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A Review on Analytical Methods for Quantification of Amphotericin-B in Marketed Dosage Form and Biological Samples

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Review Article

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ABSTRACT

Background: Amphotericin-B is an antifungal antibiotic indicated to treat infections caused by leishmaniasis. After COVID-19 outbreak amphotericin-B was preferably used to treat black fungus caused by mucormycosis in people recovering from COVID-19.

Main text: To test purity, identity and quantity of amphotericin-B in dosage forms and biological samples always needs different analytical methods such as spectroscopic, chromatographic, electro analytical methods and micro biological assays. This review depicts several published analytical methods along with instrumental conditions for the detection and quantification of amphotericin-B during the last 20 years.

Conclusion: From literature reviews, HPLC with UV detection is majorly used for quantification of amphotericin-B in pharmaceutical formulations and LC-MS for biological samples.

Keywords: Amphotericin-B; Spectroscopic method; Micro biological assay; Chromatographic method; Pharmaceutical formulations

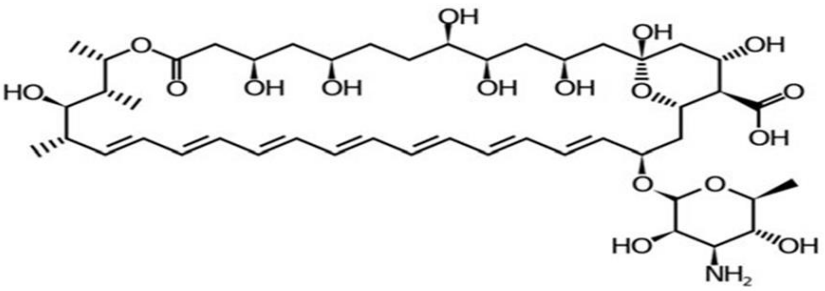
ABBREVIATIONS

HPLC: High Performance Liquid Chromatography; LC-MS: Liquid Chromatography-Mass Spectrometry; UPLC: Ultra Performance Liquid Chromatography; DMSO: Dimethylsulfoxide; LOD: Limit of Detection; LOQ: Limit of Quantification; UV: Ultraviolet; FAC: Fluorescence Activated Cell; PDA: Photodiode Array; THF: Tetra Hydro Furan; MRM: Multiple Reaction Monitoring; API ESI: Atmospheric Pressure Ionization Electron Spray Ionization; NMR: Nuclear Magnetic Resonance

INTRODUCTION

Amphotericin-B is the gold standard for treating infections and disease which is caused by lei shmaniasis. It's an anti-fungal antibiotic with alternate double bonds obtained by *streptomyces nodosus* developed as the first antifungal agent used in the treatment of various fungal injections in 1958. Chemically it is (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. Its molecular weight is 24.1 corresponding to the molecular formula of C_{47} $H_{73}NO_{17}$ and melts at 170°c. Amphotericin-B is insoluble in anhydrous alcohol, ether, benzene, toluene, slightly soluble in methanol. It is poorly soluble in water and toxicity is very high due to its hemolytic activity (Figure 1) [1].

Figure 1. Chemical structure of amphotericinB.



Amphotericin-B works by leak aging monovalent ions (Na^+, K^+, H^+, Cl^+) from the cell by binding to sterol component intracellularly and by bringing changes in the cell membrane. It also causes cytotoxicity by tangling with cholesterol. In order to make amphotericin-B penetrable to the cell wall, it is formulated as liposomes [2].

Amphotericin-B is a rare drug that has been used in the treatment of black fungus or mucormycosis. So these mucormycosis occurs primarily in patients with COVID-19 because of the use of steroids as a medication to suppress the highly immune system, in order to help COVID-19 patients caused by a known mechanism like "cytokine storm" [3-6].

Amphotericin-B is a macrocyclic ring with one side having conjugated double bond and other with OH group. Diverse techniques like High Performance Liquid Chromatography (HPLC), Ultra Performance Liquid Chromatography (UPLC), Raman spectroscopy, LC-MS, capillary electrophoresis, thin layer chromatography were used to analyze different formulations. These techniques offer a sensible and quick estimation of amphotericin -B in bulk drug, pharmaceutical formulation and biological samples [7].

LITERATURE REVIEW

Analytical methods

Spectrophotometric method: A spectrophotometric technique is used for determination of amphotericin-B in bulk and marketed formulation using Dimethylsulfoxide (DMSO) as analytical reagent for determination of amphotericin-B. In this article, the standard curve was proven to be linear at the concentration range of 2-20 µg/ml (r=0.9975) with LOD of 0.0570 μgml^{-1} and LOQ of 0.1574 μgml^{-1}. The absorbance measurement of amphotericin-B is at 415 nm. The same approach is applied to fungitericin intravenous 50 mg injection with accuracy (% recovery) of 99.69.

In alternative article they described a UV-visible spectrophotometric method for estimation of amphotericin-B in both as API and marketed dosage form. These are two primary approaches for the estimation of amphotericin-B in pharmaceuticals. The first approach is based on the use of P-Dimethyl Amino Cinnamaldehyde (PDAC) as internal standard under acidic conditions due to the presence of condensation reaction while in second approach; it is relies on the oxidative coupling reaction with DCQC (2,6-dichloroquinone N-chlorimide). The calibration curve was linear within a range of 0.5-2.5 μgml^{-1} amphotericin-B (r^2=0.9999). Beer's law was obtained over a range of 10-60 μgml^{-1} and 20-120 μgml^{-1} for first approach and second approach at a molar absorbance of 9.495 X 10^3 and 5.337 X 10^3 L $mole^{-1}$ cm^{-1}. Mariuszgagos reported a spectrophotometric analysis of dipalmitoylphosphatidylcholine bilayer containing the polyene antibiotic amphotericin-B. The primary focus of these analysis is the impact of amphotericin-b lipid layer formed with Dipalmitoyl Phosphatidyl Choline (DPPC) as well as the effect of the lipid phase on the molecular organization of amphotericin-B were investigated by using UV-VISIBLE spectrophotometry [8].

Another UV-Visible spectrophotometric technique was described for quantification of amphotericin-B in injections, lipid formulation and polymeric nanoparticles. The analytical method was performed on UV-Visible spectrometer (Thermo Fischer evolution121). UV-Visible spectrophotometric method is an easy and accurate method for assessing amphotericin-B in different novel formulation such as lipid formulation and polymeric nanoparticles. The % recovery of these method was found to be 103.5 to 106.5% for amphotericin-B (Table 1) [9].

Table 1. Spectrophotometric methods for determination of amphotericin-B.

Name of the drug	Method	Wavelength (nm)	Linearity/R^2	LOD/LOQ and Recovery
Amphotericin -B	Simple and sensitive spectrophotometric method	415	2-20 µg/ml (r=0.9975)	LOD was 0.0570 µg/ml and LOQ was 0.1574 µg/ml.; recovery 99.69%
Amphotericin -B	UV-visible spectrophotometric method	Metho-A:490 Metho-B:530	Method-A:10-60 µg/ml Metod-B:20-120 µg/ml (r^2=0.9999)	Recovery for method-A: 99.7% and method-B :99.6%
Amphotericin -B	UV-visible spectrophotometric method	408	0.312-5 µg/ml	LOD was 0.391 and LOQ was 1.187; recovery-103.5-106.5%

Flow cytofluorometric method

The flow cytofluorometry is a rapid method for testing the ampotericin-B of yeast isolates. It is a technique that uses

specific fluorochromes to quickly and thoroughly analyse a large number of cells which is suspended in a given liquid. In this article, there was a sample run on a FAC sort cytometer which has a 15-MW, 488 nm argon ion laser. The instrument parameters are forward light scatter and sideward light scatter. First, sample from each isolate's untreated control were taken. A gate containing cell clusters was removed from a cytogram made by graphing forward light scatter versus sideward light scatter. For each sample 5,000 cells were examined and results were captured as fluorescence histograms. The Becton Dickinson software was used to calculate the geometric mean of fluorescence for the specific population [10-15].

Raman spectroscopy

Resonant Raman spectroscopy uses laser vibration to produce chemical signature of the components of aqueous humor that are incredibly precise. The main motto of this study is to examine the potential for non-invasively monitoring amphotericin-B levels in the eye's aqueous humor. To test whether amphotericin-B at therapeutic quantities may be evaluated with resonant Raman spectroscopy *in vitro*, we should employ a model anterior chamber. A kryptoion laser source at 406.7 nm was used as excitatory which is used to connect to Raman microscope and Raman microscope from Kaiser optical systems was used to gather the Raman spectra. Subsequently, we should gather the spectral data from a rabbit that had been given amphotericin-B intravenously for 5 days to determine whether the spectral peaks in anterior chamber should be *in vivo*. Raman spectra of amphotericin-B obtained a peak at 1557 cm^{-1}.

Chromatographic method

An easy and accurate Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) technique was developed for detection of amphotericin-B in bulk and pharmaceutical dosage form. These technique was carried out on Luna C_{18} column (250 × 4.6 mm; 5 µ) with a mobile phase consisting of acetonitrile: Tetrahydrofuran: o-phosphoric acid (60:30:10 V/V/V) at a specific flow rate 1.0 ml min^{-1}. Liquid chromatographic separation was carried out on a shimadzu HPLC system equipped with a LC-20 AD pump, rheodyne model-7161 injector with 50 µl loop volume. Detection is carried out at 287 nm. The retention time of amphotericin-B is 10 mins. The evolved technique has been validated in terms of linearity, precision, accuracy, stability and system suitability parameters.

A High Performance Liquid Chromatography Equipped with Ultraviolet Detection (HPLC-UV) technique for estimation of amphotericin-B in bulk and dosage form. Separation was performed on the spheri-5, cyano column (30 × 4.6 mm, × 5 µm) and acetonitrile: sodium acetate buffer (72:28, v/v with pH-4) is used as a mobile phase. The analysis is done by a ultra violet detector at 408 nm and the flow rate was kept at 1.2 ml/min. The retention time for amphotericin-B was 5.9 mins. Linearity was observed in the concentration range of 0.039 to 40 µg/ml for amphotericin-B. Amphotericin B has been exposed to the stress conditions such as oxidation, acid and base hydrolysis and thermolysis.

In another study reported that the RP-HPLC PDA method where the separation was performed on a C_{18} with Photodiode Array (PDA) detector at 408 nm at a specific flow rate of 1.0 ml/min in isocratic elution using a acetic acid: Acetonitrile (40:60 v/v) as the mobile phase. The retention time of amphotericin-B for this specific method was less than 4 mins. These technique produce a linear response with a regression coefficient of 0.9998 in the concentration range of 1-20 µg/ ml [16].

Another High Performance Liquid Chromatography (HPLC) technique has been employed to determine amphotericin-B

in nose to brain nanoliposomes. The method was performed on zorba x C_{18} (250 mm × 4.6 mm, 5 µm) column by using binary mixture mixture of ultra purified water and organic modifiers of acetonitrile: methanol: THF (75:17:8 v/v/v) as mobile phase. The sample was detected at 383 nm with a flow rate of 1.0 ml min^{-1} for the mobile phase. The calibration curve shows linear for the concentration of 0.53 to 7.09 µg/ml. It is a simple, quick and selective HPLC technique of quantifying amphotericin-B and it can be used for analyze of large number of samples to check the quality of drugs. The method was validated and all the results are satisfactory. Marciela B Montenegro reported an HPLC-DAD method for development and validation of amphotericin-B. The drugs were separated based on the thermo BDS hypersil C_{18} (200 mm × 4.6 mm;5 µm) column and mobile phase consisting of organic phase (methanol: Acetonitrile in 41:18) and aqueous phase (2.5 mmol L^{-1} of disodium edentate, pH 5.0) using isocratic elution at a flow rate 1.0 ml/min and uv detection performed at 383 nm. These technique should be linear over a range of 0.05-0.12 and % recovery should be 98.08-99.96%. Determination of amphotericin–B in nano scale bilayer membrane with spectroscopic method was done by peter L. Hargreaves. In this paper, the nano disk particles were used as a distinct bilayer membrane environment in which capable of solubilizingamphotericin-B, allowing research into antibiotic aggregation [17].

Amphotericin-B in human plasma was analyzed through the High Performance Liquid Chromatography (HPLC) technique and the drug is extracted from plasma by using methanol. The column used for separation was X Bridge C_{18} (150 × 4.6 mm) reversed phase column. The separation was achieved by using acetic acid (0.73%): Acetonitrile (60:40 v/v). The column temperature need to be kept at 30ºC and the flow rate was set at 1.0 ml/min. For preparation of amphotericin-B stock solution, HPLC grade methanol is used. The sample were prepared and extracted by spiking the plasma with the help of methanol (680 µl). So these samples were centrifuged at 2000 g for 5 mins and the supernatant was introduced into the column. The outcome indicated that the technique was linear for the concentration of 50-1000 $ngml^{-1}$ (r^2=0.9999). The recovery of this method was reported to be 88% for amphotericin-B (Table 2).

Table 2. Determination of amphotericn-B using High Performance Liquid Chromatography (HPLC).

Study	Mobile phase	Column	Flow rate (ml/ min)	Injection volume (µl)	Retention time (mins)	Wave length (nm)
Validated RP-HPLC method for the estimation of amphotericin-B in bulk and pharmaceutical dosage form	Acetonitrile/tetrahydrofuran/o-phosphoric acid (60:30:10 v/v/v)	C_{18}	1	20	7.72	287
Development and validation of stability indicating HPLC-UV method for the determination of amphotericin-B in bulk and dosage form	Acetonitrile: Sodium acetate buffer (28:72 v/v)	Cyano column	1.2	35	5.9	408
Determination of amphotericin B in PLA-PEG blend nanoparticles by HPLC-PDA	Acetic acid: Acetonitrile (40:60 v/v)	C_{18} reverse phase	1	20	-	408
Development and validation of a fast and selective HPLC method for the determination of amphotericin B in nose to brain nanoliposome	Binary (A) acetonitrile/methanol (B) THF/acetonitrile (1:1 v/v)	C_{18}	1	10	6.8	383
Methodology development and validation of amphotericin b stability by HPLC-DAD	Methanol: acetonitrile (41:18 v/v)	C_{18}	1	20	-	383

Rapid quantitative evaluation of amphotericin B in human plasma by validated HPLC method	Acetic acid/acetonitrile (60:40 v/v)	C_{18}	1	20	2.7	408
Capsule phase micro extraction combined with chemo metrics for the HPLC determination of amphotericin B in human serum	Citric acid: methanol (60:40 v/v)	C_{18}	0.6	10	5.5	380
Simultaneous determination of purity and potency of amphotericin B by HPLC	Methanol/acetonitrile /tetrahydrofuran (41:18:10)	C_{18}	1	20	-	383
Validated RP-HPLC method for the estimation of amphotericin B in bulk and pharmaceutical dosage form	Acetonitrile/tetrahydrofuran/o-phosphoric acid (60:30:10 v/v/v)	C_{18}	1	20	7.7	287
Fabric phase sorptive extraction combined with HPLC-UV for the quantitation of amphotericin B in human urine	Citric acid/methanol (60:40 v/v)	C_{18}	0.6	10	-	380
Purity determination of amphotericinB, colistin sulfate and tobrmmycin sulfate in a hydrophilic suspension by HPLC	acetonitrile/methanol /citric acid (30:10:60 v/v/v)	C_{18}	0.8	-	-	408

Microbiological assay method

An agar diffusion bioassay for amphotericin-B in nasal spray solution has been published by using a media of *Candida albicans* and *Saccharomyces cerevisiae* as the test organism. The log curve for the dose-response relationship is assumed to be linear within the concentration range of 1.54-60.0 μgm^{-1} for bioassay approach. An Amphotericin B level in serum was measured by using high performance liquid chromatography. These technique was linear within a concentration range of 0.05-2.0 μgml^{-1} (Table 3).

Table 3. Characteristic parameters of microbiological assay method.

S.No	Technique/test organism	Concentration range(µg/ml)	LOD (µg/ml)	LOQ (µg/ml)	Application
1	*S.Cerevisiae*	1.54-60	1.54	15	Nasal spray solution
2	Paecilomycesvarioti	0.05-2.0	-	-	Serum
3	Diffusion plate bioassay/Paecilomycesvarioti	0.01-0.02	0.01	-	-

Liquid chromatography-mass spectrometry method

It is a rapid and sensitive Liquid Chromatography-Mass Spectrometry (LC-MS) technique for estimation of amphotericin-B in rabbit tears by using negative mode of ionization. The natamycin is used as internal standard. Both the drugs (amphotericin-B and natamycin) have been extracted from the rabbit tear sample by utilizing a solid phase extraction technique. These technique was performed by using a phenomenex Luna 3 µm CN column (100 × 2 mm, 3 µm) and a mobile phase comprised of 3.5 mM ammonium acetate (ph 4): Methanol (10:90 v/v) flowing at a rate of 0.3 ml/min. A detection was carried out on the API ESI source and Multiple Reaction Monitoring (MRM) mode using the transitions m/z 922.7→183.3 m/z for amphotericin-B and 664.4→137.2 m/z for the internal standard. The results showed that the technique was linear within a concentration range of 0.1-3.2 μgml^{-1}. Lastly, the technique has been successfully used in the study of ocular pharmacokinetic study of amphotericin-B in the NZ rabbit. Ignace C. Rose boom developed and validated High Performance Liquid Chromatography-Tandem Mass Spectrometry (HPLC-MS/MS) for estimation of antiparastic and antifungal drug amphotericin-B in human skin tissue. In this technique, natamycin employed as an internal standard and the chromatographic parameters are Gemini C_{18} analytical column (50 × 2.0 mm, 5 µm particles). The mobile phase comprised of a 0.1% formic acid blend in water at a flow rate of 0.4 ml/min.

The detector used in this method was tandem mass spectrometry [18].

Ultra performance liquid chromatography

An Ultra Performance Liquid Chromatography (UPLC) methodology was developed and validated for measurement of amphotericin-B in plasma after the adminstration of liposomal amphotericin-B. Column used for separation was an acquity uplc BEH C_{18} column (2.1 × 50 mm, 17 µm) and the detector was a Photodiode Array (PDA) detector. The separation was accomplished by using mixture of acetonitrile: 0.05 M ammonium acetate aqueous buffer (pH 5.8) with acetonitrile (90:10 v/v) as a mobile phase. The flow rate was 0.5 ml/min. This method has a short run time (5 mins) and low injection volume (3 µl). The method is used to create an analytical method for determining the concentration of non-liposomal and liposomal fractions after LAMPHOTERICIN-B administration. So this technique is linearly within a concentration range of 0.5-50.0 $mgml^{-1}$ and accuracy ranges from 97.6% to 112.1%. Ultra Performance Liquid Chromatography Tandem Mass Spectrometer (UPLC-MS/MS) technique has been developed to monitor amphotericin-B in Saudi ICU patients. In order to measure the concentration of Amphotericin-B, a waters aquity UPLC MS/MS system, BEH shield RP18 column and positive mode electro spray ionization source are utilized. The evolved Ultra Performance Liquid Chromatography (UPLC) technique within a concentration range of 200-4000 $ngml^{-1}$.

RESULTS AND DISCUSSION

Thin layer chromatography

A TLC method has been described for determination of amphotericin-B by using particular direct bioautography. Separation was carried out with silica gel in stationary phase and chloroform: Methanol: Borate buffer (4:5:1 v/v) as mobile phase. Densitometry evaluation was performed with a CAMAS TLC scanner-2 at 385 nm. The drug was obtained linearly within a concentration range of 250-500 mg. The direct bioautography method is very sensitive technique for estimation of amphotericin-B. In another approach demonstrated that it is a simple and quick technique for determining the untrapped drug in the liposomal formulation by thin layer chromatography. In this study, it determines the three drugs for liposomal formulation doxorubicin, amphotericin-B, curcumin. Compared to these drugs the amphotericin–B is very less sensitive. The assay was done by densitometer evaluation at 254 nm (Table 4) [19].

Table 4. Characteristics features of LC-MS, UPLC and TLC.

S.No	Mobile phase	Detection (nm)	Concentration range	LOD	LOQ
1	3.5 mM ammonium acetate (pH4)/methanol (10:90 v/v)	-	0.1-3.2 µg/ml	-	0.1
2	Chloroform: Methanol: Borate buffer (4:5:1 v/v)	385	250-500 ng/ml	0.8 ng	-

Capillary electrophoresis

Capillary electrophoresis technique evolved for the detection of amphotericin-B in human blood serum by combining with preparative isoelectric focusing. The technique was linear throughout a concentration range of 0.3 to 600 $ngml^{-1}$ and a recovery rate was expected to be 93-98%. In another article, it describes a micellar electro kinetic capillary electrophoresis involving diode array detector for determination of amphotericin-B after filtration through polyvinylidenedifluoride and polyethersulfone. In these method, the drug is linear and exhibit high correlation factor (r^2=0.994) (Table 5).

Table 5. Characteristics of capillary electrophoresis.

S.No	Sample	Instrumental parameters	Detector	Linearity range	%Recovery
1	Human blood serum	-	uv-detector	0.3-600 ng/ml	93-98
2	Pharmaceutical solutions	Protein analysis buffer, temp-20°c	DAD detector; 375 and 450 nm	-	-

Electrochemical method

Cyclic voltammeter was used to examine the phosphatidylcholine bilayer model membranes that included ergo sterol. So that the Amphotericin-B is crucial for the formation of ion channels in both sterol free and cholesterol containing phosphatidylcholine Bilayer [20].

Atomic absorption spectroscopy

In this study, laser microspectrofluorimetry and nuclear magnetic spectroscopy 31p were used to measure the intracellular pH of *Candida albicans* as yeast. This technique is used to test the impact of Amphotericin-B on intracellular pH has been studied on cell sap. Atomic absorption was used to measure the changes in internal k^+ concentrations concurrently with 31p NMR studies to observe H^+ & K movements. Earlier measurements of K leakage has been made with lower concentration and could not have been as accurate.

CONCLUSION

In this review, the analytical method has been described for amphotericin-B showed that spectrophotometric, Raman spectroscopy; microbiological assay, chromatography, atomic absorption and capillary electrophoresis have been adapted to the determination of amphotericin-B in pharmaceuticals and biological samples. Therefore HPLC with UV detection is widely used for pharmaceutical formulation whereas, LC with tandem mass spectrometric detector mainly employed for biological samples.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable for this section.

CONSENT FOR PUBLICATION

Not applicable for this section.

AVAILABILITY OF DATA AND MATERIAL

All data will be available upon request.

COMPETING INTERESTS

“The authors declare that they have no competing interests" in this section.

AUTHORS' CONTRIBUTIONS

CHB was main contributor in literature survey and drafting the manuscript. GG was main contributor in literature correction and editing the manuscript. RG was main contributor in adding tabular data and adding references. All author's had read and approved the manuscript.

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