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DESIGN AND VALIDATION OF NOVEL UV-VISIBLE SPECTROSCOPIC METHOD FOR ASSAY OF AMPHOTERICIN B

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ABSTRACT: The objective of this research was to design and validate an alternative accurate method for quantitative determination of Amphotericin B. Amphotericin B deoxycholate life-saving drug belongs to the polyene class approved for the treatment of progressive and potentially fatal fungal infections. Amphotericin B was estimated at 382 nm and the percentage recovery was found to be 100.2 ± 0.34. The method was tested and validated for various parameters according to the ICH guidelines. 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 less than 2% for the routine analysis of drug. The limit of detection and limit of quantitation were 0.5µg/ml and 1.5µg/ml respectively. Results shows that the developed method is simple, reproducible and successfully for the estimation of Amphotericin B without the interference of common excipients.

INTRODUCTION: Amphotericin B deoxycholate belongs to the polyene class of antifungals. It was initially designed for the treatment of local mycotic infections and later approved for the treatment of progressive and potentially fatal fungal infections. It's a life-saving drug in the treatment of serious systemic fungal infections and is still the most widely used despite the development of a series of new antifungal agents, especially the second-generation triazoles and the echinocandins. Broad-spectrum antifungal activities and minor fungal resistance are the most important pharmacological characters that encourage continuous usage of Amphotericin B [1-5].

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Chemically, Amphotericin B molecular formula is $C_{47}H_{73}NO_{17}$ and structural formula is [1R-(1R*, 3S*, 5R*, 6R*, 9R*,11R*, 15S*, 16R*, 17R*, 18S*, 19E, 21E, 23E, 25E, 27E,29E, 31E, 33R*, 35S*, 36R*, 37S*)]-33-[(3-Amino-3, 6-dideoxy-β-D-mannopyranosyl) oxy]-1,3,5,6,9,11,17,37-octahydroxy - 15, 16, 18 – trimethyl – 13 - oxo-14, 39-dioxabicyclo[33.3.1] nonatriaconta-19, 21, 23, 25, 27, 29, 31-heptaene-36-carboxylic acid. It has high molecular weight and possesses both lipophilic (polyene hydrocarbon chain) and hydrophilic (polyhydroxyl chain) features [6-8].

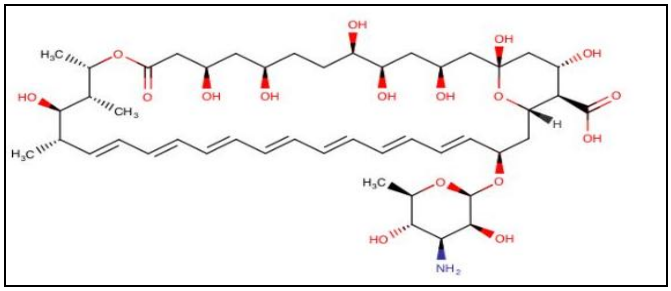

FIG. 1: CHEMICAL STRUCTURE OF AMPHOTERICIN B

Its amphoteric nature is responsible for low solubility in both aqueous and organic medium. Amphotericin B classified in the Biopharmaceutical Classification System (BCS) as a class IV compound with limited solubility and permeability properties. Therefore, the oral bioavailability of Amphotericin B is very poor (0.3%) (9-10). Ergosterol is the primary sterol in the cytoplasmic membrane of fungus, and the hydrophobic portion of the molecule binds to it. This link causes the plasma membrane to produce pores and channels which allow the extravasation of proteins and carbohydrates along with electrolytes like potassium, ammonium, and phosphate from the intracellular medium, ultimately leading to cell death [11–14]. Several analytical techniques like, High Performance Liquid Chromatography (HPLC), Reverse phase high performance liquid chromatography (RP-HPLC), Ultra Performance Liquid Chromatography (UPLC), Raman spectroscopy, LC-MS, capillary electrophoresis, thin layer chromatography and Spectroscopy have been reported for quantitative analysis of Amphotericin B but some of these methods are complexes, costlier and time consuming [15-16]. To overcome all these difficulties spectrophotometric analysis can be used as rapid, promising and reliable method for quantitative analysis of Amphotericin B. So, the aim of this study is to develop a new simple, rapid, reliable and precise UV spectrophotometric method for analysis of Amphotericin B.

EXPERIMENTAL:

Materials: Pure Amphotericin B drug was obtained as a gift sample from Ramsons Pharmaceutical private limited, Amritsar. All chemical and reagents used in this study were of analytical grade and the reagent solutions were prepared using double distilled water. A Shimadzu Corporation UV-1800spectrophotometer & electronic balance was used.

Methods

Determination of Maximum Absorption & Preparation of Calibration Graph of Amphotericin B: The stock solution was prepared by dissolving 20 mg of pure sample of Amphotericin B drug in 40 ml of phosphate buffer pH 6.8and then volume made up to 100 ml. The prepared solution was sonicated on bath sonicator to get a clear standard stock solution. The effect of dilution on absorption maxima was studied by diluting the above solution to 20µg/ml and scanned both the stock and diluted samples from 200-400 nm. Aliquots of different concentration of Amphotericin B solution were made up with phosphate buffer pH 6.8.

Validation of Analytical Method:

Accuracy: The accuracy of an analytical procedure expresses the closeness of reference value and the observed value. Accuracy was investigated by using different concentration of Amphotericin B in 3 replicates of each concentration by two different methods. In first method, accuracy was reported as percentage recovery by the assay of known amount of the drug with excipients. Accuracy was also determined by change the concentration of Amphotericin B. The respective absorbance of diluted samples was determined against phosphate buffer pH 6.8 as blank.

Precision: The precision of an analytical procedure expresses the degree of scatterness between a series of measurements obtained from multiple sampling of the same homogeneous sample and duplicate prepared samples under the prescribed conditions.

Repeatability: Repeatability expresses the precision under the same operating conditions. Repeatability was investigated by using different concentrations of Amphotericin B in 3 replicates each.

Intermediate Precision: Intermediate precision expresses within different days and different analyst. Amphotericin B was analysed in three independent replicates on the same day in morning and evening (intra-day accuracy and precision) and on three consecutive days (inter-day accuracy and precision). The relative standard deviations of intra-day and inter-day values were calculated. The Relative standard deviation of analysis of Amphotericin B by different analysts was also calculated.

Linearity and Range: The linearity of an analytical procedure is its ability (within a given range) to test results which are directly proportional to the concentration (amount) of analyte in the sample.

Aliquots of different concentration for Amphotericin B were prepared up to the highest concentration, till linearity was observed and absorbance was recorded. The range of an analytical procedure was the interval between the upper and lower concentration of Amphotericin B.

Robustness: The robustness of an analytical procedure is a measure of its capacity to remain unaffected by minute, but deliberate variations in method parameters and provides a proof of its reliability during normal usage. This was done by altering the concentration of sodium hydroxide (replaces the 0.1N sodium hydroxide by 0.01N sodium hydroxide).

Limit of Detection and Limit of Quantitation: The limit of detection (LOD) and Limit of quantitation (LOQ) were calculated according to ICH guidelines. The LOD and LOQ were separately determined based on standard deviation of response of the calibration curve

$$LOD = 3.3\sigma/s$$

$$LOQ = 10\sigma/s$$

Where σ represents the standard deviation of Y-intercept & s is the slope of calibration curve.

RESULT AND DISCUSSION:

Determination of Wavelength & Calibration Graph of Amphotericin B: The U.V scan the standard solution of Amphotericin B was done for the range 200-400 nm and absorption maximum was determined at 382nm as shown in **Fig. 2** and **Fig. 3.** The effect of dilution on absorption maxima was studied by diluting the above solution to 20µg/ml and observed parameter show no change in absorbance maxima on diluting the solution from 200µg/ml to 20µg/ml solution, which confirmed at 382 nm. Amphotericin B solution was found to follow Beer's law in concentration range of 0-18µg/ml. The correlation coefficient was found to be 0.999 and shown in **Fig. 4**.

FIG. 2: UV SCAN OF THE STANDARD SOLUTION OF AMPHOTERICIN B

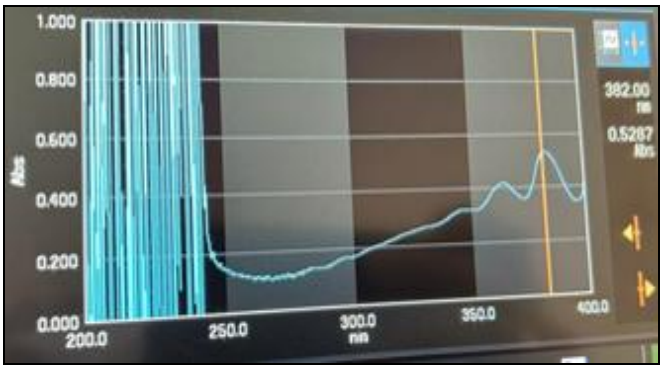

FIG. 3: UV SCAN OF THE DILUTED STANDARD SOLUTION OF AMPHOTERICIN B

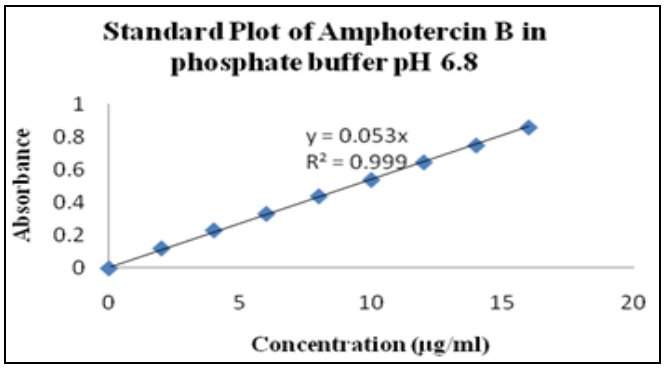

FIG. 4: CALIBRATION GRAPH OF AMPHOTERICIN

Result of the Validation of Analytical Method of Amphotericin B:

Accuracy: The mean percentage recovery values were close to the taken theoretical concentration and their %R.S.D. (0.33) values were within the acceptable range as per ICH guidelines. This indicates that the method has high accuracy. The validity and reliability of the proposed method was further accessed via recovery studies by the standard addition method. These results revealed

that any small change in the drug concentration in the solutions could be accurately determined by the proposed analytical methods. The evaluated data is summarized in **Table 1** and **2.**

TABLE 1: RESULT FOR ACCURACY OF AMPHOTERICIN B

Amphotericin B taken (µg/ml) with excipient	Amphotericin B recovered (%)*± S.D	%R.S.D
5	100.2±0.34	0.34

*Every reading is average of 3 samples.

TABLE 2: STANDARD ADDITION OF AMPHOTERICIN B FOR ACCURACY

Amphotericin B taken (µg/ml)	Amphotericin B recovered (%)*± S.D	%R.S.D
2.5	99.12±0.49	0.49
5	101.1±0.35	0.34
7.5	100.03±0.22	0.22

*Every reading is average of 3 samples.

Precision: Precision was investigated by studying the repeatability and intermediate precision. In repeatability results indicated the excellent precision under the same operating conditions. Intermediate precision express with in laboratory variation in inter- day, intra-day and different analysts. The % R.S.D. values for proposed analytical method were within the acceptable range as per ICH guidelines. The evaluated data is summarized in **Table 3, 4** and **5.**

TABLE 3: RESULTS FOR REPEATABILITY PRECISION OF AMPHOTERICIN B

Amount of drug taken (µg/ml)	Average amount of Amphotericin B found* (µg) ± S.D	%R.S.D
5	5.03±0.018	0.36
10	10.04±0.031	0.31
15	15.06±0.034	0.22
20	19.98±0.080	0.40

*Every reading is average of 3 samples.

TABLE 4: RESULTS FOR INTERMEDIATE PRECISION OF AMPHOTERICIN B FOR INTER AND INTRADAY STUDY

Amount of drug taken (µg/ml)	Average amount of drug found in Intraday studies* (µg/ml)		Average amount of drug found in Inter days studies* (µg/ml)			Precision (Intraday)		Precision (Inter day)	
5	Morning	Evening	1st day	2nd day	3rd day	S.D	% R.S.D	S.D	% R.S.D
	5.01	4.99	5.02	5.05	5.01	0.023	0.46	0.046	0.91

*Every reading is average of 3 samples.

TABLE 5: RESULTS FOR INTERMEDIATE PRECISION OF AMPHOTERICIN B BY DIFFERENT ANALYST

Type of analysis	Amount of drug taken (µg/ml)	Average amount of drug found*(µg/ml)	S.D	%R.S.D
Analyst 1	5	5.01	0.039	0.99
Analyst 2	5	4.99	0.045	0.90

*Every reading is average of 3 samples.

Linearity and Range: Amphotericin B aliquots were scanned for absorbance at wavelength 382nm. The linearity was observed in concentration range of 0-40µg/ml with R2 value 0.999 shown in **Fig. 5.**

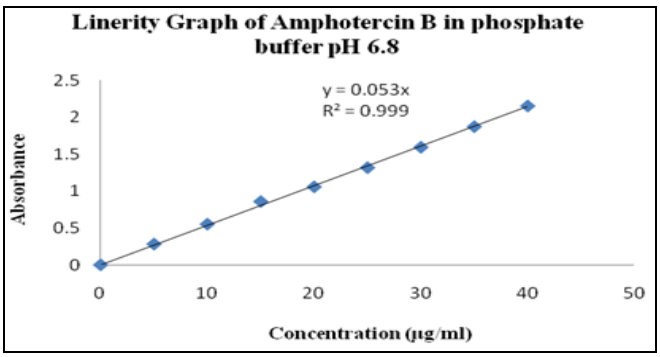

FIG. 5: LINEARITY GRAPH OF AMPHOTERICIN B

Robustness: The evaluation of robustness was performed by changing the concentration of sodium hydroxide (replacing 0.1N sodium hydroxide with 0.01N sodium hydroxide). This study found no significant variations by stated changes during the analysis of Amphotericin B. The data evaluated is summarized in **Table 6.**

TABLE 6: RESULTS FOR ROBUSTNESS OF AMPHOTERICIN B

Type of analysis		Amount of drug taken (µg/ml)	Average amount of drug found* (µg) ± S.D	%R.S.D
Concentration	Volume used (ml)			
By 0.1N sodium hydroxide	5	5	5.02±0.019	0.38
	10	5	4.97±0.017	0.34
	15	5	5.01±0.034	0.68
By 0.01N sodium hydroxide	10	5	4.98±0.042	0.84

Limit of Detection and Limit of Quantitation: The LOD & LOQ were 0.5 and 1.6µg/ml by using equations respectively.

CONCLUSION: All parameters of validation were found according to ICH guidelines i.e % R.S.D value is less than 2. So, this shows the analytical method is simple, sensitive and rapid and it can be conveniently employed for the routine analysis and the quality control of Amphotericin B.

Analysis of authentic samples containing Amphotericin B showed no interference from the common additives and excipients. Hence, recommended procedure is well suited for the assay and evaluation of drugs in pharmaceutical preparations.

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CONFLICTS OF INTEREST: Nil

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