RESEARCH ARTICLE

Method Development and Validation of Indigenously Isolated Caspofungin Acetate and its related Substances using RP-HPLC Method

Kiran K Gannamani, Sudhakar Chintakula*, Suresh Maddila

Department of Chemistry, School of science, GITAM (Deemed to be University), Visakhapatnam, Andhra Pradesh, India

***Received:** 12th October, 2023; Revised: 05th November, 2023; Accepted: 29th November, 2023; Available Online: 25th December, 2023*

ABSTRACT

Caspofungin is prepared from a precursor PneumocandinB_0 derived from a fungus *Glarea lozoyensis.* Prepared crude caspofungin acetate has many impurities, namely, A, B, D, E and PneumocandinB_0, and these impurities vary slightly in chemical or structural composition and need to be eliminated by purification. The present study involves the preparation of caspofungin acetate from PneumocandinB_0, followed by purification and method development for detecting caspofungin and its related substances."We used a high-tech liquid chromatograph system, which included components like the Agilent 1260 infinity quaternary pump module, a sophisticated MWD detector, and the Empower 3 data handling system. To separate and analyze substances, we employed an analytical column made of stainless steel. This column was 150 mm long and had an internal diameter of 3.0 mm. It was filled with octadecyl silane chemically bonded to porous silica particles, each with a tiny diameter of 3 micrometers. The test method underwent validation to ensure it could accurately identify specific elements, detect minimal quantities, establish the lowest measurable amounts, maintain linearity, demonstrate precision, achieve accuracy, and suitably handle sample solutions and the system." and the developed test method is suitable and can be introduced into the routine use for the detection of caspofungin acetate and its related substances.

Keywords: Caspofungin acetate, Method development, Validation.

International Journal of Pharmaceutical Quality Assurance (2023); DOI: 10.25258/ijpqa.14.4.12

How to cite this article: Gannamani KK, Chintakula S, Maddila S. Method Development and Validation of Indigenously Isolated Caspofungin Acetate and its related Substances using RP-HPLC Method. International Journal of Pharmaceutical Quality Assurance. 2023;14(4):893-899.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Caspofungin acetate is a parenteral antifungal medication administered to individuals with invasive aspergillosis who cannot tolerate other antifungal medications or whose infection is resistant. It is the first echinocandin, a brand-new class of antifungals, to be made available in the United States.[1] Caspofungin is widely recognized for its effectiveness in combatting *Candida* species as a fungicide.[2-7] What makes it stand out is its ability to remain effective even against *Candida* strains that have developed resistance to azoles or polyenes. This is because caspofungin works by specifically targeting the fungal cell wall, rather than the cell membrane, which makes it a reliable option in treating these infections.[8-11]

The precursor PneumocandinB_0 (Figure 1), which comes from the fungus *Glarea lozoyensis*, is used to make capsofungin acetate (Figure 2).[12] The prepared crude form of capsofungin contains a number of impurities, including impurity A (Figure 3), impurity B (Figure 4), impurity D (Figure 5), and impurity E (Figure 6). These contaminants, which differ significantly in chemical or structural composition from -10,12- dimethyltetradecanamide" and PneumocandinB0, must be removed through purification. The creation of caspofungin acetate from PneumocandinB_0, followed by its purification and the invention of a method for its detection.

MATERIALS AND METHODS

Method Development for Determination and Validation of Caspofungin Acetate and its Related Substances

After the purification of caspofungin acetate, a specific, selective, linear, accurate, and stability-indicating reverse phase HPLC technique was established to quantify caspofungin acetate and its related chemicals.[5]

Instruments

The study utilized a sophisticated High-Performance Liquid Chromatograph system featuring components such as the Agilent 1260 Infinity Quaternary Pump module and MWD Detector (equivalent to Agilent 1260 Infinity). For data management, the system incorporated the Empower 3 software, which is comparable to Waters' software. The analytical column used was a stainless steel column, measuring 150 mm in length with a 3.0 mm internal diameter. This column was

**Author for Correspondence: schintak@gitam.edu*

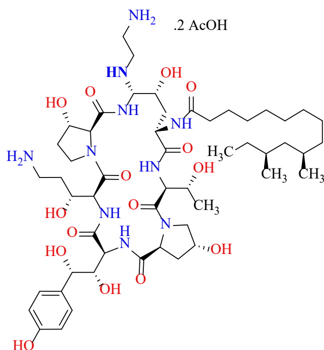

Figure 1: Caspofungin acetate

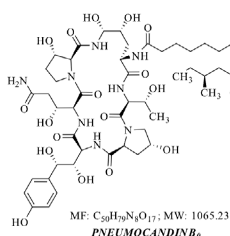

Figure 2: Pneumocandin B0

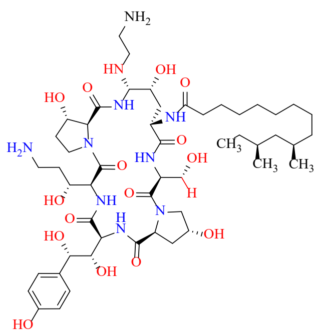

Figure 3: Caspofungin IMP-A

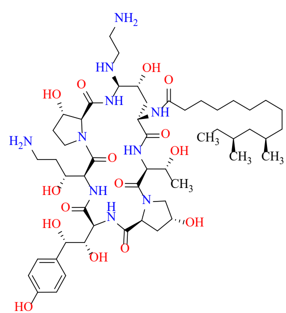

Figure 4: Caspofungin IMP-B

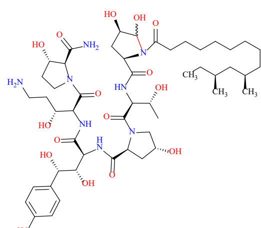

Figure 5: Caspofungin IMP-D

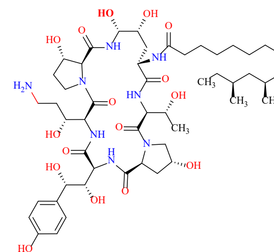

Figure 6: Caspofungin IMP-E

packed with Octadecyl silane chemically bonded to porous silica particles, each having a diameter of 3 µm. (Make: YMC).

Preparation of Solutions

Solution A: Approximately 1.0 mL of perchloric acid and 0.75 g of sodium chloride were carefully placed into a 1000 mL volumetric flask. They were then dissolved and further diluted with water until the flask was filled to its 1000 mL mark.

Solution-B: Acetonitrile was used

Making an Aqueous Solution of Sodium Acetate, 0.01 M

A quantity of approximately 0.8203 grams of Sodium acetate was introduced into a 1000 mL volumetric flask. This substance was dissolved and then further diluted with water until the flask was completely filled. The pH level was subsequently adjusted to a precise value of 4.0 ± 0.05 using glacial acetic acid.

Preparation of Diluent

An air-free blend was prepared by combining a 0.01 M aqueous solution of Sodium acetate with Acetonitrile in a volumetric ratio of 4 parts sodium acetate solution to 1 part Acetonitrile.
Blank: Diluent was used as a blank

Chromatographic conditions

The method established for detecting caspofungin acetate involves the following specified conditions, where YMC hydrosphere C18 (150 × 4.6) mm 3 µm¸ or equivalent was used as chromatography column with gradient pump operation setting where the initial volume of mobile phase -B was 33% till 14.5 minutes, then 50% till 35 minutes and 80% up to 50 minutes followed by 33% at 70 minutes. The liquid rate flow was adjusted as 1.0 mL/minute with 10 µL sample injection volume at 30°C of column oven and 4°C of sample tray temperature with a runtime of 70 minutes.

Impurity Mixture

Approximately 1-mg of each impurity A, B, D, E, and PneumocandinB0 was carefully weighed and then placed into a 20 mL volumetric flask. These substances were dissolved, followed by dilution with an appropriate diluent to fill the flask, and thorough mixing was performed. Subsequently, 1-mL of this prepared solution was transferred into a 10 mL volumetric flask, again diluted to its capacity with the same diluent, and mixed thoroughly.

System Suitability Solution

An amount of approximately 5.5 mg of caspofungin acetate standard, which had been dried beforehand, was carefully placed into a 10 mL volumetric flask. Following this, 1-mL of

an impurity mixture was introduced, dissolved, and further diluted with an appropriate diluent to fill the flask. The solution was thoroughly mixed.

Test Solution

A quantity of 55 mg of the sample was accurately weighed and then transferred into a clean, dry 100 mL volumetric flask. It was dissolved, diluted with an appropriate diluent to reach the flask's capacity, and thoroughly mixed. It's important to note that the prepared solution should be used for injection within 14 hours of its preparation.

Reference Solution

Approximately 55 milligrams of the Caspofungin acetate standard were carefully placed into a 100 mL clean and dry volumetric flask. Afterward, they were dissolved, further diluted with an appropriate diluent to reach the flask's capacity, and thoroughly mixed. Following this, 1-mL of this solution was transferred into another clean, dry 100 mL volumetric flask, diluted to volume with diluent, and mixed thoroughly. Lastly, 1-mL of this solution was moved into a 10 mL clean, dry volumetric flask, diluted to volume with diluent, and mixed well.

Note: Caspofungin acetate storage condition at -70 ± 10°C. For the thawing process, caspofungin acetate solid samples during routine testing can be transferred from a -70°C freezer to a 2 to 8°C refrigerator to thaw for about 20 minutes and then transferred to room temperature to stand for 1-hour

Validation[13-17]

System suitability

"Six separate injections were made into the chromatograph for the system suitability solution, diluent, sensitivity solution, and standard solution replicates. The resulting chromatograms were recorded. On a daily basis, the relative mean and standard deviation were documented to assess the suitability and consistency of the system."

Specificity

Solutions for each known related chemical were made and administered separately to validate the retention time. Following the protocol, solutions were prepared, including the caspofungin control sample, spiked samples containing known related chemicals at specified levels, and a diluent. Subsequently, these solutions were injected into an HPLC system, and the evaluation of peak purity was performed using Waters Empower software.

LoD and LoQ, respectively

The LoD and LoQ values for caspofungin acetate and its associated compounds were established by assessing the peak response of the analyte. To verify the precision of the anticipated LoD and LoQ concentrations for both the related substances and caspofungin acetate, solutions containing these substances were meticulously prepared at the expected concentrations. Each of these solutions was then injected into the HPLC system six times, adhering to the prescribed methodology.

Precision

System precision was assessed by injecting the standard solution into the HPLC six times, following the established procedure. To gauge method precision, six separate sample solutions of caspofungin acetate were meticulously prepared, each from a single batch. These solutions were spiked with known related substances at the specified levels and subsequently injected into the HPLC system in accordance with the prescribed methodology. Additionally, intermediate precision was determined by having another analyst prepare sample solutions from the same batch used in method precision. These solutions were injected separately on a different day using distinct systems and columns.

Linearity

Solutions were prepared using caspofungin acetate and its associated compounds, covering a concentration range from 5 to 120% of the specified level. For each of these solution variations, six replicates were individually injected into the HPLC system. Response (Area) vs concentration (Amount) was used to plot a calibration curve. The graph was used to determine the y-intercept and correlation coefficient (r^2).

Accuracy

To assess accuracy, solutions containing caspofungin acetate and its related substances were meticulously prepared in triplicate. These solutions were prepared at levels including the LoQ, 50, 100, and 150% of the specified level. Subsequently, each of these solutions was individually injected into the HPLC system for analysis. The recovery percentage was ascertained by entering the area values of the peak obtained in the calibration curve equation to get the values of recovered concentration.

Solution stability

To evaluate the stability of the solutions, including the mobile phase, standard solutions, and sample solutions, an initial preparation and analysis were conducted. Subsequently, these solutions were analyzed at various time intervals to assess their stability over time. Solution stability was also analyzed by keeping them at room temperature and under refrigerator conditions (5 ± 3°C).

Robustness

System suitability and standard solutions were prepared following the test method. Additionally, a sample solution was prepared, spiked with known related substances at the specification level. This prepared sample solution was then intentionally subjected to varying conditions during injection into the HPLC system. These varied conditions involved changes in the flow rate, allowing us to assess both the system's suitability and the method's resilience to these deliberate variations (± 10%), column oven temperature (± 5°C), wavelength (± 2 nm), buffer pH variation (± 0.2 unit), organic variation in mobile phase - A (± 2% absolute) and column (Batch / Lot variation) from the methodology values.

Forced degradation

To demonstrate the specificity of the established method and confirm its stability, forced degradation tests were conducted on caspofungin acetate. These stress degradation tests encompassed various conditions, including hydrolysis (acid and base), oxidation, photolysis, and heat. Acid degradation involved treating the drug with 0.5 M HCl at 50°C for 30 minutes, while base degradation utilized 0.5 M NaOH at room temperature for 30 minutes. Additionally, peroxide degradation was conducted with 0.2% H_2O_2 for 20 minutes at room temperature. The standard drug solution was subjected to heat degradation at 60°C for 120 hours. To investigate photolytic degradation, the drug was exposed to both white fluorescent light for 1.2 million lux hours and UV light at 200-watt hours/meter square. These tests aimed to assess the drug's resilience under these challenging conditions and to establish the method's specificity.

RESULTS AND DISCUSSION

System Suitability

The retention time for caspofungin acetate was determined to be 20.5 minutes, and the corresponding results for retention time (RT) and relative retention time (RRT) have been presented in the Table 1. The RT and RRT are given for information only and may vary slightly based on system configuration and column usage.

Specificity

In the chromatogram of the diluent, there were no detectable peaks at the retention time corresponding to caspofungin acetate or its related compounds (Figure 7). In addition, when the chromatograms of related compounds and the spiked sample were examined, it was clear that these connected chemicals were separated clearly from closely eluting peaks of caspofungin acetate and related substances (Table 2). The peak purity data obtained from known related drugs indicated the absence of co-eluting peaks, demonstrating that the peaks were uniform in the spiked sample, control sample, and spiked sample with dilution. In light of these observations, it can be reasonably concluded that the test method is highly specific in detecting related compounds in caspofungin.

Detection limit and quantification limit

The data demonstrates that the signal-to-noise ratio satisfies the acceptance criteria for both caspofungin acetate and its related substances at the LoD. Additionally, the percentage Relative Standard Deviation (%RSD) meets the acceptance criteria for both caspofungin acetate and its related substances at LoD and LoQ (Table 3). Consequently, the test method exhibits precision in quantifying related substances within the caspofungin acetate drug substance. Furthermore, it's noteworthy that the LoQ values for caspofungin acetate and its related substances are comfortably below 50% of the specification level. Detailed results can be found in the Table.

Table 1: System suitability of the caspofungin acetate and its related substances

S. No.	*Name*	*RT (min)*	*RRT#*	*Correction factor*
1	Impurity A	19.8	~ 0.97	1.48
2	Caspofungin	20.5	= 1.00	1.00
3	Impurity B	23.55	~ 1.15	1.32
4	Impurity E	30.26	~ 1.48	1.28
5	Impurity D	32.57	~ 1.59	1.08
6	Pneumocandinb0	34.34	~ 1.67	1.01

*Relative retention time

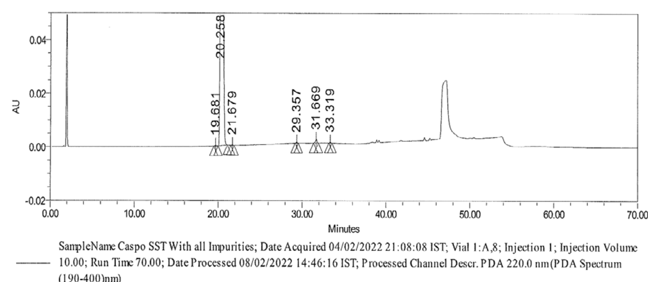

Figure 7: Chromatogram showing separated impurities along with the Caspofungin acetate

Table 2: Specificity of the caspofungin acetate and its related substances in the spiked sample

Spiked sample				
Name	*Retention time (minutes)*	*RRT*	*Peak purity*	
			Purity angle	*Purity threshold*
Impurity-A	19.68	~ 0.97	6.162	7.717
Caspofungin acetate	20.25	= 1.00	0.125	0.255
Impurity-B	21.67	~ 1.15	6.770	8.613
Impurity-E	29.35	~ 1.48	4.862	6.740
Impurity-D	31.66	~ 1.59	1.666	2.021
Pneumocandin-Bo	33.31	~ 1.67	4.647	7.020

Linearity

The correlation coefficient for both caspofungin acetate and its related substances exceeds 0.990. This indicates that the response of caspofungin acetate and each of its related substances remains linear across the entire range from the LoQ to 150% of the specification level (Table 4).

Precision

Considering variations among different analysts, day-to-day testing, differences between analytical systems, and variations between chromatographic columns, the results obtained from both Method precision and Intermediate precision demonstrate the robustness of the method in identifying related compounds within caspofungin acetate drug material. You can find the detailed results presented in Table 5.

Accuracy

Based on the recovery data, it can be concluded that the test method exhibits a satisfactory level of accuracy when determining related compounds within caspofungin acetate

Table 3: LoD and LoQ of the caspofungin acetate and its related substances

Name	Area		%RSD		Conc. (µg/mL)		Conc.(% w/w)	
	LoD	LoQ	LoD	LoQ	LoD	LoQ	LoD	LoQ
Impurity-A	1144.33	2817.33	2.857	0.915	0.1095	0.2737	0.02	0.05
Caspofungin acetate	1417.5	3549.5	0.6951	0.488	0.0922	0.2306	0.02	0.05
Impurity-B	1166.83	2962.67	1.28	0.45	0.0994	0.2486	0.02	0.05
Impurity-E	1081.83	3260.17	0.81	0.36	0.1076	0.2690	0.02	0.05
Impurity-D	1307.5	3262.5	0.9	0.3	0.0965	0.2413	0.02	0.05
Pneumocandin-Bo	1411.0	3540.8	0.6	0.4	0.0983	0.2457	0.02	0.05

Table 4: Linearity of the caspofungin acetate and its related substances

Name	LoQ	80%	100%	120%	150%	Slope	Intercept	Correlation coefficient	Response factor
Impurity-A	0.2737	0.4379	0.5474	0.6568	0.8211	10166.01	-3.66795	0.999973	1.52
Caspofungin acetate	0.2306	0.3689	0.4612	0.5534	0.6918	15502.8	-1.5821	0.999999	1.0
Impurity-B	0.2486	0.3977	0.4972	0.5966	0.7458	11866.29	14.31791	0.999958	1.30
Impurity-E	0.2690	0.4304	0.538	0.6456	0.807	12126.65	12.26207	0.99999	1.28
Impurity-D	0.2413	1.1582	1.4478	1.7373	2.1717	13510.97	10.8271	0.999999	1.147
Pneumocandin-Bo	0.2457	0.3931	0.4914	0.5896	0.7371	14322.81	-10.9419	0.999999	1.08

Table 5: Precision of the caspofungin acetate and its related substances

	Method precision			Intermediate precision			Overall precision		
Name	Mean	SD	%RSD	Mean	SD	%RSD	Mean	SD	%RSD
Impurity-A	0.1107	0.0012	1.09	0.113	0.002	1.35	0.112	0.002	1.45
Impurity-B	0.1102	0.0017	1.56	0.108	0.001	1.31	0.109	0.002	1.72
Impurity-E	0.1088	0.0020	1.88	0.113	0.002	1.37	0.111	0.003	2.50
Impurity-D	0.3860	0.0070	1.81	0.371	0.010	2.65	0.379	0.011	2.95
Pneumocandin-Bo	0.1078	0.0016	1.49	0.108	0.001	1.37	0.108	0.001	1.36

Table 6: Accuracy of the caspofungin acetate-related substances

	At LoQ level			At 80% level			At 100% level			At 150% level		
Name	Mean	SD	% RSD	Mean	SD	%RSD	Mean	SD	% RSD	Mean	SD	%RSD
Impurity-A	91.73	0.32	0.35	100.97	0.68	0.67	98.67	0.21	0.21	97.27	0.15	0.16
Impurity-B	95.40	0.62	0.65	97.93	0.55	0.56	99.10	0.40	0.40	98.63	0.45	0.46
Impurity-E	96.70	0.53	0.55	98.50	0.40	0.41	98.93	0.38	0.38	99.47	0.40	0.41
Impurity-D	100.7	0.15	0.15	98.53	0.35	0.36	99.50	0.40	0.40	98.03	0.50	0.51
Pneumocandin-Bo	97.83	0.60	0.62	96.87	0.65	0.67	95.53	0.85	0.89	95.40	1.15	1.21

Table 7: Stability of the standard solutions of caspofungin acetate and related substances

Time	Caspofungin Acetate		Impurity A		Impurity B		Impurity E		Impurity D	
	Area	%Difference	Area	%Difference	Area	%Difference	Area	%Difference	Area	%Difference
Initial	7150	-	5585	-	5960	-	6560	-	19500	-
After 6 hours	7148	0.03	5580	0.09	5961	0.02	6558	0.03	19510	0.05
After 8 hours	7148	0.03	5584	0.02	5955	0.08	6550	0.15	19525	0.13
After 10 hours	7140	0.14	5583	0.04	5950	0.17	6552	0.12	19530	0.15
After 12 hours	7135	0.21	5584	0.02	5952	0.13	6555	0.08	19540	0.21
After 14 hours	7133	0.24	5579	0.11	5949	0.18	6550	0.15	19550	0.26
After 15 hours	7130	0.28	5580	0.09	5948	0.20	6553	0.11	19560	0.31
After 19 hours	7125	0.35	5582	0.05	5952	0.13	6551	0.14	19575	0.38

Table 8: Robustness of different parameters on caspofungin acetate and its related substances

Parameter	*Variation*	*RRT*				
		Impurity-A	*Impurity-B*	*Impurity-E*	*Impurity-D*	*Pneumocandin Bo*
STEP	-	0.97	1.07	1.45	1.56	1.64
Flow rate	-10%	0.97	1.06	1.43	1.55	1.65
	+10%	0.98	1.07	1.43	1.54	1.63
STEP	-	0.97	1.07	1.44	1.55	1.63
Column lot variation STP**	-	0.97	1.06	1.45	1.53	1.62
STEP	-	0.97	1.07	1.45	1.54	1.65
Column oven temperature	-5°C	0.96	1.05	1.44	1.54	1.65
	+5°C	0.98	1.06	1.43	1.56	1.65
STEP	-	0.97	1.07	1.45	1.55	1.65
Wavelength variation	-2 nm	0.97	1.07	1.45	1.55	1.65
	+2 nm	0.97	1.07	1.45	1.55	1.65
STEP	-	0.97	1.07	1.43	1.55	1.65
Organic variation in mobile phase-A	-2% absolute	0.96	1.06	1.42	1.52	1.63
	+2% absolute	0.98	1.07	1.40	1.57	1.67

drug substance across the entire range from the LoQ to 150% of the specification level (Table 6).

Stability of mobile phase, standard and sample solutions

Based on the provided data, we can infer that the Standard solution remains stable for a duration of 19 hours under refrigerated conditions (5 ± 3°C). Similarly, the Sample solution also exhibits stability for up to 19 hours under the same refrigerated conditions. Furthermore, the data indicates that the mobile phase maintains its stability for a minimum of 24 hours (Table 7).

Robustness

The system suitability outcomes met the stipulated requirements under various conditions as per the test procedure. Additionally, upon examining chromatograms of samples containing known related compounds subjected to the robustness criteria mentioned earlier, it was observed that, unlike the standard testing procedure (STP) condition, there is minimal variation in the RRTs of related drugs (Table 8). Therefore, the testing method proves to be dependable for detecting related compounds in caspofungin acetate drug material across the specified range of variations for each of the aforementioned criteria.

CONCLUSION

The preparation and purification of caspofungin acetate derived 1.6 g of pure product with a percentage yield of 70%. However, the yield percentage may vary with the impurity profile of crude. The established method has undergone validation encompassing accuracy, linearity, specificity, LoD and LoQ, precision, stability of the system, standard and sample solutions. The outcomes of these investigations have demonstrated adherence to the predefined acceptance criteria. From the gathered data, it can be confidently asserted that the method exhibits specificity, selectivity, and accuracy. As a result, this methodology is suitable for routine quantitative analysis of related substances in caspofungin acetate.

ACKNOWLEDGMENT

We acknowledge GITAM University for providing PhD registration to Mr Kiran Kumar for the doctorate award.

AUTHOR CONTRIBUTIONS

Each of the authors made substantial contributions to this manuscript, actively engaged in the review and editing process, and provided their approval for the final draft to be published.

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