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RESEARCH ARTICLE
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DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR DETERMINATION OF ERTUGLIFLOZIN IMPURITY PROFILE

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ABSTRACT
Several procedures were conducted to develop and validate an analytical method and impurity profile for new antidiabetic drug. In method development, reverse-phase high-performance liquid chromatography (RP-HPLC) employed a Jasco, Finepack C18 column (250 × 4.6 mm, 5 µm). The selected mobile phase consists of mobile phase A: (0.1% OPA+0.1%TFA) and mobile phase B: Mix acetonitrile and Methanol in the ratio of 40:60 (v/v), both were mixed in the proportion of 85:15, with a flow rate of 1.0 mL/min. Detection occurred at 260 nm. System appropriateness was validated with a tailing factor below 1.5 for principal peaks, theoretical plates exceeding 2000, and relative standard deviations (RSD) under 2.0% for both intra-day and inter-day precision. The validation results indicated an accuracy with recovery percentages ranging from 98.4 to 100.2. Linearity was assessed by linear regression analysis. Linearity was observed within the range of 50-150 µg/mL, with correlation coefficients of 0.999. Additionally, solution stability was confirmed, exhibiting less than a 2.0% variation in peak area over 48 hours at room temperature. The chromatographic analysis showed that Impurity I had a retention time of 5.71 minutes with a purity of 99.54%, while Impurity II exhibited a retention time of 13.47 minutes with a purity of 99.25%. whereas, the chromatographic analysis of Impurity III revealed that it had a retention time of 22.70 minutes, with a purity of 88.74%.

KEYWORD: Analytical Method Development, Ertugliflozin, Impurity Profile, RP-HPLC, System Suitability

INTRODUCTION
Ertugliflozin is a selective inhibitor of sodium-glucose co-transporter 2 (SGLT2) utilised in the management of type 2 diabetes mellitus. Ertugliflozin, an SGLT2 inhibitor, functions by inhibiting glucose reabsorption in the kidneys, facilitating the elimination of surplus glucose via urine and aiding in the reduction of blood sugar levels. It is frequently used with other antidiabetic medicines to improve therapeutic efficacy and tackle various facets of diabetes care. [1-3] In the pharmaceutical sector, guaranteeing the quality and efficacy of drug products is essential. This entails not only the precise measurement of the drug's concentration but also the identification and quantification of any impurities that may impact the drug's safety and efficacy. Consequently, the establishment and verification of a reliable analytical method for Ertugliflozin in its combination dose form are crucial for fulfilling regulatory standards and guaranteeing the drug's quality. [4-7]
The formulation of an analytical method for Ertugliflozin entails creating a procedure that can consistently and precisely quantify the medication inside a complicated mixture, such as a mixed

dosage form. High Performance Liquid Chromatography (HPLC) is frequently utilised for this purpose owing to its exceptional precision, sensitivity, and capacity to isolate the medication from other formulation components. [8-10]

Upon development, the analytical method must undergo comprehensive validation to ascertain its reliability and precision. Validation entails evaluating multiple parameters, such as specificity, linearity, accuracy, precision, and the detection and quantification limitations. This guarantees that the procedure yields reliable and precise outcomes under diverse settings and complies with the requirements established by regulatory bodies. [11-13]

Alongside measuring Ertugliflozin, it is essential to characterise any potential contaminants in the combination dosage form. Impurity profiling ensures the drug's safety for use and adherence to regulatory norms. Methods like HPLC can identify and quantify these contaminants. [14-15]

Through the development and validation of an analytical method and the profile of impurities, the pharmaceutical industry may ascertain that Ertugliflozin in its combination dosage form adheres to quality and safety criteria, eventually enhancing patient outcomes through effective and dependable therapy.A study of the current literature reveals that few High Performance Liquid Chromatography (HPLC) methods have been documented for the quantification of Ertugliflozin, with only a handful of publications focussing on its analysis in bulk and dose forms.

HPLC has proven to be an essential instrument for the regular analysis and separation of pharmaceuticals, whether in isolation or in conjunction with other chemicals or degradation products, and is extensively utilised in pharmaceutical formulations. Due to the lack of comprehensive techniques for Ertugliflozin testing, it is essential to establish a novel, straightforward, and dependable isocratic Reverse Phase HPLC (RP-HPLC) approach. This method seeks to deliver precise and reproducible quantification of Ertugliflozin in both bulk and pharmaceutical preparations. The proposed approach has been validated in accordance with International Conference on Harmonisation (ICH) requirements, confirming its precision, accuracy, linearity, and reproducibility. [16-17]

MATERIALS AND METHODS

MATERIALS

The study utilised Acetonitrile, Methanol, and DMSO, all produced by Merck Life Science. Furthermore, Potassium Dihydrogen Orthophosphate is procured from S D Fine Chem. Ltd, Mumbai. The research utilised various instruments, including a Jasco 4600 Series Quaternary HPLC and a Jasco Double Beam UV-Visible Spectrophotometer (V-630). A Chemiline pH Meter (101) was employed for pH measurements, and a Shimadzu Balance (AY-120) was utilised for accurate weighing. A Rolex Sonicator (UCB-40) was employed for sonication activities.

METHODS

Pre- Analysis Study of Drug

1. Melting Point Determination [21]

The melting point of Ertugliflozin was measured utilising the capillary tube method. A Thiele tube filled with a liquid paraffin solution contained a small quantity of pure medication, which was introduced into a capillary tube sealed at one end by flame. The sample-filled capillary is secured with thread to the thermometer and suspended in Thiele's tube, then heated until the drug powder melts. The melting point of the pure medication powder was recorded.

2. Identification of standard drug by FTIR Spectroscopy [22]

The drug compatibility study was performed by FTIR technique. The Ertugliflozin samples were scanned over wave number range of 500-4000 cm-1 with diffraction reflectance scanning technique.

3. UV Spectroscopy: (Determination of λ maximum) [23]

A precisely measured amount of the pure medicinal powder Ertugliflozin was placed into a 100 ml calibrated volumetric flask. A minimal quantity of methanol was incorporated, and the solution was subjected to sonication for 30 minutes utilising an Equitron sonicator (EquitronEquipments Pvt. Ltd.). The volume was subsequently adjusted to the mark with methanol. The resultant solution was filtered using Whatman filter paper to yield a clear solution, which constituted the 100 ppm standard stock solution. The UV spectra of this stock solution was obtained within the wavelength range of 200-400 nm, and the λ max of the medication was ascertained utilising methanol as the blank.

4. Calibration curve [24]

A calibration curve for Ertugliflozin in methanol was established by preparing a standard stock solution, which involved precisely weighing a designated amount of pure Ertugliflozin powder with a Shimadzu BL22OH balance. The measured medication was placed into a 100 ml calibrated volumetric flask, and a small quantity of methanol was subsequently added. The amalgamation was subjected to sonication for 30 minutes utilising an Equitron sonicator (EquitronEquipments Pvt. Ltd.). Following sonication, the solution volume was adjusted to 100 ml with methanol. The resultant solution was subsequently filtered using Whatman filter paper to yield a clean 100 ppm standard stock solution of Ertugliflozin.

Method Development by RP-HPLC

Trial	Chromatographic conditions	Observation	Conclusion
Experiment 1 The eluent employed was a mobile phase, and detection was performed with a UV detector. The analysis was conducted at an optimised flow rate of 1 mL/min and a detection wavelength of 260 nm. The cumulative duration for each analysis was 45 minutes.	JASCO system with model PU 4180 RHPLC pump, Rheodyne sample injection port (20 µl), JASCO UV-4075 UV-VIS detector, and ChromNAV CFR chromatography software (version 2.0)	Placebo interference was detected at Ertugliflozin impurity-II at a retention time of 21.62 minutes. The assay yielded a result of 99.53%.	To eliminate placebo interference, it is necessary to optimise the procedure by adjusting the column temperature from 40°C to 45°C.
Trial 2 All chromatographic conditions are identical, except for Variation in column temperature in the aforementioned technique		During the partial verification, it was noted that with prolonged sequence runs, peak tailing increased and consistency in retention times of column-to-column impurities was absent.	It is necessary to modify the chromatographic parameters and uniformity.
Trial 3 Modified chromatographic		There was no interference at the retention time of the	This experiment has been finalised and assessed for recovery,

parameters. Preparation of Mobile Phase-A: Complete buffer as mobile Phase-A. Preparation of Mobile Phase-B: Acetonitrile and methanol were combined in an 85:15 (v/v) ratio and subjected to sonication for degassing.		analyte peak and contaminants; the limit of quantification (LOQ) was established according to ICH guidelines, and the signal-to-noise (S/N) ratio exceeded 10. Assay yielded 92.90%, tailing factor measured at 1.1, and theoretical plates calculated as 27,308.	specificity, and system suitability, confirming that all parameters meet the acceptance criteria. No interference is observed in the blank and placebo during the retention period of the analyte peak. All contaminants are segregated from the analyte peak. Therefore, the same procedure is employed for partial validation.

OPTIMIZED METHOD

Mobile phase A: (0.1% OPA+0.1%TFA)

Dilute 1 mL of ortho phosphoric acid and 1 mL of trifluoro acetic acid to 1000 mL of HPLC grade water, sonicate to dissolve and filter through 0.45µ membrane filter.

Mobile phase B: Mix acetonitrile and Methanol in the ratio of 40:60 (v/v) respectively.

Diluent: Combine water with methanol in a volumetric ratio of 85:15.

The chromatographic analysis was conducted under the specified conditions: The stationary phase utilised was a Jasco, Finepack C18 column of 250 mm × 4.6 mm with a particle size of 5 µm. The flow rate was sustained at 1.1 mL/min, with detection occurring at a wavelength of 260 nm. The overall duration was 45 minutes, with the column temperature maintained at 35°C. Injections of 20 µL were administered, and the auto sampler temperature was maintained at 25°C to guarantee uniform and precise sample management.

Preparation of solutions [24]

The blank solution was formulated utilising the diluent. To prepare the Ertugliflozin standard stock solution (1000 ppm), 25 mg of Ertugliflozin was accurately weighed and placed into a 25 mL volumetric flask, dissolved in 15 mL of diluent using sonication, then diluted to the mark and vortexed for 2 minutes. To prepare the assay standard solution, 4 mL of the stock solution was transferred into a 25 mL flask, diluted with a diluent, and vortexed.

To prepare the RS standard solution (0.25 ppm), 2 mL of a 150 ppm standard solution was diluted to a final volume of 50 mL. Subsequently, 1 mL of this dilution was transferred to a 25 mL flask, further diluted, and mixed thoroughly. The placebo solution was formulated by weighing 2 g of placebo into a 25 mL flask, adding 15 mL of diluent, sonicating for 15 minutes, diluting, and vortexing. For the sample preparation, 2 g of the sample was measured into a 25 mL flask, combined with 15 mL of diluent, sonicated, diluted, and vortexed. The chromatographic parameters were established, the column was equilibrated, and a blank injection was documented. Five replicate injections of the Ertugliflozin standard (for assay) and six replicate injections of the Ertugliflozin standard (for reference standard) were conducted to evaluate system appropriateness.

Injection Profile: [for Assay of Ertugliflozin]

The injection profile for the assay of Ertugliflozin was executed in the subsequent sequence: A solitary injection of the diluent was initially administered, succeeded by five injections of the standard

solution. Subsequently, a single injection of the placebo solution was administered, followed by two injections of the sample solution. To finalise the procedure, a single injection of the bracketing standard solution was administered to verify the accuracy and consistency of the assay.

Injection Profile: [for RS of Ertugliflozin]
The assay's injection profile comprised the subsequent sequence: A solitary injection of the diluent was administered first, succeeded by six injections of the standard solution. This was followed by one injection of the placebo for Ertugliflozin and a single injection of the sample solution. The procedure was finalised with a single injection of the bracketing standard solution to guarantee the reliability and uniformity of the test outcomes.

Evaluation of system suitability for Assay [28]
The USP tailing for the Ertugliflozin peak from the initial injection of the standard should not exceed 2.0. Theoretical plates for the Ertugliflozin peak from the initial injection standard should be no less than 2000. The % RSD for five replicate injections of the Ertugliflozin peak should not exceed 2.0.

Evaluation of system suitability for RI [28]
The USP tailing for the Ertugliflozin peak from the initial injection of the standard should not exceed 2.0. Theoretical plates for the Ertugliflozin peak from the initial injection standard should be no less than 2000. The % RSD for six replicate injections of Ertugliflozin peak should not exceed 5.0. Upon successful system suitability, inject one placebo solution of Ertugliflozin, followed by two injections of the sample solution for assay, and one injection for the reference standard (RS) of Ertugliflozin into the chromatograph. If the assay and RS analyses are conducted concurrently, prioritise the assay injection for the RS. Document the chromatograms and quantify peak regions.

Calculations:
Calculate % assay of Ertugliflozin by using the following formula.

$$\% 40: \text{Assay} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{100}{LC}$$

Total Impurities: Sum of Known + unknown impurities

VALIDATION OF RP-HPLC METHOD [28]
Validation is a process of "establishing documented evidence which provides a high degree of assurance that a specific activity will consistently produce a desired result (or) product meeting its pre-determined specifications and quality attributes".

Method Validation

1. Specificity and Selectivity
To ensure specificity of the optimized procedure, chromatogram of blank was recorded. The selectivity was also assessed by peak purity test to confirm that the Gaussian peak of analyte was due to single constituent. The method's specificity was determined by resolution of analyte peak from the nearest resolving peak.

2. Linearity
The linearity calibration curve (peak area vs. concentration) of Ertugliflozin was checked over the concentration ranges from 50% to 150%. The linearity was evaluated by linear regression analysis.

3. Limit of Detection (LOD)
LOD was calculated based on the data derived from the linearity parameter. Each of the six replicate analyses yielded estimated values for the slope and y-intercept from the linearity plot. The mean of

the slope (S) and the standard deviation of the y-intercept (σ) were computed. The parameters for LOD were calculated using the following formulae, derived from the response and regression equation based on the slope:
LOD = 3.3 σ \ S
Where; σ = Standard deviation Response,
S = Slope of Linearity curve

4. LOQ accuracy Preparation:
The Limit of Quantitation was derived from the results of the linearity parameter. Each of the six replicate analyses yielded estimated values for the slope and y-intercept from the linearity plot. Weighed 2 g of the sample into a 25 mL volumetric flask, added 15 mL of diluent, 2 mL of Ertugliflozin impurity combination (1 ppm), and sonicated for 15 minutes. Dilute to volume with diluent and vortex for 2 minutes to ensure thorough mixing.
Mean of slope (S) and standard deviation of the y intercept (σ) was calculated. From these results, the parameters LOQ was performed using below equation (based on the response and regression equation using slope):
LOQ = 10 σ \ S
Where; σ = Standard deviation of Response, S = Slope of Linearity curve

5. Recovery (Accuracy)
The recovery analysis was conducted utilising placebo and standard addition techniques. Samples were examined at three distinct concentration levels to determine the % recovery.
To prepare Ertugliflozin Impurity-I at a concentration of 200 ppm, 2 mg of the impurity was measured into a 10 mL volumetric flask, dissolved in 5 mL of 0.1% ortho phosphoric acid and 0.1% trifluoroacetic acid, sonicated for 2 minutes, and subsequently diluted to volume with the diluent, followed by vortexing for 2 minutes. To achieve 50% accuracy, 2 g of the sample was measured into a 25 mL flask, combined with 15 mL of diluent, 100 µL each of Ertugliflozin Impurity-I and Impurity-II, and 50 µL of Impurity-III, sonicated for 15 minutes, and subsequently diluted to volume and vortexed. To achieve 100% accuracy, 2 g of the sample was combined with 416 µL of Impurity-I, 400 µL of Impurity-II, and 210 µL of Impurity-III in a similar manner. For the Ertugliflozin impurity combination (about 1 ppm), 100 µL of Impurity-I, 100 µL of Impurity-II, and 50 µL of Impurity-III were transferred into a 20 mL volumetric flask, diluted with a diluent, and vortexed for 2 minutes.

6. Precision
Precision study was performed for two means: Repeatability and Intermediate precision.

7. Repeatability
Sample solution of Ertugliflozin (1000 µg/mL) was prepared and chromatograms were recorded. Peak area of the Stage-III, 1- Hydroxy, Keto Unspecified impurity were measured from the same concentration solution six replicates and %RSD was computed.

8. Intermediate Precision
Replication at varying days with distinct time intervals: Samples of a solution containing Ertugliflozin (1000 µg/mL) were produced. Evaluated the prepared solution according to the testing protocol. Six distinct sets of sample preparations were analysed on various days. The mean peak area of Stage-III, 1-Hydroxy, and keto. An unspecified impurity was calculated along with its standard deviation and percentage relative standard deviation (%RSD).

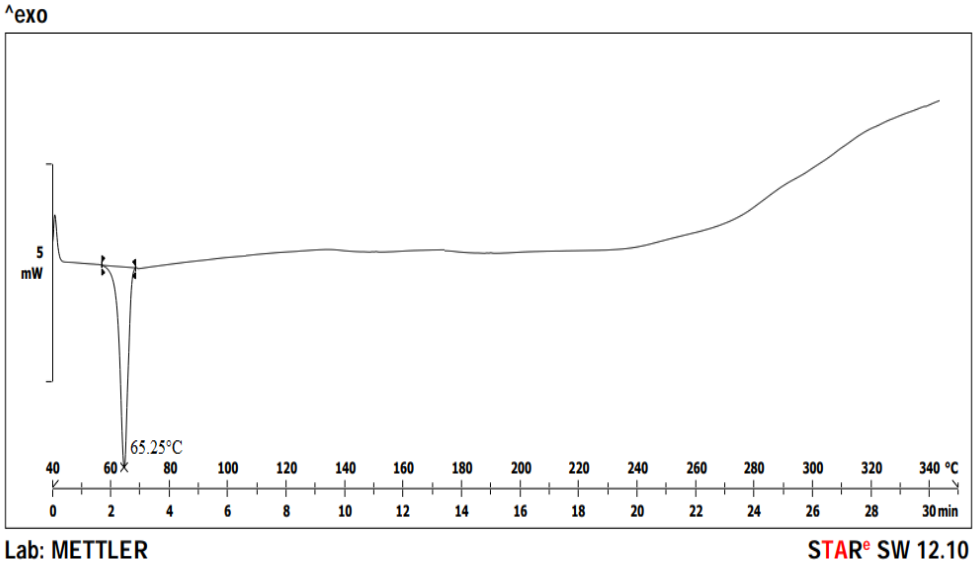
RESULTS AND DISCUSSION

Pre- analysis study of Ertugliflozin

1. Melting Point Determination

Determination of melting point of Ertugliflozin was found to be 65-66^{0}C. Melting point of pure drug Ertugliflozin was found to be 65.25°C by using Differential Scanning Calorimetry (Mettler Toledo). [65-66°C- Reported in the literature].

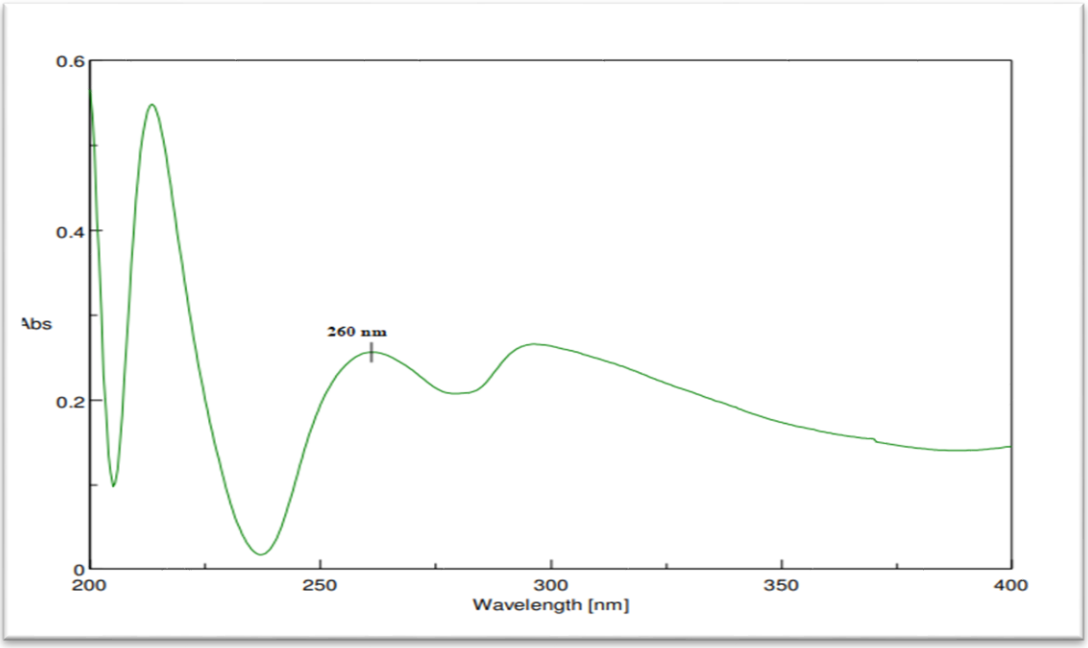
Figure 1: DSC graph of Ertugliflozin.



2. UV Spectroscopy

The UV spectra were recorded with UV spectrophotometer. The lambda maximum of Ertugliflozin (20 µg/ml) in methanol was found to be 260nm.

Figure 2: Lambda maximum for ERT



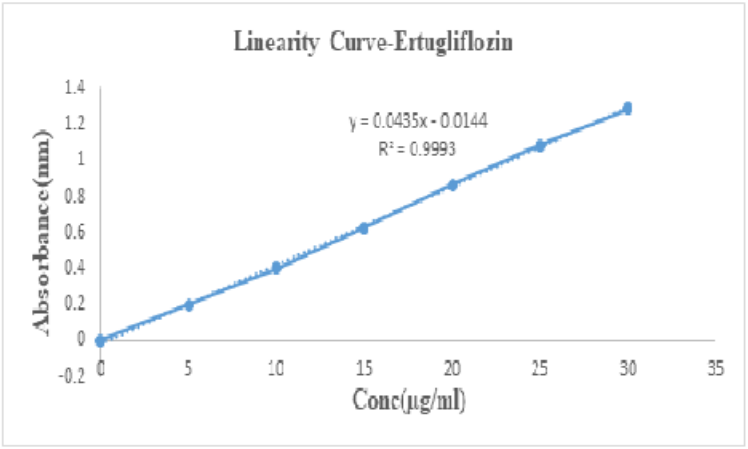
3. Calibration curve

Based on the study it is established that for related compound method development 260nm was selected for further work and method finalization.

Figure 3: A- Absorbances at conc. of 5-30µgm/ml,B- Linearity Curve-Ertugliflozin

Concentrations (µgm/ml)	Absorbance
0	0
5	0.201
10	0.403
15	0.625
20	0.865
25	1.085
30	1.286

(A)



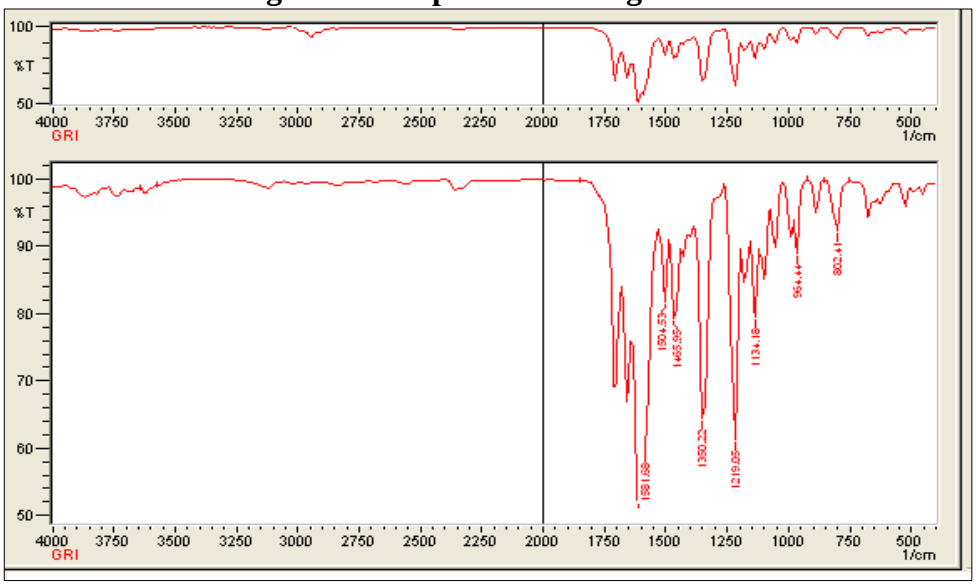
(B)

4. FTIR Spectroscopy

The infrared spectrum of Ertugliflozin is illustrated in the figure, and the table delineates the assignments for the characteristic bands in the IR spectrum.

The FTIR spectrum reveals a signal at 1581.68 cm−1, indicative of a keto group, and signals at 1350.22 cm−1, signifying the existence of C-O groups. The stretching frequency at 802.41 cm−1 indicates the existence of the C-Cl group. The absence of signal in the range of 3400 to 3000 cm−1 supports the nonexistence of OH and NH protons in the structure.

Figure 4: IR Spectra of Ertugliflozin



RP-HPLC

Reverse phase chromatography recommended for the ionic and adequate to non-polar Substances; hence, it was chosen for additional investigation. Reversed-phase chromatography is very efficient and appropriate due to its superior reproducibility, efficiency, and analyte stability. The C18 column exhibits lower polarity compared to the C8 columns. Jasco, Finepack C18 (250 mm × 4.6 mm, 5 µm) was selected for the analysis of Ertugliflozin and its impurities. The gradient software was chosen for additional examination.

Figure 5: A- Chromatogram for Blank,B- Chromatogram for Placebo, C- HPLC

Chromatogram of Standard Ertugliflozin

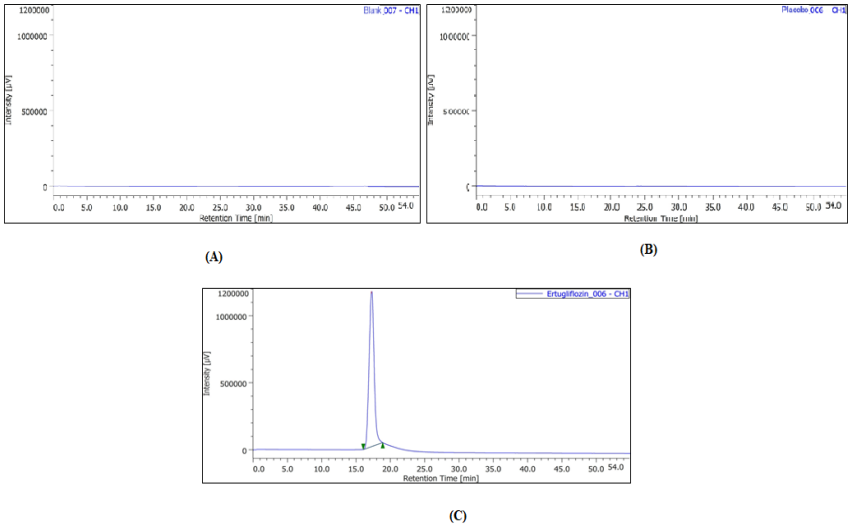


Figure 6: A-50ppm, B-80 ppm, C-90 ppm, D-100 ppm, E-110 ppm

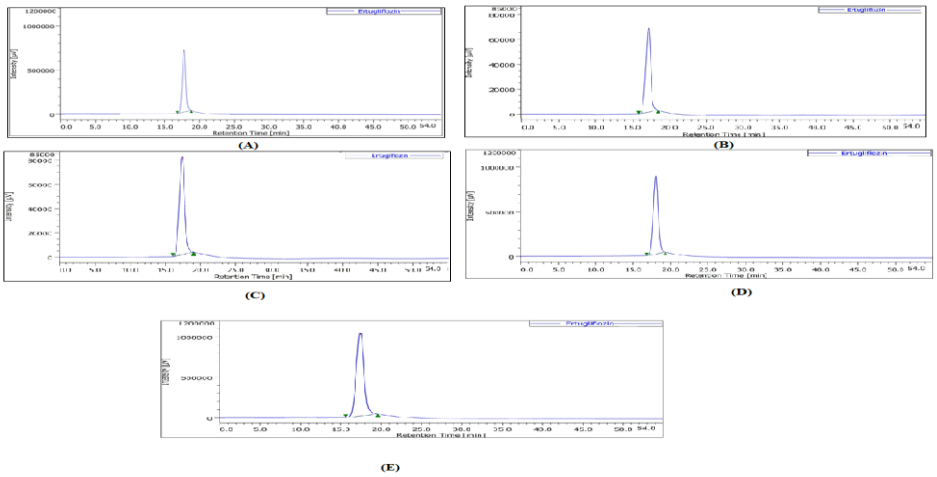


Figure 7: Overlay Chromatogram for Sample

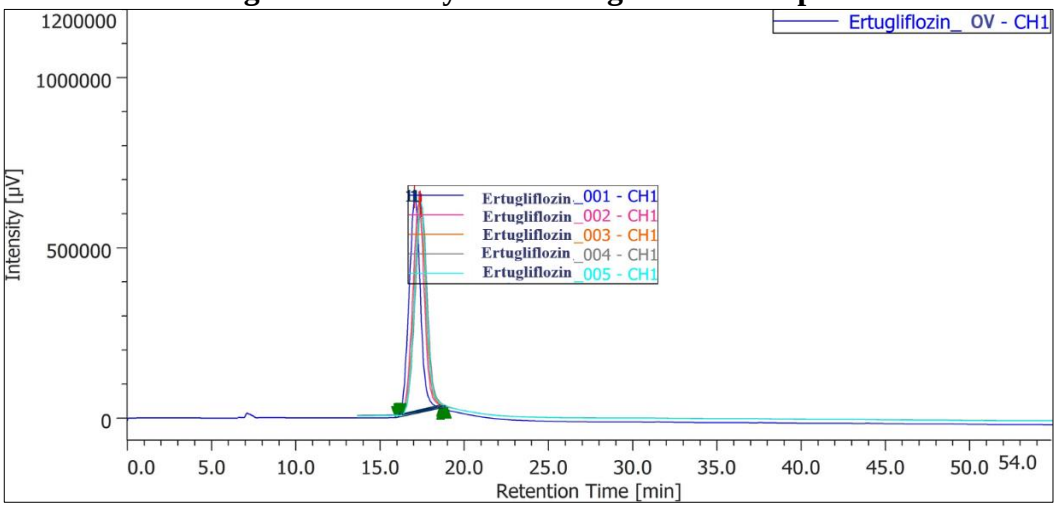
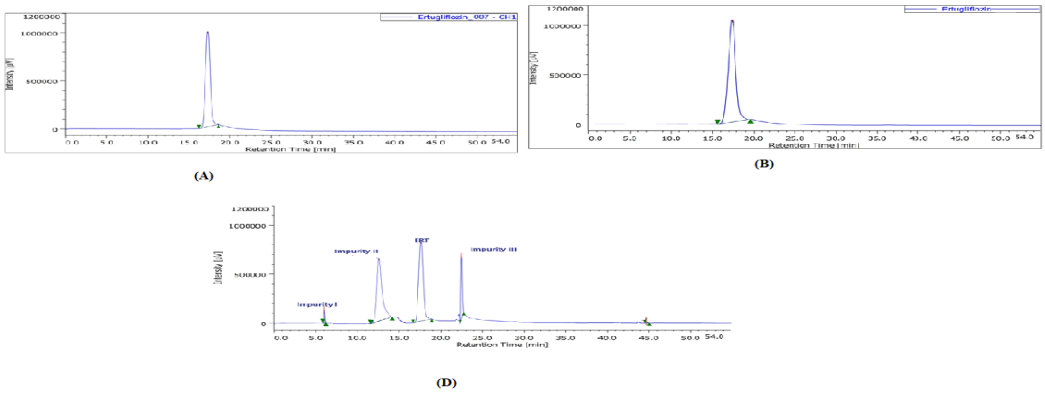


Figure 8: A- Chromatogram for Standard – RS, B- Chromatogram for Sample, C- Chromatogram for Impurity spiked sample



System Suitability

Table 1: System Suitability

System Suitability		
Sr. No.	**Sample**	**Area Response**
1	Injection 1	3527.915
2	Injection 2	3522.120
3	Injection 3	3528.665
4	Injection 4	3530.487
5	Injection 5	3529.596
Mean		3527.757
%RSD		0.2
Tailing factor		1.1
Theoretical plates		39548

Precision

Table 2: System Precision

System Precision		
Sr. No	**Sample**	**Area response**
1	Injection 1	4527.913
2	Injection 2	4522.122
3	Injection 3	4528.66
4	Injection 4	4530.486
5	Injection 5	4529.598
6	Injection 6	4526.387
Mean		4527.52767
%RSD		0.1
Tailing factor		1.2
Theoretical plates		39874

Method Precision:

Standard solution preparation for assay:

Refer system suitability parameter

Sample solution Preparation_1 for Assay:

Weighed 2g of sample to a 25 mL volumetric flask, added 15 ml diluent and sonicated for 15 minutes. Diluted to volume with diluent and vortex for 2 minutes to mixed well.

Table 3: Preparation of Standard solution for assay

S. No.	Sample	Sample weight (g)	Vol. flask (mL)	Diluted to volume with
1	**Sample** 2	2.024	25	Diluent
2	**Sample** 3	2.024		Diluent
3	**Sample** 4	2.025		Diluent
4	**Sample** 5	2.025		Diluent
5	**Sample** 6	2.024		Diluent

Remaining 5 samples were prepared in same as above procedure as per sample preparation_1 and injected to a HPLC system.

Table 4: %Assay of Ertugliflozin

S. No.	Sample Preparation	Ertugliflozin %Assay
1	**Sample** 1	94.1
2	**Sample** 2	93.8
3	**Sample** 3	93.9
4	**Sample** 4	94.2
5	**Sample** 5	93.7
6	**Sample** 6	93.8
Mean		93.92
SD		0.2547
%RSD		0.2

Linearity for Ertugliflozin standard (Assay):

The linear calibration curve (peak area vs concentration) of Ertugliflozin was evaluated throughout concentration ranges from 50% to 150%.

Ertugliflozin standard stock solution preparation: (concentration: 2000 ppm)

Table 5: Linearity for Ertugliflozin

Sr. No.	Linearity Level (%)	Pipette out of Ertugliflozin standard from stock	Volumetric flask (mL)	Diluted to volume with	Final concentration (ppm)
1	50	1.0	25	Diluent	80
2	80	1.6	25		128
3	90	1.8	25		144
4	100	2.0	25		160
5	110	2.2	25		176
6	150	3	25		240

Each level was injected in to the HPLC system and peak area was measured. Peak area vs. Concentration (on X-axis concentration and on Y-axis peak area) was plotted. Correlation coefficient was calculated.

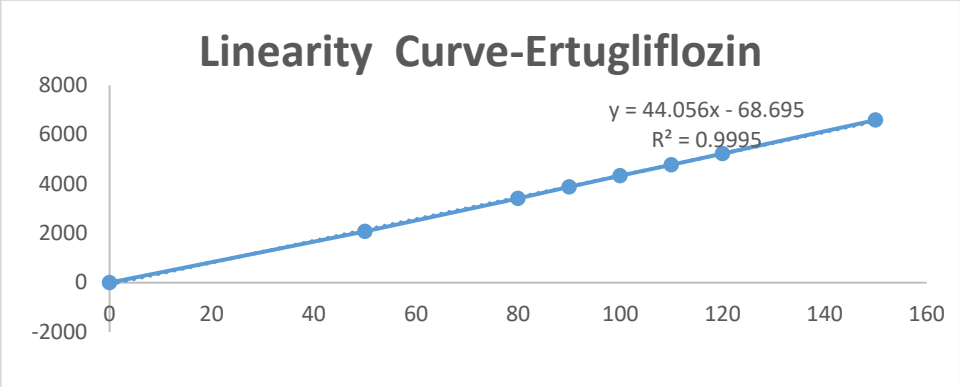
Table 6:

S. No.	Linearity Level (%)	Concentration (ppm)	Area
1	50	69.4967	2069.9
2	80	117.1948	3415
3	90	133.0941	3874
4	100	148.9935	4336
5	110	164.8928	4776.8

6	150	220.8475	6498
% Intercept			0.2

Linearity Curve:

Figure 9: Linearity Curve-Ertugliflozin



Accuracy for Ertugliflozin - Assay:
Ertugliflozin standard stock solution preparation: (concentration: 2000 ppm)
Refer system suitability parameter
Accuracy 50% Preparation:
Weighed accurately 2 g of placebo to a 25 mL volumetric flask, added 15 mL of diluent, added 1 mL of standard solution (2000 ppm), sonicated for 15 minutes, diluted to volume with diluent and vortex the solution to mixed well for 2 minutes.

Accuracy at 100%:
Accuracy 100%_1 preparation:
Weighed accurately 2g of placebo to a 25 mL volumetric flask, added 15 mL of diluent, added 2 mL of standard solution (2000ppm), sonicated for 15 minutes, diluted to volume with diluent and vortex the solution to mixed well for 2 minutes. 2 more preparations have been prepared in same manner as above.

Table 7: Accuracy at 100%

S. No	Levels %	Wt. of placebo taken (g)	Volumetric flask (mL)	Added Standard stock solution (conc. 1987.418ppm) (mL)	Diluted to volume with
1	100	2	25	2	Diluent
2	100	2	25	2	

Prepared 2 test solutions for 100% and injected each sample and analyzed in HPLC.

Accuracy 150% preparation:
Weighed accurately 2g of placebo to a 25 mL volumetric flask, added 15 mL of diluent, added 3 mL of standard solution (2000 ppm), sonicated for 15 minutes, diluted to volume with diluent and vortex the solution to mixed well for 2 minutes

Acceptance criteria:
Individual and mean % recovery for Ertugliflozin at each level should be between 97.0 and 103.0
% RSD at each accuracy level should be not more than 2.0
Calculated % Recoveries for each individual weight at each level and results are tabulated below.

Table 8: Accuracy at 50,100% and 150%

% Level (about)	Sample	Mean Area Response	mcg/mL added	mcg/mL Recovered	%Recovery	Mean %Recovery	%RSD
50	1	2048.887	77.4967	76.9703	99.2	99.3	NA
	1	4337.672		149.3417	100.2		
100	2	4334.974		149.2469	100.2	100.2	0
	3	4338.737	148.9935	149.3791	100.2		
150	1	6480.451	228.4902	224.586	98.4	98.4	NA
% RSD					0.8		

Solution stability:

Acceptance criteria:

The % difference of area response for Assay Ertugliflozin standard should be NMT ± 2.0% with respect to initial and further time points.

Table 9: Solution stability

Ertugliflozin Standard - AY		
Time intervals (Hrs.)	**Area Response**	**% difference**
Initial	4327.9	NA
9	4330.4	0.1
14	4324.1	-0.1
19	4328.8	0.0
31	4338.6	0.2

Table 10: Solution stability

Ertugliflozin sample-AY		
Time intervals (Hrs.)	**Area Response**	**% difference**
Initial	4049.5	NA
14	4013.2	-0.9
27	4018.8	-0.7

System suitability and system precision Related Substances:

Blank preparation: Diluent as blank

Ertugliflozin standard stock solution Preparation: (concentration: 1000 ppm)

Weighed 25 mg of Ertugliflozin standard WS/RS to a 25 mL volumetric flask, added 15 mL of diluent and sonicated for 2 minutes. Diluted to volume with diluent and vortex for 2 minutes to mixed well.

Standard solution preparation: (concentration: 160 ppm)

Pipetted 4 mL of Ertugliflozin standard stock solution (997.594 ppm) to a 25 mL volumetric flask, diluted to volume with diluent and vortex for 2 minutes to mixed well.

Standard solution preparation for RS: (concentration: 0.25 ppm)

Pipetted 8 µL of Ertugliflozin standard solution (159.615 ppm) to a 50 mL volumetric flask, diluted to volume with 30 mL diluent and vortex for 2 minutes to mixed well.

Placebo solution Preparation [For Ertugliflozin]:

Weighed 2.00299 g of placebo in 25 mL volumetric flask, added 15 mL of diluent and sonicated for 15 minutes. Diluted to volume with diluent and vortex for 2 minutes to mix well.

Acceptance criteria for system suitability:
The % RSD of Ertugliflozin peak from 6 replicate injections standard preparation should be NMT 5.0.
USP tailing factor/asymmetry Ertugliflozin peak from first injection of standard preparation should be NMT 2.0
USP plate count/theoretical plates of Ertugliflozin peak from first injection of standard should be NLT 2000.

Acceptance criteria for system precision:
The %RSD of the retention time for the Ertugliflozin peak obtained from 6 injections of standard preparation should be NMT 1.0
The %RSD of the area response for the Ertugliflozin peak obtained from 6 injections of standard preparation should be NMT 5.0

Table 11: System Suitability

System Suitability		
S. No.	**Sample**	**Area Response**
1	Injection 1	5.888
2	Injection 2	5.885
3	Injection 3	5.844
4	Injection 4	5.972
5	Injection 5	6.037
6	Injection 6	6.144
Mean		5.961
%RSD		1.4
Tailing factor		1.0
Theoretical plates		63524

Table 12: System Precision

System Precision			
S. No	**Sample**	**Rt (minutes)**	**Area response**
1	Injection 1	16.164	5.888
2	Injection 2	16.182	5.885
3	Injection 3	16.183	5.844
4	Injection 4	16.239	5.972
5	Injection 5	16.270	6.037
6	Injection 6	16.249	6.144
Mean		16.214	5.961
%RSD		0.2	1.4
Tailing factor		1.0	
Theoretical plates		63547	

Method Precision (RS):
Standard solution preparation for RS: (concentration: 0.25 ppm) Refer system suitability parameter RS 5.6

Table 13: Impurity stock preparation

Name of the impurity	Weight (mg)	Vol. Flask (mL)	Added (mL)	Sonicated (minutes)	Diluted to volume with	Potency/ Purity (%)	Final Conc. (ppm)
Ertugliflozin impurity-I	1.045	5	2 mL of 0.1%OPA+ 0.1%TFA	2	0.1%OPA + 0.1%TFA	94.40	191.63
Ertugliflozin impurity-II	2.014	10	5 mL of methanol		Methanol	96.70	205.101
Ertugliflozin impurity-III	1.910	5	2 mL of diluent		diluent	98.70	388.88

Sample solution Preparation_1 for RS:

Weighed 2 g of sample to a 25 mL volumetric flask, added 15 ml diluent, 0.208 mL of Ertugliflozin impurity -I, 0.195 Ertugliflozin impurity -II and 0.103 Ertugliflozin impurity –III and sonication for 15 minutes. Diluted to volume with diluent and vortex for 2 minutes to mixed well.

Table 14: Sample solution Preparation_1 for RS

S. No.	Sample	Sample weight (g)	Vol. flask (mL)	Diluent added mL	Imp-I added mL	Imp-II added mL	Imp-II added mL	Sonication for	Dilute to volume with
1	Preparation 2	2.002	25	15	0.210	0.197	0.105	20	diluent
2	Preparation 3	2.017		15	0.210	0.197	0.105	20	diluent
3	Preparation 4	2.030		15	0.210	0.197	0.105	20	diluent
4	Preparation 5	2.004		15	0.210	0.197	0.105	20	diluent
5	Preparation 6	2.010		15	0.210	0.197	0.105	20	diluent

Remaining 5 samples were prepared as per same procedure of sample preparation_1 and injected to a HPLC system.

Table 15: Sample solution Preparation of Impurity-I, Impurity-II and Impurity-III

	Sample Preparation	Impurity-I %	Impurity-II %	Impurity-III %
1	Preparation 1	1.022	1.043	0.887
2	Preparation 2	1.045	1.079	0.945
3	Preparation 3	1.078	1.045	1.086
4	Preparation 4	1.022	1.056	1.012
5	Preparation 5	1.056	1.075	1.048
6	Preparation 6	1.078	1.011	1.013
Mean		1.055	1.050167	1.0515
SD		0.01	0.01	0.05
%RSD		1.1	1.0	1.6

Accuracy (RS):

LOQ accuracy Preparation:

Weighed 2g of sample to a 25 mL volumetric flask, added 15 mL of diluent, 2 mL of Ertugliflozin impurity mixture (1ppm) and sonicated for 15 minutes. Diluted to volume with diluent and vortex for 2 minutes to mixed well.

Table 16: % Recovery of Impurity-I

% Recovery of Impurity-I							
% Level (about)	**Sample**	**Mean Area Response**	**mcg/mL Added**	**mcg/mL Recovered**	**%Recovery**	**Mean %Recovery**	**%RSD**
LOQ	1	1.215	0.07	0.065	83.5	83.5	NA
50	1	13.214	0.6692	0.72	92.8	92.8	NA
	1	33.256		1.7124	106.9		
	2	34.125		1.2458	107.4		
	3	33.124		1.5124	106.2		
100	4	33.658	1.4944	1.5689	106.5	106.8	0.5
	5	34.154		1.6124	107.3		
	6	33.785		1.6845	106.4		
200	1	58.451	3.998	2.8124	91.5	91.5	NA
% Mean for 9 levels					100.944444		
% RSD for 9 levels					9.5		

Table 17: % Recovery of Impurity-II

% Recovery of Impurity-II							
% Level (about)	Sample	Mean Area Response	mcg/mL Added	mcg/mL Recovered	%Recovery	Mean % Recovery	%RSD
LOQ	1	6.124	0.081	0.0912	117	117	NA
50	1	55.745	0.82	0.8528	103.8	103.8	NA
	1	109.852		1.6941	104.9		
	2	111.221		1.7145	106.3		
	3	110.014		1.6923	105		
100	4	112.523	1.55	1.7378	107.3	105.8	0.8
	5	111.758		1.7101	105.3		
	6	110.412		1.7045	105.8		
200	1	247.744	3.23	3.8178	115.3	115.3	NA
% Mean for 9 levels					107.855556		
% RSD for 9 levels					9.5		

Table 18: % Recovery of Impurity-III

% Recovery of Impurity-III							
% Level (about)	**Sample**	**Mean Area Response**	**mcg/mL Added**	**mcg/mL Recovered**	**%Recovery**	**Mean %Recovery**	**%RSD**
LOQ	1	2.6	0.0777	0.08	104.9	104.9	NA
50	1	25.2	0.7779	0.77	99	103.8	NA
	1	46.9		1.42	89.2		
	2	51.6		1.57	98.1		
	3	53.1		1.61	100.9		
100	4	54.2	1.6025	1.65	103	105.8	0.8
	5	54.6		1.66	103.9		
	6	55.4		1.68	105.3		
200	1	116.4	3.2674	3.54	108.6	108.6	NA
% Mean for 9 levels					101.433333		
% RSD for 9 levels					9.5		

Linearity – RS (Impurities and standard):
The linearity calibration curve (peak area vs. concentration) of impurities were checked over the concentration ranges from LOQ to 200%. The linearity was evaluated by linear regression analysis.

Standard solution preparation:
Refer system suitability parameter
Impurity stock preparation :(Ertugliflozin Impurity-I, impurity-II)
Refer method precision (RS) parameter
Table for Linearity sample preparation:

Table 19: Linearity – RS (Impurities and standard)

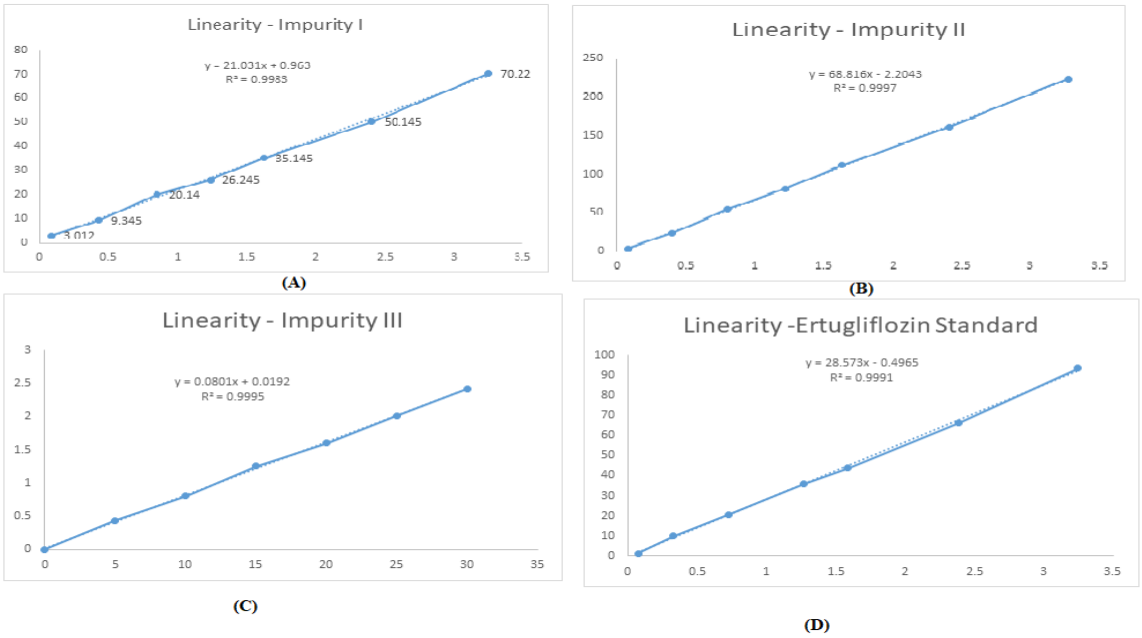
S. No.	Level %	Vol. flask (mL)	Ertugliflozin standard (from stock 159.615 ppm)	Impurity-1 (from stock 191.63 ppm)	Impurity-II (from stock 205.100 ppm)	Conc.
1	LOQ	50	0.027	0.022	0.020	0.0800
3	25	10	0.027	0.022	0.020	0.4000
4	50	10	0.052	0.045	0.040	0.8000
5	80	10	0.080	0.065	0.060	1.2800
6	100	10	0.100	0.085	0.080	1.6000
7	150	5	0.075	0.063	0.059	2.4000
8	200	5	0.102	0.085	0.080	3.2000

Each level was injected in to the HPLC system and peak area was measured. Peak area vs. Concentration (on X-axis concentration and on Y-axis peak area) was plotted. Correlation coefficient was calculated.

Table 20: Linearity – RS (Impurities and standard)

S. No.		Ertugliflozin Impurity-I		Ertugliflozin Impurity-II		Ertugliflozin Standard	
	Linearity Level (%)	Conc. (ppm)	Area	Conc. (ppm)	Area	Conc. (ppm)	Area
1	LOQ	0.0845	3.012	0.078	4.017	0.0762	1.275
2	25	0.4268	9.345	0.4002	23.719	0.331	9.885
3	50	0.8487	20.14	0.8104	54.67	0.73	20.815
4	80	1.2356	26.245	1.2206	80.997	1.2669	35.825
5	100	1.6189	35.145	1.6308	111.508	1.5861	43.862
6	150	2.4046	50.145	2.4102	161.783	2.3842	66.238
7	200	3.2477	70.22	3.2716	223.773	3.2461	93.508
Correlation Coefficient		0.998		0.999		0.999	
Slope		21.63		68.744		28.684	
Intercept		-0.059		-1.732		-0.245	
% Intercept		-0.2		-1.5		-0.6	

Figure 10: A-Linearity - Impurity I, B-Linearity - Impurity II, C- Linearity - Impurity III,D-Linearity -Ertugliflozin Standard



Specificity:

Table 21: Individual impurities preparations for Ertugliflozin

Sr. No.	Impurity name	Pipette out from the stock conc. (mL)	Vol. flask (mL)	Diluted to volume with	Final Conc. (ppm)
1	**Ertugliflozin Imp-I**	0.083	10	Diluent	1.6
2	**Ertugliflozin Imp-II**	0.078	10		1.6
3	**Ertugliflozin Imp-III**	0.041	10		1.6

Each impurity transferred to HPLC vial and injected in to the HPLC system.

Impurity spiked sample preparation:

Weighed 2 g of sample to a 25 mL volumetric flask and added 15 mL of diluent, 208 µL of Ertugliflozin Impurity-I, 195 µL of Ertugliflozin impurity-II, 103 µL of Ertugliflozin impurity-III and sonicated for 15 minutes. Diluted to volume with diluent and vortex for 2 minutes to mixed well.

Table 22: Ertugliflozin impurities stock preparation

Name of the impurity	Weight taken (mg)	Vol. flask (mL)	Diluted to volume with	Potency/ Purity (%)	Final Conc. (ppm)
Impurity I	5.123	25	Methanol	100.0	163.94
Impurity II	2.181	10	Methanol	100.0	218.10
Impurity III	2.173	10	Methanol	97.6	212.08

Table 23: Individual impurities preparations Ertugliflozin

Sr. No.	Impurity name	Pipette out from the stock conc. (mL)	Vol. flask (mL)	Diluted to volume with	Final Conc. (ppm)
1	Related compound-B	0.098	10	Diluent	1.6
2	Related compound-C	0.074	10	Diluent	1.6
3	Related compound-D	0.075	10	Diluent	1.6

Table 24: Specificity

Specificity		
		Retention Time (minutes)
Blank		Not detected
Placebo	Ertugliflozin	Not detected
	Ertugliflozin AS Std.	16.94
	Ertugliflozin RS Std.	18.22
	Ertugliflozin	16.32
Impurity	Ertugliflozin Impurity-I	5.75
	Ertugliflozin Impurity-II	12.84
	Ertugliflozin Impurity-III	22.78

API stability:

Table 25: API stability

Ertugliflozin Standard - RS		
Time intervals (Hrs)	Area Response	% difference
Initial	6.888	NA
11	6.838	-0.7
17	7.055	2.4
21	7.101	3.1
29	6.792	-1.4

Sample

Interval	**Ertugliflozin**		**Ertugliflozin impurity -III**	
	Area	% difference	Area	% difference
Initial	4240.828	NA	23.469	NA
6	4224.018	-0.4	24.581	4.7
12	4205.253	-0.8	23.253	-0.9
22	4174.986	-1.6	23.543	0.3

Table 26: Impurity spiked sample

Interval	Ertugliflozin		Ertugliflozin Imp-I		Ertugliflozin Imp-II		Ertugliflozin Imp-III	
	Area	% difference	Area	% difference	Area	% difference	Area	% difference
Initial	4304.871	NA	34.748	NA	109.937	NA	70.409	NA
6	4300.077	-0.1	34.648	-0.3	111.311	1.2	73.856	4.9

12	4295.887	-0.2	34.783	0.1	111.045	1.0	73.737	4.7
22	4287.440	-0.4	34.898	0.4	111.158	1.1	67.516	-4.1

Observation

The stability study of the aforementioned solution indicates that Impurity-III is unstable upon its introduction into the sample. Therefore, it is advised that Impurity-III be made freshly and that a fresh impurity-spiked sample be injected.

Characterization of Impurities

Table 27: Characterization of Ertugliflozin Impurity 1, Impurity II

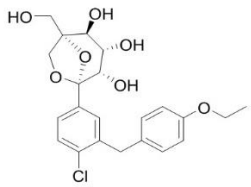
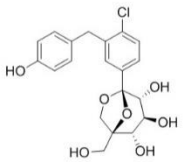
Characterization of Impurities	**Impurity I**	**Impurity II**
	Identified impurity peak 1 is ((S)-5-(tert-Butoxy)-2-(2-(2-((S)-2-((tert-butoxycarbonyl)amino)-3-(1-trityl-1H-imidazol-4-yl)propanamido)-2-methylpropanamido)-2-methylpropanamido)-5-oxopentanoyl)glycine.	Identified impurity peak II is (1S,2S,3S,4R,5S)-5-(4-Chloro-3-(4-(2-hydroxyethoxy)benzyl)phenyl)-1-(hydroxymethyl)-6,8-dioxabicyclo[3.2.1]octane-2,3,4-triol. It is further characterized by Chromatographic purity, IR and1HNMRstudy.
Chemical Structure- Ertugliflozin Impurity I		
Molecular Formula	$C_{22}H_{25}ClO_7$	$C_{20}H_{21}ClO_7$
Molecular Weight	436.89	
IUPAC Name	(1S,2S,3S,4R,5S)-5-(4-Chloro-3-(4-ethoxybenzyl)phenyl)-1-(hydroxymethyl)-6,8-dioxabicyclo[3.2.1]octane-2,3,4-triol; (1S,2S,3S,4R,5S)-5-(4-Chloro-3-(4-ethoxybenzyl)phenyl)-1-(hydroxymethyl)-6,8-dioxabicyclo[3.2.1]octane-2,3,4-triol;	
Storage condition	Preservewellclosedvial.Storeat2-8ºC	Preservewellclosedvial.Storeat2-8ºC

Figure11: Chromatogram of Impurity I

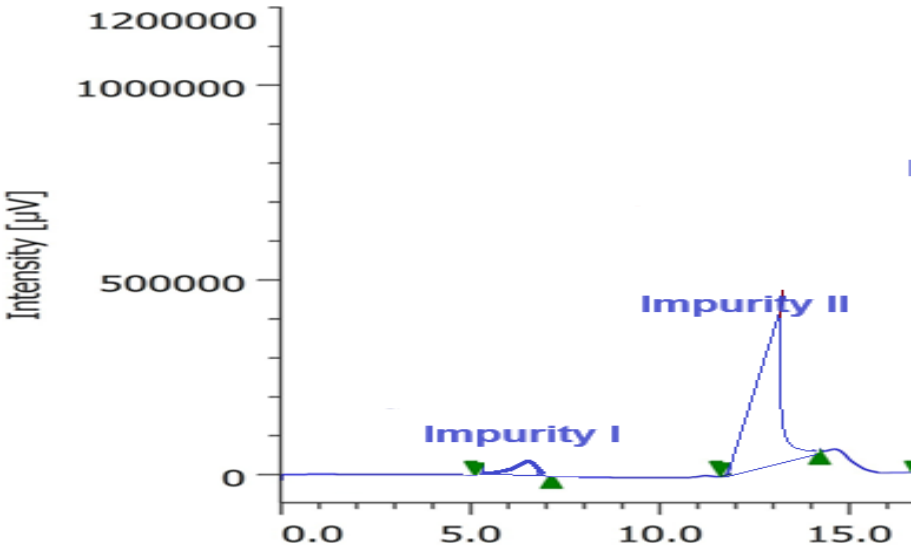
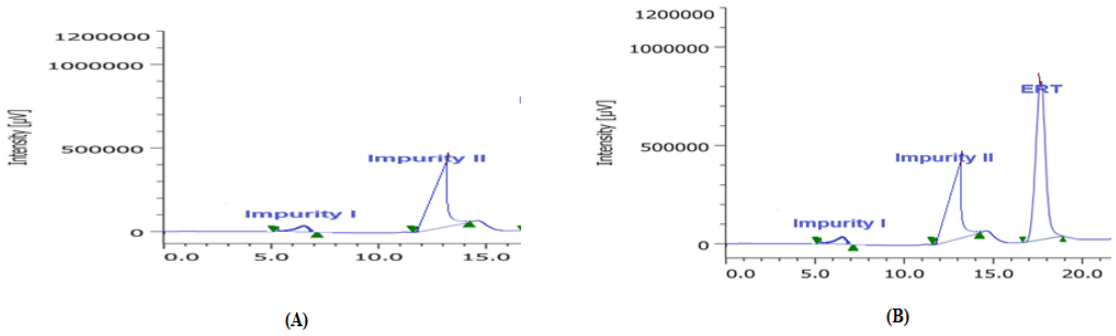


Figure 12: A- Chromatogram of Impurity I, B- Chromatogram of Impurity II



Conclusion

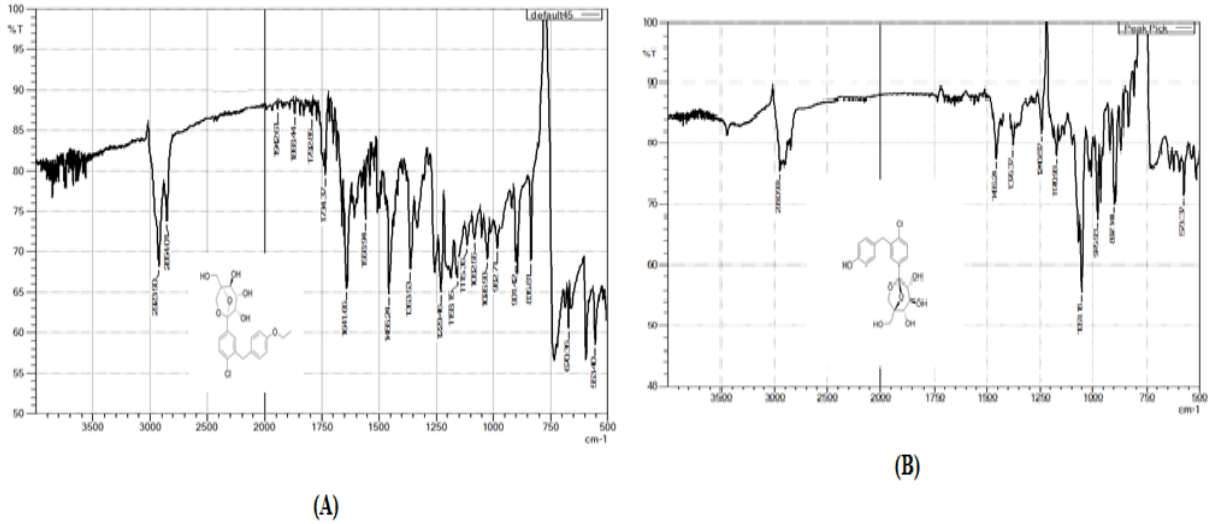
The chromatographic analysis showed that Impurity I had a retention time of 5.71 minutes with a purity of 99.54%, while Impurity II exhibited a retention time of 15.47 minutes with a purity of 99.25%.

FTIR SPECTRUM

The IR spectra of Impurity I displays multiple distinctive absorption bands associated with various functional groups. The C-Cl stretching vibration occurs at 836.81 cm^{-1}. The C-O stretching vibrations are observed at two distinct wave numbers: 1082.55 cm^{-1} and 1229.46 cm^{-1}, signifying the existence of C-O bonds in varying settings. The O-H bending vibration is observed at 1363.53 cm^{-1}. The IR spectrum data elucidate the chemical structure of Impurity I, emphasising the existence of distinct functional groups including C-Cl, C-O, and O-H.

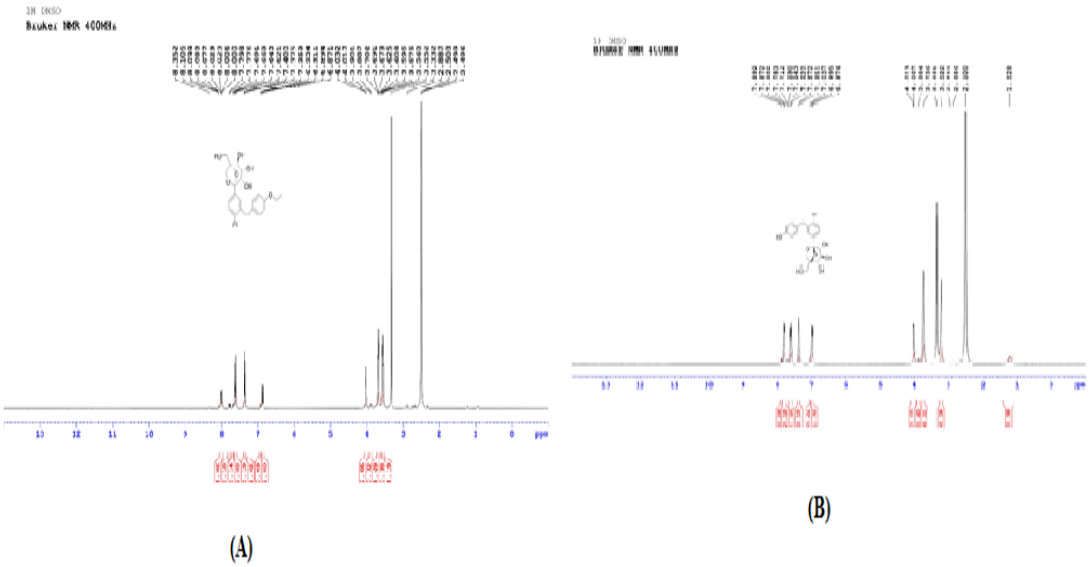
The IR spectra of Impurity II reveals the existence of many functional groups. The C-Cl stretching vibration is detected at 573.37 cm^{-1}, indicative of a chlorine-containing molecule. The C-O stretching vibration is observed at 1061.78 cm^{-1}, indicating the existence of an oxygen-containing functional group. The O-H bending vibration is observed at 1376.37 cm^{-1}, signifying the existence of hydroxyl groups. These spectrum characteristics aid in elucidating the chemical composition of Impurity II.

Figure 13: A- IR Spectrum of Impurity I, B- IR Spectrum of Impurity II



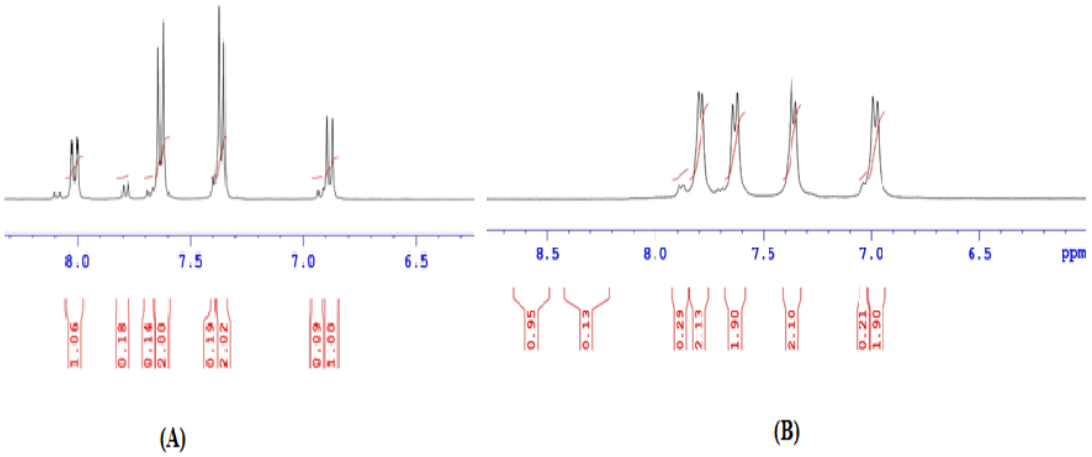
(A) (B)

Figure 14: A-1HNMR of Impurity I, B-1HNMRof Impurity II



(A) (B)

Figure 15: A- Zoomspectrumof1HNMRofImpurity I, B- Zoomspectrumof1HNMRofImpurity II
Zoomspectrumof1HNMRofImpurity I



Interpretation:
^{1}H NMR: δ 1.24 (3H, t, *J* = 7.0 Hz), 3.06 (1H, d, *J* = 10.2 Hz), 3.22 (1H, t, *J* = 10.2 Hz), 3.52 (1H, d, *J* = 10.2 Hz), 3.62-3.72 (2H, 3.67 (s), 3.67 (s)), 3.74-3.85 (4H, 3.79 (s), 3.79 (s), 3.80 (s), 3.80 (s)), 3.96-4.07 (2H, 4.01 (q, *J* = 7.0 Hz), 4.01 (q, *J* = 7.0 Hz)), 6.56 (1H, dd, *J* = 1.3, 0.5 Hz), 6.81 (2H, ddd, *J* = 8.8, 1.9, 0.6 Hz), 7.20-7.34 (3H, 7.26 (dd, *J* = 8.3, 1.3 Hz), 7.28 (ddd, *J* = 8.8, 1.0, 0.6 Hz)), 7.50 (1H, dd, *J* = 8.3, 0.5 Hz).

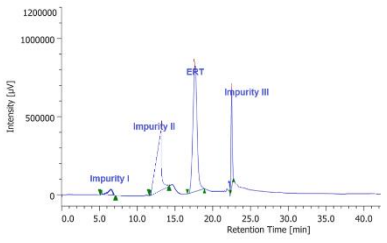
Conclusion: Based on the above characterization data by IR spectrum and 1H NMR technique it is concluded that identified impurity peak 1.

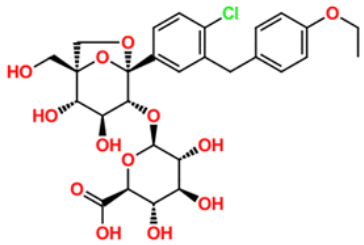
Zoom spectrum of 1HNMR of Impurity II

Interpretation:
^{1}H NMR: δ 3.32 (1H, dd, *J* = 10.2, 10.0 Hz), 3.50-4.01 (10H, 3.57 (d, *J* = 10.2 Hz), 3.58 (t, *J* = 5.8 Hz), 3.58 (t, *J* = 5.8 Hz), 3.67 (s), 3.67 (s), 3.73 (s), 3.73 (s), 3.82 (d, *J* = 10.0 Hz), 3.84 (d, *J* = 14.8 Hz), 3.94 (d, *J* = 14.8 Hz)), 4.02-4.14 (2H, 4.08 (t, *J* = 5.8 Hz), 4.08 (t, *J* = 5.8 Hz)), 6.57 (1H, dd, *J* = 1.0, 0.5 Hz), 6.82 (2H, ddd, *J* = 8.8, 1.6, 0.6 Hz), 7.21-7.34 (3H, 7.27 (dd, *J* = 8.3, 1.0 Hz), 7.28 (ddd, *J* = 8.8, 1.0, 0.6 Hz)), 7.51 (1H, dd, *J* = 8.3, 0.5 Hz).

Conclusion: Based on the above characterization data by IR spectrum and 1H NMR technique it is concluded that identified impurity peak II.

Table 28: Characterization of Ertugliflozin Impurity III

Characterization of Impurities	Impurity III	Chromatogram of Impurity III
	Identified impurity peak III is (2S,3R,4S,5S)-2-(4-Chloro-3-(4-ethoxybenzyl)phenyl)-6,6-bis(hydroxymethyl)-2-methoxytetrahydro-2H-pyran-3,4,5-triol.	

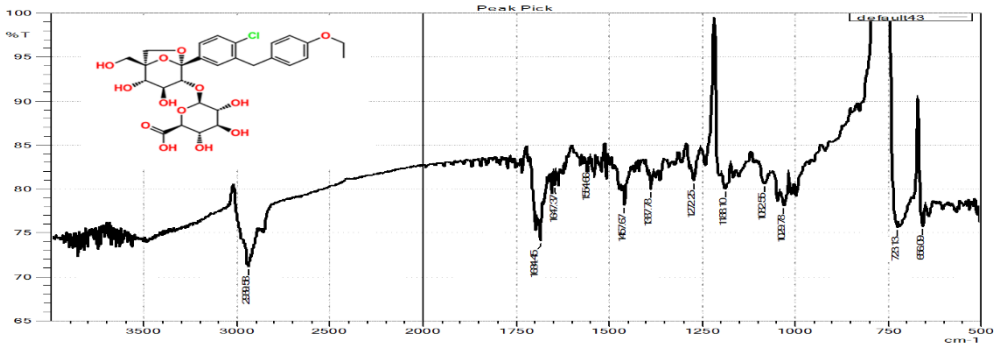
Chemical Structure- Ertugliflozin Impurity I		
Molecular Formula	$C_{23}H_{29}ClO_8$	
Molecular Weight	468.9	
IUPAC Name	(2S,3R,4S,5S)-2-(4-Chloro-3-(4-ethoxybenzyl) phenyl)-6,6-bis(hydroxymethyl)-2-methoxytetrahydro-2H-pyran-3,4,5-triol.	
Storagecondition	Preservewellclosedvial.Storeat2-8ºC	

Conclusion

The chromatographic analysis of Impurity III revealed that it had a retention time of 22.70 minutes, with a purity of 88.74%.

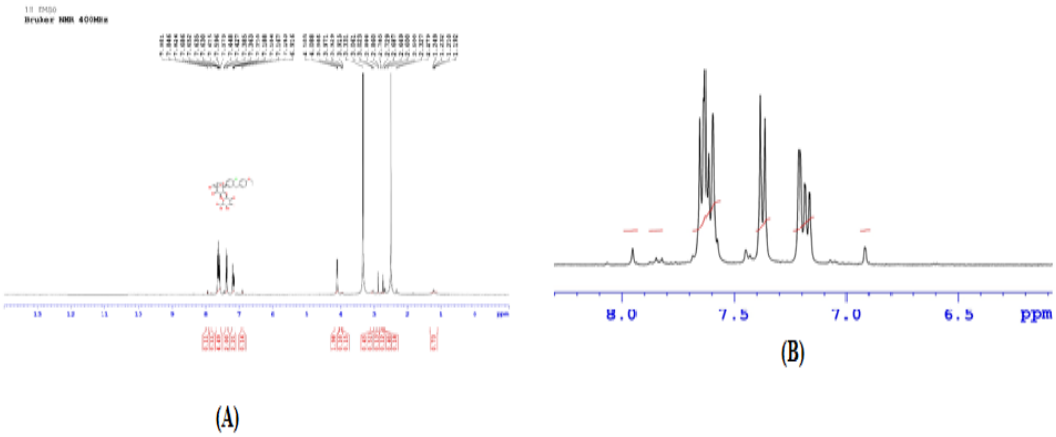
FTIR Spectrum

Figure16: IR Spectrum of Impurity III



The IR spectra of Impurity III displays significant absorption bands associated with several functional groups. The C-Cl stretching vibration is detected at 666.09 cm^{-1}, signifying the presence of a chlorine atom in the molecule. The C-O stretching vibration is observed at 1082.55 cm^{-1}, indicating the existence of an oxygen-containing functional group. The O-H bending vibration is detected at 1367.78 cm^{-1}, indicative of hydroxyl groups. The spectrum characteristics yield significant insights into the chemical structure of Impurity III.

Figure 17: A- 1HNMRof Impurity III, B- Zoom spectrum of 1HNMR of Impurity III
Figure 18: 1HNMR of Impurity III



Interpretation:
^{1}H NMR: δ 1.24 (3H, t, *J* = 7.0 Hz), 3.05-3.32 (5H, 3.11 (d, *J* = 10.2 Hz), 3.23 (t, *J* = 10.2 Hz), 3.27 (s)), 3.53 (1H, d, *J* = 10.2 Hz), 3.62-3.72 (2H, 3.67 (s), 3.67 (s)), 3.76-3.86 (4H, 3.81 (s), 3.81 (s), 3.81 (s), 3.81 (s)), 3.96-4.07 (2H, 4.01 (q, *J* = 7.0 Hz), 4.01 (q, *J* = 7.0 Hz)), 6.57 (1H, dd, *J* = 1.0, 0.5 Hz), 6.81 (2H, ddd, *J* = 8.8, 1.9, 0.6 Hz), 7.21-7.34 (3H, 7.27 (dd, *J* = 8.3, 1.0 Hz), 7.28 (ddd, *J* = 8.8, 1.0, 0.6 Hz)), 7.51 (1H, dd, *J* = 8.3, 0.5 Hz).

Conclusion: Based on the above characterization data by IR spectrum and 1H NMR technique it is concluded that identified impurity peak III.

CONCLUSION

The suggested high-performance liquid chromatography (HPLC) method is specific for identifying contaminants associated with ertugliflozin found in ertugliflozin. The linear curves generated for ertugliflozin and its associated impurities. The values for the Limit of Detection (LOD) and the Limit of Quantification (LOQ) have been deemed satisfactory. The results of the recovery phase were deemed satisfactory. The proposed technique yielded replicable outcomes. This method is useful for the detection and quantification of contaminants in ertugliflozin.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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