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Analytical Method Development and Validation of Posaconazole for Injection

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Abstract
The development and validation of analytical methods for pharmaceutical compounds, such as Posaconazole, are critical for ensuring safety, efficacy, and quality in injectable formulations. Posaconazole, a triazole antifungal agent, is widely used for treating and preventing invasive fungal infections, especially in immunocompromised patients. This study presents a robust, sensitive, and specific method for the quantitative analysis of Posaconazole in its injectable form, utilizing high-performance liquid chromatography (HPLC). Key parameters including accuracy, precision, specificity, linearity, limit of detection (LOD), and limit of quantitation (LOQ) were carefully evaluated to validate the method in accordance with ICH guidelines. The developed method exhibited a high degree of precision and accuracy, with linearity observed over a specified concentration range. The LOD and LOQ values confirmed the method's sensitivity, ensuring reliable detection and quantification of Posaconazole even at low concentrations. This validated method is suitable for routine quality control analysis of Posaconazole injections, ensuring the drug's consistency, safety, and compliance with regulatory standards.

Keywords: Method development, HPLC, Posaconazole, Injection

Introduction

A triazole, posaconazole has only just entered the market. Inhibiting 14a-demethylase (CYP51), an enzyme critical to yeast and mold ergosterol production, is the typical mechanism of action of azoles [1-4]. Posaconazole is an alternative to other antifungal drugs that may be used in salvage treatment. It is a broad-spectrum azole that targets a wide variety of fungus, including species of Aspergillus and Candida [5-6].

The area under the curve (AUC) did not rise with dosages over 800 mg of posaconazole, while it is well-tolerated when taken orally at doses of 600–800 mg daily. Taking it with fatty meals might increase its AUC by 400%. Posaconazole is metabolized by glucuronization, despite the fact that it is an inhibitor of CYP3A4. In comparison to other triazoles, such voriconazole, it has a much better and smaller drug interaction profile, which means that its potential for drug interactions is limited. Measuring blood levels may be crucial, particularly in those with impaired absorption, including cancer patients or those after abdominal surgery, because of the known varied systemic availability of the chemically similar itraconazole. Intestinal resorption may be affected by mucositis or major abdominal surgery as posaconazole can only be taken orally [7-13].

One popular analytical method for measuring pharmaceuticals in dose forms is high-performance liquid chromatography, or HPLC. In order to reliably estimate posaconazole in dosage form, this work seeks to develop and verify a novel HPLC analytical technique that is fast, sensitive, robust, inexpensive, and well-designed.

First, dissolve the active ingredient in solvent. Then, inject the mixture into the high-performance liquid chromatography (HPLC) system. Finally, separate the components according to how they interact with the stationary phase. A UV-Vis detector monitors the absorbance of posaconazole in the eluent, allowing for its detection. In order to determine the concentration of posaconazole in the sample, the peak area or height is linked with a standard calibration curve [14-26].

Chromatographic parameters, such as mobile phase composition, column type, and detection wavelength, were fine-tuned to maximize the recently established approach. The method's accuracy, precision, and specificity were confirmed by validation that adhered to the requirements set forth by the International Conference on Harmonization (ICH) Q2 R1. Its injections containing posaconazole were accurately measured using the procedure [27-35].

Material and Methods

Instrumentation

a. A high-performance liquid chromatography (HPLC) system equipped with either the Waters 2695 separation module or the Waters e2695 separation module along with a 2489 UV-visible detector (Waters Alliance or equivalent), or a Shimadzu 2010 CHT system with a UV-visible detector or an equivalent model.
b. Data handling system: Empower Pro or LC Solutions, or an equivalent system.
c. Analytical column: A stainless steel column, 250 mm in length and 4.6 mm in internal diameter, packed with octadecyl silane-bonded porous silica particles with a diameter of 5.0 µm. Recommended: Zorbax SB C18 (250 x 4.6 mm, 5.0 µm).

Preparation of Diluent Orthophosphoric Acid Solution

Transferred 5 mL of Orthophosphoric acid into a 100 mL clean, dry volumetric flask and dilute to volume with water and mix well.

Preparation of pH 3.5 Buffer

Weigh approximately 1.37 g of sodium dihydrogen phosphate monohydrate and dissolve it in 1000 mL of water. Mix thoroughly and adjust the pH of the solution to 3.5 ± 0.05 using a dilute solution of orthophosphoric acid. Filter the resulting solution through a 0.45 µm membrane filter and degas it.

Preparation of Mobile Phase

- Mobile Phase –A: 100% buffer solution.
- Mobile Phase – B: Prepared a degassed mixture of methanol and Acetonitrile in the ratio of 40:60 v/v.

Preparation of diluent

Prepared a degassed mixture of Acetonitrile and water in the ratio of 50:50 v/v.

Chromatographic conditions

- Instrument : Shimadzu HPLC or equivalent.
- Column : Zorbax SBC 18, (250X4.6) mm, 5.0µm or equivalent.
- Pump mode : Gradient
- Flow rate : 1.0 mL/minute
- Detection : UV, at 262 nm
- Injection Volume : 10 µL
- Column oven temperature : 30°C
- Data acquisition time : 60 minutes

Table 1: Chromatographic conditions

Time (min)	Mobile Phase –A (%v/v)	Mobile Phase –B (%v/v)
T 0.01	50	50
T 1.0	50	50
T 40.0	20	80
T 45.0	20	80
T 46.0	50	50
T 60.0	50	50

Preparation of solution:

Blank solution: Diluent.

Standard stock solution:

Accurately weigh approximately 30 mg of Posaconazole standard and transfer it to a clean, dry 50 mL volumetric flask. Add about 20 mL of diluent and sonicate until completely dissolved. Then, dilute the solution to the final volume with the diluent. Next, transfer 4 mL of this solution into a 20 mL volumetric flask, dilute it to the mark with diluent, and mix thoroughly.

Standard solution: (600 PPM of Posaconazole)

Accurately transferred 3 mL of standard stock solution into a 100 mL clean, dry volumetric flask, diluted to volume with diluents and mixed well.

Placebo solution

Precisely transferred 3 mL of the standard stock solution into a clean, dry 50 mL volumetric flask, then diluted to the mark with diluent and mixed thoroughly

Sample solution

Carefully measured and transferred 3 mL of the standard stock solution into a clean, dry 50 mL volumetric flask, then brought the volume up to the mark with diluent and mixed thoroughly.

Procedure

The chromatographic conditions were established as previously outlined, and the column was equilibrated with the mobile phase until a stable baseline was achieved.

The blank solution (diluent) was injected twice into the chromatograph, with the second injection being recorded as the blank chromatogram.

Following this, the standard solution was injected six times into the chromatograph, and the resulting chromatograms were recorded to measure the peak area

3.4.9 Evaluation of system suitability:

The USP tailing factor for the Posaconazole peak from the first injection of the standard solution must not exceed 2.0. Additionally, the USP tailing factor for the Posaconazole peak from the first injection of the standard solution should not be less than 5000. It should also not exceed 5.0.

Next, inject the placebo solution into the chromatograph and record the resulting chromatogram. Afterward, inject the sample solution into the chromatograph and document the chromatogram.

The blank (diluent) and placebo chromatograms were analyzed for any extraneous peaks. Any corresponding peaks noted in the sample solution chromatograms were disregarded. The retention time (RT) for the Posaconazole peak is approximately 28 minutes (for reference only).

Note: Peaks with relative retention times (RRT) around 0.068 and 0.072 should be disregarded, as these correspond to Betadex Sulfobutyl Ether Sodium and Edetate Disodium

Table 2: Elution Order

S.No.	Name	#RRT
1	PSK-I 4-(4-(4-(4-(((3R, 5R)-5-((1H-1,2,4-triazole 1-yl) methyl)-5-(2,4-diflurophenyl) tetrahydrofuraon-3-yl)methoxy) phenyl) piperazin-1-yl) phenyl)-2-((2S,3S)-2-(benzyloxy) pentan-3-yl)-2,4-dihydro-3H-1,2,4-triazol-3-one	~0.20
2	PSK-2 2-((2S,3S)-2-hydroxypentane -3yl)-4-(4-(4-(4-hydroxy phenyl) piperazin-1 yl) phenyl)-2-4-dihydro-3H-1,2,4-triazol-3-one)	~1.59
3	Posaconazole	= 1.00

#Relative Retention Time

RRT's for the process related impurity given for information only and to be integrated but not reported.

Note-2: The RT and RRT may vary slightly based on system configuration and column usage.

Calculation:

$$Any\ individual\ unspecified\ degrdation\ product = \frac{AU}{AS}\ x\ \frac{\text{Ws}}{50} x\ \frac{4}{20}\ x \frac{3}{100} x\ \frac{50}{Sv} x\ \frac{P}{LC}$$

Total degradation products (%) = sum of all any individual unspecified impurities

Where,

Au = Area count of peaks due to any individual unspecified degradation Product

Au	=	Area count of peaks due to any individual unspecified degradation product in the chromatogram of sample solution.
As	=	Average area counts of Posaconazole peak from six replicate injections of standard solution.
Ws	=	Weight of Posaconazole standard taken in mg.
Sv	=	Volume of sample taken in mL.
P	=	Percent potency of Posaconazole standard (on as is basis).
LC	=	Label claim of Posaconazole in Posaconazole injection (18mg/mL).

Validation parameters

Based on the information provided, the test method has been validated, demonstrating its suitability for producing consistent and reproducible results across the specified range for the determination of related substances, as outlined by their specification limits in routine drug product testing. The following sections detail the validation parameters that have been assessed for identifying related substances in the Posaconazole injection:

a. Specificity
b. Limit of detection and limit of quantification
c. Linearity
d. Precision (System precision, method precision and intermediate precision).
e. Accuracy (Recovery)
f. Range
g. Stability of standard, sample solution, and mobile phase
h. Robustness
i. System suitability

Specificity

Procedure: Solutions for blank, placebo, standard, and sample were prepared and injected into the system following the test procedure. Additionally, all individual impurities were prepared and injected.

Identification

Solutions of the Posaconazole standard and the Posaconazole injection sample were prepared according to the test method and subsequently injected into the HPLC following the established methodology.

Placebo interference

The placebo solution was prepared according to the test method and injected into the HPLC following the established methodology. Additionally, a blank (diluent) was also injected as per the procedure.

Peak Purity

Individual solutions of known related substances, including Betadex Sulfobutyl Ether Sodium, Sodium Metabisulfite, Sodium Ascorbate, Polysorbate 80, Arginine, and L-Cystine, were prepared and injected to verify their retention times. Solutions of the Posaconazole injection sample (control sample) and a sample spiked with known related substances at their specification levels (spiked sample) were prepared and injected into the HPLC according to the established methodology. Peak purity was assessed using Waters Empower 3 software.

Forced degradation

Samples of Posaconazole injection and their corresponding placebo were subjected to various stress conditions as outlined below. Solutions resulting from each stressed condition were prepared and injected into the HPLC following the established methodology. Peak purity was evaluated using Waters Empower 3 software.

a. Acid Degradation.
b. Base Degradation.
c. Peroxide Degradation.
d. Thermal Degradation.
e. Photolytic Degradation.
f. Humidity Degradation.

Limit of Detection and Limit of Quantitation:

Procedure:

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) for Posaconazole were established based on the slope, standard deviation, and visual assessment of the responses from the linearity study conducted across a range of 1% to 150% of the standard concentration. To confirm the precision of the predicted LOD and LOQ concentrations for Posaconazole, solutions containing the analyte at these concentrations were prepared, and each solution was injected six times into the HPLC, following the specified test method conditions.

Precision

System Precision

Standard solution was prepared as per the test method and injected six times into HPLC as per methodology.

Method Precision

Six sample solutions were prepared individually using placebo spiked with Posaconazole drug substance at 0.2% level as per test method and injected each solution into HPLC as per methodology.

Intermediate Precision (Ruggedness)

The sample was analyzed using a separate solution from the same batch, prepared as outlined in the method precision section. This analysis was conducted by a different analyst utilizing a different HPLC system and column on a different day. The results obtained were then compared to those from the method precision assessment.

Accuracy

Procedure: Samples were prepared API was spiked to the placebo at a concentration of 0.05 mg/mL to 0.15 mg/mL of Posaconazole.

Linearity

Procedure: Samples were prepared from 25 % to 150% with respect to test concentration of 0.1 mg/mL and injected into the system as per the developed test procedure.

Solution stability

Procedure: Sample and standard solutions were prepared and injected as per procedure.

Assay of Posaconazole

Product details

Details of Posaconazole concentration of standard and sample solutions are shown below

Table 3: Assay of Posaconazole

Assay	**Posaconazole Concentration (µg/mL)**	
Strength	Standard	Sample
300 mg/vial	120	115

Based on the information provided, the test method has been validated to demonstrate its capability to produce consistent and reproducible results within the specified limits for the assay of Posaconazole during routine testing of the drug product, as detailed in this report.

The validation parameters that have been evaluated for the assay of Posaconazole in Posaconazole injection are:

1. Specificity

2. Linearity
3. Precision (System precision, method precision and intermediate precision)
4. Accuracy (Recovery)
5. Range
6. Stability of standard, sample solutions and mobile phase
7. Robustness
8. System Suitability

Method Validation

Specificity

Solutions of the Posaconazole standard and Posaconazole injection sample were prepared following the test method and subsequently injected into the HPLC according to the established methodology.

Placebo interference:

The placebo solution was prepared and injected into the HPLC following the specified methodology. Additionally, a blank solution was injected in accordance with the procedure

Known impurity interference and peak purity:

Solutions of the Posaconazole injection unspiked sample (control sample) and the sample spiked with known related substances at specification levels (spiked sample) were prepared in triplicate according to the test method and injected into the HPLC following the established methodology.

Forced Degradation:

The Posaconazole injection samples were subjected to the following stress conditions. Solutions corresponding to each stressed condition were prepared and subsequently injected into the HPLC according to the established methodology. Peak purity was evaluated using Waters Empower 3 software.

a. Acid degradation.
b. Base degradation.
c. Peroxide degradation.
d. Thermal degradation.
e. Photolytic degradation.
f. Humidity degradation.

Linearity

Procedure: A range of solutions was prepared using the Posaconazole standard, covering concentration levels from 50% to 150% of the test concentration. Each of these solutions was then injected into the HPLC following the prescribed methodology.

Precision

Procedure

System Precision

Standard solution -1 was prepared as per test method and injected six times into HPLC**.**

Method Precision

Six sample solutions were prepared individually using single batch of Posaconazole injection as per test method and injected each solution into HPLC as per methodology.

Intermediate precision (ruggedness)

Sample solutions from the same batch were analyzed, prepared separately in accordance with the method precision guidelines. This analysis was conducted by a different analyst using a different column and HPLC system on a separate day. The results obtained were then compared with those from the method precision evaluations

Accuracy

Procedure

Sample solutions were prepared in triplicate by incorporating an equivalent amount of placebo present in the Posaconazole injection, spiked with known quantities of the Posaconazole drug substance. The recovery levels examined were 50%, 75%, 100%, 125%, and 150% of the test concentration, and the analysis was conducted according to the established methodology.

Range

Procedure

The range of the analytical method can be determined from the linearity, precision, and accuracy data. The reported range is expressed as a percentage concerning the test concentration

Robustness

Standard Solution I and Standard Solution II were prepared according to the test method and injected into the HPLC under deliberately varied conditions to assess system suitability and the method's robustness against such variations. The varied conditions include change in flow rate by ±10%, column oven temperature by ±5%, wavelength by ±5 nm, pH of buffer by ±0.2 units, organic composition in mobile phase by ±2% absolute from the methodology values**.**
Standard solution –I and standard solution – II were prepared as per the test procedure and injected into HPLC as per methodology.

Analytical Method Validation: Related substance

The PSK-I 4-(4-(4-(4-(((3R, 5R)-5-((1H-1,2,4-triazole 1 yl) methyl)-5-(2,4 diflurophenyl) tetrahydrofuraon-3-yl) methoxy) phenyl) piperazin 1 yl) phenyl)-2((2S,3S)-2-(benzyloxy) pentan-3-yl)-2,4-dihydro-3H-1,2,4 triazol-3-one and PSK-2 2-((2S,3S)-2-hydroxypentane -3yl)-4-(4-(4-(4 hydroxy phenyl) piperazin-1yl) phenyl) 24-dihydro-3H-1,2,4-triazol-3-one) are the related substances observed in drug hence need to develop a technique for the estimation of these related substance. Hence method was developing and validated. the details are discussed as under; The diluents and Buffer and Mobile phase were used for the method is Orthophosphoric Acid Solution, 3.5 Phosphate buffers and 100% buffer, methanol and acetonitrile 40:60 v/v respectively. These buffers and combination of mobile phase is capable to elute and separate the peaks of aforementioned related substance. In trial-1 the phosphate buffer of 3.5 pH, mobile phase-A (100% buffer) and Mobile Phase-B (100% acetonitrile), diluents (degassed solution of acetonitrile and water in the ratio 50:50% v/v) and the following chromatographic condition used to develop the method.

Chromatographic conditions:

Instrument	: Shimadzu HPLC
Column	: Zorbax SBC18, 250 X 4.6 mm 5 micrometer
Flow	: 1 ml per minute
Pump mode	: Gradient
Detector	: UV at 262 Nm
Injection volume	: 10 microliter
Column temperature	: 30 ºC
Auto sample temperature	: 25 ºC
Run time	: 60 minutes
Mode of elution	: Isocratic in the ratio of 30:70

Due to the above conditions the peak of Posaconazole was eluted at 11.6 minutes. PSK 1 and PSK 2 specific from Posaconazole and unknown impurity elutes at RT 23.7 minutes. The retention time of Posaconazole was more hence the mobile phase and column conditions were further optimizes.

In trilal-2 The mobile phase ratio was changed to 20:80% v/v instead of 30:70 v/v only, remaining mobile phase and chromatographic conditions were the same as trial 1.

The following Observation s were withdrawn

- RT of Posaconazole was 5.6 minutes.

- Retention of PSK1 and PSK 2 were observed at 14.2 and 29.6 minutes but the peak shapes were not satisfactory.

Hence method was further optimized to get better peak shapes by altering flow rate and mobile phase composition. The trial-3 was initialed with mobile phase ratio selected was acetonitrile and buffer sodium dihydrogen phosphate monohydrate of 3.5 pH of 75:25% v/v. ratio and the Chromatographic conditions:

Instrument	: Shimadzu HPLC or equivalent
Column	: Zorbax SBC18, 250 x 4.6 mm 5 micrometer or equivalent
Flow	: 1.2 ml per minute
Pump mode	: Gradient
Detector	: UV at 262 Nm
Injection volume	: 10 microliter
Column temperature	: 40 °C
Auto sample temperature	: 30 °C
Run Time	: 60 minutes
Mode of elution	: Isocratic in the ratio of 35:65%v/v

Due to the above changes the RT of Posaconazole was reduced to 4.7 minutes and PSK 1 was 16.2 and PSK 2 was 2.4 minutes respectively. Conclusion: out of three mobile phases in mobile phase composition ratio acetonitrile and buffer is 35:65% v/v resolution and peak shapes were satisfactory. Based on this observation same methodology was finalize.

Individual Parameters Optimization Summary

Optimization of mobile phase

The phosphate buffer (pH 3.5) was adopted for determination of assay of Posaconazole in Posaconazole injection. Finalized buffer is as follows

As per the API vendor assay methodology, gradient program was given and runtime was 55 minutes, to reduce the run time method development was started with isocratic ratio of buffer and acetonitrile ratio of 50:50. To get the sufficient response and peak shape mobile phase ratio was altered to 60:40 ratios 65:35 and 75:25, 70:30. In all the ratios in 65:35 ratios symmetrical peak was observed and main peak resolved from all other peaks. Hence this ratio was finalized for determination of assay of Posaconazole and Posaconazole injection.

a. Optimization of column

Column mentioned in the AP method was Hypersil BDS 24 X 4.6 mm 5-micron column changed to Zorbax SB C18 250 X 4.6 mm, 5 micron Zorbax SB C18 column was equivalent to Hypersil BDS asymmetrical peaks were observed and resolution between Posaconazole and its related impurities were good. Hence this column was finalized for determination of assay of Posaconazole in Posaconazole Injection.

b. Fixing of injection volume

The injection volume mentioned in the Posaconazole assay method was 10 microliter with this injection volume peak shape and response were good, symmetrical peak was observed hence,10 micro liter injection volume was adopted for determination of assay of Posaconazole in Posaconazole Injection.

c. Column temperature

The column temperature mentioned in Posaconazole related substance method was 30°C. At this column temperature Posaconazole specific from all its impurities and peak shape was good and same only adapted for determination of assay of Posaconazole in Posaconazole injection**.**

d. Optimization of Diluent

The diluent mentioned in Posaconazole related substance method was 11 of water acetonitrile. Posaconazole is easily soluble in water and acetonitrile mixture, so water acetonitrile in the ratio 11 is adopted as diluent. By using this diluent peak response was good and symmetrical peak was observed. Hence 11 ration of water and acetonitrile was adopted as diluent for determination of Posaconazole in Posaconazole injection.

e. Optimization of concentration

The concentration was finalized as 0.1 mg per ml with this concentration peak shape was good. Hence, the concentration of 0.1 mg/mL was finalized for the determination of assay of Posaconazole in Posaconazole injection.

f. Optimization of wavelength

Wavelength mentioned in Posaconazole API method was 262nm. At this nm maximum absorbance was observed. Since the Posaconazole was having maximum absorbance at 262 nm hence, same was adopted for determination of assay of Posaconazole in Posaconazole injection.

g. Optimization of pump mode

The isocratic pump mode was adapted for determination of assay of Posaconazole in Posaconazole Injection.

g. Fixing up system suitability Parameters

From the chromatogram of standard solution. Following system suitability parameter were fixed.

- USP plate counts for Posaconazole peak from first injection of standard solution should not be less than 2000.
- Percentage RSD of Posaconazole peak areas from five replicate injections of standard solution should be not more than 2.0.
- The USP tailing factor for Posaconazole from first injection of standard solution should be not more than 2.0.

Based on the method development results the finalized assay method for determination of Posaconazole in Posaconazole Injection

The optimized chromatographic conditions and gradient program as stated in experimental section (6.4.3.6). The prepared standard, placebo and sample solutions were loaded into the HPLC instrument and the method was validated using the parameters viz. system Specificity, Limit of detection and limit of quantification, Linearity, Precision), Accuracy, Range, Stability of standard/sample solution/ mobile phase, Robustness, and system suitability.

Specificity

Specificity is the ability of method to measure the targeted analyte in presence of other which are expected to be present

Identification

Acceptance criteria Retention time of drug peak obtained from Posaconazole injection sample should be analogous with that of standard.

Table 4: Results of Retention time of Posaconazole

Name	Retention Time (Minutes)	
	Standard	**Sample**
Posaconazole	28.377	28.466

Conclusion

Retention time of Posaconazole peak obtained from Posaconazole injection sample is comparable with the standard.

Placebo interference

Acceptance standards: During the drug's and its linked chemicals' retention times, no peak should be seen.

Observation

Posaconazole and its known related drugs' retention times did not show any peaks in the chromatogram.

Conclusion

According to the study's findings, there is no interaction with the placebo when it comes to identifying related chemicals in injections of posaconazole.

Peak purity

Acceptance Standards Posaconazole at its peak purity and any known related chemicals should meet certain acceptance standards.

Table 5: Results of Peak Purity

Retention times of known related substances, Betadex Sulfobutyl ether				
Name			Retention time (minutes)	
PSK-2			5.771	
PSK-1			45.128	
Betadex Sulfobutyl ether			1.951	
Name	Peak Purity of Posaconazole			
	Purity angle		Purity Threshold	
Control sample/diluted	0.076		0.253	
Spiked Sample				
Name	Retention time (minutes)	Relative Retention time (minutes)	Peak Purity	
			Purity angle	Purity Threshold
PSK -2	5.787	0.20	0.456	0.687
Posaconazole	28.481	1.00	0.077	0.252
PSK - 1	45.178	1.59	0.935	1.215

*Posaconazole peak purity was considered from spiked sample/diluted.

Conclusion

The separated known related substances from the other known related compounds, Betadex Sulfobutyl Ether Sodium and Posaconazole peak, are shown in the chromatogram of the spiked sample.

The peak purity data for Posaconazole in both spiked and control samples confirm the absence of co-eluting peaks, demonstrating that the peaks are homogeneous.

As a result, the test procedure for identifying similar compounds in injections of posaconazole is accurate.

Forced Degradation

Acceptance criteria: The posaconazole peak should be free from any co-eluting peaks and must display homogeneity. Additionally, the peak purity should meet the necessary standards for approval.

Table 6: Results of forced degradation parameters

Degradant	**Degradation condition**	**% Assay**	**Total impurities (% w/w)**	**Mass balance**	**Peak Purity**	
					Purity Angle	**Purity Threshold**
Un-degraded sample	-	98.2	0.142	-	0.096	0.358
Acid Degradation	5M HCl /85°C/180 minutes	93.8	3.308	98.7	0.091	0.333
Base Degradation	5M NaOH/85°C/180 minutes	97.7	0.196	99.5	0.091	0.344
Peroxide Degradation	30% H_2O_2/85°C/5 minutes	85.2	7.77	94.5	0.084	0.298

Thermal Degradation	50°C/72 hours	97.8	0.150	99.6	0.096	0.355
Photolytic Degradation	White Fluorescent light 1.2 million lux hours and UV light, 200 watt-hours/square meter	98.0	0.205	99.9	0.095	0.355
Humidity Degradation	90% RH/25°C/72 hours	97.9	0.145	99.7	0.094	0.356

Table 7: Known/unknown related substances formed in forced degradation studies

Degradation condition	**Related substance formed/increased**			
	Known	**%w/w**	**Unknown**	**%w/w**
Un-degraded sample	PSK - 2	0.017	RRT 0.60	0.020
			RRT 0.90	0.014
			RRT 1.32	0.46
	PSK – 1	ND	RRT 1.35	0.026
			RRT 1.53	0.019
Acid Degradation (5M HCl /85°C/180 minutes)	PSK -2	0.012	RRT 0.22	0.070
			RRT 0.34	0.422
			RRT 0.35	0.084
			RRT 0.36	0.054
			RRT 0.38	0.032
			RRT 0.44	0.029
			RRT 0.47	0.025
			RRT 0.54	0.412
			RRT 0.56	0.488
			RRT 0.58	0.170
			RRT 0.59	0.130
			RRT 0.60	0.026
	PSK - 1	ND	RRT 0.66	0.060
			RRT 0.72	0.250
			RRT 0.74	0.588
			RRT 0.75	0.193
			RRT 1.06	0.057
			RRT 1.10	0.018
			RRT 1.12	0.082
			RRT 1.32	0.062
			RRT 1.35	0.024
			RRT 1.53	0.020
Base degradation (5M NaOH/85°C/180 minutes)	PSK -2	0.021	RRT 0.61	0.028
			RRT 0.72	0.028
			RRT 0.90	0.014
	PSK 1	ND	RRT 1.32	0.056
			RRT 1.35	0.029
			RRT 1.53	0.20
Peroxide degradation \ 30% H_2O_2/85°C/5 minutes	PSK 2	0.013	RRT 0.16	0.142
			RRT 0.30	5.717

Degradation condition	Related substance formed/increased			
	Known	**%w/w**	**Unknown**	**%w/w**
			RRT 0.35	1.581
			RRT 0.60	0.018
	PSK 1	ND	RRT 0.72	0.231
			RRT 1.32	0.043
			RRT 1.35	0.013
			RRT 1.53	0.019
Thermal Degradation (50°C/72 hours)	PSK 2	0.019	RRT 0.60	0.019
			RRT 090	0.013
	PSK 1	ND	RRT 1.32	0.045
			RRT 1.35	0.031
			RRT 1.53	0.023
Photolytic Degradation (White Fluorescent Light, 1.2 million lux hour and UV light 200 watt-hours/square meter)	PSK 2	0.021	RRT 0.60	0.013
			RRT 0.72	0.033
			RRT 0.90	0.019
			RRT 1.10	0.018
			RRT 1.32	0.055
			RRT 1.35	0.028
			RRT 1.53	0.018
Humidity Degradation (90% RH/25°C/72 hours)	PSK 2	0.017	RRT 0.60	0.013
			RRT 0.90	0.022
			RRT 1.32	0.050
	PSK 1	ND	RRT 1.35	0.022
			RRT 1.53	0.021

The drug product shows susceptibility to degradation under acidic and peroxide conditions, as revealed by the stress tests conducted to break down posaconazole injection. However, it remains stable under other stress conditions. All degradation conditions were determined to be satisfactorily satisfied with the percentage degradation achieved for the samples strained under different settings employing an assay and related substance methods. The analysis also confirmed the absence of co-eluting peaks and that the Posaconazole peak is homogenous. This indicates that the test procedure is specific and effective for identifying the relevant compounds in Posaconazole injections.

Limit of detection and Limit of quantitation

The predicted LOD and LOQ concentrations were accurately confirmed by preparing Posaconazole solutions at the expected concentrations and injecting each solution into the HPLC approximately six times, following the test procedure guidelines.

Table 8: Results of LOD and LOQ

Injection ID	Posaconazole	
	LOD	**LOQ**
1.	2761	8315
2.	2888	8391
3.	2715	8380
4.	2819	8402
5.	2747	8353
6.	2741	8339
Statistical Analysis		

Mean	2779	8363
SD	64	33
%RSD	2.3	0.4
Concentration Levels		
Concentration (µg/mL)	0.107	0.324
Concentration (%w/w)	0.010	0.030

Posaconazole's reported LOQ values above fall below 50% of the specified standard. As a result, the procedure for measuring relevant chemicals in posaconazole injection is accurate.

Precision

System precision

Acceptance criteria

The six replicate injections of the standard Posaconazole solution should produce peak areas with a percentage relative standard deviation (RSD) of no more than 5.0%.

Table 9: Results of System Precision

Results	
Injection ID	**Area of Posaconazole**
1.	86930
2.	97394
3.	87630
4.	88034
5.	87426
6.	87648
Statistical Analysis	
Mean	87510
SD	365
%RSD	0.4
95% confidence interval (±)	383

Method Precision: Acceptance criteria

The % RSD for the Posaconazole results obtained from the six determinations should be less than 10.0%.

The % recovery should fall between 85.0% and 115.0% for specification levels up to 0.2%, and between 90.0% and 110.0% for specification levels above 0.2%.

Table 10: Results of Method Precision

Sample ID	**Posaconazole**	
	%w/w	**% Recovery**
Sample -1	0.206	101.5
Sample -2	0.206	101.5
Sample -3	0.206	101.5
Sample -4	0.206	101.5
Sample -5	0.206	101.5
Sample -6	0.207	102.0

Statistical Analysis		
Mean	0.206	101.6
SD	0.000	0.2
% RSD	0.0	0.2
95% confidence interval (±)	0.000	0.2

The outcome demonstrated the accuracy of the test procedure for identifying related compounds in injections of posaconazole.

Intermediate Precision (Ruggedness)

Table 11: Results of Intermediate precision

Sample ID	Posaconazole			
	Method Precision		Intermediate Precision	
	%w/w	% Recovery	%w/w	% Recovery
Sample – 1	0.206	101.5	0.203	101.0
Sample – 2	0.206	101.5	0.203	100.5
Sample – 3	0.206	101.5	0.203	101.0
Sample – 4	0.206	101.5	0.203	101.0
Sample – 5	0.206	101.5	0.204	101.5
Sample – 6	0.207	102.0	0.204	101.5
Statistical Analysis				
Mean	0.206	101.6	0.203	101.1
SD	0.000	0.2	0.001	0.4
% RSD	0.000	0.2	0.5	0.4
95% confidence interval (±)	0.000	0.2	0.001	0.4
Overall Statistical Analysis (%w/w)				
Mean	0.205			
SD	0.002			
% RSD	1.0			
95% Confidence interval (±)	0.001			

Posaconazole in injection form could be precisely quantified using this test method because the precision findings met the predefined acceptance requirements.

Accuracy

The measurement of the discrepancy between the true value and the measured value is called accuracy.

Table 12: Results of accuracy

Posaconazole (for LOQ Level)			
Level/Sample ID	**Amount added (%w/w)**	**Amount found (%w/w)**	**% Recovery**
LOQ/Sample ID - 1	0.0304	0.0311	102.3
LOQ/Sample ID – 2	0.0304	0.0314	103.3
LOQ/Sample ID – 3	0.0304	0.0314	103.3

Spiked level (%)	Volume of stock solution taken (mL)	Dilution (mL)	Spiked sample area	Amount added (µg/mL)	Amount found (µg/mL)	(%) Recovery of spiked sample
50% PREP-1	2.5	50	1073470	0.0496	0.0495	99.8

50% PREP-2	2.5	50	1073452	0.0496	0.0495	99.8
100% PREP-1	5	50	2153840	0.0992	0.0994	100.1
100% PREP-2	5	50	2151609	0.0992	0.0993	100.0
150% PREP-1	7.5	50	3217191	0.1488	0.1484	99.7
150% PREP -2	7.5	50	3222411	0.1488	0.1484	99.9

Since the test method's accuracy findings fulfilled the predefined acceptance requirements, it was deemed reliable for quantifying posaconazole in injectable form.

Linearity

The measurement's proportionality to the analyte concentration within the given range is known as linearity.

Acceptance criteria: At least 0.999 should be the R2 value.

Table 13: Results of Linearity and Range

Posaconazole				
% Level (Approximate)	**Concentration (µg/mL)**	**Area**	**Statistical Analysis**	
LOQ	0.324	8315	Slope	25707
10	0.360	9313		
15	0.540	14066	Intercept	436
20	0.720	18693		
25	0.901	24316	Residual sum of squares	597
50	1.801	47426		
75	2.702	69616		
100	3.602	93854	Correlation coefficient	0.9999
125	4.503	116348		
150	5.404	138579		

Posaconazole has a correlation coefficient greater than 0.990. Posaconazole thus responds linearly from LOQ to 150% of the standard concentration level.

Solution stability

Standard solution stability

Table 14: Results of Standard solution stability

Standard stability time	**Standard area**	**% Variation**
0 hours	2141083	NA
2 hours 30 minutes	2141597	-0.02
4 hours	2141994	-0.04
7 hours	2141707	-0.03
10 hours	2148161	-0.33
17 hours	2155606	-0.68

Sample Solution stability

Table 15: Results of Sample Solution stability

Sample stability time	Standard area	% Variation
0 hours	2250381	NA
3 hours	2249836	0.02
6 hours	2256239	-0.26
12 hours	2265515	-0.67

Assay of Posaconazole

The test for posaconazole injection was determined using the reverse phase isocratic HPLC technique. The table below provides specifics on the concentration of Posaconazole in the sample and standard solutions.

Table 16: The details of the standard and sample solutions

Assay	Posaconazole Concentration (µg/mL)	
Strength	Standard	Sample
300 mg/vial	120	115

Known impurity interference and peak purity

Table 17: Results of Known impurity interference and peak purity

Retention times of known Related substances, Betadex Sulfobutyl ether and Edetate			
Name	**Retention Time (minutes)**		
PSK -2	2.359		
PSK -1	17.610		
Betadex Sulfobutyl ether	Not Detected		
Edetate	Not Detected		
Name	**Peak Purity of Posaconazole**		
	Purity Angle	**Purity Threshold**	
Control sample	0.030	0.248	
Spiked Sample	0.055	0.245	
Sample ID	**Assay (% Labeled amount)**	**Statistical analysis**	
Control Sample – 1	100.6	**Mean**	100.9
Control Sample -2	100.8	**SD**	0.31
Control Sample -3	101.2	**% RSD**	0.3
Control Sample - 1	100.1	**Mean**	99.8
Control Sample -2	99.6	**SD**	0.29
Control Sample -3	99.6	**% RSD**	0.3
Sample ID	**Mean Assay (% Labeled amount)**	**% difference w.r.t unspiked sample**	
Control Sample	100.9	1.1	
Spiked Sample	99.8		

The retention lengths of betadex sulfobutyl ether sodium and edetate disodium, two recognized related compounds, indicate that they were all separated from the posaconazole peak. The absence of interference from known related medications is further demonstrated by comparing the variation in the mean assay of Posaconazole obtained from the analysis of control samples (triplicate) with that of spiking samples, which was determined to be within acceptable limits. Data on the purity of the posaconazole peak for both the spiked and un-control samples revealed that the peak is homogeneous and does not exhibit co-eluting peaks. Thus, it may be concluded that no known related substance interferes with the assay of posaconazole in posaconazole injection.

Forced Degradation

Table 18: Forced degradation study

Degradation Mechanism	Degradation Condition	% Assay	Total impurities (%w/w)	Mass Balance	Peak Purity of Posaconazole	
					Purity angle	Purity threshold
Undegraded sample	-	98.2	0.142	-	0.102	0.262
Acid Degradation	5M HCl/85°C/180 minutes	93.8	3.308	98.7	0.111	0.261
Base Degradation	5M NaOH/85°C/180 minutes	97.7	0.196	99.5	0.111	0.262
Peroxide Degradation	30% H2O2 /85°C/5 minutes	85.2	7.777	94.5	0.111	0.261
Thermal Degradation	50°C/72 hour	97.8	0.150	99.6	0.104	0.263
Photolytic Degradation	White Fluorescent light, 1.2 million Lux hours and UV light, 200 watt-hours/square meter	98.0	0.205	99.9	0.106	0.263
Humidity degradation	90% RH/25°C/72 hours	97.9	0.145	99.7	0.107	0.262

A review of peak purity data from a degradation sample analysis indicates that the peak of posaconazole is homogeneous and free of coeluting peaks. It follows that the test protocol is specific to the injection form of posaconazole assay.

Acceptance Criteria

- A correlation coefficient of 0.9990 or above is required.
- % Y intercept should not be more than 2.0.

Table 19: Results of linearity

% Level (Approximate)	Concentration (µg/mL)	Average area of Posaconazole	Statistical analysis	
50	57.565	1127570	Slope	19515
80	92.103	1793611	Intercept	-5217
90	103.616	2009857	% Y-Intercept	-0.2
100	115.129	2231085	Residual sum of Squares	8668
110	126.642	2467165		
120	138.155	2686247	Correlation Coefficient	0.9999
150	172.694	3375146		

Posaconazole has a linear peak response within the concentration range of 50% to 150% of the test concentration.

Precision

System precision

Acceptance Criteria

The percent RSD of the posaconazole peak regions derived from six replicate injections of standard Solution-I should not exceed 2.0. The results are shown in the table below.

Table 20: Results of system precision

No of replicates	Area of Posaconazole
1	2434908
2	2436413
3	2438032
4	2438392
5	2438235
6	2439668
Statistical analysis	
Mean	2437608
SD	1682
%RSD	0.1
95% Confidence Interval (±)	1765

Method Precision

Table 21: Results of Intermediate Precision

No of replicates	Assay (% labeled Amount)
1	100.5
2	101.6
3	101.8
4	101.7
5	100.8
6	101.6
Statistical Analysis	
Mean	101.3
SD	0.5
%RSD	0.5
95% confidence interval (±)	0.5

The test outcome proved how accurate the protocol was for the assay of injectable posaconazole.

Intermediate Precision (Ruggedness)

Table 22: Results of Intermediate Precision

No. of replicates	Assay (% Labeled amount)	
	Method Precision	**Intermediate Precision**
1.	100.5	99.6
2.	101.6	99.6
3.	101.8	99.9
4.	101.7	100.0
5.	100.8	99.8
6.	101.6	100.3
Statistical Analysis		
Mean	101.3	99.9
SD	0.5	0.3
%RSD	0.5	0.3
95% confidence interval (±)	0.5	0.3
Overall Statistical Analysis		
Mean	100.6	
SD	0.9	
%RSD	0.9	
95% confidence interval (±)	0.6	

The results show that the test method for the analysis off drug in Posaconazole injection is reliable with respect to changes from day to day, system to system, column to column, and analyst.

Accuracy

Table 23: Results of Accuracy

Concentration/Sample ID	Amount added (mg)	Amount found (mg)	% Recovery	Statistical analysis	
50% level sample 1	35.180	35.414	98.9	**Mean**	**98.9**
50% level sample 2	35.940	35.364	98.4	**SD**	**0.5**
50% level sample 3	35.720	35.479	99.3	**%RSD**	**0.5**
75% level sample 1	53.820	52.838	98.2	**Mean**	**98.1**
75% level sample 2	53.740	52.726	98.1	**SD**	**0.1**
75% level sample 3	53.820	52.820	98.1	**%RSD**	**0.1**
100% level sample 1	71.820	70.487	98.1	**Mean**	**98.2**
100% level sample 2	71.430	70.168	98.2	**SD**	**0.1**
100% level sample 3	71.600	70.61	98.3	**%RSD**	**0.1**
125% level sample 1	89.450	88.013	98.4	**Mean**	**98.3**
125% level sample 2	89.530	87.992	98.3	**SD**	**0.1**
125% level sample 3	89.750	88.122	98.2	**%RSD**	**0.1**
150% level sample 1	107.51	105.453	98.1	**Mean**	**98.1**
150% level sample 2	107.62	105.648	98.2	**SD**	**0.1**
150% level sample 3	107.57	105.520	98.1	**%RSD**	**0.1**
Overall Statistical Analysis					
Mean	98.3	SD	0.3	95% confidence interval (±)	0.2

The recovery findings showed that the accuracy of the test procedure is satisfactory.

The analytical method's range for posaconazole assay in injection is between 50% and 150% of the test concentration.

System suitability

Table 24: System suitability

Injection ID	Area of Posaconazole
1	2540105
2	2545940
3	2549905
4	2544292
5	2553671
Statistical Analysis	
Mean	2546783
SD	5213
%RSD	0.2
USP Plate Count	5153
USP Tailing	1.3
Standard Agreement	0.99

Conclusion

Triazole antifungal agents like posaconazole are often used to people with impaired immune systems to treat or prevent invasive fungal infections. This research introduces a particular, sensitive, and robust HPLC-based methodology for the quantitative evaluation of injectable Posaconazole. In order to verify the technique, it was scrutinized for critical characteristics in compliance with ICH criteria. These parameters included precision,

accuracy, specificity, linearity, limit of detection (LOD), and limit of quantification (LOQ). Linearity was noted across a specific concentration range, indicating that the suggested approach was very precise and accurate. Reliable detection and quantification of Posaconazole, even at low concentrations, were ensured by the LOD and LOQ values, which proved the method's sensitivity. Consistent, safe, and regulatory compliant Posaconazole injections may be assured with this proven method's regular quality control analysis. System suitability results met the acceptance criteria as mentioned in the test method.

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