Nanotechnology Perceptions
ISSN 1660-6795
www.nano-ntp.com

Design and Method Validation of Amphotericin-B in Human Plasma by A RP-HPLC Method

Tanvir Yusuf Shaikh[1], Suvarna Manoj Bhadane[2], Shriram Bairagi[3], Pusuluri Siva Krishna[4], Mayble Mary Lyngkhoi[5], Pavankumar D Chopade[6], Prabhakar Vishvakarma[7], Vikas Kumar Pal[8*]

[1]Associate Professor, Smt. Sharadchandrika Suresh Patil College of Pharmacy, Chopda
[2]Associate Professor, R. G. Sapkal Institute of Pharmacy, Anjaneri, Trimbakeshwar Road, Nashik, MH
[3]Professor, YNP College of Pharmacy, Asangaon, Palghar
[4]Assistant Professor, Department of Pharmaceutical Analysis, Vignan Pharmacy College, Vadlamudi
[5]Assistant Professor, University of Science and Technology Meghalaya, Kling Road, Baridua, Ri-Bhoi Meghalaya
[6]Assistant Professor, Oriental College of Pharmacy Sanpada, Navi Mumbai, Maharashtra
[7]Associate Professor, Department of Pharmaceutics, Krishna Institute, Bijnor, Uttar Pradesh
[8]Assistant Professor, Krishna Institute of Pharmacy and Sciences, Kanpur, Gram Amiliha Post Tatiyganj, Mandhana Kanpur, Uttar Pradesh
Email id: vikaskumarpal02@gmail.com

A novel RP-HPLC technique was created for the quantification of amphotericin-B in human plasma that is straightforward, sensitive, accurate, and exact. An extraction procedure was optimised using a full factorial design. The volume of the deproteinating agent, centrifugation speed, centrifugation time, and centrifugation temperature were found to have a significant impact on all results ($P<0.0001$). Following deproteinisation, the medication was examined using a UV detector on a C18 (150 x 4.6 mm, 5 µm) column. Acetonitrile and phosphate buffer (pH 3) in a 60:40 (v/v) ratio at a flow rate of 1.0 ml/min make up the mobile phase. Linearity was determined to be 0.9998 when the standard calibration curve was built in the concentration range of 5 µg/ml to 30 µg/ml. The internal standard was irbesartan. Amphotericin-B and the internal standard were found to have retention times of 5.42 and 2.89 minutes, respectively. There was no discernible interference peak. An alternate technique for analysing amphotericin-B in plasma samples is high performance liquid chromatography, which has been effectively

shown to be quick and sensitive.
Keywords: RP-HPLC, Validation, amphotericin-B, Human plasma

1. Introduction

An antifungal called amphotericin B is used to treat leishmaniasis, fungal infections in neutropenic patients, and cryptococcal meningitis in HIV-infected patients. Depending on the fungus's susceptibility and the concentration found in bodily fluids, it can be either fungistatic or fungicidal. [1] By attaching itself to sterols (ergosterol) in the cell membrane of vulnerable fungi, the medication works. Because of the transmembrane channel that is created and the resulting alteration in membrane permeability, intracellular components can leak out. Amphotericin B and azoles work by targeting ergosterol, the main sterol in the fungal cytoplasmic membrane. [2] A polyene called amphotericin B forms an irreversible bond with ergosterol, which compromises the integrity of the membrane and eventually causes cell death.

However, this antifungal medication's clinical use is restricted due to adverse effects and poor absorption, which stem from its low water solubility (<0.001 mg/mL).6. AmB's low solubility is also the primary cause of its reported side effects, which include polyuria, hypokalaemia, hypomagnesaemia, and renal failure. [3, 4] As a result, a lot of work has gone into making AmB more soluble in water. [5] In order to create a micellar dispersion for encapsulating AmB and increasing its solubility, Fungizone® (Bristol-Myers Squibb, Rueil-Malmaison, France) was initially introduced as a colloidal dispersion in combination with sodium deoxycholate (DOC). [6] Nevertheless, the nephrotoxicity and infusion toxicity, which could be attributed to the dimeric form produced with DOC through a technological procedure involving pH shifts, nevertheless commonly result in problematic side effects. Commercial products that encapsulate AmB with hydrogenated soybean lecithin, phosphatidylcholine, and cholesterol (polyaggregate forms) have already reached the market. [7] These include a lipid complex (Abelcet®, Sigma-Tau, Gaithersburg, MD, USA) and a liposomal product (Ambisome®, Astellas, Tokyo, Japan). Higher dosages of these formulations result in a decreased toxicity incidence and good acceptance. [8, 9]

No pharmacopoeia2 recognises amphotericin-B as an official medication. According to a literature review, methods for determining the presence of amphotericin-B in human plasma have been reported using LC-MS/MS, UV Spectroscopy, and HPLC. [10, 11] However, these current techniques were costly and required several extraction stages. For the extraction and measurement of Amphotericin-B in plasma samples utilising HPLC with a UV detector, a new bioanalytical technique that is simpler, easier, faster, and more cost-effective must be developed and validated. Additionally, the extraction procedure must be as quick and easy as possible. Despite being well-established, the extraction process is influenced by the protein separation procedure, the volume of the extracting solvent, the choice of deproteinising agent, and the ease of extraction. [12, 13] Short analysis time, sensitivity, robustness, accuracy, precision, sharpness of the peak, and other economic factors should also be taken into account during the estimation procedure. Appropriate extraction conditions, buffer selection, pH, UV detector wavelength and flow rate, and stationary and mobile phases can all help attain these criteria. For proper elution, a somewhat volatile buffer with a pH that is appropriate for the drug's pKa value should be employed; to shorten the retention duration, the mobile phase's

composition and flow rate should be changed. [14, 15] The current work set out to create and verify a bioanalytical HPLC technique for the determination of amphotericin-B in human plasma that was sensitive, straightforward, easy, quick, repeatable, accurate, and cost-effective.

2. METHODOLOGY

Materials: Amphotericin-B was obtained as gift sample from Yarrow Chem. Pvt. Ltd, Maharashtra, India. HPLC grade acetonitrile, methanol and orthophosphoric acid were obtained from Merck, Mumbai, India Limited. HPLC grade water was obtained from MOLYCHEM, Thane, India. The blank human plasma was obtained from Blood bank.

Instrumentation: The HPLC system (Cyberlab LC 100) consisting of binary gradient pump, Microsorb-MV 100-5 C- 18 column (250 x 4.6mm, 5 µm), UV detector was employed for analysis. Chromatographic data was acquired using WS-100 Workstation software. Microsorb MV 100-5 C-18 column (150mm×4.6 mm, 5µm) was used as a stationary phase. A Millipore glass filter (Millipore filter cellulose nitrate gridded with 0.22 µ size and 47 mm diameter) assembly attached with vacuum pump was used to filter mobile phase.

Chromatographic Conditions: The isocratic mobile phase consisting of a mixture of phosphate buffer (pH 3.0 adjusted with ortho-phosphoric acid) and acetonitrile in the ratio of 60:40 (v/v) was used on Microsorb MV 100-5 C-18 column (250mm×4.6 mm, 5µm) as a stationary phase. The flow rate of the mobile phase was 1.0 ml/min. Detector signal was monitored at a wavelength of 311 nm using UV detector while keeping the 10 min run time for chromatographic analysis. Prior to the injection of the drug solution, the column was equilibrated for at least 30 min. The column temperature was kept ambient and injection volume was 20 µl.

Preparation of sample: A 100 ml volumetric flask containing 10 mg of Amphotericin-B working standard was filled with around 25 ml of diluent, and the mixture was sonicated for 20 minutes to create the Amphotericin-B standard stock solution. Using diluent, the volume was increased to 100 ml. A Nylon 66 membrane filter with pores of 0.45µm was used to filter this mixture. [16] The stock solution was diluted with the mobile phase to create the following dilutions. The study's internal standard (IS) was irbesartan. To create 100 µg/ml concentrations, a precisely weighed quantity of irbesartan (10 mg) was added to a 100 ml glass volumetric flask, and the volume was adjusted with mobile phase. Additionally, the right amount of irbesartan was diluted to create a functioning concentration. [17] To obtain calibration concentrations of 5, 10, 15, 20, 25, and 30 µg/ml, 10 µl aliquots of each spiking solution were added to 490 µl of plasma. In a similar manner, quality control samples containing 15, 20, and 25 µg/ml were made. Using a linear regression analysis, the calibration curve was used to determine the concentrations of the quality control and sample. [18]

Extraction: To extract Amphotericin-B from human plasma, a solvent deproteinisation method was employed. After transferring 490 µl of plasma to two millilitre micro centrifuge tubes, 10 µl of Amphotericin-B working solution and 10 µl of IR were added. The pre-optimized deproteinising chemical was introduced after 30 seconds of vortexing. The micro centrifuge tubes containing the samples were centrifuged at a predetermined speed for the ideal

temperature and duration. After that, the liquid supernatant was moved to an HPLC vial that had been previously labelled for examination. [19]

Optimization of extraction process: The amount of deproteinising agent, centrifugation speed, centrifugation time, and centrifugation temperature may all be significant factors in drug extraction. Thus, DoE (Design Expert® v.10.0.6.0 software) was used to optimise these four criteria in this study. ACN was employed as a deproteinising agent in this investigation. Two different amounts of the deproteinising agent—500 µl and 1000 µl—were used. The supernatant was separated using two distinct centrifugation speeds, such as 10,000 and 12,000 rpm. Similar to this, two distinct levels of centrifugation time and temperature were examined: 7.5 and 10 minutes, while 5 and 7.5 degrees Celsius, respectively. [20, 21]

The use of experimental design made it simpler to optimise independent variables and their interactions. The effect of many variables at once can be studied with the aid of experiment design. The central composite design is used in this study to optimise the independent variables. Four independent variables with two levels were employed in this investigation, indicating that a total of 16 runs were needed to complete the experiment. As independent variables, the centrifugation speed (B), time (C), temperature (D), and volume of the deproteinising agent (A) were employed. These independent variables were projected at two different levels, such as minimum (−1) and maximum (+1). A total of 30 experimental runs were conducted, and the results, such as the percentage of drug extracted from plasma (TR1), the number of theoretical plates (TR2), and the drug tailing factor (TR3), were entered into software to examine how the independent variables interacted and to optimise the system. Table 1 lists the parameters and concentrations utilised in the core composite design for the extraction of quetiapine from human plasma, while Table 2 lists the experimental runs' composition. [22, 23] The Quadratic Equation and analysis of variance (ANOVA) were used to estimate the significance of the independent variables.

$$RS = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2$$

Where, TR is the response, β represents as regression coefficients and A, B, C and D are, volume of deproteinizing agent, speed of centrifugation, time of centrifugation and temperature of centrifugation respectively.

Table 1: Central Composite Design for Extraction of Amphotericin-B From Human Plasma

Independent factors	Levels	
	-1	+1
Volume of deproteinating agent (µl)	500	1000
Speed of centrifugation (rpm)	10000	12000
Time of centrifugation (min)	7.5	10
Temperature of centrifugation (ºC)	5	7.5

Table 2: Experimental Runs Obtained from Central Composite Design

Run	Independent Variables			
	Volume of deproteinating agent (µl)	Speed of centrifugation (rpm)	Time of centrifugation (min)	Temperature of centrifugation (ºC)
1	500	10,000	7.5	5
2	1000	10,000	7.5	5
3	500	12,000	7.5	5
4	1000	12,000	7.5	5
5	500	10,000	10	5
6	1000	10,000	10	5
7	500	12,000	10	5
8	1000	12,000	10	5
9	500	10,000	7.5	7.5
10	1000	10,000	7.5	7.5
11	500	12,000	7.5	7.5
12	1000	12,000	7.5	7.5
13	500	10,000	10	7.5
14	1000	10,000	10	7.5
15	500	12,000	10	7.5
16	1000	12,000	10	7.5
17	250	11,000	8.75	6.25
18	1250	11,000	8.75	6.25
19	750	8,000	8.75	6.25
20	750	14,000	8.75	6.25
21	750	11,000	6.125	6.25
22	750	11,000	13.25	6.25
23	750	11,000	8.75	3.75
24	750	11,000	8.75	8.75
25	750	11,000	8.75	6.25
26	750	11,000	8.75	6.25
27	750	11,000	8.75	6.25
28	750	11,000	8.75	6.25
29	750	11,000	8.75	6.25
30	750	11,000	8.75	6.25

Method Validation by HPLC: The designed and optimised approach was validated in accordance with USFDA requirements. The following procedures, including selectivity,

linearity, accuracy, precision, recovery, and stability, were used to validate the method. [24]

Selectivity: The lack of protein and/or other impurity interference with the drug peak's retention period is a measure of selectivity. Rat blank plasma samples and drug-containing plasma (LLQC, LQC, MQC, and HQC samples) were extracted using the same method as described above, and six replicate injections of each were used for HPLC analysis. [25]

Linearity: Eight distinct concentrations, ranging from 5 µg/ml to 30.0 µg/ml, and blank samples (plasma devoid of medication) were used to create the calibration figure. Three duplicates of each concentration were made. To determine the correlation coefficient and linear regression, drug concentration was plotted against drug/IS peak area ratio.

Accuracy: Three distinct quality control concentrations, including LQC, MQC, and HQC, were used to measure accuracy. Nominal concentrations and average back-calculated concentrations were used to determine the percent accuracy using the procedure provided in Equation. [26] Six replicate injections of each quality control concentration were used to calculate the average back calculated concentration.

Percent accuracy= BC/NC×100

Where, BC represents the average back calculated concentration and NC represents the nominal concentration of drug. [27]

Precision: Both intra- and inter-day precision studies were carried out. Three quality control levels—LQC, MQC, and HQC—were taken into consideration in this investigation. Six duplicates of each were used twice a day (morning and evening) to perform intra-day precision of the established approach for these three quality controls. For these three quality controls, an inter-day precision study was also conducted using six replicates of each, twice on two separate days (i.e., one and two days). [28, 29] The percentage relative standard deviation (%RSD) was computed and the drug/IS peak area ratio was assessed.

Recovery: Analytical standards of the same concentration (un-extracted samples) were compared with the absolute recovery of Amphotericin-B from spiking human plasma samples (extracted samples). The mean percentage recovery was computed in this investigation at three distinct concentration levels, including LQC, MQC, and HQC. The extracted and un-extracted samples were examined in triplicate for every concentration level, and the mean was determined. The recovery percentage approaching 100% shows that the process is reliable and that the chosen solvent has an extremely high extraction efficiency. [30, 31, 32] Eqn provides the formula used to determine the recovery percentage.

Percent recovery = AE/AU×100

Where, AE is the peak area of extracted samples and AU is the peak area of un-extracted samples.

Limit of detection and limit of quantitation: Amphotericin-B's limit of detection and limit of quantitation were established using serial dilutions of known concentration at signal-to-noise ratios of 3:3 and 10:1, respectively. [33]

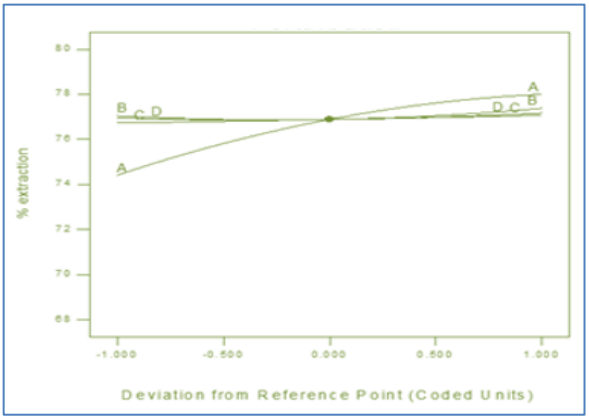
Stability studies: Amphotericin-B stability in human plasma was assessed using LQC and HQC samples under various stress scenarios. [34, 35] The formula provided in Eqn was used

to determine the percentage of medication that was still present in the plasma.

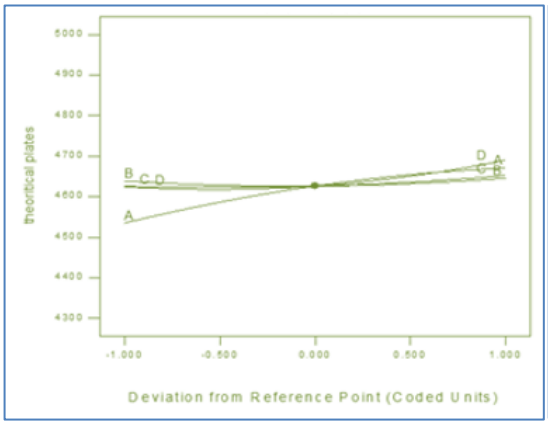
Percent drug remaining = final concentration/initial concentration×100.

3. RESULT AND DISCUSSION

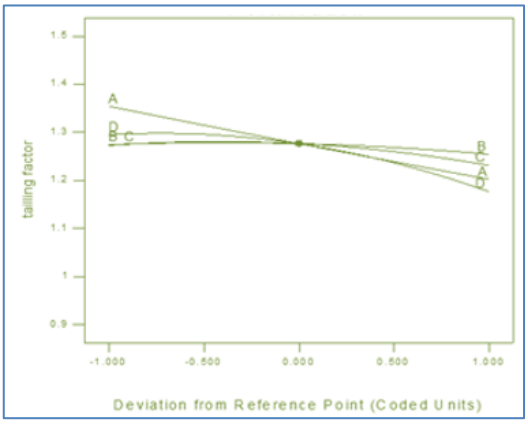
Optimisation of Extraction of Drug from Plasma: Using the central composite design, the Design Expert® was utilised to optimise the extraction process. Analysis was done on the primary and complicated effects of independent factors on responses. Using a quadratic equation and perturbation plots created using the program, the impact of independent variables on responses was evaluated, as seen in Figure 1.



A



B



C

Fig 1: Effect of Factors, Volume of Deproteinising Agent (A), Speed of centrifugation (B), Time of Centrifugation (C) And Temperature of Centrifugation (D) On % Drug Extraction (TR1), Number Of Theoretical Plates (TR2) And Tailing Factor (TR3)

The volume of deproteinising agent (A) shown a significant effect on the percentage of drug extraction from plasma (TR1), showing that the volume of deproteinising agent increased the

percentage of drug extraction. Likewise, the percentage of drug extraction is marginally reduced by centrifugation temperature (D) and speed (B). However, the drug's ability to be extracted from the plasma was unaffected by centrifugation time (C). The number of theoretical plates is not affected by centrifugation speed (B) or time (C), according to the perturbation plot of the number of theoretical plates (TR2). However, the number of theoretical plates is increased by the volume of deprotenizing agent (A) and slightly increased by centrifugation temperature (D). The tailing factor (TR3) is reduced by the volume of the deproteinising agent (A), centrifugation time (C), and centrifugation temperature (D); nevertheless, the tailing factor is unaffected by centrifugation speed.

ANOVA was used to analyse the responses, which included the percentage of drug extraction (TR1), the number of theoretical plates (TR2), and the drug tailing factor (TR3). The letters A, B, C, and D stand for the volume of the deproteinising agent, centrifugation speed, centrifugation time, and centrifugation temperature, respectively. Below are the equations for the response factors.:

% drug Extraction (TR1) = + 75.33 +1.86 * A + 0.15 * B + 0.17 * C + 0.13 * D + 0.27 * AB -0.55 * AC -0.28 * AD - 0.15 * BC -0.27 * BD -0.074 * CD -0.63 * A2+0.31 * B2 + 9.954 E-002 * C2 +0.14 * D2

Number of theoretical Plates (TR2) = +4512.23 +66.54 * A +3.26 * B +13.54 * C +32.76 * D -28.45 * AB +15.00 * AC +29.43 * AD +2.65 * BC -36.53 * BD +24.11 * CD -23.62 * A2+16.39 * B2 +12.45 * C2 +31.76 * D2

Tailing Factor (TR3) = +1.23 -0.065 * A -0.014 * B -0.023 * C -0.055 * D +0.023 * AB+2.501E-002 * AC+8.654E-003 * AD+0.039 * BC-1.250E-002 * BD-0.015 * CD+1.875E-003 * A2 -0.014 * B2-0.024 * C2-0.065 * D2

Validation of optimised factors: By contrasting the observed and predicted responses, the responses derived from optimised independent factors, such as the volume of the deproteinating agent, centrifugation speed, centrifugation time, and centrifugation temperature, were verified. Table 3 indicates that the discrepancy between the observed and expected responses was within ±2.11%. It was determined that the desirability value was 0.765. The value of the desirability, which is a measure of the method's suitability, was found to be close to 1.0, indicating a robust extraction process.

Table 3: Optimized Independent Variables

Response	Predicted Results	Observed Result	Residual Values
% extraction of Drug from plasma	75.11	77.62	-2.11
Number of theoretical plates	4845	`4732	1.01
Tailing factor	0.978	0.968	1.01

Validation of Bioanalytical Method: In accordance with USFDA requirements, the procedure was validated. With a short run time of 5.42 minutes for amphotericin-B and 2.89 minutes for internal standard irbesartan, the validated method was easy to use, cost-effective, and quick. The spiked blank plasma sample did not exhibit a peak on or close to the drug and internal standard retention times, according to specificity experiments. Fig. 2 displays the drug and

internal standard chromatographs.

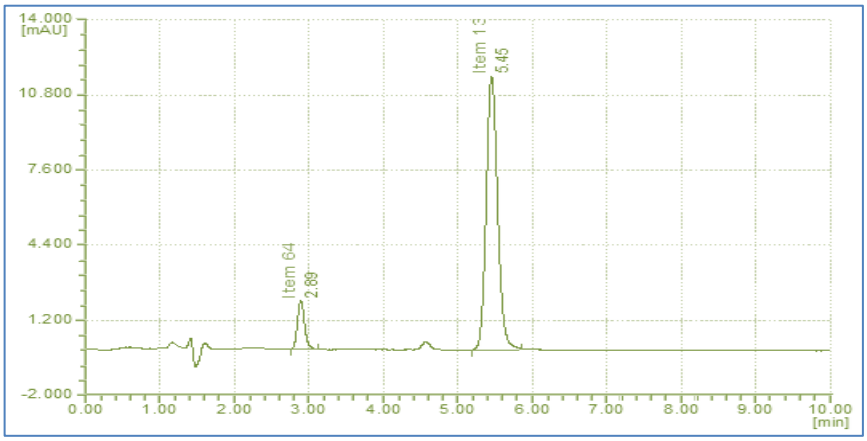


Figure 2: Chromatogram Of Amphotericin-B And IS

A substantial connection of 0.9989 was revealed by the correlation coefficient value derived from linearity. Using a calibration plot, the linear regression was determined to be y=0.8236x+0.0562. where x represents the drug concentration and y represents the drug/IS peak area ratio. With a %RSD ranging from 2.53% to 4.56%, the accuracy results indicated that the approach was accurate. As indicated in Table 4, the percentage accuracy was also determined to be within the range of 97.21 to 100.04%. According to precision results, this method's %RSD ranged from 2.11% to 3.49%.

Table 4: Precision And Accuracy Results of Proposed Method (n=6)

Quality Control Level	Nominal concentration (µg/ml)	Precision (% RSD)		Accuracy	
		Intra-day	Inter-day	% Accuracy	% RSD
LQC	5	3.49	3.02	97.21	4.56
MQC	10	3.33	2.11	99.04	2.53
HQC	25	2.92	3.22	100.04	3.46

In comparison to un-extracted samples of the same concentration, the mean recovery of valsartan from spiked human plasma (extracted) samples was found to be between 75.11 and 77.62%, with percent RSD values ranging from 0.45 to 0.71 percent. Table 5 reports the results of the recovery.

Table 5: Recovery From Human Plasma (n=3)

Quality Control Level	Nominal concentration (µg/ml)	Percent mean recovery (%)		% RSD
		Mean	SD	
LQC	5	75.11	0.53	0.71
MQC	10	77.62	0.51	0.64
HQC	25	76.34	0.37	0.45

The results showed that the limits of quantitation and detection were 15 ng/ml and 4.9 ng/ml, respectively. Table 6 reports the results of stability studies that were examined at two levels, such as LQC and HQC. According to the stability investigations, amphotericin-B proved stable.

Table 6: Results of Stability Studies

Quality Control Level	Percent Drug (%)	
	Bench Top Stability	Long Term Stability
LQC	97.8	96.3
HQC	99.7	98.1

4. CONCLUSION

Design Expert version software was used to successfully build and optimise an HPLC technique. Plots of perturbations have shown a high significant effect of independent variables. The DoE is a very effective tool for optimising independent variables for the creation of a bioanalytical method, according to the study's findings. In this way, the method described was sensitive enough for the quantitative determination of AB in different biological tissues.

References

1. Chandrasekar, P. Diagnostic challenges and recent advances in the early management of invasive fungal infections. Eur. J. Haematol. 2010, 84, 281–290.
2. Zaragoza, R.; Peman, J.; Salavert, M.; Viudes, A.; Sole, A.; Jarque, I.; Monte, E.; Roma, E.; Canton, E. Multidisciplinary approach to the treatment of invasive fungal infections in adult patients. Prophylaxis, empirical, preemptive or targeted therapy, which is the best in the different hosts? Ther. Clin. Risk Manag. 2008, 4, 1261–1280.
3. Maertens, J.; Groll, A.H.; Cordonnier, C.; de la Camara, R.; Roilides, E.; Marchetti, O. Treatment and timing in invasive mould disease. J. Antimicrob. Chemother. 2011, 66 (Suppl. S1), i37–i43.
4. Paramythiotou, E.; Frantzeskaki, F.; Flevari, A.; Armaganidis, A.; Dimopoulos, G. Invasive fungal infections in the ICU: How to approach, how to treat. Molecules 2014, 19, 1085–1119.
5. Garey, K.W.; Rege, M.; Pai, M.P.; Mingo, D.E.; Suda, K.J.; Turpin, R.S.; Bearden, D.T. Time to initiation of fluconazole therapy impacts mortality in patients with candidemia: A multi-institutional study. Clin. Infect. Dis. 2006, 43, 25–31.
6. Zheng, Y.J.; Xie, T.; Wu, L.; Liu, X.Y.; Zhu, L.; Chen, Y.; Mao, E.Q.; Han, L.Z.; Chen, E.Z.; Yang, Z.T. Epidemiology, species distribution, and outcome of nosocomial Candida spp. bloodstream infection in Shanghai: An 11-year retrospective analysis in a tertiary care hospital. Ann. Clin. Microbiol. Antimicrob. 2021, 20, 34.
7. Yang, Z.T.; Wu, L.; Liu, X.Y.; Zhou, M.; Li, J.; Wu, J.Y.; Cai, Y.; Mao, E.Q.; Chen, E.Z.; Lortholary, O. Epidemiology, species distribution and outcome of nosocomial Candida spp. bloodstream infection in Shanghai. BMC Infect. Dis. 2014, 14, 241.
8. Nett, J.E.; Andes, D.R. Antifungal Agents: Spectrum of Activity, Pharmacology, and Clinical Indications. Infect. Dis. Clin. N. Am. 2016, 30, 51–83.
9. Andes D, Safdar N, Marchillo K and Conklin R. Pharmacokineticpharmacodynamic comparison of amphotericin B (AMB) and two lipid-associated AMB preparations, liposomal AMB and AMB lipid complex, in murine candidiasis models. Antimicrobial Agents and Chemotherapy

2006; 50: 674–684.
10. Vishvakarma P, Mandal S, Pandey J, Bhatt AK, Banerjee VB, Gupta JK. An Analysis of The Most Recent Trends in Flavoring Herbal Medicines in Today's Market. Journal of Pharmaceutical Negative Results. 2022 Dec 31:9189-98.
11. Mandal S, Vishvakarma P, Mandal S. Future Aspects and Applications of Nanoemulgel Formulation For Topical Lipophilic Drug Delivery. European Journal of Molecular & Clinical Medicine. 2023;10(01):2023.
12. Mandal S, Vishvakarma P. Nanoemulgel: A Smarter Topical Lipidic Emulsion-based Nanocarrier. Indian J of Pharmaceutical Education and Research. 2023;57(3s):s481-98.
13. Prabhakar V, Agarwal S, Chauhan R, Sharma S. Fast dissolving tablets: an overview. International Journal of Pharmaceutical Sciences: Review and Research. 2012;16(1):17.
14. Egger P, Bellmann R and Wiedermann CJ. Determination of amphotericin B, liposomal amphotericin B, and amphotericin B colloidal dispersion in plasma by high-performance liquid chromatography. Journal of Chromatography B, Biomedical Sciences and Applications 2001; 760: 307–313.
15. Eldem T and Arican-Cellat N. Determination of amphotericin B in human plasma using solid-phase extraction and high-performance liquid chromatography. Journal of Pharmaceutical and Biomedical Analysis 2001; 25: 53–64.
16. Echevarría I, Barturen C, Renedo MJ and Dios-Viéitez MC. Highperformance liquid chromatographic determination of amphotericin B in plasma and tissue. Application to pharmacokinetic and tissue distribution studies in rats. Journal of Chromatography A 1998; 819: 171–176.
17. Gallis HA, Drew RH and Pickard WW. Amphotericin B: 30 years of clinical experience. Reviews of Infectious Diseases 1990; 12: 308–329.
18. Hosotsubo H and Hosotsubo K. Improved high-performance liquid chromatographic determination of amphotericin B in human serum and plasma. Journal of Pharmaceutical and Biomedical Analysis 1989; 7: 975–979.
19. Lacroxi C, Wojciechowski F and Danger P. Simultaneous determination of itraconazole, hydroxylitraconazole, and amphotericin B in human plasma by HPLC with photodiode array detection. Annales de biologic Clinique 1995;53(5):293-297.
20. Abu Rustum M. Determination of amphotericin B in human plasma by reversed phase high-performance liquid chromatography using a short octyl column. J Liquid Chromato 1990;13(20):3985-4003.
21. Hulsewede JW. Comparision of High performance liquid chromatography and bioassay for the determination of 5-fluorocytosine in serum. Inter National J Med Micro 1994;281(4):513-518.
22. Margosis M and Aszalos A. Quantitation of amphotericians by reverse-phase high- performance liquid chromatography J Pharma Scien 1984;73(6):835-838.
23. Lupashevskaya DP, Bershtein IY, Raigorodskaya VY and Birman GS. Spectrophotometric analysis of polyene antibiotics. 1977:;2(1):33-36.
24. Glynn Raymond G. Quantification of amphotericin B in aqueous solutions by high- performance liquid chromatography. LC-GC 1989;7(1):58-60.
25. Lue LP, Hadman ST and Vancura A. Liquid chromatographic determination of amphotericin B in different pharmaceuticals. Journal of AOAC International 2002; 85: 15–19.
26. Grudal I, Nadeau P, Brajtburg J and Medoff G. Application of differential spectra in the ultraviolet-visible region to study the formation of Amphotericin B-sterol complexes. Biochi Biophy Acta 1980;602(2):260-268.
27. Ganiere Monteil C, Marie-France K, Iooss P, Thomas L and Larousse C. Quantitation of Amphotericin B in plasma by second- derivative spectrophotometry. J Pharma Biomed Anal 1998;17(3):481-485.
28. Olson JA, Adler-Moore JP, Schwartz J, Jensen GM and Proffitt RT. Comparative efficacies,

toxicities, and tissue concentrations of amphotericin B lipid formulations in a murine pulmonary aspergillosis model. Antimicrobial Agents and Chemotherapy 2006; 50: 2122–2131.

29. Nickos Botsoglou A, Dimitrios Feltoutris J, George Papageorgiou E, Florou-Paneri P and Antonios Mantis J. Rapid determination of Amphotericin B in serum and urine by third- order derivative spectrophotometry. J Pharma Scien 1996;85(4):402-406.
30. Zhu H, Pang H, Hu C and Feng F. Content Determination of amphotericin B and liposomal amphotericin B by turbidimetric method. ZhongguoKangshengsu Zazhi 2005;30(12):752-755.
31. ICH Harmonised Tripartite Guideline. Validation of Analytical Procedures: Methodology, ICH Topic Q2B. The European Agency for the Evaluation of Medicinal Products: London, 1996.
32. Sánchez-Brunete JA, Dea MA, Rama S, Bolás F, Alunda JM, Raposo R, Méndez MT, Torrado-Santiago S and Torrado JJ. Treatment of experimental visceral leishmaniasis with amphotericin B in stable albumin microspheres. Antimicrobial Agents and Chemotherapy 2004; 48: 3246–3252
33. Lamothe J. Activity of amphotericin B in lipid emulsion in the initial treatment of canine leishmaniasis. The Journal of Small Animal Practice 2001; 42: 170–175.
34. Murray HW. Treatment of visceral leishmaniasis in 2004. The American Journal of Tropical Medicine and Hygiene 2004; 71: 787–794.
35. Olliaro PL, Guerin PJ, Gerstl S, Haaskjold AA, Rottingen JA and Sundar S. Treatment options for visceral leishmaniasis: a systematic review of clinical studies done in India, 1980–2004. The Lancet Infectious Diseases 2005; 5: 763–774.