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THE UNIVERSITY OF IOWA COLLEGE OF PHARMACY

Division of Pharmaceutics and Translational Therapeutics

Chemical stability of amphotericin B in lipid-based media

by

Éverton do Nascimento Alencar

December, 2017

PPGNANOFARMA

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“I am not what happened to me, I am what I choose to become.”

Carl Jung

ABSTRACT

This thesis is part of research efforts with the intent of applying chemical stability and drug degradation kinetics knowledge to lipid-based systems, as amphotericin B (AmB) usually requires a lipid-based nanotechnological delivery system to overcome its biopharmaceutical issues. The aim of this thesis was to determine the instability pathway and degradation kinetics of AmB in oil solutions, since stability of drug in delivery systems are chemically complex to be investigated due to their multi-phased aspect. This thesis was driven on the hypothesis that AmB´s toxicity might be associated to the drug degradation instead of what is reported by the literature, which is mainly related to the drug´s aggregation state. The experimental research herein was conducted in order to investigate the chemical instability pathways and kinetics of AmB in oils, aiming to generate knowledge that can be useful on a larger context involving microemulsions currently studied by our research group. For this purpose, reaction mixtures containing AmB were prepared using different solvents and co-solutes. Samples were stored under different conditions of temperature and light exposure. AmB aggregation state in the reaction mixtures was investigated by UV-Vis spectroscopy. The degradation pathway of AmB in oil was investigated. The results revealed that the use of hydrogen donator antioxidants decreased the degradation of AmB under thermal stress. Also, the AmB degradation under dark conditions was not linearly temperature dependent. Additionally, the use of radical initiator greatly increased the degradation of AmB in methanol. Based on the obtained results, adsorption and aggregation did not appear to play an important role in the AmB degradation in oil. Under dark conditions, the most likely pathway for AmB degradation in oil was autoxidation, whereas under light exposure, the most likely degradation pathway was light catalyzed oxidation. Empirical degradation schemes were drawn and differential equations were developed to explain the AmB loss process. A kinetic model was successfully used to describe the AmB loss in different solvents under dark environment. The model proved that AmB undergoes a complex degradation pathway, once a simple autocatalytic model could not describe its loss. In fact, a reversible loss probably related to hydrolysis might be involved, as demonstrated by the scheme and the kinetic model. A second model successfully described the AmB loss under light exposure. AmB loss in this condition showed to be of pseudo-first order. Due to the complex degradation pathway, the kinetics of the different processes leading to the AmB degradation could not be distinguished. The information generated by this research will help to predict AmB instability in microemulsion systems, once the data of AmB degradation in aqueous phase and surfactants will be associated. Additionally, further efforts on trying to generate and isolate the degradants here suggested can help on the investigation of the toxicity related to degradation products.

Keywords: Amphotericin B; Microemulsions; Drug Degradation; Oxidation; Chemical Kinetics.

RESUMO

Esta tese foi gerada como parte dos esforços em gerar conhecimentos de estudos de estabilidade química e cinética de degradação que possam ser aplicados a microemulsões contendo Anfotericina B (AmB). O objetivo desta tese foi determinar as vias de degradação da AmB em óleo, visto que microemulsões são multifásicas e de composição complexa. Esta tese mostra esforços experimentais em determinar as vias de degradação da AmB em óleo, assim como determinar o esquema de degradação e o modelo matemático que reflita o comportamento de degradação desta molécula em solução lipídica. Diferentes soluções contendo AmB em óleo foram preparadas e armazenadas em diferentes condições de temperatura e luz, assim como presença e ausência de co-solutos. Estudos analíticos foram feitos por Espectrofotometria UV-Vis e por método validado em Cromatografia Líquida de Alta Pressão. Foi possível observar que o uso de antioxidantes doadores de hidrogênio no meio reacional aumentou a estabilidade de AmB sob estresse térmico ao longo do tempo. Entretanto, a cinética de degradação da AmB em triglicerídeo de cadeia média não foi linearmente dependente da temperatura. A adição de um iniciador radicalar aumentou a degradação da AmB em metanol. Adicionalmente, baseado em estudos de adsorção e agregação, foi possível observar que estes fenômenos não estão diretamente relacionados a degradação da AmB em óleo. Em armazenamento protegido da luz, a via de degradação mais provável por ser responsável pela perda de AmB do meio reacional é a auto-oxidação. Enquanto sob luz visível e UV, a oxidação catalisada pela luz é predominante. Modelos cinéticos foram construídos baseados nestas evidências experimentais. O modelo complexo para condições protegidas de luz mostrou que a AmB sofre não somente auto-oxidação, mas perda reversível, que pode estar relacionada à hidrólise por água residual. O modelo para degradação sob exposição à luz foi de pseudo-primeira ordem. Os modelos teóricos representaram os dados experimentais adequadamente e as constantes cinéticas foram estimadas. Entretanto, devido a degradação ocorrer por vias de degradação múltiplas e concomitantes, os parâmetros cinéticos de cada processo envolvido não foram estimados. As informações geradas por esta tese ajudarão a estimar adequadamente a cinética de degradação em sistemas de liberação complexos quando associados aos dados cinéticos nas demais fases em que o fármaco esteja presente. Além disto, este estudo sugeriu possíveis produtos oriundos da oxidação da AmB. Portanto, estudos de identificação e síntese devem ser realizados a fim de investigar a relação da toxicidade da AmB e seus produtos de degradação.

Palavras-chave: Anfotericina B; Microemulsões; Estabilidade química; Cinética de degradação; Oxidação.

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This was not the work of a 4 years period. I can definitely say this Thesis is, at the moment, the work of my life. Indeed, this Thesis was not only developed through laboratory experiments performed solely by me, but by through a network of collaborators, involving colleagues of the more distinguished academic degrees, undergraduates, master and doctorate students, and Doctors. These collaborations happened frequently overseas, through days and nights of work among Brazil, The United States and France.

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CHAPTER 1

Introduction, Peer collaborated efforts and Objectives

1.1. Introduction

Amphotericin B (AmB) is an antifungal and antiprotozoal drug that was originally isolated from *Streptomyces nodosus*. It is a macrolide composed of a macrocyclic lactone ring containing 37 carbons atoms (Figure 1).

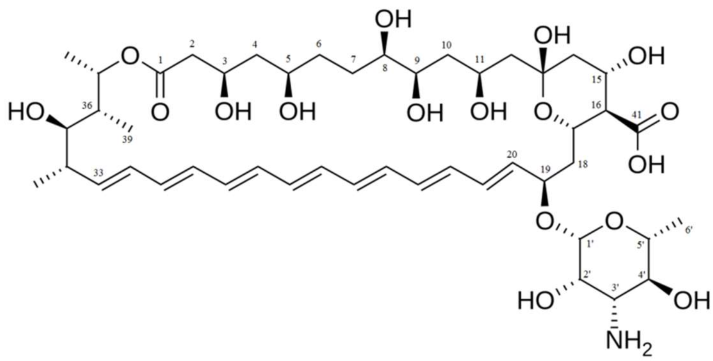

Figure 1 – Structure of amphotericin B showing the numbering of carbon atoms in the molecule

A heptaene from carbon 20 to carbon 33 provides a non-polar characteristic to the molecule (Driver *et al.*, 1990), whereas the presence of seven hydroxyl groups and one carboxyl group provides some polarity. As a result, this molecule is amphiphilic (Andreoli, 1974). AmB contains a carboxyl group (pK_a 5.5) and a primary amine group (pK_a 10), the later attached to a mycosamine ring (3-Amino-3,6-dideoxy-β-D-mannopyranose) which is connected to the main macrocyclic chain by an *o*-glycoside linkage at carbon 19. The anionic carboxylic acid and cationic protonated amino group result in a zwitterion with amphoteric characteristic (Chéron *et al.*, 1988; Hellgren *et al.*, 1995).

Clinically, AmB is used for the treatment of invasive fungal infections, and it has a broad spectrum of action, including strains of *Candida* spp. and *Aspergillus* spp., and other filamentous fungi (Klepser, 2011). However, high nephrotoxicity has limited its use to second-line treatment when first course with azoles or echinocandins (regarding patients' conditions and specific infection) fails to eliminate the infections (Fanos e Cataldi, 2000; Walsh *et al.*, 2008; Pappas *et al.*, 2009). Additionally, AmB is used to treat visceral leishmaniasis, an infectious disease caused by protozoan parasites of the genus *Leishmania*. Leishmaniasis is a public health issue in many tropical, subtropical and Mediterranean countries as well as rural southern United States. Untreated infections can be fatal. The first choices of treatment for the diseases are antimonial agents. However, in many countries, AmB is considered first choice of treatment due to *Leishmania* resistance to antimonial drugs (Chattopadhyay e Jafurulla, 2011; Mosimann *et al.*, 2018).

Although AmB is considered a useful drug for systemic fungal infections and visceral leishmaniasis (Chattopadhyay e Jafurulla, 2011), severe toxicity usually attributed only to AmB aggregated forms interaction to human cells cholesterol molecules hinders its clinical use. The infusion of traditional formulations (amphotericin B deoxycholate) can cause shaking, fever, vomiting, tachypnea and may require co-administration of antipyretics, antihistamines, antiemetics and hydrocortisone to reduce the side effects during treatment (Klepser, 2011). On the other hand, administration of lipid formulations of AmB are associated to reduced toxicity and enables the use of this drug for prophylactic use (Hann e Prentice, 2001; Mosimann *et al.*, 2018).

Lipid-based AmB delivery systems are currently being developed to reduce dosage, increase potency, reduce cost and enhance bioavailability and pharmacokinetics profiles (Hann e Prentice, 2001). Recent *in vitro* and *in vivo* studies have focused on the use of AmB microemulsions to treat fungal infections and leishmaniasis (Darole *et al.*, 2008; Damasceno *et al.*, 2012; Silva *et al.*, 2013; Morais *et al.*, 2016). Microemulsions are colloidal dispersions of two immiscible phases stabilized by a surfactant interfacial film, which are characterized as isotropic, clear and thermodynamically stable (Mouri *et al.*, 2014). Microemulsion droplets range in size from 10 to 100 nm, and therefore, can be used for parenteral administration (Date e Nagarsenker, 2008).

Regardless of the delivery system, stability studies are required to optimize storage conditions and shelf-life. Drug product stability means the maintenance of all product performance attributions during manufacture, distribution, storage and product use. Product performance attributes include chemical integrity, physical integrity, therapeutic effectiveness, safety, sterility, utility and sensory acceptability. Chemical stability relates to maintaining chemical potency and purity, and limiting the generation of degradation products (covalent transformations). Physical stability relates to organoleptic characteristics, phase changes, non-covalent changes such as aggregation, polymorphism, coalescence and droplet size growth. Last, therapeutic and safety stabilities relates to the maintenance of unchanged therapeutic effects and prevention of toxicity increase, respectively (Ansel *et al.*, 2005). Furthermore, the knowledge of drug degradation kinetics and products of degradation related to the specific lipid medium can provide information regarding optimum manufacture conditions, excipient-drug stability for compounding optimization and toxicity.

Despite extensive research on trying to improve stability of delivery systems, whether is by adding conservatives, or by freeze-drying in order to remove water from systems; and regardless the extensive efforts on AmB delivery systems research, very few studies have been reported on its chemical stability. In particular, the identity and toxicological safety of AmB degradation products and the kinetics of their formation in various environmental conditions and drug delivery systems has not been reported. Studies have demonstrated that AmB toxicity is reduced in lipid-based delivery systems (Brime *et al.*, 2003; Damasceno *et al.*, 2012; Wasko *et al.*, 2012; Mosimann *et al.*, 2018). However, it is unclear whether reduced toxicity is due to reduced dimer and AmB-aggregate concentrations or changes in the pattern and rate of degradation product formation.

Although being an old molecule and having widespread use for leishmaniasis and fungal infections in lipid-based systems (such as the liposomal AmBisome®, and the widely studied emulsioned systems) the limited data on AmB degradation only concerns aqueous media and organic solvents (Hamilton-Miller, 1973; Beggs, 1978; Lamy-Freund *et al.*, 1985; Hung *et al.*, 1988). Thus, not only is necessary to investigate the influence of the degradation products of AmB on its toxicity, but how this drug behaves in lipid-based media and which degradation pathway is the main responsible for its degradation. This way, it would be possible to control or prevent drug degradation in those formulations, once different processes may be predominant in different type of media.

In this context, High Performance Liquid Chromatography (HPLC) is a useful technique for the separation and quantification of low degradant concentration in complex mixtures. However, in order to obtain reliable results, method validation must be performed (Kazakevich e Lobrutto, 2007). Validation is a standardized process for reducing error to ensure accurate and precise measurements with adequate sensitivity, selectivity and linearity above the limits of detection and quantification (Ich, 1996; Fda, 2001; Skoog *et al.*, 2007).

This thesis will show current efforts in evaluating the chemical stability of amphotericin B in lipid-based media, since besides the work being done on development, characterization and *in vitro* and *in vivo* studies regarding lipid-based systems containing amphotericin B, little is known regarding its chemical stability in different media and how this stability might play a role on aggregation, pharmacological activity and toxicity.

1.2. Peer Collaborated Efforts

The Graduate Program in Pharmaceutical Nanotechnology is a Ph.D. program composed of researchers from several Brazilian Universities, with expertise in production, characterization and evaluation of nanotechnological delivery systems for diverse routes. Part of the work herein presented was done in collaboration with Master and Doctorate students from Brazil and France, specially the work related to nanotechnology.

In order to increase the knowledge of drug stability applied to microemulsions, the experimental research of this thesis was developed at The University of Iowa – College of Pharmacy – Division of Pharmaceutics and Translational Therapeutics under the guidance of Prof. Lee. E. Kirsch, Ph.D., who has generously joined collaborative efforts to Prof. Socrates Egito, Ph.D. (Federal University of Rio Grande do Norte (UFRN), member of the Graduate Program in Pharmaceutical Nanotechnology to advise me on the development of this project.

1.3. Thesis Structure

As a doctorate student from this program, my initial efforts regarding research were towards development and characterization of delivery systems with amphotericin B and also natural oils in the field of nanotechnology. However, as a need to provide valuable information regarding amphotericin B stability, my research switched in the very beginning of the doctorate studies mainly to the stability studies of this drug aiming to provide valuable and needed information to be applied in future studies involving amphotericin B incorporated in lipid-based systems. Based on this context, this thesis was organized in the following way:

Chapter I provides literature background, peer collaborative efforts, a general explanation of the Thesis structure and the Objectives of the work.

Chapter II presents the efforts to develop and validate an analytical method to quantify amphotericin B in oils, as well as efforts to determine the predominant pathway of degradation of this drug in different lipid-based reaction mixtures.

In Chapter III, the quantification of AmB degradation kinetics are presented using the suggested empirical model for substrate loss under different environmental conditions.

Furthermore, in the Appendix, additional collaboration work regarding microemulsions containing amphotericin B, analytical methods of analysis for quantifying and characterizing natural oils, and papers regarding the development of nanostructured delivery systems based on natural products are shown. Although covered by the scope of the Graduate Program in Pharmaceutical Nanotechnology, these papers are not the focus of this thesis. However, they are shown as a parallel project I was involved as a student on the area of expertise of this program and contributed to my education towards obtaining the degree of Doctor in Pharmaceutical Nanotechnology.

1.4. Objectives

General Objective

The **long-term research goals** involved in this research were to determine the chemical instability pathways and kinetics of AmB in complex drug delivery systems currently studied by our research group, mainly emulsions and microemulsions, and to determine if the generation of degradation products was associated to the drug toxicity. However, due to the complex nature of these systems and the different degradation behavior a drug may have in different media, the **objective of this thesis** was to determine the instability pathway and degradation kinetics of AmB in oil solutions.

Specific objectives

The specific objectives pursued in this work, as explored along the next chapters, were:

Specific Aim 1: **Quantify AmB from oil solutions.**

An extraction method was developed to recover AmB from oil for posterior quantitation. A HPLC/UV-Vis method able to accurately measure low concentrations of AmB was developed and validated.

Specific Aim 2: **Determine if adsorption and aggregation play a role on AmB loss and if AmB undergo oxidation**

Adsorption testing was performed to determine if this process accounts for AmB loss observed by analytic methods. In addition, aggregation of AmB was assessed along stress testing to determine if different aggregation forms of this drug play a role on AmB degradation kinetics in lipid media.

Stress testing under oxidative conditions were performed with AmB in oils solutions in order to determine if the drug degrades under different oxidative pathways.

Specific Aim 3: **Determine degradation rates and predict degradation scheme and model for AmB loss**

A degradation scheme for AmB loss was suggested according to the conditions tested. An empirical model was developed, and kinetic parameters were determined.

CHAPTER 2

This chapter contains a research paper manuscript about the validation of a HPLC method for quantifying AmB extracted from oil samples, AmB solubility determination in medium chain triglycerides and the role of media on the degradation of AmB, as well as an attempt to determine the degradation pathway of AmB in oils and reaction mixtures.

The manuscript focusses on determining the primary pathway of AmB degradation based on the hypothesis that autoxidation is the main pathway in organic solvents. However, the manuscript presents efforts to investigate alternate pathways that could be responsible for substrate loss detectable by the developed analytical method. As well as shows different investigations into the oxidation theory, such as using reaction mixtures containing antioxidants with different mechanisms of action, investigating the influence of the type of solvent and presence/absence of light.

We intend to submit this research article to the Journal of Pharmaceutical and Biomedical Analysis upon completion and editing of this manuscript.

Studies on amphotericin B instability in lipid-based media, Part I: Identification of degradation pathways as function of mixture conditions

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ABSTRACT

The aim of this work was to determine the predominant degradation pathways of amphotericin B (AmB) as a function of different organic reaction mixtures in oil and storage conditions in lipid-based solutions. For this purpose, UV-Vis Spectroscopy and HPLC analytical methods were developed and HPLC method validated in order to accurately quantify AmB. In this study, the method described proved to be reproducible and accurate with linearity (R^2 = 0.996) from 3.2 to 52.0 nM using a UV detector. LOD and LOQ were 1.33 and 4.45 nM, respectively. AmB was successfully extracted from medium-chain triglyceride based oil prior HPLC analysis and AmB solubility in medium-chain triglyceride was determined to be approximately 1.0 µM. Different reaction mixtures were done using different solvents, antioxidants and radical initiator. Samples were stored under different conditions of temperature and light exposure. AmB aggregation state in reaction mixtures was investigated by UV-Vis spectroscopy. The degradation pathway of AmB in oil was investigated. Use of hydrogen donator antioxidants decreased the degradation of AmB under thermal stress. AmB degradation under dark conditions was not temperature dependent. Use of radical initiator increased the degradation of AmB in methanol greatly. Based on the obtained results, adsorption and aggregation did not appear to play a role in AmB degradation in oil. The degradation pathways of AmB were related to oxidation processes and were found to be light-dependent. Under dark conditions, the most likely pathway for AmB degradation was autoxidation. Whereas under light exposure, the most likely degradation pathway was light catalyzed oxidation.

Keywords: Amphotericin B; Autoxidation; Drug Degradation;

1. INTRODUCTION

Amphotericin B (AmB) is an antibiotic synthesized by *Actinomycetes* microorganisms (mainly *Streptomyces spp.*) that was first introduced as an antifungal agent [1]. AmB contains a carboxyl group (pKa 5.5) and a primary amine group (pKa 10), the later attached to a mycosamine ring (3-Amino-3,6-dideoxy-β-D-mannopyranose) which is connected to the main macrocyclic chain by an o-glycoside linkage at carbon 19. The anionic carboxylic acid and cationic protonated amino group result in a zwitterion with amphoteric characteristic [2, 3]. Additionally, the presence of seven hydroxyl groups on the aliphatic chain and a heptaene on the macrocycle provide an amphiphilic characteristic to the molecule [4] (Figure 1).

FIGURE 1

To this date, no investigative studies have been performed in order to determine the degradation pathway of AmB in oil. However, studies have been performed in aqueous media, as well as in organic solvents. Hung and Collaborators [5], performed a study using a factorial design to determine which variables would influence on AmB degradation. The study involved different conditions as dependent variables, including temperature, light, ionic strength, oxygen, surfactant concentration and pH. Although none of the conditions involved oil as solvent, the study suggested that AmB is more stable in darkness, in the absence of oxygen and at low temperature, no further investigations or discussion regarding degradation pathway was done.

Recent study from our research group showed that in aqueous media, at extreme conditions, such as high and low pH, hydrolysis is the most predominant degradation pathway. On the other hand, oxidation plays a bigger role on the degradation of this drug at neutral pH (Unpublished data), as also suggested by Lamy-Freund and collaborators [6], which performed studies in a mixture of aqueous and organic media to investigate the generation of free radicals by Electron Spin Resonance (ESR). The authors also monitored the oxygen consumption throughout the experiment. The study supported the hypothesis of degradation by autoxidation at this reaction media.

Furthermore, the aggregational state of AmB, which has been widely associated to this drug´s toxicity [7], affects directly the total loss of AmB substrate in aqueous media, as mentioned by Lamy-Freund and collaborators [8]. The study showed that aggregated form in water/DMSO mixture had slower degradation rates than AmB in its monomeric state, evidence of the influence of aggregation in the kinetics of degradation. However, the study did not quantify AmB directly, instead they only investigated degradation by determining free radical formation.

As observed in the literature, there is a lack of information regarding amphotericin B chemical stability in lipid-based media. The studies above performed in aqueous media did not discussed possible other oxidation reactions other than autoxidation to be involved in the degradation of AmB. Furthermore, it is still unclear if aggregation plays a role on different AmB degradation pathways in lipid-based media. For this reason, a great amount of information is still needed before accurately inferring the relation between amphotericin B instability and its pharmacological and toxicological effects In lipid media. Thus, the aim of this work was to determine the predominant degradation pathway of AmB as a function of different reaction mixtures and storage conditions in lipid-based solutions. For this purpose, analytical methods were developed and validated in order to accurately quantify AmB.

2. MATERIALS AND METHODS

2.1 Chemicals

Amphotericin B (Product number: A4888, Purity ≥ 80 % (HPLC)), carbamazepine, methanol CHROMASOLV® for HPLC, butylated hydroxytoluene (BHT), benzylic alcohol, chloroform, butanol, peanut oil and azobisisobutyronitrile (AIBN) were acquired from Sigma Aldrich (St. Louis, MO, USA). Soybean oil was obtained from Spectrum Chemical (New Brunswick, NJ, USA). Acetonitrile HPLC grade, dimethyl sulfoxide (DMSO) and ethylenediaminetetraacetic acid, disodium salt (EDTA) were purchased from Fisher Scientific (Hampton, PA, USA). Propyl gallate and α-tocopherol were obtained from Acros (NJ, USA). Miglyol® 812 was a gift from Cremer Oleo GambH & Co. KG (Hamburg, Germany). HPLC grade deionized water (Nanopure Infinity - Barnstead thermolyne, MN, USA) was used throughout the experiments. All other chemicals were ACS reagent grade.

2.2 Instrumentation

HPLC analyses were carried out using a Waters Alliance 2695 separation module (Waters Corporation, MA, USA) coupled to a Waters 2487 Dual λ Absorbance detector set at 406 nm for AmB and at 284 nm for Carbamazepine. The column consisted in a Hypersil BDS C18 of 250 mm x 4.6 mm (Thermo Scientific, MA, USA). The mobile phase consisted of 5 mM Na_2EDTA aqueous solution and acetonitrile (65:35, v/v). Flow rate was 0.5 mL/min. Temperature of the sample holder and the column were set at 4 °C and 25 °C, respectively. HPLC method was adapted from previous studies [Unpublished Article].

UV absorbance Scans were performed using a Hewlett Packard 8453 diode-array UV-Visible spectrophotometer. The method was adapted from previous studies from our research group [9]. A quartz cuvette with a path length of 1 cm was used and the solutions were scanned in the range of 250 to 500 nm. Data were analyzed using UV-Visible ChemStation software. A.05.04.

2.3 HPLC method validation

The validation of the HPLC method followed the U S Food and Drug Administration guidance for Industry with adaptations [10].

2.3.1 Calibration Curve

A stock solution was prepared by weighing 20 mg of AmB and transferring the drug to a 100 mL volumetric flask that had its final volume completed with methanol. The stock solution was 174.0 µM based on 80.00 % purity of bulk drug and it was used to prepare calibration standards by series dilution. The solutions had concentrations of 3.2, 6.5, 13.0, 26.0 and 52.0 nM in methanol:DMSO (3:2, v/v). AmB solutions were kept in vials protected from light during the experiment.

2.3.2 System suitability

The suitability of the system was assessed by analyses of 6 injections of AmB at 13.0 nM. The criterion of acceptance was ± 2 % for the coefficient of variation percent (% CV) for retention times and ± 10% for both the peak area and peak height.

2.3.3 Linearity

The linearity was verified by investigating if the solutions concentration range is where analyte response is linearly proportional to concentration. The AmB peak area was considered for plotting the linearity graph and the analysis was based on the least square regression method. Linearity curves were performed in three different days.

2.3.4 Precision/Accuracy

The precision studies were performed by intermediate precision (between-day) and repeatability (within-day). 3.2, 13.0 and 52.0 nM solutions were used in both tests. Repeatability was performed by injecting triplicate samples of the studied solutions. Intermediate precision was performed by injecting the three samples in four consecutive days. Results were expressed as the average ± standard deviation and the coefficient of variance was used to assess the precision. Accuracy was determined by calculating the percent nominal mean of six repetitions for concentrations 3.2, 13.0 and 52.0 nM.

2.3.5 Limits of detection and quantification:

Limit of detection (LOD) and limit of quantification (LOQ) were obtained based on calibration curve data (slope and standard deviation) using AmB solutions in methanol:DMSO (3:2, v/v), as described in previous section.

2.4 Extraction of AmB from oil solution

A liquid/liquid separation method was adapted from the literature to extract the AmB from oil (Miglyol® 812) solutions for HPLC analysis [11]. A 1.0 mL aliquot of AmB oil solution was mixed with 4.0 mL of DMSO. The mixture was stirred on vortex for 2 minutes and allowed to separate for 2 hours at 25 °C while protected from light. After separation, 2.0 mL from the bottom phase were removed from the tube and transferred to a vial containing 3.0 mL of methanol. The resultant solution was mixed and analyzed by HPLC. The calibration standards were prepared in a solvent composed of 3 parts of methanol and 2 parts of DMSO as the resultant solution from extraction.

Extraction yield was calculated comparing the extracted sample to a control AmB solution in DMSO, which was prepared at the same theoretical AmB concentration of the extracted sample at the analytical step. This solution was analyzed by HPLC. Extraction time requirement was determined measuring AmB recovery periodically over 54 hours of extraction. Samples were stored at 25 °C and kept protected from light during the extraction. The experiment was conducted in duplicate and the samples were analyzed by HPLC three times.

2.5 Determination of solubility of AmB in medium-chain triglyceride

The solubility of amphotericin B in medium-chain triglyceride (Miglyol® 812) was determined. A mixture containing an excess of AmB in oil (1mg in 25 mL) was placed in microcentrifuge tubes and kept under magnetic stirring for 52 hours at 25 °C. Samples were prepared in duplicate. Each sample was centrifuged at 8000g for 45 min at 25 °C. The supernatants were collected, extracted and analyzed by HPLC in triplicate.

2.6 Reaction mixtures preparation

A series of reaction mixtures containing AmB at 0.8 µg/mL (0.86 µM) were prepared by diluting a stock solution of AmB 160 µg/mL (174.0 µM) in methanol with Miglyol® 812, peanut oil and soybean oil. Reaction mixtures involved the use of co-solutes, such as the radical initiator AIBN (0.7mg/mL) and the antioxidants propyl gallate (5.0 mg/mL), α-tocopherol (1.0 mg/mL) and BHT (2.5 mg/mL). Besides using the oils mentioned above as solvents, methanol was used in order to overcome drawbacks related to the use of oil, such as solute solubility in oil, need for extraction of compounds for posterior HPLC analysis or its spectroscopic properties. The studies in methanol involved the same co-solutes; However, the influence of antioxidant concentration was evaluated by increasing the concentration of BHT from 10 to 150 mg/mL. In addition, AIBN was added to the stock solution of AmB in methanol (160 µg/mL) in order to initiate AmB degradation and observe possible formation of degradants detectable by the HPLC method. AmB 0.8 µg/mL reaction mixtures at ambient temperature in benzylic alcohol, butanol, chloroform, methanol, Miglyol® 812 and octanol were used in the study of the effect of AmB aggregation state.

Furthermore, carbamazepine was used as a model drug that is known to degrade by oxidation and it has its degradation scheme elucidated [12]. Reaction mixtures of carbamazepine 1 µg/mL and AIBN 0.7mg/mL in the solvents mentioned previously were made in order to investigate the effectiveness of this radical initiator at the work concentration.

All reaction mixtures were submitted to different storage temperatures (ambient temperature, 30, 40, 50, 60, 70 and 80 °C) depending upon the study. Reaction mixtures were placed in transparent glass vials, always protected from light, with the exception to the samples submitted to photodegradation study.

The study of adsorption involved only the reaction mixture of AmB 0.8 µg/mL in Miglyol® 812 stored at 60 ºC in scintillation vials containing different amount of glass beads to analyze if drug loss changed with increase of surface area. The surface areas in contact with the reaction mixture were 26.4 cm^2, 34.9 cm^2 and 43.3 cm^2.

Reaction mixtures were sampled periodically up to 312 hours and analyzed by HPLC and/or UV-Vis Spectroscopy. The oil solutions were submitted to drug extraction prior HPLC analyses, according to section 2.4 and BHT 2.5 mg/mL was added to the DMSO during extraction to quench any possible further oxidation reaction until analysis. All samples were stored at 4 °C, when not analyzed immediately to quench further reactions from happening. Reaction mixtures are described as Table S1 from Supplementary material.

3. RESULTS AND DISCUSSION

3.1 Method validation

AmB 13.0 nM solution in methanol:DMSO (3:2, v/v) was analyzed 6 times for the suitability study. Figure 2 shows chromatograms of the sample and the blank. AmB peak area, peak height and retention time from the 6 replicates of AmB 13.0 nM solution are described in Table 1. AmB peak was well-resolved from elution time of non-retained components.

TABLE 1

The number of theoretical plates was calculated using six replicates based on the Equation (1), where t_r is the retention time (16.2 min) and W is the peak width. The calculated number of theoretical plates was 5184, which is superior to 2000, minimum recommended according to the system suitability test limits [13].

$$N = 16\left(\frac{t_r}{W}\right)^2 \quad (1)$$

The linear regression showed good linearity in a range from 3.2 to 52.0 nM, providing a correlation coefficient of 0.996 ± 0.003. Additionally, the slope value was 216.36 ± 5.15 and the intercept was 639.92 ± 96.41. Figure 3 shows the calibration plot used for linearity assessment.

The precision is the amount of scatter in the results obtained from multiple analyses of a sample. The samples showed low coefficient of variation percentage on the within-day precision evaluation, which means the method has adequate precision in analyses performed in the same day with the sample concentration range (from 3.2 to 52.0 nM) (Table 2). Regarding the between-day precision it was possible to observe that the lower concentration (3.2 nM) had the higher % CV of all three samples; However, this is expected since inter–day variations on the HPLC system, such as flow or small temperature changes could have an effect on analyses of very low concentrated samples. All % CV in the within- and between-day precision in this study were ≤ 13 %, which meet the FDA [10] requirement of coefficient of variance ≤ 15% in order to consider a method precise. However, in research studies it is also usual to determine acceptances criteria that meet the analytical purposes of the method based on multiple research studies, especially when developing a method to detect very low concentrations [14, 15]. Since all samples had acceptable coefficients of variance, it can be inferred that the method's precision was considered satisfactory, presenting low random errors.

The accuracy is the closeness of the measured value to the true value (recovery). The recoveries of AmB samples at concentrations of 3.2 nM, 13.0 nM and 52.0 nM were 72.75, 87.57 and 101.86 %, respectively (Table 2). Based on FDA acceptance criteria for recovery (15 % variation from the real concentration), only the 13.0 nM and 52.0 nM samples recovery (87.57 and 101.86%) meet the criteria since they are with 15 % variation from 100 %. The lower concentration sample (3.2 nM) had a low recovery rate, showing low accuracy of the method for concentrations as low as 3 nM.

TABLE 2

The limit of detection represents the lowest concentration of AmB capable of producing a distinguishable response from the noise, typically, three times the noise level. The LOD was calculated based on ratio of standard deviation of the response (SD) and the slope estimated from the calibration curve of the standards multiplied by 3 (Eq. (2)). The LOQ was calculated based on the same approach; However, the ratio was multiplied by 10 for this parameter (Eq. (3)).

$$LOD = \frac{SD \times 3}{Slope} \quad (2) \qquad\qquad LOQ = \frac{SD \times 10}{Slope} \quad (3)$$

The calculated LOD was 1.33 nM. The limit of quantification is the lowest concentration of the drug that can be accurately and precisely quantified; this value usually corroborates accuracy values for lower concentrations. The calculated LOQ in this validation was 4.45 nM. LOQ was calculated using SD of the intercept of calibration curves (three repetitions) and since low concentration samples showed high % CV in the precision analyses, these samples increased the intercept's SD, increasing LOQ. Although the method presented itself as linear from 3.2 to 52.0 nM based on average values, it is appropriate to work with solutions in the concentration range from 4.45 to 52.0 nM, once the LOQ represents the lowest amounts that can be precisely quantified. This result is also corroborated by the low recovery percent (accuracy) of the 3.2 nM AmB solution, indicating that samples with concentrations in this proximity will not be accurately quantified. In summary, the validation parameters analyzed in this project show that the method is adequate for detecting low concentrations of AmB (from 4.45 to 52.0 nM).

3.2 AmB extraction and determination of solubility of AmB in medium-chain triglyceride:

The yield of extraction was calculated according to Equation (4), where: AmB_{Oil} is the peak area of AmB from solution in oil after extraction and AmB_{DMSO} is the peak area of the control solution in DMSO.

$$Extraction\ yield = \frac{AmB_{Oil} \times 100}{AmB_{DMSO}} \quad (4)$$

The separation process using DMSO was successfully able to extract amphotericin B from Miglyol® 812 allowing drug from solutions in oil to be analyzed by HPLC. Based on calculation, relative extraction yield was approximately 81.0 %. All analytical concentration values of extracted samples in this study are represented as corrected values in respect to the dilutions and the yield of the extraction process, representing true sample concentration.

Extraction results for 0, 1, 2, 4, 8, 30 and 54 hours were approximately 81.3, 82.0, 79.3, 82.7, 80.2, 79.5, 81.8 %. Analysis of variance (ANOVA) showed no significant difference of these values at the $p < 0.05$ level for AmB oil solution samples extracted at all times. This result is relevant to future stability studies of amphotericin B in oil since is possible to note that once the drug is kept away from stress conditions, the extraction process may be conducted up to 50 h after experimental protocol involving the drug solution without any significant concentration change.

The HPLC method was used to analyze AmB oil solutions in order to determine the solubility of the drug in medium-chain triglyceride (Miglyol® 812). The samples contained a surplus of AmB and only the soluble content was analyzed periodically. Concentration values calculated based on the linear equation were found to be 1.03 ± 0.01 μM and 1.01 ± 0.02 μM for the duplicate. Regarding AmB in medium-chain triglyceride, the approximate AmB concentrations used for this study were < 1.0 μM.

3.3 Determination of AmB degradation pathway

3.3.1 Effect of adsorption on AmB loss of substrate

Adsorption can be chemically defined as the increase of density of fluids in the vicinities of interfaces or the change in concentration of a substance at the interface as compared with the neighboring phases [16]. This phenomenon has been described as responsible for the loss of substrate of different drugs due to adsorption to the surface of the solution´s container. Reports have been made regarding drugs, such as the anthracycline antibiotics, to strongly adsorb to medical devices during treatment [17, 18]. However, to this date it was still unclear if adsorption played a role on the loss of substrate of AmB in oil.

Figure 4 shows a plot of AmB concentration *versus* time, where reaction mixtures of AmB in Miglyol® 812 were stored in glass vials and exposed to different surface areas by adding different amounts of glass beads. Adsorption experiments in glass, other than other materials, are important once marketable formulations, such as Fungizone are stored in glass vials and degradation experiments are performed using several laboratory glassware.

FIGURE 4

At 60 °C, AmB appeared to degrade at the same rate regardless of the glass surface area, suggesting that this drug does not adsorb to this type of container material at given storage conditions, once it would be expected to find a difference at the remaining amount of AmB overtime according to the surface area if adsorption played a relevant role on the loss this drug. Differently, Mizutani and Mizutani (1977) observed the adsorption of several other drugs to glass surfaces using controlled pore glass as reference. Reports on the adsorption of epinephrine, atropine, physostigmine, insulin, and other drugs have been made [19].

3.3.2 Determination of aggregation state of AmB in reaction mixtures

In order to determine the aggregation state of AmB in methanol and Miglyol® 812, full-wavelength scan spectra were obtained from UV-Vis spectroscopy. This determination followed the understanding that the aggregation state of this drug can influence degradation rates and perhaps degradation pathways, as demonstrated by Lamy-Freund and collaborators [8], which studied the effect of different AmB aggregation forms on autoxidation in water:DMSO mixture. Monomeric and aggregated AmB UV-Vis scans are widely known and stablished by the literature [7, 8, 20]. AmB in its monomeric form shows four characteristics peaks with maximum absorbance at around 406-409 nm, whereas the aggregated AmB shows maximum absorbance at around 327 nm [20].

As observed for different organic solvents (Figure 5), AmB at 0.8 µg/mL can appear in different aggregation forms. AmB in methanol shows characteristic monomeric behavior, whereas AmB in chloroform appears to be in its aggregated form. AmB scan in Miglyol® 812 obtained from UV-Vis spectroscopy does not provide sustainable information to assure its aggregation state. Thus, for the purposes of further investigation, methanol was used as main solvent for investigations involving aggregation, once this organic solvent allows the identification of AmB aggregation state in UV-Vis spectroscopy.

FIGURE 5

3.3.3 Effect of radical initiator on AmB degradation

Autoxidation is known to happen in three stages: initiation, propagation and termination. It happens autocatalytically through free-radical intermediates. Molecular oxygen in its triplet state (ground state) is involved. The direct reaction between triplet state oxygen and unsaturated substrates is spin forbidden, once those compounds are mostly in singlet state. In order for autoxidation to happen, the spin barrier needs to be overcome by initiating mechanisms, such as the presence activated oxygen species or free radicals [21].

Based on AmB chemical moieties, oxidation of the polyene group and formation of epoxides is a likely degradation pathway depending upon the media and storage conditions. AmB had been proved to autoxidize in different solvents [6].

However, is was still unclear if this pathway is responsible for AmB loss in oil. The use of radical initiators to induce autoxidation at faster rates is a method of great relevance in the investigations of drug degradation pathways [22]. As suggested by the literature, AIBN can be used as a radical initiator in stress studies in temperatures starting at 40 °C [22]. Figure 6 shows a concentration time profile of AmB 0.8 µg/mL in Miglyol® 812. However, no significant change was observed between the drug decay of the reaction mixtures with and without the radical initiator. This might indicate that drug impurities already generate enough radicals to saturate AmB, and the excess of free radicals generated by AIBN have no influence on increasing degradation process.

FIGURE 6

Since other literature sources mention that temperatures higher than 40 °C should be used for complete decomposition of AIBN and radical formation, [23] different temperatures were tested (Figure 7).

FIGURE 7

Additionally, carbamazepine was used as a control drug in the oxidation by radical initiator experiment. This drug has its degradation scheme by oxidation known and reported in the literature [12]. Figure 8 shows that the addition of AIBN to a reaction mixture containing carbamazepine in Miglyol® 812 changed the degradation rate of this drug at 50°C, proving the effectiveness of this radical initiator in this oil. It is possible to observe that after 24h, carbamazepine concentration in the presence of

AIBN decayed 50% from the initial concentration (1.0 µg/mL), whereas in the the sample without AIBN, carbamazepine concentration remained almost unchanged over the course of 24h at 50°C, showing that AIBN is indeed effective at this given temperature in Miglyol® 812 to induce drug oxidation. From this experiment, it can be confirmed that AmB in Miglyol® 812 was not considerably susceptible to oxidation by radical initiator addition at low and mild temperatures (Figure 7). However, even without the use of AIBN, AmB degraded in oil, suggesting that the autocatalyzed oxidation in oil happened. This hypothesis was further explored in the following section by adding antioxidants to the reaction mixtures.

FIGURE 8

3.3.4 Effect of antioxidants on AmB degradation in Miglyol® 812

As described extensively by the literature, antioxidants can be classified based on how they are capable of stopping or preventing oxidation reactions and may be used to help understand the oxidation mechanism taking place in reaction mixtures. Antioxidants can act in different ways, such as quenching reactive oxygen, transferring electron, transferring hydrogen and chelating metals [24]. Figure 9 shows the concentration time profile of AmB in reaction mixture with different antioxidants. It was possible to observe that all studied antioxidants were capable of reducing the degradation of this drug, confirming that autoxidation is one of the degradation pathways of AmB in oil based on the mechanisms of the antioxidants.

FIGURE 9

After 72h, α-tocopherol was able to increase the remaining concentration of AmB in the reaction mixture from 16% to 48%. This molecule is considered a radical scavenger and acts a chain-breaking antioxidant. Each tocopherol molecule donates a hydrogen, generating a new non-radical and a tocopherol-derived radical, which reacts with another radical from the medium. Thus, each tocopherol molecule is able to eliminate two free-radicals [25]. It is noteworthy that its activity is temperature dependent. Its action is limited and non-existent above 100 and 150 °C, respectively [26]. By its effectiveness, it is possible to infer that in Miglyol, AmB or its impurities generate free radicals, undergoing autoxidation.

BHT and propyl gallate are both synthetic phenolic antioxidants. And similar to α-tocopherol, they donate hydrogen atoms, converting a radical to a non-radical and converting themselves in radicals that will react with other radicals to make stable compounds [27]. Although both antioxidants were effective at given concentrations, temperatures and reaction mixtures, propyl gallate was able to reduce the degradation of AmB greatly. The effectiveness of phenolic antioxidants may depend upon activation energy, rate constants, oxidation-reduction potential, volatility, heat susceptibility and solubility in the media, as well as the composition of the media [26]. Thus, proper choice of antioxidants for different media and conditions should take in account their mechanisms, but their effectiveness may vary greatly and should be determined experimentally. Further data on how these antioxidants effected AmB degradation in other oils are shown in Supplementary material.

3.3.5 Effect of antioxidant concentration

Concentration and solubility should be taken in consideration when choosing an antioxidant either for formulation or for degradation studies, as mentioned in the previous section. Based on solubility properties, BHT was chosen for further studies in methanol. Figure 10 shows a concentration time profile of AmB 0.8 μg/mL in methanol with different BHT concentrations.

FIGURE 10

It was possible to observe that BHT concentration did not affect considerably the loss of AmB in the studied conditions, showing that the amount of substract (BHT) needed for antioxidation did not affect the loss of AmB on the studied concentrations of BHT. Based on the assumption that most radicals were eliminated by hydrogen donation and covalent reaction with generated BHT-radicals and also that autoxidation depending upon peroxide radicals was fully terminated, we might consider that BHT did not stopped all AmB loss due to inherent lack of effectiveness of BHT on preventing autoxidation at given condition or that AmB is undergoing other degradation processes during autoxidation that also lead to loss of chromophore signal (reaction on the polyene).

3.3.6 Effect of different solvents on AmB Degradation

Media effect on chemical reactivity that leads to drug degradation can be approached according to different parameters, such as intermolecular forces, chemical interactions and solvent polarity [28]. Those parameters could be properly used when comparing aqueous media or organic solvents with known chemical properties. Regarding oils (non-pure solvents), their variable chemical composition makes it difficult for standardization of those parameters on the literature. Thus, the effect of different oils on a solute degradation may be better explained in terms of oil chemical composition. Considering that autoxidation happened in the studied conditions, as discussed in the effect of antioxidants on AmB loss, the reaction happened at different rates in different solvents (Figure 11).

FIGURE 11

An increase in AmB loss was observed in Soybean oil when compared to peanut oil. Although both oils are mainly composed of unsaturated fatty acids, soybean oil has a higher percentage of these compounds [29, 30]. Thus, making the media more susceptible to free radical generation under elevated temperatures. As observed in Figure 11, at 72h of storage at 50 °C protected from light, AmB appeared to be lost greatly in Miglyol ® 812, compared to Soybean oil and Peanut oil. Miglyol ® 812 is a mixture of medium-chain triglycerides (mainly caprylic/capric acids), making this oil less susceptible to be oxidized by free radicals. Different from the other oils, all radicals generated by AmB and/or its impurities (around 20% according to the fabricant) are likely to be consumed by AmB molecule itself, due to better oil stability, increasing AmB loss, whereas for the other oils, unsaturated molecules can also be oxidized by the consumption of free radicals.

AmB was more stable in methanol at the studied conditions (Figure 11). That might be related to the chemistry principles of the autoxidation reaction and oxygen reactivity. Autoxidation is determined by triplet oxygen (ground state/ unpaired spin) being homolytically broken and converted to free radical or acting as a biradical [24]. Computational mechanistic studies by Shayan and Vahedpour (2012) have shown that methanol reactivity with singlet state oxygen (responsible for other oxidation processes, such as, light catalyzed, see topic 3.3.10) is higher and generates more stable products than reaction with triplet state oxygen [31]. Therefore, smaller reactivity of triplet oxygen (the most found in oxygen molecules) with methanol in autoxidation conditions reduces AmB degradation.

3.3.7 Effect of oxidation on the aggregation state of AmB

UV-Vis spectroscopic studies were conducted in methanol in order to determine if AmB aggregation state changes overtime during oxidation. AmB 0.8 µg/mL and AIBN in methanol were stored at 50°C and periodically analyzed. Figure 12 shows that AmB absorbance decreased overtime and no aggregation state changes were observed by UV-Vis spectroscopy. The apparent peak increase at 350 nm was not due to aggregation change, but to the absorbance of AIBN, as shown in the same figure.

FIGURE 12

In addition, when a blank was run with AIBN (Figure 13.B), a non-typical scan presented itself (regarding aggregation state standard scans); However, in order to observe if aggregation species were present, DMSO was added to convert all possible aggregates to monomers and no spectrum change was observed, besides intensity due to the dilution, showing that the loss of AmB overtime in methanol does not happen due to change in aggregation state. To prove DMSO effectiveness in converting aggregational species, an AmB solution in water (18.5 µg/mL) was done and analyzed (Figure 13.A), where AmB presented itself as aggregated and a second solution of the same concentration containing DMSO was done (Figure 13.A) and AmB was converted to monomers due to DMSO presence in the solution.

FIGURE 13

3.3.8 Generation of degradation products by oxidation

Oil samples went through extraction in order to have their AmB content quantified by HPLC during previous studies. It is possible that detectable degradants were not extracted by the method developed for AmB. In order to simulate degradation generation by autoxidation without the influence of the extraction, a reaction mixture of AmB (160 μg/mL) and AIBN 0.7 mg/mL in methanol was analyzed by HPLC (Figure 14). AmB concentration was higher than the previous mentioned in this study in order to provide more substrate to generate higher amounts of degradants. Methanol was used instead of oil since by using that solvent no extraction step is needed.

FIGURE 14

We did not detect any new peaks being formed while AmB concentration decreased overtime in the presence of AIBN at 406nm. Additionally, the sample was analyzed in a wide range of wavelengths to investigate the formation of degradants with absorbance at different wavelength, and no new peaks were observed. By this result we can infer that the chromophore (polyene) was modified during degradation since there is no new peak being detected at 406 nm. It is possible that after oxidation, the heptaene chain of AmB formed a pentaene epoxide or different structures, based on the changes displayed by pentaene macrolides after oxidation [32].

3.3.9 Effect of temperature on the oxidation of AmB

Temperature usually affect chemical reactions because at higher temperatures a larger fraction of molecules possesses more energy than energetic reaction barriers.

Figure 15 shows the concentration time profile of AmB 0.8 μg/mL in Miglyol ® 812 at a range of temperature from 30 to 80 °C. Typically, reaction rates of drug degradation follows Arrhenius kinetics, which is a linear dependence of the natural log of the reaction rate versus the reciprocal of the absolute temperature [28]. Although AmB degradation increase with the temperature [33], In Figure 18 it is possible to observe overlap on the concentration of AmB overtime within different temperatures, showing clearly that there is no linear dependence on the loss of AmB regarding temperature change. The same behavior was observed for AmB in methanol (See Figures S1, S2, S3 and S4 from supplementary material).

FIGURE 15

Non-linear Arrhenius kinetics may be attributed to different parameters, such as pH shift or complex multistep degradation pathways. Additionally, peroxides, the primary intermediates in autoxidation propagation reactions are unstable at high temperatures and more stable at mild temperatures; moreover oxygen solubility decreases with increasing temperature [34].

3.3.10 Effect of light on the oxidation of AmB

Oxidation catalyzed by light is another oxidation reaction that might be responsible for AmB degradation. All studies until now were done under protection of light once this parameter can initiate oxidation reactions differently. Figure 16 shows the UV spectra of AmB in methanol under no thermal stress (25 °C) in two distinct environments, one protected from light and other exposed to light (UV and visible rays simultaneously, of 8 W/m² and 16800 lx of potency, respectively). As previously demonstrated, AmB was fairly stable over the course of 72h in methanol at low temperatures in the absence of any oxidation initiator (Figure 16.A). On the contrary, when exposed to light, AmB started to degrade immediately and after 72h of light exposure almost all AmB degraded.

FIGURE 16

Light catalyzed oxidation, or photosensitized oxidation is not autocatalytic as autoxidation. This process is dominated by singlet oxygen, which are highly oxidizing species that can directly react with organic species (spin allowed reactions). However, triplet oxygen from the environment (mostly responsible for temperature dependent degradation processes and autoxidation) must be converted to singlet oxygen in the presence of light [21].

Light catalyzed oxidation cannot be inhibited by chain-breaking antioxidants (BHT, propyl gallate and others), but by singlet oxygen quenchers (such as β-carotene). The rate of photosensitized oxidation is usually faster than that of autoxidation, once singlet state substrates react much faster with singlet oxygen than with triplet oxygen [21].

4. CONCLUSIONS

In this study, the method described proved to be reproducible and accurate with linearity (R^2 = 0.996) from 3.2 to 52.0 nM using a UV detector. AmB was successfully extracted from medium-chain triglyceride based oil prior HPLC analysis. The degradation pathway of AmB in oil was investigated. Adsorption did not appear to play a role in AmB degradation in oil. Based on our results, the aggregation state of AmB in oil is still unclear, thus it was not possible to infer if different aggregation forms change the degradation pathway of this drug. The solvents played a role on AmB degradation, where in methanol the drug appeared to be lost in a typical autoxidation process, whereas in medium-chain triglyceride the initiation of the autoxidation to happen fast, thus, not being detected in the performed experiments. The degradation pathways were proven to be related to oxidation processes and were found to be light-dependent and further investigations will be conducted in order to assess the kinetics of the process. Under dark conditions, the most likely pathway for AmB degradation was autoxidation. Whereas under light exposure, the most likely degradation pathway was light catalyzed oxidation.

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Tables

Table 1 – Average values of system suitability parameters with standard deviation and coefficient of variance percent

Sample	AmB 13.0 nM		
	Retention time (min)	**Peak area**	**Peak Height**
Average (n=6)	16.20	2669.50	110.50
S.D.	0.02	189.46	4.54
%CV	0.13	7.09	4.11

Notes: S.D.: Standard deviation; %CV: Coefficient of variance percent

Table 2 – Peak area averages with standard deviation and coefficient of variance percent obtained from between- and within-day precision and recovery % from accuracy analyses of amphotericin B standard solutions

AmB Concentration (nM)	**Between-day precision (n=4)**			**Within-day precision (n=3)**			**Accuracy (n=6)**
	PA	S.D.	% CV	PA	S.D.	% CV	Recovery %
3.2	655.83	81.12	12.37	674.66	10.50	1.55	72.75
13.0	2768.25	172.39	6.23	3151.67	198.65	6.23	87.57
52.0	11279.67	276.43	2.45	11869.33	96.20	0.81	101.86

PA: Average peak area; S.D.: Standard Deviation; % CV: Coefficient of variance.

Figures

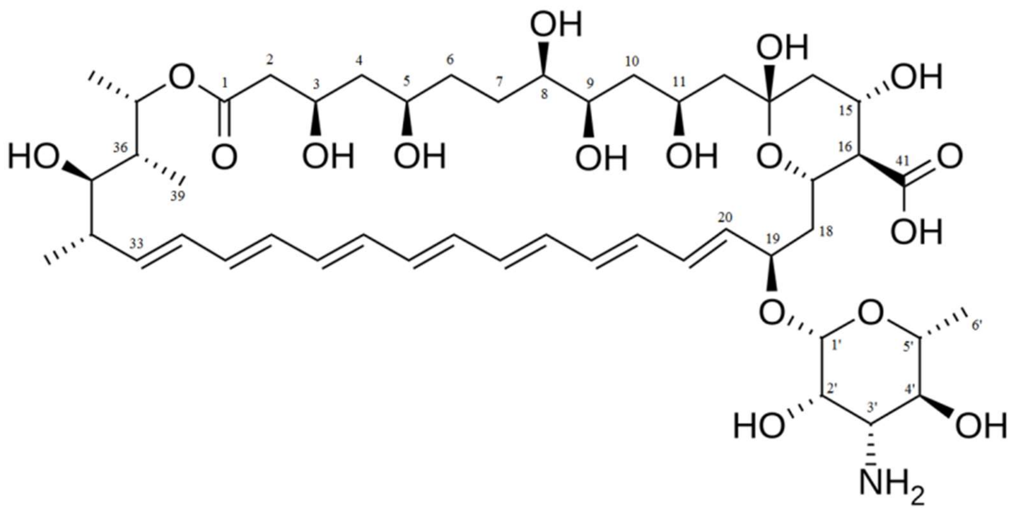

Figure 1 – Structure of amphotericin B showing the numbering of carbon atoms in the molecule.

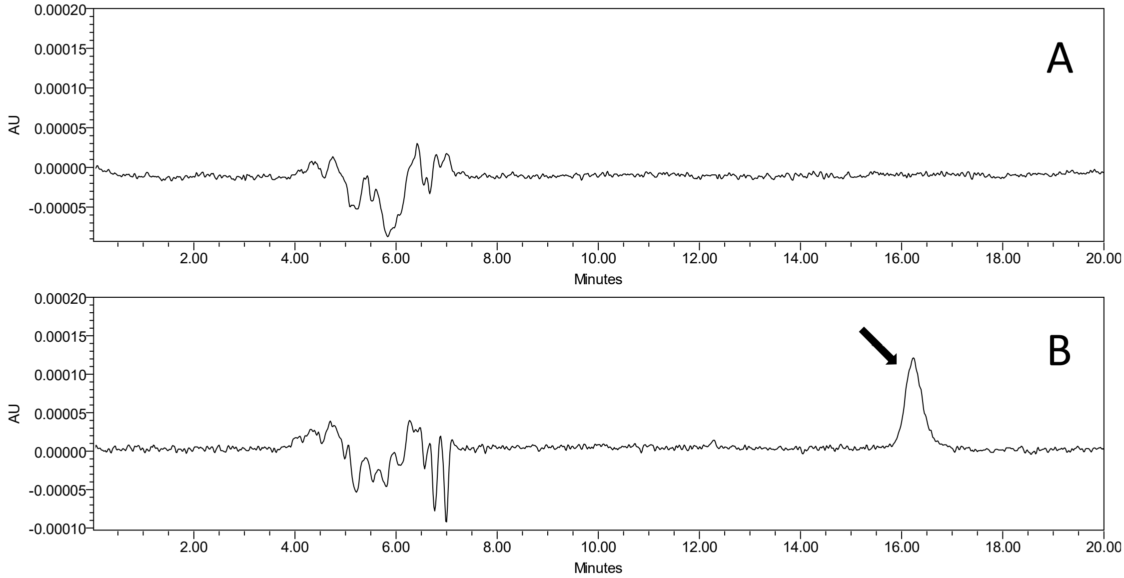

Figure 2 – Chromatograms obtained by isocratic elution using HPLC. A: blank (methanol:DMSO (3:2, v/v)); B: AmB 13.0 nM standard solution in methanol:DMSO (3:2, v/v).

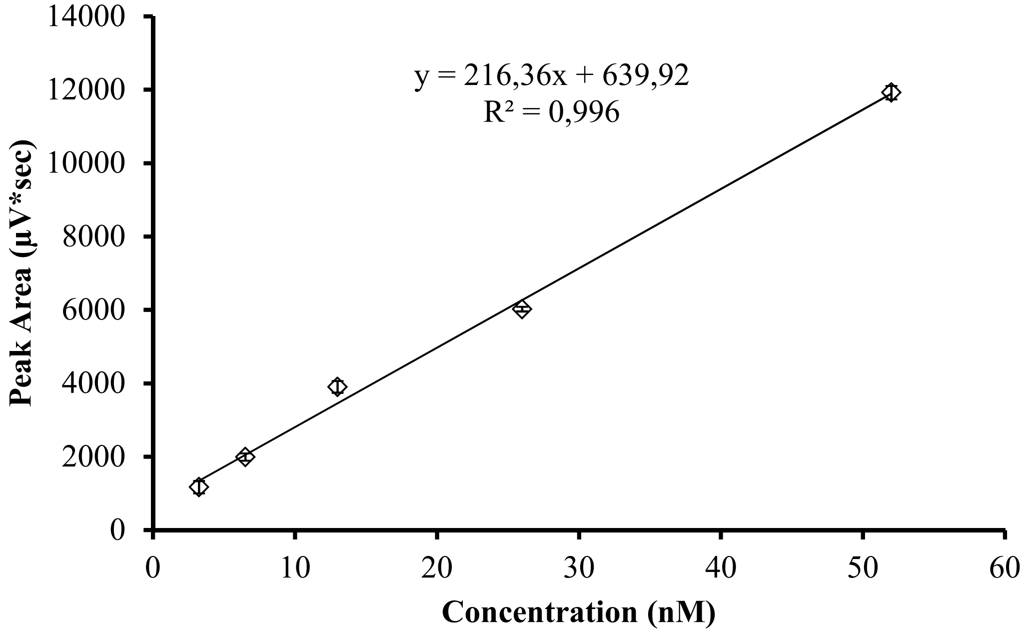

Figure 3 – Calibration curve of amphotericin B standard solutions in methanol:DMSO (3:2) (n=3).

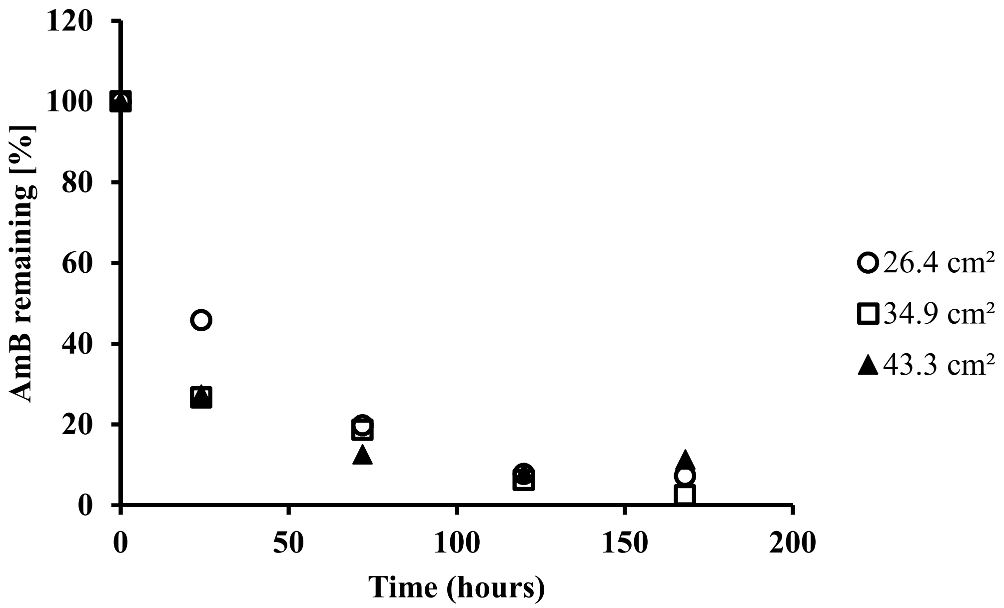

Figure 4 – AmB loss *versus* time plot. AmB concentration of 0.8 µg/mL in Miglyol® 812. Different surface areas were obtained by adding glass beads to the vial. 26.4 cm² (no beads), 34.9 cm² (30 glass beads), 43.3 cm² (60 glass beads).

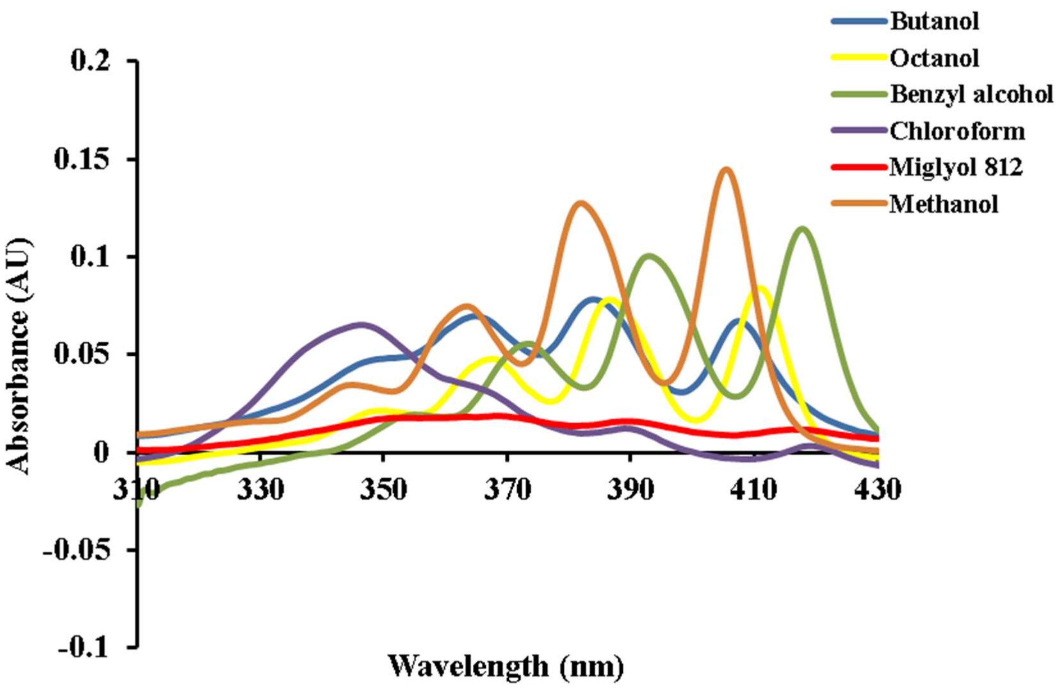

Figure 5 – Scan spectra of AmB 0.8 µg/mL in different organic solvents.

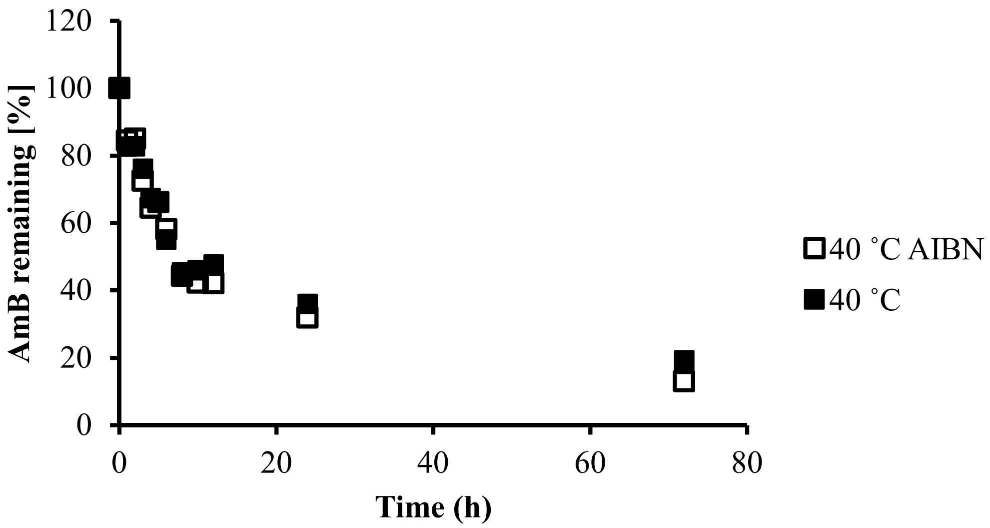

Figure 6 – AmB loss *versus* time plot. AmB concentration of 0.8 µg/mL in Miglyol® 812 at 40°C, with and without AIBN 0.7 mg/mL.

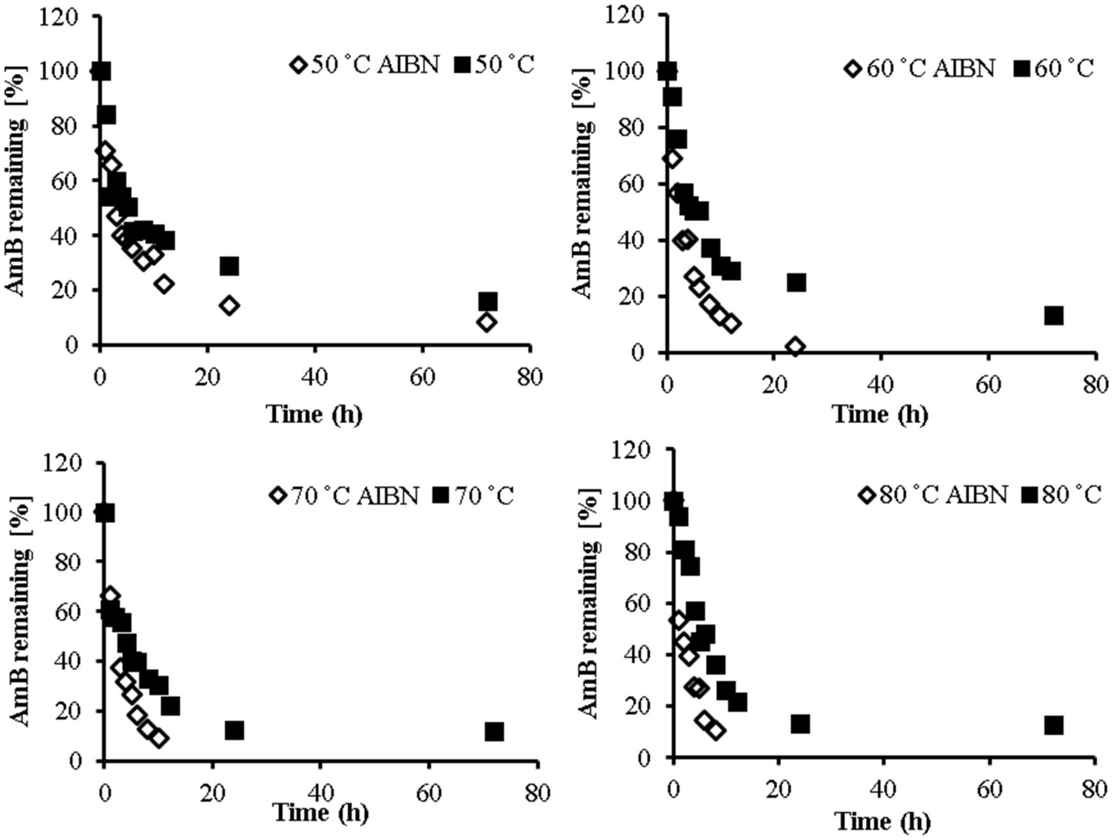

Figure 7 – AmB loss *versus* time plot. Reaction mixture of AmB0.8 µg/mL in Miglyol® 812 at different temperatures, with and without AIBN 0.7 mg/mL.

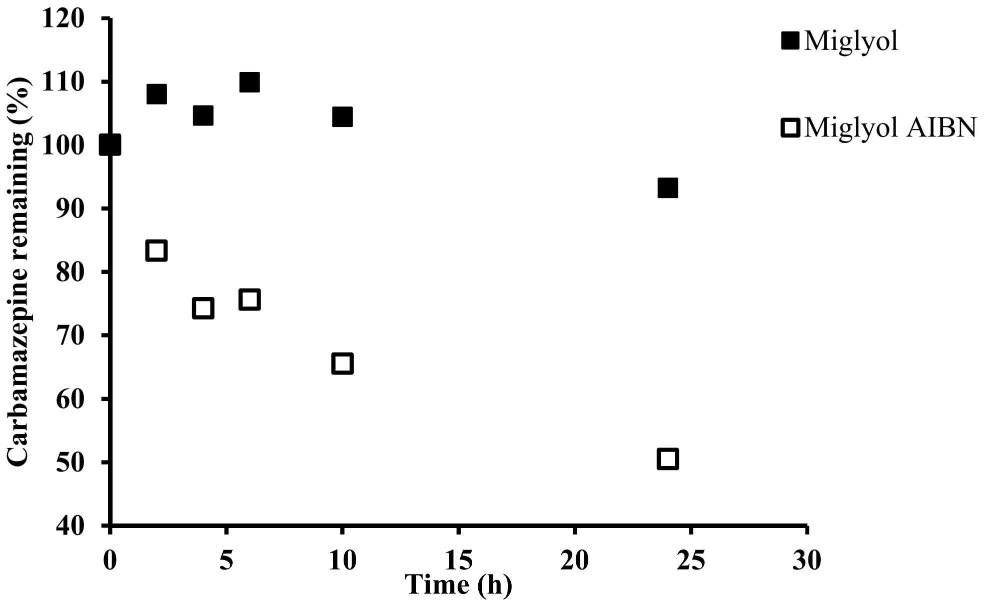

Figure 8 –Carbamazepine loss *versus* time plot. Reaction mixture of Carbamazepine 1.0 µg/mL in Miglyol® 812 at 50°C, with and without AIBN 0.7 mg/mL.

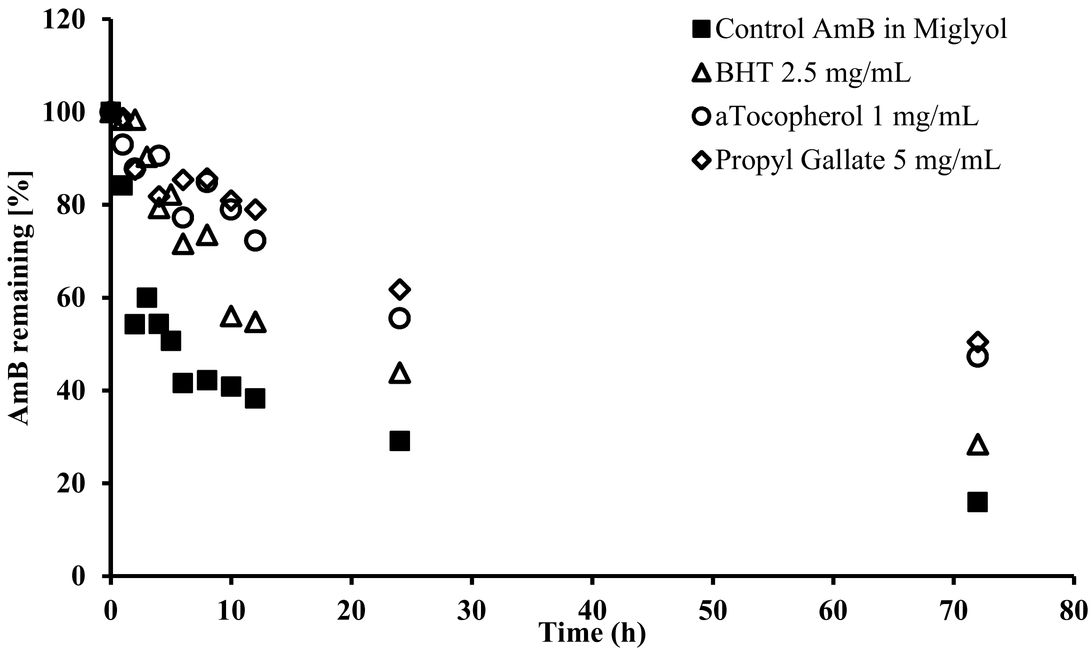

Figure 9 – AmB 0.8 µg/mL in Miglyol® 812 at 50°C (Control). Other samples were added of different antioxidants (BHT 2.5 mg/mL, α-tocopherol 1 mg/mL and propyl gallate 5mg/mL).

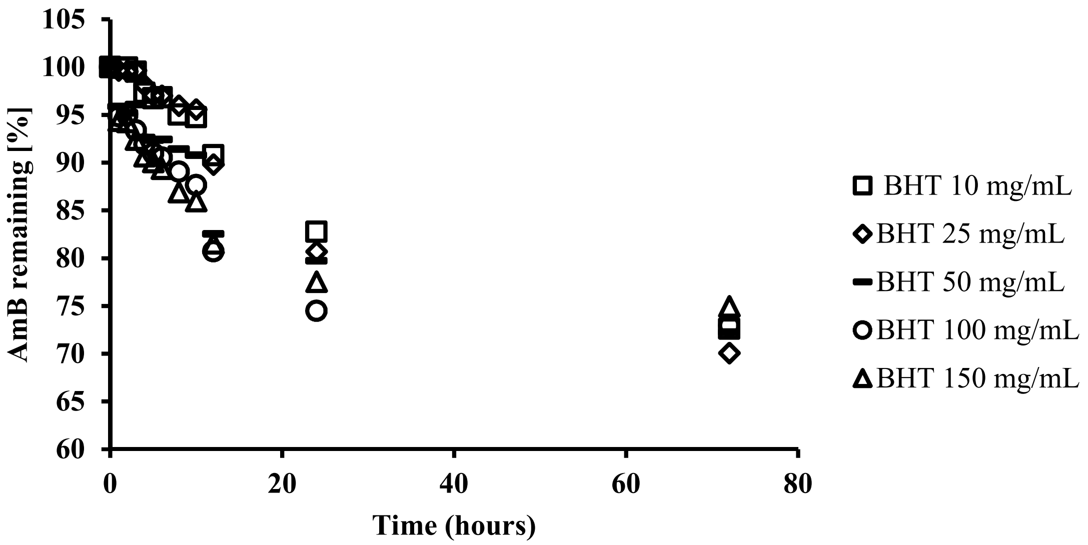

Figure 10 – AmB 0.8 μg/mL in methanol at 50°C added of different concentrations of BHT (10, 25, 50, 100 and 150 mg/mL).

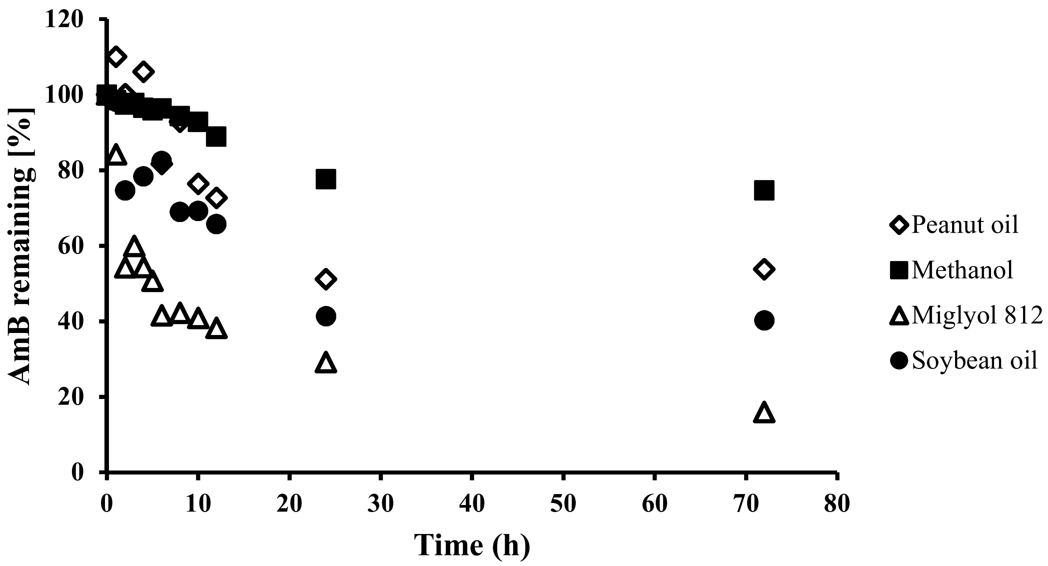

Figure 11 – AmB loss *versus* time plot. AmB concentration of 0.8 μg/mL in different solvents at 50 °C. Miglyol® 812, Soybean oil and Peanut oils were used for comparison among oils and methanol was used as reference organic solvent.

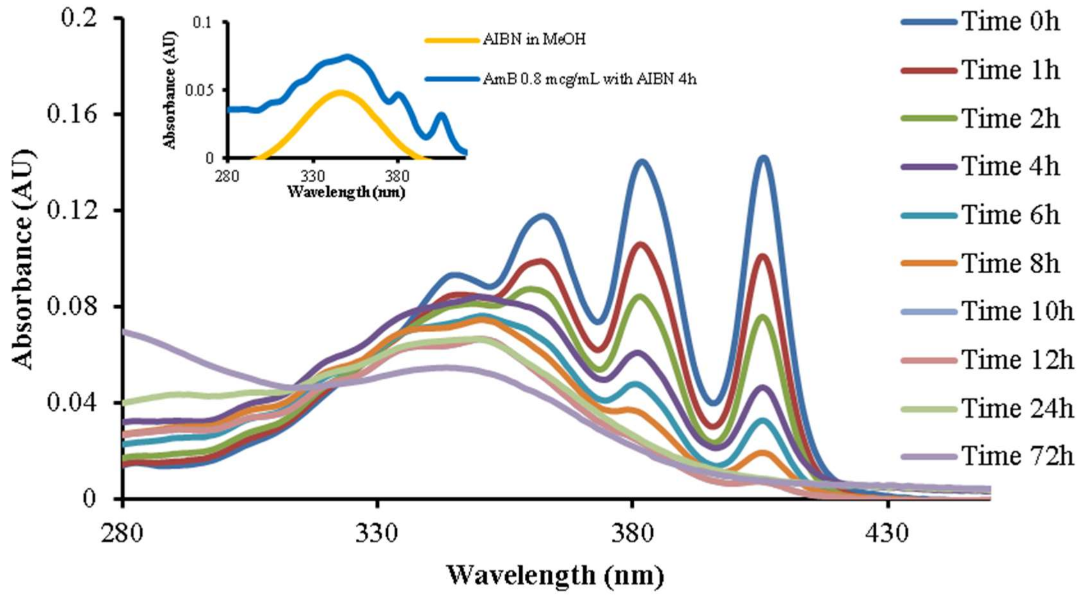

Figure 12 – Wavelength scan spectra of AmB 0.8 µg/mL in methanol stressed with AIBN 0.7 mg/mL at 50°C for over 72h. Wavelength scan (280 to 400 nm) of AIBN in methanol.

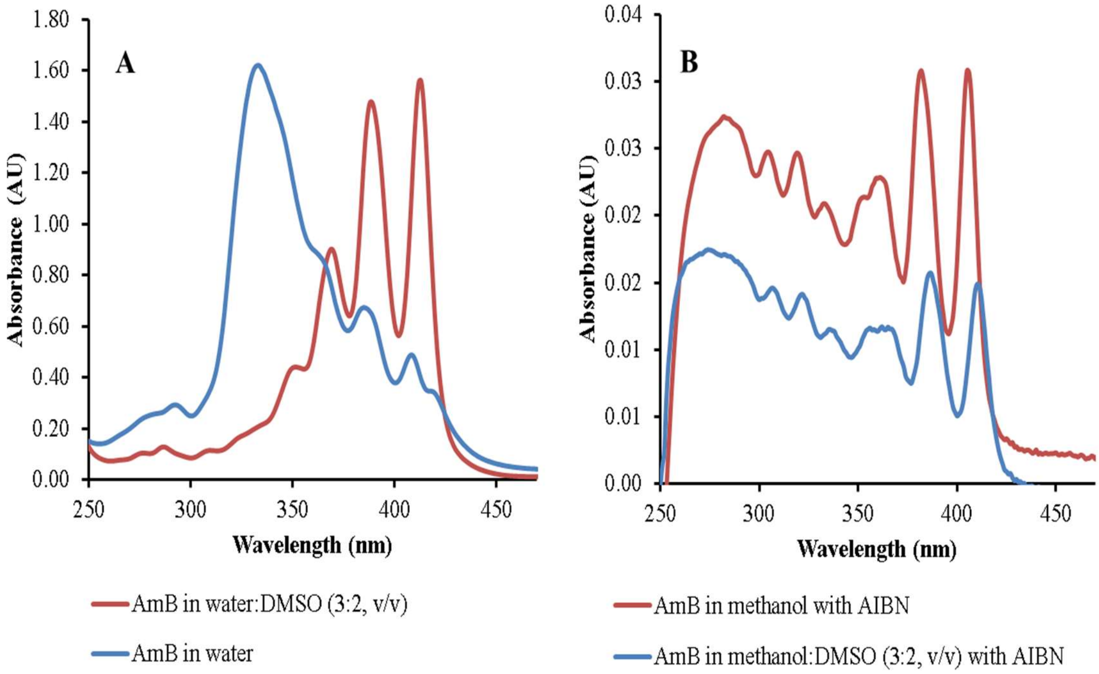

Figure 13 – Wavelength scan spectra of AmB. (A) Blue: AmB 18.5 µg/mL in water (aggregated state), Red: AmB in water 18.5 µg/mL:DMSO (3:2, v/v) (monomeric state). (B) Red: AmB 0.8 µg/mL and AIBN 0.7 mg/mL in methanol stressed at 50 °C for 4h. Blue: AmB 0.8 µg/mL and AIBN 0.7 mg/mL in methanol stressed at 50 °C for 4h diluted in DMSO (methanol:DMSO (3:2, v/v)) in order to observe if the sample aggregates after oxidized.

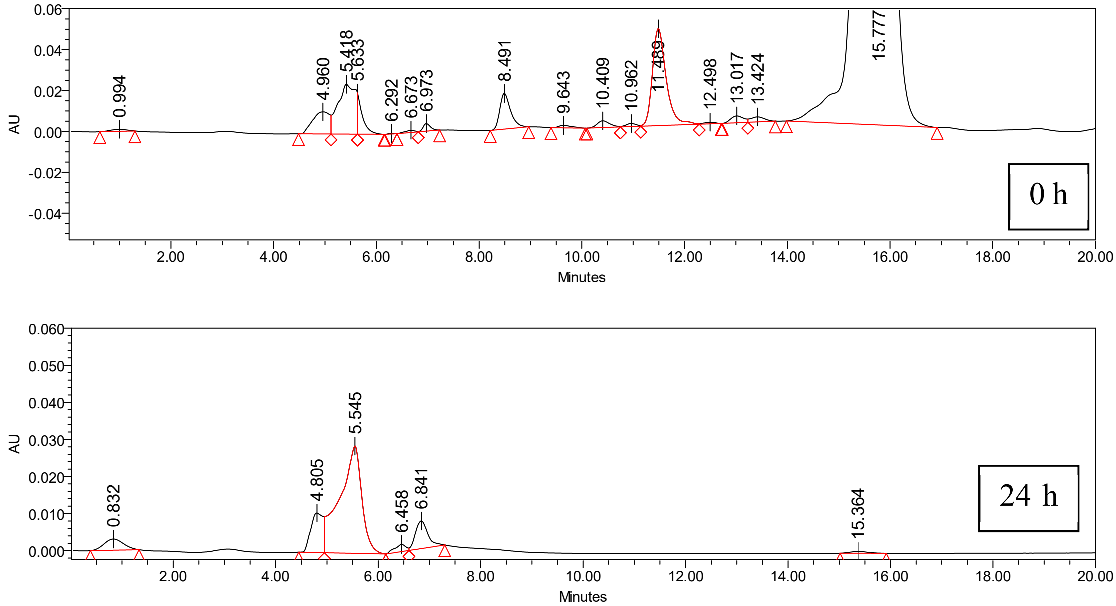

Figure 14 – Chromatogram of reaction mixture containing 160.0 µg/mL AmB and AIBN 0.7 mg/mL in methanol stressed at 50 °C at starting of reaction (0h) and after 24 hours.

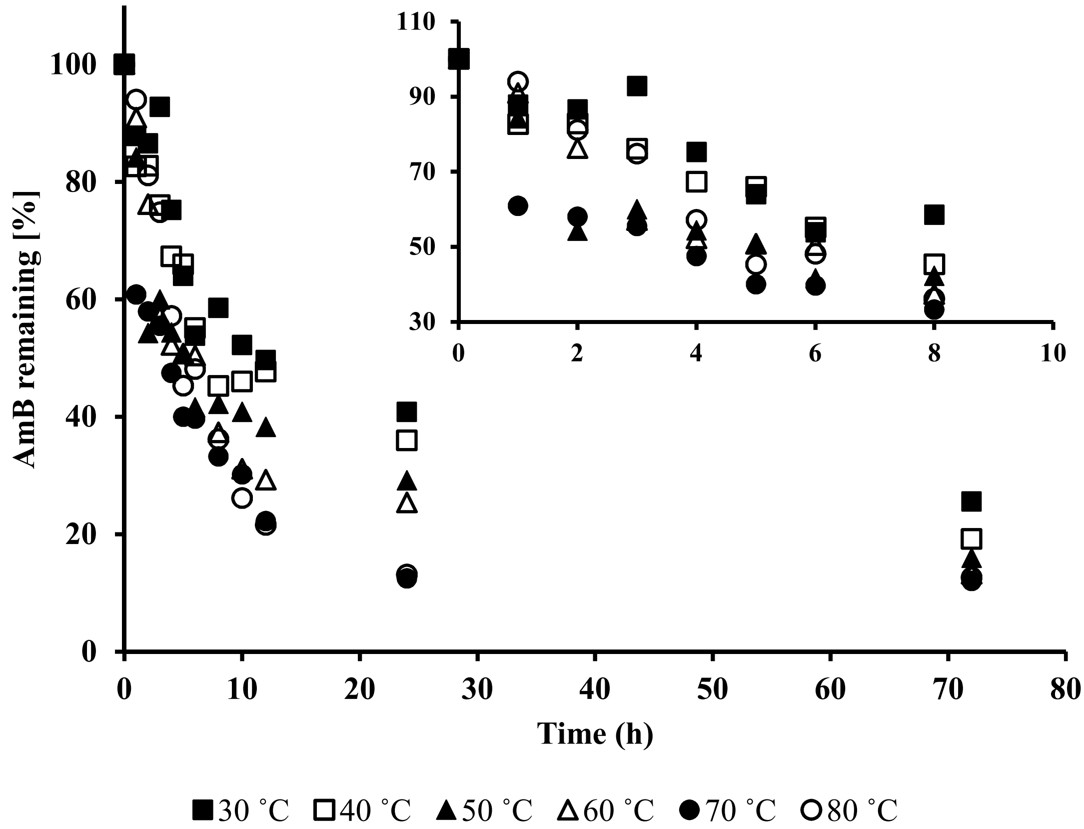

Figure 15 – The concentration time profiles of AmB (0.8 μg/mL) in Miglyol® 812 stressed at 30, 40, 50, 60, 70 and 80 °C for 72 hours.

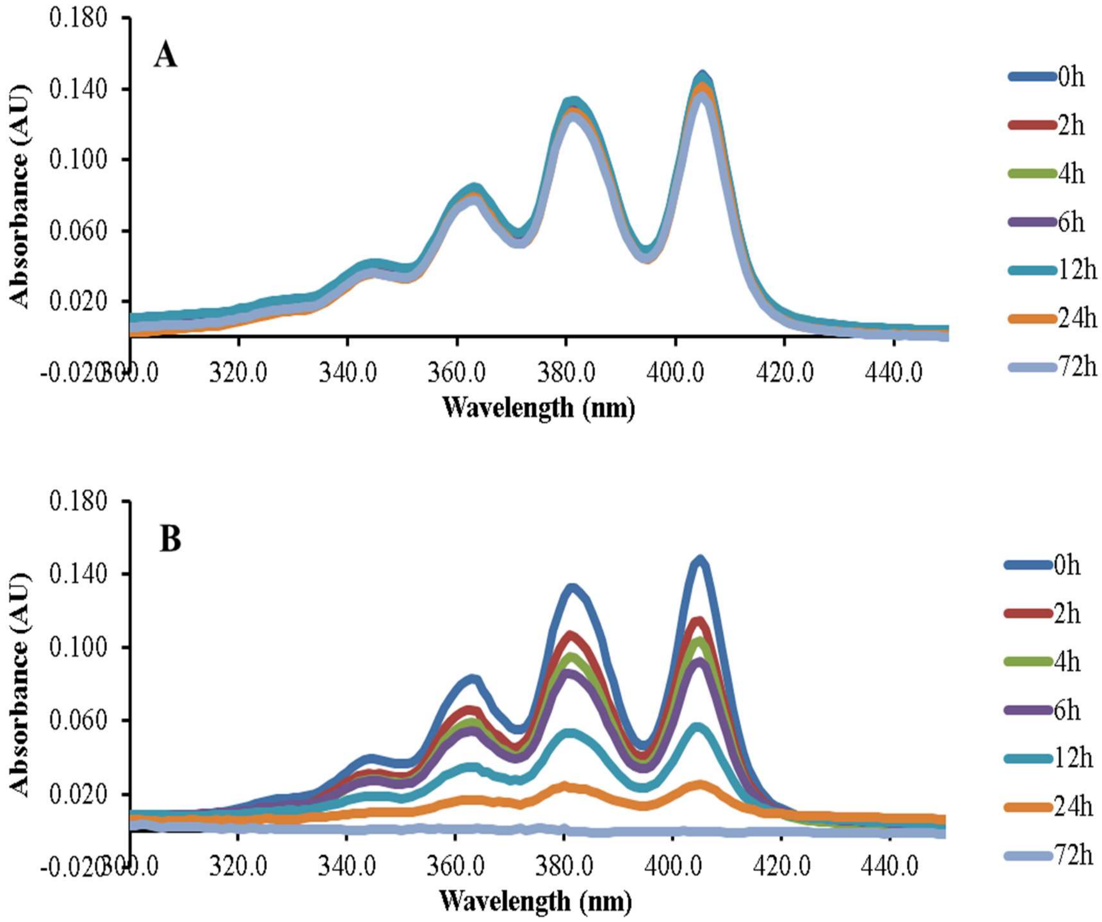

Figure 16 – Full- wavelength scan spectra of AmB 0.8 µg/mL from 0 to 72 h at 25 °C. A: sample protected from light. B: sample exposed to light (UV and visible rays simultaneously, of 8 W/m² and 16800 lx of potency, respectively).

SUPPLEMENTARY MATERIAL

Tables

Table S1 – Reaction mixtures used for different amphotericin B degradation pathway investigations

Substrate		Solvent	Temperature	Co-solute		Pathway test
Drug	Concentration			Type	Concentration	
Amphotericin B	0.8 µg/mL	Miglyol® 812	60 °C	-	-	Adsorption
Amphotericin B	0.8 µg/mL	Miglyol® 812	AT	-	-	Aggregation
Amphotericin B	0.8 µg/mL	Methanol	AT	-	-	Aggregation
Amphotericin B	0.8 µg/mL	Methanol	50 °C	AIBN	0.7 mg/mL	Aggregation under oxidation condition
Amphotericin B	0.8 µg/mL	Butanol	AT	-	-	Aggregation
Amphotericin B	0.8 µg/mL	Octanol	AT	-	-	Aggregation

Amphotericin B	0.8 µg/mL	Benzylic alcohol	AT	-	-	Aggregation
Amphotericin B	0.8 µg/mL	Chloroform	AT	-	-	Aggregation
Amphotericin B	0.8 µg/mL	Miglyol® 812	30 – 80 °C	-	-	Temperature effect
Amphotericin B	0.8 µg/mL	Methanol	30 – 50 °C	-	-	Temperature effect
Amphotericin B	0.8 µg/mL	Miglyol® 812	50 °C	AIBN	0.7 mg/mL	Effect of radical initiator
Amphotericin B	0.8 µg/mL	Miglyol® 812	50 °C	BHT	2.5 mg/ mL	Effect of antioxidant
Amphotericin B	0.8 µg/mL	Miglyol® 812	50 °C	propyl gallate	5.0 mg/mL	Effect of antioxidant
Amphotericin B	0.8 µg/mL	Miglyol® 812	50 °C	α-tocopherol	1.0 mg/mL	Effect of antioxidant
Amphotericin B	0.8 µg/mL	Methanol	50 °C	AIBN	0.7 mg/mL	Effect of radical initiator
Amphotericin B	0.8 µg/mL	Methanol	50 °C	propyl gallate	5.0 mg/mL	Effect of antioxidant

Amphotericin B	0.8 μg/mL	Methanol	50 °C	α-tocopherol	1.0 mg/mL	Effect of antioxidant
Amphotericin B	0.8 μg/mL	Methanol	50 °C	BHT	10.0 mg/mL	Effect of antioxidant concentration
Amphotericin B	0.8 μg/mL	Methanol	50 °C	BHT	25.0 mg/ mL	Effect of antioxidant concentration
Amphotericin B	0.8 μg/mL	Methanol	50 °C	BHT	50.0 mg/mL	Effect of antioxidant concentration
Amphotericin B	0.8 μg/mL	Methanol	50 °C	BHT	100.0 mg/mL	Effect of antioxidant concentration
Amphotericin B	0.8 μg/mL	Methanol	50 °C	BHT	150.0 mg/mL	Effect of antioxidant concentration
Amphotericin B	0.8 μg/mL	Soybean oil	50 °C	AIBN	0.7 mg/mL	Effect of radical initiator

Amphotericin B	0.8 µg/mL	Soybean oil	50 °C	BHT	2.5 mg/ mL	Effect of antioxidant
Amphotericin B	0.8 µg/mL	Soybean oil	50 °C	propyl gallate	5.0 mg/mL	Effect of antioxidant
Amphotericin B	0.8 µg/mL	Soybean oil	50 °C	α-tocopherol	1.0 mg/mL	Effect of antioxidant
Amphotericin B	0.8 µg/mL	Soybean oil	50 °C	-	-	Effect of antioxidant
Amphotericin B	0.8 µg/mL	Peanut oil	50 °C	AIBN	0.7 mg/mL	Effect of radical initiator
Amphotericin B	0.8 µg/mL	Peanut oil	50 °C	BHT	2.5 mg/ mL	Effect of antioxidant
Amphotericin B	0.8 µg/mL	Peanut oil	50 °C	propyl gallate	5.0 mg/mL	Effect of antioxidant
Amphotericin B	0.8 µg/mL	Peanut oil	50 °C	α-tocopherol	1.0 mg/mL	Effect of antioxidant
Amphotericin B	0.8 µg/mL	Peanut oil	50 °C	-	-	Effect of antioxidant
Amphotericin B	160.0 µg/mL	Methanol	50 °C	-	-	Degradant generation
Amphotericin B	160.0 µg/mL	Methanol	50 °C	AIBN	0.7 mg/mL	Degradant generation

Carbamazepine	1.0 µg/mL	Miglyol® 812	50 °C	-	-	Radical initiator effectiveness
Carbamazepine	1.0 µg/mL	Miglyol® 812	50 °C	AIBN	0.7 mg/mL	Radical initiator effectiveness
Carbamazepine	1.0 µg/mL	Soybean oil	50 °C	-	-	Radical initiator effectiveness
Carbamazepine	1.0 µg/mL	Soybean oil	50 °C	AIBN	0.7 mg/mL	Radical initiator effectiveness

Figures

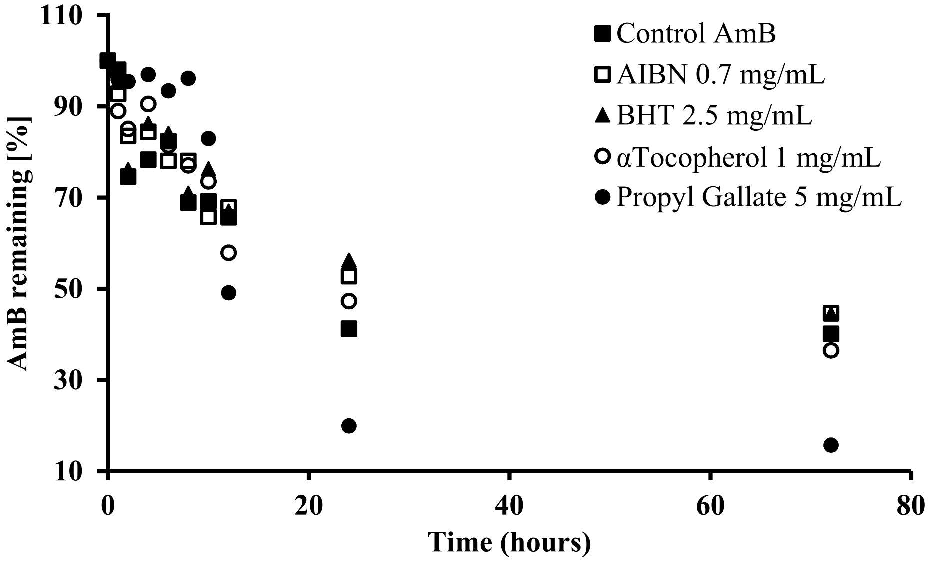

Figure S1 – AmB 0.8 µg/mL in Soybean oil at 50°C (Control). Other samples were added of AIBN 0.7 mg/mL and different antioxidants (BHT 2.5 mg/mL, α-tocopherol 1 mg/mL and propyl gallate 5mg/mL).

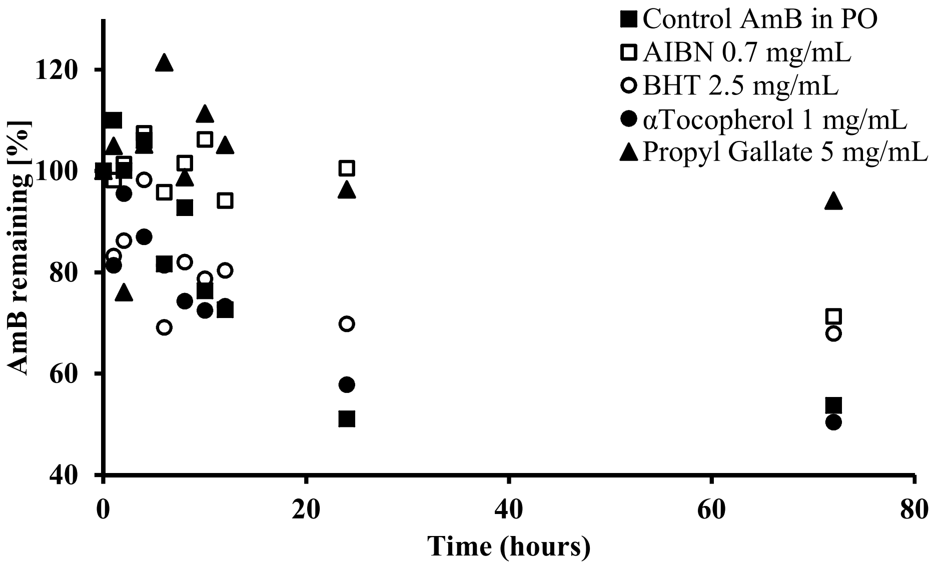

Figure S2 – AmB 0.8 μg/mL in Peanut oil at 50°C (Control). Other samples were added of AIBN 0.7 mg/mL and different antioxidants (BHT 2.5 mg/mL, α-tocopherol 1 mg/mL and propyl gallate 5mg/mL).

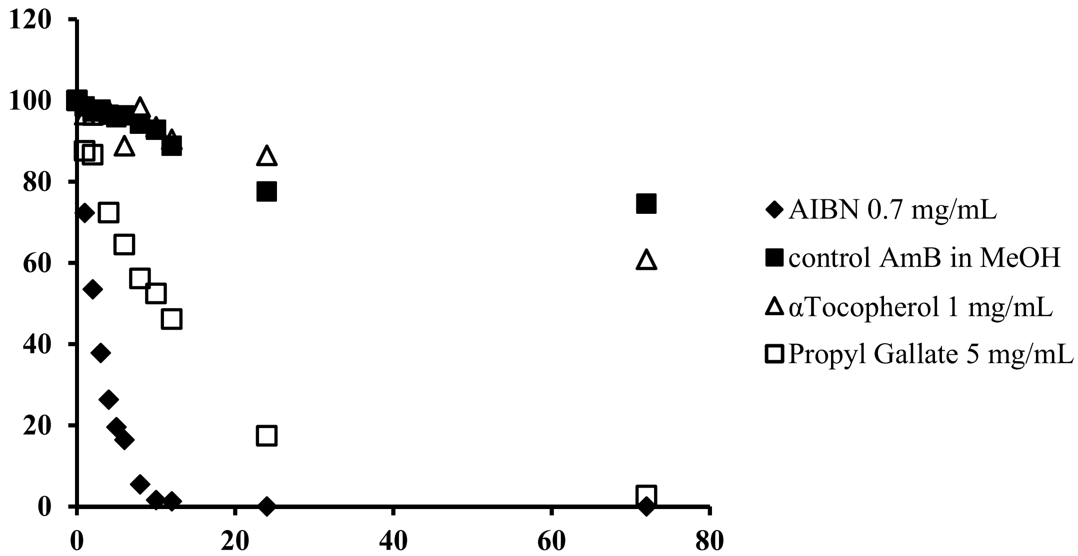

Figure S3 – AmB 0.8 μg/mL in methanol at 50°C (Control). Other samples were added of AIBN 0.7 mg/mL and different antioxidants (α-tocopherol 1 mg/mL and propyl gallate 5mg/mL).

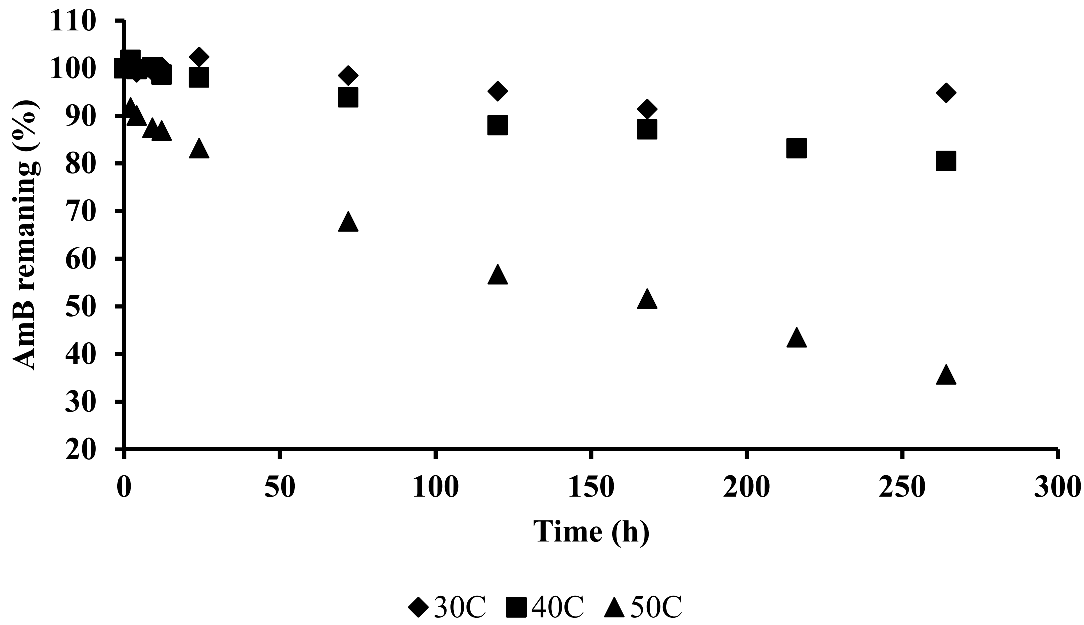

Figure S4 – The concentration time profiles of AmB 0.8 µg/mL AmB in methanol stressed at 30, 40 and 50 °C for 264 hours.

HIGHLIGHTS

- Amphotericin B in medium chain triglyceride does not adsorb to glass surface.
- Amphotericin B does not change its aggregation state after autoxidation in medium chain triglyceride.
- Amphotericin B undergoes autoxidation in oil.
- Amphotericin B undergoes light catalyzed oxidation under light exposure.

CHAPTER 3

This chapter contains a research paper manuscript regarding the degradation kinetics of AmB in different oils and in organic solvents. The work features degradation scheme based on data and literature evidence, as well as a mathematic model for attempting to predict kinetic parameters. The suggested model generated empirical data, which were tested against the experimental data to investigate how the model fits the experimental results. Such results were showed for different conditions, such as temperatures and solvents.

We intend to submit this research article to the Journal of Pharmaceutical and Biomedical Analysis upon completion and editing of this manuscript.

Studies on amphotericin B instability in lipid-based media, Part II: Kinetics of amphotericin B degradation

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ABSTRACT

The aim of this study is to determine a degradation scheme for amphotericin B degradation under different environmental conditions, such as light exposure, different temperatures and solvents, as well as to estimate the kinetic parameters involved based on an empirical degradation model. For those purposes we recollect experimental data and findings from the previous work (Part I) for development of schemes and models. Differential equations were developed to explain AmB loss process. A kinetic model was successfully used to describe AmB loss in different solvents under dark environment. Under this condition the loss of AmB involved autoxidation as already described in previous studies and confirmed by this model. In addition, AmB loss undergoes a complex degradation pathway, once a simple autocatalytic model could not describe its loss. In fact, a reversible loss probably related to hydrolysis might be involved, as demonstrated by the scheme and the kinetic model. A second model described AmB loss under light exposure successfully. AmB loss in this condition showed to be pseudo-first order, corroborating literature data on photocatalytic degradation of pharmaceutical molecules. Due to complex degradation pathway, the kinetics of the different processes leading to AmB degradation could not be distinguished.

Keywords: Amphotericin B; Autoxidation; Photocatalytic oxidation; Drug Degradation.

1. INTRODUCTION

Amphotericin B (AmB) is an antifungal and antiprotozoal drug that was originally isolated from *Streptomyces nodosus*. Chemically, this molecule has a macrocyclic lactone ring composed of 37 carbons, with a heptaene chain from carbon 20 to carbon 33, providing a non-polar characteristic to the molecule [1]. The opposite side has seven hydroxyl groups and one carboxyl group providing some polarity to AmB. As a result, this molecule has amphiphilic properties [2]. An amino sugar group (3-Amino-3,6-dideoxy-β-D-mannopyranose) is connected to the main macrocyclic ring by an *o*-glycoside bond. AmB is a zwitterion with amphoteric properties due to the presence of two ionizable groups, a carboxyl (pK_a 5.5) and primary amine (pK_a 10) [3, 4]. Figure 1 shows AmB chemical structure.

FIGURE 1

To overcome drawbacks such as low solubility and toxicity, this drug has been delivered in lipidic carries opposed to its traditional formulation with deoxycholate (micellar system) [5]. Although being an old molecule, little is known regarding its degradation scheme and kinetics in lipid media. Chemical stability, hence, degradation and kinetic studies are of great relevance when it concerns safety and efficacy of drug products [6, 7]. When it comes to AmB, its degradation/toxicity relation has not been fully elucidated, as its toxicity is mostly attributed to its aggregation state. However, if drug degradation plays a role on toxicity is still a matter to be discussed more widely. To begin the study of this hypothesis, studies involving AmB behavior in separate solvents that compose lipidic complex drug delivery systems are required. Such studies start with elucidating possible degradation pathways and then investigating degradation kinetics and degradants structures.

Studies by Lamy-Freund, Ferreira and Schreier demonstrated that AmB undergoes autoxidation in a mixed media composed of Water/DMSO. This study investigated only the formation of free radicals opposite from loss of substrate [8]. Rickards and Smith [9] studied the autoxidation of polyenes of the filipin complex and lagosin. These molecules are similar in structure to AmB as they are macrocyclic lactone rings with polyene chains in one of their sides. However, different from AmB, they contain pentaene chains whether than heptaene. The study found that concentrated solutions of lagosin, at dark conditions, were susceptible to loss, which was slightly prevented by the addition of Butylated Hydroxyanisole (BHA). The study also confirmed conversion from pentaene groups to tetraene after degradation with epoxide formation [9].

In a previous study conducted by our group in which AmB was in methanol and oil solutions, we showed that autoxidation is only one of the possible oxidation pathways that this drug can undergo, depending upon storage conditions [10]. It was also discovered that aggregation state does not change along stress testing and that adsorption does not play a role on AmB loss when soluble in oil. The study showed that AmB loss was increased in methanol in the presence of a radical initiator (AIBN) and that in the presence of several antioxidants the loss of AmB decreased in different oils. Antioxidants responsible for hydrogen donation and radical scavenging were used. These results confirmed that autoxidation is one of the degradation pathways of AmB in oil under light protection. However, the degradation of AmB in oil was not temperature dependent, suggesting that multiple and complex degradation pathways could be involved. In the light of this hypothesis, another condition was studied. When exposed to light, at ambient temperature and no radical initiator, a fast rate degradation reaction took place. Thus, this drug can also undergo a different oxidation mechanism, photosensitized or photocatalytic oxidation [10].

This study takes place after the Article titled “Studies on amphotericin B instability in lipid-based media, Part I: Identification of degradation pathways as function of mixture conditions”, as it provides further investigation on amphotericin B instability phenomena in lipid-based media. The aim of this study is to determine a degradation scheme for AmB degradation under different storage conditions in different oil reaction mixtures as well as to estimate the kinetic parameters involved based on an empirical degradation model. For that purpose, we herein recollect experimental data and findings from the previous work (Part I) for development of schemes and models.

2. METHODS

2.1 Determination of degradation scheme and model for substrate loss under dark and light exposure

A proposed degradation scheme was proposed for AmB loss under dark conditions, at 0.86 µM in different solvents, stored from 30 to 80 °C. The fraction of AmB loss under these conditions was calculated according to Equation 1, where AmB0, AmBt and AmB∞ were initial AmB concentration, AmB concentration at each given time point and remaining AmB concentration at the end of the degradation plot, respectively.

$$Fraction\ of\ AmB\ loss = 1 - \left(\frac{AmB_0 - AmB_t}{AmB_0 - AmB_\infty}\right) \quad (1)$$

The plot of AmB loss fraction in Miglyol® 812 and Methanol under the conditions above versus time are shown in Figures 2 and 3, respectively.

FIGURE 2

FIGURE 3

A proposed scheme was developed, as well as the differential equations for the loss of AmB under these conditions based on empirical estimation and degradation profile analysis. An empirical model was developed according to the degradation profiles mentioned above and previous studies regarding degradation pathways in the same conditions. Figure 3 shows a loss of AmB consistent with autocatalytic degradation, consistent with sigmoidal profiles displayed by autoxidation reactions. This is consistent with the information provided in previous studies in methanol [10]. On the other hand, the profile displayed in Figure 2 for AmB in Miglyol® 812 does not show the characteristic sigmoidal shape, instead is more consistent with log-linear biphasic degradation. Degradation scheme for fitting both types of profiles is shown and discussed in the results section.

Similarly, the fraction of AmB loss in methanol under light exposure at 25°C was calculated according to Equation 1. Degradation scheme, differential equation and degradation model based on empirical estimation were also developed and are described in the results section.

2.2 Model parameters estimation method

The Advanced Modeling and Simulation Tool Kit (AMASTK, UI Copyright 2012) was used for nonlinear optimization, simulation and kinetic parameter estimation. AMASTK was developed by Dr. Stephen Stamatis in R, an open access programming language and software environment, which was used for data analysis, calculations and graphical display. A package for R named deSolve was used to integrate the ordinary differential equations (ODE) described in the previous sections according to each condition described [11]. Another package (FME) was necessary to fit the experimental data to model output concentration as a function of time [12].

3. RESULTS AND DISCUSSION

3.1 Model for substrate loss under dark storage

The fraction of AmB versus time profile of AmB in medium chain triglyceride shown in Figure 2 is not consistent with traditional autoxidation sigmoidal profiles. However, according to previous studies, it is known that AmB undergo autoxidation in this solvent at 50°C [10]. On the contrary, degradation profile of AmB in methanol (Figure 3) is sigmoidal; However, empirical models consistent with only reversible loss of substrate or autocatalysis did not fit properly the observed data for both solvents. Thus, these profiles may be representative of a complex degradation scheme involving multiple pathways, including autoxidation [13]. A proposed scheme describing the loss of AmB in this condition is shown in Figure 4.

FIGURE 4

The proposed scheme involves both autoxidation, where an oxidized fraction of AmB is also included as substrate (AmB_{ox}) and a reversible loss compatible with possible hydrolytic process due to residual moisture in the oil sample. Hydrolysis experimental conditions were not previous investigated for oil solutions; However, the literature reports that oils stored under different humidity levels show different values of peroxide values and acid index, as those indexes increase with increasing humidity and they are related to increased chemical instability [14]. Additionally, drug degradation depend on water activity on the solvent, which might result from different characteristics of the reactions such as temperature and the solvent itself, besides the amount of residual water on the medium [15].

The differential equation that represents the AmB loss is shown in Equation 2. The initial fraction of AmB_{ox} was a fix parameter used to determined kinetic parameters and UC is used for Unknown Compounds.

$$\frac{dfAmB}{dt} = -(k_1 + k_{ox})\,(AmB)\,(AmB_{ox}) + k_{-1}\,(UC) \quad (2)$$

The proposed differential equation based on the empirical degradation scheme was fed to the Advanced Modeling and Simulation Tool Kit (AMASTK). Estimated rate constants are shown in Table 1, values were optimized by fitting observed data to the model predicted using AMASTK.

TABLE 1

AmB loss in Miglyol® 812 from 30 ºC to 80º did not show to be linear, as the rate constants did not increase proportionally with the temperature (Table 1). Chemical processes that do not follow Arrhenius kinetics may be explained by several factors, such as: phase transitions; change in oxygen solubility in the media over a different range of temperature; and multi-step reaction pathways, as one of reaction steps of pathway might depend on a temperature barrier to be overcome, the reactions can occur differently at different temperature range [16].

Observed data, are in good agreement with the predicted model. Figures 5 and 6 shows that the model was suitable for either degradation profiles of AmB in Miglyol 812 and methanol, suggesting that AmB indeed undergo different pathways in different solvents and that this model can account for both complex pathway adequately.

FIGURE 5

FIGURE 6

The model was also applied to determining theoretical fraction values of AmB overtime for different oils used in the study. The predicted model was in agreement with the observed data of AmB in Peanut oil (Figure 7). The plot shows data points that did not fit the predicted model adequately. However, this is probably due to experimental scatter, once the predicted values line follows the overall degradation trend of the observed data. Similarly, Figure 8 shows the plot of the predicted values from the model and observed data of AmB loss in Soybean oil.

FIGURE 7

FIGURE 8

3.2 Model for substrate loss under light exposure

According to literature, photochemical oxidation follows first order kinetics [17, 18]. Although having a similar reaction mechanism to autoxidation, the reaction initiated by light happens at faster rates, thus, showing no characteristic initiation lag decay, as in the autoxidation processes. The result is a pseudo-first order reaction [17, 18].

Accordingly, Figure 9 is AmB 0.86 µM degradation scheme at 25 °C in methanol stored under light exposure (Visible and UV-light). The scheme shows a direct loss of AmB to unknown degradation products under light.

FIGURE 9

The loss of AmB under this condition can be described by the differential equation 3.

$$\frac{dfAmB}{dt} = -k_{ox}(AmB) \quad (3)$$

Rate constant estimation showed a k_{ox} of 0.076 h^{-1} that represents the overall rate of AmB loss at this condition. The model was in good agreement with the observed data as shown in Figure 10.

FIGURE 10

Photodegradation of AmB is phenomenon already well established in the literature [19, 20]; However, its kinetic study and its difference from oxidation degradation profiles under light protection was not well stablished in the literature. Previous study showed the difference in degradation pathway between light catalyzed oxidation and autoxidation [10]. The empirical models developed in this work support the difference between these processes in non-aqueous solvents, since they showed to be in agreement with our experimental data.

3.3 AmB degradation products by oxidative pathways

Based on the results obtained from previous study [10] and on the good agreement of the empirical models with the observed degradation data on this study, it is well stablished that AmB undergoes oxidation processes. As hypothesized and showed on the degradation scheme for AmB loss under light protection, a complex degradation pathway may be involved with its loss; However, autoxidation is one of the processes that can be confirmed to happen. In addition, under light catalysis, this drug is also oxidized.

Based on AmB moieties, different chemical groups could be target of chemical degradation reactions [21]. Based on basic chemistry, the group most likely to be oxidized first on AmB is the conjugated polyene [22]. Gagós and Czernel [22] induced AmB oxidation in aqueous media, at different pH and suggested possible degradants based on fluorescence studies. The study is in agreement with the findings of Rickards and collaborators [9], which determined that the polyene macrolides lagosin and filipin undergo oxidation and that the target group of the reaction is the polyene chain.

Polyene chain in AmB molecule goes from carbon 20 to carbon 33 [1], where the central double bonds are less reactive due to resonance. Thus, the double bonds of the two ends of the polyene chain are more susceptible to oxidation. The prime product of this oxidation process generates epoxide groups, regardless of the medium, as shown by Rickards and Collaborators [9]. Nevertheless, if the medium is aqueous or an organic medium with high humidity levels, the epoxide can hydrolyze and generate dialcohols, as described for oxidation in aqueous medium by Gagós and Czernel [22].

Further spectroscopic, chromatographic and drug synthesis studies are required to accurately isolate, identify and synthetize drug degradation products from AmB reaction mixtures in different conditions.

4. CONCLUSIONS

An empirical degradation scheme was developed and differential equations were developed to explain AmB loss process. A kinetic model was used to describe AmB loss in different solvents under dark environment with success. Under this condition the loss of AmB involved autoxidation as already described in previous studies and confirmed by this model. In addition, AmB loss undergoes a complex degradation kinetics, once a simple autocatalytic model could not describe its loss. In fact, a reversible loss probably related to hydrolysis might be involved, as demonstrated by the scheme and the kinetic model. A second model described AmB loss under light exposure successfully. AmB loss in this condition undergoes a pseudo-first order kinetics, corroborating literature data on photocatalytic degradation of pharmaceutical molecules. Due to complex degradation pathway, the kinetics of the different processes leading to AmB degradation could not be distinguished.

5. ACKNOLEDGMENTS

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Tables

Table 1 – Estimated rate constants for amphotericin B degradation at different reaction conditions under light exposure (obtained by using AMASTK®, for estimations and optimization)

Solvent	Temperature	k_1 (h^{-1})	k_{-1} (h^{-1})	k_{ox} (h^{-1})
Miglyol® 812[a]	30 °C	0.130	0.082	0.127
Miglyol® 812 [a]	40 °C	0.151	0.102	0.142
Miglyol® 812 [a]	50 °C	0.218	0.099	0.139
Miglyol® 812 [a]	60 °C	0.154	0.034	0.249
Miglyol® 812 [a]	80 °C	0.158	0.007	0.204
Methanol	50 °C	0.021	0.016	0.204
Peanut oil	50 °C	0.037	0.015	0.214
Soybean oil	50°C	0.072	0.010	0.250

[a] Medium chain triglyceride marketable mixture of capric and caprylic acids.

Figures

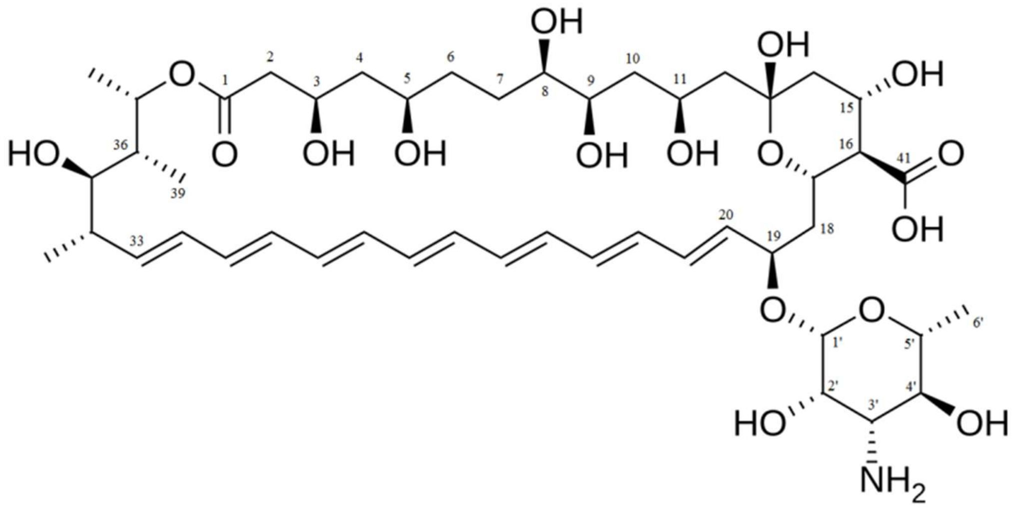

Figure 1 – Molecular Structure of amphotericin B

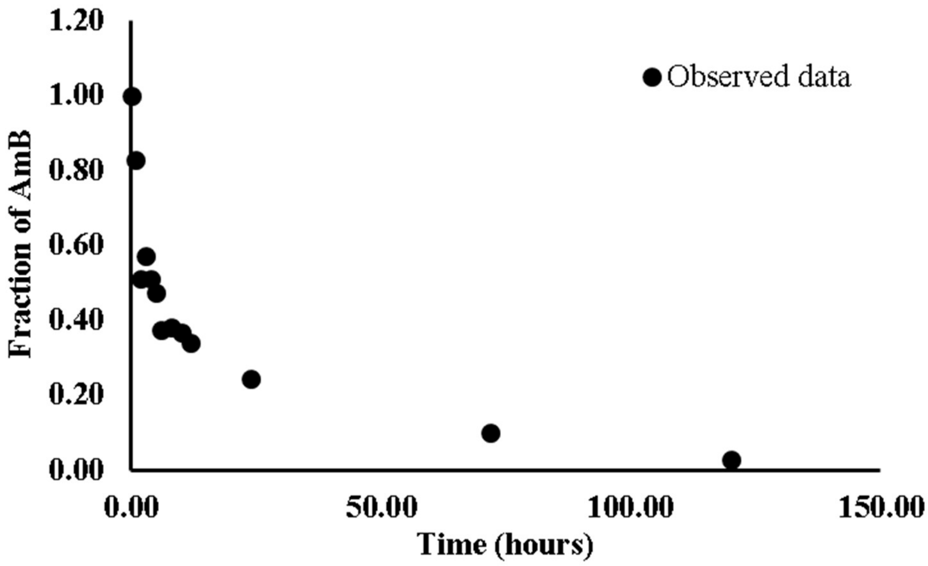

Figure 2 – Fraction of AmB loss versus time, where initial AmB concentration was 0.86 µM in Miglyol® 812, stored under dark conditions at 50 °C.

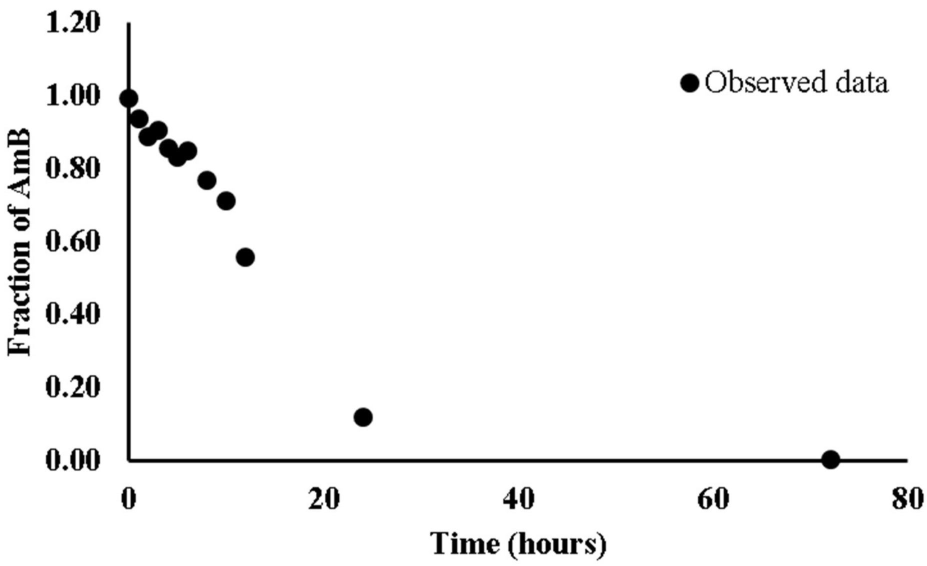

Figure 3 – Fraction of AmB loss versus time, where initial AmB concentration was 0.86 μM in methanol, stored under dark conditions at 50 °C

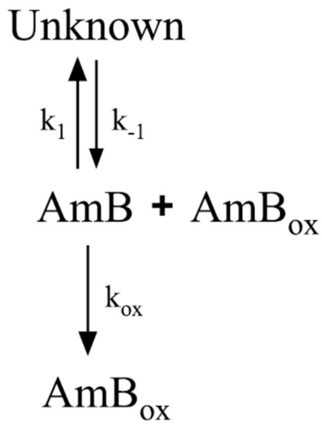

Figure 4 – Proposed AmB degradation scheme under dark storage

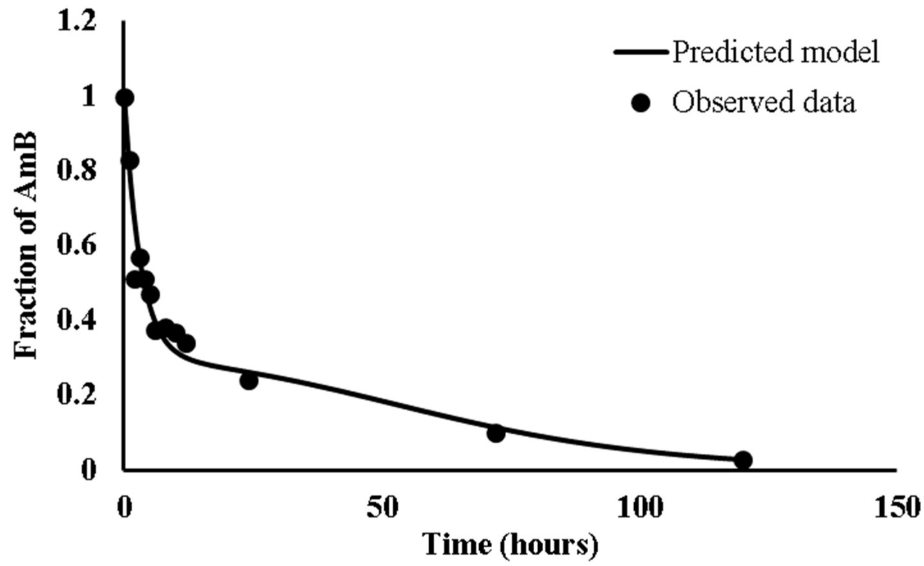

Figure 5 – Fraction of AmB loss versus time, where initial AmB concentration was 0.86 µM in Miglyol® 812, stored under dark conditions at 50 °C, with observed data and predicted model values.

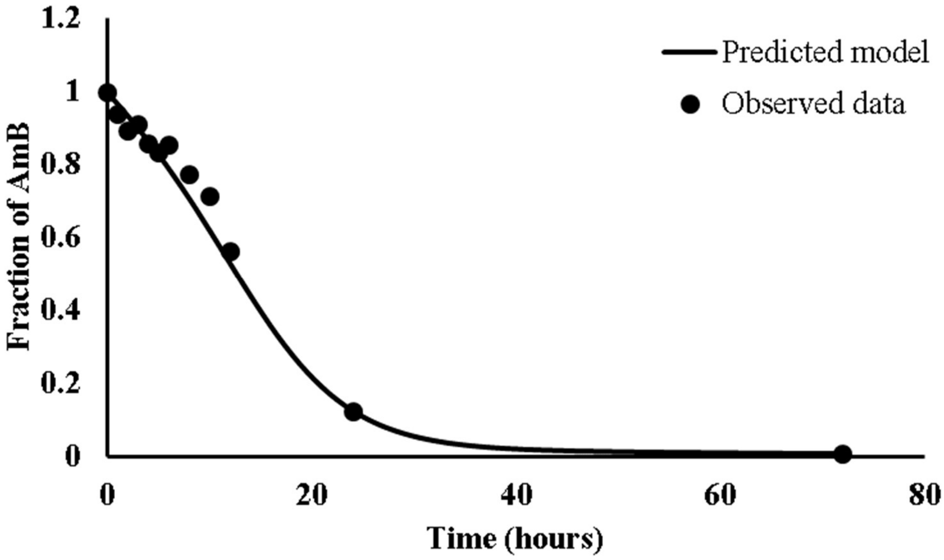

Figure 6 – Fraction of AmB loss versus time, where initial AmB concentration was 0.86 μM in methanol, stored under dark conditions at 50 °C, with observed data and predicted model values.

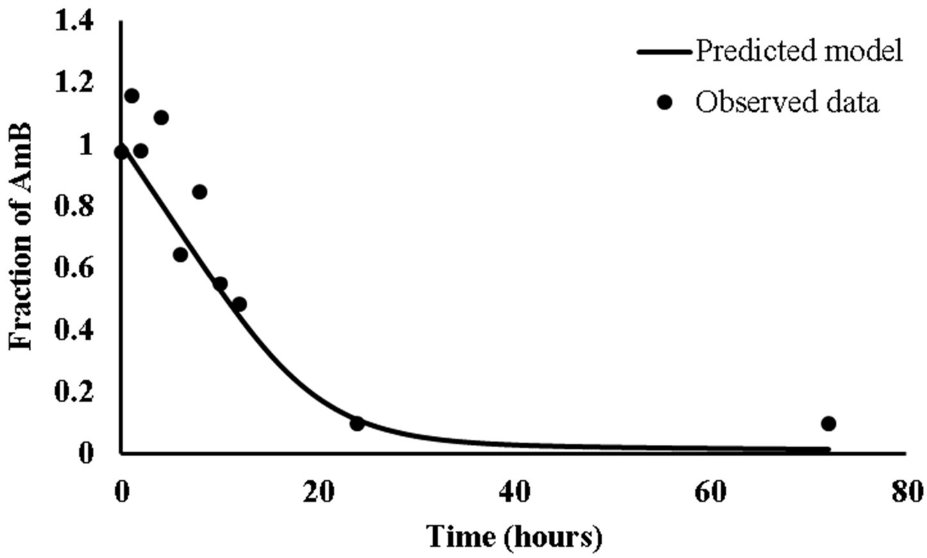

Figure 7 – Fraction of AmB loss versus time, where initial AmB concentration was 0.86 μM in peanut oil, stored under dark conditions at 50 °C, with observed data and predicted model values.

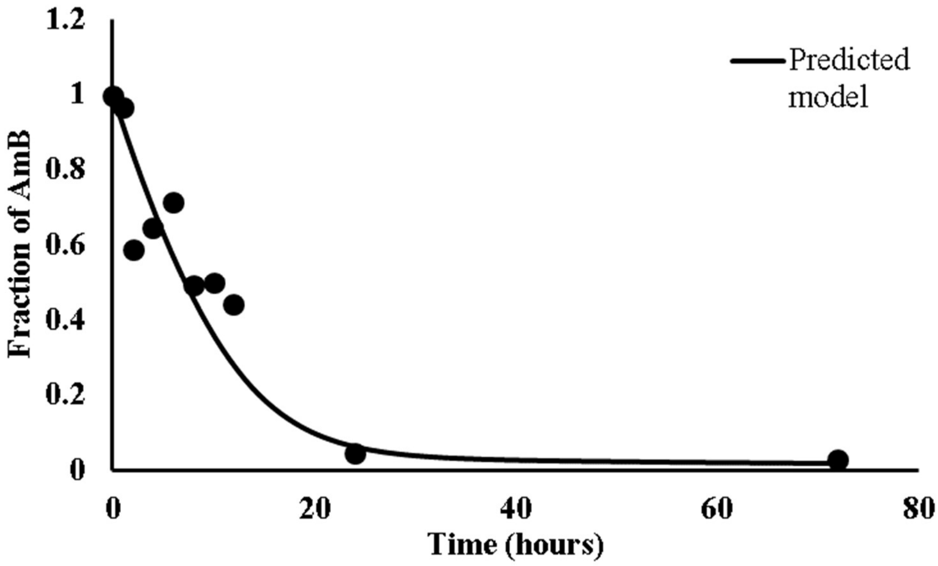

Figure 8 – Fraction of AmB loss versus time, where initial AmB concentration was 0.86 μM in soybean oil, stored under dark conditions at 50 °C, with observed data and predicted model values.

$$\mathrm{AmB} \xrightarrow{k_{ox}} \mathrm{AmB}_{ox}$$

Figure 9 – Proposed AmB degradation scheme in methanol under light exposure.

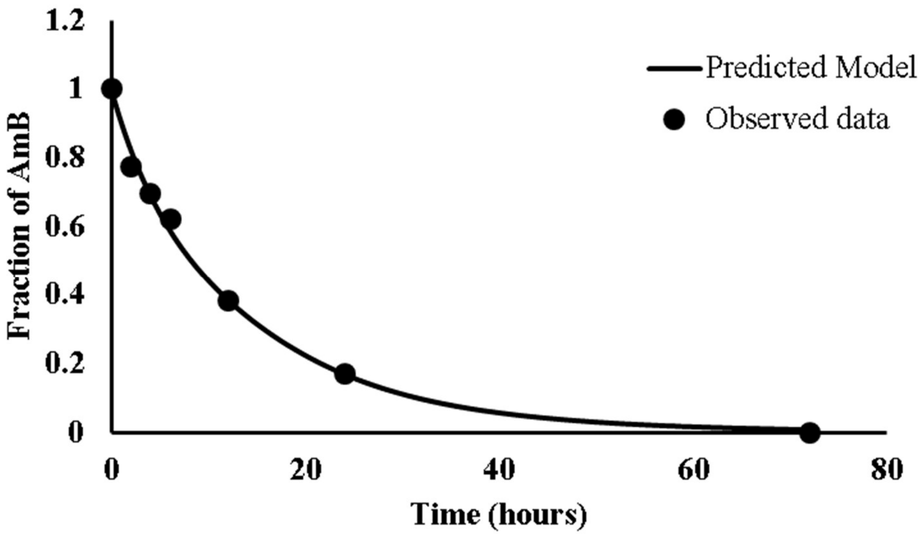

Figure 10 – Fraction of AmB loss versus time, where initial AmB concentration was 0.86 µM in methanol, stored under light exposure (UV and visible rays simultaneously, of 8 W/m² and 16800 lx of potency, respectively) at 25 °C, with observed data and predicted model values.

HIGHLIGTHS

- Amphotericin B degradation undergoes a complex pathway;
- Degradation scheme for amphotericin B loss in oil under dark environment involves autocatalysis and reversible loss of substrate;
- Amphotericin B loss in methanol under light undergoes a pseudo-first order kinetics;
- Oxidative degradation product formation from amphotericin B might involve epoxide formation in the polyene chain;

CONCLUSIONS

This thesis initially reviewed background concepts, usage, pharmacology, in-vitro and in-vivo studies and further information regarding microemulsions containing AmB and its freeze-drying as a method for stability improvement. The introduction showed the information recollected from the literature regarding nanotechnological use of AmB that driven the experimental section of this research, once we had limited information on the literature from AmB chemical instability itself and no chemical kinetics information to this moment in lipid-based systems.

From chapter 2, an analytical method in HPLC was successfully developed and validated. The method proved to be reproducible and accurate with linearity (R^2 = 0.996) from 3.2 to 52.0 nM using a UV detector. LOD and LOQ were 1.33 and 4.45 nM, respectively. AmB was successfully extracted from medium-chain triglyceride based oil prior HPLC analysis.

The degradation pathway of AmB in oil was investigated. Adsorption to glass surface and aggregation did not appear to play a role in AmB degradation in medium chain triglyceride. The degradation pathways were related to oxidation processes, as hypothesized initially based on AmB moieties and the media used. Under protection from light in different oils, the most likely pathway for AmB degradation was autoxidation. The loss of AmB was not temperature dependent, suggesting a complex degradation pathway involving not only autoxidation. Whereas under light exposure, the most likely degradation pathway was light catalyzed oxidation, as investigated in methanol using UV-Vis spectroscopy.

In chapter 3, empirical degradation schemes were proposed and differential equations were developed to explain AmB loss process. A kinetic model was used to describe AmB loss under protection from light in different solvents. The model was in good agreement with the observed data and it was based on the biexponential reversible loss and autocatalytic loss of AmB. The autocatalytic loss was showed to happen in the autoxidation experiments. However, a simple autocatalytic model did not describe the data generated. Thus, it was proved by the model that AmB undergoes a complex degradation kinetics, as hypothesized initially by the absence of temperature-dependence. Additionally, the biexponential kinetic model, suggestive of reversible loss, is probably related to hydrolysis due to residual water from the solvents.

A second model described AmB loss under light exposure successfully. AmB loss in this condition showed to be pseudo-first order. Constant rates described in the model were estimated. However, due to complex degradation pathway, the kinetics of the different processes leading to AmB degradation could not be distinguished.

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APPENDIX

As the main area of expertise of my research laboratory in Brazil and as part of the continuous studying on development and nanotechnology, collaborations studies are often conducted and I took part on other researches along with developing my thesis.

This section of the Thesis is dedicated to show the publications in scientific journals I was involved with as main and/or co-author in the area of nanotechnology during the period of time of execution of this PhD research. They were of great importance on my intellectual learning and developing as a doctoral student in Pharmaceutical Nanotechnology and could not go unnoticed on this document. However, they were not the main objective of this Thesis. Thus, they will not be further discussed.

Article

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Chemical Characterization and Antimicrobial Activity Evaluation of Natural Oil Nanostructured Emulsions

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The aim of this work was to investigate the antimicrobial activity of nanostructured emulsions based on copaiba (*Copaifera langsdorffii*) resin-oil, copaiba essential oil, and bullfrog (*Rana catesbeiana* Shaw) oil against fungi and bacteria related to skin diseases. Firstly, the essential oil was extracted from copaiba resin-oil and these oils, along with bullfrog oil, were characterized by gas chromatography combined with mass spectrometry (GC-MS). Secondly, nanostructured emulsion systems were produced and characterized. The antimicrobial susceptibility assay was performed, followed by the Minimum Inhibitory Concentration (MIC) determination, the bioautography assay, and the antibiofilm determination. Strains of the genera *Staphylococcus*, *Pseudomonas*, and *Candida* were used. The CG-MS analysis was able to identify the components of copaiba resin-oil, copaiba essential oil, and bullfrog oil. The MIC assay in association with the bioautography revealed that some esters of palmitic and oleic acids, α-curcumene, α-himachalene, isothujol, and α-fenchene—probably inhibited some strains. The nanostructured emulsions based on copaiba resin-oil and essential oil improved the antimicrobial activity of the pure oils, especially against *Staphylococcus* and *Candida*, resistant to azoles. The bullfrog oil nanostructured emulsion showed a lower antimicrobial effect when compared to the copaiba samples. However, bullfrog oil-based nanostructured emulsion showed a significant antibiofilm activity ($p < 0.05$). Given the significant antimicrobial and antibiofilm activities of the evaluated oils, it may be concluded that nanostructured emulsions based on copaiba and bullfrog oils are promising candidates for the treatment of infections and also may be used to incorporate other antimicrobial drugs.

Keywords: Nanostructured Emulsion, Copaiba (*Copaifera langsdorffii*) Oil, Bullfrog (*Rana catesbeiana*) Oil, Antimicrobial Activity.

1. INTRODUCTION

Natural oils have been used in popular medicine as antimicrobial agents for treatment of various infections.[1] Nowadays, due to the substantial number of drugs resistant to microorganisms, these oils and other natural products have become scientifically recognized, encouraging the introduction of new products originated from animal and vegetable sources in the market.[2] Among these products, bullfrog (*Rana catesbeiana* Shaw) and copaiba (*Copaifera langsdorffii*) oils are widely used in popular medicine.

Copaiba oil is extracted from trees known as Copaibeiras (*Copaifera* spp.), which are distributed in South America

*Author to whom correspondence should be addressed.

1533-4880/2015/15/880/009 doi:10.1166/jnn.2015.9187 molecules

MDPI

Article

New Trends on Antineoplastic Therapy Research: Bullfrog (*Rana catesbeiana* Shaw) Oil Nanostructured Systems

Lucas Amaral-Machado [1,2,3], Francisco H. Xavier-Júnior [1], Renata Rutckeviski [1], Andreza R. V. Morais [1], Éverton N. Alencar [1], Teresa R. F. Dantas [1], Ana K. M. Cruz [4], Julieta Genre [1], Arnóbio A. da Silva-Junior [5], Matheus F. F. Pedrosa [5], Hugo A. O. Rocha [4] and Eryvaldo S. T. Egito [1,2,3,*]

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Abstract: Bullfrog oil is a natural product extracted from the *Rana catesbeiana* Shaw adipose tissue and used in folk medicine for the treatment of several diseases. The aim of this study was to evaluate the extraction process of bullfrog oil, to develop a suitable topical nanoemulsion and to evaluate its efficacy against melanoma cells. The oil samples were obtained by hot and organic solvent extraction processes and were characterized by titration techniques and gas chromatography mass spectrometry (GC-MS). The required hydrophile-lipophile balance and the pseudo-ternary phase diagram (PTPD) were assessed to determine the emulsification ability of the bullfrog oil. The anti-tumoral activity of the samples was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for normal fibroblast (3T3) and melanoma (B16F10) cell lines. Both extraction methods produced yielded around 60% and the oil was mainly composed of unsaturated compounds (around 60%). The bullfrog oil nanoemulsion obtained from PTPD presented a droplet size of about 390 nm and polydispersity = 0.05 and a zeta potential of about −25 mV. Both the bullfrog oil itself and its topical nanoemulsion did not show cytotoxicity in 3T3 linage. However, these systems showed growth inhibition in B16F10 cells. Finally, the bullfrog oil presented itself as a candidate for the development of pharmaceutical products free from cytotoxicity and effective for antineoplastic therapy.

Keywords: bullfrog oil; nanomedicine; tumor cells; nanocarrier molecules

MDPI

Article

Thermo-Oxidative Stability Evaluation of Bullfrog (*Rana catesbeiana* Shaw) Oil

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Abstract: Bullfrog oil (BO), a natural product obtained from recycling of adipose tissue from the amphibian *Rana catesbeiana* Shaw, has been recently evaluated as a therapeutic activity ingredient. This work aimed to evaluate the long-term and accelerated thermal oxidative stabilities of this product, which is a promising raw material for emulsion technology development. BO was extracted from amphibian adipose tissue at 70 °C with a yield of 60% ± 0.9%. Its main fatty acid compounds were oleic (30.0%) and eicosapentaenoic (17.6%) acids. Using titration techniques, BO showed peroxide, acid, iodine and saponification indices of 1.92 mEq·O_2/kg, 2.95 mg·KOH/g oil, 104.2 g I_2/100 g oil and 171.2 mg·KOH/g oil, respectively. In order to improve the accelerated oxidative stability of BO, synthetic antioxidants butylhydroxytoluene (BHT) and buthylhydroxyanisole (BHA) were used. The addition of BHT increased the oxidation induction time compared to the pure oil, or the oil containing BHA. From the results, the best oil-antioxidant mixture and concentration to increase the oxidative stability and allow the oil to be a stable raw material for formulation purposes was derived.

Keywords: BO; natural products; fatty acids; physicochemical properties; oxidative stability; thermal stability

1. Introduction

In the last few years, new biological active products derived from animals have been used as raw material for the food and pharmaceutical industries [1,2]. The bullfrog (*Rana catesbeiana* Shaw) is an amphibian widely used in the meat and leather market [3]. Bullfrog meat has a high nutritional value due to its high content of essential amino acids and low lipid quantity [4]. Normally, the adipose tissue of the bullfrog has been considered a waste product by the food industry, nevertheless it can be reused by biotechnological processing for the production of natural oil, with applications in nutritional, food, cosmetic, and pharmaceutical areas [3].

In Brazilian folk medicine, BO has been regarded as a natural remedy for the treatment of immune and inflammatory diseases [5]. This oil has several advantages for therapeutic applications, including greater biocompatibility and biodegradability, lower toxicity, and a more sustainable process

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Freeze-Dried Microemulsion Containing Amphotericin B for Leishmaniasis Treatment: An Overview

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REVIEW

Visceral leishmaniasis is a neglected tropical disease that can be fatal if left untreated. Amphotericin B (AmB) has been quite effective against visceral leishmaniasis, but its conventional micellar form, Fungizon®, is nephrotoxic. On the other hand, the less toxic AmB liposomal formulation, AmBisome®, is very expensive. An alternative to solve these drawbacks is to develop a new lipid formulation such as microemulsions (MEs) containing AmB. Additionally, it is desired to remove the water from such systems in order to reduce instabilities related to microbiological contamination and hydrolysis. The aim of this study was to provide a comprehensive overview on the current knowledge regarding AmB-loaded MEs for the treatment of leishmaniasis. Furthermore, the influence of the freeze-drying process to this system was evaluated. This review also discusses the advances regarding leishmaniasis such as general features, drug-resistance parasites, current treatment and new approaches from colloidal nanocarries for treating leishmaniasis. Likewise, it was emphasized the treatment with AmB addressing its molecular characteristics, mechanism of action, resistance mechanism and overviewing the clinical use of current available formulations. An approach on the ME as nanocarriers for AmB was carried out discussing some aspects on the ME structure, physicochemical characteristics, formation theories, advantages, and studies from the literature and from our preliminary results involving AmB-loaded ME for leishmaniasis. Moreover, it was also provided a theoretical overview on the freeze-drying process taking into account aspects such as its steps, cryoprotectant, parameters that would interfere on the process and design of experiment. Furthermore, studies on the freeze-dried AmB-load ME from our group and from the literature were approached.

Keywords: Drug Delivery Systems, *Leishmania*, Lyophilization, Amphotericin B, Colloidal Nanocarries.

CONTENTS

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1. INTRODUCTION

Leishmaniasis is a neglected tropical disease caused by 20 different species that belong to the genus *Leishmania*, which is transmitted by the bite of a female mosquito of the genus *Phlebotomus* and *Lutzomyia*, commonly known as sandflies.[1,2] In vertebrates, this parasite multiplies inside the cells of the mononuclear phagocyte system acquiring the intracellular growth ability. Moreover, it is capable to escape of the host immune response due to the complement activation. The clinical manifestations of leishmaniasis recognized by the World Health Organization are divided into three main forms: cutaneous,

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Review

Freeze-drying of emulsified systems: A review

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ABSTRACT

Colloidal systems such as emulsions, microemulsions and nanoemulsions are able to transport active molecules, enhance their solubility and stability and minimize their side effects. However, since they are dispersions with an aqueous continuous phase they have some disadvantages such as the risk of microbiological contamination, degradation by hydrolysis, physico-chemical instability and loss of pharmacological activity of the drug. Freeze drying, in which the water is removed from the preparation by sublimation under vacuum, has been suggested as a means to resolve these problems. Lyophilized products are very stable and are easy to transport and store. However, there is very little information in the literature about the application of this technique to emulsified systems. The aim of this review is to evaluate the lyophilization process as a tool for increasing the shelf life of emulsified systems such as emulsions, microemulsions and nanoemulsions. In addition, the mechanism of cryoprotection and the techniques that can be used to characterize the freeze-dried systems are discussed.

Contents

Abbreviation: FD, freeze-drying.

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REVIEW ARTICLE

Microemulsion systems containing bioactive natural oils: an overview on the state of the art

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ABSTRACT
Natural oils are extremely complex mixtures containing compounds of different chemical nature. Some of them have physiological or therapeutic activities that may act either alone or in synergy. Therefore, they are used in the pharmaceutical, agronomic, food, sanitary and cosmetic industries. Today, the interest in bioactive natural oils is growing due to their immense potential to prevent and treat numerous human diseases. Formulation in microemulsions (MEs) containing natural oils appeared suitable to improve pharmaceutical and biopharmaceutical properties of bioactive compound derivatives from these oils. Microemulsion systems are thermodynamically stable, transparent, and are isotropic dispersions consisting of oil and water stabilized by an interfacial film of surfactants, typically in combination with a cosurfactant. They can protect labile compounds from premature degradation, control release, increase solubility and hence enhance the bioavailability of poorly bioavailable compounds. The aim of this work was to review the various advantages of bioactive compounds presented in natural oil loaded ME systems to be used as delivery systems. First, the state of the art of the parameters involved in the ME formation, including the basic concepts of the physicochemical formulation of the ME systems, and the main aspects of production and the energy responsible for their formation were reported. The second section describes the use of ME systems and reviews the recent applications of natural oil-loaded in the ME systems as the bioactive compound in the formulation.

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Microemulsion; natural oils; plant oils; delivery systems; bioactive compounds

Introduction

Microemulsion (ME) has attracted much interest for several years in terms of delivery and target potential[1–3]. Microemulsions are transparent, optically isotropic, and thermodynamically stable-phase transition systems, which possess low surface tension and small droplet size[4,5]. These systems are formed by two immiscible liquids (water and oil) mixed to form a single phase stabilized by an interfacial film of alternating surfactant and cosurfactant molecules[6,7]. MEs can appear in at least three major microstructures: swollen micellar (oil-in-water, O/W), reverse micelles (water-in-oil, W/O) and bicontinuous structures[8,9].

Microemulsions have many advantages as drug delivery systems, including improved appearance, high stability, easiness of preparation and small droplet size, resulting in a large surface area from which the active substances can partition and be absorbed or permeate through membranes[1,6,8,10]. Also, such systems possess the ability to enhance the bioavailability of poorly soluble drugs by maintaining them in a molecular dispersion, consequently allowing for controlled or sustained release of their active agent[1,11]. Microemulsions form spontaneously (zero energy input). Therefore, they are easy to manufacture (not process dependent) and scale-up[9,12,13]. These special properties of the ME offer a high potential for numerous practical applications, including enhanced oil recovery, pharmaceutical and cosmetic formulations, edible coatings for food, and other industrial applications[7,14].

Recently, MEs containing natural oils have been of increasing interest to researchers and have shown great potential in industrial applications. MEs' utility lies in their ability to incorporate a large amount of active natural oil products in the continuous or dispersed phase, which are otherwise difficult to formulate[6,15–17]. Natural oil products are extremely complex mixtures containing compounds of various chemical natures, which act either alone or in synergy with other compounds, giving a global therapeutic activity when incorporated in formulations[18–22]. These oils are used widely to the prevention and treatment of very severe diseases including cancer, Alzheimer's, and cardiovascular diseases, as well as their bioactivity as spasmolythic, revulsive, anti-inflammatory, analgesic and acaricide, antibacterial, antiviral, antipsoriatic, antioxidant, and antidiabetic agents[17,23–28].

Due to the therapeutic advantages and the complex composition of the natural oil, various formulation approaches including carrier technology such as MEs offer an intelligent approach for the delivery of bioactive compounds from the natural oil products. Therefore, the aim of this work was to review the various advantages of natural oils loaded ME systems to be used as delivery systems for these bioactive compounds. The present review is divided into two sections. First, the state of the art of parameters involved in the ME formation, including the basic concepts of the physicochemical formulation of the ME systems, and the main aspects of production and the energy responsible for their formation were reported. The second section describes the use of ME systems and reviews the recent applications of natural oil-loaded in the ME systems as the bioactive compound in the formulation.

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Article

Article

Development of a Gas Chromatography Method for the Analysis of Copaiba Oil

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Abstract

A rapid, simple, precise and economic method for the quantification of main compounds of copaiba resin and essential oils (*Copaifera langsdorffii* Desf.) by gas chromatography (GC) has been developed and validated. Copaiba essential oil was extracted by hydrodistillation from the copaiba resin. Resin derivatization allowed the identification of diterpenes compounds. A gas chromatography–mass spectroscopy (GC/MS) method was developed to identify compounds composing the copaiba resin and essential oil. Then the GC/MS method was transposed to be used with a flame ionization detector (FID) and validated as a quantitative method. A good correlation between GC/MS and GC/FID was obtained favoring method transposition. The method showed satisfactory sensitivity, specificity, linearity, precision, accuracy, limit of detection and limit of quantitation for β-caryophyllene, α-humulene and caryophyllene oxide analyses in copaiba resin and essential oils. The main compounds identified in copaiba essential oil were β-bisabolene (23.6%), β-caryophyllene (21.7%) and α-bergamotene (20.5%). Copalic acid methyl ester (15.6%), β-bisabolene (12.3%), β-caryophyllene (7.9%), α-bergamotene (7.1%) and labd-8(20)-ene-15,18-dioic acid methyl ester (6.7%) were diterpenes identified from the derivatized copaiba resin. The proposed method is suitable for a reliable separation, identification and quantification of compounds present in copaiba resin and essential oil. It could be proposed as an analytical method for the analysis of copaiba oil fraction in raw and essential oil parent extracts and after they have been incorporate in pharmaceutical formulations.

Introduction

Compounds obtained from vegetable sources are usually complex mixtures of plant's secondary metabolites bearing protective activity against microorganisms and animal predators. They were found to have pharmacological activities motivating their used in folk medicine since ancient times (1, 2). Among natural compounds of interest, different extracts of copaiba oil show biological activities that would be worth to be used in medicine. In folk medicine, the

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Experimental design approach applied to the development of chitosan coated poly(isobutylcyanoacrylate) nanocapsules encapsulating copaiba oil

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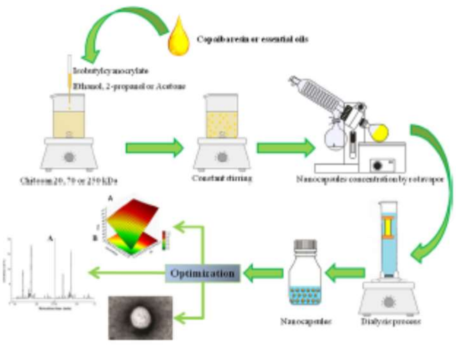
HIGHLIGHTS

- Copaiba oil-loaded chitosan decorated nanocapsules was produced.
- Nanocapsules size and zeta potential were optimized by experimental design.
- Chitosan was used as a stabilizer for the nanocapsules production.
- pH and the temperature of polymerization influenced both the size and zeta potential.
- Copaiba oil was efficiently encapsulated and showed all compounds of the parent oil.

GRAPHICAL ABSTRACT



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ABSTRACT

The aim of this work was to develop, characterize and optimize the natural copaiba oil-loaded chitosan decorated poly(isobutylcyanoacrylate) nanocapsules. These innovatively obtained natural-based systems were developed by an original method of interfacial polymerization of isobutylcyanoacrylate using chitosan as a stabilizer for the nanocapsules. A preliminary study investigated the influence of the molecular weight of chitosan, the type of copaiba oil extract and the solvent phase. Nanocapsules could only be produced with copaiba resin oil, with size ranging from 300 to 1200 nm. Nanocapsule size and zeta potential were then optimized by two-level three-variable full-factorial experimental design. Samples showed

Abbreviations: $CO_{dispersed-phase}$, amount of copaiba oil found in the dispersed media of the nanocapsules; CO_{total}, total amount of copaiba oil used in the preparation; Adj R^2, adjusted determination coefficient; F_{model}, F-value of the model; F_{Tab}, tabulated F- value; $F_{Tabresidues}$, tabulated F- value of the residues; $F_{residues}$, F- value of the residues; R^2, coefficient of determination; x_1, pH of the polymerization medium; x_2, temperature of polymerization; x_3, concentration of chitosan in the polymerization medium; Y_1, predicted droplet size (nm).

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