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High-Throughput Lc-Ms/Ms Technique For Reliable Measurement Of Posaconazole In Human Plasma

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ABSTARCT:

A rapid and sensitive LC-MS/MS bioanalytical method was developed and validated for quantifying Posaconazole in human plasma using posaconazole D5 as the internal standard, per USFDA guidelines. Chromatographic separation employed a Waters Atlantis dC18 column (3µ, 4.6×50 mm) with a mobile phase of acetonitrile:5 mM ammonium formate containing 0.01% formic acid (95:5 v/v) at 0.6 mL/min flow rate, yielding retention times of 2.00 min for both analyte and IS. The method demonstrated excellent selectivity with no interference, linearity from 0.80 to 801.48 ng/mL, and robust in-system suitability (area ratio and S/N at LLOQ within limits). Precision and accuracy across six QC replicates met acceptance criteria, with consistent recovery at low, mid, and high levels, negligible matrix effects, and stability up to 18 hr for stock solutions, 3 days frozen for working solutions, 2 days/20 hr bench-top for spiked plasma, and 5 freeze-thaw cycles. This validated method supports reliable pharmacokinetic assessments and therapeutic drug monitoring of Posaconazole in clinical plasma samples. This LC-MS/MS approach addresses limitations of existing methods by offering superior sensitivity, high throughput, and cost-effectiveness for Posaconazole quantification, vital for ensuring therapeutic efficacy in antifungal therapy amid variable bioavailability challenges.

Keywords: Posaconazole, LC-MS/MS, bioanalytical method, human plasma, validation.

INTRODUCTION:

Posaconazole, a broad-spectrum triazole antifungal agent, is widely used for treating and preventing invasive fungal infections in immunocompromised patients, particularly through pharmaceutical dosage forms such as injections and oral formulations. Accurate quantitative estimation of Posaconazole in these dosage forms is essential to ensure drug safety, efficacy, and quality control during manufacturing and stability studies. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) stands out as a preferred bioanalytical technique due to its high sensitivity, selectivity, and ability to handle complex matrices.

Developing a robust LC-MS/MS method involves optimizing chromatographic conditions, mass spectrometric parameters, and sample preparation to achieve reliable quantification across a wide concentration range. This approach adheres to International Council for Harmonisation (ICH) guidelines, focusing on parameters like linearity, precision, accuracy, limits of detection (LOD), and quantification (LOQ). Such methods enable precise analysis even at low concentrations, supporting routine quality assurance in pharmaceutical production.

Validation confirms the method's reproducibility, specificity, and stability under various conditions, making it suitable for pharmacokinetic studies and regulatory submissions. LC-MS/MS methods for Posaconazole have demonstrated high throughput and robustness, often using protein precipitation or solid-phase extraction for sample cleanup. This research presents a validated LC-MS/MS method tailored for Posaconazole in pharmaceutical dosage forms, advancing reliable bioanalysis in clinical and industrial settings.

MATERIALS AND METHODS:

➢ Chemicals and Reagents:

Posaconazole (98.0%) and the internal standard Posaconazole-D5 (99.19%) were obtained from Vivan Life Sciences and Simson Life Sciences (Mumbai, India). Ammonium formate (AR grade) and formic acid (AR grade) were sourced from Sigma-Aldrich (India). LC–MS grade methanol, acetonitrile, and ammonia were procured from Fisher Scientific (India). DMSO (LR grade) was used for preparation of primary stock solutions, while Milli-Q water was used for all aqueous solutions. The sample diluent consisted of methanol–water (50:50, v/v), and the rinsing solution comprised acetonitrile–water (50:50, v/v). Additional aqueous solutions of 1% and 2% formic acid were prepared for solid-phase extraction (SPE) procedures.

➢ Instrumentation and Chromatographic Conditions:

Quantification of posaconazole and Posaconazole-D5 was performed using a UPLC–MS/MS system equipped with electrospray ionization. Mass spectrometer tuning was carried out by direct infusion of analyte and internal standard solutions to identify optimal precursor and product ions. The instrument was operated in multiple reaction monitoring (MRM) mode with optimized voltages, collision energies, and gas flows for both compounds. Chromatographic separation was achieved using a mobile phase composed of acetonitrile and aqueous ammonium formate (5 mM, 0.01% formic acid). Samples were prepared using SPE on Strata X-C (30 mg/1 mL) cartridges following an optimized protocol involving methanol conditioning, water equilibration, acidified wash steps, and elution with ammonia-containing acetonitrile.

➢ Mobile Phase and Elution:

The aqueous component of the mobile phase consisted of 5 mM ammonium formate containing 0.01% formic acid, prepared by dissolving ammonium formate in water and adding formic acid before sonicating to clarity. The final mobile phase was a mixture of acetonitrile and this buffer at a ratio of 95:5 (v/v). For SPE elution, acetonitrile containing 10% ammonia was used, providing efficient recovery of both the analyte and internal standard. Acidified aqueous solutions (1% and 2% formic acid) were employed during washing steps to improve matrix removal and enhance extraction cleanliness.

➢ MS/MS Parameters:

MS/MS parameters were optimized through direct infusion of tuning solutions for both posaconazole and Posaconazole-D5. Q1 and Q3 scans were recorded to determine the most abundant precursor-to-product ion transitions for use in MRM mode. Source-dependent parameters—including ion spray voltage, nebulizer and auxiliary gas flow rates, and source temperature—were adjusted to maximize signal intensity and stability. Compound-dependent settings such as declustering potential and collision energy were optimized individually for the analyte and internal standard. The finalized MS/MS settings were applied consistently for all sample analyses.

➢ Preparation of Standards and Quality Control Samples:

Primary stock solutions of posaconazole were prepared by accurately weighing the analyte, correcting for purity, dissolving the required amount in 1 mL of DMSO, and diluting to 2 mL with methanol, yielding a final concentration of 1,024,987.25 ng/mL. From this stock, an intermediate solution was prepared using methanol–water (50:50, v/v), which served as the source for preparing serial spiking solutions. These spiking solutions were subsequently used to generate plasma calibration standards covering the concentration range of 2.62–784.12 ng/mL through bulk spiking into blank plasma at a ratio of 50 µL spiking solution to 950 µL plasma.

Quality control (QC) samples were prepared independently from separate stock dilutions to ensure assay accuracy and precision. High-, medium-, and low-concentration QC samples were designed to represent approximately 75–85%, 45–55%, and 30% of the upper calibration standard, respectively, while the LLOQ QC corresponded to 1% of the lowest calibration standard. Final plasma QC concentrations were 590.23 ng/mL (HQC), 277.41 ng/mL (MQC), and 7.82 ng/mL (LQC). The internal standard Posaconazole-D5 was prepared similarly to the analyte stock, diluted with methanol–water to form the intermediate solution, and further diluted to obtain a working solution that provided a final concentration of 250 ng/mL in plasma.

➢ Sample Extraction Procedure:

Sample preparation was performed using an optimized solid-phase extraction (SPE) method employing Strata X-C (30 mg/1 mL) cartridges. Each plasma sample (190 µL) was spiked with 10 µL of calibration or QC spiking solution and 50 µL of internal standard working solution. The mixture was acidified with 400 µL of 1% formic acid in water and vortex-mixed thoroughly. SPE cartridges were conditioned with 1 mL of methanol followed by 1 mL of water. The prepared samples were then loaded onto the cartridges and washed sequentially with 1 mL of 2% formic acid and 1 mL of water to

remove matrix components. Analytes were eluted using 250 µL of acetonitrile containing 10% ammonia. The eluates were collected, transferred to autosampler vials, and injected into the UPLC–MS/MS system for analysis.

BIOANALYTICAL METHOD VALIDATION:

Method validation was performed according to FDA and EMA bioanalytical method validation guidelines.

- **System Suitability:**

System suitability testing was conducted at the start of each analytical batch to verify consistent instrument performance. For this purpose, one medium quality control (MQC) sample, one lower limit of quantification (LLOQ) sample, one blank, and one upper limit of quantification (ULOQ) sample were independently extracted as per the optimized protocol. Following extraction, the MQC sample was injected six times consecutively, interspersed with single injections of LLOQ, ULOQ, and blank samples. The acceptance criteria required that the % relative standard deviation (%RSD) of the analyte-to-internal standard area ratios for six consecutive MQC injections remain ≤ 5.0% and the signal-to-noise (S/N) ratio of the LLOQ be ≥ 5, confirming system precision and sensitivity prior to sample analysis.

- **Selectivity:**

Selectivity was assessed to demonstrate the method's ability to specifically quantitate Posaconazole and the internal standard (IS) in the presence of endogenous plasma components. Blank human plasma samples from six different sources—including one hemolysed and one lipemic lot—were subjected to the extraction procedure alongside a calibration standard and at least two sets of QC samples. The chromatograms of extracted blanks were examined for interfering peaks at the retention times of the analyte and IS and compared with those of calibration and QC samples. Acceptability criteria required interference at the analyte retention time to be less than 20% of the LLOQ response and interference at the IS retention time to be less than 5% of the IS response at LLOQ.

- **Calibration Curve:**

Calibration standards were prepared in K_3EDTA human plasma and comprised a blank (matrix only), blank with IS, and eight non-zero standards ranging from 2.62 ng/mL to 784.61 ng/mL. Peak area ratios of analyte to IS were plotted against nominal concentrations to generate calibration curves. Linearity was evaluated using regression analysis, with the correlation coefficient (r^2) required to be greater than 0.99. Accuracy demands were set such that the lowest calibration standard (STD A) was within 80–120% of nominal, and all other standards were within 85–115%.

- **Accuracy and Precision:**

Intra- and inter-day accuracy and precision were determined by analyzing QC samples at four concentration levels (LLOQ, LQC, MQC, and HQC) over at least three independent runs on different days. Each concentration level was tested in six replicates per batch. Precision was expressed as %RSD, with acceptance criteria ≤ 20% for LLOQ and ≤ 15% for LQC, MQC, and HQC. Accuracy was evaluated as the mean percentage of the nominal concentration, with required ranges of 80–120% for LLOQ and 85–115% for other QC levels.

- **Sensitivity:**

Sensitivity was established at the LLOQ concentration by analyzing six replicate LLOQ samples alongside calibration standards and QC samples (six replicates each of LQC, MQC, and HQC) across three analytical runs. Acceptance criteria included a signal-to-noise ratio ≥ 5, %RSD ≤ 20%, and mean concentration within 80–120% of the nominal value.

- **Matrix Factor:**

Matrix effect was evaluated to assess variability from endogenous plasma components. Analyte stock solutions were diluted to LQC and HQC levels. Nine replicates of each level were processed using the SPE method.

Table 1: Aqueous Sample Preparation for Matrix Factor

LQC/HQC Volume	Internal Standard (IS) Volume	Elution Solution Volume	Final Volume
10 µL	50 µL	190 µL	250 µL

- **Percent Recovery:**

Recovery was calculated by comparing the peak areas of extracted plasma QC samples at LQC, MQC, and HQC levels to those of unextracted reference standards representing 100% recovery. Six replicates per QC level were processed. The method was considered acceptable if %RSD was ≤ 15% and %recovery was ≥ 40% at each QC level.

Table 2: Sample Preparation for Percent Recovery

SS Volume (LQC/HQC)	IS Volume	Elution Volume	Final Volume
10 µL	50 µL	190 µL	250 µL

➢ **Dilution Integrity:**

Dilution integrity was verified to confirm the assay's capability to accurately quantify samples above the ULOQ after dilution. Spiking solutions (SS DI 1/2 and SS DI 1/5) were prepared at concentrations 2 to 5 times higher than the HQC, then diluted appropriately with screened K_3EDTA plasma and processed using the validated procedure. Acceptance criteria required %RSD ≤ 15% and mean concentrations within 85–115% of nominal.

➢ **Stability Studies:**

• **Short-Term Stock and Working Solution Stability:** Six aliquots of freshly prepared stock (SS CS 01 & CS 08) and IS were kept at ambient temperature (stock) or for 6 h (working solution). Fresh stock and working solutions were prepared, and six MQC replicates were analyzed.

Table 3: Preparation for Stock and Working Solution

Parameter	Analyte Volume	IS Volume	Elution Volume	Final Volume
Stock	10 µL	50 µL	190 µL	250 µL
Working	10 µL	50 µL	190 µL	250 µL

- **Long-Term Stock and Working Solution Stability:** Six aliquots were stored at 2–8°C for 4 days and analyzed against freshly prepared stock and working solution samples using MQC replicates. Stability was accepted if %RSD was ≤ 15% and the percent difference was within ±15%.
- **Bench Top Stability:** QC samples at LQC and HQC levels were kept at ambient temperature for 24 hours and compared with freshly prepared samples. Acceptance criteria were %RSD ≤ 15%, %difference within ±15%, and mean accuracy within 85–115% of nominal.
- **Freeze–Thaw Stability:** Samples underwent five freeze-thaw cycles between -20°C and -70°C and were analyzed alongside fresh samples. Acceptance criteria matched those of bench-top stability.
- **Long-Term Stability in Biological Matrix:** QC samples stored at -20°C to -70°C for the study duration were analyzed against fresh calibration standards and QCs, meeting the same acceptance criteria for accuracy and precision.

RESULTS & DISCUSSION:

➢ **System suitability:**

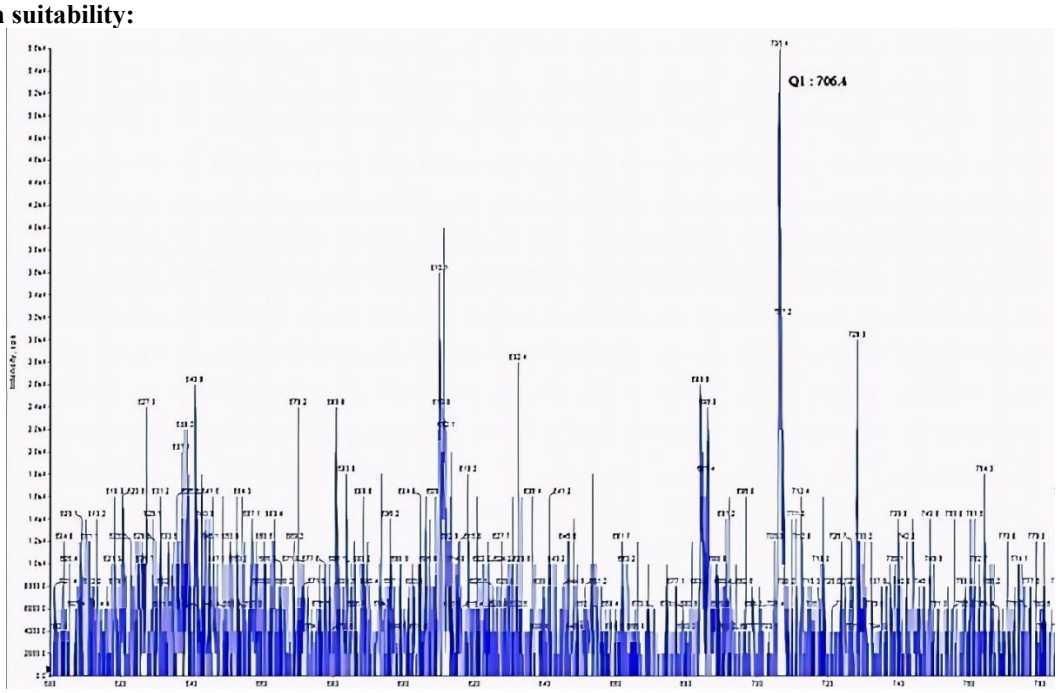

Figure 1: Product ion spectrum Q1 scanning

Figure 1 shows the Q1 product ion spectrum of posaconazole demonstrated a predominant precursor ion at m/z 706.4, corresponding to the protonated molecule $[M+H]^+$. This confirms proper ionization of posaconazole under positive ESI conditions and provides the optimal precursor ion for further method development steps such as collision-induced dissociation (CID) and MRM transition selection. The strong and stable signal at m/z 706.4 indicates that the tuning parameters (ion spray voltage, declustering potential, gas flow, and temperature) were appropriately optimized for maximum sensitivity.

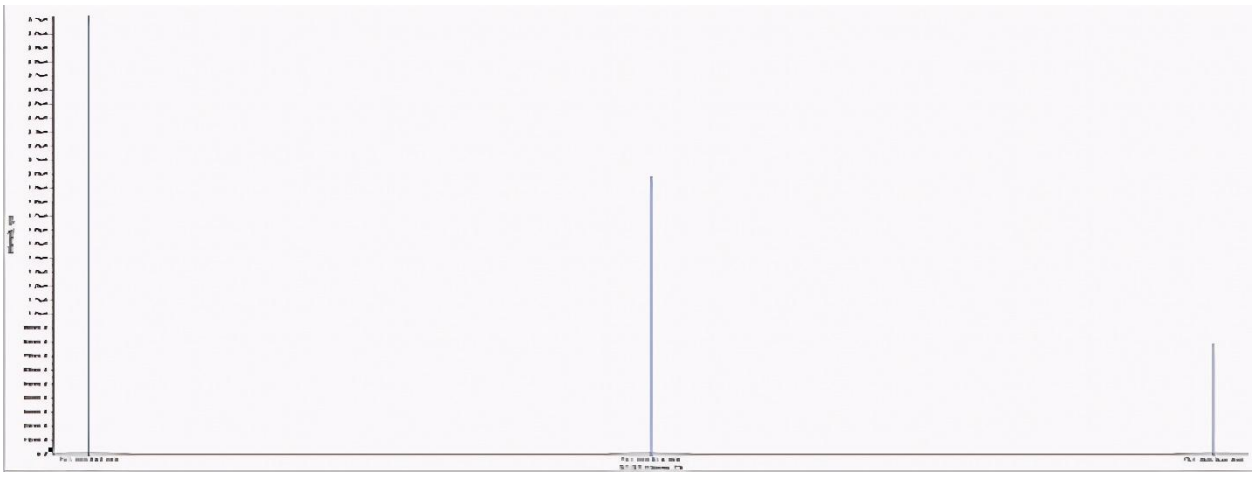

Figure 2: The multiple reaction monitoring (MRM) scan Q1 and Q3 of Posaconazole (3pairs)

Figure 2 shows the Multiple Reaction Monitoring (MRM) transitions selected for posaconazole during LC-MS/MS method development. The precursor ion observed in Q1 was m/z 701.3, which represents a stable protonated molecular ion of posaconazole under the optimized ionization conditions. Three product ion transitions were monitored in Q3, yielding the following ion pairs: 701.3/683.4 and 701.3/614.3 and 701.3 /644.2 respectively.
These product ions were generated by collision-induced dissociation (CID) of the precursor ion and represent characteristic fragment ions of posaconazole. Monitoring multiple MRM transitions enhances both selectivity and sensitivity of the bioanalytical method. Among these, one transition is typically chosen as the quantifier ion (highest intensity and most stable), while the others serve as qualifier ions for confirmation of analyte identity.
The presence of all three transitions with strong and reproducible signals confirms proper fragmentation behavior and supports their suitability for reliable LC-MS/MS quantification of posaconazole in biological samples.

Table 4: Tuning Parameters of Posaconazole and Posaconazole-D5

Parameter	**Posaconazole**	**Posaconazole-D5**	**Description**
Polarity	+	+	Both detected in positive mode, indicating stable protonated ion formation.
Declustering Potential (DP)	194.12 V	188.65 V	Slightly higher DP for posaconazole reflects small differences in ion transmission due to isotopic variation.
Entrance Potential (EP)	10.19 V	10.00 V	Similar EP values indicate comparable ion focusing behavior.
Collision Energy (CE)	41.47 V	46.82 V	Internal standard requires slightly higher energy for optimal fragmentation.
Dwell Time	200 ms	200 ms	Identical dwell times ensure synchronized acquisition and improved precision.

Posaconazole and Posaconazole-D5 were optimized under positive ESI mode, showing similar ionization and fragmentation behavior. The analyte required slightly higher declustering potential (194.12 V) compared to the internal standard (188.65 V), while collision energies were adjusted to obtain stable product ions. Source parameters such as curtain gas (25), ion spray voltage (5500 V), temperature (500 °C), and nebulizer/drying gases (GS1: 55, GS2: 60) were optimized to enhance desolvation and ensure stable ion formation. The selected MRM transitions were 701.30 → 683.40 for posaconazole and 706.30 → 688.40 for Posaconazole-D5, representing their respective protonated precursor ions and dominant fragment ions. These optimized conditions collectively provide high sensitivity, stable response, and reliable

quantification in the LC-MS/MS method.

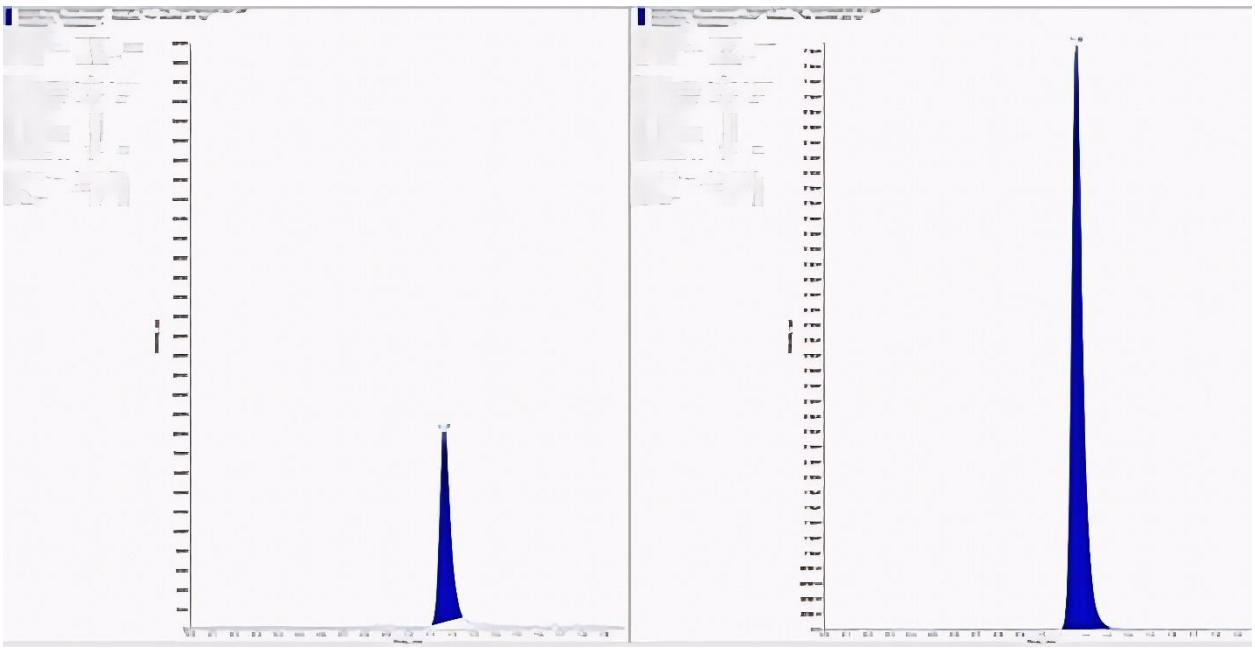

Figure 3: Chromatogram of Posaconazole and Posaconazole D5 (CS01) with IS

Figure 3 shows the chromatogram of Posaconazole and its internal standard, Posaconazole-D5, for the calibration standard CS01. Both analytes exhibit well-defined and sharp peaks with no interfering signals near their respective retention times, indicating good selectivity of the LC-MS/MS method even at low concentration levels. The consistent response of the internal standard confirms stable ionization and reliable instrument performance during analysis.

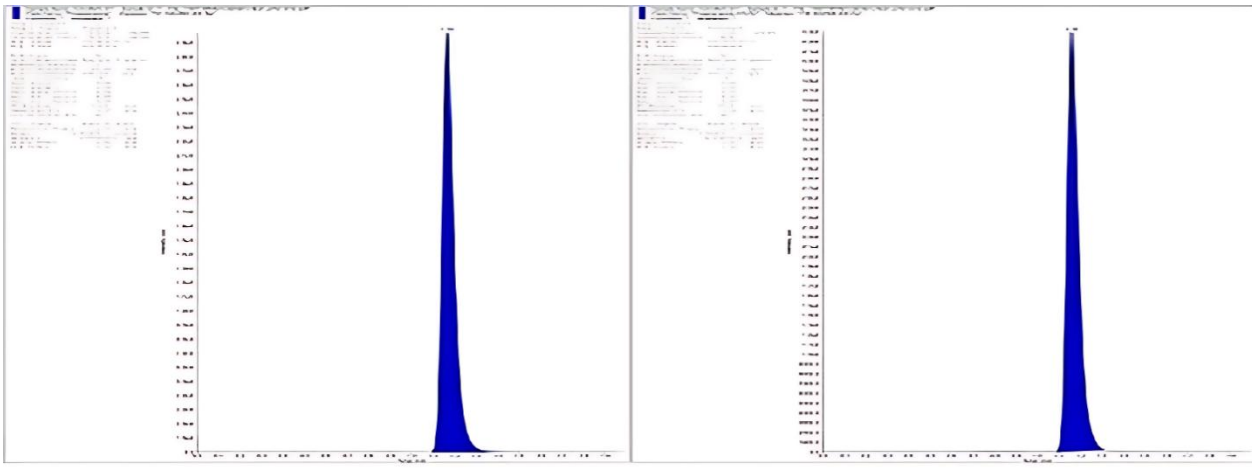

Figure 4: Chromatogram of Posaconazole and Posaconazole D5 (CS08) with IS

Figure 4 displays the chromatogram for calibration standard CS08, representing a higher concentration level. The peaks for both Posaconazole and Posaconazole-D5 are more intense yet remain symmetrical and free from co-eluting interferences. The consistent retention times between CS01 and CS08 demonstrate good chromatographic reproducibility across the calibration range. The proportional increase in peak area with concentration indicates proper linearity and suitability of the method for quantitative analysis.

Table 5: Optimization of Chromatographic Conditions

Chromatographic Condition	Trial-01	Trial-02	Trial-03	Trial-04	Trial-05
Analytical Column	Phonics dC8 (2.3 × 50 mm)	Phonics dC8 (4.6 × 50 mm)	Waters Atlantis dC8 (4.6 × 100 mm)	Waters Atlantis dC8 (4.6 × 50 mm)	Waters Atlantis dC18 (4.6 × 50 mm)
Injection Volume	10 µL	10 µL	10 µL	10 µL	10 µL
Flow Rate	1.0 mL/min	0.8 mL/min	0.8 mL/min	0.6 mL/min	0.6 mL/min
Autosampler Temperature	5°C	5°C	5°C	5°C	5°C
Column Oven Temperature	50°C	50°C	50°C	50°C	50°C
Detector	API 3500 MS/MS	API 3500 MS/MS	API 3500 MS/MS	API 3500 MS/MS	API 3500 MS/MS
Mobile Phase Composition	ACN : 5 mM Ammonium Formate with 0.01% formic acid (50:50)	ACN : 5 mM Ammonium Formate with 0.01% formic acid (70:30)	ACN : 5 mM Ammonium Formate with 0.01% formic acid (80:20)	ACN : 5 mM Ammonium Formate with 0.01% formic acid (90:10)	ACN : 5 mM Ammonium Formate with 0.01% formic acid (95:5)
Run Time	2.00 min	2.00 min	2.00 min	2.00 min	2.00 min

Table 6: Summary of Chromatographic Optimization Trials (1–5)

Trial	Column & Size	Flow Rate (mL/min)	Mobile Phase Composition	Injection Volume	Autosampler Temp (°C)	Column Temp (°C)	Result / Observation
1	Phonics dC8 (2.3 × 50 mm)	1.0	ACN : 5 mM Ammonium Formate (50:50)	10 µL	5	50	Very low analyte peak area; poor response, insufficient retention and ionization.
2	Phonics dC18 (4.6 × 50 mm)	0.8	ACN : 5 mM Ammonium Formate (70:30)	10 µL	5	50	Low response; weak peak intensity, insufficient sensitivity.
3	Waters Atlantis dC8 (4.6 × 100 mm)	0.8	ACN : 5 mM Ammonium Formate (80:20)	10 µL	5	50	Very poor peak sharpness; low analyte intensity; suboptimal separation.
4	Waters Atlantis dC8 (4.6 × 50 mm)	0.6	ACN : 5 mM Ammonium Formate (90:10)	10 µL	5	50	Improved response, but peak shape still not fully satisfactory.
5	Waters Atlantis dC18 (4.6 × 50 mm)	0.6	ACN : 5 mM Ammonium Formate (95:5)	10 µL	5	50	Best response and peak shape; suitable for quantitative analysis.

Table 7: Spiking Solution Check Results

Sr. No.	Sample Name	Analyte Peak Area (counts)	Analyte Nominal Concentration (ng/mL)	IS Peak Area (counts)	Area Ratio	Calculated Concentration (ng/mL)	Accuracy (%)
1	SSC CS1	6,550	2.62	271,465	0.0241	2.59	98.89
2	SSC CS2	13,540	5.25	265,784	0.0509	5.36	102.16

3	SSC CS3	43,513	17.49	258,649	0.1682	17.52	100.15
4	SSC CS4	151,832	58.30	267,566	0.5675	58.87	100.97
5	SSC CS5	336,116	145.74	242,983	1.3833	143.37	98.37
6	SSC CS6	903,222	364.36	253,005	3.5700	369.87	101.57
7	SSC CS7	1,499,148	628.21	248,448	6.0341	625.09	99.50
8	SSC CS8	1,804,939	785.26	241,853	7.4630	773.10	98.45
9	SSC LLOQ	5,952	2.63	248,449	0.0240	2.57	97.85
10	SSC LQC	19,572	7.85	255,674	0.0766	8.02	102.18
11	SSC MQC	695,350	276.33	252,750	2.7511	285.05	103.16
12	SSC HQC	1,372,946	587.95	242,762	5.6555	585.88	99.65

The spiking solution check demonstrated that all calibration standards and QC levels showed accurate and consistent recovery, with accuracy values ranging from 97.85% to 103.16%. These results fall within the acceptable bioanalytical validation limits (±15% for all levels and ±20% for LLOQ), confirming that the spiking solutions, stock dilutions, and preparation procedures are accurate, stable, and reliable for use in calibration curve and QC sample preparation.

Table 8: System Suitability Test Results

Sample Name	**Analyte Peak Area**	**Analyte RT (min)**	**IS Peak Area**	**IS RT (min)**	**Area Ratio (Analyte/IS)**	**Analyte S/N Ratio**
SST MQC-01	801,220	1.17	295,282	1.16	2.7134	19,128.35
SST MQC-02	756,506	1.17	276,421	1.17	2.7368	17,810.57
SST MQC-03	755,254	1.17	279,980	1.17	2.6975	14,651.44
SST MQC-04	750,852	1.17	277,385	1.16	2.7069	19,040.43
SST MQC-05	743,844	1.17	273,113	1.17	2.7236	22,868.06
SST MQC-06	742,019	1.17	273,560	1.16	2.7125	21,570.83
SST LLOQ	3,777	1.16	159,132	1.15	0.0237	132.62
Mean (MQC)	758,282.50	1.17	279,290.17	1.17	2.7151	—
SD (MQC)	21,836.39	0.0000	8,234.91	0.0055	0.0136	—
%RSD (MQC)	2.88	0.00	2.95	0.47	0.50	—

The system suitability test confirmed that the LC–MS/MS method is robust and reliable for Posaconazole analysis. Retention times for the analyte and internal standard were highly consistent (1.17 min) with %RSD between 0.00–0.47%, demonstrating excellent chromatographic reproducibility. Peak areas and area ratios showed low variability (%RSD ≤ 2.95%), indicating precise instrument performance. Signal-to-noise ratios were high for MQC samples (>14,000) and sufficient for LLOQ samples (132.62), confirming the method's sensitivity across the quantifiable range. Overall, these results demonstrate that the chromatographic system is stable, precise, and suitable for plasma analysis of Posaconazole.

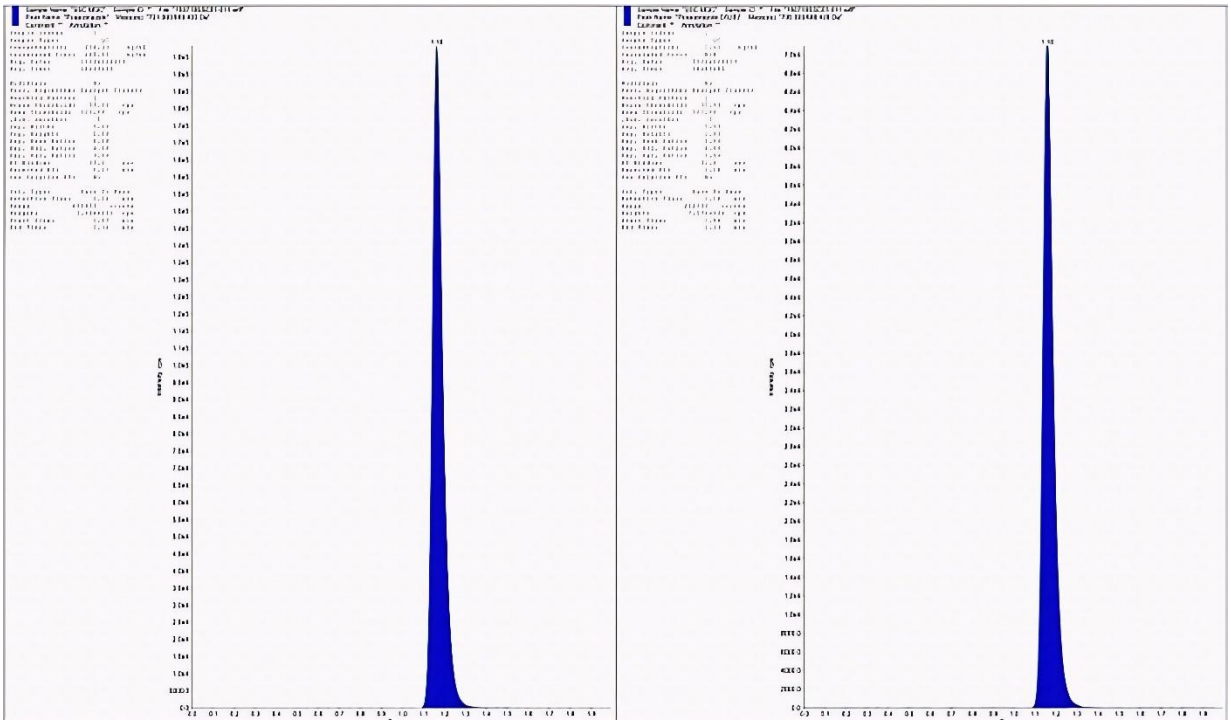

Figure 5: Chromatogram of MQC with IS

The chromatogram of the MQC sample with internal standard (Fig. 5) demonstrates consistent analyte response across six consecutive injections. The %RSD of the area ratio of analyte to internal standard was within the predefined acceptance limit, indicating high precision and reproducibility of the analytical system. Furthermore, the signal-to-noise (S/N) ratio observed for the LLOQ sample met the acceptance criteria, confirming that the method is sufficiently sensitive to reliably quantify the analyte at the lower limit of quantification.

- **Selectivity:**

Table 9: Selectivity Results

Sample ID	Analyte RT (min)	Area at Analyte RT	IS RT (min)	Area at IS RT
LLOQ	1.16	4,294	1.16	158,789
Blank	0.00	0	0.00	0
LLOQ	1.16	4,294	1.16	158,789
Blank	0.00	0	0.00	0
LLOQ	1.16	4,294	1.16	158,789
Blank	0.00	0	0.00	0

(Repeated LLOQ and Blank samples were analyzed for selectivity assessment.)

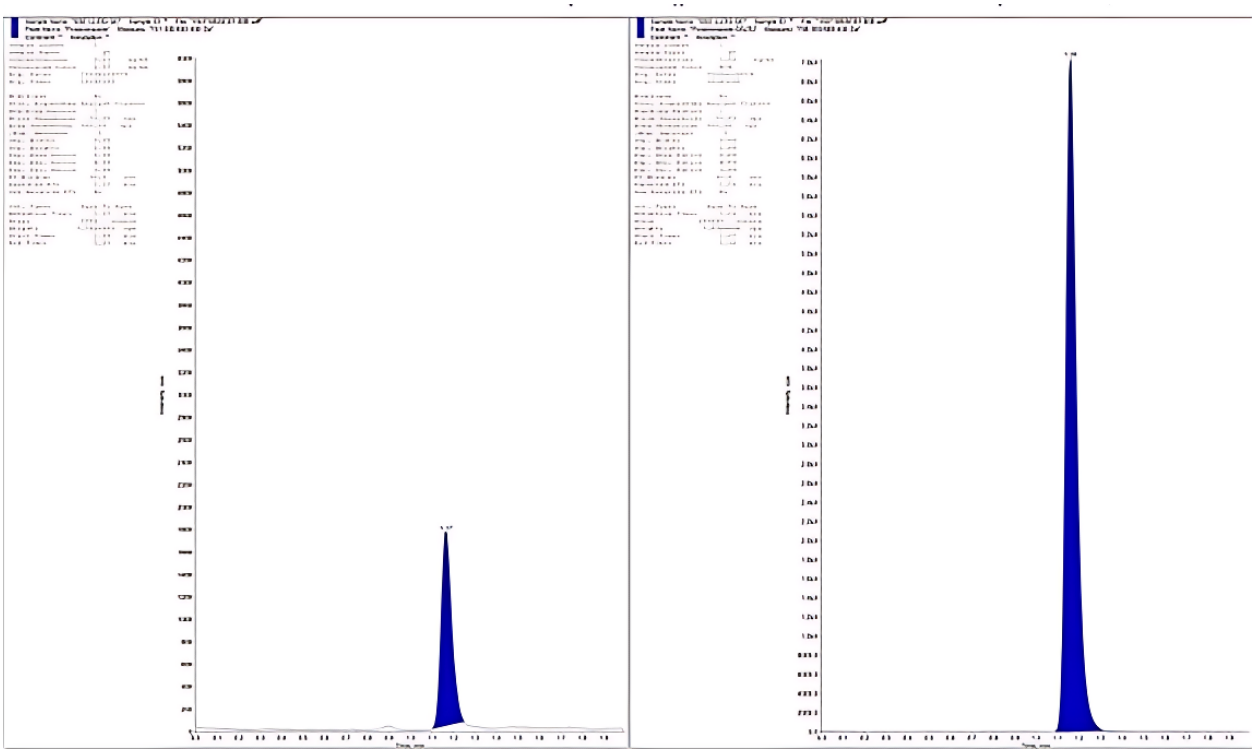

Figure 6: Chromatogram of LLOQ with Internal Standard (IS)

The method demonstrated excellent selectivity, with no interfering peaks observed at the retention times of Posaconazole or the internal standard in blank plasma samples. LLOQ samples showed clear, well-defined peaks, confirming that the method can accurately differentiate and quantify the analyte and internal standard without interference from endogenous matrix components.

- **Calibration Curve Standards:**

Table 10: Calibration Curve Results

Sr. No.	Spiking Solution ID	Nominal Concentration (ng/mL)	Calculated Concentration (ng/mL)	% Accuracy
1	SS CS-8	785.26	785.26	100.00
2	SS CS-7	628.21	645.54	102.76
3	SS CS-6	364.36	357.65	98.16
4	SS CS-5	145.74	146.73	100.68
5	SS CS-4	58.30	59.12	101.41
6	SS CS-3	17.49	17.07	97.61
7	SS CS-2	5.25	5.16	98.20
8	SS CS-1	2.62	2.65	101.18

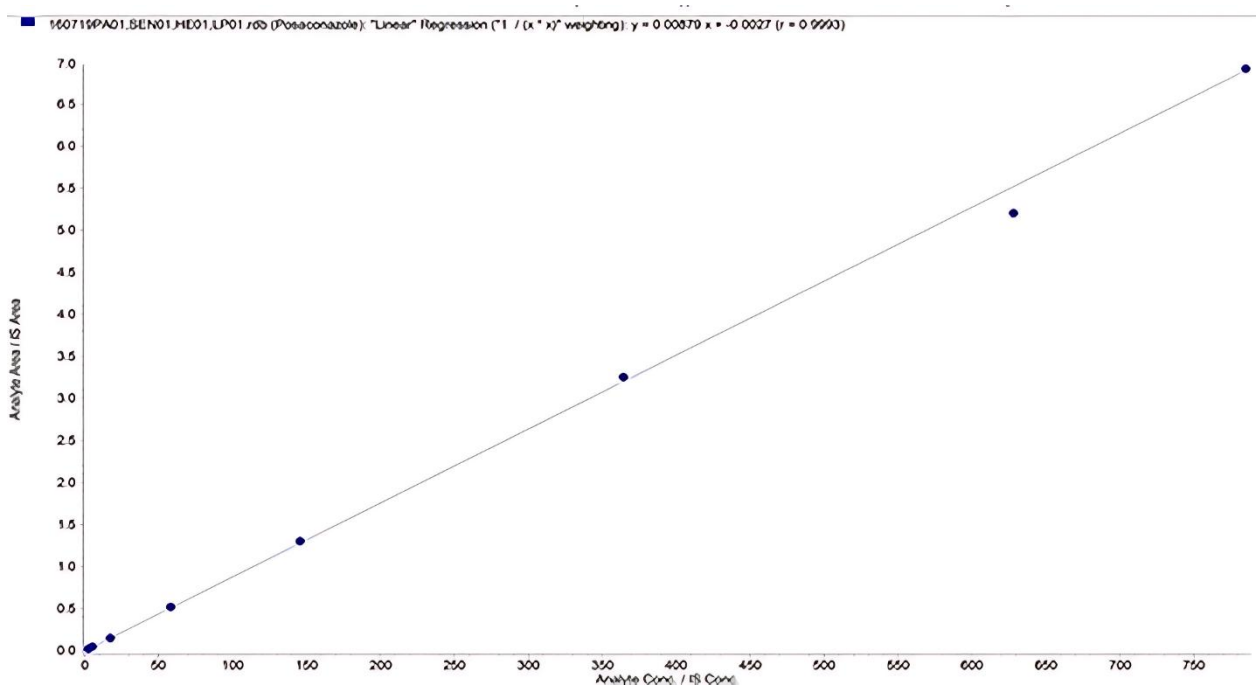

Figure 7: Calibration Curve Showing Linearity

The calibration curve for Posaconazole showed excellent linearity over the concentration range of 2.62–785.26 ng/mL. Calculated concentrations closely matched nominal values, with accuracy between 97.61% and 102.76%, confirming that detector response is proportional to analyte concentration and that the method provides reliable quantification across the analytical range.

- **Accuracy and Precision:**

Table 11: Accuracy and Precision Results

STD ID	Nominal Concentration (ng/mL)	Calculated Concentration (ng/mL)	% Accuracy
CS-01	2.62	2.65	101.18
CS-02	5.25	5.16	98.20
CS-03	17.49	17.07	97.61
CS-04	58.30	59.12	101.41
CS-05	145.74	146.73	100.68
CS-06	364.36	357.65	98.16
CS-07	628.21	645.54	102.76
CS-08	785.26	785.26	100.00

The method exhibited high precision and accuracy across all QC levels and calibration standards. Calculated concentrations were within ±5% of nominal values, and replicate injections showed low %RSD, confirming reproducibility. Intra- and inter-run assessments indicated accuracy between 97.61% and 102.76%, with precision (%RSD) consistently between 2% and 3%, meeting regulatory standards. These results validate the method as reliable, precise, and suitable for quantitative analysis of Posaconazole in plasma samples.

- **Sensitivity:**

Table 12: Sensitivity Test Results (LLOQ)

Sr. No.	RUN 1 (ng/mL)	RUN 2 (ng/mL)	RUN 3 (ng/mL)
Acquisition Batch ID	160719PA01, SEN01	170719PA02, SEN02	170719PA03, SEN03
Nominal Concentration (LLOQ, ng/mL)	2.62	2.62	2.62
1	2.77	2.81	2.69
2	2.89	2.73	2.72
3	2.84	2.76	2.58
4	2.88	2.71	2.58
5	2.80	2.72	2.60

6	2.94	2.73	2.74
Mean	2.85	2.74	2.65
SD	0.0625	0.0367	0.0733
%SD	2.19	1.34	2.77
Accuracy (%)	108.78	104.58	101.15

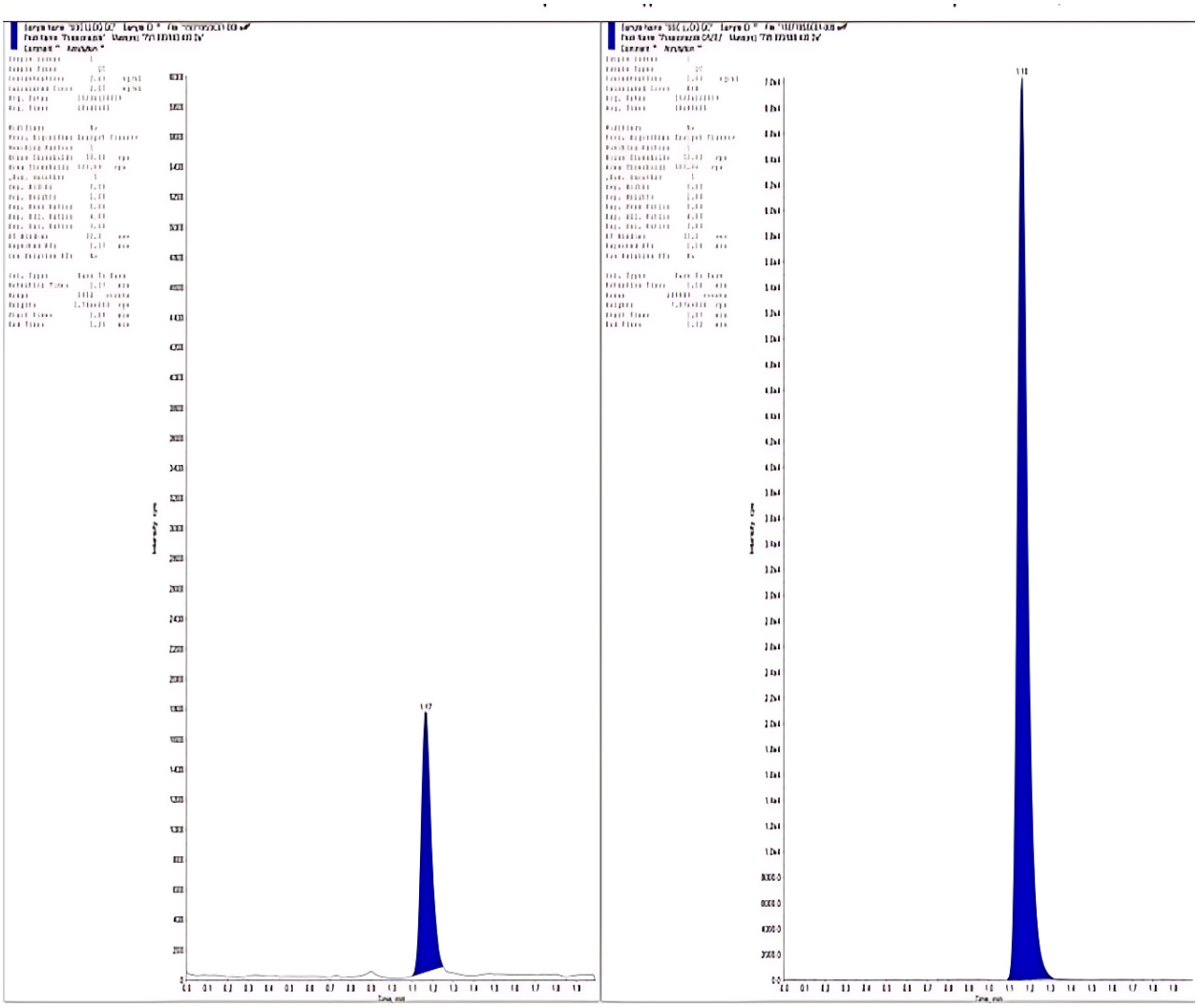

Figure 8: Chromatogram of LLOQ with IS

The sensitivity of the method was evaluated using the LLOQ samples at a nominal concentration of 2.62 ng/mL. The observed mean concentrations for three independent runs ranged from 2.65 to 2.85 ng/mL, with %SD values between 1.34% and 2.77%, demonstrating excellent precision at the LLOQ level. The calculated accuracy ranged from 101.15% to 108.78%, which is within the acceptable bioanalytical limits (±20% for LLOQ). These results confirm that the method is sufficiently sensitive and reliable for quantifying the analyte at the lower limit of quantification.

- **Matrix Factor:**

Table 13: Matrix Factor Results for LQC Concentration

Sr. No.	Unextracted Drug Area	Aqueous Drug Area	Matrix Factor (Drug)	Unextracted IS Area	Aqueous IS Area	Matrix Factor (IS)	IS-Normalized Matrix Factor
1	16,795	17,024	0.99	128,712	130,460	0.99	1.00
2	17,190	16,986	1.01	128,431	128,456	1.00	1.01
3	17,154	16,964	1.01	129,004	129,618	1.00	1.01
4	17,256	17,466	0.99	130,394	130,106	1.00	0.99

5	17,581	17,198	1.02	129,436	130,488	0.99	1.03
6	17,441	17,100	1.02	132,937	126,545	1.05	0.97
7	16,980	17,052	1.00	129,451	128,002	1.01	0.99
8	16,157	17,218	0.94	131,396	131,564	1.00	0.94
Mean	—	—	0.99	—	—	—	—
SD	—	—	0.0276	—	—	—	—
%RSD	—	—	2.79	—	—	—	—

Table 14: Matrix Factor Results for HQC Concentration

Sr. No.	Unextracted Drug Area	Aqueous Drug Area	Matrix Factor (Drug)	Unextracted IS Area	Aqueous IS Area	Matrix Factor (IS)	IS-Normalized Matrix Factor
1	622,799	630,484	0.99	130,938	131,331	1.00	0.99
2	641,819	633,428	1.01	134,082	132,912	1.01	1.00
3	624,238	647,372	0.96	134,196	137,884	0.97	0.99
4	635,890	638,094	1.00	133,492	134,064	1.00	1.00
5	653,742	654,527	1.00	135,876	134,348	1.01	0.99
6	641,250	648,285	0.99	135,323	135,593	1.00	0.99
7	648,795	645,852	1.00	134,789	137,037	0.98	1.02
8	635,751	645,048	0.99	134,760	135,928	0.99	1.00
Mean	—	—	1.00	—	—	—	—
SD	—	—	0.0104	—	—	—	—
%RSD	—	—	1.04	—	—	—	—

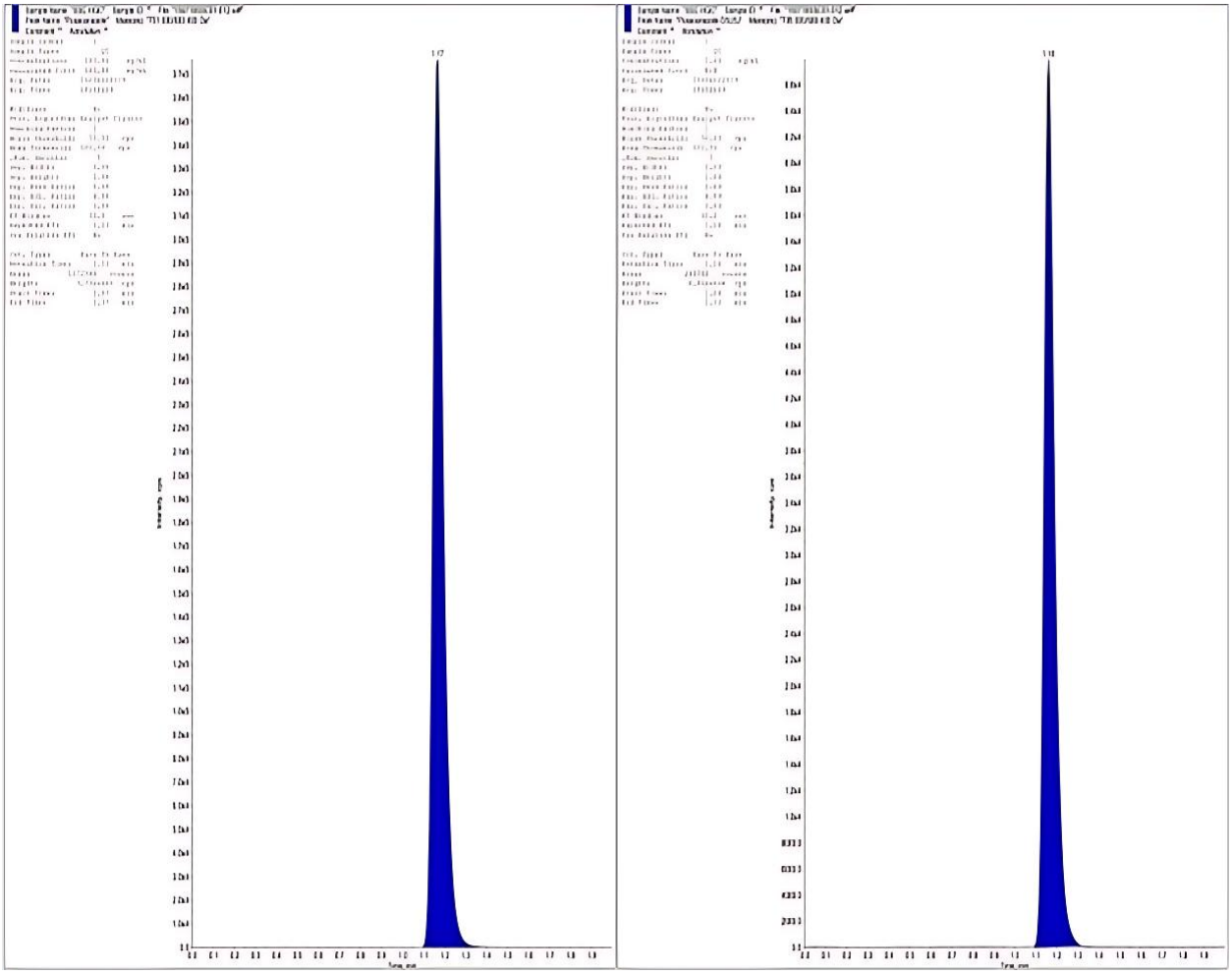

Figure 9: Chromatogram of HQC with Internal Standard

The matrix factor was assessed to evaluate potential ion suppression or enhancement from plasma components. For LQC, the IS-normalized matrix factor ranged from 0.94 to 1.03, with a mean of 0.99 and %RSD of 2.79%, while for HQC it ranged from 0.98 to 1.02, with a mean of 1.00 and %RSD of 1.04%. These results indicate minimal matrix effects, well within the accepted ±15% limit, and demonstrate that the internal standard effectively compensates for minor variations, confirming the robustness and reliability of the method across the concentration range.

- **Percent Recovery:**

Table 15: Percent Recovery of Posaconazole and Posaconazole-D5 at Different QC Levels

QC Level	Posaconazole Peak Area (Extracted)	Posaconazole Peak Area (Unextracted)	% Recovery (Posaconazole)	Posaconazole-D5 Peak Area (Extracted)	Posaconazole-D5 Peak Area (Unextracted)	% Recovery (Posaconazole-D5)
LQC	25,213.50 ± 528.72	26,278.33 ± 712.83	95.95	342,897.67 ± 6,308.23	317,002.83 ± 8,705.72	108.17
MQC	824,205.83 ± 11,951.79	852,682.67 ± 30,254.41	96.66	307,140.50 ± 4,604.40	290,122.83 ± 10,627.99	105.87
HQC	1,620,222.50 ± 36,814.98	1,683,686.33 ± 28,040.86	96.23	293,304.83 ± 7,193.64	272,962.50 ± 4,592.01	107.45
Average	—	—	96.28 ± 0.36	—	—	107.16 ± 1.18

The percent recovery of Posaconazole was evaluated at LQC, MQC, and HQC levels by comparing extracted samples with unextracted reference solutions. Recovery ranged from 95.95% to 96.66%, with an average of 96.28% and a low %RSD of 0.37%, demonstrating excellent extraction efficiency and reproducibility. Similarly, the internal standard (Posaconazole-D5) showed recovery between 105.87% and 108.17%, with an average of 107.16% and a %RSD of 1.10%, indicating consistent and reliable extraction. These results confirm that both Posaconazole and its internal standard are effectively recovered from plasma, supporting the accuracy, robustness, and overall reliability of the bioanalytical method.

- **Dilution Integrity:**

Table 16: Result of Dilution Integrity

Dilution	Calculated Concentration (ng/mL)	% Accuracy
1:2	1,254.26 ± 10.24	106.66
1:5	3,160.46 ± 12.59	107.51

The back-calculated concentrations for both 1:2 and 1:5 dilution samples were within acceptable limits. The %RSD values (0.82% and 0.40%) and % mean accuracy (106.66% and 107.51%) demonstrate that the method can accurately quantify samples exceeding the upper limit of quantification after appropriate dilution.
Dilution integrity results showed that back-calculated concentrations for samples diluted 1:2 and 1:5 maintained accuracy over 106%, with very low variability (%RSD ≤0.82%). This verifies that samples above the quantifiable range can be diluted without loss of accuracy or precision.

- **Stability Studies:**

• Stock & Working Solution Stability:

Short-Term Stock Solution Stability (Ambient Temperature):

Table 17: Short-Term Stock Solution Stability of Posaconazole and Posaconazole-D5 at Ambient Temperature

Parameter	Fresh (Mean ± SD)	Stability (Mean ± SD)	% Difference
Posaconazole Peak Area Ratio (Analyte/IS)	2.9621 ± 0.0208	2.9842 ± 0.0111	0.60
Posaconazole-D5 IS/Analyte Ratio	0.3363 ± 0.0039	0.3373 ± 0.0019	0.35

Stock solutions of Posaconazole and Posaconazole-D5 were stable up to ~21 hours at ambient temperature, with minimal variation in analyte/internal standard response.

Long-Term Stock Solution Stability (2–8°C):

Table 18: Long-Term Stock Solution Stability of Posaconazole and Posaconazole-D5 at 2–8°C

Parameter	Fresh (Mean ± SD)	Stability (Mean ± SD)	% Difference
Posaconazole Peak Area Ratio (Analyte/IS)	2.8822 ± 0.0183	2.8930 ± 0.0099	0.52
Posaconazole-D5 IS/Analyte Ratio	0.3452 ± 0.0026	0.3462 ± 0.0020	0.53

Stock solutions were stable for 3 days at 2–8°C, supporting reliable use for long-term experiments.

Short-Term Working Solution Stability (Ambient Temperature):

Table 19: Short-Term Working Solution Stability of Posaconazole and Posaconazole-D5 at Ambient Temperature

Concentration / Parameter	Fresh (Mean ± SD)	Stability (Mean ± SD)	% Difference
Posaconazole LLOQ (52.60 ng/mL)	0.0306 ± 0.0003	0.0303 ± 0.0005	-0.74
Posaconazole ULOQ (15,655.77 ng/mL)	8.7353 ± 0.0850	8.7299 ± 0.0665	-0.38
Posaconazole-D5 IS/Analyte Ratio	0.3355 ± 0.0039	0.3380 ± 0.0034	0.79

Working solutions were stable for ~21 hours at ambient temperature, suitable for routine sample preparation.

Long-Term Working Solution Stability (Ambient Temperature):

Table 20: Working Solution Stability of Posaconazole and Posaconazole-D5 at Ambient Temperature

Concentration / Parameter	Fresh (Mean ± SD)	Stability (Mean ± SD)	% Difference
Posaconazole LLOQ	0.0307 ± 0.0003	0.0306 ± 0.0008	-0.36
Posaconazole ULOQ	8.5102 ± 0.0255	8.4942 ± 0.0383	-0.24
Posaconazole-D5 IS/Analyte Ratio	0.3463 ± 0.0018	0.3473 ± 0.0017	0.53

Working solutions were stable for ~17 hours and 53 minutes at ambient temperature.

- **Bench-Top Stability:**

Table 21: Plasma QC Samples

QC Level	Nominal (ng/mL)	Calculated (ng/mL)	% RSD	% Mean Accuracy
LQC	7.85	8.37 ± 0.092	1.09	106.62
HQC	587.95	619.70 ± 4.39	0.71	105.40

Posaconazole was stable in plasma for ~20 hours at ambient temperature, allowing safe storage and analysis within 2 days of collection.

- **Freeze-Thaw Stability**

Table 22: Freeze–Thaw Stability of Posaconazole in Plasma QC Samples

QC Level	Nominal (ng/mL)	Calculated (ng/mL)	% RSD	% Mean Accuracy
LQC	7.85	8.24 ± 0.228	2.76	104.97
HQC	587.95	614.05 ± 4.30	0.70	104.44

Posaconazole remained stable after five freeze-thaw cycles at -20°C, confirming that repeated freezing and thawing does not affect sample integrity.

Stability studies confirmed that stock solutions of Posaconazole and its internal standard remain stable for approximately 21 hours at room temperature and for at least 3 days refrigerated at 2–8°C, with minimal changes in response. Working solutions were stable for around 21 hours short-term and nearly 18 hours long-term at ambient temperature, with low variation. Plasma QC samples retained stability for about 20 hours on the bench, maintaining accuracies above 105% and low %RSD values. Furthermore, the analyte remained stable after five freeze-thaw cycles, with accuracy around 104–105% and %RSD within acceptable limits. These stability data reinforce the method's suitability for routine bioanalysis under typical laboratory conditions.

CONCLUSION:

From the above executed research it can be concluded that, the validated LC–MS/MS method for Posaconazole demonstrated high sensitivity, selectivity, accuracy, and precision across the entire analytical range. System suitability and chromatographic optimization confirmed robust and reproducible retention times, peak shapes, and instrument response. Calibration curves were linear with excellent correlation, and QC samples showed consistent accuracy and precision, including at the LLOQ level. Minimal matrix effects, high extraction recovery, and maintained stability under various conditions—including bench-top, freeze-thaw, and long-term storage—further support the reliability of the method. Overall, the method is robust, reproducible, and fully suitable for the quantitative determination of Posaconazole in plasma for routine bioanalytical applications.

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REFERENCES:

1. Bharti Mittu AC, Chauhan P. Analytical method development and validation: a concise review. J Anal Bioanal Tech. 2015;6(1):1–5.
2. Breaux J, Jones K, Boulas P. Analytical methods development and validation. Pharm Technol. 2003:6–13.
3. Khopkar SM. Basic concepts of analytical chemistry. New Delhi: New Age International; 1998.
4. Schreiber A. Analysis of pesticides in food samples using the SCIEX Triple Quad™ 3500 system. Food Anal Simplified. 2017:5.
5. Moein MM, El Beqqali A, Abdel-Rehim M. Bioanalytical method development and validation: critical concepts and strategies. J Chromatogr B. 2017 Feb 1;1043:3–11.
6. Kalakuntla RR, Kumar KS. Bioanalytical method validation: a quality assurance auditor viewpoint. J Pharm Sci Res. 2009 Sep 1;1(3):1.
7. Zheng YZ, Wang S. Advances in antifungal drug measurement by liquid chromatography–mass spectrometry. Clin Chim Acta. 2019 Jan 25.
8. Groll AH, Walsh TJ. Posaconazole: clinical pharmacology and potential for management of fungal infections. Expert Rev Anti Infect Ther. 2005 Aug 1;3(4):467–87.
9. Chawla G, Ranjan C. Principle, instrumentation, and applications of UPLC: a novel technique of liquid chromatography. Open Chem J. 2016 May 6;3(1).
10. SCIEX. The theory of HPLC: chromatographic parameters. E-Learning for the Analytical Chemistry Community. LC/GC, Chromacademy. 2014:1–21.
11. HPLC Channel. The theory of HPLC and instrumentation of HPLC.
12. De Hoffmann J, Charette J, Stroobant V. Mass spectrometry: principles and applications. New York: John Wiley & Sons; 1996. p. 91–97.
13. Van Eeckhaut A, Lanckmans K, Sarre S, Smolders I, Michotte Y. Validation of bioanalytical LC–MS/MS assays: evaluation of matrix effects. J Chromatogr B. 2009;877(23):2198–2207.
14. Kalakuntla RR, Kumar KS. Bioanalytical method validation: a quality assurance auditor viewpoint. J Pharm Sci Res. 2009 Sep 1;1(3):1.
15. USFDA. Guidance for industry: bioanalytical method validation in pharmaceutical development. U.S. Department of Health and Human Services; 2013.
16. Tiwari G, Tiwari R. Bioanalytical method validation: an updated review. Pharm Methods. 2010 Oct 1;1(1):25–38.
17. Food and Drug Administration. Guidance for industry: bioanalytical method validation. US Department of Health and Human Services, CDER, CVM; 2013 Sept.
18. Cunliffe JM, Noren CF, Hayes RN, Clement RP, Shen JX. A high-throughput LC–MS/MS method for the quantitation of posaconazole in human plasma: implementing fused core silica liquid chromatography. J Pharm Biomed Anal. 2009 Aug 15;50(1):46–52.
19. Reddy TM, Tama CI, Hayes RN. A dried blood spots technique-based LC–MS/MS method for the analysis of posaconazole in human whole blood samples. J Chromatogr B. 2011 Nov 15;879(30):3626–38.
20. Alffenaar JW, Wessels AM, Van Hateren K, Greijdanus B, Kosterink JG, Uges DR. Method for therapeutic drug monitoring of azole antifungal drugs in human serum using LC/MS/MS. J Chromatogr B. 2010 Jan 1;878(1):39–44.
21. Shen JX, Krishna G, Hayes RN. A sensitive liquid chromatography and mass spectrometry method for the determination of posaconazole in human plasma. J Pharm Biomed Anal. 2007 Jan 4;43(1):228–36.
22. Baietto L, D'Avolio A, Ventimiglia G, De Rosa FG, Siccardi M, Simiele M, et al. Development, validation, and routine application of a HPLC method with single mass detector for quantification of itraconazole, voriconazole, and posaconazole in human plasma. Antimicrob Agents Chemother. 2010 Aug 1;54(8):3408–13.
23. Franceschi L, D'Aronco S, Furlanut M. Development and validation of a LC–MS/MS method for determination of voriconazole and posaconazole in serum samples. J Bioanal Biomed. 2011;3:92–7.
24. Nagaraju C, Ray UK, Vidavalur S. Development and validation of chiral HPLC method for the identification and quantification of enantiomer in posaconazole drug substance. Chem Sci. 2018;7(3):506–14.
25. Wadsworth JM, Milan AM, Anson J, Davison AS. Development of a LC–MS/MS method for simultaneous measurement of voriconazole, posaconazole, and itraconazole. Ann Clin Biochem. 2017 Nov;54(6):686–95.
26. Khalil HA, El-Yazbi AF, Belal TS, Hamdy DA. High performance liquid chromatographic assay for simultaneous determination of posaconazole and vincristine in rat plasma.
27. DrugBank. Drug profile for posaconazole.