Analytical methods for determination of Amphotericin B in biological samples: a short review

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Keywords: Amphotericin B, Biological samples, Plasma, Human CSF, Piroxicam, HPLC

Abstract

Determination of Amphotericin B in biological samples is of great importance, not only in clinical and forensic toxicology, but also in drug testing and pharmacokinetic profile. Human biological samples including organs, tissues, biofluids such as blood and their derivatives, are increasingly important resources for biomedical research. They can help us to understand how a diagnosis can be carried out, categorized and treatment can be further imitated for a whole variety of medical condition and are particularly important when studying pharmacokinetic profile of drugs in animal models. This review paper is a compilation of last 60 years research articles from 1954 to 2013 that describe procedures for the detection of Amphotericin B, in enormous variety of biological samples. The efficient analytical techniques assigned for accurately analyzing and quantifying drug in biological samples are HPLC and LC MS/MS (hyphenated techniques). Furthermore, this review provides all basic information regarding the sample preparation, type of biological sample and all detail concerning the determination through chromatography.

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Date of Submission: Oct 8, 2014  Date of Acceptance: Oct 12, 2014  Date of Publishing: Nov 3, 2014

How to cite this paper:
Introduction

Amphotericin B (AmB) is a polyene antifungal antibiotic produced by Streptomyces nodosus and used in clinical practice since the 1960s. Two amphotericins (A and B) were isolated in the 1950s from Streptomyces nodosus, an aerobic bacterium, from a soil sample from Venezuela’s Orinoco River Valley by Gold and coworkers in the 1950s. Amphotericin B having molecular formula of \( C_{47}H_{73}NO_{17} \) and chemical structure as shown in Figure 1. It has seven conjugated double bonds, an internal ester, a free carboxyl group, and aglycoside side chain with a primary amino group. AmB bind to ergosterol in the fungal membrane forming a channel in the membrane with the hydrophilic surface facing the interior of the channel. This permits leakage of intracellular components (\( K^+ \), \( Na^+ \), \( H^+ \) and \( Cl^- \)), leading to fungal death.

![Figure 1: Chemical structure of Amphotericin B](image)

AmB is poorly soluble in water and has limited oral absorption. Limited use of AmB is confined due to its dose dependent nephrotoxicity which is frequent and severe. But still remains the most effective drug in treatment of systemic fungal infections and regained wide clinical applications. In recent years, new formulations such as liposomal amphotericin B have been developed that have significantly minimized nephrotoxicity and side effects. Currently, several lipid-based formulations of AmB have been developed such as a liposomal formulation (AmBisome®), a lipid complex of AmB with phospholipids (ABELCET®) and a colloidal dispersion of AmB with cholesteryl sulfate (Amphotec®) and they are now commercially available. These formulations have been developed to improve tolerability for the patient, but may show considerably different pharmacokinetic characteristics as compared conventional AmB. However, developing a safe and effective drug/delivery system is not only part of the picture; its determination in biological sample as well as in pharmaceutical dosages form with aid of most accurate method plays a significant role in formulation development with clinical relevance. Physicochemical and pharmacokinetic properties of a drug are another point of consideration for selection of suitable method for its determination in sample. Physicochemical and pharmacokinetic properties of AmB are summarized in Table 1 and Table 2.

Table 1: Physicochemical properties of Amphotericin B

<table>
<thead>
<tr>
<th>Category</th>
<th>Polyene Antibiotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAS Number</td>
<td>1397-89-3</td>
</tr>
<tr>
<td>ATC Number</td>
<td>A01AB04; A07AA07; G01AA03; J02AA01.</td>
</tr>
<tr>
<td>Empirical Weight</td>
<td>924.08</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>924.08</td>
</tr>
<tr>
<td>Solubility</td>
<td>N,N-dimethylformamide 2-4 mg/mL</td>
</tr>
<tr>
<td></td>
<td>N,N-dimethylformamide + hydrochloric acid 60-80 mg/mL</td>
</tr>
<tr>
<td></td>
<td>Dimethyl sulfoxide 30-40 mg/mL</td>
</tr>
<tr>
<td></td>
<td>Water at pH 2 or pH 11 0.1 mg/mL</td>
</tr>
<tr>
<td>Melting Point</td>
<td>170°C</td>
</tr>
<tr>
<td>pKa</td>
<td>5.7 (carboxyl) and 10.0 (amine)</td>
</tr>
<tr>
<td>logP</td>
<td>0.8</td>
</tr>
<tr>
<td>IR Bands</td>
<td>1010, 1065, 1038, 1103, 1183, 1126 cm⁻¹ (KBr disk)</td>
</tr>
<tr>
<td>UV ( \lambda ) max</td>
<td>406, 382, 363 and 345 nm</td>
</tr>
</tbody>
</table>

Table 2: Pharmacokinetics of Amphotericin B

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorption</td>
<td>Oral &lt;5%, Intramuscular poor</td>
</tr>
<tr>
<td>Half-life</td>
<td>Initial phase 24-48 h, Terminal phase 15 d</td>
</tr>
<tr>
<td>Apparent volume of distribution</td>
<td>Overall 4 L/kg, Central compartment 0.44 L/kg, Fast compartment 0.35 L/kg, Slow compartment 3.20 L/kg</td>
</tr>
<tr>
<td>Urinary recovery at 24 h</td>
<td>3%</td>
</tr>
<tr>
<td>Binding to ( \beta )-lipoproteins</td>
<td>91-95%</td>
</tr>
</tbody>
</table>

Determination of Amphotericin B in biological samples

Several assay methods are reported in the literature for the determination of AmB in biological samples while accounted with their individual limitations. Microbiological techniques have been used extensively for the determination of AmB in serum.
But not have been widely followed confining to relatively less précised, difficult to standardize and slow in comparison to instrumental analytical methods and when there is a need for rapid clinical feedback of the AmB levels. Other method which can be opted is spectrophotometric assay \(^{[14-17]}\) but there are chances of interferences due to the presence of heme and other colored substances in estimation of AmB. The most widely used methods include HPLC methods which are used for determination of AmB in biological fluids. In some methods internal standard are not employed, which requires careful sample preparation in order to reduce the chances of errors.\(^{[18-22]}\) while other methods has internal standards as one of the component which increases the running cost of assay\(^{[23-35]}\) such as Piroxicam, Natamycin, p-nitroaniline etc. Problem associated with HPLC methods are large volume of plasma is needed for studies which is difficult and comes restriction to quantification of AmB in plasma or serum and are not ideal for other biological samples because of higher limits of quantification and long chromatographic run times. Advanced hyphenated technique such as LC-MS/MS is an alternative to HPLC methods for determination of AmB in other biological samples generally which has better selectivity and sensitivity than HPLC methods\(^{[36-38]}\). The data for determination of AmB in biological samples for pharmacokinetics as well as for toxicological studies have been appended in proceeding pages in Table 3 with important process variables which impact on rapidity, accuracy and limit of detection (LOD) for quantification of drug.

**Conclusion**

Bio-analytical methods are the most powerful analytical tool for screening and identification of AmB in biological matrices as compared to microbiological methods. They are more sensitive, have low detection limit, accurate, précised and high recovery. However, adequate sample preparation is a key prerequisite aspect of successful quantitative and qualitative analysis. Among all of the published methods, the most frequently used method for quantification of AmB is HPLC coupled to mass spectrometer or UV detector. The LC-MS/MS methods are considered as most appropriate method for quantification of AmB in other biological samples. The ultimate goal is to obtain results with more and more precision and accuracy and at increasingly lower concentration levels of the AmB being determined in biological samples. Its a continues process, with advancement of technology, new analytical methods could be developed in future, but in current status HPLC methods are most commonly used for plasma and serum samples with exception to low concentration of AmB in certain biological samples/fluids.

**Acknowledgements**

None.

**Funding**

None.

**Competing Interests**

None declared.

**References**

12. Halde C, Newcomer VT, Wright ET, Sternberg TH. An evaluation of amphoteri-


Table: 3 Analytical methods of determination of Amphotericin B in biological samples

<table>
<thead>
<tr>
<th>S. No</th>
<th>Method</th>
<th>Biological sample</th>
<th>Internal Standard (IS)</th>
<th>Experimental condition</th>
<th>LOD</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Spectro-photometric Method</td>
<td>Serum, urine</td>
<td>-</td>
<td>System: Shimadzu UV-160A Sample preparation: solvent acetonitrile added to one mL of serum or urine sample, then centrifuged and UV scan with blank water. Detection: 402 nm</td>
<td>0.2 to 2.4 µg/mL</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>HPLC</td>
<td>Human plasma</td>
<td>-</td>
<td>System: Waters HPLC Alliance system on an e2695 separations module with a Waters 2998 photo-diode array (PDA) detector. Column: Waters XBridge&lt;sup&gt;TM&lt;/sup&gt; C&lt;sub&gt;18&lt;/sub&gt; reversed-phase column (150 x 4.6 mm, 3.5 µm) Mobile phase: acetic acid (0.73%): acetonitrile (60:40 v/v). Flow rate: 1.0 mL/min Injection volume: 20 µl Detection: 408 nm</td>
<td>9.98 ng/mL</td>
<td>18</td>
</tr>
<tr>
<td>3</td>
<td>HPLC</td>
<td>Human plasma, Mice liver, spleen and kidneys</td>
<td>-</td>
<td>System: Jasco PU-1580 pump, a Gilson 231 XL auto-sampler fitted to a 100 µL sampling loop and a Jasco UV-1575 UV–visible detector. Column: Thermo Hypersil BDS C&lt;sub&gt;18&lt;/sub&gt; reverse-phase column (250 x 4.6 mm, 5µm) Mobile phase: acetonitrile : acetic acid: water (52:4.3:4) Flow rate:1 mL/min Detection: 406 nm</td>
<td>0.016 µg/mL</td>
<td>19</td>
</tr>
<tr>
<td>4</td>
<td>HPLC</td>
<td>Human, dog and rabbit Serum and cerebrospinal fluid</td>
<td>-</td>
<td>System: Liquid chromatograph (model no. ALC/GPC 200; Waters Associates) equipped with a model 6000 solvent delivery system, a model U6K Universal injector, a model 440 absorbance detector. Column: MicroBondapak C&lt;sub&gt;18&lt;/sub&gt; column (300 x 4 mm) Mobile phase: Methanol was mixed with the EDTA solution (8:2 v/v) Injection volume: 300 µL Flow rate: 2.5 mL/min Detection: 405 nm</td>
<td>0.02 µg/mL</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>HPLC</td>
<td>Sheep (Plasma, lung lymph fistulas and tracheotomy)</td>
<td>-</td>
<td>System: Modular HPLC (4-L250, L-6200, AS-4000; Hitachi) Column: Lichrospher RP-18e column (250 x 4mm) Mobile phase: 10 mM EDTA,2K-methanol (25:75 v/v) Detection: fluorescence with excitation and emission set at 405 nm</td>
<td>10 to 5,000 ng/mL</td>
<td>21</td>
</tr>
<tr>
<td>6</td>
<td>HPLC</td>
<td>Brains, livers, and kidneys of mice</td>
<td>-</td>
<td>System: Kontron 560 HPLC system consisting of a Kontron 525 pump, Kontron UV detector 430, Auto-sampler T360, and a Kontron column heater Column: Nucleosil 120 C&lt;sub&gt;18&lt;/sub&gt; (125 x 4.6 mm, 3 µm) Mobile phase: eluent A (methanol, acetonitrile, and 0.00125 M EDTA (500:350:300) and eluent B (tetrahydrofuran 100%). Injection volume: 20 µL Flow rate:1.2 mL/min Detection: 430 nm</td>
<td>840 ng/mL</td>
<td>22</td>
</tr>
<tr>
<td>7</td>
<td>HPLC</td>
<td>Rat Piroxicam</td>
<td>Column: Reversed-phase ODS column HiQsil. C&lt;sub&gt;18&lt;/sub&gt; (150 x 4.6 mm, 5 µg/mL)</td>
<td></td>
<td></td>
<td>23</td>
</tr>
<tr>
<td>Method</td>
<td>Sample Type</td>
<td>Column</td>
<td>Mobile Phase</td>
<td>Injection Volume</td>
<td>Flow Rate</td>
<td>Detection</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td>8 HPLC</td>
<td>Human serum</td>
<td>Disperse yellow 42</td>
<td>acetonitrile : 0.02 M EDTA disodium salt at pH 5.0 (40:60 v/v)</td>
<td>10 µl</td>
<td>1.0 mL/min</td>
<td>406 nm</td>
</tr>
<tr>
<td>9 HPLC</td>
<td>Human serum</td>
<td>p-nitroaniline</td>
<td>Mobile phase: acetonitrile: methanol: 5mM EDTA disodium salt (187:449:354 v/v)</td>
<td>50 µl</td>
<td>1.5 mL/min</td>
<td>386 nm</td>
</tr>
<tr>
<td>10 LC</td>
<td>Dog and human cerebrospinal fluid</td>
<td>Nystatin</td>
<td>Mobile phase: 0.01 M sodium acetate buffer, pH 7.4: acetonitrile (60:40 v/v)</td>
<td>100 µl</td>
<td>0.5 mL/min</td>
<td>410 nm</td>
</tr>
<tr>
<td>11 HPLC</td>
<td>Rat (Plasma, liver, spleen and kidney)</td>
<td>Piroxicam</td>
<td>Mobile phase: acetonitrile : acetate acid (10%) : water (41:43:16)</td>
<td>100 µl</td>
<td>1 mL/min</td>
<td>405 nm</td>
</tr>
<tr>
<td>12 HPLC</td>
<td>Human plasma</td>
<td>Berberine</td>
<td>Mobile phase: acetonitrile 0.03M: ammonium acetate (44:56 v/v pH 4.8)</td>
<td>15 µl</td>
<td>0.8 mL/min</td>
<td>407 nm</td>
</tr>
<tr>
<td>13 HPLC</td>
<td>Plasma and sputum</td>
<td>Piroxicam</td>
<td>Mobile phase: acetonic acid and 10 mM acetate buffer (44:51:5 v/v)</td>
<td>80 µL</td>
<td>1 mL/min</td>
<td>407 nm</td>
</tr>
<tr>
<td>14 HPLC</td>
<td>Rat plasma</td>
<td>α-naphthol</td>
<td>Mobile phase: Acetonitrile : water: acetic acid (44:51:5 v/v)</td>
<td>50 µL</td>
<td>1 mL/min</td>
<td>407 nm</td>
</tr>
<tr>
<td>15 HPLC</td>
<td>Human plasma</td>
<td>1-amino-4-nitroanaphthalene</td>
<td>Mobile phase: Disodium edetate (20 mM) (45:55 v/v) at pH 5.0</td>
<td>100 µL</td>
<td>1 mL/min</td>
<td>407 nm</td>
</tr>
<tr>
<td></td>
<td>HPLC</td>
<td>Sample Type</td>
<td>Analyte</td>
<td>System:</td>
<td>Column:</td>
<td>Mobile phase:</td>
</tr>
<tr>
<td>----</td>
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</tr>
<tr>
<td>16</td>
<td>HPLC</td>
<td>Rat Plasma</td>
<td>Lornoxicam</td>
<td>Shimadzu Model, LC-20AT HPLC pump, Rheodyne</td>
<td>Hypersil ODS, C_{18} (250 x 4.6 mm, 5 µm)</td>
<td>10 mmol phosphate buffer: acetonitrile. (65:35 v/v)</td>
</tr>
<tr>
<td>17</td>
<td>HPLC</td>
<td>Rabbit</td>
<td>1,1-diphenyl-2-pikryl-hydrazyle</td>
<td>HPLC setup comprised a Waters model 501 pump, a Waters 717 plus auto-sampler, and a Spectro flow 757 absorbance detectors.</td>
<td>Nova-Pack C_{18} column (150 x 4.6 mm, 4 µm) using a pH 7.5</td>
<td>Mobile phase: methanol : acetonitrile mixture (5:95 v/v)</td>
</tr>
<tr>
<td>18</td>
<td>HPLC</td>
<td>Human Plasma</td>
<td>4-nitro-1-naphthylamine</td>
<td>Hewlett Packard 1050 series degasser, pump, a Jones Chromatography Column Heater model 7965, a Waters 717 auto-sampler, Shimadzu SPD 10A ultraviolet detector.</td>
<td>Waters symmetry C_{18} column (150 x 4.6 mm, 5 µm) attached to Waters symmetry C18 guard column (20 x 3.9 mm, 5 µm)</td>
<td>Mobile phase: Gradient elution of acetonitrile from 20% to 42% plus 0.1 M acetate buffer (pH 4.2) solution from 80% to 58% in 10.5 min.</td>
</tr>
<tr>
<td>19</td>
<td>HPLC</td>
<td>Human serum</td>
<td>3-nitropheno1</td>
<td>Hitachi AS4000 Intelligent AutoSampler, a Hitachi L-6200A Intelligent Pump, and an ABI Kratos Spectroflow 758A UV/VIS Detector</td>
<td>Mobile phase: acetonitrile : water containing 0.05N sodium acetate, anhydrous (34:66 v/v).</td>
<td>Injection volume: 20 µL</td>
</tr>
<tr>
<td>20</td>
<td>LC/MS/MS</td>
<td>Rabbit tears</td>
<td>Natamycin</td>
<td>Perkin-Elmer Series 200 HPLC system</td>
<td>Luna 3µ CN column (100 x 2 mm, 3 µm)</td>
<td>Mobile Phase: Methanol: Ammonium acetate (3.5 mM, adjusted to pH 4 with acetic acid) (90:10 v/v)</td>
</tr>
<tr>
<td>21</td>
<td>LC/MS/MS</td>
<td>Human CSF</td>
<td>Rifaximin</td>
<td>Agilent 1200 system (Agilent Technologies).</td>
<td>Sunfire C_{18} column (50 x 2.1 mm, 5 µm)</td>
<td>Mobile phase: 0.1% Formic acid: methanol (40:60 v/v)</td>
</tr>
<tr>
<td>22</td>
<td>LC/MS/MS</td>
<td>Plasma, Urine, Fecal</td>
<td>Natamycin</td>
<td>PE Sciex and API 3000 (for free amphotericin B) or API 3+ (for total amphotericin B) triple quadrupole mass spectrometer</td>
<td>Waters Symmetry C_{18} column (150 x 3.0 mm, 5 µm)</td>
<td>Mobile phase: methanol : water: acetic acid (68:6:29.4/1.96 v/v/v)</td>
</tr>
</tbody>
</table>

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