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"Development and Validation of a UV Spectrophotometric Analytical Method for Amphotericin B"

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ABSTRACT
Amphotericin B Drug 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 Amphotericin B, and it is still in use today. The development of many innovative Amphotericin B formulations as lipid formulations and polymeric nanoparticles utilising UV-spectrophotometer technique has been discussed in the literature; however, specifics of peak characteristics and validation data are provided. The goal of this study wa7896 to provide an effective approach for validating Amphotericin B 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 = 0.0826x-0.0763(r^2=0.9996) at 384.8nm. Method accuracy showed % accuracy value for all the three concentration levels ranged from 98.37 % to 98.41% 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 9. 468(µg/ml) and 28.69(µg/ml) respectively. The suggested approach is straightforward and may be considered a strategy that is routinely practicable for estimating Amphotericin B.

Keywords: UV-Spectrophotometer, Amphotericin B, Method validation, Methanol, Phosphate buffer solution 7.5pH

1. Introduction
Amphotericin B first isolated from Streptomyces nodosus in 1955, is a polyene macrolide antibiotic providing antifungal activity against the widest spectrum of fungal infections such as Aspergillosis, Candidiasis, Blastomycosis, Coccidioidomycosis, Cryptococcosis and Histoplasmosis and also in certain protozoan infections such as Leishmaniasis. It has no major problems of resistance and remains the gold standard till date Amphotericin B has high molecular weight and possesses both hydrophobic (polyene hydrocarbon chain) and hydrophilic (polyhydroxyl chain) domains. This amphoteric nature is responsible for its poor solubility in both aqueous and organic solvents Amphotericin B falls in the Biopharmaceutical Classification System (BCS) as a class IV compound with limited solubility and permeability properties. Thus, the oral bioavailability of Amphotericin B is very low (0.3%). Amphotericin B is water soluble at a pH below 2 or above 11 and at this pH, the value is about 0.1 mg/ml. The molecule is not stable under such extreme conditions and forms salts which shows better solubility but have less activity than the basic compound. Amphotericin B binds to ergosterol, the principal steroid alcohol in fungal membranes, thereby disturbing membrane function to the point of outpouring of cellular contents. The ergosterol molecule of fungi features a cylindrical three-dimensional structure, in contrast to cholesterol, the major sterol in mammalian membranes that feature a sigmoid form. Binding to a sterol leaves the amphoteric Amphotericin B molecule with its hydrophilic edge unbalanced relative to the larger hydrophobic portion of the complex creating areas of local tension inside the membrane. Thus, Amphotericin B acts its mechanism on a broad spectrum of fungal species[1] Analytical chemistry is a scientific field focused on determining the chemical composition of samples. Its primary concern lies in both qualitative and quantitative analysis of materials. Qualitative methods provide information about the identity of atomic or molecular species and functional groups present in the sample, while quantitative methods yield numerical data concerning the relative amounts of these components. This discipline plays a crucial role in studying the chemical composition, structure, and behaviour of matter. Chemical analysis, which involves applying various processes to identify or quantify substances, components of solutions or mixtures, and the structures of chemical compounds, serves as a core aspect of analytical chemistry. The purpose of chemical analysis is to gather and interpret valuable chemical information relevant to a wide range of societal contexts. Analytical chemistry plays a vital role in the entire lifecycle of drug development, spanning from discovery to safety, formulation, quality control, packaging, storage, marketing, and more. Ensuring the excellent quality and purity of drugs or dosage forms intended for

human use is essential, as they directly impact human life and behaviour. Thus, the thorough analysis of such drugs is carried out using analytical methods. The development and validation of new analytical methods for sample testing form the heart of analytical chemistry. For a long time, analytical chemistry has played a significant role in the advancement of science and technology. Its scope is vast, encompassing a wide array of natural, chemical, and instrumental techniques and procedures.[2] Validation is an analytical procedure providing the performance aspect of the procedure. The results from method validation can be used to determine the quality, reliability and consistency of analytical results. Various literature has given insight on the estimation of several formulations of Amphotericin B as a liposome, lipid formulations and polymeric nanoparticles using UV-VIS spectrophotometry, but details on peak characteristics and validation data were not described. Moreover, the literature survey revealed that no such method validation had been developed in UV VIS spectrophotometry in the pure form of Amphotericin B. This study aims to establish a new and standard spectrophotometric method for the estimation Amphotericin B. [1]

2. Materials and Methods

2.1. Reagents and Chemicals: -

Amphotericin B (5mg), API was obtained from Bharat serum and vaccine limited Ambernath, and Methanol (25ml), was from S d fine-chem. limited Mumbai. UV Spectrophotometer (Shimadzu, UV 1800 Software: UV Probe 2.51) was used for the development of an analytical method.

2.2. Selection of wavelength for analysis of Amphotericin B: -

In order to ascertain the wavelength of maximum absorption of Amphotericin B, 5:5 methanol and buffer solution as a blank and amphotericin standard solution (50 PPM) was scanned from 400 nm to 200 nm. Absorption maxima were determined for drug. Amphotericin B showed maximum absorbance at 384.8nm shown in results.

2.3. Preparation of standard stock solution: -

In order to prepare stock solution, weighed accurately 5mg amphotericin B and transferred into 10 ml volumetric flask, added 5ml of methanol, 3ml buffer solution and sonicated to dissolve the standard completely and diluted up to the mark with buffer (100 PPM). Further diluted 0.5 mL to 50 mL with buffer (50 PPM).

3. Validation of the Method

The process of validating an analytical method aims to confirm that the developed method's performance characteristics meet the standard requirements for its intended analytical application. The UV-VIS Spectrophotometric method was validated according to the International Conference on Harmonization (ICH) guidelines (2005). The following characteristics were considered for validation: Linearity, Accuracy, Precision, Limit of detection & Limit of quantitation.

3.1. 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 study involved creating standard solutions of with concentrations ranging from 5µg/ml to 50µg/ml. A calibration graph was then constructed for each concentration of amphotericin. The linearity was determined by taking 10 different concentrations (5ug/ml, 10 ug/ml, 15ug/ml, 20ug/ml, 25ug/ml, 30ug/ml, 35ug/ml, 40ug/ml, 45ug/ml, 50ug/ml). The linearity was determined at conc. Range of 5µg/ml to 50µg/ml and R2 obtained at 0.9996.

3.2. 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 of developed method was established in three replicates at three different level of concentration (80ug/ml, 100ug/ml, 120ug/ml). The % recovery was ranged from 98-102% indicating the accuracy of method.

3.3. Precision: -

Precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous test under the prescribed conditions. Inter-day precision was determined by taking three different concentrations (10ug/ml, 15ug/ml, 20ug/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.

3.4. 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 quantities 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.

3.5. Limit of quantitation (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.

4. Results and Discussion

After scanning amphotericin B with a concentration of 50µg/ml using UV-VIS Spectrophotometer within the wavelength range of 200 to 800nm against 5:5 methanol and buffer solution as a blank show maximum absorption peak at 384.8nm in the resulting spectra as shown in figure. However, in this study, 384.8nm was selected to identify amphotericin B.

4.1. Linearity: -

Ten points standardization curve was obtained in a concentration range from 5ug/ml to 50ug/ml. The response of the drug was found to be linear within the investigation concentration range and the linear regression equation was y = 0.0826x - 0.0763 with correlation coefficient 0.9996.

Table 1: Concentration vs absorbance table for linearity study of Amphotericin B

Concentration(µg/ml)	Absorbance(nm)
5	0.002
10	0.093
15	0.173
20	0.255
25	0.342
30	0.415
35	0.502
40	0.581
45	0.661
50	0.758

4.2 Accuracy: -

The percentage accuracy for the developed method for all the three concentration levels ranged from 80% to 120% with standard deviation ranging from ± 0.0172 to ± 0.0172 (Table2) showed that a small change in the concentration of the drug could be accurately determined with high accuracy.

4.3 Precision: -

The repeatability (inter-day) and intermediate precision (intra-day) precision studies (Table 3 and Table 4) of the developed method confirmed that the method is precise and reliable

4.4 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 9.468 (µg/ml) and 28.69 (µg/ml) respectively (Table 5).

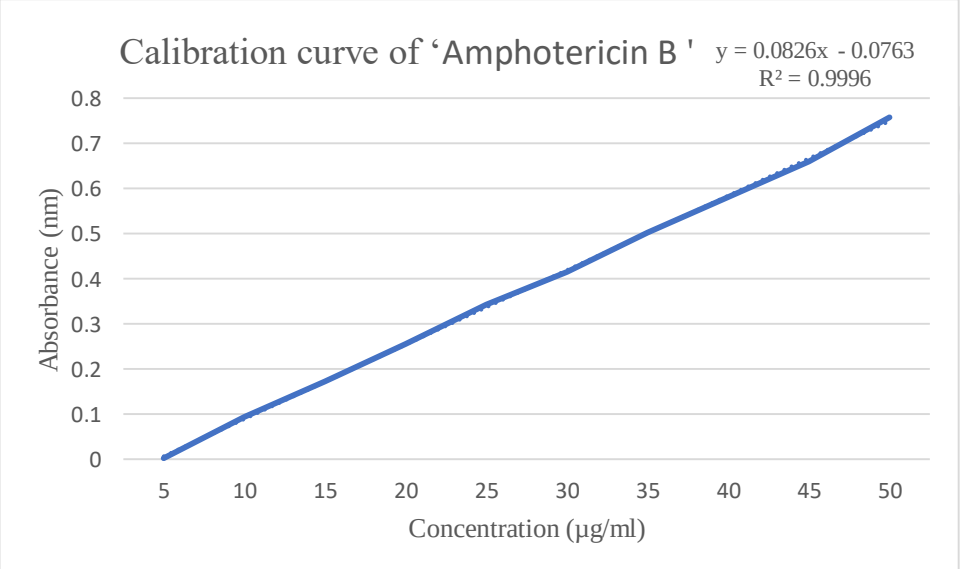

Figure1: Calibration curve of Amphotericin B at 384.8n

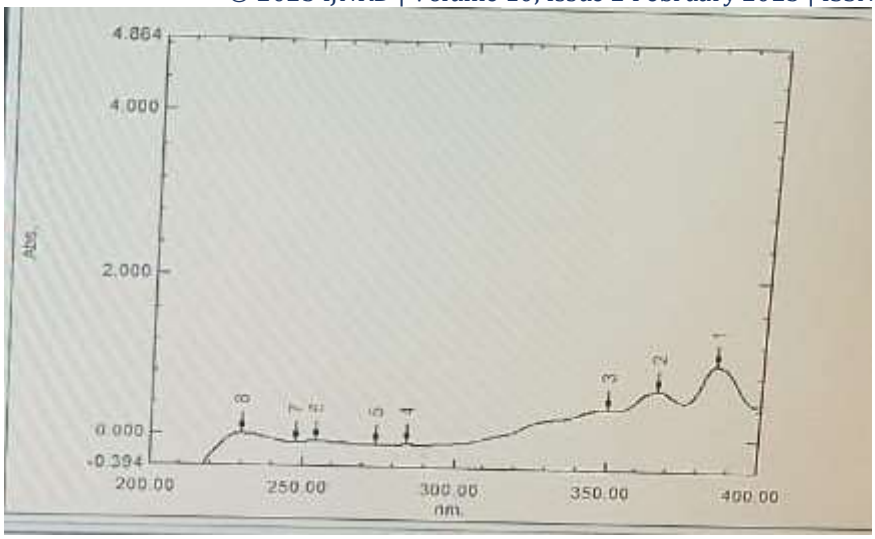

Figure2: UV-spectrum of Amphotericin B in 5:5 methanol and buffer solution

Table 2: Accuracy: -

CV	Absorbance	Recovery concentration	Added concentration	% Recovery	% RSD
80%	0.347	15.74	16	98.37	0.0172
100%	0.434	19.68	20	98.40	0.0172
120%	0.521	23.62	24	98.41	0.0172

Table 3: Intra-day precision:

Precision	**Mean**	**SD**	**%RSD**
Day1	7.0611	0.0116	1.6
Day 2	7.0415	0.0212	0.3

Table 4: Inter-day precision:

Precision	**Mean**	**SD**	**%RSD**
Morning	7.001	0.0095	0.13
Afternoon	6.999	0.0078	0.11
Evening	7.030	0.0342	0.48

Table 5: LOD and LOQ:

LOD	9.468
LOQ	28.69

5. CONCLUSION:

The UV-VIS spectroscopy method for determination of amphotericin concentration was validated as per ICH guidelines and it meets to specific acceptance criteria in respect of linearity, accuracy, intraday and inter-day precision as well as detection limit, quantification limit. The proposed method is simple, reliable and could be regarded as economically viable techniques and can be used for the routine quality control method for Amphotericin B.

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