

A Rapid and Reliable Rp-Hplc Bioanalytical Method for Determination of Ensifentrine in Human Plasma

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ABSTRACT

Ensifentrine in human plasma was quantified using a simple, dependable, and accurate method. The sample was prepared using the protein precipitation technique (PPM), and the chromatogram was created using an Agilent C18 column (250 x 4.6 x 5 µm). Tenueligiptin is an internal standard (IS) for ensifentrine isolation; the temperature did not change at thirty degrees Celsius; the most effective wavelength was 348 nm; ensifentrine retention times were 3.406 minutes and 2.363 minutes (IS). The standard curve for ensifentrine doses between 0.5 and 20 ng/mL was linear ($R^2 > 0.995$). Every analytical validation parameter was computed using the ICH criteria. The recommended bioanalytical technique fulfilled recovery, accuracy, precision, and other validation requirements. It was reliable, strong, and insightful. The drug of interest and internal standard peaks were symmetrical, well separated, and there was no plasma interference. They had an appropriate tailing factor as well. This approach may be very helpful for therapeutic drug monitoring (TDM), bioequivalency studies, pharmacokinetics investigations, toxicity, and biomedical research.

Keywords: API ,HPLC,LOD,LOQ,Ensifentrine

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INTRODUCTION

AN OVERVIEW OF THE FIRST CHAPTER:

Bioanalytical approaches provide a fresh way to find and measure a molecule of interest in a matrix¹. These methods are used to assess drug and metabolite levels in body fluids. Numerous techniques are available for measuring coalesces. When selecting analytical

procedures, factors such concentration levels, the chemical characteristics of the analyte, the specimen matrix, the cost of the analysis, the speed of the experiment, quantitative or qualitative measurement, the necessary precision, and the equipment are all taken into account. Selectivity, accuracy, precision, recovery, sensitivity, stability, and other elements that impact the quality of the findings are considered while validating bioanalytical procedures³.

DEVELOPMENT OF METHOD:

Definition of a method development plan



Obtaining background information



Development of laboratory methods



Creation of the test protocol



Definition of the methods validation protocol



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Validation of laboratory procedures



Generation of validated test methods



Validation report 4

Sample preparation: Blood, plasma, serum, and urine are common biological media that include analytical components. Typically, a patient's vein is punctured and blood is drawn using a 5-7 mL hypodermic syringe. Tubes containing venous blood are treated with an anticoagulant, such as heparin or potassium EDTA salt. Plasma is prepared by centrifugation at 4000 rpm for 15 minutes. Thirty to fifty percent of the original volume must be collected. **3**

Storage conditions for the biological samples:

Biological samples must be preserved because processing biological materials takes time. Samples must be kept in a freezer to avoid degradation. Protein samples are treated with substances like sodium azide and 2mercaptoethanol that prevent cellular respiration in order to prevent the growth of germs.

First biological sample treatment: To discover and improve sample preparation techniques, use analyte and internal standard physicochemical variables such structure, partition coefficient, pH, functional groups, dissociation constant, polarity, and solubility.

Liquid-liquid extraction (LLE): Important ideas in liquid-liquid extraction (LLE) include differential solubility and subatomic particle dispersion between organic and aqueous (original material) components. Fifth. Liquid-liquid extraction is the process of transferring a specific material from one liquid component to another. **6**

Solid phase extraction (SPE), a highly selective method of specimen preparation, binds the analyte to a solid substrate. After the interferences are eliminated, the analyte is selectively eluted. SPE performance is influenced by the volume of the loaded sample, the kind and amount of sorbent used, and the volume and composition of the washing and elution solutions.

Protein precipitation is a commonly used technique for protein extraction in biological research. An organic modifier can start the precipitation process. This alteration might be salt. Changes in pH can also cause precipitation.

Protein solubility is impacted by this. The sample is centrifuged, and the supernatant is put into the LC system. After that, it is dissolved in the proper solvent. The specimen then converges. Protein precipitation is often used with SPE5 to get a pure extract.

Methodology evolution: Mixtures can be separated using HPLC, a chromatographic technique. In natural chemistry and research, it is used to separate and purify a material's constituent parts.

1) Normal phase chromatography: Polar stationary and nonpolar flexible phases are combined in normal phase chromatography (NPC) to create polar mixtures.

2) Reversed phase chromatography: This method combines a polar dense phase with a non-polar stationary phase.

1) Mobile Phase: The stage may be moved. In these frameworks, the mobile phase is often a mixture of two or more distinct solvents, either with or without additional chemicals or naturally occurring dissolvable modifiers.

1a) Buffer determination: Analytes are identified by their hydrophobicity in switch stage HPLC. How effectively the analyte is maintained depends on its hydrophobicity. An analyte's ability to maintain itself is reduced when ionization causes it to lose its hydrophobicity.

1b) pH selection: There are two groups: low pH (1–4) and generally acceptable pH (4–7). In terms of curiosity, each spectrum has a certain function. Low pH has the advantage of strengthening the process while needing less filling area.

2. The Stationary Phase: Selecting the right stationary stage can boost method development's efficacy. For example, if a C8 stage retains fewer analytes than a C18 stage, it could be more effective.

2a) Column Length: Using whatever is available is a common error made by chromatographers. A typical C18 section is 250 cm by 4.6 mm. These components function well in many different setups.

2b) Internal Diameter: By selecting a shorter section with the appropriate stage, run times may be reduced and the optimal portable stage and elution configuration can be quickly determined.

Gradient Programming: Using a portable stage angle is the simplest and least complex way to design a methodology. Start with a low dissolvable quality and work your way up to a higher grade.

4) The Detector: UV-obvious detectors are the most widely used because they can detect a wide variety of analytes and are very selective for certain combinations.

5) Chromatogram

a) Retention: Analytes are safe to keep. If this happens, increase the solubility. There are more natural dissolvables in the portable stage, according to the changeover phase study.

b) Peak Shape: When employing reverse phase HPLC to isolate significant combinations, this is a prevalent problem. Always use high-quality silica phases to prevent issues.

ANALYZE THE STRATEGY

Technique validation is the process of determining if a newly developed analytical technique is suitable for its intended use, in accordance with ICH guidelines.

The following conditions must be met for bioanalytical verification:

1. Precision.

2. Accuracy.
3. Powerful
4. Limitations on Quantification
5. The boundaries of detection.
6. Hardiness
7. Compliance
8. Dependability
9. The matrix effect.

Table: 1 Criteria for Bio Analytical Verification:

Parameters	Definition	Method	Acceptance criteria
Accuracy	The closeness of comprehension between the esteem that is recognized as either a traditional genuine esteem or an accepted reference esteem, and the esteem found is communicated by the exactness of the expository system. This is also referred to as trueness ² .	It is advised to measure using a minimum of three concentrations within the predicted concentration range and a minimum of five determinations per concentration.	The nominal value or LLOQ (lower limit of quantification) should not differ by more than 20%. The mean value should be within 15%.
Precision	The precision of a methodical approach conveys the degree of comprehension (level of dissemination) between the arrangement of estimates obtained from several examinations of the same homogenous example under the suggested conditions ³ .	measured at least three concentrations and with a minimum of five determinations per concentration.	Precision shouldn't be more than 15%.CV (coefficient variation) or LLOQ shouldn't be more than 20%.
Robustness	A logical strategy's vigor is a measure of its capacity to remain unaffected by minor variations in approach parameters and provides an indication of its unwavering quality during routine application.		
Limit of quantification(LOQ)	The most insignificant measure of analyte in an example, which can be quantitatively selected with appropriate precision and accuracy, is depicted as the quantification uttermost compasses of a single consistent methodology ¹ .		. The analyte response should have an accuracy of 80–120% and a precision of no more than 20%.
Limit Of detection(LOD)	The lowest amount of analyte in an example that can be identified but not truly quantified under		

	stated exploratory circumstances is the discovery limit of a single explanatory approach.		
Ruggedness	The sensitivity of a technique to minor changes that may arise during normal analysis, such as slight variations in pH values, mobile phase composition, or temperature, is measured by its ruggedness.		
Linearity	An investigation strategy's linearity is its ability to obtain test results that are directly related to the example's analyte focus. The range of the investigative method is the difference between the analyte's upper and lower fixation in the case where it has been demonstrated that the scientific method has the proper degree of linearity, accuracy, and precision.	A blank sample, a zero sample, and six to eight non-zero samples encompassing the anticipated range, including LLOQ, should be included.	At least 10% of the anticipated maximum concentration should be the LLOQ. The upper limit of quantification, or ULOQ, need to be at least twice the anticipated Cmax value. R2 (regression coefficient) > 0.98.
Stability	During the method validation procedure, an analyte's stability under different circumstances must also be investigated. Stability experiments must be conducted in conditions that are likely to occur while handling and analyzing actual specimens. reeze-Thaw stability: An analyte's stability should be assessed following three freeze-thaw cycles. Short-Term Temperature Stability: An analyte's stability in a biological matrix at room temperature should be investigated. Long-term stability: An analyte's stability in the matrix should endure from the moment the specimen is collected until the final day of the experiment.	carried out using three aliquots at the target temperature for 24 hours following three freeze-thaw cycles at HQC (higher quality control) and LQC (lower quality control). Before analysis, three aliquots of each of the HQC and LQC should be thawed at room temperature and stored for four to twenty-four hours. The concentration of stability samples will be compared with the mean of back-calculated values of the standards from the first day of long-term stability testing, as determined by storing three aliquots of HQC and LQC under the identical circumstances intended for study samples.	The stability sample should be within 15%.

Matrix effect	The total impact of every sample component—aside from the analyte—on the measurement of quantity 4.	Three blank specimens will be retrieved from each of at least six batches of matrix that are being screened. In the aforementioned extracted blank specimens, Formatrix effect MQC (middle quality control), LQC, and HQC spiking dilutions are likewise spiked, as is internal standard dilution.	
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LITERATURE REVIEW :

Author(s) & Year	Objective	Method/Technique	Optimized Chromatographic Conditions	Key Findings	Conclusion
Mohan Goud Vanga et al. (2025)	To develop and optimize an RP-UPLC method using an Analytical Quality by Design (AQbD) approach for pharmaceutical analysis.	RP-UPLC method developed using Design-Expert® software and Central Composite Design (CCD); method validated as per ICH guidelines.	Column: ACQUITY UPLC HSS C18 SB; Mobile Phase: 0.01 N KH ₂ PO ₄ (pH 5.4): Acetonitrile (66.4:33.6, v/v); Flow Rate: 0.27 mL/min; Detection: PDA at 272.0 nm; Temperature: Ambient; Elution: Isocratic.	Statistical analysis confirmed a significant model with normal distribution. The method showed excellent linearity ($r^2 = 0.9997$) over the concentration range of 3.75–22.5 µg/mL, with LOD of 3.3 µg/mL and LOQ of 10 µg/mL.	The validated AQbD-based RP-UPLC method was found to be robust, accurate, and suitable for the analysis of bulk drugs and pharmaceutical formulations, with successful statistical comparison of results.

THE DRUG'S PROFILE :

The drug is called ensifentrine.

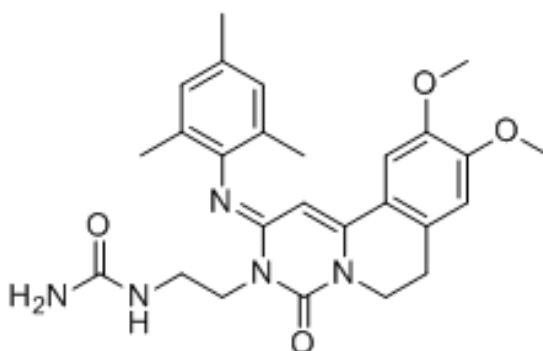


Figure :1 Structure of Ensifentrine

Patients with chronic obstructive pulmonary disease (COPD) are given ensifentrine, a phosphodiesterase inhibitor.

Organization:

CAS Number : 1884461-72-6

IUPAC Name : {2-[(2E)-9,10-dimethoxy-4-oxo-2-[(2,4,6-trimethylphenyl)imino]-2H,3H,4H,6H,7H-pyrimido[4,3-a]isoquinolin-3-yl]ethyl}urea

Average Molecular Weight : 477.565 Monoisotopic: 477.237604498

Molecular formula. : C₂₆H₃₁N₅O₄

Look : Powder

Physical Condition : Solid

Water Solubility : 0.0218 mg/mL

Log p : 3.27

pK values : pK_a: 5.23 (Basic), 14.74 (Acidic).

As a maintenance drug, ensifentrine may help adult patients with chronic obstructive pulmonary disease (COPD).

Pharmacodynamics :

Ensifentrine promotes bronchodilation in people with asthma and chronic obstructive pulmonary disease (COPD). Ensifentrine has been demonstrated to reduce proinflammatory cytokines such granulocyte-macrophage colony-stimulating factor (GM-CSF) in cystic fibrosis bronchial epithelial cells.

Mechanism of action : Ensifentrine primarily targets the PDE3 and PDE4 enzymes. When both PDE3 and PDE4 are suppressed, intracellular levels of cAMP and/or cGMP increase. This has a synergistic effect on airway smooth

muscle contraction and reduces the inflammatory response.

Absorption: C_{max} occurred 0.6–1.5 hours following the dose.

Distribution : The distribution volume is 2700 L and 1820 L for healthy people and 8150 L and 5490 L for COPD sufferers.

Protein binding : Proteins are bound 90% of the time.

Metabolism : Ensifentrine undergoes two primary metabolic processes: oxidation (hydroxylation and O-demethylation) and conjugation (glucuronidation). At physiologically relevant concentrations, ensifentrine was mostly metabolized by CYP2C9 and, to a lesser extent, CYP2D6.

Route of elimination: The primary method of ensifentrines' removal is through feces. After being administered, less than 0.3% of the 3 mg of nebulized ensifentrine was eliminated in urine. The half-life is between 10.6 and 12.6 hours.

METHODS AND MATERIALS :

Materials :

API: Ensifentrine API obtained as a gift sample from Spectrum Laboratories in Kukatpally, Hyderabad; and internal standard from Spectrum Laboratories in Kukatpally.

Human plasma provided by Spectrum Laboratories in Hyderabad;

Chemical and Reagents

Table 2 : Chemicals and Reagents :

Sr. No.	Chemicals	Quality
1	Acetonitrile	HPLC grade
2	Water	HPLC grade
3	Methanol	HPLC grade
4	Potassium di hydrogen orthophosphate	AR grade
5	Orthophosphoric acid	AR grade
6.	Millipore membrane 0.2 um	-
7	Ensifentrine	-

Equipment and Instruments

Table 3 : Equipment and Instruments

Sr. No.	Instruments	Make and model
1	UV Spectrophotometer	Microprocessor UvVisibleSingleBeam
2	HPLC	Waters Alliance 2695
3	Analytical weighing Balance	Sortorious, ScaletecBsa224s-Cw
4	Ultrasonicator	Lab Man
5	pH Meter	Lab Man

6.	Hot air oven	Sisco
7	Vortex meter	Remi

METHODS FOR TESTING AND ANALYZING

Prepare a solution.

Each sonication solution was made, utilized just once, and stored at room temperature.

The next section discusses buffer preparation techniques and possible remedies.

Preparation of diluent :

A 50:50 acetonitrile and water solution was used as the diluent to create a diluted (v/v) solution based on the drug's solubility.

Preparation of Buffer (v/v): In a 1000 mL volumetric flask, add 1.36 g of potassium dihydrogen orthophosphate to create the buffer (v/v): 0.01N KH₂PO₄ Buffer. To degas and sonicate, add around 900 milliliters of milli-Q water. Finally, dilute the orthophosphoric acid with water until the pH reaches 4.8.

Keeping prescription drugs available.

To create the standard, weigh out 1.25 mg of ensifentrine and transfer it to a 100 mL dry, clean volumetric flask. After adding 3/4 volume diluent and sonicating for five minutes, dilute to the desired volume. Filter the mixture with 0.5µm Hplc nylon filters to remove the 25 µg/ml ensifentrine.

KH₂PO₄ Buffer Preparation (V/V, 0.01N) Fill a 1000 mL volumetric flask with precisely 1.36 g of potassium dihydrogen orthophosphate. Degas about 900 milliliters of milli-Q water before sonication. Finally, dilute a solution of orthophosphoric acid with water until the pH is 4.8.

Preparing the Stock Solution:

Common Preparation:

To make the standard, weigh and transfer 1.25 mg of ensifentrine into a 100 mL dry, clean volumetric flask. Add the remaining diluents to get the desired volume after adding 3/4 of the diluent and sonicating for five minutes. Filter the mixture using Hplc nylon 0.5µm filters to remove the 25 µg/ml Ensifentrine.

Standard Working Solution: Use pipettes to transfer 0.05 mL, 0.1 mL, 0.15 mL, 0.4 mL, 1.0 mL, 1.2 mL, 1.6 mL, and 2.0 mL into eight distinct 10 mL volumetric flasks.

CHOOSING AN INTERNAL STANDARD:

Selecting an internal standard: Bioanalysis often suggests internal standards. Labeled information systems are

inappropriate in many situations because they are typically too expensive to operate and difficult to access through approved channels. Internal standards (IS) are also developed using analyte structural homologs. Nonetheless, the selected information systems (IS) must have the following characteristics:

1. The system is really stable.
2. There is no interaction between the analyte and the interstitial substance (IS).
3. The extraction efficiencies of analyte and internal standard (IS) are comparable.
4. Analytical variability is reduced since analyte behavior is consistent before and after sample preparation.
5. An overall increase in process reliability.

Teneligliptin Standard Internal Stock Solution

After weighing, add 2 mg of Teneligliptin working standards to a 100 mL dry, clean volumetric flask. Use diluents to measure the final volume after adding three-quarters of the diluent and sonicating for five minutes. Teneligliptin (20 ppm/µg/ml) is filtered using HPLC nylon 0.5µm filters.

To get a final concentration of 0.08µg/ml ISD, mix 0.5 ml of solution, working stock dilutions of the analyte, and spike blank plasma.

establishing quality control (QC) samples and calibration curve (CC) standards.

To make quality control (QC) samples, working stock analyte dilutions were mixed with blank plasma.

The standard value, or LLOQ, is 0.5 ng/mL.

1.0 ng/mL is Standard 2.

1.5 ng/mL is Standard 3.

Standard-4: 4.0 ng/ml

Standard-5/MQC is 10.0 ng/ml.

Standard-6 is 12.0 ng/ml.

Standard-7/HQC is 16.0 ng/ml, and

The Standard-8/ULOQ is 20.0 ng/ml.

Table no-4 CC spiking solutions of Ensifentrine:

Spiking solution	Pipette out ML	make up in ML	spiking in ML	make upon ML	final conc in ng/ml
Standard-1	0.050	50	0.05	2.5	0.5
Standard-2	0.100	50	0.05	2.5	1

Standard-3	0.150	50	0.05	2.5	1.5
Standard-4	0.400	50	0.05	2.5	4
Standard-5	1.000	50	0.05	2.5	10
Standard-6	1.200	50	0.05	2.5	12
Standard-7	1.600	50	0.05	2.5	16
Standard-8	2.000	50	0.05	2.5	20

AN EXTRACTION METHOD FOR BIO SAMPLE ANALYSIS.

In this experiment, the protein precipitation technique was used to extract 500µl of internal standard, 50µl of ensifentrine, and 0.75µl of plasma from rabbit plasma. In the next step, teneligliptin was used as an internal standard. After adding 1.2 mL of acetonitrile, the mixture was vortexed for two minutes, cyclomixed for fifteen seconds, then centrifuged for five minutes at 3200 rpm to remove the supernatant. After being filtered, ten microliters of the material were submitted to an HPLC for analysis.

Data analysis

The data was collected and evaluated using the Analyst software version 2.2. A verified Excel sheet was used to construct statistics such as mean, SD, and % CV for the analytical data collected during technique validation.

VALIDATION METHODOLOGY IN BIOANALYTICAL METHOD: -

System Suitability Parameter: The element that establishes the system's appropriateness.

To ensure that the test combination meets the specifications of a liquid chromatographic system, system suitability tests are performed. System suitability testing is limited by acceptance requirements that must be fulfilled before sample analysis.

Methodology: The study begins with six MQC quality control samples from a single vial.

Acceptance criteria include a percentage CV of area ratio of less than 5.00% and a percentage CV of retention time (RT) of less than 2.00%.

Automation of Carryover Samplers :

Analyte residue from a previous sample that is still in the analysis equipment may cause carry-over, or a change in the reported concentration. To ascertain carry-over during validation, compare blank samples to the calibration standard at the ULOQ level.

Methodology: High-performance liquid chromatography (HPLC) was used to assess the possibility of carry-over. The following samples were injected one after the other in order to measure carryover.

A blank is a mobile phase solution that uses water as the solvent.

ULOQ stands for Blank, Standard Quality Control, and Lower Standards (AQ-LLOQ).

Acceptance criteria: After aqueous or extracted ULOQ, the carryover area response for consecutive bulk injections of RS or STD must be less than 20% of the equivalent standard area for aqueous or extracted LLOQ.

Biomatrix specificity and screening.

Specificity is the capacity of a bioanalytical technique to differentiate analytes from other molecules, such similar compounds.

Methodology:

Six samples of standard and LLOQC sample solutions are injected in order to evaluate specificity.

Acceptance requires a minimum of 80% of matrix lots (Biological Sample) that satisfy anticoagulant standards and a proportion of interfering peaks in STD Blk at the analyte retention time that is $\leq 20.00\%$ of the LLOQ.

Sensitivity

While LLOQ is the lowest concentration of a sample that may be recognized within an acceptable range, sensitivity is frequently characterized in terms of detection/determination capabilities.

Methodology:

Six lower drug doses (LLOQ) are administered in order to evaluate sensitivity.

Acceptance criteria: For the samples to be deemed acceptable, at least 67% (4 of 6) of them must have an LLOQ sensitivity of 80.00% to 120.00%.

Looking at the matrix factor.

A matrix effect is a change in analyte reactivity caused by one or more unknown components in the sample matrix. When validating a procedure, consider the matrix influence of many independent sources or lots.

Methods: To assess the matrix effect, at least three replicates of low and high QCs (LQC and HQC) should be analyzed, each utilizing a matrix from at least six distinct sources or lots.

Precision (percent coefficient of variation) should not exceed 15%, and accuracy should be within $\pm 15\%$ of the nominal concentration.

Linearity (range and calibration curves).

The analytical platform's response to the nominal analyte concentration is displayed by the calibration curve. To create calibration standards, a fixed quantity of analytes is injected into a matrix. Calibration standards should be

made using the same biological matrix that was used to make the research materials.

Methodology: The calibration range is established by injecting six calibration standard concentrations, removing blank and zero samples, and utilizing the sample regression model to ascertain the concentration-response relationship.

Acceptance criteria: Except for the LLOQ (STD 1) standard, all CC standards should have an accuracy percentage between 85.00 and 115.00.

The accuracy percentage should be between 80.00 and 120.00%, according to the LLOQ standard.

Linearity in respect to time.

A technique's linearity ruggedness indicates how vulnerable it is to minor alterations that may occur during normal analysis.

Methodology: -With the exception of blank and zero samples, six calibration standard concentrations are injected in order to ascertain the calibration range. Next, use the sample regression model approach to ascertain the concentration-response relationship.

Acceptance criteria: All CC standards, with the exception of LLOQ (STD 1), need a percentage accuracy of 85.00 to 115.00. The LLOQ standard calls for an accuracy percentage between 80.00 and 120.00 percent.

Accuracy and Precision (interday)

Verify the quality controls both during and between runs to ensure accuracy and precision. The same tests and data should be used to evaluate accuracy and precision.

Methodology: For every analytical run, six duplicates of each QC concentration level were used.

Acceptance criteria: Except for the LLOQ, which must be within $\pm 20\%$, accuracy must be within $\pm 15\%$ of nominal concentration at all concentration levels.

Exceptional accuracy and precision over time.

The same runs and data should be utilized to evaluate accuracy and consistency.

Methodology: Six injections of QC samples are made at each QC concentration level during each analytical run.

Accuracy must be within $\pm 15\%$ of the nominal concentration at all concentration levels, except for the LLOQ, which requires $\pm 20\%$. The precision (%CV) of the concentrations found at each level should not exceed 15%, with the exception of the LLOQ, which should not exceed 20%.

Recuperation

Peak areas from synthetic plasma samples were compared to extracted blank plasma spiked with standards that had the same area and a known drug dosage in order to assess recovery.

Methodology: Teneligliptin recoveries at LQC, MQC, and HQC levels showed the improved extraction efficiency of the bioanalytical approach. Efficiency by assessing the interference between the extracted and unextracted samples after giving the primary drug to the six LQC, MQC, and HQC samples.

To enter, you need to meet the following requirements:

At all QC levels, the recovery percentage CV shouldn't be more than 15%. For every quality control level, the average recovery percentage CV should be less than 20%.

The Internal Recuperation Standard

Peak areas in produced plasma samples are compared to Internal Standards from the same location as well as extracted blank plasma that has been spiked with a known pharmaceutical dosage.

Methodology: The IS recoveries after six cycles of injecting the six samples and comparing interference with extracted and unextracted samples showed the bioanalytical technique's exceptional extraction performance.

Acceptance criteria: Each QC level's CV of recovery must be less than 15.00%. All quality control levels must have an average recovery percentage CV of less than 20.00%.

The reliability of reinjection

Replication evaluates how repeatable the process is.

Quality control techniques are frequently used to evaluate precision and accuracy. The repeatability of any samples that may be reinjected (due to instrument interruptions or other reasons, such as equipment malfunction) should be evaluated and documented in the study's Validation Report or Bioanalytical Report.

Methodology: Repeatability was evaluated following six injections of QC samples and the determination of acceptable limits.

Acceptance criteria: The LLOQ QC sample should be between 80.00 and 120.00%, and the percentage mean accuracy for LQC, MQC, and HQC samples should be between 85.00 and 115.00%.

Frequency :

to ensure that analyte concentrations are unaffected by any activities performed during sample preparation, processing, analysis, or storage conditions. The stability of matrix samples and long-term stock solutions was assessed at -80 ± 5 °C and -28 ± 5 °C for 37 days. The biological matrix is supplemented with varying quantities of QC samples (HQC and LQC).

Acceptance criteria: The mean concentrations for each QC level must be within $\pm 15\%$ of the nominal values.

TALKS AND RESULTS: DISCUSSION AND RESULTS

METHOD DEVELOPMENT

The ensifentrine method estimate was calculated using drug solubility and Pka Value in compliance with the most recent ICH recommendations.

The parameters of chromatography are changed.

The ensifentrine test was developed by carefully examining the impact of several parameters by changing one parameter at a time while maintaining the same values for all other variables. As a result, the following studies were initiated. A high purity advance C18 column served as the stationary phase in this investigation. The mobile phase and flow rate to establish a baseline and identify peaks. Many line separation experiments were conducted on the components by varying the frequently used solvents, compositions, and flow rates. To get the optimum drug separation under isocratic conditions, mixtures of widely used solvents, including as water, methanol, and acetonitrile, with or without buffers in various

combinations, were assessed as mobile phases on a C18 stationary phase. The chromatographic peaks were crisp, resolved, and tail-free, indicating the ideal ratio of 60:40 v/v between acetonitrile and 0.1% OPA buffer. A mobile phase flow rate of 1.0 mL/min was determined to be appropriate.

Optimal approach:

Conditions of chromatography

Mobile phase : Acetonitrile: 0.1% OPA 40:60v/v

Flow rate : 1.0ml/min

Column : Agilent 250mm x 4.6mm , 5 μ m

Detector wavelength: 348nm

Column temperature : 30°C

Injection volume : 10 μ L

Run time : 5.0min

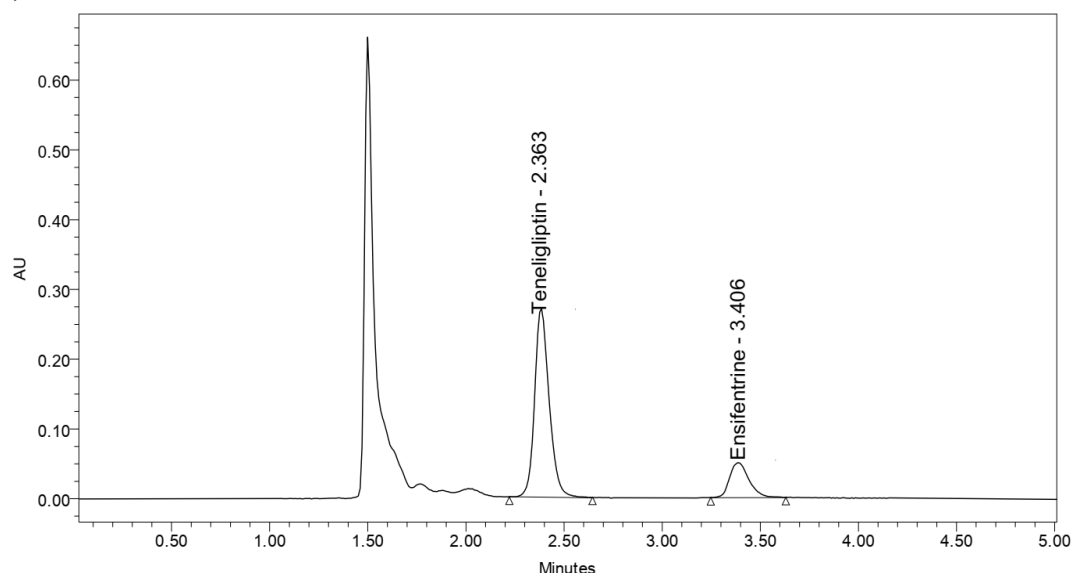


Fig no 2: Chromatogram of Optimized Chromatogram

Table no 5: Observation of Optimized Chromatogram

	Peak Name	RT	Area	USP Plate Count	USP Tailing	USP Resolution
1	Teneligliptin	2.363	268326	2973	1.20	
2	Ensifentrine	3.406	88923	3468	1.10	3.7

Observation: The internal standard and ensifentrine eluted with high resolution (IS) at 2.363 and 3.406 minutes, respectively. This method was optimized and has to be verified because the tailing factor and plate count were so high. All system-specific parameters, including plate count and tailing factor, were within acceptable bounds, and drugs were eluted with adequate retention time and resolution.

Techniques for Validation

Ensifentrine's system's efficacy

This system suitability method makes it possible to regularly provide accurate and repeatable data to regulatory bodies by guaranteeing that the HPLC equipment is operating as intended. Tests to ascertain signal stability, carryover, and instrument responsiveness are part of this method.

Table no 6: System Suitability of Ensifentrine :

Analyte	Ensifentrine	ISTD	Teneligliptin			
Sample Name		Analyte Area	Analyte RT (min)	ISTD Area	ISTD RT (min)	Area Ratio
AQ MQC		88868	3.43	264866	2.38	0.3355
AQ MQC		89582	3.44	270655	2.38	0.3310
AQ MQC		89189	3.44	259786	2.37	0.3433
AQ MQC		88999	3.45	266436	2.37	0.3340
AQ MQC		89208	3.45	265522	2.38	0.3360
AQ MQC		88656	3.46	265123	2.39	0.3344
MEAN			3.443		2.378	0.33570
SD			0.0093		0.0084	0.004120
%CV			0.27		0.35	1.23

We looked at ensifentrine resolution, tailing factor, and plate count. The ICH criteria call for a tailing factor of less than two, a resolution of more than two, and a plate count of more than 2000. All system-appropriate parameters were within the allowed range and passed. The retention time (RT) CV must be less than 2%.

Transfer of ensifentrine auto samplers

Tracking the carryover back to the injection valve and eliminating it by switching from partial loop injection to complete loop injection allowed for a more thorough cleaning of the sample flow channel. the mass of analyte injected at the assay's lower limit of quantification (LLOQ) and the detection method's absolute sensitivity.

Table 7: Auto sampler carryover of Ensifentrine .

Sample ID	Peak Area		% Carryover	
	Drug	ISTD	Drug	ISTD
Unextracted samples				
RS	0	0	N/A	N/A
AQ ULOQ	176522	265743	0.00	0.00
RS	0	0		
AQ LLOQ	3736	265352	N/A	N/A
Extracted samples				
STD Blk	0	0	N/A	N/A
ULOQ	172970	262165	0.00	0.00
STD Blk	0	0		
LLOQ	3636	263182	N/A	N/A

Discussion: After two injections, less than 20% of the recovered LLOQ standard area remains unextracted.

Specificity is the capacity to precisely assess the analyte in the presence of expected variables.

Biomatrix Screening and Specificity

Table 8: Ensifentrine Biological Matrix Specificity and Screening.

S.No.	Parameters	Response		% Interference		Pass/Fail
		Drug	ISTD	Drug	ISTD	

1	STD Blk1	0	0	0.00	0.00	Pass
2	LLOQ1	3612	268812			
3	STD Blk2	0	0	0.00	0.00	Pass
4	LLOQ2	3553	267175			
5	STD Blk3	0	0	0.00	0.00	Pass
6	LLOQ3	3575	258866			
7	STD Blk4	0	0	0.00	0.00	Pass
8	LLOQ4	3592	261484			
9	STD Blk5	0	0	0.00	0.00	Pass
10	LLOQ5	3568	275015			
11	STD Blk6	0	0	0.00	0.00	Pass
12	LLOQ6	3614	262584			

Observation: Using this method, we were unable to identify any interfering peaks in the blank and placebo samples during the medication retention periods. As a result, this strategy was called "particular."

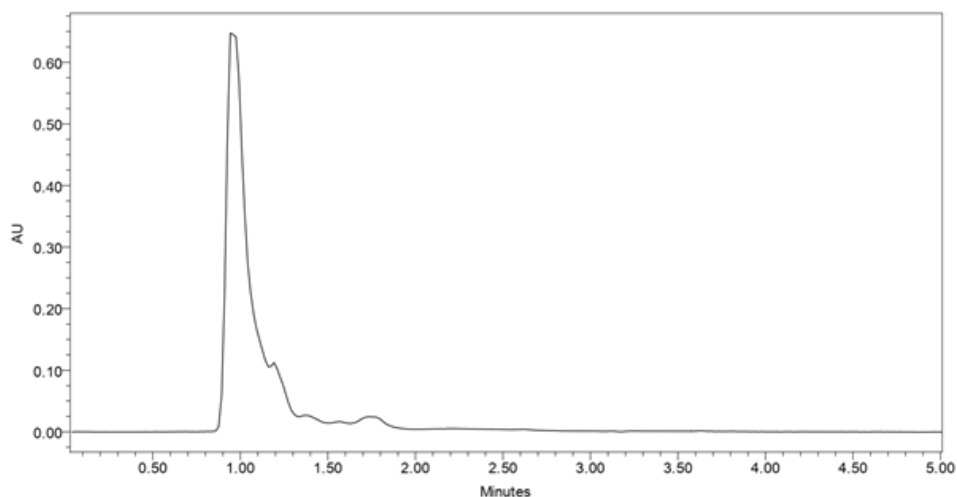


Figure 3 Typical Chromatogram of a Sample of Blank Plasma

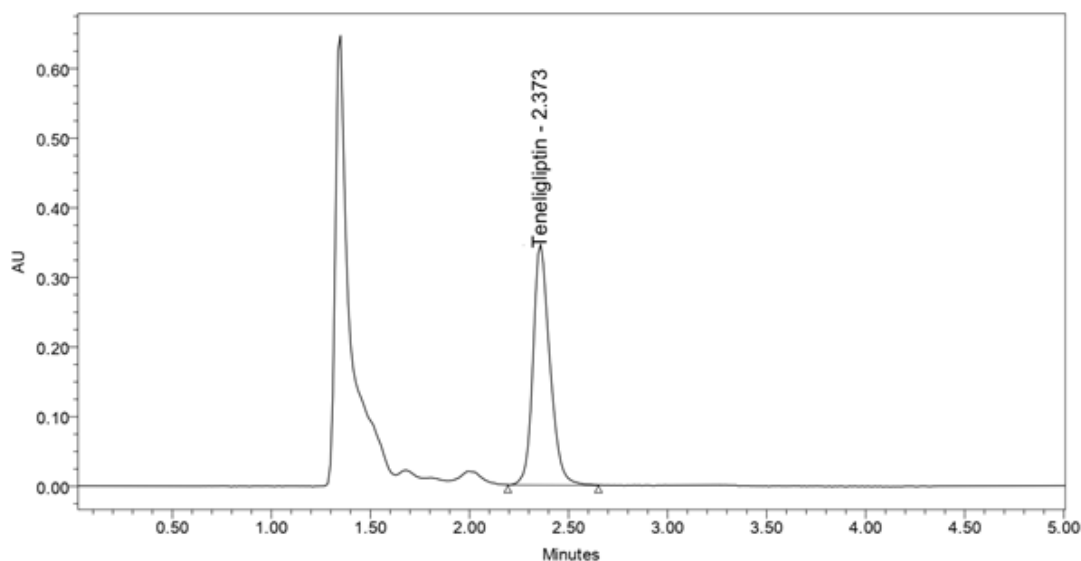


Figure 4: Blank Plasma Representative Chromatogram with Internal Standard

Sample Discussion: The analyte and internal standard response zones span less than 20% and 5% of the LLoq Area, respectively. We were unable to identify any conflicting peaks in the blank and placebo during the medication retention periods using this strategy. It was said that this process was special.

Sensitivity

The definition of sensitivity is "the lowest analyte concentration that can be measured with acceptable accuracy and precision, i.e., LLoQ."

Table 9: Sensitivity of Ensifentrine.

Analyte	Ensifentrine	Teneligliptin
S No.	LLOQ	
	Nominal Concentration (ng/mL)	
	0.500	
	Nominal Concentration Range (ng/mL)	
	(0.400-0.600)	
	Calculated Concentration (ng/mL)	
1	0.497	
2	0.501	
3	0.511	
4	0.508	
5	0.492	
6	0.494	
n	6	
Mean	0.5005	
SD	0.00766	
% CV	1.53	
% Mean Accuracy	100.10	
No. of QC Failed	0	

Discussion: The mean of six injections was 100.10% ensifentrine, which was within acceptable limits. The range of LLOQ concentration was 80–120%. The system

sensitivity was passed using this approach since the Sensitivity% CV limit was less than "20%." 1.53% of ensifentrine was the coefficient of variance.

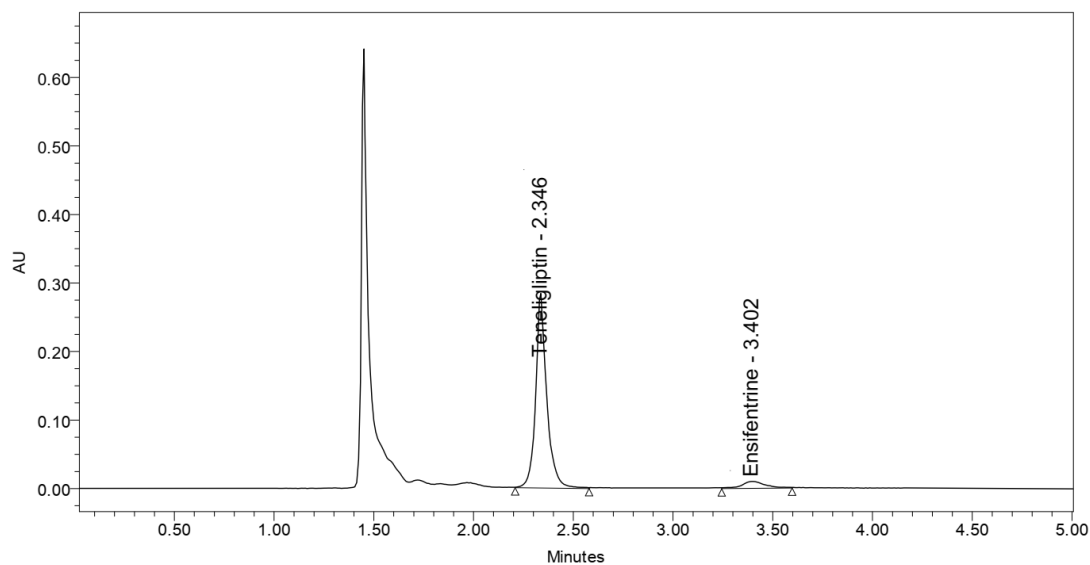


Figure5: Chromatogram of Sensitivity (LLOQ)

Table no 10: Matrix factor evolution of Ensifentrine (absence of matrix factor)

Analyte	Ensifentrine	ISTD	Teneligliptin
S. No.	Plasma Lot No.	HQC	LQC
		Nominal Concentration (ng/mL)	
		16.000	1.500
		Nominal Concentration Range (ng/mL)	
		(13.600-18.400)	(1.275-1.725)
		Calculated Concentration (ng/mL)	
1	LOT1	15.800	1.489
		15.987	1.425
		16.100	1.423
2	LOT2	16.060	1.521
		16.034	1.502
		16.002	1.502
3	LOT3	16.012	1.501
		16.029	1.508
		16.066	1.499
4	LOT4	16.122	1.425
		16.119	1.526
		16.145	1.504
5	LOT5	16.152	1.506
		16.098	1.512
		16.099	1.532
6	LOT6	15.986	1.521
		16.050	1.521

		16.069	1.532
n		18	18
Mean		16.0517	1.4972
SD		0.08126	0.03555
% CV		0.51	2.37
% Mean Accuracy		100.32	99.81
No. of QC Failed		0	0

Discussion: Ensifentrine's %Mean was 100.32% and 99.81%, while its %CV was 0.51% and 2.37% of HQC and LOQ, respectively. Given that the CV limit was below "20%," the system matrix passed. To assess the Matrix,

QC samples were injected into six batches at both high and low doses.

Linearity

Table 11: Linearity of Ensifentrine:

Analyte	Ensifentrine						ISTD	Teneligliptin
	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8
	Nominal Concentration (ng/mL)							
	0.500	1.000	1.500	4.000	10.000	12.000	16.000	20.000
	Nominal Concentration Range (ng/mL)							
	(0.400-0.600)	(0.850-1.150)	(1.275-1.725)	(3.400-4.600)	(8.500-11.500)	(10.200-13.800)	(13.600-18.400)	(17.000-23.000)
	Back Calculated Concentration (ng/mL)							
P&A1	0.504	1.012	1.504	3.98	10.10	11.98	15.59	20.01
P&A2	0.501	1.009	1.498	3.95	10.05	11.82	15.94	20.08
P&A3	0.511	0.989	1.478	3.99	10.03	12.01	15.99	20.00
n	3	3	3	3	3	3	3	3
Mean	0.5054	1.0033	1.4934	3.9727	10.0600	11.9367	15.8400	20.0310
SD	0.00501	0.01250	0.01373	0.02421	0.03606	0.10214	0.21794	0.04279
%CV	0.99	1.25	0.92	0.61	0.36	0.86	1.38	0.21
% Mean Accuracy	101.09	100.33	99.56	99.32	100.60	99.47	99.00	100.16

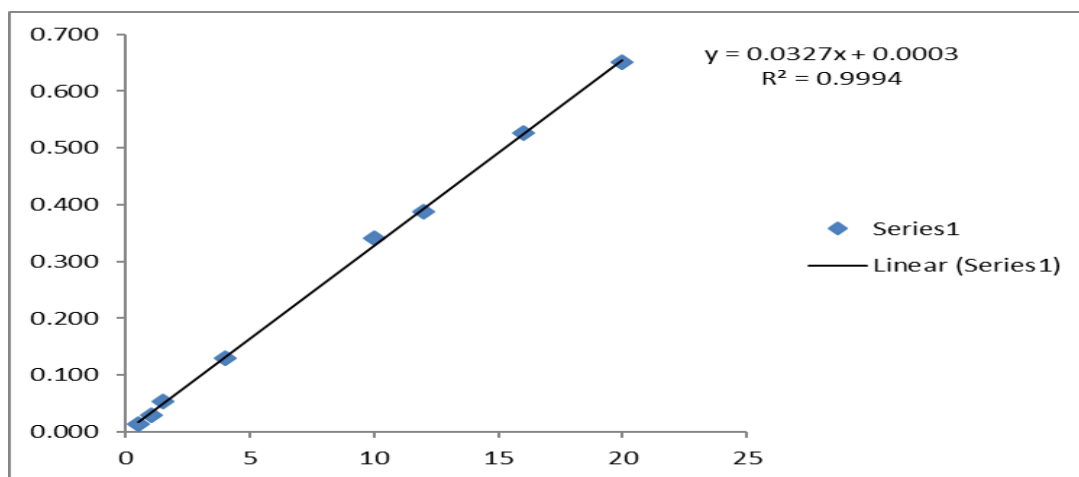


Figure 6: Typical Calibration Curve for Ensifentrine Regression Analysis

Discussion: It was shown that the calibration was linear across the ensifentrine concentration range of 0.5–20 ng/ml. The coefficient correlation (r^2) value was more than 0.999 in each case. This demonstrates that the results are linear and that there is a strong connection between the peak area ratios for each analyte concentration.

Acceptance Criteria:

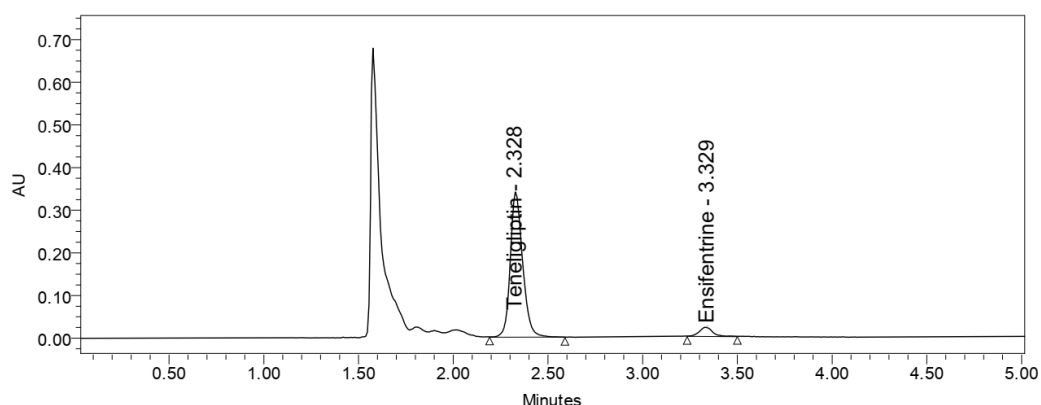
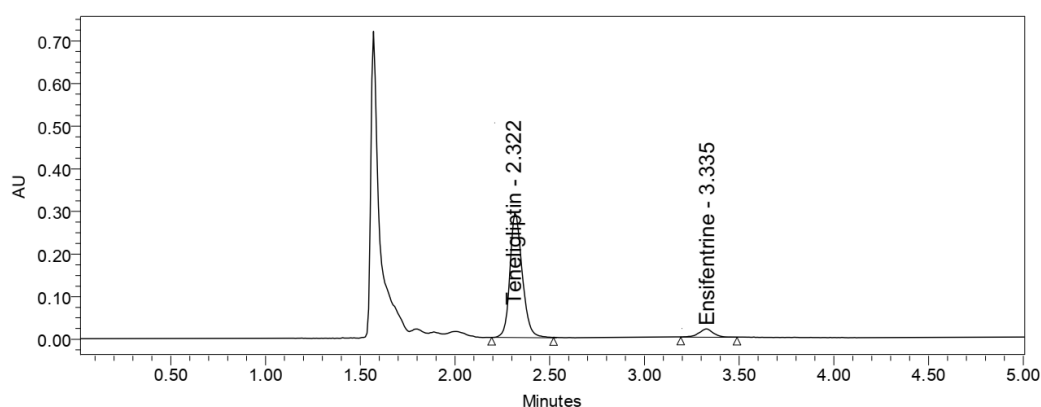
The correlation coefficient (r^2) should be at least 0.98.

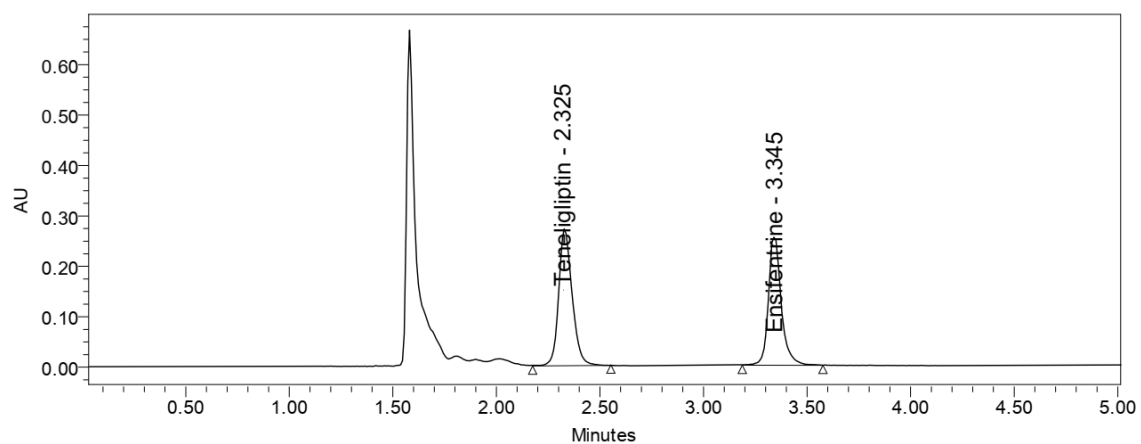
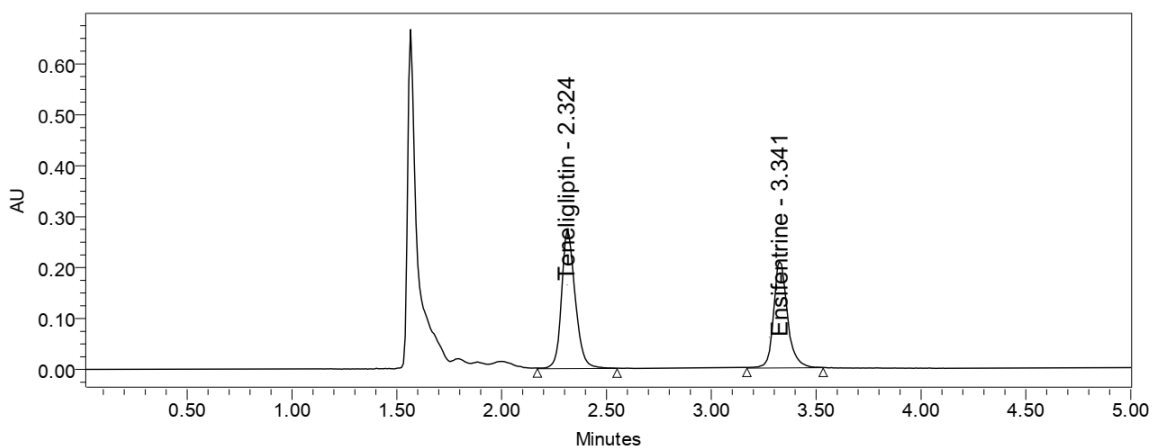
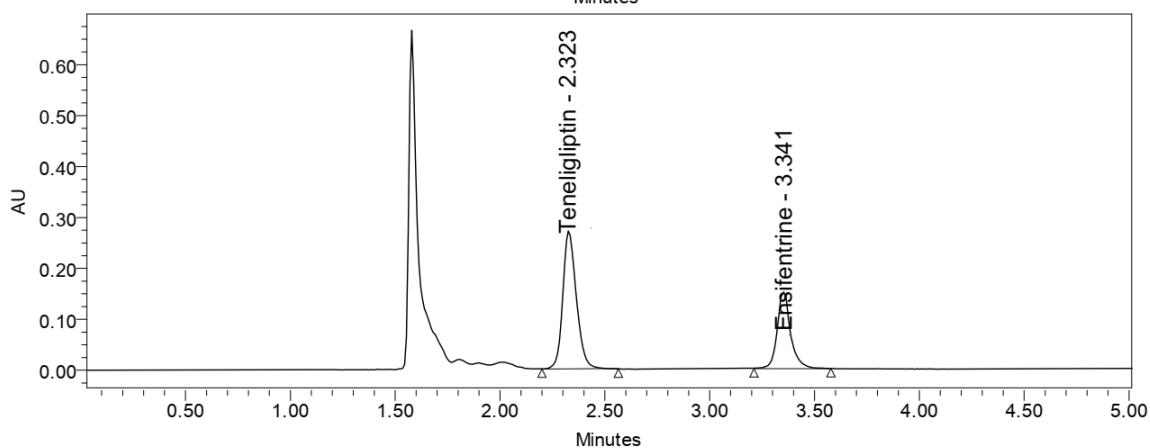
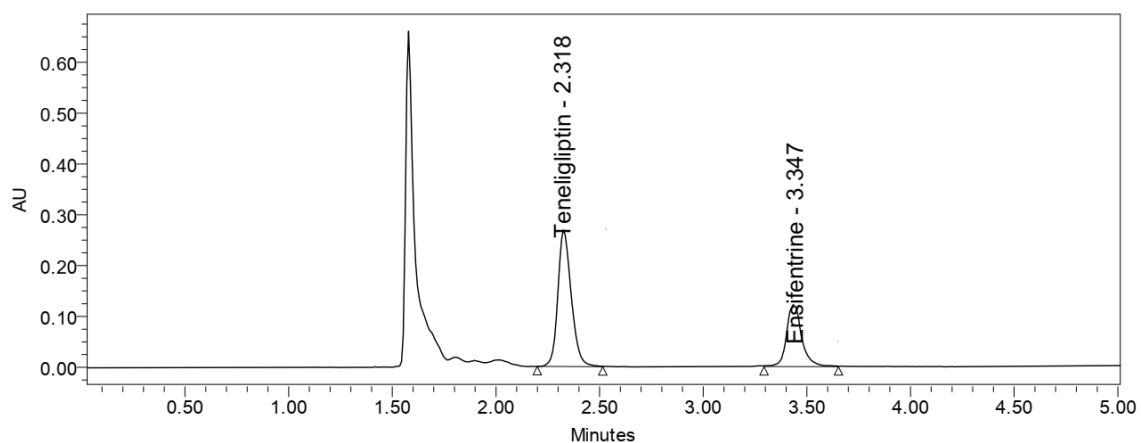
The nominal value and the LLOQ may differ by 20%.

For standards other than LLOQ, a variance of $\pm 15\%$ from the nominal value is allowed.

Every two conceptual CCs must have the aforementioned qualities.

The criteria must be met by 75%, or at least 6 non-zero CCs, including the LLOQ and maximum concentration.





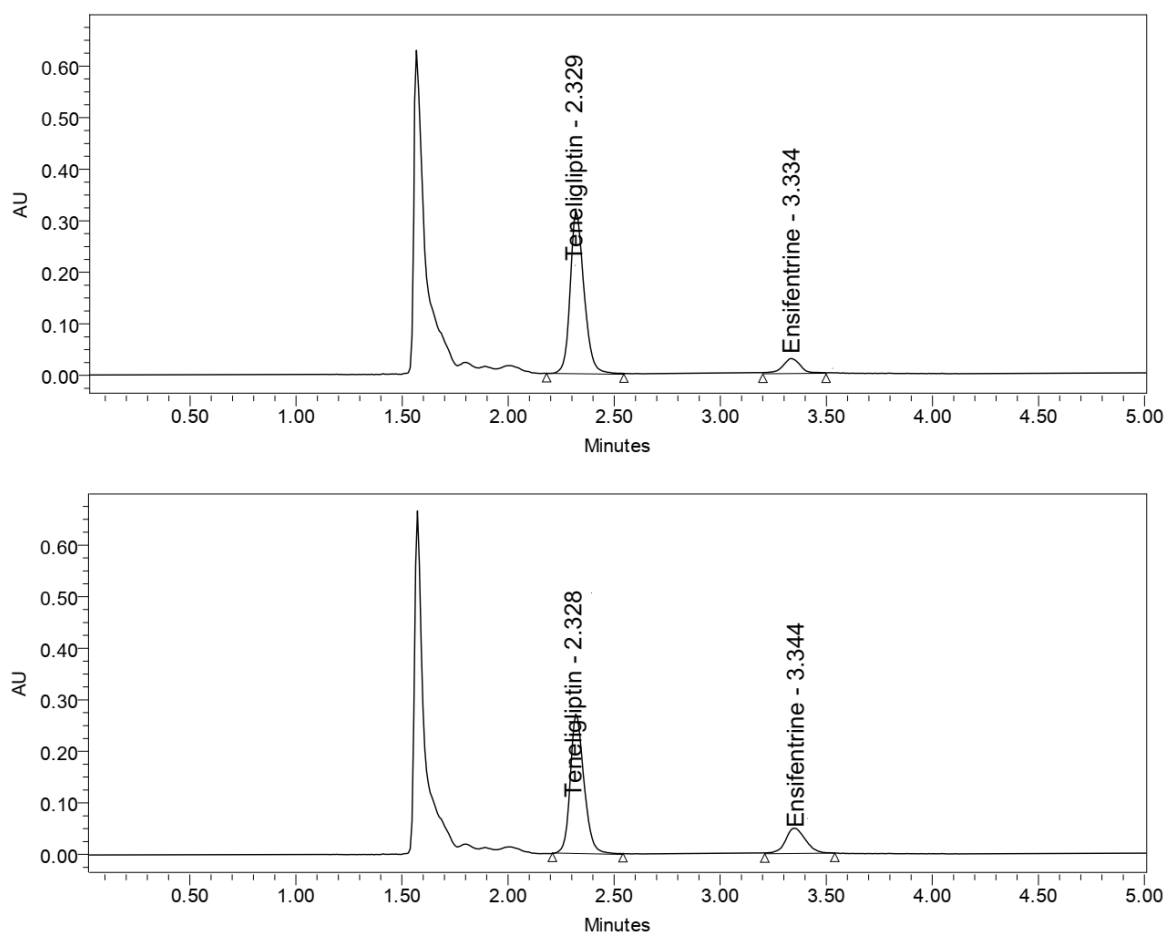


Figure 7: CC and Qc Sample of Chromatograms

Precision and accuracy (intra-day runs of Ensifentrine)**Table no12:** precision data for intra-day runs of Ensifentrine

Analyte	Ensifentrine		Teneligliptin	
	HQC	MQC1	LQC	LLOQ QC
	Nominal Concentration (ng/mL)			
	16.000	10.000	1.500	0.500
	Nominal Concentration Range (ng/mL)			
	(13.600-18.400)	(8.500-11.500)	(1.275-1.725)	(0.400-0.600)
	Back Calculated Concentration (ng/mL)			
	15.69	10.60	1.523	0.502
	15.94	9.63	1.504	0.518
	15.27	10.25	1.509	0.509
	16.35	9.24	1.512	0.482
	16.40	9.99	1.512	0.510
	15.39	10.20	1.524	0.535
n	6	6	6	6

Mean	15.8400	9.9850	1.5140	0.5093				
SD	0.47615	0.48492	0.00792	0.01752				
%CV	3.01	4.86	0.52	3.44				
% Mean Accuracy	99.00	99.85	100.93	101.87	15.26	10.21	1.521	0.498
15.30	10.23	1.456	0.478					
16.07	10.12	1.413	0.499					
16.28	10.23	1.502	0.505					
16.34	10.04	1.519	0.507					
16.25	10.26	1.517	0.510					
n	6	6	6	6				
Mean	15.9167	10.1812	1.4880	0.4995				
SD	0.50146	0.08616	0.04414	0.01150				
%CV	3.15	0.85	2.97	2.30				
% Mean Accuracy	99.48	101.81	99.20	99.90	15.96	10.26	1.498	0.491
16.01	9.26	1.504	0.495					
15.36	9.99	1.508	0.501					
16.00	10.11	1.478	0.510					
16.29	10.19	1.510	0.516					
16.19	10.26	1.569	0.507					
n	6	6	6	6				
Mean	15.9688	10.0108	1.5112	0.5033				
SD	0.32425	0.38192	0.03060	0.00944				
%CV	2.03	3.82	2.02	1.88				
% Mean Accuracy	99.81	100.11	100.74	100.67				
Between Batch Precision and Accuracy								
n	18	18	18	18				
Mean	15.9085	10.0590	1.5044	0.5041				
SD	0.41777	0.34967	0.03179	0.01314				
%CV	2.63	3.48	2.11	2.61				
% Mean Accuracy	99.43	100.59	100.29	100.81				

Rugged Precision and Accuracy (inter-day runs of Ensifentrine)**Table no 13:** precision data for inter-day runs of Ensifentrine .

Analyte	Ensifentrine		ISTD	Teneligliptin
	HQC	MQC1	LQC	LLOQ QC
	Nominal Concentration (ng/mL)			

	16.000	10.000	1.500	0.500
	Nominal Concentration Range (ng/mL)			
	(13.600-18.400)	(8.500-11.500)	(1.275-1.725)	(0.400-0.600)
	Calculated Concentration (ng/mL)			
Different Column	16.230	10.260	1.498	0.505
	16.020	10.120	1.499	0.509
	16.037	10.102	1.507	0.516
	16.115	10.240	1.513	0.518
	16.163	10.192	1.511	0.501
	16.152	10.126	1.519	0.501
n	6	6	6	6
Mean	16.1195	10.1733	1.5078	0.5083
SD	0.07986	0.06705	0.00821	0.00737
% CV	0.50	0.66	0.54	1.45
% Mean Accuracy	100.75	101.73	100.52	101.67
Different Analyst	16.017	10.056	1.503	0.497
	15.059	10.158	1.510	0.492
	16.000	10.124	1.532	0.519
	16.150	10.150	1.515	0.509
	16.489	10.160	1.500	0.523
	16.184	10.019	1.508	0.517
n	6	6	6	6
Mean	15.9832	10.1112	1.5114	0.5095
SD	0.48571	0.05965	0.01135	0.01258
% CV	3.04	0.59	0.75	2.47
% Mean Accuracy	99.89	101.11	100.76	101.90
No. of Failed QC's	0	0	0	0

Discussion: To evaluate intraday and interday accuracy and precision, six duplicates were inspected at five different QC levels (LLOQ, LQC, MQC, and HQC). Six duplicate analyses of ensifentrine at four concentration levels (LLOQ, LQC, MQC, and HQC) were used to assess the approach's accuracy and precision. Both intraday and interday plasma sample accuracy were remarkable, with mean percent accuracy ranging from 99.43% to 100.81% for intraday and 99.89% to 101.90% for interday. Analyte

and plasma samples had precision (%CV) values of 2.11-3.84% intraday and 0.59%-3.04% interday.

Acceptance Requirements:

Precision should be between 15% and 20% for LLOQ conc for low, medium, and high QC concentrations.

Recovery of Ensifentrine: QC concentrations at low, mid, and high levels should be between $\pm 15\%$ to $\pm 20\%$ of typical values.

Table 14: Recovery of Ensifentrine.

S No.	HQC		MQC1		LQC	
	Un extracted Response	Extracted Response	Un extracted Response	Extracted Response	Un extracted Response	Extracted Response

1	e	136751	89399	88954	13908	13848
2	138331	137423	89204	89125	13910	13897
3	138934	137927	89313	88950	13944	13834
4	137581	136813	89674	89262	13892	13832
5	138323	137427	89125	89166	13916	13894
6	138082	137471	89876	89155	13980	13828
n	6	6	6	6	6	6
Mean	138332	137302	89432	89102	13925	13856
SD	480.55	445.46	288.85	124.93	31.84	31.72
% CV	0.35	0.32	0.32	0.14	0.23	0.23
% Mean Recovery	99.26		99.63		99.50	
Overall % Mean Recovery	99.463					
Overall SD	0.1907					
Overall % CV	0.19					

Recovery - Internal standard**Table no 15:** Recovery of Teneligliptin (IS)

S.No.	Un extracted Area Ratio	Extracted Area Ratio
1	267280	264882
2	265843	265671
3	271014	262802
4	267471	266452
5	265985	265538
6	266477	265139
n	6	6
Mean	267345.0	265080.7
SD	1915.24	1238.82
% CV	0.72	0.47
% Mean Recovery	99.15	

Discussion: Peak areas from produced plasma samples were compared to extracted blank plasma that had been spiked with standards that had the same area and a known ensifentrine dosage in order to calculate recovery.

Acceptance Standards:

For each QC level, the C.V% of mean analyte and ISTD recoveries must be less than 15%. The minimum and maximum recovery percentages should deviate by no more than 25%.

Robust linearity :**Table No. 16:** Ensifentrine Rugged Linearity

Analyte	Ensifentrine					ISTD	Teneligliptin	
	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8
	Nominal Concentration (ng/mL)							

	0.500	1.000	1.500	4.000	10.000	12.000	16.000	20.000
	Nominal Concentration Range (ng/mL)							
	(0.400-0.600)	(0.850-1.150)	(1.275-1.725)	(3.400-4.600)	(8.500-11.500)	(10.200-13.800)	(13.600-18.400)	(17.000-23.000)
	Calculated Concentration (ng/mL)							
Different Column	0.502	1.026	1.507	4.010	10.260	12.260	16.500	19.780
Different Analyst	0.512	1.001	1.497	4.152	10.129	12.136	17.930	19.926
n	2	2	3	2	2	2	2	2
Mean	0.5072	1.0135	1.5020	4.0810	10.1945	12.1980	17.2150	19.8530
SD	0.00686	0.01768	0.00707	0.10041	0.09263	0.08768	1.01116	0.10324
%CV	1.35	1.74	0.47	2.46	0.91	0.72	5.87	0.52
% Mean Accuracy	101.43	101.35	100.13	102.03	101.95	101.65	107.59	99.27

Discussion: Six dosages of calibration standards (0.5 ng/ml to 20 ng/ml) are used to define the calibration range, excluding blank and zero samples. Ensifentrine's coefficient of correlation was found to be 0.999, and the

calibration curves appeared to be linear. A technique's linearity ruggedness indicates how susceptible it is to minor alterations that may occur during normal analysis.

Reinjection Reproducibility :

Table No. 17: Ensifentrine Reinjection Reproducibility

Analyte	Ensifentrine			
	HQC	MQC1	LQC	LLOQ QC
	Nominal Concentration (ng/mL)			
	16.000	10.000	1.500	0.500
	Nominal Concentration Range (ng/mL)			
	(13.600-18.400)	(8.500-11.500)	(1.275-1.725)	(0.400-0.600)
	Calculated Concentration (ng/mL)			
	15.250	10.320	1.452	0.509
	15.650	9.989	1.458	0.511
	15.860	9.680	1.503	0.519
	16.240	10.560	1.524	0.499
	16.023	10.178	1.503	0.508
	16.330	10.266	1.578	0.497
n	6	6	6	6
Mean	15.8922	10.1655	1.5030	0.5072
SD	0.40046	0.30220	0.04624	0.00811
% CV	2.52	2.97	3.08	1.60
% Mean Accuracy	99.33	101.66	100.20	101.43
No. of Failed QC'S	0	0	0	0

Stabilities: Long term stock solution stability**Table no 18:** stability of Ensifentrine (zero days).

Analyte	Ensifentrine	ISTD	Teneligliptin
S No.		HQC	LQC
		Nominal Concentration (ng/mL)	
		16.000	1.500
		Nominal Concentration Range (ng/mL)	
		(13.600-18.400)	(1.275-1.725)
		Calculated Concentration (ng/mL)	
1		15.690	1.487
2		15.570	1.469
3		16.033	1.680
4		16.247	1.459
5		16.006	1.582
6		16.317	1.549
n		6	6
Mean		15.9771	1.5377
SD		0.29681	0.08466
% CV		1.86	5.51
% Mean Accuracy		99.86	102.51
No. of Failed QC'S		0	0

Matrix samples stability at -28±5 °C for 37 days**Table no 19:** Matrix samples stability at -28±5 °C for 37 days of Ensifentrine

Table no 19: Matrix samples stability at -28±5 °C for 37 days of Ensifentrine				
Analyte	Ensifentrine			
S No.	HQC		LQC	
	Nominal Concentration (ng/mL)			
	16.000	16.000	1.500	1.500
	Nominal Concentration Range (ng/mL)			
	(13.600-18.400)	(13.600-18.400)	(1.275-1.725)	(1.275-1.725)
	Calculated Concentration (ng/mL)			
	Comparison Samples	Stability Samples	Comparison Samples	Stability Samples
1	15.883	16.250	1.436	1.425
2	15.560	15.789	1.506	1.501
3	16.035	15.989	1.504	1.510
4	16.025	16.124	1.590	1.547
5	16.070	16.058	1.498	1.500
6	16.110	16.097	1.497	1.509

n	6	6	6	6
Mean	15.9472	16.0512	1.5052	1.4987
SD	3.76431	0.15465	0.04916	0.04001
% CV	23.60	0.96	3.27	2.67
%Mean Accuracy	99.67	100.32	100.34	99.91
% Mean Stability	100.65		99.57	
Failed QCS	0	0	0	0

Matrix samples stability at -80±5 °C for 37days**Table no 20: Matrix samples stability at -80±5 °C for 37 days of Ensifentrine**

Table no 26: Matrix samples stability at 30-45 °C for 57 days of Ensifentrine				
Analyte	Ensifentrine			
S No.	HQC		LQC	
	Nominal Concentration (ng/mL)			
	16.000	16.000	1.500	1.500
	Nominal Concentration Range (ng/mL)			
	(13.600-18.400)	(13.600-18.400)	(1.275-1.725)	(1.275-1.725)
	Calculated Concentration (ng/mL)			
	Comparison Samples	Stability Samples	Comparison Samples	Stability Samples
1	15.960	16.020	1.487	1.501
2	15.998	16.071	1.499	1.476
3	16.078	16.054	1.510	1.524
4	15.014	15.470	1.500	1.510
5	16.060	16.150	1.500	1.521
6	16.097	16.112	1.480	1.492
n	6	6	6	6
Mean	15.8678	15.9795	1.4960	1.5040
SD	0.42145	0.25368	0.01071	0.01823
% CV	2.66	1.59	0.72	1.21
%Mean Accuracy	99.17	99.87	99.73	100.27
% Mean Stability	101.09		100.24	
Failed QCS	0		0	

15. SUMMARY AND CONCLUSION

For the simultaneous measurement of ensifentrine in human plasma, a simple, precise, and accurate approach was created and verified. The protein precipitation technique (PPM) was used to prepare the sample. The chromatogram utilized was the Agilent C18 250 x 4.6 x 5

µm. At a flow rate of 1.0 ml/min, the mobile phase was pushed through the column. It was composed of acetonitrile and buffer 0.1% OPA in a 60:40 ratio. The Internal Standard [IS] for ensifentrine separation is teneligliptin. At 30°C, the temperature did not change. 348 nm was found to be the ideal wavelength. The ensifentrine retention times were 3.406 and 2.363 minutes (IS) ,

correspondingly. For ensifentrine values between 0.5 and 20 ng/ml, the standard curve was linear ($R^2 > 0.995$). ICH requirements were followed in the establishment of each analytical validation parameter. The created bioanalytical technique surpassed the guidelines' accuracy, precision, recovery, and other validation requirements, making it robust, selective, and dependable. There was no plasma interference, and the peaks generated for the drug of interest and the internal standard were distinct from one another, symmetrical, and had the proper tailing factor. Therapeutic drug monitoring (TDM), bioequivalency research, pharmacokinetics studies, toxicology, and biological investigations might all benefit greatly from this method.

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17. CONFLICT OF INTEREST : The authors declare no conflict of interest.

18. BIBLIOGRAPHY

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