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# *In vitro in vivo* relations for the parenteral liposomal formulation of Amphotericin B: A biorelevant and clinically relevant approach

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## Abstract

There is limited information on how to perform *in vitro* release tests for intravenously administered parenteral formulations and how to relate the *in vitro* release with an *in vivo* pharmacokinetic parameter after the administration of the formulation. In this study, the effect of hydrodynamics (using sample and separate and continuous flow conditions) and medium components (synthetic surfactants, albumin and buffers) on the release of Amphotericin B from the liposomal Ambisome® formulation were investigated. Pharmacokinetic modeling of plasma concentration profiles from healthy subjects administered Ambisome® was used to estimate the *in vivo* release rate constant of drug from the formulation in order to compare it with the *in vitro* release profiles. With the estimated *in vivo* and *in vitro* release rate constants, release profiles were generated. Two approaches were followed: comparison of *in vivo and in vitro* release rate constants and comparison of the area under the percent release-time curve from observed in vitro release data and simulated *in vivo* release data. Albumin was found to be most critical factor for the release of the drug by having a negative effect on the amount of Amphotericin B released. The release profiles obtained with the sample and separate method in both Krebs Ringer buffer- and Phosphate Saline buffer - albumin 4.0% w/v were predictive of the *in vivo* release profiles in healthy subjects. Determining the factors affecting drug release from parenteral formulations and relating the release profiles to a pharmacokinetic parameter *in vivo* supports the development of *in vitro in vivo* relations for parenteral products.



**Keywords:**

Amphotericin B; liposomes; parenteral; formulation; in vitro; release; pharmacokinetics;

## 1. Introduction

The timescale of therapeutic effect of parenterals can be controlled, to a certain extent, by the type of the formulation (e.g. suspensions, liposomes). Liposomes, which are the focus of this study, are vesicles formed by one or more phospholipid bilayer(s) with an internal aqueous phase and a typical size ranging from 25 nm to 2.5 μm that could encapsulate or integrate drugs in their structure [1]. There is a lack of regulatory guidance with specific test conditions for *in vitro* release tests for liposomes. Shah et. al. [2] recommended the use of the flow through cell dialysis adapter in the flow through cell apparatus. The Food and Drug Administration (FDA) guidelines for liposomal products only states that a validated release test should be performed with a suitable release medium (e.g. plasma, or simulated physiological or non-physiological medium) and with suitable agitation [3]. *In vitro* release from liposomes has been studied using several methods including dialysis and sample and separate methods [4-7]. In sample and separate methods, a critical step is the separation of the released drug from the liposomes. Ultracentrifugation can be used, but the long times required to pellet small liposomes (< 100 nm) makes this technique unsuitable to capture a snapshot of drug release for construction of a release profile [8-13]. Solid phase extraction (SPE) provides a quicker separation and the drug still entrapped in the liposomes can also be quantified, making it possible to calculate the release based on the quantity of drug remaining in the formulation; this approach is particularly suitable if the released drug has degradation or solubility issues [14, 15].

For the development of an *in vitro* release test for liposomes, the first step is to consider selection of relevant conditions: a suitable release medium based on the physicochemical properties of the drug, suitable hydrodynamics and an adequate dialysis membrane with an appropriate molecular weight cut-off (MWCO) if needed. *In vitro* hydrodynamics would relate to the agitation applied in the form of predominantly rotational flow, provided by e.g. a magnetic stirrer or by a predominantly linear flow (e.g. the flow through cell apparatus) [2].

Amphotericin B (AmB) is a polyene anti-fungal antibiotic, which is highly protein bound *in vivo* [16]. Ambisome® is a commercially available liposomal parenteral formulation of AmB. Ambisome® liposomes have a diameter less than 100 nm and consist of a unilamellar bilayer with AmB intercalated within the membrane, where the drug is an integral part of the liposomal structure [17]. Ambisome® is administered by intravenous infusion and indicated for treatment of severe systemic mycoses [18]. Such patients can be critically ill and frequently exhibit hypoalbuminaemia.

The release of polydiacetylene (a colorimetric compound) from liposomes with the same charge and of similar composition as Ambisome® (negatively charged liposomes of dimyristoylphosphatidylcholine and polymerized 10, 12-pentacosadiynoic acid), using hexadecyltrimethylammonium bromide (CTAB; cationic surfactant), sodium lauryl sulfate (SLS; anionic surfactant) and Triton 100X (non-ionic surfactant), has been reported [19]. CTAB produced the fastest release followed by Triton 100X. The release with SLS was minimal but the addition of NaCl increased the amount released; as for charged surfactants, an increase in the ionic strength decreased the critical micellar concentration (CMC), while non-ionic surfactants were not affected [20]. Therefore, the buffer used in the release test is another factor to investigate. Mechanistically, it has been reported that surfactant monomers partition into the surface of the liposomes, then surfactant-saturated vesicles and lipid-saturated micelles start to coexist followed by the lipids forming mixed micelles with the surfactants eventually leading to liposomal disruption [21-23].

Whereas *in vitro* release tests are frequently conducted for quality control purposes, *in vitro* release test conditions which reflect the *in vivo* performance are desirable. For parenteral formulations administered intravenously such as liposomes, pharmacokinetic (PK) models of formulated and released drug circulating concurrently could be exploited to estimate the *in vivo* release profile, in order to guide *in vitro* release test development.

In previous studies, biorelevant media representing the plasma albumin concentration [24, 25] and media able to provide clinically relevant AmB solubility values using synthetic surfactants [25] have been developed. These media were developed based on the AmB active pharmaceutical ingredient (API) and tests are needed to investigate how these media will affect the release from the liposomal formulation. Ambisome® liposomes are negatively charged [17] and this will define how surfactants, depending on their charge, will interact with them.

The aims of this study were a) to investigate how media composition including synthetic surfactants, buffers, and protein content (bovine serum albumin (BSA) concentration), and hydrodynamic conditions affect the release of AmB from Ambisome® liposomes and b) using PK modelling of published data of AmB plasma concentrations from healthy subjects to estimate *in vivo* release rates and area under the curve of the percent released-time profile, in order to identify clinically relevant *in vitro* test conditions for a parenteral liposomal formulation using Ambisome® as model formulation.

## 2. Materials and Methods

### 2.1. Materials

AmB analytical standard (87.8%), methanol (MeOH) high performance liquid chromatography (HPLC) grade, formic acid mass spectrometry grade, NaOH, $MgCl_2$, $CaCl_2$, CTAB, and $NaHCO_3$ were obtained from Sigma Aldrich (Germany); AmB API powder (85%) from Cayman Chemical (USA); BSA protease free powder fraction V, dimethyl sulfoxide (DMSO), dextrose, SLS, $Na_2HPO_4$, $NaH_2PO_4$, $KH_2PO_4$, NaCl and KCl from Fisher Scientific (USA); Tween 80 from Amresco (USA); GF/D (pore size 2.7 µm, 25 mm diameter) and GF/F (pore size 0.7 µm, 25 mm diameter) filters from Whatman (UK); regenerated cellulose (RC) filters 0.45 µm 13 mm diameter from Cronus (UK); cellulose ester dialysis tubing of 300 kDa MWCO

from Spectrum Labs® (USA) and Sep – Pak® Vac 3cc (500 mg) tC18 SPE column from Waters (Massachusetts, USA).

### 2.2. Sample treatment of AmB from release media

The SPE method to separate liposomal AmB from released AmB was a modification of the method reported by Egger et al [15]. Briefly, the SPE column was conditioned with 1.0 mL of MeOH followed by 1.0 mL of water. 1.0 mL of sample was passed through the column and the eluate was collected in a clean vial (liposomal AmB), the column was washed with 2.0 mL of water and collected in the same tube, (remaining liposomal AmB in the column). 1.0 mL of methanol was passed through the column to elute the AmB retained in the column (released AmB). In the case of samples with proteins, samples were treated as described previously [24]. Briefly, proteins were precipitated by adding 2 volumes of methanol to 1 volume of the sample followed by mixing in a vortex for 30 seconds and then centrifuged for 10 min at 12,000 rpm and 5°C in an Eppendorf centrifuge. Supernatant was filtered through a 0.45 μm RC filter before injection to the HPLC.

### 2.3. Chromatographic conditions for the analysis of AmB from release media

The chromatographic method to quantify AmB was described previously [24]. Briefly, AmB was quantified by HPLC with a C18 Waters Sunfire column (Ireland) 150 x 46 mm 5μm at 25°C. The mobile phase was formate buffer (50 mM; pH = 3.2): MeOH (25:75, v/v); the flow rate was 1 mL/min and AmB was detected at $\lambda = 406$ nm. The UV spectrum was recorded from 300 to 450 nm. Freshly prepared standard solutions (0.5 – 15 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 limit of detection (LoD) and the limit of quantification (LoQ) were 0.12 and 0.37 μg/mL, respectively.

### 2.4. *In vitro* release studies of AmB from Ambisome® formulation

144 The factors investigated for the development of the *in vitro* release studies were: i) the
145 composition of the release media: type of buffer, BSA concentration and synthetic surfactant
146 concentration, and ii) the hydrodynamic conditions in terms of the method used i.e. sample and
147 separate (bottle/stirrer) or continuous flow (flow through cell apparatus) method.

148 **2.4.1. Sample and separate method (bottle/stirrer setup)**

149 Ambisome® powder (0.5 mg AmB) was placed into a 100 mL glass bottle (56 mm diameter/
150 105 mm height; Duran, Germany) with 30 mL of release medium and stirred with a magnetic
151 stirrer (in a Variomag multipoint stirring plate) at 37°C. Release studies were performed based
152 on a two level factorial design of experiments (DoE). The composition of release media and
153 agitation conditions used in the DoE are shown in Table 1, the combination of all the factors
154 resulted in eight experimental setups.

155 The agitation rates in the bottle/stirrer setup were selected based on the linear velocity of the
156 stirrer edge, which at 130 rpm (10.2 cm/s) is comparable to the linear flow velocities in
157 vein/arteries and at 380 rpm (29.5 cm/s) to flow velocities in the aorta (Table 2).

158 The concentration of SLS was as described previously [25] to produce clinically relevant AmB
159 solubility values (PBS SLS 1.4 mM, KRB SLS 1.5 mM, PBS SLS 60.0 mM BSA 4.0% w/v
160 and KRB SLS 30.0 mM BSA 4.0% w/v). In addition to the experimental conditions described
161 in Table 1, release studies were also performed in KRB with CTAB and Tween 80 without
162 BSA at low agitation. The concentration selected was the CMC + 5% CMC of the surfactant
163 in KRB (CTAB CMC = 0.2 mM [25], Tween 80 = 10.0 µM [29]) resulting in test
164 concentrations of 0.2 mM for CTAB and 10.5 µM for Tween 80. Sampling times were 1, 2, 4,
165 6, 8, and 12 h and after sample treatment (SPE and protein precipitation; section 2.3), samples
166 were injected to the HPLC and the % AmB released over time was calculated. All experiments
167 were performed in triplicate.

### 2.4.2. Continuous flow method (flow-through cell apparatus)

AmB release studies were carried out in a flow-through cell dissolution apparatus (Sotax CE7 smart connected to a Sotax piston pump CP7, Sotax, Aesch, Switzerland) operated in the closed mode [30]. A 5mm ruby glass bead was positioned at the bottom of the cell (large cell: 22.6 mm diameter). The dialysis membrane was placed into the flow through cell apparatus dialysis adapter and Ambisome® powder (0.5 mg AmB) was placed into the membrane with 1.0 mL of the release medium. Glass fibre filters (GF/D, GF/F) were positioned at the top of the cell.

Release studies were performed considering a) biorelevant conditions and b) conditions using synthetic surfactants. The biorelevant release studies were based on a two level factorial DoE, where the velocity and BSA concentration in KRB (2.0 and 4.0% w/v, representing hypoalbuminaemic and healthy subjects, respectively) were the factors investigated. Velocities used were considered biorelevant: "Low velocity" (flow rate: 8 mL/min)) is comparable to capillary flow and "High velocity" (flow rate: 35 mL/min) is comparable to intermediate capillary-vein flow (Table 2). 36 mL of release medium were used in order to simulate the equivalent volume available on administration of 1 mg/kg of AmB as Amphotericin B® to a 70 kg subject (assuming 5 L of blood volume). Furthermore, as the 36 mL volume used does not allow for distribution as would happen *in vivo*, it represents an extreme case in terms of available volume.

For studies performed in media with synthetic surfactants PBS SLS 1.4 mM was the release medium and the effect of velocity was investigated [medium velocity: 16 mL/min, high velocity: 35 mL/min] and 50 mL of medium were used in order to achieve sink conditions (3x saturation solubility) [25].

Samples were taken for up to 12 h and, after sample treatment (if necessary), were injected to the HPLC and the %AmB release over time was calculated. All experiments were performed in triplicate at 37°C.

### 2.5. Release data treatment

Data treatment was previously described [24]. Briefly, for the studies with the sample and separate method, %AmB released over time was calculated based on the percent of AmB still entrapped in the liposomes at the time of sampling ($\%AmB_{liposomal}$) (Eq 1) to construct the calculated $\%AmB_{released}$ profile.

$$\%AmB_{released} = \%AmB_{initial} - \%AmB_{liposomal} \quad \text{(Eq 1)}$$

where $\%AmB_{initial}$ is the mass of AmB placed into the reservoir initially (100%) and $\%AmB_{released}$ is the calculated AmB percent released at time $t$. There was no correction for degradation for these profiles based on the assumption that the AmB still in the liposome cannot be subject of degradation [31].

For the studies with the continuous flow method, %AmB released over time was corrected for degradation using the degradation rate constant with Eq 2 to construct the calculated $\%AmB_{released}$ profile.

$$\%AmB_{released} = \%AmB_{released_{(obs)}} + k_{deg} * AUC_{0-t} \quad \text{(Eq 2)}$$

where $\%AmB_{released}$ is the corrected AmB percent released accounting for degradation, $\%AmB_{released_{(obs)}}$ is the AmB percent released at time *t*, $AUC_{0-t}$ is the Area Under the Curve of the observed concentration – time curve from time 0 to time $t$ and $k_{deg}$ is the degradation rate constant obtained from the degradation experiments.

First order curve fitting (Eq 3) was performed on the $\%AmB_{released}$ profiles in order to obtain the release rate constant ($k_{rel}$) (GraphPad Prism 7, GraphPad Software, Inc, USA).

$$\%AmB_{released} = \%AmB_{released}max * (1 - e^{-k_{rel}t}) \quad \text{Eq 3.}$$

where $t$ is time and $\%AmB_{released}max$ is the maximum AmB amount released. The coefficient of determination ($R^2$) and Akaike information criterion (AIC) were calculated.

$AUC_{0-12h}$ was calculated for all the $\%AmB_{released}$ profiles.

### 2.6. Atomic Force Microscopy (AFM)

To further investigate the effect of proteins and surfactants on the liposomes, AFM studies were performed. Ambisome® liposomes were incubated in the following media: KRB, KRB BSA 4.0% w/v, KRB SLS 1.5 mM BSA 4.0% w/v (for 30 min) and in KRB CTAB 0.2 mM, KRB Tween 10.5 µM and KRB SLS 1.5 mM (for 5 min; a shorter period of incubation was set in order to reflect the fast release of AmB from the liposomes observed in the absence of BSA). After the incubation, samples were centrifuged for 30 min at 13,300 rpm in an Eppendorf centrifuge, the supernatant was discarded and the pellet was dried under vacuum. The pellets were diluted with 1 mL of HPLC water, and then 10 µL of the liposomal solution was placed on a freshly cleaved mica surface (1.5 cm × 1.5 cm; G250-2 Mica sheets 1″ × 1″ × 0.006″; Agar Scientific Ltd., Essex, UK). The sample was then air-dried for ~30 min and imaged immediately by scanning the mica surface in air under ambient conditions using a Bruker MultiMode 8 Scanning Probe Microscope (Bruker, Billerica, Massachusetts, USA) operated on Peak Force QNM mode. The AFM measurements were obtained using ScanAsyst-air probes (Bruker, Billerica, Massachusetts, USA); the spring constant was calibrated by thermal tune (Nominal 0.4 N $m^{-1}$) and the deflection sensitivity calibrated using a silica wafer. AFM scans were acquired at a resolution of 512 × 512 pixels at scan rate of 1 Hz, and produced topographic images of the samples in which the brightness of features increases as a function of height. The

raw image data were processed using Bruker Nanoscope Analysis (version 1.5), and height images were flattened to remove sample tilt and scanner bow. The surface roughness ($R_a$) of each substrate was determined by using Nanoscope Analysis' algorithm to analyse several scans of the surface from different locations (n = 20). AFM images were collected from random spot surface sampling (at least four areas).

## 2.7. PK modeling for the estimation of the *in vivo* AmB release rate constant from plasma concentration profiles

### 2.7.1. Data for PK modeling of Ambisome® following administration to healthy subjects

Published data of plasma concentration profiles from healthy subjects administered Fungizone® (AmB deoxycholate formulation, molar fractions: sodium deoxycholate and AmB, 0.7 and 0.3 respectively; 5 subjects, 0.6 mg/kg, 2h infusion time [32, 33]) and Ambisome® (5 subjects, 2.0 mg/kg, 2h infusion time [34]) where the liposomal and released/non-liposomal AmB were quantified and digitalized with Webplot digitalizer 3.8 software. AmB plasma concentrations obtained after Fungizone® administration, lipid-bound AmB and released AmB from the liposomes after the administration of Ambisome®, will be referred as free AmB, liposomal AmB and released AmB, respectively.

### 2.7.2. Workflow for PK modeling and estimation of *in vivo* release profile

The workflow for the PK modeling to estimate the *in vivo* release rate constant of AmB from Ambisome® ($k_{rel-iv}$) and for model optimization are shown in Figures 1 and 2, respectively.

PK parameters for released AmB were estimated based on the parameter estimates of AmB following Fungizone® administration.

Compartmental modeling was performed with the excel add-in PKSolver [35] and the estimation of $k_{rel-iv}$, the optimization of the models and the simulations were performed with

Berkeley Madonna® 8.3.23 software. The $R^2$ was obtained from observed plasma concentration profiles vs predicted plasma concentration profiles of both liposomal and released AmB. The *in vivo* elimination rate constant from liposomal AmB models ($k_{elL}$) comprised the sum of the rate constants of liposomal AmB elimination ($k_{10L}$) and *in vivo* AmB release ($k_{rel-iv}$), i.e. $k_{elL} = k_{10L} + k_{rel-iv}$.

### 2.8. Evaluation of the *in vitro* tests using the PK model

The evaluation of the capacity of the AmB *in vitro* release tests to predict the *in vivo* release was explored in two parts:

Part A. *In vitro* release rate constants ($k_{rel}$) (from the profiles that fitted a first-order release profile) were compared to $k_{rel-iv}$ (mean +/- 1 standard deviation -Table 6).

Part B. *In vivo* release profiles were simulated using $k_{rel-iv}$, using the same dose and available volume as was used in the *in vitro* release tests to facilitate comparison with *in vitro* data. Three simulated % AmB released profiles were generated using $k_{rel-iv}$ (mean +/- 1 standard deviation), followed by calculation of $AUC_{0\text{-}12h}$ for each profile.

### 2.9. Statistical analysis

Pareto charts, based on the DoE analysis, were constructed for the identification of significant factors affecting the $AUC_{0\text{-}12h}$ obtained from the *in vitro* release tests. A factor was significant when the standardized effect (bars) was larger than the line for statistical significance level ($\alpha = 0.05$) (vertical line). An independent means t – test was performed to compare 2 independent means: for the continuous flow studies with PBS SLS 1.4 mM at medium and high velocity; in the AFM studies, data were compared against the control sample [KRB control (centrifugation/vacuum)]; and for $AUC_{0-12h}$ values from simulated *in vivo* (as described in section 2.8 part B) and observed *in vitro* AmB release profiles. A $p<0.05$ was considered

significant. Additionally, the 90% confidence interval (90% CI) for the ratio of the $AUC_{0-12h}$ geometric means of the measures for the observed *in vitro* and predicted *in vivo* $\ln AUC_{0-12h}$ were calculated for in vitro data where the $AUC_{0-12h}$ were not significantly different to the in vivo $AUC_{0-12h}$ data. Data analysis, creation and analysis of the DoE were performed with the statistical software Statgraphics Centurion XVII (USA) and the 90% CI were calculated with IBM SPSS Statistics 25 (USA).

## 3. Results and discussion

### 3.1. *In vitro* release studies of AmB from Ambisome®

#### 3.1.1. Sample and separate method

*In vitro* release profiles of AmB from Ambisome® using the sample and separate method are shown in Figure 3 and their corresponding $AUC_{0-12h}$ values are presented in Table 3.

In media with synthetic surfactants, the release is almost complete at the first sampling point (1 h) regardless of the buffer or the surfactant tested. Consequently, the statistical analysis of release rates could not be performed for the release profiles in synthetic surfactants using the sample and separate method. As it is observed *in vivo* that liposomal AmB is in circulation for considerably more than 1 h [34], ~100% release from the liposomes at 1 h would not be considered a clinically relevant profile. The statistical analysis of $AUC_{0-12\mathrm{h}}$ of the release profiles obtained with the sample and separate method (Figure 4a) shows that the buffer used to prepare the medium does not have any effect on the release, while BSA concentration and agitation had a negative and positive effect on AmB release, respectively.

The interaction between BSA concentration and agitation was significant, revealing that even though agitation does not affect the release of AmB in media containing BSA 4.0% w/v, at

high agitation conditions in media with BSA 2.0% w/v, the $AUC_{0-12h}$ is higher than using low agitation. The positive effect of agitation on the release of AmB from the liposomal formulation could be attributed to the increased suspension or dispersal of the liposomes and thus exposure to the medium, and/or the increased mechanical stress exerted on the liposomes (i.e. collision with the bottle wall or the magnetic stirrer). It is interesting that in higher agitation conditions the release of AmB from the liposomes in media with a lower BSA concentration (2.0% w/v), was higher than in the media with a higher BSA concentration (4.0% w/v). BSA seems to provide some kind of protective effect to the liposome, as the release of AmB from Ambisome® did not change significantly between both agitation conditions when BSA 4.0% w/v was present in the media. Further studies for the characterization of this interaction of BSA with the Ambisome® liposomes would provide a mechanistic understanding of the release process of AmB from the liposomal formulation.

The release profiles of AmB from liposomes in PBS BSA 2.0% w/v and KRB BSA 2.0% w/v at low agitation and PBS BSA 4.0% w/v and KRB BSA 4.0% w/v at high agitation showed first order release and the parameters from the first order fitting are listed in Table 4.

### 3.1.2. Continuous flow method

*In vitro* release profiles of AmB from Ambisome® obtained using the continuous flow method are shown in Figure 5 and their corresponding $AUC_{0-12h}$ values are presented in Table 3.

The release of AmB from the liposomes in media incorporating synthetic surfactant (SLS) was slower than that observed with the sample and separate method. The slower release observed with this setup could be attributed to the use of the dialysis membrane.

The statistical analysis (Figure 4b) showed that the Velocity had a positive effect on the AmB release from the liposomes. The BSA*Velocity interaction had a negative effect on the AmB release as the release is higher in using the low velocity conditions in the medium with BSA

4.0% w/v, whereas the BSA concentration on its own was not a significant factor for the release. The release data in PBS SLS 1.4 mM show that $AUC_{0-12h}$ is not statistically similar when a high velocity is used compared to the medium velocity. The release profiles of AmB from liposomes in KRB BSA 4.0% w/v medium at high velocity and in PBS SLS 1.4 mM at both medium and high velocities showed first order release and the parameters from the first order fitting are listed in Table 4.

### 3.2. AFM studies

Figure 6 shows the images obtained from the AFM and Table 5 contains the parameters of the liposome characteristics measured by AFM.

Liposomes could not be seen on the samples from media with SLS and CTAB (Figure 6c and 6e), probably due to quick disruption of the liposomes in the presence of these surfactants in the media, as revealed also by the complete AmB release at the first sampling point in these media with the sample and separate method (Figure 3). Liposomes were found in the sample with Tween 80 (Figure 6f) as expected by the slightly slower release in this medium. The liposomes in the medium with Tween 80 appear to be larger in size and more irregular shaped than the control sample, which could reflect occurrence of the reported mechanism of surfactant-liposome interaction, with surfactant-saturated vesicles and lipid-saturated micelles, which increase the size of the liposomes prior to liposomal disruption [21-23]. The presence of BSA in the media with SLS results in an alteration of the interaction of the SLS surfactant with the liposomal structure, as liposomes were present in this sample, revealing the interference of the surfactant by BSA (Figure 6d) [36-38]. The liposomes in the sample with BSA and SLS were larger in size than those observed in the corresponding sample without SLS, possibly due to changes in BSA structure on interaction with SLS, altering the form it interacts with the liposome or due to aggregation. Aggregation can be observed in the sample with BSA only

(Figure 6b) as in the sample with Tween 80, probably due to the same process described for SLS BSA. The diameter and surface roughness of the liposomes were statistically significantly different to the control sample in KRB BSA 4.0% w/v (for the samples in KRB CTAB 0.2 mM and KRB SLS 1.5 mM a statistical comparison was not performed as liposomes were not present in these samples, revealing that the charged surfactants are able to disrupt the liposomes quickly). These parameters (diameter and surface roughness) were not statistically significantly different compared to the control sample for the samples in KRB SLS 1.5 mM BSA 4.0 % w/v and KRB Tween 80, revealing that the interaction between SLS and BSA changes the way that these molecules interact with the liposomes and that the non-ionic surfactant is slightly less aggressive to the liposomes than the charged ones. Based on these results it could be considered advisable to conduct AFM studies of liposomal size and integrity in a range of dissolution media being considered for development of clinically relevant release testing; as observed in the current work the effect of the media examined on liposomal size and integrity broadly aligns with the release profiles observed.

### 3.3. PK modeling of *in vivo* release profiles in healthy subjects

Observed and predicted *in vivo* liposomal and released AmB plasma profiles are shown in Figure 7. Table 6 shows the PK parameters obtained from compartmental modeling before and after model optimization, and the estimated value of $k_{rel-iv}$.

The liposomal AmB profile for healthy subjects was described by the model developed ($R^2$ = 0.99). The model for liposomal AmB did not change after the optimization as the only relevant parameter is the $k_{elL}$ which includes $k_{rel-iv}$ and $k_{10L}$. The $k_{rel-iv}$ value was set to be lower than $k_{elL}$ and the difference of the value with $k_{elL}$ was $k_{10L}$. After the optimization, the elimination rate constant of released drug ($k_{10}$) was higher than the initial value. The difference in the half – life of elimination for AmB from Fungizone® (0.17 ± 0.14 h; calculated after poly-

exponential fitting) [34] and from Ambisome® (0.66 h; calculated from the PK modeling in the current study), reflects the difference in parameter estimates from the models fitted to the data obtained following administration of each formulation. As $k_{10}$ was an optimized parameter in the current study, the decrease in the amount of drug in plasma could also be due to distribution. A limitation of the model used in the current study is that peripheral release of AmB is not included. To our knowledge, the peripheral release kinetics are not known. In a situation where peripheral liposomal release was expected to notably impact the plasma concentrations, the PK model could be developed to include the peripheral release step. In the current work, given the relatively low rate constant reflecting redistribution of AmB from the peripheral to the central compartment (Table 6) it is unlikely that any peripheral release will have a significant impact on the $k_{rel-iv}$estimate in the current model. It is also possible that the lack of a peripheral release step may have partly promoted an underestimation of V1; although AmB is highly protein bound, a slightly higher V1 for the free AmB than for the liposomal AmB might be expected. Therefore, as with all models, there is continued scope for further model optimisation. However, for the purposes of illustrating the application of PK modelling in informing the development of a biorelevant in vitro release test method, the current model approach is deemed sufficient.

## 3.4. Evaluation of clinical relevance of the *in vitro* release tests

### 3.4.1. Part A. Comparison of *in vitro* and *in vivo* release rate constants

The *in vitro* $k_{rel}$ (obtained from the first order fitting of the *in vitro* release profiles) and the $k_{rel-iv}$ of healthy subjects (obtained from the PK modeling of liposomal and released plasma concentration profiles [34]) are presented in Table 4. Further details on the fittings are available in the "Supplementary data" file.

The *in vitro* $k_{rel}$ values are trending higher than the *in vivo* release rate. However, this analysis assumes a first-order release rate *in vivo* and is hindered by the lack of first order release *in vitro* in several of the test conditions. The *in vitro* $k_{rel}$ estimated from the study in PBS BSA 4.0% w/v at high agitation conditions was statistically similar to the $k_{rel-iv}$ of healthy subjects, however this result needs to be interpreted with caution as the coefficient of variation for the *in vitro* $k_{rel}$ is 75% and the constants could be considered statistically similar due to this high variability. The release rate constants estimated from the studies in media with BSA 2% w/v in low agitation conditions are borderline in terms of being statistically similar to in vivo values (p-value 0.056-0.058), suggesting potential for further refining in vitro conditions to generate release profiles similar to in vivo release profiles.

### 3.4.1. Part B. Comparison of *in vivo* and *in vitro* $AUC_{0-12h}$

The $AUC_{0-12h}$ of the *in vitro* release profiles and the *in vivo* simulated profile (obtained from the developed PK model) are presented in Table 3.

The simulated %AmB $AUC_{0-12h}$ was 165.18 ± 11.49 (%AmB*h) for healthy subjects. The t-test results show that the *in vitro* $AUC_{0-12h}$ calculated from the %AmB released profiles obtained in media with BSA 4.0% w/v with the sample and separate method were statistically similar to the *in vivo* $AUC_{0-12h}$. With the continuous flow method, the most promising results were generated in media with BSA 2% w/v in low velocity conditions. Despite the fact that the t-test comparisons show that some results were statistically similar, if the 90% CIs are compared against the usual bioequivalence interval (80% - 125%) [39], for all the 90% CIs from sample and separate method with BSA 4.0% w/v, either the lower or upper bound was within 80 - 125% which leaves the test as inconclusive but with room for improvement (ideally increasing the number of subjects tested). Interestingly, with the continuous flow method, BSA 2% w/v in low velocity conditions resulted in an $AUC_{0-12h}$ which could be considered similar

to the in vivo AUC based on standard bioequivalence comparisons. Along with the $k_{rel}$ data in Table 4, this suggests scope to further investigate the interplay between hydrodynamics (agitation/velocity) and BSA concentration to identify suitable clinically relevant dissolution conditions.

## 4. Conclusions

There is a lack of guidance for *in vitro* release testing of parenteral formulations. In this work, factors including medium components and hydrodynamic/agitation conditions were tested to understand how they affect drug release from a liposomal formulation for intravenous administration (Ambisome®). Regarding hydrodynamics, in both setups tested (sample and separate and continuous flow), an increase in the agitation/velocity resulted in significant increase of AmB release. The characterization of drug release from liposomes after direct contact of synthetic surfactants with the liposomes (sample and separate method) was not possible due to fast disruption of the liposomes. The use of the dialysis membrane in the continuous flow setup could overcome this issue and allows the use of simple media with synthetic surfactants for the characterization of release from these formulations. The presence of proteins (BSA) is a critical factor affecting release of drugs with high protein binding (such as AmB) with an increasing BSA concentration generally leading to a decrease in drug release. A novel approach for the estimation of the *in vivo* release rate constant from liposomes was developed through PK modeling. An *in vitro- in vivo* relation was developed, with $AUC_{0-12h}$ of *in vitro* release profiles in media with BSA 4.0% w/v with the sample and separate method being statistically similar to the *in vivo* calculated $AUC_{0-12h}$. Establishing an *in vitro- in vivo* relation by using clinically relevant release testing and PK modeling is of high importance in order to improve the efficiency of the development and the quality evaluation of such formulations.

## Acknowledgments

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548

549 **Tables**

550 **Table 1**. Levels and factors investigated with the sample and separate method for the release
551 studies of AmB from Ambisome®.

| **Factors in KRB (no synthetic surfactants added)** | | | |
|---|---|---|---|
| Level | BSA %w/v | Buffer | Agitation (rpm) |
| -1 | 2.0 | PBS | 130 (Low Agitation) |
| +1 | 4.0 | KRB | 380 (High Agitation) |
| **Factors in media with synthetic surfactant (SLS)** | | | |
| Level | BSA %w/v | Buffer | Agitation (rpm) |
| - 1 | 0.0 | PBS | 130 (Low Agitation) |
| + 1 | 4.0 | KRB | 380 (High Agitation) |

552

553 **Table 2**. *In vivo* (bloodstream) and *in vitro* (flow through cell apparatus with the large cell: 22.6
554 mm diameter) flow rates and velocities [26-28].

| ***In vivo*** ***(bloodstream)*** | | | ***In vitro*** **(flow through cell apparatus)** | |
|---|---|---|---|---|
| Blood vessel | Flow rate (mL/min) | Velocity (cm/s) | Flow rate (mL/min) | Average linear velocity (cm/s) |

| | | | | |
|---|---|---|---|---|
| Arteries | 3.0 - 26.0 | 4.9 - 19.0 | 3.0 - 26.0 | 0.01 - 0.11 |
| Veins | 1.2 - 4.8 | 1.50 - 7.80 | 1.2 - 4.8 | 0.00 - 0.02 |
| Coronary artery | 35.0 | - | 35.0 | 0.15 |
| Capillaries | - | 0.03 | 7.0 | 0.03 |
| Aorta | - | 30.0- 40.0 | 9655.0 | 40.00 |
| Vena cave | - | 15.00 | 3620.0 | 15.00 |

555

556

**Table 3**. %AmB $AUC_{0-12h}$ calculated for all the *in vitro* release profiles: sample and separate and continuous flow investigating the effect of buffers, BSA concentration, surfactants and agitation and statistical analysis for the comparison of *in vitro* $AUC_{0-12h}$ and simulated $AUC_{0-12h}$ (based on in vivo release rate constant, $k_{rel-iv}$) ($AUC_{0-12h}$ = 165.18 ± 11.49 %AmB*h). [for sample and separate; LA: low agitation, HA: high agitation. For continuous flow; LV: low velocity, MV: medium velocity, HV: high velocity] (Mean ± SD; n = 3).

| **Sample and separate method (Bottle/stirrer setup)** | | | | | | |
|---|---|---|---|---|---|---|
| **Buffer** | **BSA (%w/v)** | **Agitation/Velocity** | **Surfactant** | $AUC_{0-12h}$(%AmB ∗ h) | ***t-test* comparison (p value)** | **90% CI (healthy subjects)** |
| PBS | 2.0 | LA | - | 296.04 ± 24.89 | <0.05 | |
| KRB | 2.0 | LA | - | 327.34 ± 23.63 | <0.05 | |
| PBS | 4.0 | LA | - | 176.35 ± 36.09 | 0.70 | 80.20 - 138.51 |
| KRB | 4.0 | LA | - | 162.14 ± 29.63 | 0.81 | 77.03 - 122.87 |
| PBS | 2.0 | HA | - | 401.98 ± 28.82 | <0.05 | |
| KRB | 2.0 | HA | - | 409.86 ± 69.55 | <0.05 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| PBS | 4.0 | HA | - | 173.78 ± 24.78 | 0.65 | 85.87 - 127.55 |
| KRB | 4.0 | HA | - | 146.79 ± 8.11 | 0.09 | 79.65 - 99.27 |
| PBS | 0.0 | LA | SLS | 1140.67 ± 0.78 | <0.05 | |
| KRB | 0.0 | LA | SLS | 1112.47 ± 1.37 | <0.05 | |
| PBS | 4.0 | LA | SLS | 1136.05 ± 5.95 | <0.05 | |
| KRB | 4.0 | LA | SLS | 1138.21 ± 2.3 | <0.05 | |
| KRB | 0.0 | LA | Tween 80 | 1107.72 ± 5.25 | <0.05 | |
| KRB | 0.0 | LA | CTAB | 1137.93 ± 3.23 | <0.05 | |
| PBS | 0.0 | HA | SLS | 1150 | | |
| KRB | 0.0 | HA | SLS | 1117.67 ± 8.98 | <0.05 | |
| PBS | 4.0 | HA | SLS | 1135.18 ± 6.79 | <0.05 | |
| KRB | 4.0 | HA | SLS | 1150 | | |

| Continuous Flow method (Flow through cell apparatus) | | | | | | |
|---|---|---|---|---|---|---|
| KRB | 2.0 | LV | - | 174.38 ± 15.63 | 0.46 | 91.71-121.27 |
| KRB | 4.0 | LV | - | 376.23 ± 13.76 | < 0.05 | |
| KRB | 2.0 | HV | - | 745.35 ± 97.47 | <0.05 | |
| KRB | 4.0 | HV | - | 408.91 ± 80.85 | < 0.05 | |
| PBS | 0.0 | MV | SLS | 442.33 ± 129.39 | 0.06 | 176.82 - 382.93 |
| PBS | 0.0 | HV | SLS | 694.36 ± 124.82 | < 0.05 | |

**Table 4**. Parameters obtained after fitting (first order equation model) of %AmB released profiles from Ambisome® with the sample and separate and the continuous flow method and statistical comparison of *in vitro* release rate constants and *in vivo* release rate constants (estimated with the PK model for healthy subjects, $k_{rel-iv}$ = 0.025 ± 0.002 $h^{-1}$) [for sample and separate; LA: low agitation, HA: high agitation. For continuous flow; LV: low velocity, MV: medium velocity, HV: high velocity. * = statistically similar] (Mean ± SD, n = 3).

| **Buffer** | **BSA (%w/v)** | **Agitation/ Velocity** | ***In vitro*** $k_{rel}$ **($h^{-1}$)** | $\%AmB_{released}max$ | **$R^2$** | **AIC** | ***t-test* comparison (p value)** |
|---|---|---|---|---|---|---|---|
| | Sample and separate | | | | | | |
| PBS | 2.0 | LA | 0.117 ± 0.040 | 56.07 ± 10.04 | 0.93 ± 0.04 | 35.01 ± 2.81 | 0.056 |
| KRB | 2.0 | LA | 0.214 ± 0.083 | 44.96 ± 4.88 | 0.94 ± 0.06 | 31.99 ± 9.93 | 0.058 |
| PBS | 4.0 | HA | 0.321 ± 0.245 | 21.90 ± 4.37 | 0.85 ± 0.11 | 30.01 ± 7.03 | 0.17* |
| KRB | 4.0 | HA | 0.127 ± 0.021 | 25.09 ± 3.50 | 0.86 ± 0.04 | 29.36 ± 3.11 | 0.01 |
| | Continuous flow | | | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| KRB | 4.0 | HV | 0.467 ± 0.162 | 43.10 ± 10.56 | 0.86 ± 0.03 | 66.54 ± 7.51 | 0.04 |
| PBS SLS 1.4 mM | 0.0 | MV | 0.725 ± 0.102 | 41.87 ± 12.27 | 0.93 ± 0.10 | 49.69 ± 8.84 | 0.00 |
| PBS SLS 1.4 mM | 0.0 | HV | 1.547 ± 0.523 | 60.66 ± 9.09 | 0.97 | 54.91 ± 3.18 | 0.00 |

**Table 5**. Parameters of liposomes obtained from AFM from the samples prepared with the media components investigated in the *in vitro* release studies

| **Sample** | **Diameter (nm)** | **Surface Roughness (nm)** | **Density ($\mu m^{-2}$)** |
|---|---|---|---|
| KRB control (centrifugation/vacuum) | 69.4 ± 18.9 | 12.9 ± 1.6 | 11.9 |
| KRB BSA 4.0% w/v | 29.0 ± 2.6 | 4.1 ± 0.2 | 4.3 |
| KRB SLS 1.5 mM | No Particles | | |
| KRB SLS 1.5 mM BSA 4.0% w/v | 100.0 ± 27.4 | 10.0 ± 3.1 | 3.3 |
| KRB CTAB 0.2 mM | No Particles | | |
| KRB Tween 10.0 µM | 81.4 ± 7.7 | 11.6 ± 2.4 | 6.4 |

**Table 6**. PK parameters from the compartmental modeling and model optimization from liposomal and released AmB after administration to healthy subjects.

| | | **PK parameters** | |
|---|---|---|---|
| **Population** | | Healthy subjects | |
| **AmB form** | | Free | Liposomal |
| **V1 (L)** | | 4.830 | 4.820 |
| **V2 (L)** | | 32.486 | 2.552 |
| $\boldsymbol{k_{10}}$ **(h$^{-1}$)** | *initial* | 0.539 | 0.155* |
| | *optimized* | 1.052 ± 0.301 | 0.129 ± 0.002* |
| $\boldsymbol{k_{12}}$ **(h$^{-1}$)** | | 4.955 | 0.285 |
| $\boldsymbol{k_{21}}$ **(h$^{-1}$)** | | 0.737 | 0.538 |
| **R$^2$** | *initial* | 0.19 | 0.99 |
| | *optimized* | 0.92 ± 0.06 | 0.99 |
| $\boldsymbol{k_{rel-iv}}$ **(h$^{-1}$)** | | - | 0.025 ± 0.002 |

* For the liposomal AmB, $\boldsymbol{k_{10}}$ refers to $\boldsymbol{k_{10L}}$ **; Similarly V1(L), V2(L), k$_{12}$, k$_{21}$ and k$_{10}$ refer to the relevant parameters for liposomal AmB in the "Liposomal" column i.e. Comp1L, Comp2L, k$_{12}$L, k$_{21}$L and k$_{10}$ L respectively.**

**Figure captions**

**Figure 1**. Workflow for the PK modeling of free AmB (Fungizone® administration) and liposomal AmB (Ambisome® administration) in order to estimate $k_{rel-iv}$ (*in vivo* release rate constant).

**Figure 2**. Compartmental PK modeling of liposomal AmB and free AmB for the estimation of $k_{rel-iv}$.

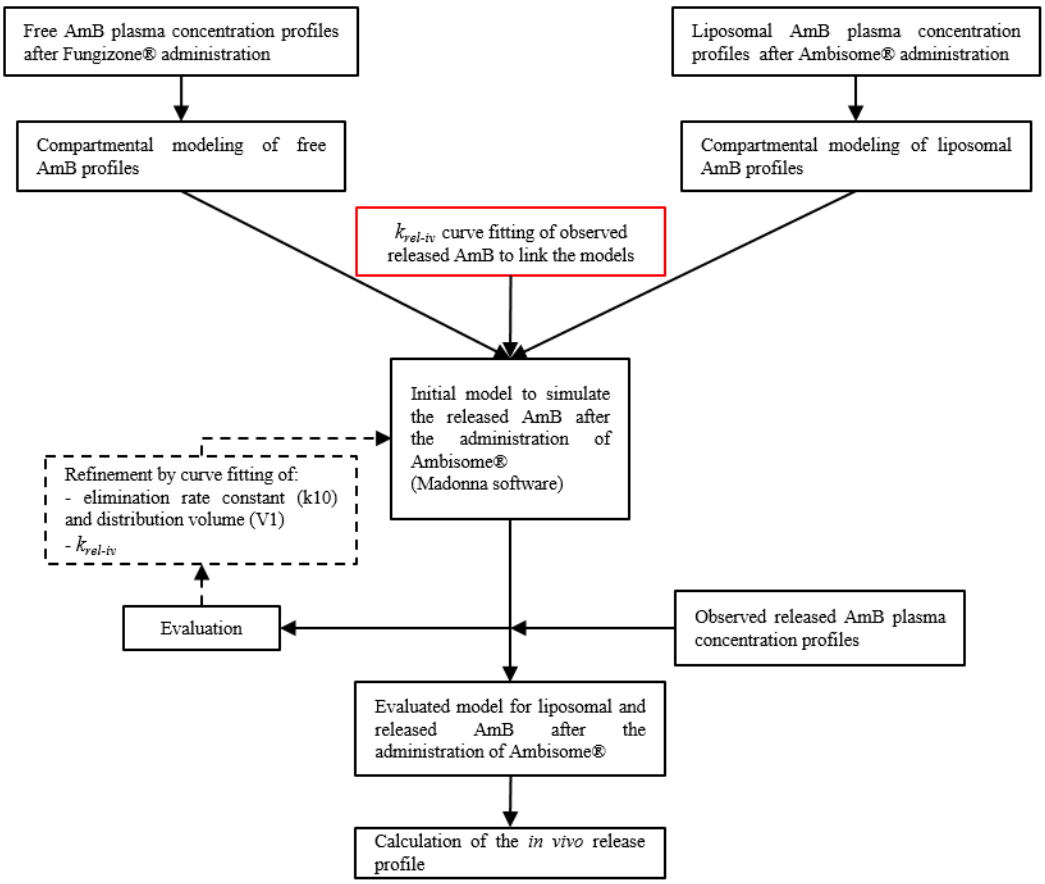
**Figure 3**. %AmB released as a function of time using the sample and separate method at 37°C to investigate the effects of buffer, agitation, composition including a) BSA concentration and b) type of synthetic surfactant and BSA 4.0% w/v presence) on AmB release (Mean ± SD; n = 3).

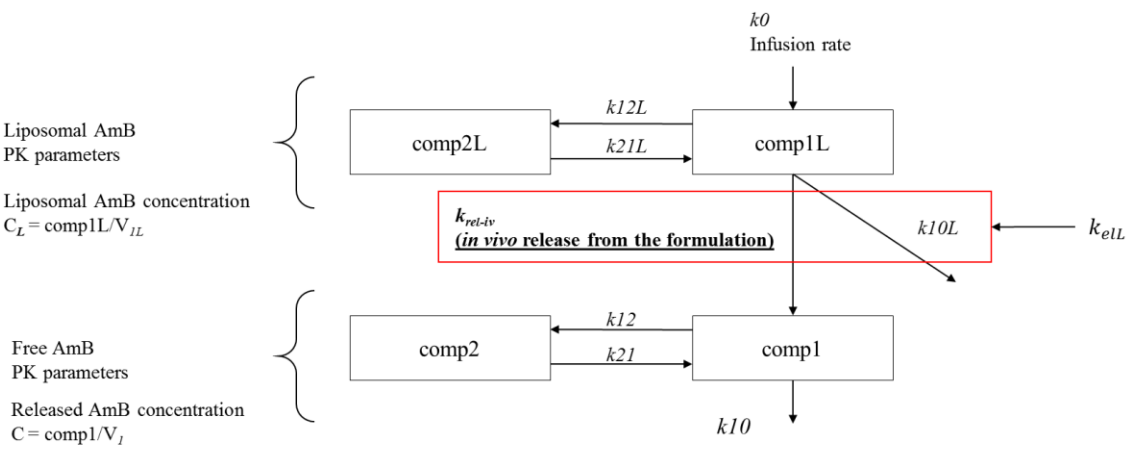
**Figure 4**. Pareto charts for the estimated effects of the main factors and 2 level interactions of the analysis of $AUC_{0-12h}$ from a) sample and separate method and b) continuous flow method. A factor was significant when the estimated effect (horizontal bars) was larger than the standardized effect (vertical line).

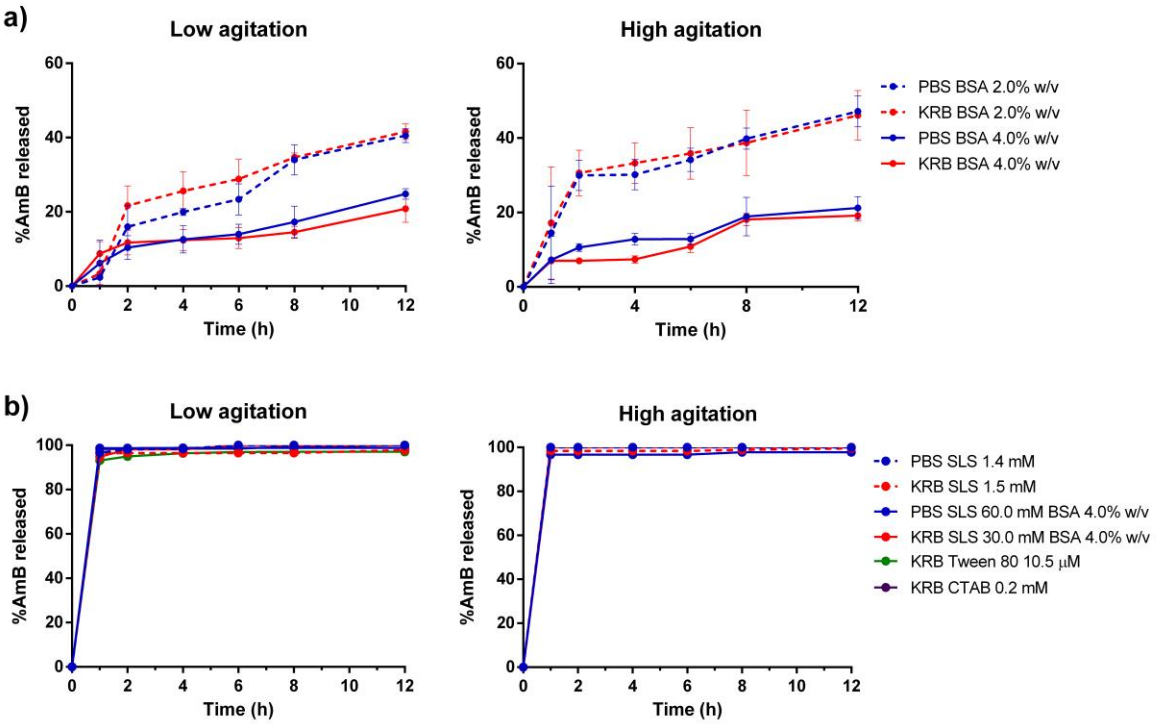
**Figure 5**. %AmB released as a function of time with the continuous flow method at 37°C in KRB to investigate the effects of BSA concentration and velocity, and in PBS SLS 1.4 mM to investigate the effect of velocity on AmB release (Mean ± SD; n = 3).

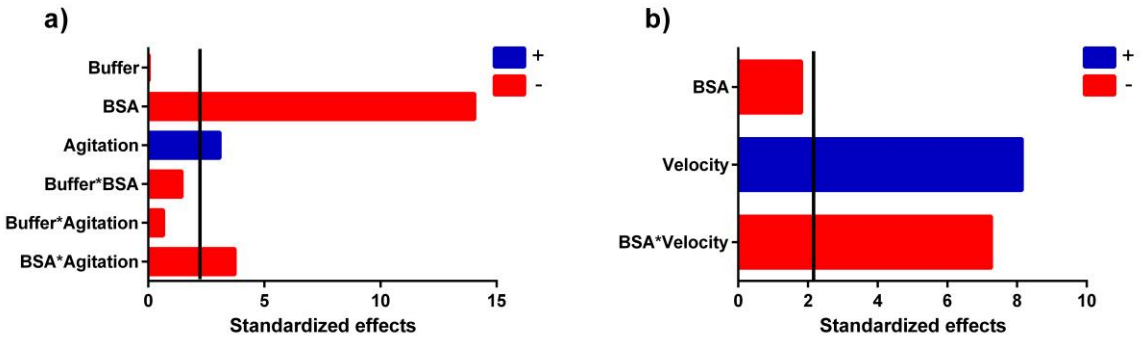
**Figure 6**. AFM images to evaluate the effect of media components on Ambisome® liposomes. a) KRB, b) KRB BSA 4.0% w/v, c) KRB SLS 1.5 mM, d) KRB SLS 1.5 mM BSA 4.0% w/v, e) KRB CTAB 0.2 mM and f) KRB Tween 80 10.0 µM. The scale bar represents 200 nm.

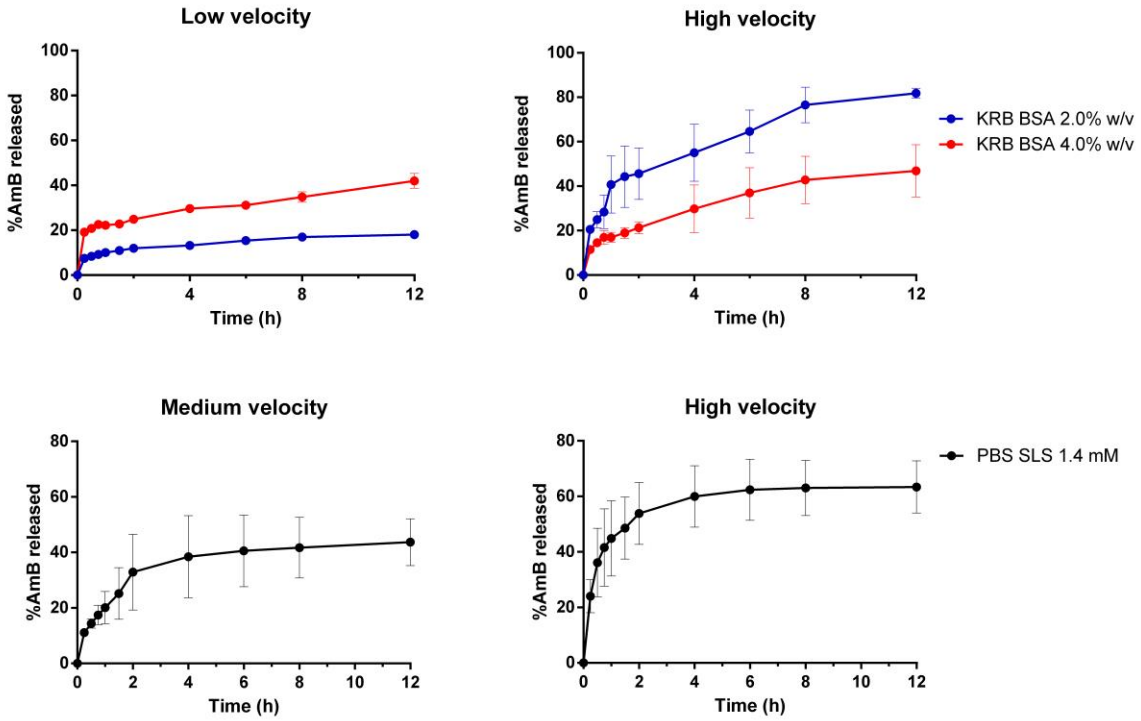
**Figure 7**. Observed and predicted liposomal and released AmB plasma profiles simulated with the optimized models. Healthy subjects' data (Bekersky et al, n = 5, [31]), a) Liposomal AmB, b) released AmB. Blue points and line: observed data; red solid lines: mean of the prediction, red dotted lines: standard deviation of the prediction.

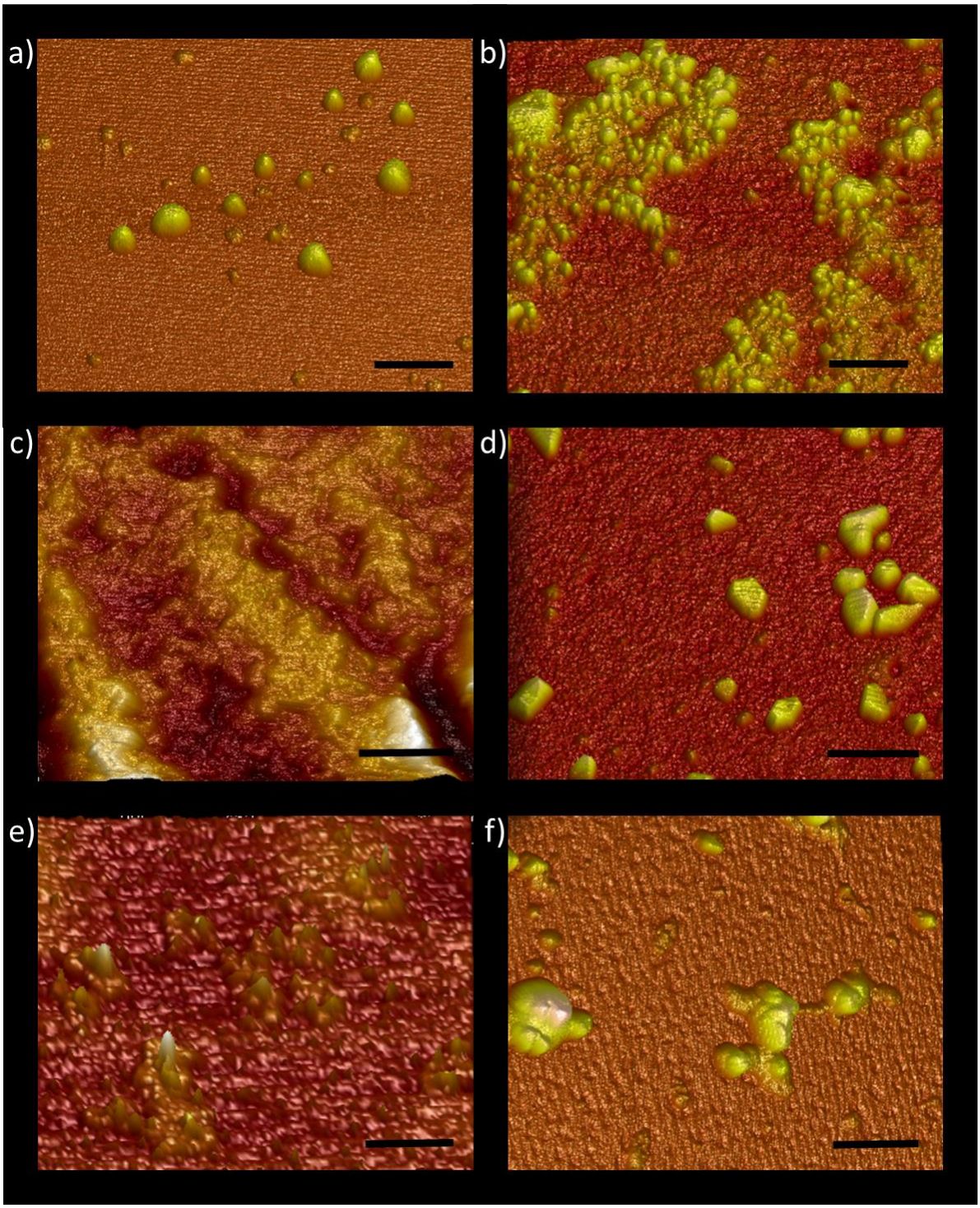

Free AmB plasma concentration profiles after Fungizone® administration
Liposomal AmB plasma concentration profiles after Ambisome® administration
Compartmental modeling of free AmB profiles
Compartmental modeling of liposomal AmB profiles
$k_{rel\text{-}iv}$ curve fitting of observed released AmB to link the models
Initial model to simulate the released AmB after the administration of Ambisome® (Madonna software)
Refinement by curve fitting of:
- elimination rate constant (k10) and distribution volume (V1)
- $k_{rel\text{-}iv}$
Evaluation
Observed released AmB plasma concentration profiles
Evaluated model for liposomal and released AmB after the administration of Ambisome®
Calculation of the *in vivo* release profile

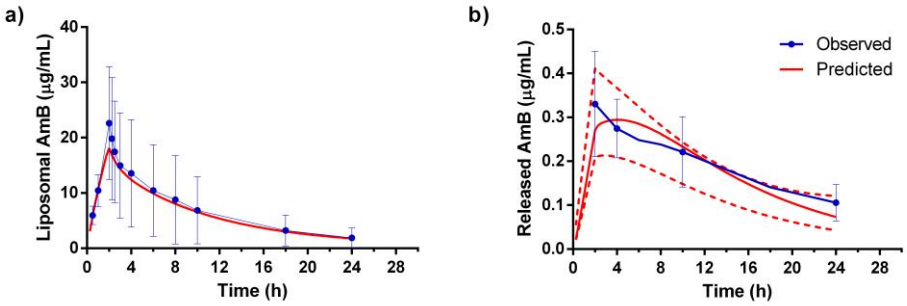

k0
Infusion rate
Liposomal AmB
PK parameters
Liposomal AmB concentration
$C_L$ = comp1L/$V_{1L}$
comp2L
k12L
k21L
comp1L
$k_{rel-iv}$
(in vivo release from the formulation)
k10L
$k_{elL}$
Free AmB
PK parameters
Released AmB concentration
C = comp1/$V_1$
comp2
k12
k21
comp1
k10


a)
Low agitation
High agitation
%AmB released
Time (h)
PBS BSA 2.0% w/v
KRB BSA 2.0% w/v
PBS BSA 4.0% w/v
KRB BSA 4.0% w/v
b)
Low agitation
High agitation
%AmB released
Time (h)
PBS SLS 1.4 mM
KRB SLS 1.5 mM
PBS SLS 60.0 mM BSA 4.0% w/v
KRB SLS 30.0 mM BSA 4.0% w/v
KRB Tween 80 10.5 μM
KRB CTAB 0.2 mM


a)
Buffer
BSA
Agitation
Buffer*BSA
Buffer*Agitation
BSA*Agitation
+
-
0
5
10
15
Standardized effects
b)
BSA
Velocity
BSA*Velocity
+
-
0
2
4
6
8
10
Standardized effects


Low velocity
%AmB released
Time (h)
High velocity
%AmB released
Time (h)
KRB BSA 2.0% w/v
KRB BSA 4.0% w/v
Medium velocity
%AmB released
Time (h)
High velocity
%AmB released
Time (h)
PBS SLS 1.4 mM


a)
b)
c)
d)
e)
f)


a)
Liposomal AmB (µg/mL)
40
30
20
10
0
0
4
8
12
16
20
24
28
Time (h)
b)
Released AmB (µg/mL)
0.5
0.4
0.3
0.2
0.1
0.0
Observed
Predicted
0
4
8
12
16
20
24
28
Time (h)