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DEVELOPMENT AND VALIDATION OF STABILITY-INDICATING ASSAY RP-HPLC METHOD FOR THE ESTIMATION OF ANIDULAFUNGIN AND RELATED COMPOUNDS IN PARENTERAL DOSAGE FORM

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ABSTRACT

RP HPLC method has been developed for the detection of Anidulafungin and to separate its related compounds. Anidulafungin was separated on a Zodiac C18 (150 × 4.6mm × 3.0µm) analytical column. The solvent system used for assay was Trifluoroacetic acid buffer: Acetonitrile (52:48 %v/v) of pH 4.7 at 1.5 ml/min flow rate and the detection at 300nm wavelength. The solvent system used for related compounds separation was phosphate buffer as Mobile phase A and Acetonitrile as Mobile phase B at 0.8 ml/min flow and the detection at 220nm wavelength and injection volume of 50 µL whose elution is gradient. The method has been validated according to the ICH guidelines for linearity, accuracy, the limit of detection, the limit of quantification, solution stability and robustness. The linear regression coefficient was found to be 1.000 with a slope of 28225.77 and an intercept is 35762.79. The accuracy was determined by a three different level recovery study and the mean % recovery was 101.0-101.5 which is within the specified limits. LOD and LOQ values were found to be 0.083 and 0.250ppm, respectively. The method developed was robust, simple and accurate. It is therefore appropriate for routine analysis.

Keywords: Anidulafungin, RP HPLC, Related Compounds

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INTRODUCTION

Anidulafungin belongs to the drug class Echinocandins which shows very good results for the treatment of various fungal infections.[1,2] It is a semi-synthetic lipopeptide of Echinocandin B_o, which is the fermentation product of Aspergillus nidulans.[3] It is approved by FDA in the year 2006 for the treatment of fungal infections. It is white to off-white powder which is available in lyophilized form which has to be reconstituted with 30 mL of water during its use.[4-6] The route of administration is intravenous.[3] The innovator brand for the drug is Vicuron Pharmaceuticals. It is available in the trade names Eraxis, Ecalta etc. It is freely soluble in methanol and its solubility is about 0.0564 mg/ml. The plasma half-life of the drug is 40-50 h. The pKa value for Anidulafungin is 9.46 for strongest acid and -3.5 for strongest basic. It has a logP value of 2.9. It is effectively used in the treatment of invasive candidiasis and esophageal candidiasis. Side-effects on Anidulafungin include stomach pain, diarrhoea, dizziness, constipation and vomiting.[7,8] It acts by inhibiting the glucan synthase which is present in fungal cells and the formation of 1, 3 β-D glucan synthase which is an important element of fungal cell wall formation, which leads to osmotic instability and cell death. It is active in-vitro against both Aspergillus and Candida species, which are resistant to Fluconazole and Amphotericin B.[9] When Anidulafungin and Fluconazole are compared, results show that Anidulafungin is more effective than Fluconazole for the treatment of invasive candidiasis and show the safe drug profile, similar to that of Fluconazole.[10] The main aim of this work is to develop a simple HPLC method for determining the assay in less retention time compared to the

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previous methods, to separate the related compounds[11] of Anidulafungin properly in less retention time and to validate the proposed methods.

EXPERIMENTAL

Materials

Anidulafungin working standard and Anidulafungin impurities A and B are supplied by Omene Life Sciences Pvt. Ltd. 100 mg/vial Anidulafungin injection marketed formulation Canidula was obtained from Gufic Biosciences Limited. Chemicals were obtained from Merck. The HPLC method for assay and related compounds (RCs) were developed and validated by using HPLC with UV/PDA detector of Agilent technologies (1260 Infinity) and Waters (e2695). The outputs of signals were monitored by Empower and Open Lab software. The column used was Zodiac C18 (150 × 4.6mm × 3.0µm).

Preparation of Solutions

For Assay

1N Sodium hydroxide solution was prepared, 1ml of Trifluoro acetic acid was transferred into 1000ml of water, pH was adjusted to 4.7 with 1N NaOH solution was used as a buffer.

Mobile Phase

Mixture of Buffer and Acetonitrile in 52:48% v/v ratio., Methanol is used as diluent. Placebo solution: Taken 1 vial and flipped off the seal then reconstituted with 30 ml of water then pipette out 4ml of the above solution to 25ml volumetric flask then diluted to volume with diluent.
Standard stock solution-1 and solution-2: Accurately 33.7mg of the standard was weighed and dilutions were made.

Standard Final Solution-1 and Solution-2

5ml of standard stock solution was taken in 25ml of the volumetric flask, 2ml of water was added and diluted.

Sample Solution

Taken 1 vial and flipped off the seal then reconstituted with 30 ml of water then pipette out 2ml of the above solution to 25ml volumetric flask then diluted to volume with diluent.
Linearity stock solution: 105.0mg of the standard was accurately weighed, diluted to required volume and sonicated.

For Related Compounds (RC)

10% Orthophosphoric acid solution: Transferred 1ml of Orthophosphoric acid into 10 ml of the volumetric flask containing 5ml of water then diluted to volume with water and mix well.

Mobile Phase A

Accurately weighed and transfer about 7.0g of Sodium perchlorate monohydrate and 1.38 g of Sodium di-hydrogen Orthophosphate monohydrate diluted with water and sonicated. The pH of this solution was adjusted to 2.9 ±0.05 with 10% orthophosphoric acid, filtered and sonicated. Mobile phase B used was Acetonitrile

Placebo Solution

Taken 1 vial and flipped off the seal then reconstituted with 30 ml of water then pipetted out 3ml of the above solution to 20ml volumetric flask then diluted to volume with diluent.
Standard stock solution-1 and solution -2: Accurately 13.0 mg of the standard was weighed and then diluted to volume with diluent. Standard final solution-1 and solution-2: 3ml of standard stock solution was taken in 25ml of the volumetric flask, 3ml of water was added and diluted to volume with diluent.
Sample solution: Taken 2 vials and flipped off the seal then reconstituted with 30 ml of water then pipetted out 3ml of the above solution to 20ml volumetric flask then diluted to volume with diluent.

System suitability solution: 6.0 mg of the standard was accurately weighed, transferred into 10ml of volumetric flask. 0.5ml of 0.001N NaOH solution was added and kept aside for 5 mins. Later it was neutralized with 0.5ml of 0.001N Hydrochloride solution diluted to volume with diluent and mixed well. (same for Related compound C solution preparation)

Related Compound A Stock Solution
2.0 mg of related compound A was accurately weighed. 5.0ml of water was added, sonicated and diluted to volume with water. Related compound A final solution: 1.0 ml of Related compound A stock solution was pipetted out in 20ml of volumetric flask and diluted to volume with diluent.

Related Compound B Stock Solution
2.0 mg of related compound B was weighed. 5.0ml of 0.1% of Dimethylformamide (DMF) in Tetrahydrofuran (THF) was sonicated to dissolve and diluted to volume with 0.1% of DMF in THF solution and mixed well. Related compound B final solution: 1.0 ml of Related compound B stock solution was pipetted out in 20ml of volumetric flask and diluted to volume with diluent.
LOQ establishment solution: 3.0ml of the standard solution was pipetted out into 50ml of volumetric flask and diluted to volume with diluent.

LOD Solution
3.3ml of the standard solution was pipetted out into a volumetric flask and diluted to volume with diluent.
Linearity stock solution: 12.5mg of the standard was accurately weighed, 60ml of diluent was added sonicated to dissolve and diluted to volume with diluent.

Analytical Method Development
For Assay
The mobile phase of Trifluoroacetic acid buffer: Acetonitrile (52:48 % v/v) of pH 4.7 is used for the method development trials. Trials were done by using the same mobile phase composition and by changing the runtime to obtain the proper peak shape, asymmetry, and theoretical plate count. The mode of separation employed is isocratic.

For Related Compounds (RC)
The mode of separation employed was gradient flow. So Mobile phase A and B were employed. Trials were done by using Mobile phases A and B changing the run time and flow rate of each mobile phase in gradient method at different time intervals to obtain the proper separation of the related compound peaks from the standard peaks.

Method Validation
The proposed method for both assay and RC were validated according to ICH Q2 (R1) guidelines.[12] Following parameters were assessed as system suitability and system precision, specificity, precision, linearity, accuracy, solution stability, LOD and LOQ, robustness.

RESULTS AND DISCUSSION

Analytical Method Development for Assay
An HPLC method was developed for determining the assay by using the mobile phase composition Trifluoroacetic acid: Acetonitrile by isocratic elution method at a column temperature and autosampler temperature of 25°C respectively. The column used was Zodiac C18 (150x4.6) mm, 3.0µm (P.No: ZLS.C18.46.150.0310). The flow rate was 1.5 ml/min with an injection volume of 10L. The wavelength was 300nm and the run time was 20 min. Anidulafungin peak was observed at the retention time 8.6 min and the tailing factor for Anidulafungin was 0.8. Figure-1 shows the Anidulafungin peak at 8.6 min. The method was validated according to the ICH guidelines.

Analytical Method Development for RC
An HPLC method was developed for determining the related compounds of Anidulafungin by using phosphate buffer as Mobile phase A and Acetonitrile as Mobile phase B. The column used was Zodiac

C18 (150x4.6) mm, 3µm (P.No.: ZLS.c18.46.150.0310). The column temperature and auto sample temperature were maintained at 45°C and 25°C respectively. The flow rate was 0.8 ml/min with an injection volume of 50 µL. The wavelength was 220 nm with a run time of 70 minutes. The elution method was gradient. Below Table-1 gives the data of the gradient flow. The anidulafungin was observed at the retention time 33.16 min. The below Fig.-2 shows the retention time of the main analyte and the well-resolved impurities.

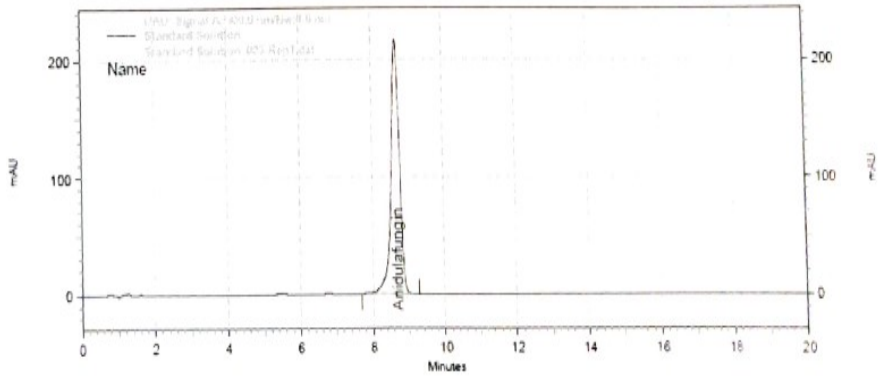

Fig.-1: Sample Chromatogram of Anidulafungin for Assay Method

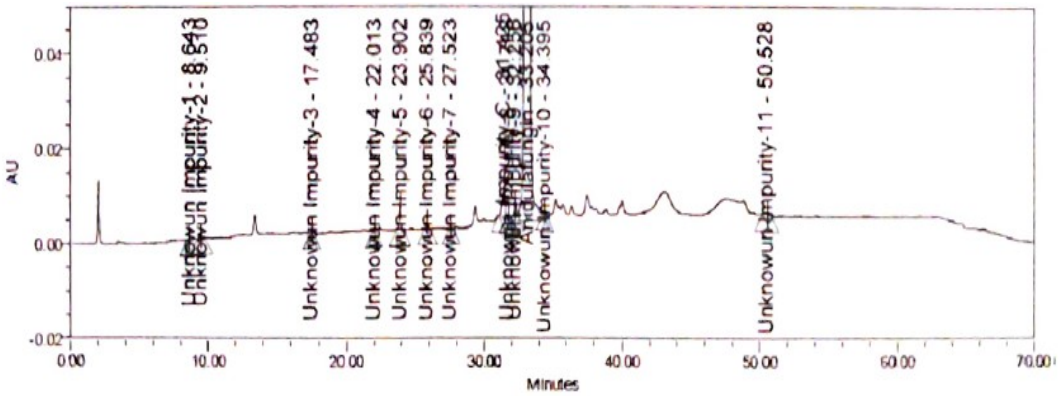

Fig.-2: Sample Chromatogram of Anidulafungin and its Impurities

Table-1: Gradient Flow Details

Time (min)	Flow Rate (ml/min)	Mobile Phase-A (%)	Mobile Phase-B (%)
0.01	0.8	100	0
2	0.8	100	0
10	0.8	85	15
40	0.8	20	80
60	0.8	20	80
65	0.8	100	0
70	0.8	100	0

Method Validation for Assay

System Suitability and System Precision

System suitability and system precision were performed and the results obtained explain that the method meets the acceptance criteria as the results obtained were within the limits. The tailing factor obtained was 0.8 which is less than 2.0, % RSD of 6 replicate injections was 0.3 which is less than 2.0. Theoretical plates were 7916 which is not less than 2000.

Specificity

Specificity was performed and there was no interference found due to blank and placebo solution at the retention time of the standard solution.

Precision
Precision was performed intraday and interday. The overall mean was found to be 101.6. The overall SD and %RSD were found to be 0.89 and 0.9 which is not more than 2.0.

Linearity
Linearity was performed by injecting 5 injections over the range of 50% to 150% of the standard concentration level. The linear regression equation was found to be y = 28226x – 35763 and the regression coefficient was found to be 1.000. The correlation coefficient was found to be 1.000.

Accuracy
For determining accuracy triplicate injections of 50% to 150% range of test concentrations are injected. The individual recovery at each level was found to be 101.5, 101.1 and 101.0 which are within the range 98.0 to 102.0% and the %RSD was found to be 0.1 which is not more than 2.0.

Solution Stability
To perform solution stability the standard solutions and sample solutions were prepared and stored at RT and RF conditions, at different time intervals of 24 hours and 32 hours each solution was injected. The obtained similarity factor values are between the acceptable range of 0.98 to 1.02. The % difference values of sample solution at 24 hours RT and RF conditions were found to be 1.6 and -0.6 respectively. The % difference values of sample solution at 32 hours RT and RF conditions were found to be 2.2 and -0.8 respectively.

LOD and LOQ Determination
LOD and LOQ Values were found to be 3.06 µg/ml and 9.27 µg/ml respectively.

Robustness
Robustness was done by variations in flow rate, temperature, mobile phase organic variation and pH. The results obtained met the system suitability acceptance criteria values. Results for Flow rate variation and temperature variation in assay are given in Table-2, the result of mobile phase organic variation and pH variation in assay are given in Table-3.

Table-2: Results for flow rate variation and temperature variation in assay

Parameter	Low	Actual	High	Parameter	Low	Actual	High
Flow	1.3 ml/min	1.5 ml/min	1.7 ml/min	Temperature	20 ºC	25 ºC	30 ºC
Main peak RT	9.37	8.32	7.29	Main peak RT	7.68	8.32	8.23
Tailing factor	0.8	0.8	0.8	Tailing factor	0.8	0.8	0.8
Theoretical plates	8543	7916	7506	Theoretical plates	7255	7916	8552
% RSD	0.1	0.3	0.2	% RSD	0.1	0.3	0.1
Similarity factor	0.99	0.99	0.99	Similarity factor	0.99	0.99	0.99

Table-3: Results for Mobile Phase Organic Variation and pH Variation in Assay

Parameter	Low	Actual	High	Parameter	Low	Actual	High
Solution-A:Solution-B (%v/v)	544: 456	520: 480	472: 528	pH	4.5 ±0.05	4.7 ±0.05	4.9 ±0.05
Main peak RT	11.83	8.32	4.77	Main peak RT	8.63	8.32	8.73
Tailing factor	0.9	0.8	0.9	Tailing factor	0.9	0.8	0.9
Theoretical plates	8519	7916	6982	Theoretical plates	7953	7916	8015
%RSD	0.1	0.3	0.1	% RSD	0.2	0.3	0.1
Similarity factor	1.00	0.99	1.00	Similarity factor	0.99	1.00	1.00

Method Validation for RC
System Suitability and System Precision
System suitability and system precision were performed. The tailing factor and % RSD for three replicate injections were found to be 0.8 and 0.1which are not more than 2.0 and 10.0 respectively. The resolution

between Related compound-C and Anidulafungin was found to be 5.6 which is not less than 2.0. The system was considered as suitable and precise as the results meet the acceptance criteria.

Specificity

Specificity was performed and no interference was found between blank and placebo at the retention time of anidulafungin peak and individual impurities.

Linearity

Linearity was performed by injecting 6 injections over the range of LOQ to 150% from linearity standard stock solution. Regression equation obtained was y = 15947024.02x + 3999298.360. The correlation coefficient and regression coefficient values were found to be 1.000 respectively.

Method Precision

Method precision was performed by injecting six replicate sample solutions. The % RSD values of single max. Related compound and total impurities were found to be 1.5 which was within the limits specified.

LOD and LOQ Determination

LOD and LOQ values were found to be 0.083 ppm and 0.250 ppm respectively. The S/N values of LOD and LOQ were found to be 5.9 which was more than 3.0 and 14.0 which was more than 10.0 respectively which are within the acceptance criteria limits.

Solution Stability

The results obtained for standard solution stability at both RT and RF conditions showed that obtained similarity factor values are within the acceptance criteria range of 0.95-1.05. The results obtained for sample solution stability at both RT and RF conditions depicted that % difference values of single maximum related compound and % difference of total impurities are within the specified acceptance limits. Figure-3 shows the chromatograms of sample and standard solution at respective stored RT and RF conditions.

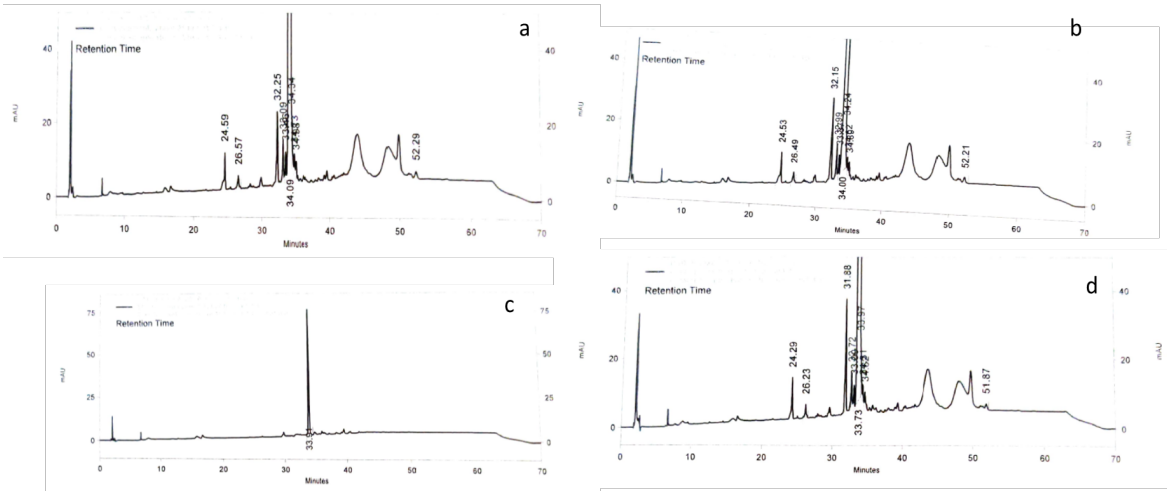

Fig.-3: (a.) Chromatogram of Sample solution for 24 hours at RF condition, (b.) Chromatogram of Sample solution for 24 hours at RT condition, c. Chromatogram of Standard solution for 48 hours at RF condition, d. Chromatogram of sample solution for 48 hours at RT

Robustness

Robustness was done by variations in flow rate, column oven temperature and pH. The results obtained met the system suitability acceptance criteria values. Results for Flow rate variation in RC are given in Table-4, Column oven temperature variation and pH variation in RC are given in Table-5.

Table-4: Results for Flow rate variation in RC

Parameter	Low	Actual	High
Flow rate	0.6 ml/min	0.8 ml/min	1.0 ml/min
RT	35.84	33.74	32.37

Tailing factor	1.8	0.8	0.8
Resolution between Related compound-C and Anidulafungin	5.0	5.6	4.7
% RSD	0.2	0.1	0.1
RRT	1.00	1.00	1.00
Related compound-A	11.04	10.59	9.30
Related compound-B	48.13	11.84	42.73
Related compound-C	34.01	32.34	30.69

Table-5: Results for Column Oven Temperature and pH Variation in RC

Parameter	Low	Actual	High	Parameter	Low	Actual	High
Temperature	40ºC	45ºC	50ºC	pH	2.7±0.05	2.9±0.05	3.1±0.05
RT	33.71	33.72	33.64	RT	33.65	33.74	33.85
Tailing factor	0.9	0.9	0.9	Tailing factor	1.1	0.8	1.1
Resolution between Related compound-C and Anidulafungin	5.8	6.2	5.2	Resolution between Related compound-C and Anidulafungin	4.1	5.6	4.2
% RSD	0.8	0.1	1.6	% RSD	0.4	0.1	0.3
RRT	1.00	1.00	1.00				
Related compound-A	10.73	10.55	10.37	RRT	1.00	1.00	1.00
Related compound-B	44.94	44.37	43.83	Related compound-A	10.27	10.59	10.53
Related compound-C	31.99	31.97	31.89	Related compound-B	44.59	44.84	44.91
				Related compound-C	31.89	32.34	32.11

CONCLUSION

A simple, sensitive, robust and effective Stability Indicating Assay and RC Method Development and Validation of Anidulafungin Injection by HPLC has been developed. The proposed method is found to be robust and this method can effectively be used in the quality control lab for the routine analysis of this product. Further, this HPLC method was successfully validated as per ICH Q2 (R1) guidelines and proved to be precise, linear, sensitive, accurate and robust. This method is time-saving (20 min), it can be used for determining the assay of anidulafungin. In this method impurities are separated properly so, this method can be used for the intended purpose.

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