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Original Research Article

RP-HPLC Bioanalytical Method Development and Validation of Posaconazole

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Abstract: A simple, quick, sensitive, accurate, and exact high-performance liquid chromatography (HPLC) method with UV detection was created and tested to find out how much posaconazole is in human plasma. The chromatographic separation was done on a C18 column (250 × 4.6 mm) with a mobile phase of acetonitrile and water (55:45, v/v) at a flow rate of 0.8 mL/min. The measurement was at 262 nm. During the development of an analytical method, the technique showed good linearity in the concentration range of 5–100 ppm with a regression score of 0.9954. Acetonitrile was used to separate the proteins in plasma samples so that drugs could be pulled out. With a regression coefficient (r^2) of 0.9955, bioanalytical confirmation found linearity over the range of 0.15–50 ppm. Posaconazole was found to have an average recovery rate of 85.63% from plasma. It was found that accuracy and precision were good enough. Testing for stability revealed that even small deliberate adjustments to the chromatographic settings did not affect the process. Posaconazole's stability in human plasma was confirmed by stability tests showing that %CV values fell within regulatory limits for freeze-thaw, short-term, and long-term stability. The proven method is fine for normal bioanalytical uses.

Keywords: RP-HPLC, Posaconazole, Bioanalytical, Method development and validation.

INTRODUCTION

Posaconazole is a triazole antifungal drug that works against many different kinds of fungi. It is frequently employed to treat and prevent invasive fungal infections, particularly in individuals with compromised immune systems (1). It can strongly fight fungi of the genus Aspergillus, Candida, and other species that are important to

public health (2). For pharmacokinetic study and therapeutic drug monitoring, it's very important to acquire an exact measurement of posaconazole in biological matrices because its therapeutic window is small, its bioavailability changes, and it may interact with other drugs (3). A lot of different diagnostic methods have been recorded for finding out the amount of posaconazole there is, including high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC–MS/MS) (4). Even though LC-MS/MS techniques are very sensitive, they are often associated with high running costs, difficult sample preparation, and limited availability in clinical and routine quality control labs (5). For bioanalytical uses, though, reverse-phase high-performance liquid chromatography (RP-HPLC) with UV detection is a cheap, reliable, and easy-to-use replacement (6). A simple and dependable RP-HPLC method for measuring posaconazole in human plasma needs to be created so that regular bioanalysis can be done more easily (7). Bioanalytical technique validation must follow regulatory guidelines, which say that selectivity, linearity, accuracy, precision, recovery, robustness, stability under different circumstances, and other factors must be checked (8). Proper sample preparation, such as protein precipitation, makes the extraction process even more efficient and consistent (9). The goal of this work is to come up with a simple, quick, accurate, and precise RP-HPLC method with UV detection that follows normal bioanalytical validation rules so that posaconazole can be measured in human plasma (10). The accepted method is supposed to work for pharmacokinetic studies and routine checks on how well therapeutic drugs are working (11).

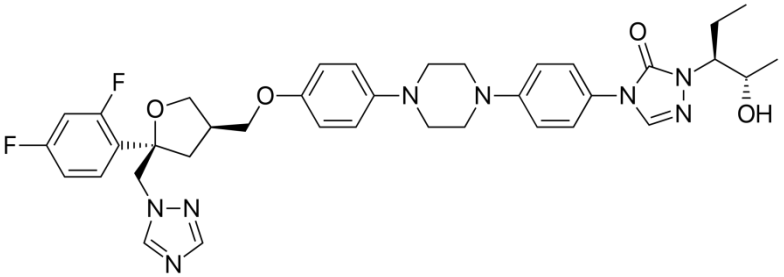

Figure 1: Structure of Posoconazole

MATERIALS AND METHODS

Instruments and Chemicals:

A pure sample of posaconazole was acquired from Brick Pharmaceuticals. Throughout the investigation, analytical-grade water and HPLC-grade acetonitrile were utilized. Every chemical and reagent used was pure enough for chromatographic analysis. The Agilent 1120 Compact LC system was utilized to perform the chromatographic analysis. A Shimadzu UV-1700 UV/Visible spectrophotometer was used to perform UV spectrophotometric measurements. An LC/GC analytical balance was used to weigh the samples, and a Lifecare ultrasonic bath was used to help prepare them.

Conditions for Optimization of Chromatographic Method:

To get the best mobile phase, the resolution, peak symmetry, and peak area for posaconazole were measured. Different mobile phase mixtures were tested, such as acetonitrile (HPLC grade) and water (HPLC grade) in different ratios and methanol and water. Acetonitrile: water at a flow rate of 0.8 ml/min turned out to be the best of these because it gave uniform, well-resolved peaks for posaconazole. Chromatographic detection was done at 262 nm, which is posaconazole's highest absorbance. The chromatogram had a good peak shape and a fair resolution of the standard drug. The tailing factor was within the acceptable range. Posaconazole's retention period was found to be 4.683 minutes. The optimized mobile phase was used to find the system suitability parameters. These were shown to be within the appropriate range (12, 13).

Mobile Phase Preparation:

The mobile phase was HPLC-grade acetonitrile and HPLC-grade water mixed together in a 55:45 (v/v) ratio. It was filtered through a 0.45-µm membrane filter and sonicated in an ultrasonic bath for 15 minutes before being used (14).

Standard Stock Solution Preparation:

After 2.5 mg of the working standard was carefully weighed, it was put into a 25 mL volumetric flask to make a standard stock solution of posaconazole. About 10 mL of HPLC-grade methanol was added, and then the mixture was sonicated for 20 minutes to make sure that everything dissolved completely. Then, 25 mL of HPLC-grade water was added to the mixture to get a final concentration of 100 ppm. The solution that was made was filtered using a nylon-66 membrane filter with a pore size of 0.45 µm. The stock solution was properly mixed with methanol to make the working standard solutions that came next (15-17).

Human Blood Plasma Separation:

The human blood plasma was given by Neon Laboratory. Blood samples were taken using purple-top EDTA tubes. The samples were then spun in a centrifuge for 20 minutes at 4°C at 3000 rpm. After spinning, the plasma was carefully separated using a clean pipetting method. 1.0 mL of plasma was then

placed into a 1.5 mL Eppendorf tube that was marked with the right tracking number and labeled as plasma (18, 19).

Sample Solution Preparation:

The sample solutions were made by mixing 100 µL of working standard solutions of posaconazole at 0.150, 0.5, 1.5, 2.5, 3.5, and 5 ppm with 0.90 mL of rat plasma. 1.0 mL of acetonitrile was added to the mixture as a precipitating agent to make plasma proteins form, and it was then vortexed for a long time. The samples that were centrifuged for 15 minutes at 3000 rpm at 2–4°C for 15 minutes showed end concentrations of 1.5, 5, 15, 25, 35, and 50 ppm. Once the clear supernatant was properly sorted, chromatographic analysis was done (20).

Spiking of Posaconazole in plasma

To achieve the necessary calibration concentrations, posaconazole was spiked in plasma by adding suitable volumes of the posaconazole working standard solutions to blank plasma. To guarantee that the medication was distributed evenly, precisely measured volumes of the standard solutions were combined with plasma samples. Following centrifugation and protein precipitation with acetonitrile, the spiking plasma samples were collected for RP-HPLC analysis of the clear supernatant (21, 22).

Table 1: Human Blood Plasma Spiking of Posaconazole

Sr. No	Concentration (ppm)	Volume of Spiking (ml)	Volume of Plasma (ml)	Final volume (ml)	Final concentration (ppm)
1	05	0.1	0.9	1	0.5
2	10	0.1	0.9	1	1.0
3	15	0.1	0.9	1	1.5
4	25	0.1	0.9	1	2.5
5	35	0.1	0.9	1	3.5
6	50	0.1	0.9	1	5.0

Method Development and Validation:

When bioanalytical methods are validated, it means that the data they give us about biological things is correct. Selectivity, sensitivity, accuracy, precision, consistency, recovery, linearity, and stability are critical validation features. To show that the method used is correct, it is necessary to confirm each substance that is measured in a biological matrix. The current RP-HPLC bioanalytical method was created and validated after its selectivity, accuracy, precision, recovery, calibration curve linearity, and analyte stability in treated plasma samples were all tested (23).

Selectivity: Blank plasma samples from at least six different sources were tested to see if they would interfere with the selection test. The retention time of posaconazole showed no peaks that could have interfered, which means that the method had enough specificity (24).

Calibration Curve: Calibration curves over a concentration range of 5–100 ppm were possible by plotting the peak area against the standard concentration. The data, which showed strong linearity over the tested range, are summed up in Table 2 (24).

Quality Control Samples Preparation: Quality control (QC) samples were made with three different amounts of concentration: low quality control (LQC, 1.5 ppm), medium quality control (MQC, 25 ppm), and high quality control (HQC, 50 ppm) (24).

Accuracy and Precision: The method's precision was checked by repeating measurements of the LQC, MQC, and HQC samples three times each. Both intraday and interday accuracy were tested. a. To find out how accurate and precise the three different amounts were within the same batch, they were each measured twice on the same day. b. To check inter-batch accuracy and precision, the same amounts were analyzed twice on different days (24).

Recovery: Comparing the analytical reactions of extracted plasma samples at low, medium, and high QC levels with those of unextracted reference solutions—which indicated 100% recovery—was how recovery investigations were carried out (24).

Stability Studies

Stability of posaconazole in plasma was assessed under different conditions, including freeze–thaw, short-term, and long-term stability. For freeze–thaw stability, samples underwent three cycles and were analyzed after the third cycle. Short-term stability was evaluated by keeping three aliquots each of LQC and HQC samples at room temperature for 8 hours, followed by analysis. Long-term stability was determined by storing at least three aliquots of LQC and HQC samples under specified conditions for 15 days and subsequently analyzing them (9).

RESULT AND DISCUSSION:

Posaconazole in plasma was estimated using the developed RP-HPLC bioanalytical technique, which underwent a rigorous validation process for selectivity, linearity, accuracy, precision, recovery, and stability. The acquired results verify that the technology is appropriate for regular bioanalysis.

Chromatographic Performance and Selectivity

The RP-HPLC chromatogram of posaconazole (Figure 2) demonstrated a well-resolved and symmetrical peak with a consistent retention time, indicating adequate chromatographic separation. Analysis of blank plasma samples (Figure 4) showed no internal interference at the retention time of posaconazole, confirming the selectivity of the method. Furthermore, the chromatogram of posaconazole spiked in blank plasma (Figure 5) exhibited clear and distinct peaks without matrix interference, validating the method's specificity.

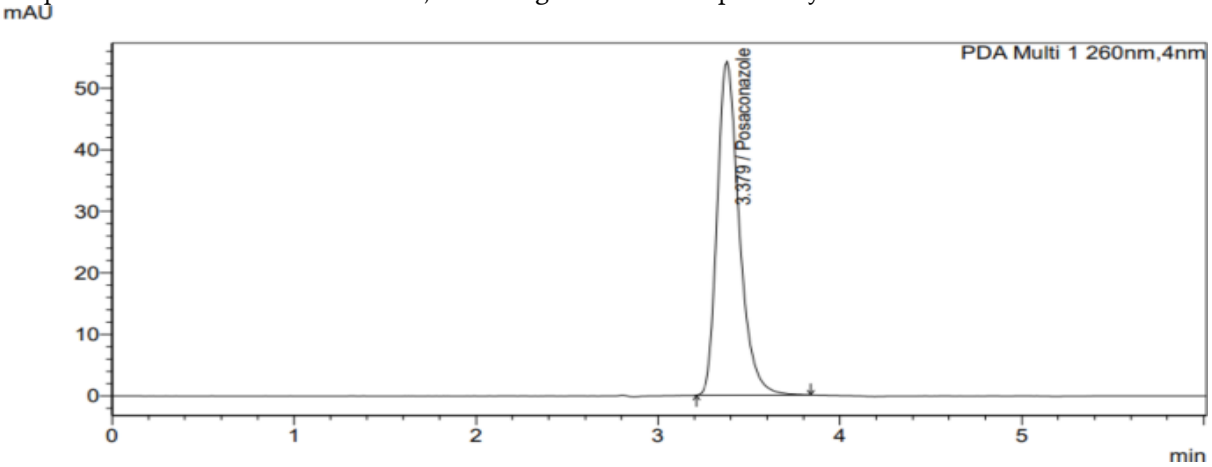

Figure 2: Posaconazole RP-HPLC chromatogram showing its retention time (RT).

Linearity and Calibration Curve

The method's repeatability was tested in the range of 0.5 to 5.0 ppm. The calibration curve formed a good correlation coefficient and a linear reaction when peak area and nominal concentration were plotted against each other. The regression results in Table 2 show that the peak area increases in direct proportion to the posaconazole concentration. The linear link is backed up by the posaconazole calibration curve spiked in plasma (Figure 3), which shows that the method can be used for quantitative analysis in the range that was looked at.

Table 2: Standard Posaconazole Calibration Data for Linearity

Sr. No	Concentration (ppm)	Peak Area (mAU)	Standard Deviation (SD)
1	0.5	296797	0.215
2	1.0	724593	0.0325
3	1.5	1129352	0.0227
4	2.5	1603231	0.0743
5	3.5	1992591	0.0518
6	5.0	2511290	0.0725

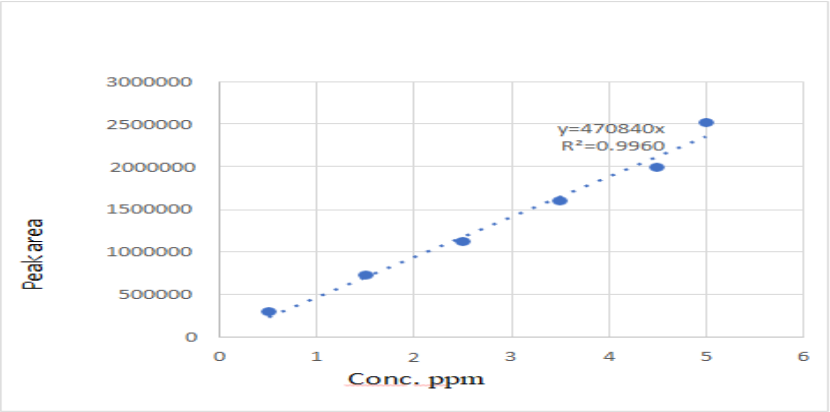

Figure 3: Posaconazole Calibration curve of spiked in plasma

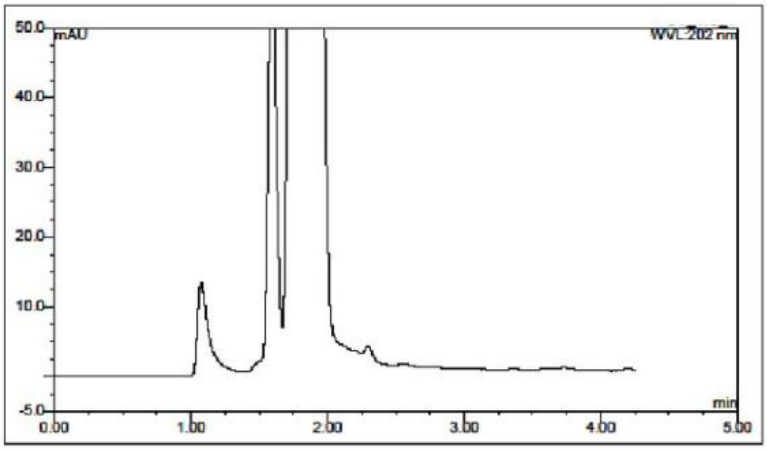

Figure 4: Blank Chromatogram of plasma

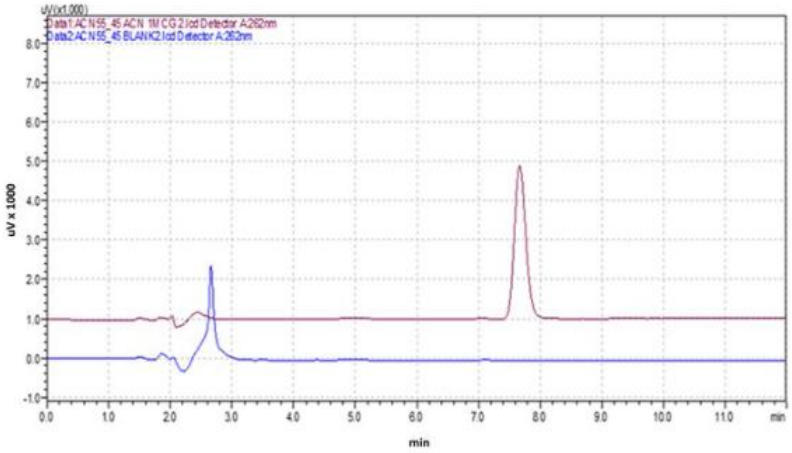

Figure 5: Chromatogram of the Posaconazole with Blank

Accuracy and Precision:

Quality control samples at three concentration levels—LQC (0.5 ppm), MQC (2.5 ppm), and HQC (5.0 ppm)—were used to test how accurate and precise the work was. Intra-batch (within-day) accuracy and precision data can be seen in Table 3. The %CV values were within the right range, and the %precision values ranged from 97.12% to 99.92%, demonstrating that the method could be used multiple times with consistent results. The inter-batch (between-day) precision and accuracy results in Table 4 showed that the method worked the same on all days. The accuracy rates were between 96.56% and 98.98%, and the CV rates were well within the standards set by the government. These results indicate that the method can accurately and precisely measure posaconazole in plasma.

Table 3: Data of Accuracy and Precision for posaconazole in plasma

Quality Control Sample	Amount. Added (ppm)	Peak Area	Amount found (ppm)	% Accuracy	% C.V

LQC	0.5	296796	0.49	98.15	0.2280
	0.5	296489	0.48	97.12	
	0.5	296199	0.49	98.15	
MQC	2.5	1129352	2.46	98.43	0.4407
	2.5	1139126	2.47	98.88	
	2.5	1139489	2.48	98.82	
HQC	5	2511290	4.96	99.92	0.6179
	5	2512671	4.89	98.47	
	5	2509612	4.87	97.94	

Table 4: For Posaconazole in plasma Inter batch Accuracy and Precision

Quality Control Sample	Amount. Added (ppm)	Peak Area*	Amount found (ppm)	% Accuracy	% C.V
LQC	0.5	269796	0.48	98.00	0.2391
	0.5	296482	0.47	98.45	
	0.5	296191	0.49	96.90	
MQC	2.5	1129352	2.49	96.56	0.2449
	2.5	1139484	2.46	96.98	
	2.5	1139216	2.47	98.80	
HQC	5	2511296	4.89	98.98	0.6052
	5	2512671	4.87	98.81	
	5	2509612	4.86	98.44	

Recovery

Posaconazole recovery from plasma was assessed at low, medium, and high concentrations. According to Table 5, the extraction efficiency was constant across the tested concentrations, with the mean recovery ranging from 85.26% to 87.31%. The reproducible recovery values indicate that the protein precipitation method using acetonitrile is suitable for plasma sample preparation.

Table 5: For Posaconazole in plasma Recovery study

Conc. (ppm)	Peak Area (Extracted)	Peak Area (Un-extracted)	% Recovery
0.5	296796	339896	87.31%
2.5	1129352	1318789	85.63%
5	2511290	2945278	85.26%

Stability Studies

To maintain analyte integrity during analysis, the stability of posaconazole in plasma was evaluated under various handling and storage circumstances. After three freeze-thaw cycles, posaconazole remained stable, according to freeze-thaw stability results (Table 6), with %CV within acceptable bounds and % purity values near nominal concentrations. Studies of short-term stability carried out for eight hours at room temperature (Table 7) showed no change in concentration, suggesting sufficient stability during standard sample handling. Posaconazole was stable in plasma for 15 days, with % purity values reaching 98% and %CV values within acceptable bounds, according to long-term stability tests (Table 8).

Table 6: For Posaconazole in plasma Freeze and Thaw stability

Conc. (ppm)	Peak Area	Conc. Found	% Purity	S.D	% C.V
0.5	296421	0.48	98.72	0.3295	0.2430
2.5	1129322	2.49	99.33	0.2468	0.4486

Table 7: For Posaconazole in plasma Short term temperature stability

Conc. (ppm)	Peak Area	Conc. Found (ppm)	% Purity	S.D	% C.V
0.5	296221	0.48	98.70	0.2100	0.2114
2.5	1129355	2.48	99.12	0.4661	0.4761

Table 8: For Posaconazole in plasma Long Term Stability

Conc. (ppm)	Peak Area	Conc. Found (ppm)	% Purity	S.D	% C.V
0.5	296421	0.49	99.1	0.31121	0.2225
2.5	1129322	2.47	98.82	0.2900	0.4982

CONCLUSION

Following the USFDA's bioanalytical validation rules, a simple, sensitive, and reliable RP-HPLC bioanalytical method for measuring posaconazole in plasma was successfully created and confirmed. The method was able to reliably and accurately distinguish between concentrations in the range that was tested. It also showed good predictability, precision, recovery, and stability. A simple protein precipitation process made it possible to consistently and reliably remove posaconazole from plasma. All of the validation factors were found to be within the limits set by the government, and the validated method was shown to be correct, exact, and able to be used more than once with the same results. The suggested method is good for studying the pharmacokinetics, toxicokinetics, bioavailability, and bioequivalence of posaconazole pill formulations because it works well with plasma samples and is generally reliable. It can also be used to measure the amount of posaconazole in rat plasma.

DECLARATIONS:

Consent for publication:

All the authors approved the manuscript for publication.

Competing interests:

All authors declare no competing interests.

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