Investigation and simulation of dissolution with concurrent degradation under healthy and hypoalbuminaemic simulated parenteral conditions- case example Amphotericin B

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Abstract

Guidance on dissolution testing for parenteral formulations is limited and not often related *in vivo* performance. Critically ill patients represent a target cohort, frequently hypoalbuminaemic, to whom certain parenteral formulations are administered. Amphotericin B (AmB) is a poorly soluble, highly protein-bound drug, available as lipid-based formulations and used in critical illness. The aim of this study was to develop media representing hypoalbuminaemic and healthy plasma, and to understand and simulate the dissolution profile of AmB in biorelevant media. Dissolution media were prepared with bovine serum albumin (BSA) in Krebs-Ringer buffer, and tested in a flow through cell apparatus and a bottle/stirrer setup. Drug activity was tested against *Candida albicans*. BSA concentration was positively associated with solubility, degradation rate and maximum amount dissolved, and negatively associated with dissolution rate constant and antifungal activity. In the bottle/stirrer setup, a biexponential model successfully described simultaneous dissolution and degradation, and increased in agitation reduced the discriminatory ability of the test. The hydrodynamics provided by the flow-through cell apparatus was not adequate to dissolve the drug. Establishing discriminating test methods with albumin present in the dissolution media, representing the target population, supports future development of biorelevant and clinically relevant tests for parenteral formulations.

Keywords:

biorelevant; dissolution; albumin; hypoalbuminaemia; solubility; degradation; amphotericin B

1. Introduction

The parenteral administration route is utilized when a quick or a depot effect is needed, when the patient cannot take oral formulations for systemic therapy or when the physicochemical properties of the drug make it impossible to be delivered by any other route [1]. Formulations such as microspheres, liposomes, nanoparticles and emulsions (among others) have been developed to be able to meet the requirements of a long or a sustained exposure. The dissolution test is an *in vitro* test designed to characterize the dissolution/release of the drug from a formulation and hopefully, predict the behaviour of the drug *in vivo*. There are 3 main methods to assess dissolution/release from controlled release parenterals that have been described extensively in the literature: Sample and separate, Continuous flow and Dialysis methods [2-6].

Several factors may influence the dissolution of a formulation *in vivo*. As the ultimate goal of the dissolution test is to ensure clinical performance, these factors should be reflected in the dissolution test [7]. Biorelevant dissolution testing takes into consideration the characteristics of the site of administration *in vivo* that may impact on the dissolution and release of a drug from a formulation. This involves the composition and the physicochemical properties of the medium and the hydrodynamics where the drug will be released [8].

For parenterals administered intravenously the release medium is blood, consisting of 2 fractions, the cellular fraction and plasma. Plasma is a fluid that contains ions and biomolecules. Albumin is the major circulating protein in human plasma (up to the 60% of plasma proteins). The normal reference value of plasma albumin for a healthy subject is 40 g/L ± 10% [9]. Albumin is the most relevant protein in terms of drug administration as it is a carrier for metals, ions, fatty acids, amino acids, bilirubin, enzymes and drugs [9].

Several parenteral formulations, that are not simple aqueous solutions, can be administered in the clinical setting to patients that have significant morbidities such as cancer or critically

illness. Hypoalbuminaemia is common in the critical ill patients (affecting approximately 50% of patients), and while there is no reference value for hypoalbuminaemia, it can be considered when the plasma albumin levels are lower than < 25 g/L [9]. Low levels of serum albumin may affect pharmacokinetics and pharmacodynamics of highly protein bound drugs. With a decrease in the protein levels in plasma there is more unbound drug in circulation which would lead to an increased pharmacological effect. On the other hand, the free drug can penetrate into tissues with a corresponding increase in the volume of distribution and a subsequent decrease in the maximum plasma concentration. [9, 10]. Release/dissolution of poorly soluble, highly protein bound drugs from parenteral formulations, and associated local drug concentrations are likely to be influenced by protein concentration at the site of release. Therefore, *in vitro* dissolution tests that simulate the *in vivo* environment are needed for parenteral formulations that are not aqueous solutions, which take into account the likely changes that arise in target patient groups, with particular reference to albumin concentration.

One drug that is administered to critically ill patients is Amphotericin B (AmB), which is still one of the most effective therapies for systemic fungal infections. In clinical practice, AmB is administered as an infusion using a multidose scheme usually lasting for several days [11]. AmB is highly bound (>95%) to plasma proteins (Low Density Lipoproteins, albumin and α1 glycoproteins [12, 13]). The major drawback of AmB is its poor water solubility (reported values: 0.09 µg/mL [14], 1.38 µg/mL [15] and 6 µg/mL [16] at pH = 7). To tackle this problem, several formulations have been developed, including Liposomal AmB (Ambisome®) and AmB in a lipid complex (Abelcet®), where AmB is within the lipid structures. Furthermore, a correlation has been observed between volume of distribution at steady state of total AmB following administration of Abelcet® and albumin concentration, in critically ill patients [17], illustrating the relevance of albumin concentration to AmB pharmacokinetics in the target patient cohort.

The aim of this study was to investigate the solubility and the dissolution of AmB in simulated plasma with albumin concentrations representing healthy subjects and hypoalbuminaemic patients, and to develop a mathematical model to describe and simulate all the processes involved in its dissolution, in order to be the basis for the development of biorelevant dissolution testing of AmB formulations.

2. Materials and Methods

2.1 Materials

AmB analytical standard (87.8%), Methanol (MeOH) High Performance Liquid Chromatography (HPLC) grade, formic acid, Sabouraud Dextrose (SBD) Broth, NaOH, $MgCl_2$, $CaCl_2$, $NHCO_3$ and NH_4HCO_2 were obtained from Sigma Aldrich (Germany); AmB active pharmaceutical ingredient (API) powder (85%) from Cayman Chemical (USA); Bovine Serum Albumin Protease Free Powder Fraction V (BSA), dimethyl sulfoxide (DMSO), dextrose, Na_2HPO_4, NaH_2PO_4, NaCl and KCl were obtained from Fisher Scientific (USA); Sabouraud dextrose (SBD) agar was obtained from Oxoid (UK), 25 mL sterile universal culture tubes were obtained from Sterilin Thermo Scientific (UK); 10 µL plastic loops from Microspec (UK); GF/D (pore size 2.7 µm, 25 mm diameter) and GF/F (pore size 0.7 µm, 25 mm diameter) filters were obtained from Whatman (UK) and regenerated cellulose (RC) filters 0.45 µm 13 mm diameter from Cronus (UK).

The yeast strain used in the microbiology experiments was *Candida albicans* SC5314 [18].

2.2. Dissolution biorelevant media composition and characterization

The dissolution media employed were Krebs-Ringer Buffer (KRB), supplemented with BSA at different concentrations according to the experiment: 1.5, 2, 3 or 4% w/v. The pH was adjusted to 7.2 – 7.3 with 0.1 M HCl using a Seven Compact pH meter (Mettler Toledo, China). The osmolality of the media with 2 and 4% w/v BSA was measured via the freezing-point

depression method with a Micro-Osmometer 3300 (Advanced Instruments, Massachusetts USA). Viscosity of all media was measured with a Bohlin Rheometer (Germany) with a shear rate 0.1 - 1.5 Pa (logarithmic scale), 20 integrations per measurement and with a delay time of 5 seconds and an integration time of 20 seconds. The geometry was a 4° and 40 mm diameter (CP 4/40) cone parallel to a plate and the experiments were conducted at 25°C in triplicate. The measurement at the closest value to the steady state was recorded as the viscosity value.

2.3. Chromatographic conditions for the analysis of AmB from biorelevant dissolution media samples

The chromatographic method to quantify AmB was a modification of the method reported by Nilsson-Ehle et al [19]. Briefly, AmB was quantified by HPLC analysis using a Hewlett Packard Series 1100 equipped with an auto sampler, temperature regulated column compartment, quaternary pump and diode array detector (DAD detector) (Agilent Technologies). The column was a C18 Waters Sunfire Column (Ireland) 150 x 46 mm 5µm. The temperature of the column compartment was set at 25°C. The mobile phase consisted of formate buffer (50 mM; pH = 3.2): MeOH (25:75, v/v); the flow rate was 1 mL/min and analysis was performed with the DAD detector at $\lambda = 406$ nm. The UV spectrum was recorded from 300 to 450 nm (where necessary for detection of the degradant). Quantification of AmB in samples was made based on calibration curves. Freshly prepared standard solutions (0.5 – 10 µg/mL) in the corresponding medium were prepared by appropriate dilution of a 500 µg/mL stock solution of AmB analytical standard in 1:1 MeOH: DMSO v/v. The 5 µg/mL standard solution in KRB – BSA 4% w/v was incubated at 37°C and was monitored every hour to check the stability of the samples for up to 24 h. The limit of detection and the limit of quantification were 0.12 and 0.37 µg/mL, respectively.

2.4. Sample treatment of AmB in the biorelevant dissolution media

Proteins were precipitated by adding 2 volumes of methanol to 1 volume of the sample followed by mixing in a vortex (Scientific Industry Inc., USA) for 30 seconds and then centrifuged for 10 minutes at 12000 rpm and 5°C (Eppendorf Heraeus Fresco 17 centrifuge, Thermo Electron LED GmbH., Germany). The supernatant was filtered through a 0.45 μm RC filter before injected to the HPLC.

2.5. Liquid chromatography – Mass Spectrometry (LCMS) studies

The identification of the mass of the molecular structures detected as peaks in the HPLC chromatograms was performed by LCMS. An excess of AmB was added to the medium (KBR-BSA 4% w/v) and after stirring for 8 h at 130 rpm [Variomag multipoint stirring plate (Thermo Electron Corporation, Germany); 15 x 6 mm magnetic stirrers (Fisherbrand, UK)] at 37°C, the undissolved drug was removed by centrifugation [3000 rpm 5 min 5°C]. The supernatant was treated for protein precipitation (section 2.4) and analysed by LCMS [Ultimate 3000 UHPLC (Dionex, USA); autosampler; quaternary pump; DAD detector; maXisHD Time-of-Flight Mass Spectrometer coupled with an electrospray source (ESI-TOF) (Bruker Daltonics, Germany)]. The conditions of the chromatography analysis were the same as previously described (section 2.3), with the exception of the injection volume being 30 μL and a split flow post column before the mass spectrometry detector to a flow rate of 0.3 mL/min. In this case, the formate buffer (50 mM) was prepared with formic acid and ammonium formate, in order to make it suitable for Mass Spectrometry (absence of sodium ions). The samples were analysed in negative mode. Data was processed using external calibration with the Bruker Daltonics software (DataAnalysisTM) as part of the overall hardware control software (CompassTM).

172 **2.6. Degradation studies of AmB in the biorelevant dissolution media**

173 In order to characterise the degradation of AmB in the dissolution media, approximately 10 mg
174 of AmB API powder was added to 50 mL of the dissolution media (KRB-BSA 1.5, 2, 3, or 4%
175 w/v) and then stirred for 1 hour at 130 rpm (in Variomag multipoint stirring plate) at 37°C. The
176 samples were centrifuged for 5 minutes at 3000 rpm and 4°C (Heraeus Biofuge Primo R
177 Centrifuge, Thermo Electron LED GmbH. Germany) to remove the undissolved AmB and the
178 supernatant was incubated at 37°C. Samples were taken at 0.5, 1, 1.5, 2, 3, 4, 5, 6 and 8 hours,
179 injected to the HPLC after sample treatment (protein precipitation; section 2.4) and the
180 concentration of AmB in the samples was calculated. All experiments were performed in
181 triplicate. A linear fit was applied to the degradation data from 4 h to the last time point, after
182 a natural logarithm transformation of the measured concentration (Excel 2013, Microsoft.
183 USA) and the degradation rate constant (k_{deg}) was calculated from the slope of the line.

184

185 **2.7. Solubility studies of AmB in the biorelevant dissolution media**

186 Approximately 2.5 mg of AmB API powder were placed in a 100 mL glass bottle (56 mm
187 diameter/ 105 mm height; Duran, Germany) with 30 mL of KRB supplemented with BSA 1.5,
188 2, 3 or 4% w/v, stirred at 130 rpm (in Variomag multipoint stirring plate) and incubated at
189 37°C. The sampling times were 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12 and 24 hours. The undissolved
190 AmB was removed by centrifugation (Eppendorf centrifuge 3000 rpm, 5 min, 5°C; following
191 validation (data not shown) centrifugation was selected as a cost-effective alternative to
192 filtration) and after sample treatment (protein precipitation; section 2.4), the samples were
193 injected to the HPLC and the concentration of AmB in the samples was calculated. All
194 experiments were performed in triplicate.

195

196 **2.8 Mass balance studies of AmB in the biorelevant dissolution media**

The total undissolved, dissolved and degraded percentages of AmB in KRB-BSA 2% and 4% w/v media were calculated. Approximately 0.3 mg of the AmB API powder were weighed on a micro balance (Sartorius SE 2-F connected to an Eliex E550 antistatic device). The AmB was placed in a 100 mL glass bottle containing 15 mL of dissolution medium (KRB with 2 or 4% w/v BSA), and then stirred at 130 rpm and incubated at 37°C. A 1 mL sample withdrawn at 24 h was centrifuged (3000 rpm, 5 min, 5°C; removal of the undissolved AmB), and after addition of methanol for the protein precipitation (section 2.4), was injected to the HPLC and the AmB concentration ($AmB\ dissolved\ at\ 24\ h$) was calculated. The remaining AmB in the bottle was dissolved with methanol (protein precipitation procedure; section 2.4), analysed and the final concentration of AmB ($AmB\ final$) was calculated. All experiments were performed in triplicate.

The total undissolved ($AmB\ T_{undissolved}$: AmB undissolved powder in the bottle), the total dissolved ($AmB\ T_{dissolved}$: AmB in solution at 24 h and AmB degraded up to 24 h) and the total degraded ($AmB\ T_{degraded}$: AmB degraded up to 24 h) percentages of AmB were calculated based on Eq (1-3):

$$AmB\ T_{undissolved} = AmB\ final - AmB\ dissolved\ at\ 24\ h \quad \text{Eq 1}$$

$$AmB\ T_{dissolved} = AmB\ initial - AmB\ T_{undissolved} \quad \text{Eq 2}$$

where $AmB\ initial$ is the mass (0.3 mg) placed into the reservoir initially (100%)

$$AmB\ T_{degraded} = AmB\ T_{dissolved} - AmB\ dissolved\ at\ 24\ h \quad \text{Eq 3}$$

2.9. Antimicrobial activity assay of AmB in the biorelevant media: MIC and MFC determination

2.9.1. Quantification of *C. albicans*. The strain was maintained on SBD agar plates. A new culture was started from a single colony in a culture tube with 5 mL of SBD broth and was incubated at 37°C overnight in a shaking incubator (Innova 44, New Brunswick Scientific,

USA), after which the optical density (OD) was measured at 600 nm (OD_{600}). The Colony Forming Units (CFU) of the culture was determined by preparing serial dilutions from 10^{-1} to 10^{-6}. 100 µL of the suspensions were plated on SBD agar plates, incubated overnight at 37°C, the number of colonies were counted and the relationship with the OD_{600} of the culture was established.

2.9.2 MIC and MFC studies: The OD_{600} of an overnight culture of *C. albicans* was measured, and diluted to a final concentration of 10^5 CFU/mL. Minimum Inhibitory Concentration (MIC) studies were performed with the following concentrations of AmB: 0, 1.5, 3, 4.5, 6, 7.5, 9 and 10.5 µg/mL for the experiments with BSA 2% w/v, and 0, 3, 4.5, 6, 7.5, 9, 10.5 and 12 µg/mL for the experiments with BSA 4% w/v. Culture medium without BSA was set as the control for these experiments. The MIC was defined as the lowest concentration of AmB at which there was no visual turbidity in the liquid broth. The Minimum Fungicidal Concentration (MFC) experiments were performed by plating 10 µL of the yeast cultures from the MIC assays on SBD agar plates and incubated for 24 h at 37°C. The MFC was defined as the lowest concentration of AmB where there was no visible growth on the agar plates. The results are expressed as the intervals where the MIC and MFC were found. The experiments were performed in duplicate.

2.10 Dissolution studies of AmB in biorelevant media:

2.10.1 Sample and separation method (bottle/stirrer)

Dissolution studies were carried in a glass bottle with a similar setup as the solubility experiments (section 2.7). 0.5 mg of AmB API powder was weighed and placed into a 100 mL glass bottle with 30 mL of the dissolution medium. Two hydrodynamic conditions were tested: low agitation (130 rpm) for KRB – BSA 0, 1.5, 2, 3 and 4% w/v and high agitation (380 rpm) for experiments with KRB – BSA 2% w/v and BSA 4% w/v. Samples withdrawn up to 24h

were treated and analysed as previously described (sections 2.7 and 2.4) and the % AmB dissolved over time was calculated. All experiments were performed in triplicate.

2.10.2 Continuous Flow method (flow through cell apparatus)

The dissolution studies were carried out in a flow-through cell dissolution apparatus (Sotax CE7 smart connected to a Sotax piston pump CP7, Sotax, Switzerland) operated in the closed mode [20]. A 5mm ruby glass bead was positioned at the bottom of the cell (small cell:12 mm diameter; large cell: 22.6 mm diameter), the conical part of the cell was filled with 1 mm glass beads and 5 mg of AmB API powder were weighed and placed on top of the glass beads. Glass fibre filters (GF/D, GF/F) were positioned at the top of the cell. Two different hydrodynamic conditions were tested: i. small cell with a flow rate of 35 mL/min (high velocity) and ii. large cell with a flow rate of 16 mL/min (low velocity). 50 mL of the dissolution medium (KRB with BSA 2% w/v or 4% w/v) were put in the reservoir under constant stirring. 0.5 mL samples were collected at specific time points up to 8 hours and volume replacement with fresh medium was made. Dissolution experiments were also performed with 0.5 mg of AmB in the two media (KRB with BSA 2% w/v or 4% w/v) under both velocity conditions and with 5 mg of AmB API in water (HPLC grade) under high velocity conditions. The samples after treatment (protein precipitation; section 2.4) were injected to the HPLC and the % AmB dissolved over time was calculated. All experiments were performed in triplicate at 37°C.

2.11 Treatment of dissolution data

The AmB dissolution profiles were corrected for degradation using the corresponding degradation rate constants (section 2.6). The concentration over time accounting for degradation ($C_{corrected}$) was calculated using Eq 4.

$$C_{corrected} = C_t + k_{deg} * AUC_{0-t} \quad \text{Eq 4.}$$

Where C_t is the observed concentration at time t, $AUC_{0\text{-}t}$ is the Area Under the Observed Concentration – Time Curve from time 0 to time t and k_{deg} is the degradation rate constant obtained from the degradation experiments.

The corrected dissolution profiles were calculated based on $C_{corrected}$ and a first order curve fitting (Eq 5) was performed in order to obtain the dissolution rate constant (GraphPad Prism 6, Graph Pad Software, Inc, USA).

$$X_{corrected} = X_{max} * (1 - e^{-k_{diss}t}) \quad \text{Eq 5.}$$

where $k_{diss}$is the dissolution rate constant, $X_{corrected}$ is the corrected percent dissolved at time t and X_{max} is the maximum corrected percent dissolved. The goodness of fit was assessed based on the correlation coefficient (R^2) and the Akaike Information Criterion (AIC).

Equation 6 was utilized to simulate the dissolution profiles ($X_{simulated}$) in the bottle/stirrer accounting for degradation. The simulations were performed using GraphPad Prism 6.

$$X_{simulated} = \frac{X_{max} * k_{diss}}{k_{diss} - k_{deg}} \left(e^{-k_{deg}t} - e^{-k_{diss}t}\right) \quad \text{Eq 6.}$$

Where X_{max} is the maximum corrected percent dissolved, k_{diss} is the dissolution rate constant and k_{deg} is the degradation rate constant.

2.12 Statistical analysis

Data were analysed with one-way ANOVA and the Tukey test was selected in order to perform pair wise multiple comparison of all groups (significance $p<0.05$).

A t- test was used to compare two experimental means (significance $p < 0.05$). The analyses were performed with Statgraphics Centurion XVII (Statpoint Technologies Inc, USA).

3. Results and Discussion

3.1 Biorelevant dissolution media simulating healthy and hypoalbuminaemic parenteral conditions

The composition of KRB and its physicochemical properties are similar to human plasma (Table 1). The biorelevance of KRB supplemented with BSA is described in terms of composition, pH, osmolality and viscosity as compared with the corresponding properties of human plasma from healthy subjects (Table 1). [21, 24]. A small variation in pH is observed due to the pH adjustment before the addition of the proteins. The addition of BSA marginally increases the viscosity of the medium ($p<0.01$) whereas the increase of the osmolality is not statistically significant ($p > 0.05$). The BSA concentration in the medium (1.5 - 4 % w/v) reflects the albumin levels in the plasma of a critically ill patient and of a healthy subject [9]. A 76% sequence identity between Human Serum Albumin (HSA) and Bovine Serum Albumin has been measured [25], justifying the use of BSA as a substitute for HSA. In addition, an *in silico* analysis utilizing molecular modeling, predicted only one favourable binding site in both HSA and BSA for AmB B [26], further supporting the biorelevance of KRB supplemented with BSA.

3.2. Identification of the AmB degradation product

During the development of the AmB quantification method, an extra peak in AmB chromatograms with a shorter retention time was noted. When the sample was incubated at 37°C, the area of this unknown peak increased with time and the AmB peak area decreased. The UV spectra of the two peaks are broadly similar with a main difference in the λ_{max} [λ_{max} for AmB: 406 nm; λ_{max} for unknown compound: 380 nm (Figure 1)], suggesting that the unknown compound is related to AmB. LCMS experiments revealed that the mass spectrum of the unknown compound has a difference in mass (corresponding to a chlorine adduct) compared to the mass of AmB (Figure 2). Based on these studies, this unknown compound is

more polar than AmB (due to its shorter retention time) and it can be suggested that it is AmB's degradation product (deg-AmB). It has been reported that AmB auto-oxidates in the presence of oxygen with formation of free radicals and epoxidation is the most probable route of degradation (but the epoxidation products have not yet been characterized) [27 - 29]. A degradation product has not been reported previously in studies where AmB was quantified. This could be due to the specific ion transition followed by the multiple reaction monitoring [30-33] and the elution of the more polar AmB degradation product in the washing step of the solid phase extraction used for the purification of the sample [31, 34-36] in these methods. Furthermore, plasma components could potentially prevent/alter the rate of AmB degradation.

3.3. Degradation studies of AmB in the biorelevant media

The degradation of AmB in dissolution media with different concentrations of BSA was assessed in order to enable the quantification of the actual AmB dissolved in the dissolution studies. Degradation data and degradation rate constants of AmB in the studied media are presented in Figure 3 and Table 2, respectively. The concentration of BSA in the medium has a statistically significant effect on the degradation rate constants of AmB ($p<0.01$). The increasing concentration of BSA results in an increase in the degradation rate constant of AmB, with the degradation rate constant being 3 times higher in the media with 4% w/v BSA compared to the one in media with BSA 1.5% w/v.

3.4. Solubility studies of AmB in the biorelevant media

The AmB solubility data for 24 h in KRB with different concentrations of BSA are presented in Figure 4. The solubility of AmB in the biorelevant media increases with an increase in BSA concentration in the medium. While drug loss through degradation facilitates further solubilisation, as the dissolution rate is faster than the degradation rate, the AmB solubility

saturation value is considered to be the point at which the concentration reaches a plateau, generally at around 10 – 12 hours (3 – 5 h for KRB BSA 1.5% w/v) (Table 2) and after this point, the AmB concentration is decreased due to degradation being the dominant process at this time when there is no longer the same excess of the drug. The deg-AmB peak was present in all samples' chromatograms and its area increased with increased mass dissolved. The increase in drug solubility mediated by albumin has been reported for another anti-fungal drug, Itraconazole, illustrating the importance of BSA concentration in the medium for poorly soluble, highly protein bound drugs [37]. These solubility values should be taken into account when developing biorelevant test conditions for an AmB formulation, with a view to developing an *in vivo / in vitro* correlation. As the patients who are going to receive AmB therapy may present with hypoalbuminaemia, albumin concentration could impact on the observed mass dissolved/released from the formulation, which will be reflected in the pharmacokinetics of the drug.

3.5. Mass Balance studies of AmB in the biorelevant media

The results of the mass balance studies of AmB in KRB with BSA 2% w/v and KRB with BSA 4% w/v are presented in Table 3. The $AmB\ dissolved\ at\ 24\ h$ is similar for both concentrations of BSA (2% w/v and 4% w/v) in the medium, but the $AmB\ T_{dissolved}$ in which the degradation of AmB is taken into account is higher in the medium with BSA 4% w/v, as the AmB degradation is higher in this BSA concentration. This supports the results in sections 3.3 and 3.4, implying that BSA has a critical effect on the degradation and solubility of AmB in the medium, confirming the faster degradation and increased mass dissolved in medium with higher BSA concentration.

3.6. Antimicrobial activity of AmB in the biorelevant media

In order to assess the effect of BSA in the activity of AmB against *Candida albicans*, MIC and MFC values were determined (Table 4). A marked effect of BSA on the activity of AmB is shown. The results for the control experiments are in agreement with what is reported in the literature for the MIC of AmB against *C. albicans* (0.06 – 1 mg/L) [36-39]. When BSA was added to the culture media, the MIC increased 10-fold in the medium with BSA 2% w/v and approximately 20-fold in the medium with BSA 4% w/v. In the absence of BSA, the MFC was fairly close to the MIC values, indicating that AmB is fungicidal. However, in the presence of BSA, the MFC was higher than the highest concentration of AmB that was tested, suggesting a fungistatic activity instead. The increase in the concentration of AmB to exert its antifungal activity has been reported before in studies where the source of albumin was HSA (human serum albumin) [40]. This could be explained by the fact that AmB is highly bound to proteins, and only the free fraction can exert a pharmacological effect. If there is more albumin in the medium, more drug will be bound to it and the concentration required to have the same efficacy will be higher. *In vivo*, AmB can also be bound to α1-acid glycoprotein [12, 13], but as this protein's blood concentration is only 0.1% w/v (24 μM) compared to the 2.0 - 4.0% w/v BSA (300 – 600 μM) (that is used to substitute HSA) its effect can be considered negligible for the purposes of this study.

3.7 Dissolution studies of AmB in biorelevant media

3.7.1 Sample and separation method (bottle/stirrer)

The AmB dissolution profiles in biorelevant media (KRB with the addition of BSA 1.5 - 4 %w/v) obtained with the bottle/stirrer setup are presented in Figure 5. Dissolution studies were performed in KRB without BSA, however neither the AmB dissolution nor the AmB degradation could be quantified due to the very low solubility, and hence minimal dissolution, of the drug in this medium. The AmB dissolution with its degradation occurring simultaneously

394 is similar at both agitation levels with a plateau value ranging from 18.55%-23.14% (low
395 agitation) and 16.21%-20.50% (high velocity). A drop in the % dissolved is observed at 24h
396 due to the degradation, with this being higher for the low agitation conditions and the low levels
397 of BSA (1.5% w/v and 2% w/v) (Figure 5a and 5b). When the dissolution profiles are corrected
398 for the degradation (Figure 5c and 5d) the continuous dissolution of AmB in the media with
399 high levels of BSA (3% w/v and 4% w/v) in both agitation conditions can be observed. At low
400 agitation conditions and at low levels of BSA in the medium the % dissolved from 12h to 24h
401 is decreased (5.49% in the medium with 1.5% w/v BSA) or remains unchanged (in the medium
402 with 2% w/v BSA). The dissolution of AmB corrected for degradation is described by a first
403 order process and the calculated dissolution rate constants are presented in Table 5. There is a
404 statistically significant decrease in dissolution rate constant (k_{diss}) between the same levels of
405 BSA (2% w/v) in high agitation conditions compared to low agitation conditions ($p = 0.033$).
406 While there is a trend towards a decrease in k_{diss} with increase in BSA concentration in the
407 medium in low agitation conditions, the differences in k_{diss} between each medium in the same
408 agitation conditions were not statistically significant ($p > 0.05$). The maximum % AmB
409 dissolved (X_{max}) is statistically similar between the same levels of BSA in the two agitation
410 conditions ($p > 0.05$). X_{max} increases with a higher concentration of BSA in the medium, with
411 this increase being statistically significant only for the low level of BSA (1.5% w/v) when
412 compared to the other three levels of BSA under low agitation ($p < 0.05$) (in agreement with
413 the mass balance studies). For the different levels of BSA under high agitation statistically
414 significant differences in X_{max} were not observed ($p > 0.05$). These results suggest that
415 agitation rate is of greater relevance when using media with lower albumin concentrations
416 probably due to the better powder dispersal and its exposure to the albumin that is present.
417 When the high agitation is applied, the discriminatory ability of the test is reduced.

418 The simulated dissolution profiles of AmB in media with different levels of BSA and under
419 both agitation conditions in the bottle/stirrer setup are presented in Figure 6. The use of a
420 biexponential function in which the dissolution (k_{diss}) and degradation rate (k_{deg}) constants
421 were incorporated results in successful prediction of AmB dissolution. Dissolution modeling
422 could be a valuable tool to provide a mechanistic understanding of drug dissolution in cases
423 where other processes, such as degradation, occur simultaneously with dissolution.
424
425 **3.7.2 Continuous Flow method (flow through cell apparatus**)
426 AmB dissolution profiles in KRB with BSA 2% w/v and BSA 4% w/v under low and high
427 velocity conditions with the flow through cell apparatus are presented in Figure 7. The
428 dissolution of AmB was very low in all cases, with the maximum % dissolved being 3.10 ±
429 0.08 % dissolved in the medium with BSA 4% w/v under low velocity after 8h and the samples'
430 concentrations did not reach the corresponding AmB solubility in the medium (Figure 4). A
431 low % AmB dissolved was also observed in the case where a lower amount of AmB was used
432 (0.5mg; data not shown) revealing that the low dissolution does not relate to the AmB amount
433 in the cell. The AmB dissolution with the flow through set up was lower than the AmB
434 dissolution with the bottle/stirrer set up (Figure 5). The theoretical average linear velocities,
435 based on flow rate and cell diameter, employed in the flow through cell apparatus were 0.07
436 cm/s (low velocity) and 0.52 cm/s (high velocity) while the outer edge of the stirrer in the bottle
437 had a rotational linear velocity of 10.2 cm/s (low agitation) and 29.5 cm/s (high agitation). With
438 the lowest velocity in the bottle/stirrer setup being at least 20 times greater than the highest
439 velocity of the flow-through cell apparatus, the reduced AmB power dispersal leading to
440 aggregation in the flow through cell compared to the bottle/stirrer set up is evident. As the
441 AmB binding to BSA is a dynamic process, selection of appropriate hydrodynamics which

facilitate interaction between BSA and solute, which in this case is a poor soluble compound with wetting issues [41], is essential.

4. Conclusions

AmB is used for the treatment of systemic fungal infections and it is highly bound to plasma proteins, including albumin. In clinical practice, AmB is used in patient cohorts that frequently exhibit hypoalbuminaemia. As hypoalbuminaemia is known to affect pharmacokinetics of highly protein bound drugs, AmB is a useful model compound to explore the development of dissolution tests that closely simulate *in vivo* conditions for parenteral therapies. In this work, we have developed biorelevant dissolution media with BSA concentrations representing hypoalbuminaemic patients and healthy subjects. BSA was shown to be a critical component in the media as the solubility and the degradation rate constant of AmB were dependent on the concentration of BSA. Accounting for concurrent degradation, dissolution over time could be modeled and simulated with the proposed approach (Equation 4-6), facilitating calculation of the total amount of AmB dissolved. The results of the two different setups for dissolution showed that the AmB powder needed a strong agitation (in terms of average linear velocity) for dissolution. Following correction of AmB dissolution to account for degraded AmB, a difference can be observed between the dissolution profiles (dependent on the BSA concentration), which is reflected in the values of the dissolution rate and maximum amount dissolved. Conversely, in the high agitation conditions, there was reduced discrimination between dissolution profiles following correction of dissolution for degraded AmB. Furthermore, the microbiological studies support the observation that the AmB is solubilised by binding to BSA, reducing the free fraction for activity and increasing the observed MIC. Establishing discriminating test methods with BSA present in the dissolution media supports future development of both biorelevant and clinically relevant tests for parenteral formulations.

For biorelevant dissolution testing for poorly soluble, highly protein bound drugs such as AmB, protein concentration should be considered as a medium component and the concentration used is critical, particularly given the relevance of the concentrations to target patient populations. Going forward it is important to include this element in biorelevant dissolution test development for release of AmB from lipid based formulations.

Acknowledgments

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5. References

[1] Ansel's pharmaceutical dosage forms and drug delivery systems, 10th edition. ed., Philadelphia: Wolters Kluwer/Lippincott Williams & Wilkins Health, 2014.

[2] A. Seidlitz, S. Nagel, B. Semmling, N. Grabow, K. Sternberg, W. Weitschies, Biorelevant Dissolution Testing of Drug-Eluting Stents: Experiences with a Modified Flow-Through Cell Setup, Dissolution Technologies, 18 (2011) 26-34.

[3] J. Shen, D.J. Burgess, In vitro dissolution testing strategies for nanoparticulate drug delivery systems: recent developments and challenges, Drug delivery and translational research, 3 (2013) 409-415.

[4] S.S. D'Souza, P.P. DeLuca, Methods to assess in vitro drug release from injectable polymeric particulate systems, Pharm. Res., 23 (2006) 460-474.

[5] J. Shen, D.J. Burgess, Accelerated in-vitro release testing methods for extended-release parenteral dosage forms, J. Pharm. Pharmacol., 64 (2012) 986-996.

[6] S. D'Souza, A Review of In Vitro Drug Release Test Methods for Nano-Sized Dosage Forms, Advances in Pharmaceutics, 2014 (2014).

[7] N. Fotaki, M. Vertzoni, Biorelevant dissolution methods and their applications in in vitro-in vivo correlations for oral formulations, Open Drug Deliv J, 4 (2010) 2-13.

[8] Q. Wang, N. Fotaki, Y. Mao, Biorelevant dissolution: methodology and application in drug development, Dissolution Technologies, 16 (2009) 6-12.

[9] M. Ulldemolins, J.A. Roberts, J. Rello, D.L. Paterson, J. Lipman, The effects of hypoalbuminaemia on optimizing antibacterial dosing in critically ill patients, Clinical pharmacokinetics, 50 (2011) 99-110.

[10] J.P. Nicholson, M.R. Wolmarans, G.R. Park, The role of albumin in critical illness, British journal of anaesthesia, 85 (2000) 599-610.

[11] A. Adedoyin, C.E. Swenson, L.E. Bolcsak, A. Hellmann, D. Radowska, G. Horwith, A.S. Janoff, R.A. Branch, A pharmacokinetic study of amphotericin B lipid complex injection (Abelcet) in patients with definite or probable systemic fungal infections, Antimicrob. Agents. Chemother., 44 (2000) 2900-2902.

[12] R. Bellmann, Clinical pharmacokinetics of systemically administered antimycotics, Current clinical pharmacology, 2 (2007) 37-58.

[13] I. Bekersky, R.M. Fielding, D.E. Dressler, J.W. Lee, D.N. Buell, T.J. Walsh, Plasma protein binding of amphotericin B and pharmacokinetics of bound versus unbound amphotericin B after administration of intravenous liposomal amphotericin B (AmBisome) and amphotericin B deoxycholate, Antimicrob. Agents. Chemother., 46 (2002) 834-840.

514 [14] M.T. Lamy-Freund, V.F. Ferreira, S. Schreier, Polydispersity of aggregates formed by the
515 polyene antibiotic amphotericin B and deoxycholate. A spin label study, Biochim. Biophys.
516 Acta, Biomembr., 981 (1989) 207-212.
517 [15] J. Mazerski, J. Grzybowska, E. Borowski, Influence of net charge on the aggregation and
518 solubility behaviour of amphotericin B and its derivatives in aqueous media, European
519 biophysics journal, 18 (1990) 159-164.
520 [16] J. Barwicz, S. Christian, I. Gruda, Effects of the aggregation state of amphotericin B on
521 its toxicity to mice, Antimicrob. Agents. Chemother., 36 (1992) 2310-2315.
522 [17] M.E. Malone, C. Gowing, M. Barry, E. Deasy, P. Kavanagh, O.I. Corrigan, D.M. D'Arcy,
523 M. Donnelly., ESICM 2012 Monday Sessions 15 October, 2012, Intensive Care Medicine, 38
524 (2012) P S154 (number 548).
525 [18] A.M. Gillum, E.Y. Tsay, D.R. Kirsch, Isolation of the Candida albicans gene for orotidine-
526 5'-phosphate decarboxylase by complementation of S. cerevisiae ura3 and E. coli pyrF
527 mutations, Molecular & general genetics: MGG, 198 (1984) 179-182.
528 [19] I. Nilsson-Ehle, T.T. Yoshikawa, J.E. Edwards, M.C. Schotz, L.B. Guze, Quantitation of
529 amphotericin B with use of high-pressure liquid chromatography, Journal of infectious
530 diseases, 135 (1977) 414-422.
531 [20] N. Fotaki, Flow-through cell apparatus (USP apparatus 4): Operation and features,
532 Dissolution technologies, 18 (2011) 46-49.
533 [21] J.T. Shepherd, The human cardiovascular system: facts and concepts, New York: Raven
534 Press, New York, 1979.
535 [22] Krebs Ringer Buffer composition (HiMedia Labs),
536 http://himedialabs.com/TD/TL1097.pdf, (accessed June 2017).
537 [23] Krebs-Ringer Modified Buffer (KRB), Cold Spring Harbor Protocols, 2014 (2014)
538 pdb.rec076406.
539 [24] P.W. Rand, E. Lacombe, H.E. Hunt, W.H. Austin, Viscosity of normal human blood under
540 normothermic and hypothermic conditions, Journal of Applied Physiology, 19 (1964) 117-122.
541 [25] A. Michnik, K. Michalik, A. Kluczewska, Z. Drzazga, Comparative DSC study of human
542 and bovine serum albumin, J. Therm. Anal. Calorim., 84 (2006) 113-117.
543 [26] A.K. Kudva, M. Manoj, B.M. Swamy, et al., Complexation of amphotericin B and
544 curcumin with serum albumins: solubility and effect on erythrocyte membrane damage, Journal
545 of Experimental Pharmacology, (2011).
546 [27] K.A. Connors, G.L. Amidon, V.J. Stella, Chemical stability of pharmaceuticals: a
547 handbook for pharmacists, John Wiley & Sons, 1986.

548 [28] S. Schreier, M.T. Lamy-Freund, Spectroscopic studies of aggregation and autoxidation
549 properties of the polyene antibiotic amphotericin B, Quim. Nova, 16 (1993) 343-349.
550 [29] M. Gagoś, G. Czernel, Oxidized forms of polyene antibiotic amphotericin B, Chem. Phys.
551 Lett., 598 (2014) 5-9.
552 [30] X. Xiong, S. Zhai, F. Liu, Determination of Amphotericin B in Human Cerebrospinal
553 Fluid by LC–MS–MS, Chromatographia, 70 (2009) 329-332.
554 [31] N.M. Deshpande, M.G. Gangrade, M.B. Kekare, V.V. Vaidya, Determination of free and
555 liposomal amphotericin B in human plasma by liquid chromatography-mass spectroscopy with
556 solid phase extraction and protein precipitation techniques, J. Chromatogr. B: Anal. Technol.
557 Biomed. Life Sci., 878 (2010) 315-326.
558 [32] W. Qin, H. Tao, Y. Chen, Z. Chen, N. Wu, Sensitive, accurate and simple liquid
559 chromatography-tandem mass spectrometric method for the quantitation of amphotericin B in
560 human or minipig plasma, J. Chromatogr. Sci., 50 (2012) 636-643.
561 [33] J.W. Lee, M.E. Petersen, P. Lin, D. Dressler, I. Bekersky, Quantitation of free and total
562 amphotericin B in human biologic matrices by a liquid chromatography tandem mass
563 spectrometric method, Therapeutic drug monitoring, 23 (2001) 268-276.
564 [34] T. Eldem, N. Arican-Cellat, Determination of amphotericin B in human plasma using
565 solid-phase extraction and high-performance liquid chromatography, J. Pharm. Biomed. Anal.,
566 25 (2001) 53-64.
567 [35] P. Egger, R. Bellmann, C.J. Wiedermann, Determination of amphotericin B, liposomal
568 amphotericin B, and amphotericin B colloidal dispersion in plasma by high-performance liquid
569 chromatography, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci., 760 (2001) 307-313.
570 [36] E. Yamamoto, K. Hyodo, N. Ohnishi, T. Suzuki, H. Ishihara, H. Kikuchi, N. Asakawa,
571 Direct, simultaneous measurement of liposome-encapsulated and released drugs in plasma by
572 on-line SPE-SPE-HPLC, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci., 879 (2011)
573 3620-3625.
574 [37] H.S. Ghazal, A.M. Dyas, J.L. Ford, G.A. Hutcheon, In vitro evaluation of the dissolution
575 behaviour of itraconazole in bio-relevant media, Int. J. Pharm., 366 (2009) 117-123.
576 [38] E.M. Johnson, J.O. Ojwang, A. Szekely, T.L. Wallace, D.W. Warnock, Comparison of In
577 Vitro Antifungal Activities of Free and Liposome-Encapsulated Nystatin with Those of Four
578 Amphotericin B Formulations, Antimicrob. Agents. Chemother., 42 (1998) 1412-1416.
579 [39] D. Ellis, Amphotericin B: spectrum and resistance, The Journal of antimicrobial
580 chemotherapy, 49 Suppl 1 (2002) 7-10.

581 [40] R.E. Lewis, N.P. Wiederhold, R.A. Prince, D.P. Kontoyiannis, In vitro pharmacodynamics
582 of rapid versus continuous infusion of amphotericin B deoxycholate against Candida species
583 in the presence of human serum albumin, J. Antimicrob. Chemother., 57 (2006) 288-293.
584 [41] B. Yu, T. Okano, K. Kataoka, G. Kwon, Polymeric micelles for drug delivery:
585 solubilization and haemolytic activity of amphotericin B, J. Controlled Release, 53 (1998) 131-
586 136.

587 **Tables**

588 Table 1. Composition and physicochemical properties of healthy human plasma and KRB;
589 Mean ± SD of measured physicochemical properties with different concentrations of BSA (n
590 = 3).

Ion	**Healthy Human Plasma**	**KRB**
	Composition (Concentration (mM); [21 - 23])	
HCO_3^-	24.80	14.99
K^+	4.60	4.56
Cl^-	99.00	127.32
Na^+	150.00	136.17
Ca^{2+}	4.70	1.00
Mg^{2+}	1.60	0.49
Inorganic phosphorus	1.51	2.00
D-Glucose	5.60	10.00
	Physicochemical properties	
pH	7.40 [21]	7.34 ± 0.03 (+BSA 1.5% w/v) 7.35 ± 0.03 (+BSA 2.0% w/v) 7.34± 0.03 (+BSA 3.0% w/v) 7.36 ± 0.01 (+BSA 4.0% w/v)
Osmolality (mOsm/L)	289.0 [21]	298.0 ± 10.4 (+BSA 2.0% w/v) 308.7 ± 2.5 (+BSA 4.0% w/v)
Viscosity (cps)	3.8 – 4.7 cP at 22°C [24]	Measurement at 25°C 3.70 ± 0.03 (+BSA 1.5% w/v) 3.79 ± 0.03 (+BSA 2.0% w/v) 3.88 ± 0.02 (+BSA 3.0% w/v)

		3.98 ± 0.02 (+BSA 4.0% w/v)

Table 2. AmB degradation rate constants (k_{deg}) and solubility values in KRB with different BSA concentrations (Mean ± SD; n = 3).

KRB-BSA (%w/v)	k_{deg} (h^{-1})	Solubility (μg/mL)
1.5	0.03 ± 0.01	13.03 ± 1.09
2.0	0.07 ± 0.00	13.80 ± 1.40
3.0	0.07 ± 0.00	15.28 ± 0.78
4.0	0.10 ± 0.02	17.56 ± 0.82

Table 3. Percentage of AmB in mass balance studies in KRB – BSA media after 24 h at 37°C (Mean ± SD; n = 3).

	KRB BSA 2% w/v	KRB BSA 4% w/v
$AmB\ dissolved\ at\ 24\ h$	39.43 ± 11.09	34.46 ± 4.29
$AmB\ final$	76.61 ± 7.98	59.21 ± 2.15
$AmB\ T_{undissolved}$	37.18 ± 3.11	24.75 ± 6.20
$AmB\ T_{dissolved}$	62.82 ± 3.11	75.25 ± 6.20
$AmB\ T_{degraded}$	23.39 ± 7.98	40.79 ± 2.15

607 Table 4. MIC and MFC (µg/mL) of AmB against *Candida albicans* in SBD broth and KRB
608 supplemented with BSA (n = 2).

Condition	MIC (µg/mL)			
	SBD-BSA 2%w/v	SBD-BSA 4%w/v	KRB-BSA 2%w/v	KRB-BSA 4%w/v
Control	0.2 – 0.4			
AmB	3 – 4.5	6.4	4.5	7.5
Condition	MFC (µg/mL)			
	SBD-BSA 2%w/v	SBD-BSA 4%w/v	KRB-BSA 2%w/v	KRB-BSA 4%w/v
Control	0.8			
AmB	> 10.5	> 12.0	> 10.5	> 12.0

609 MIC: minimal inhibitory concentration, MFC: minimal fungicidal concentration
610
611
612 Table 5. Dissolution rate constants (k_{diss}), maximum corrected AmB % dissolved (X_{max}) and
613 goodness of fit parameters (first order curve fitting; R^2, AIC) for the dissolution studies with
614 the bottle/stirrer setup (Mean ± SD; n = 3).
615

KRB-BSA (% w/v)	Agitation velocity	k_{diss} (h^{-1})	X_{max} (%)	R^2	AIC
1.5	Low	0.46 ± 0.10	26.73 ± 1.52	0.93 ± 0.01	28.93 ± 1.56
2.0	Low	0.30 ± 0.13	34.85 ± 3.29	0.98 ± 0.01	20.20 ± 3.62
3.0	Low	0.25 ± 0.10	38.33 ± 2.81	0.95 ± 0.02	32.16 ± 4.28
4.0	Low	0.20 ± 0.03	40.05 ± 3.98	0.95 ± 0.02	33.53 ± 8.04
2.0	High	0.16 ± 0.02	36.54 ± 1.25	0.99 ± 0.00	13.71 ± 2.20
4.0	High	0.16 ± 0.02	37.20 ± 5.43	0.95 ± 0.01	28.71 ± 4.57

616 R^2: correlation coefficient, AIC: Akaike Information Criterion.
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621 Figure 1. Representative UV spectra of AmB and deg-AmB.

622 Figure 2. Mass spectra of AmB and deg-AmB in KRB-BSA 4% w/v obtained by LCMS in
623 negative mode.

624 Figure 3. ln AmB concentration as a function of time in KRB with BSA 1.5-4 %w/v with the
625 bottle/stirrer set up (130 rpm) at 37°C (degradation study; Mean ± SD; n=3).

626 Figure 4. AmB concentration as a function of time in KRB with BSA 1.5-4 %w/v with the
627 bottle/stirrer set up (130 rpm) at 37°C (solubility study; Mean ± SD; n=3).

628 Figure 5. % AmB dissolved in KRB with different concentrations of BSA (%w/v) with the
629 bottle/stirrer set up at 37°C a) and b) dissolution profiles before correction for degradation; c)
630 and d) dissolution profiles after correction for degradation. (Mean ± SD; n=3) [LA: low
631 agitation; HA: high agitation].

632 Figure 6. Simulated and observed % AmB dissolved as a function of time [lines: simulated
633 profiles (obtained with Eq 6); points observed values (Mean ± SD; n=3)]

634 Figure 7. % AmB dissolved in KRB with different concentrations of BSA (%w/v) with the
635 flow through cell apparatus at 37°C (Mean ± SD; n=3) [LV: low velocity; HV: high velocity].

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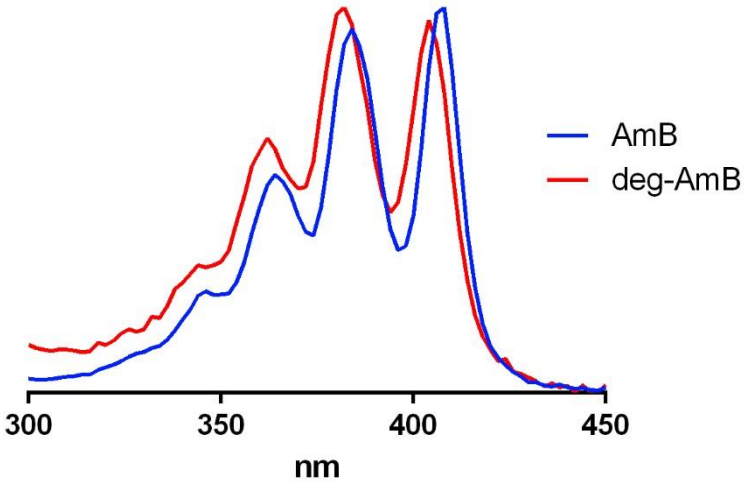

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644 Figure 1

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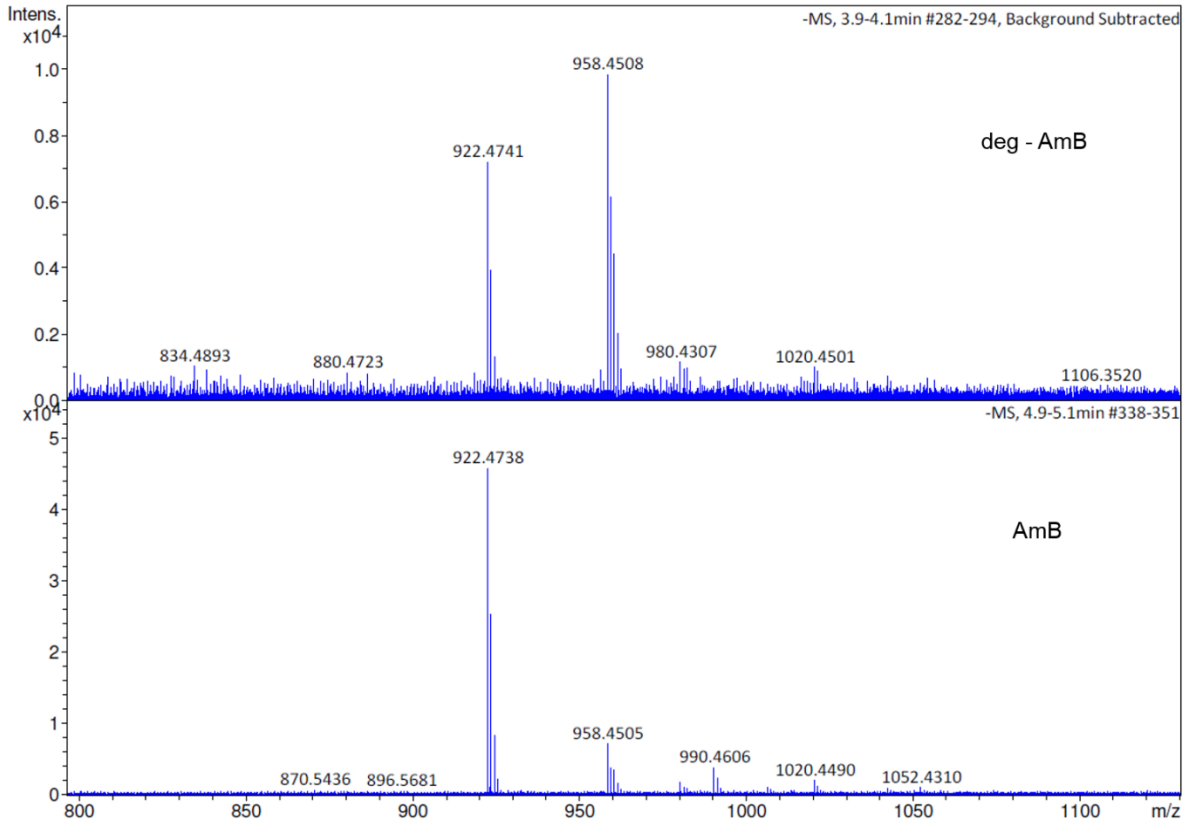
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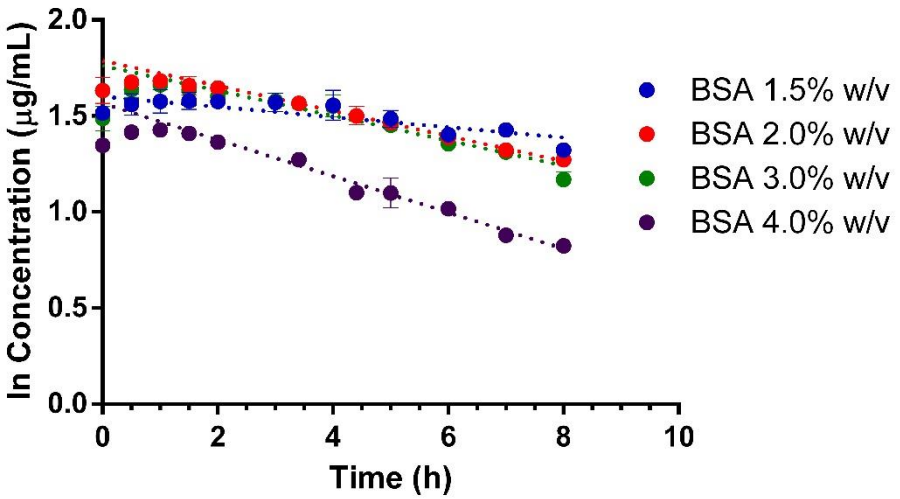
Figure 2



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652 Figure 3

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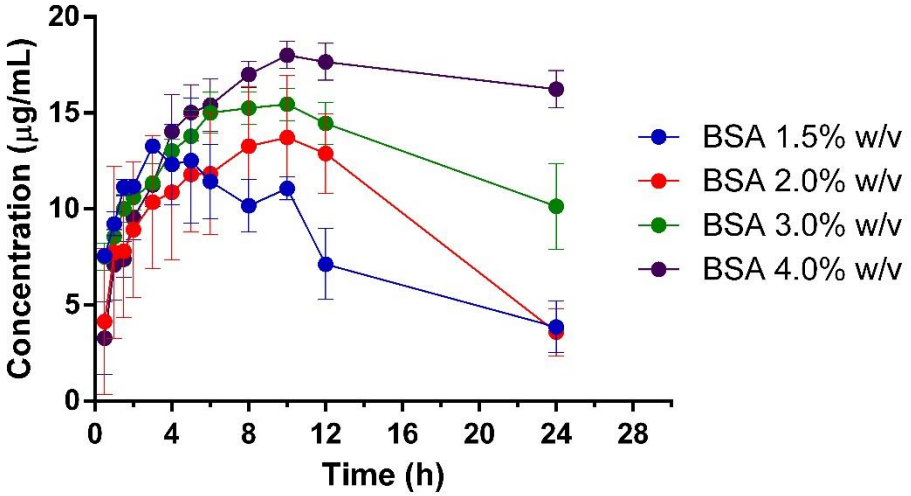

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656 Figure 4

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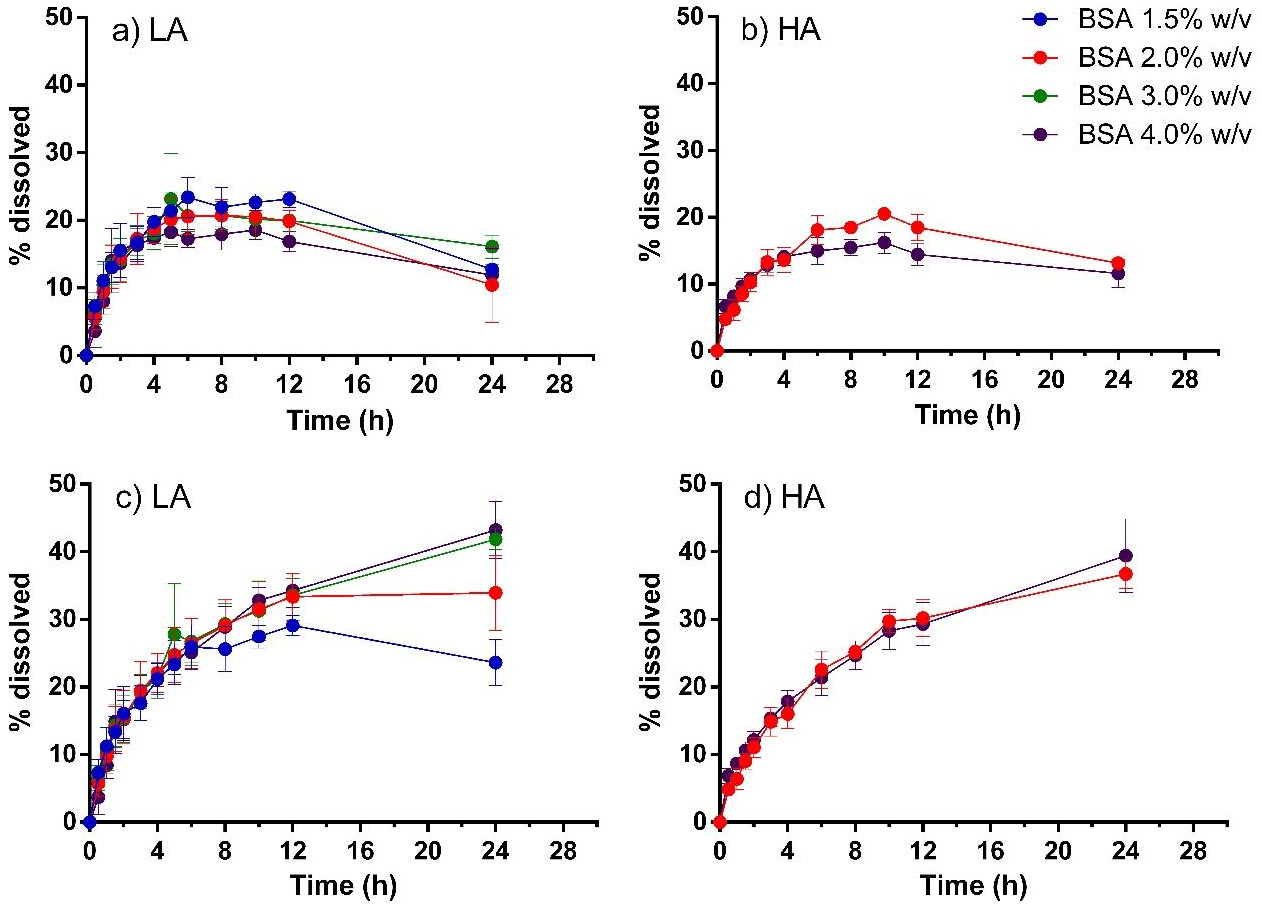

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660 Figure 5

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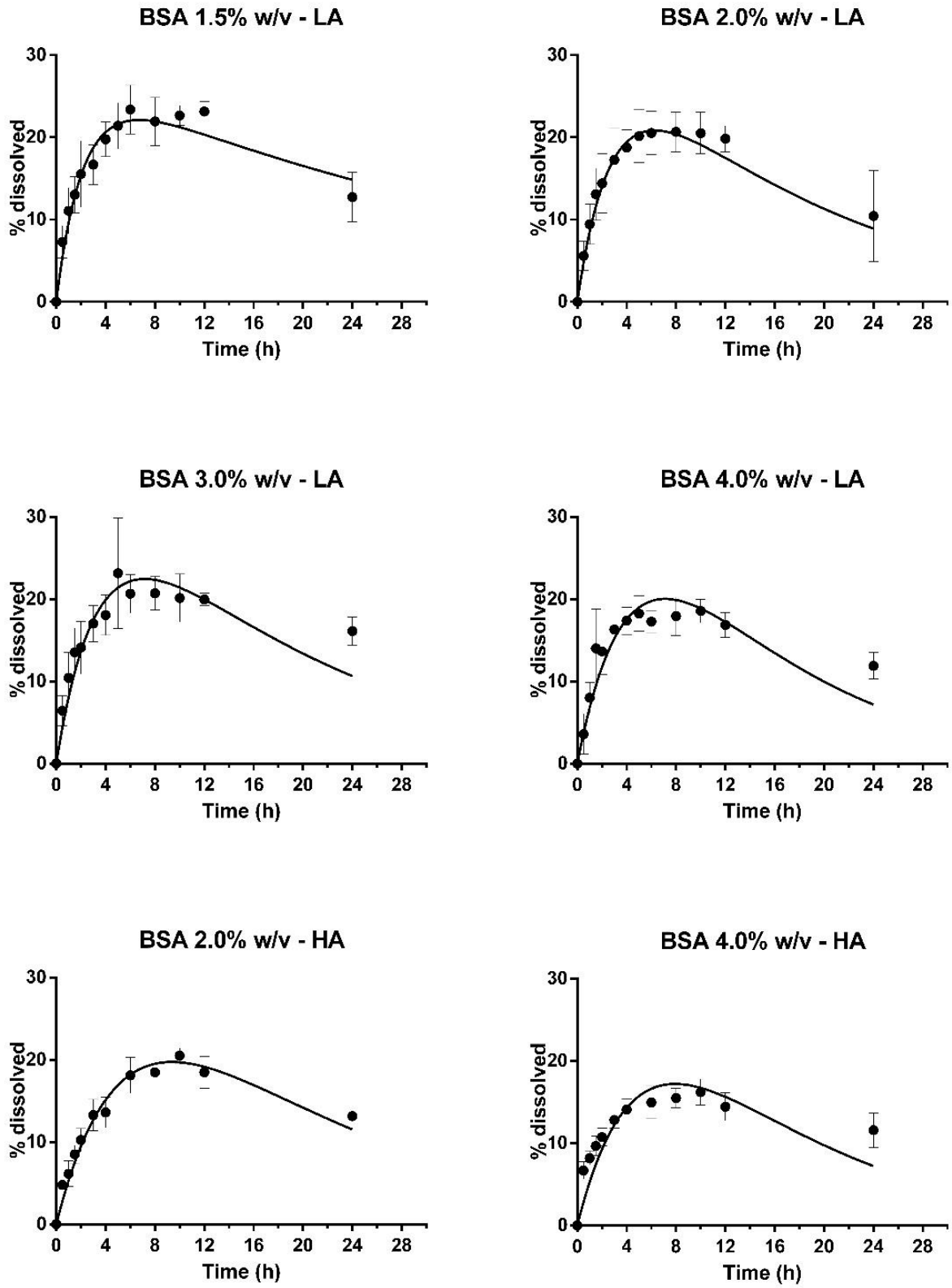

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664 Figure 6

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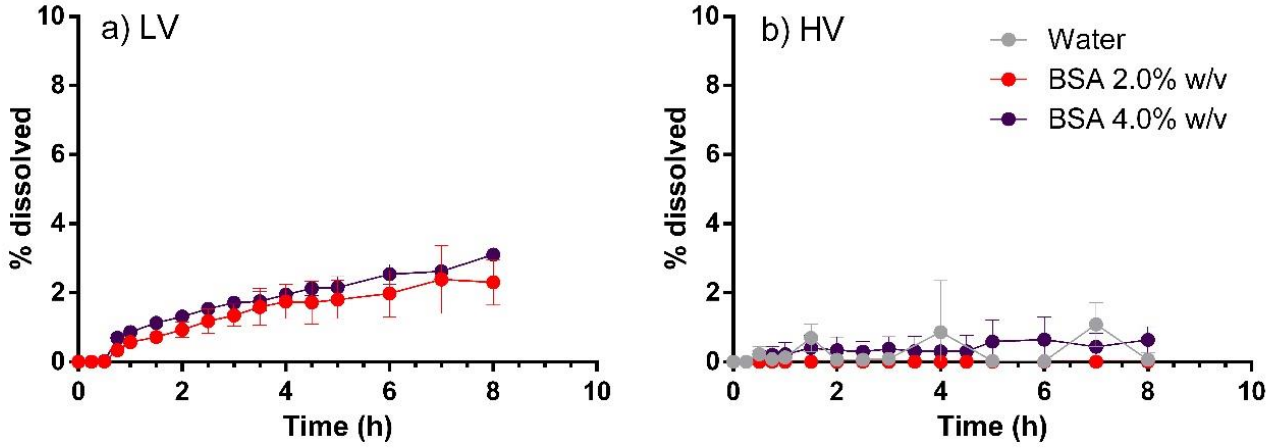

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668 Figure 7