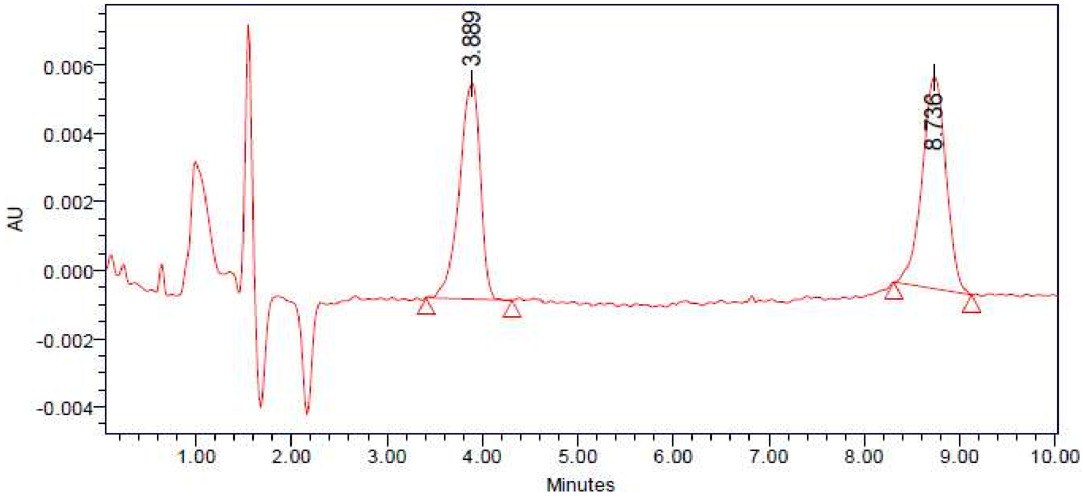

Fig 3: Chromatogram forTrail 1

Table 4: Peak Results for Trail 1

S.No	Peak Name	Rt	Area	Height	USP Resolution	USP Tailing	USPPlate count
1	Empagliflozin	3.889	96377	6320		0.81	1642
2	Metformin	8.736	107991	6192	11.26	0.94	6110

This trial shows improper base line in the chromatogram, so more trials were required for obtaining good peaks.

Trail2

Mobile phase : Phosphoricacid(pH3):Acetonitrile(65%-35%v/v)
Column : Zodiac C18 (4.6×250mm) 5µ
Flowrate : 0.7ml/min
Wavelength : 260nm
Column temp : 30ºC Injection
Volume : 10µl
Runtime : 7minutes

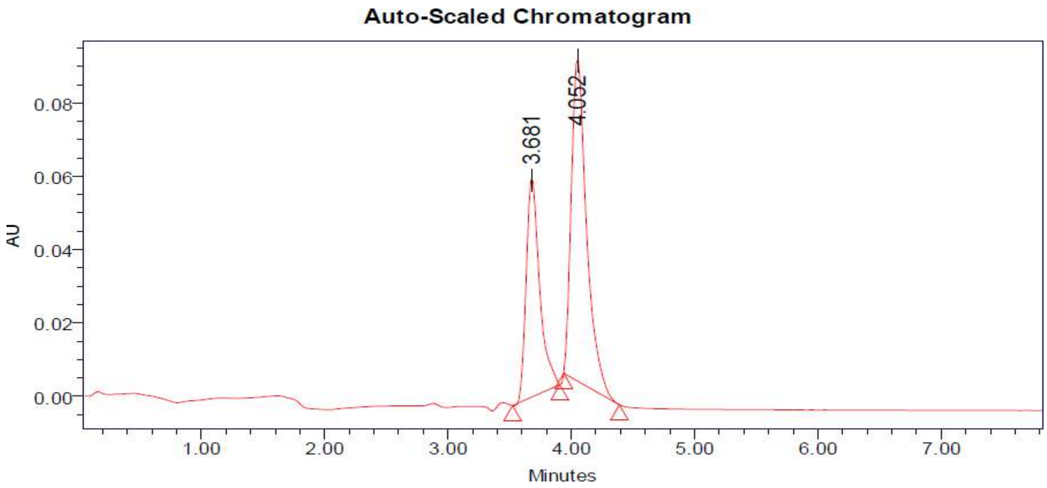

Fig 4: Chromatogram for Trail 2

Table 5: Peak Results for Trail 2

S. No	Peak name	Rt	Area	Height	USP Resolution	USP Tailing	USPplate count
1	Empagliflozin	3.681	453159	59898		1.42	5843
2	Metformin	4.052	755096	87984	1.72	1.64	5346

This trial show very less plate count, and show improper baseline in the chromatogram, so more trials were required for obtaining good peaks.

Trail 3

Mobile phase : Methanol: ACN (70:30% v/v)
Column : ZodiacC18(4.6×250mm5μm)
Flow rate : 0.8ml/min
Wavelength : 260nm
Column temp : 30ºC Injection
Volume : 10μl
Runtime : 10minutes

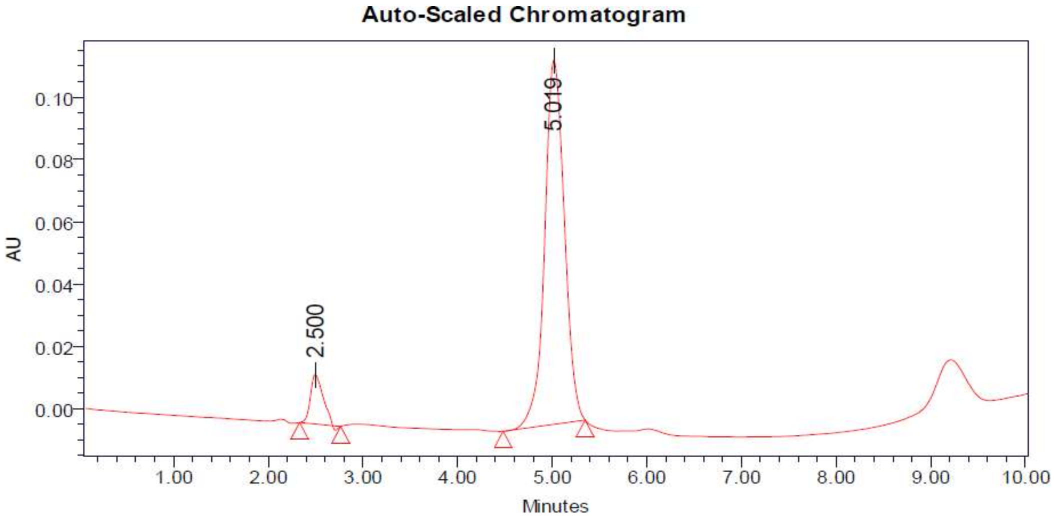

Fig 5: Chromatogram for Trail3

Table 6: Peak Results for Trail 3

S. No	Peakname	Rt	Area	Height	USP Resolution	USP Tailing	USPplatecount
1	Empagliflozin	2.500	152465	15939		1.24	1465
2	Metformin	5.019	1693743	117063	7.85	0.97	1202

This trial shows improper baseline and show less plate count in the chromatogram, so more trials were required for obtaining peaks.

Optimized Chromatogram (Standard)

Mobile phase	:Methanol:ACN(75:25v/v)
Column	:X-bridgeC18(4.6×150mm,5.0µm)
Flow rate	:1 ml/min
Wavelength	:260nm
Column temp	:35ºC InjectionVolume :10µl
Runtime	:14minutes

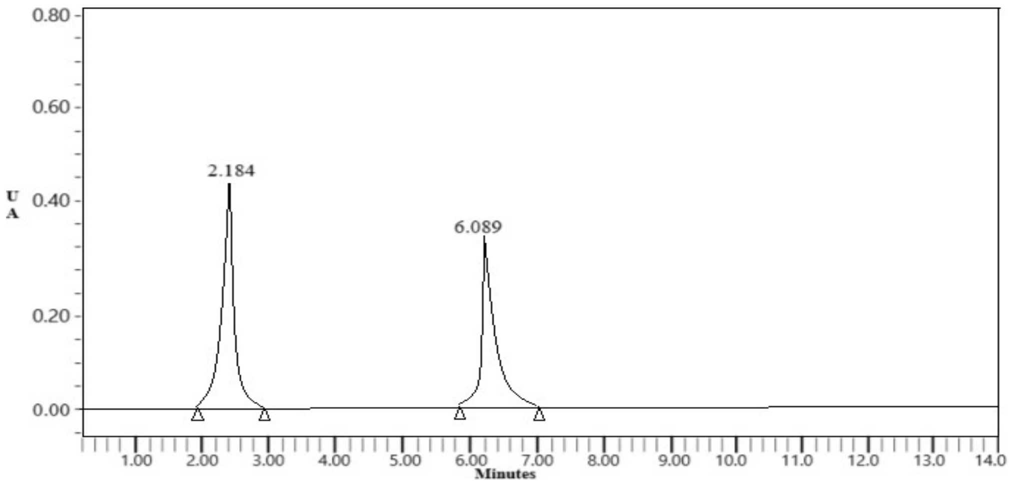

Fig 6: Optimized Chromatogram

Table 7: Peak Results for Optimized Chromatogram

S.No.	Peak name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count
1	Empagliflozin	2.184	3425413	567933		1.0	5565.5
2	Metformin	6.089	1629854	517733	2.5	1.1	5355.2

From the above chromatogram it was observed that the Empagliflozin and Metformin peaks are well separated and they shows proper retention time, resolution, peak tail and plate count. So it's optimized trial.

CONCLUSION

The developed RP-HPLC method proved to be reliable, efficient, and suitable for the simultaneous estimation of Empagliflozin and Metformin in pharmaceutical dosage forms. The validation results confirmed that the method meets the required criteria for accuracy, precision, linearity, and robustness, making it ideal for routine quality control in pharmaceutical settings. The method also demonstrated good specificity, with no interference from excipients, making it highly applicable for the analysis of Empagliflozin and Metformin combination products. Given its high efficiency, short run time, and reliability, the method can be successfully implemented in pharmaceutical laboratories for routine analysis, ensuring the quality and safety of the combination drug formulations.

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