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Development and Validation of UV-Vis Spectrophotometric Method for Estimation of Amphotericin B from Hydrogel Nanoparticle

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Abstract: ***Amphotericin B (AmB) is a polyene macrolide antibiotic that has antifungal action against the broadest range of fungal diseases as well as in some protozoan infections. There are currently no significant issues with resistance to AmB, and it is still in use today. The development of many innovative AmB formulations as lipid formulations and polymeric nanoparticles utilising UV-VIS spectrophotometry and HPLC technique has been discussed in the literature, however specifics of peak characteristics and validation data are not provided. The goal of this study was to provide an effective approach for validating AmB and to gather reliable, accurate, and consistent data on linearity, detection limit, quantification limit, accuracy, and precision using a UV-VIS spectrophotometer. The method gives a good linearity with regression of y = 0.1471x + 0.0489 (R^2 = 0.9989) at 408 nm. The repeatability (inter-day) and intermediate precision (intra-day) precision studies revealed that the method is precise and reliable where all the RSD values were <2%. Method accuracy showed % accuracy value for all the three concentration levels ranged from 91.144 % to 101.994 % that a small change in the concentration of the drug could be accurately determined with high accuracy. Limit of detection and limit of quantification were 0.0295 (µg/ml) and 0.0895 (µg/ml) respectively. The suggested approach is straightforward and may be considered a strategy that is routinely practicable for estimating AmB.***

Keywords: Amphotericin B, Antifungal, hydrogel nanoparticle UV-VIS spectrophotometry, Validation.

I. INTRODUCTION

Amphotericin B (AmB), which was first discovered in Streptomyces nodous in 1955, is a polyene macrolide antibiotic that has antifungal activity against the broadest range of fungal infections, including Aspergillosis, Candidiasis, Blastomycosis, Coccidioidomycosis, Cryptococcosis, and Histoplasmosis, as well as some protozoan infections, including Leishmaniasis.(1,2,3) For decades, the polyene fungicide amphotericin B (AMB) has been used in clinical settings to treat human fungal infections, particularly opportunistic systemic fungal infections [4]. It has uncommon antifungal resistance as well as broad-spectrum antifungal activity [5]. AMB has the potential to cause severe toxicity when used systemically, particularly nephrotoxicity and altered electrolytes hence AMB has been proposed as a topical treatment for wound infections. (6,7,8) Its low solubility in both aqueous and organic solutions is due to its amphoteric character. AmB is classified as a class IV compound with poor solubility and permeability qualities under the Biopharmaceutical Classification System (BCS). AmB has a relatively poor oral bioavailability as a result.(9)

Amphotericin B preferentially binds to ergosterol, the main element of the fungal cell membrane, as part of its antifungal mode of action. The hydrophobic (polyene hydrocarbon chain) and hydrophilic (polyhydroxyl chain) regions of the amphotericin B molecule are essential to the antifungal activity of the compound. Eight to ten amphotericin B molecules hydrophobically interact with eight ergosterol molecules through the polyene chain, forming pores made of two "barrels" of hydrogen bonded molecules that are end to end and have eight polyene monomers arranged circumferentially like staves in a barrel. The inside of the pore is exposed to the hydrophilic polyhydroxyl chains. This pore creation causes a quick efflux of K+, a stoppage of fungal glycolysis, and a subsequent efflux of Mg2+. These losses result in the inside of the fungus becoming more acidic, which precipitates the cytoplasm and ultimately leads to cell death, along with a subsequent inflow of protons into the fungus.(10,11)

An analytical process called validation provides the procedure's performance component. The quality, consistency, and reliability of analytical data may be assessed using the findings from method validation.(12)

The estimate of different AmB formulations as liposomes, lipid formulations, and polymeric nanoparticles using UV-VIS spectrophotometry and HPLC technique has been discussed in a variety of publications, however specifics on peak characteristics and validation data were left out. The purpose of this work is to develop a brand-new, internationally accepted spectrophotometric technique for AmB measurement.(13)

II. EXPERIMENTAL WORK

A. *Materials and method*

The standard drug Amphotericin B was purchased from Cipla (Mumbai). Dimethyl sulfoxide (DMSO) and ethanol were purchased from Vishal chem. Eudragit E100 polymer was obtained from Evonik (India). Chitosan was purchased from Vishal Chem . All solvents and reagent used were of analytical grade.

B. *Method of preparation*

1) *Emulsification Solvent Evaporation Method*

a) Preparation of organic phase:- An organic phase consisting of polymer (Eudragit E100) and drug (Amphotericin B) dissolved in ethanol .

b) Preparation of aqueous phase:- An aqueous phase containing a surfactant (tween 80, concentration 3%) and distilled water

c) Addition of organic phase into an aqueous phase to form an O/W type emulsion. The volume ratio of organic and aqueous phases was 1 : 9. This emulsion is broken down into nanodroplets by applying external energy through a sonicator. These nanodroplets form nanoparticles upon evaporation of the highly volatile organic solvent. The organic solvent evaporates during magnetic stirring at 300 rpm under atmospheric condition for 2 h, then centrifuge the sample to obtain the formed polymeric nanoparticle.

2) *Formulation of Nanoparticle Incorporated into Gel*

The chitosan hydrogel (HC) was obtained by dissolving chitosan in distilled water and acetic acid and it was stirred with a mechanical stirrer for 10 minutes (at 1000 rpm). During the stirring period, glycerine, methylparaben, and propylparaben were added and allowed to disperse. After the mixing stage, the polymeric nanoparticles were added into hydrogel and mix it uniformly and allowed to stand for 24 hours to release the entrapped air. This procedure resulted in a thick, yellowish, cloudy gel, with the formulation.

C. *Instrument*

UV-VIS spectrophotometer (Shimadzo) was used for the development of an analytical method.

D. *Selection of Wavelength for Analysis of Amphotericin B*

In order to ascertain the wavelength of maximum absorption of amphotericin, the stock solution of 1000 µg/ml was prepared by taking 10 mg of the drug in 10 ml of DMSO. From this stock solution, the concentration of the drug (10µg/ml) in ethanol was prepared and scanned using spectrophotometer within the wavelength range of 200 to 800nm against ethanol as blank.

E. *Preparation of Standard Stock Solution*

1 gm of formulation containing 1 mg of amphotericin was dissolved in 100ml ethanol and this was used as a stock solution. From this standard stock solution different aliquots of concentration were prepared by suitable dilutions varying in between 5 and 30 µg/ml using ethanol. The absorbance of subsequent concentration was measured at 408nm. Using the data, the calibration curve for the drug (AmB) was prepared.

III. VALIDATION OF THE METHOD

The UV-VIS Spectrophotometric method was validated according to the International Conference on Harmonization (ICH) guidelines. The following characteristics were considered for validation: linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ).

1) *Linearity:* The linearity of an analytical procedure is its property of showing the rationality that the obtained absorbance is proportional to the concentration of a sample containing the analyte. The linearity was determined by taking five different concentrations ranging from 5 to 30µg/mL investigation was carried at 408nm. The calibration curves were established by plotting the concentration versus absorbance. The regression parameters of slope, intercept and correlation coefficient were calculated by fitted to the y = mx + c.(14)
2) *Precision:* The precision of the method was determined by repeatability (inter-day) and intermediate precision (intra-day) studies. Inter-day precision was determined by taking three different concentration (2µg/ml, 4µg/ml, 6µg/ml) for three days and the percent relative standard deviation (%RSD) were calculated. For intra-day precision, the same samples were analysed for three times within the day and the %RSD was also calculated.(15)
3) *Accuracy:* The accuracy of an analytical technique reveals the closeness of agreement between the values that are accepted either as a conventional true value and the value found. The accuracy tests were carried out in triplicate in three different concentrations (8 µg/ml, 10 µg/ml, 12 µg/ml) prepared from the stock solution and their strengths were estimated by the standard curve. The criteria for acceptability of the data included accuracy within ± 2% standard deviation (SD) from nominal values and precision of within ± 2% relative standard deviation (RSD).(16)
4) *Limit of detection (LOD*): The detection limit of an analytical method is defined as the lowest amount of analyte present in a sample that can be detected but not necessarily quantitated as an exact value. The limit of detection was performed from the standard curve

$$LOD = 3.3\ S/M$$

Where, S is the standard deviation of the absorbance of the sample and M is the Slope of the calibration curve.

5) *Limit of Quantification (LOQ):* Limit of Quantification (LOQ) can be defined as the lowest amount of analyte present in a sample that can be quantitatively determined with suitable precision and accuracy . Limit of quantification was based on the standard deviation of the response and the slope of the corresponding curve using the following equation:

$$LOD = 10\ S/M$$

Where S is the standard deviation of the absorbance of the sample and M is the Slope of the calibration curve.(17)

IV. RESULTS AND DISCUSSION

After scanning formulation amphotericin B with a concentration of 10 g/ml using a UV-VIS Spectrophotometer within the wavelength range of 200 to 800 nm against ethanol as a blank, compound exhibit their greatest absorption peak at 407.29 nm in the resultant spectra, as shown in figure 1, However, 408 nm was chosen in this investigation to distinguish amphotericin B.

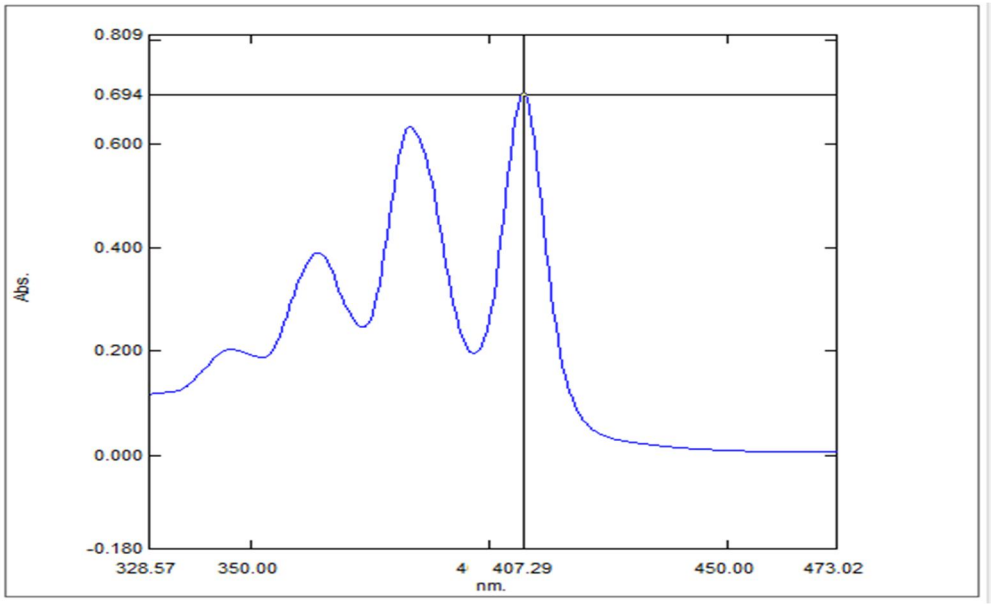

Figure 1:-UV- spectrum of formulation in ethanol to distinguish Amphotericin B

A. Linearity

Five points standardization curve was obtained in a concentration range from 5 and 30 µg/ml for AmB. The response of the drug was found to be linear within the investigation concentration range and the linear regression equation was y = 0.1471x + 0.0489 with correlation coefficient 0.9989. (Table 1, Figure 2).

Table 1:- Concentration vs absorbance table for linearity study of Amphotericin B in the hydrogel nanoparticle formulation

Concentration (µg/ml)	Absorbance (408 nm)
5ppm	0.198
10ppm	0.345
15ppm	0.482
20ppm	0.632
25ppm	0.799
30 ppm	0.924

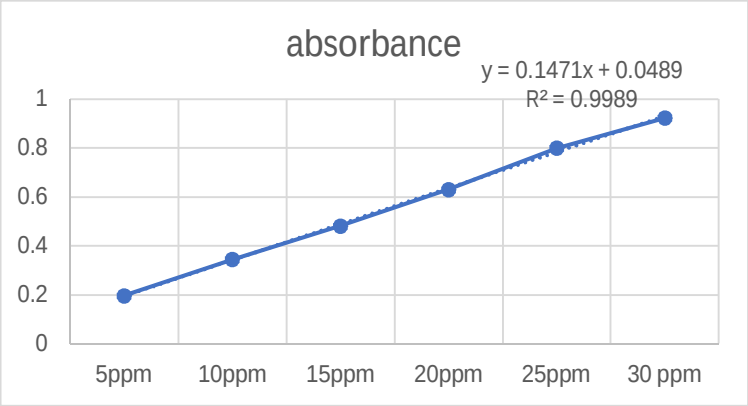

Figure 2:- Calibration curve of Amphotericin B at 408 nm

B. Precision

The repeatability (inter-day) and intermediate precision (intra-day) precision studies (Table 2 and Table 3) of the developed method confirmed that the method is precise and reliable where all the RSD values were <2%.

Table 2:- Inter-day precision

Concentration of formulation (µg/ml)	Absorbance (408 nm)			Mean ± S.D.	%Relative standard deviation (%RSD)
	1	2	3		
5	0.145	0.15	0.15	0.148333±0.002887	1.946124503
10	0.319	0.307	0.312	0.312667±0.006028	1.927840226
15	0.447	0.435	0.442	0.441333±0.006028	1.365796172

Table 3:- Intra-day Precision

Concentration of formulation (µg/ml)	Absorbance (408 nm)			Mean ± S.D.	%Relative standard deviation (%RSD)
	1	2	3		
5	0.165	0.171	0.168	0.168±0.003	1.785714286
10	0.308	0.31	0.315	0.311±0.003606	1.159341246
15	0.461	0.458	0.451	0.45666±0.005132	1.123708344

C. Accuracy

The percentage accuracy for the developed method for all the three concentration levels ranged from 91.144 % to 101.994 % (Table 4) showed that a small change in the concentration of the drug could be accurately determined with high accuracy.

Table 4:- Accuracy study of formulation

Concentration of Spiked formulation (µg/ml)	Spiked Absorbance (408 nm)	Mean ± S.D.	%RSD	% recovery
18	0.554		0.180505	91.144414
	0.555	0.554±0.001		
	0.553			
20	0.689		0.083795	101.9943
	0.688	0.689±0.000577		
	0.689			
22	0.728		0.079306	100.59122
	0.727	0.728±0.000577		
	0.728			

D. LOD and LOQ

The detection and quantitation limits as LOD and LOQ were calculated according to the formulae mentioned above. From the calculation, the LOD and LOQ values were found to be 0.0295 (µg/ml) and 0.0895 (µg/ml) respectively.

V. CONCLUSION

According to ICH recommendations, the UV-VIS spectroscopic technique for measuring amphotericin concentration has been verified, and it satisfies specified acceptance requirements for linearity, accuracy, intraday and interday precision, as well as detection limit and quantification limit. The suggested approach can be utilised for the standard quality control method for quantification of amphotericin B from hydrogel nanoparticle, since it is simple, reliable, and may be considered an economically feasible methodology.

A. Conflict of Interest

The author declares that they have no conflict of interest.

B. Acknowledgement

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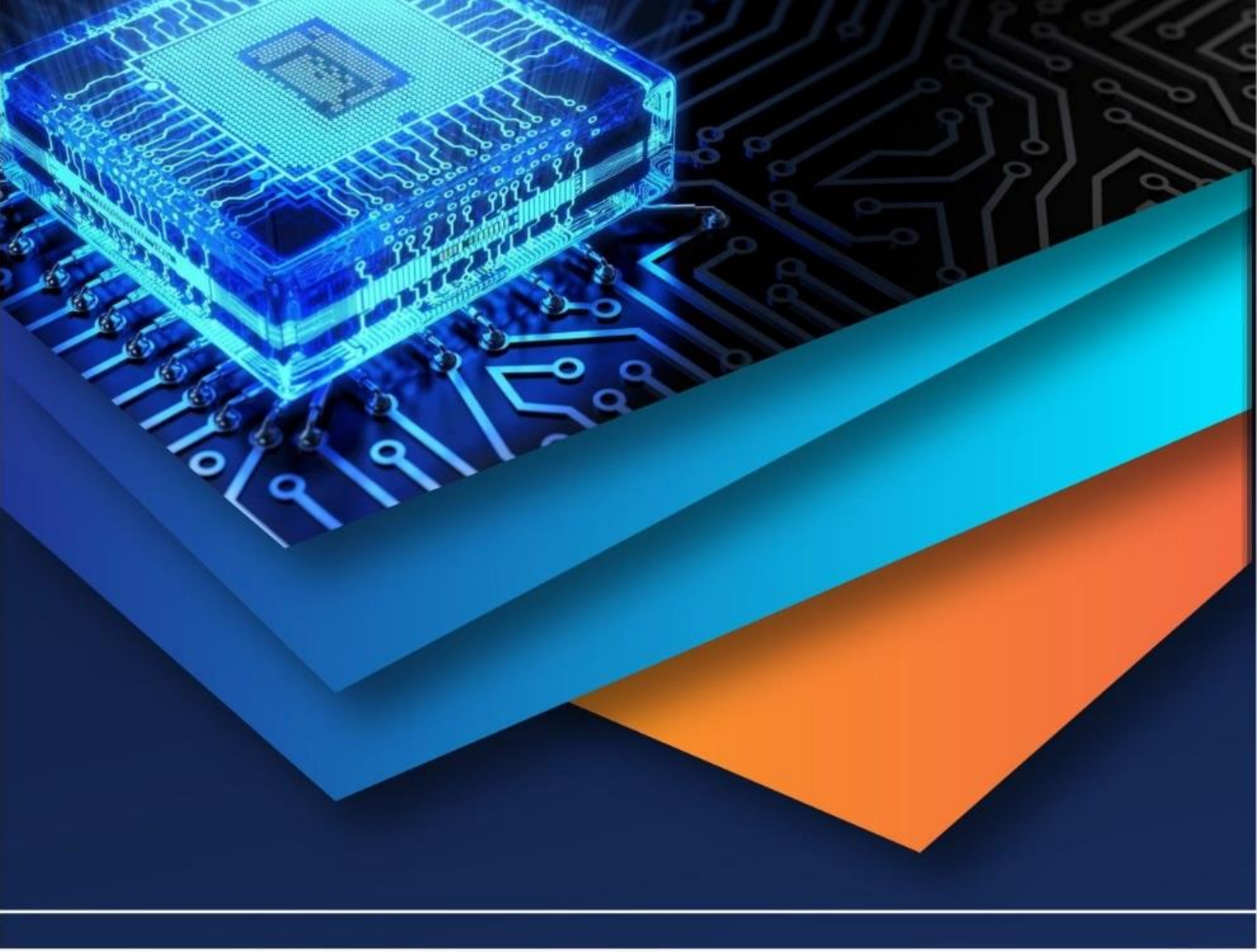

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