In vitro conditions for performance evaluation of products for intravascular administration: Developing appropriate test media using Amphotericin B as a model drug

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Abstract

Currently, there are no compendial *in vitro* release tests specifically indicated for parenteral formulations. Consideration of biorelevant and clinically relevant test media represents a valuable approach for the development of *in vitro* tests that ideally can provide information on the formulation performance *in vivo*. The aim of this study was to investigate the effect of different media components on the solubility of Amphotericin B (a poorly soluble highly protein-bound drug) in order to develop biorelevant and clinically relevant media for future *in vitro* release testing from its liposomal formulation. Three categories of media were considered in the development approach: Category 1 media: effect of albumin concentration; category 2 media: effect of biorelevant concentrations of plasma components (bile salts, phospholipids, cholesterol, albumin); category 3 media: attaining clinically relevant solubility with biorelevant and synthetic surfactants with and without albumin and setting the basis for the development of a simulated hypoalbuminaemic plasma medium. All the surfactants tested increased Amphotericin B solubility while the simultaneous presence of albumin had a negative effect on solubility. Clinically relevant media with the use of biorelevant or synthetic surfactants and albumin were developed. One medium in which the solubility of Amphotericin B was reduced was identified as potential candidate medium to simulate hypoalbuminaemic plasma. The development of biorelevant and clinically relevant media and understanding the effect of media components and their interactions, supports future development of meaningful *in vivo* predictive release tests for parenteral formulations.

Keywords:

Amphotericin B; dissolution; solubility; clinically relevant; biorelevant; degradation

1. Introduction.

Recently, the development of parenteral formulations (for drugs of low aqueous solubility and toxic drugs) has grown. Currently, there is no *in vitro* compendial method specifically assessing how changes to a formulation might result in a change in *in vivo* performance of a parenteral drug product (Allen, 2014; D'Souza and DeLuca, 2006; Seidlitz et al., 2011; Shen and Burgess, 2012, 2013). The *in vitro* dissolution/release testing used in quality control does not provide information about the dissolution/release of the drug in the environment where the formulations will be administered (such as the intravenous, intramuscular and subcutaneous routes (Allen, 2014)). Biorelevant dissolution testing refers to an attempt to mimic the conditions of the *in vivo* environment in terms of the composition and physicochemical characteristics of the *in vivo* fluids and the hydrodynamics at the site of administration (Wang et al., 2009). Recently, clinically relevant dissolution testing has been defined; the term "clinically relevant" implies the establishment of a link between a drug product quality attribute (e.g. solubility) and *in vivo* performance (e.g. *in vivo* solubility) (Abend et al., 2018; Norris, 2016). This terminology has been agreed in a workshop organised by US FDA and the International Consortium for Innovation and Quality in Pharmaceutical Development (IQ) (Abend et al., 2018). A clinically relevant approach to dissolution testing enables dissolution testing to establish safe boundaries and reject drug product batches falling outside the established safe range (Abend et al., 2018). Another aspect that is not usually covered in compendial *in vitro* release testing in terms of the media composition is the variation in *in vivo* physiological conditions induced by illness. In addition to simulating the fluid where the formulation will be administered, these physiological changes should, when appropriate, be reflected in the test medium. From previous studies, our group has developed a biorelevant test medium that simulates plasma (using Krebs Ringer Buffer (KRB) (Cold Spring Harbor Protocols, 2014) for the ionic content and bovine serum albumin (BSA) to represent the human serum albumin) which was used to evaluate the impact

of albumin concentration on the solubility and degradation of Amphotericin B (AmB) (Diaz de Leon-Ortega et al., 2018). AmB, a highly protein-bound (including to albumin and lipoproteins (Barwicz et al., 1998; Brajtburg et al., 1984; Ridente et al., 1999)) and poorly soluble drug (Torrado et al., 2008), was selected as a model drug as it is commercially available as parenteral lipid-based formulations (including Ambisome® and Abelcet®) for intravenous administration. AmB formulations can be used in patients suffering from severe systemic fungal infections. The presentation of sepsis in critically ill patients can include a reduced plasma albumin concentration (hypoalbuminaemia), thus hypoalbuminaemia is a potential feature of the vascular fluid into which AmB is administered *in vivo.* The aim of this study was to investigate the impact of different media components on the solubility and degradation of AmB to develop media able to target AmB plasma solubility (clinically relevant solubility) and to evaluate if the composition of a medium is suitable for future compendial *in vitro* release testing. Media were developed based on three categories (Figure 1). Category 1 media: biorelevant media, investigating the impact of concentration of BSA. Category 2 media: Biorelevant media, investigating the impact of biorelevant concentrations of plasma components to which AmB binds *in vivo* [cholesterol, bile salts and phospholipids, with and without BSA]. Category 3 media: Clinically relevant media; category 3a media- attaining clinically relevant solubility with physiological surfactants found in plasma (bile salts and phospholipids); Category 3b media- attaining clinically relevant solubility with synthetic surfactants (SLS, CTAB or Tween 80); Category 3c media- potential for category 3b media to be used as a basis to develop media for solubility and release studies simulating hypoalbuminaemic plasma. In advance of performing clinically relevant release testing, the development of biorelevant and clinically relevant test media (based on the active pharmaceutical ingredient (API)) is a primary step towards the development of biorelevant and clinically relevant release testing of parenteral formulations. Additionally, whereas API dissolution rates might not be considered directly

relevant to intravascular parenteral administration, API dissolution studies were performed in the current work in order to identify any factors affecting the solubility behaviour of the drug in the test media over time. This is an important step in the development of release testing of parenteral formulations, as issues relating to API precipitation, for example, may be easier to identify in a simper API dissolution test in advance of formulation release testing.

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, $MgCl_2$, $CaCl_2$, hexadecyltrimethylammonium bromide (CTAB), cholesterol (CH), $NHCO_3$ and NH_4HCO_2 were obtained from Sigma Aldrich (Germany); ethylenediaminetetracetic acid anhydrous (EDTA) from Sigma Aldrich (USA); AmB API powder (85%) from Cayman Chemical (USA); BSA protease free powder fraction V, dimethyl sulfoxide (DMSO), dextrose, sodium dodecyl sulphate (SLS), Na_2HPO_4, NaH_2PO_4, KH_2PO_4, NaCl and KCl from Fisher Scientific (USA); Tween 80 from Amresco (USA); phosphatidylcholine from egg from Lipoid GmbH (Germany); sodium taurocholate from Prodotti Chimici e Alimentaria (Italy); 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) and regenerated cellulose (RC) filters 0.45 μm 13 mm diameter from Cronus (UK).

2.1.1. Human plasma collection

Blood was drawn from healthy volunteers (having given informed consent) by median cubital vein venipuncture by a trained phlebotomist following local ethics committee approval (EIRA1, Issue 3, 11/5/2010). To act as anticoagulant, 2 mL of an EDTA 50 mg/mL solution were added to a final volume of 50 mL of blood, for a final concentration of 2 mg/mL. Plasma was separated from blood cells by centrifugation for 10 min at 2000 x g (Heraeus Biofuge

Primo R Centrifuge, Thermo Electron LED GmbH. Osterode, Germany) and the supernatant was separated into aliquots and kept at -80°C.

2.2. Sample treatment of AmB in plasma and test media

The sample treatment method was described previously (Diaz de Leon-Ortega et al., 2018). Briefly, proteins were precipitated by adding 2 volumes of methanol to 1 volume of the sample followed by mixing in a vortex mixer and centrifuged for 10 minutes at 12000 rpm at 5°C. The 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 plasma samples and test media

The chromatographic method to quantify AmB was described previously (Diaz de Leon-Ortega et al., 2018). Briefly, AmB was quantified by HPLC with a C18 Waters Sunfire column (Ireland) 150 x 46 mm 5 μm 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 λ = 406 nm. The UV spectrum was recorded from 300 to 450 nm. Quantification of AmB in samples was based on standard curves. 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 and the limit of quantification were 0.12 and 0.37 μg/mL, respectively.

2.4. Test media for evaluation of performance of parenteral drug products

Media were developed following the scheme of the 3 categories of development (Figure 1).

2.4.1. Category 1 media: investigating the impact of albumin concentration

Category 1 media consisted of KRB with varying biorelevant albumin concentrations and were developed and characterized as previously described (Diaz de Leon-Ortega et al., 2018). BSA was used as a substitute for human serum albumin as it has been reported that there is only one favourable binding site for AmB in both of them (Kudva et al., 2011).

2.4.2. Category 2 media: investigating the effect of biorelevant concentrations of plasma components on AmB solubility

Plasma components with the potential to affect the solubility of AmB *in vivo*, were added to KRB in biorelevant concentrations: bile salts 12.0 μM (mean value of the highest concentrations reported in literature (Ambros-Rudolph et al., 2007; Egan et al., 2012; Hospitals, 2014)); phospholipids 2.5 mM (Salvioli et al., 1985; Schaefer et al., 1958) and CH 4.5 mM (Abdelmagid et al., 2015). For media preparation, bile salts [sodium taurocholate was used as source of bile salts (BS)] were weighed and dissolved in KRB and then phospholipids [phosphatidylcholine from egg was used as source of phospholipids (PL)] from a stock solution of 100 mg/mL (dissolved in dichloromethane) were added; afterwards, where relevant, CH dissolved in chloroform (3.5 mg/mL) was added to the medium. Organic solvents were evaporated with a rotary evaporator consisting of a Büchi Waterbath B-480 set at 40°C and a Büchi Rotovapor R-114 (Büchi Labotechnik. Flawil, Switzerland) attached to a vacuum pump unit PC 2001 Vario (Vacuubrand GMBH. Wertheim, Germany). The pressure was decreased from 650 mbar in steps of 70 mbar every two minutes to 100 mbar, where the pressure was maintained for 10 minutes. When included in the medium, BSA (2.0% and 4.0% w/v as biorelevant concentrations) was added after the evaporation of the organic solvents. A medium with a high concentration (8.0% w/v) BSA was also tested. The compositions of the media are listed in Table 2 (Category 2 media). Osmolality, viscosity, pH and buffer capacity of these media were measured (section 2.5) and AmB solubility studies (section 2.7) were also performed in these media.

2.4.3. Category 3 media: investigating the impact of biorelevant and synthetic surfactants to achieve clinically relevant AmB solubility

Media were developed to achieve clinically relevant solubility values of AmB by using surfactants found in plasma (BS and PL) and synthetic surfactants (SLS, CTAB, Tween 80).

The effect of BSA was also evaluated (media with and without BSA 4.0% w/v). Media with synthetic surfactants were investigated for potential to simulate hypoalbuminaemic plasma.

2.4.3.1. Category 3a: Biorelevant surfactants

Attaining clinically relevant solubility with surfactants found in plasma (BS and PL), with and without BSA.

In order to evaluate the impact of BSA concentration and biorelevant surfactants on AmB solubility, media with BS, PL and BSA were prepared. The use of phosphate buffer saline (PBS) (Cold Spring Harbor Protocols, 2007) as a simpler buffer solution than KRB was also investigated as a basis for media development. A 2 level factorial design of experiments (DoE) was used to identify which factors had a significant effect on AmB solubility. Factors investigated were type of buffer: PBS or KRB; BS concentration: 3.0 or 10.0 mM; and PL concentration: 0.2 or 3.0 mM. The BS concentrations were selected to represent a wide range, including concentrations higher than the biorelevant concentration, with all investigated concentrations being higher than the critical micelle concentration of the surfactants. The PL concentration was set in order that the PL/ (BS+PL) molar fraction was lower than 0.6, which is the necessary for mixed micelles formation (Moschetta et al., 2001). Eight experimental setups resulted from the combination of these factors, BSA 4.0% w/v was added to all media, and solubility studies were performed in the 8 media. After the identification of the statistically significant factors affecting AmB solubility, the compositions of clinically relevant media were predicted with a DoE (section 2.10) which targeted AmB solubility in plasma. AmB solubility studies were then conducted in these predicted media for comparison with AmB plasma solubility values to validate the prediction. To investigate how BSA affects AmB solubility in the presence of the biorelevant surfactants, the predicted media were also prepared without BSA. Media characterization, AmB degradation and solubility studies were performed in the developed media with and without BSA (section 2.5, 2.6 and 2.7).

2.4.3.2. Category 3b: Synthetic surfactants

Attaining clinically relevant solubility with synthetic surfactants with and without BSA.

KRB and PBS were tested with 3 different types of surfactants: SLS (anionic surfactant), CTAB (cationic surfactant) and Tween 80 (non-ionic surfactant). The critical micelle concentration (CMC) of charged surfactants was determined in PBS and KRB by conductimetry (Conductivity Meter, WPA CMD 500, Scientific Laboratory Supplies Ltd, UK) to assure that the concentrations selected were above the CMC. 1 mL of a 10.0 mM solution of the surfactant in the corresponding buffer was added to either 20 mL (for SLS) or 50 mL (for CTAB) of the same buffer and the conductivity recorded. Conductivity was plotted against the surfactant concentration and the CMC was established when there was a sudden change in the slope. Measurements were performed in triplicate. Tween 80 CMC is not affected by the ionic composition of the buffer and the CMC value reported in literature was used (Sigma-Aldrich, 2019). To study the effect of the surfactants on AmB solubility, the surfactant concentrations investigated were 5.0, 50.0 and 100.0 mM with and without BSA 4.0% w/v. Single point AmB solubility experiments were conducted in all the media following the procedure described in section 2.7, with 1 mg of AmB API powder added in 10 mL of medium and sampling at 12 h, as a solubility plateau value was reached around 12 h in previous solubility determinations (Diaz de Leon-Ortega et al., 2018). The media with the surfactant resulting in an AmB solubility value closest to the AmB plasma solubility, with and without BSA, were selected to develop the clinically relevant media. Surfactant concentrations, in the range where media demonstrated the clinically relevant AmB solubility value, were investigated in order to obtain the surfactant concentration to produce clinically relevant AmB solubility by performing single point solubility experiments at 12 h in those ranges. Media characterization, degradation and solubility studies (section 2.5, 2.6 and 2.7) were performed in the developed media.

2.4.3.3. Category 3c: Potential media to simulate hypoalbuminaemic patients.

Potential for media with synthetic surfactants and BSA, to be used as a basis to develop media simulating hypoalbuminaemic plasma.

In order to identify conditions that could direct future hypoalbuminaemic medium development, clinically relevant medium in PBS from category 3b was used as a base. For these studies there was no target solubility value as a reference AmB solubility in hypoalbuminaemic plasma is not available. In order to mimic hypoalbuminaemic plasma, the use of PBS was explored with a) 2.0% w/v BSA and the corresponding concentration of surfactant developed as clinically relevant medium in category 3b, b) 2.0% w/v BSA with half of the concentration of surfactant from category 3b media and c) 4.0% w/v BSA with half of the concentration of surfactant from category 3b media. Media characterization, degradation studies (section 2.5 and 2.6) and single point solubility studies as described in section 2.4.3.2 were performed in the media.

2.5. Test media characterization

Media characterization methodology was described previously (Diaz de Leon-Ortega et al., 2018). Briefly, pH was measured in all the media following addition of all components. Osmolality 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) at 25°C in triplicate. Buffer capacity was determined by adding HCl 0.1 M until there was a change of 1 unit in the pH (Equation 1).

$$\frac{dB}{dpH} = \frac{(HCl\ volume\ (L))([HCl])}{\binom{average\ volume\ of\ sample}{over\ range\ involved}(\Delta pH)} \qquad \text{Equation 1}$$

where $\frac{dB}{dpH}$ is the buffer capacity, $[HCl]$ is the concentration of hydrochloric acid and ΔpH is the pH increment. The measurement was performed in triplicate.

The media were graded based on their complexity and received values from 1 to 4, being 1 to the simplest and 4 the most complex medium. The scale was based on the number of

components to prepare the medium (1 for PBS, 2 for KRB) and on the ease of preparation (1 for mixing the components, 2 for mixing components and evaporating solvents).

2.6. Degradation rate constant determination of AmB in plasma and in test media

The degradation rate constant determination methodology was described previously (Diaz de Leon-Ortega et al., 2018). Briefly, 3 mg of AmB API powder was added to 20 mL of plasma and then stirred for 1 hour at 130 rpm at 37°C. Samples were centrifuged for 5 minutes at 3000 rpm at 4°C and the supernatant incubated at 37°C. Samples were taken at 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0 and 24.0 hours, and analysed as described in section 2.3. AmB in the samples was determined. For dissolution media, the same procedure was followed with 10 mg of AmB API powder added to 50 mL of dissolution media and monitored for 8 hours. All experiments were performed in triplicate. A linear fit was applied to the degradation data from 4 h to the last time point, after a natural logarithm transformation of the measured concentration (Excel 2013) and the degradation rate constant (k_{deg}) was calculated as the slope of the line.

2.7. Solubility studies of AmB in plasma and in test media

Solubility study methodology was described previously (Diaz de Leon-Ortega et al., 2018). Briefly, approximately 2.5 mg of AmB API powder were placed in a 100 mL glass bottle with 30 mL of the corresponding dissolution media, stirred at 130 rpm and incubated at 37°C. The sampling times were 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0 and 24.0 hours. The undissolved AmB was removed by centrifugation and after protein precipitation and samples were analysed as described in section 2.3. Solubility studies in plasma were performed with 1.5 mg of AmB in 10 mL of plasma. Solubility studies in media with synthetic surfactants (single point solubility at 12 h) were performed with ~1 mg of AmB in 10 mL of medium. The AmB solubility saturation value was considered when the concentration reached a plateau value. An AmB solubility value in the dissolution media similar to the AmB plasma solubility was considered "clinically relevant". All experiments were performed in triplicate.

2.8. Dissolution studies of AmB in clinically relevant media from category 3a and 3b, and category 3c media, with the flow through-cell dissolution apparatus

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 (Fotaki, 2011). Tests were carried in category 3 clinically relevant media (3a and 3b) and in media from category 3c. A 5mm ruby glass bead was positioned at the bottom of the cell (small cell: 12 mm diameter; large cell: 22.6 mm diameter). 0.5 mg of AmB API powder was weighed and mixed with 6.0 g or 0.75 g of 1 mm glass beads, for the large or small cell, respectively, and were placed into the cell filling the conical part. Glass fibre filters (GF/D, GF/F) were positioned at the top of the cell. Two different hydrodynamic conditions were tested: i. High velocity: small cell with a flow rate of 35 mL/min (average linear velocity 0.52 cm/s) and ii. Low velocity: large cell with a flow rate of 16 mL/min (average linear velocity 0.07 cm/s). 50 mL of test medium were placed into the reservoir under constant stirring at 37°C. 0.5 mL samples were collected at specific time points up to 8 hours and volume was replaced with fresh medium. Dissolution studies were also performed with 5 mg (high dose) of AmB in category 3b PBS medium without BSA under both velocity conditions. Samples were treated and analysed as described in section 2.3 and the %AmB dissolved over time was calculated. All experiments were performed in triplicate.

2.9. Treatment of dissolution data

Treatment of dissolution data was described previously (Diaz de Leon-Ortega et al., 2018). Briefly, AmB dissolution profiles were corrected for degradation using the corresponding degradation rate constants with Equation 2.

$$C_{corrected(t)} = C_t + k_{deg} * AUC_{0-t} \qquad \text{Eq 2}$$

where C_t is the observed concentration at time t, AUC_{0-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 $C_{corrected(t)}$ and a first order curve fitting (Eq 3) was performed in order to obtain the dissolution rate constant (Graph Pad Prism 7, Graph Pad Software, Inc., USA).

$$X_{corrected(t)} = X_{max} * (1 - e^{-k_{diss}t}) \qquad \text{Eq 3}$$

where $k_{diss}$is the dissolution rate constant, $X_{corrected(t)}$ is the corrected percent dissolved at time t and X_{max} is the maximum corrected percent dissolved.

2.10. Statistical analysis

Equation 4 was used to analyse the DoE of the category 3a clinical relevant media development.

$$y = \mu + A + B + C + A * B + A * C + B * C \qquad \text{Equation 4}$$

where y is the response (AmB solubility), μ is the total mean; A, B and C are the main factors (buffer, BS concentration and PL concentration, respectively); $A * B$, $A * C$ and $B * C$ are the 2 level interactions. Buffer type was represented by a value of -1 for PBS and +1 for KRB. The coefficients were determined by calculating the main effects and interactions and relating them to the factor tested (by least square algorithms of DoE) and the substituted equation was used to calculate the composition of clinically relevant media (targeting AmB solubility plasma values).

To evaluate the effect of surfactants and BSA on AmB solubility in the category 3a and 3b clinically relevant media, the standardized effects for surfactants (BS-PL, SLS, CTAB and Tween 80), BSA presence and its interaction, were calculated and used to construct a Pareto chart. A factor was significant when the standardized effect (bars) was larger than the line for statistical significance level ($\alpha = 0.05$) (vertical line).

To compare degradation rate constants, a t-test was used to compare two experimental independent means and a paired t-test to compare two experimental related means (significance $p < 0.05$). A multiple linear regression model was used to investigate if the solubility and the degradation rate constant of AmB in any given medium could be related to any of the parameters of media characterization: viscosity, pH and buffer capacity (significance $p < 0.05$). Data analysis and the DoE (design and analysis) were performed with the statistical software Statgraphics Centurion XVII (USA).

3. Results and discussion

3.1. Determination of solubility and degradation rate constant of AmB in human plasma from healthy volunteers

AmB solubility in plasma reached a plateau between 3 – 12 h at a concentration of 32.52 ± 0.98 µg/mL (Figure 2) and the AmB degradation rate constant in plasma was 0.033 ± 0.002 h^{-1} (degradation half-life ~21 h) (Table 1).

3.2. Test media development for evaluation of performance of parenteral drug products

3.2.1. Category 1 media

AmB solubility values in category 1 media, obtained in our previous study, were lower (13.03 - 17.56 µg/mL) than those observed in plasma and the degradation rate constants were not statistically similar to those observed in plasma (Figure 2, Table 1) (Diaz de Leon-Ortega et al., 2018).

3.2.2. Category 2 media

The osmolality of Category 2 media is similar to the osmolality of plasma. The viscosity values are also similar to those of plasma using a similar method and range of shear rates, to those presented by Rand et al (Rand et al., 1964) for plasma at 22°C (Table 2).

On the other hand pH is only similar to plasma in the media with BSA but the buffer capacity was not as high as in plasma. BSA increased the osmolality and the buffer capacity, and kept the pH at ~7.5. However, when any other component apart from BSA was added, an increase in the pH and a decrease in the buffer capacity were observed (Table 2).

Addition of BSA, in a concentration range from 1.5 to 4.0% w/v in KRB buffer (category 1 biorelevant media), was reported to increase AmB solubility (Diaz de Leon-Ortega et al., 2018). The addition of biorelevant plasma components, to which AmB is bound *in vivo*, to the medium would be expected to increase AmB solubility bringing it closer to its plasma solubility. AmB concentration profiles in category 2 media show a decrease in AmB solubility compared to its solubility in KRB BSA 4.0% w/v (category 1 media) and plasma (Figure 2). In the concentrations utilized for media development, PL and CH are not soluble (NCBI, 2019; Sigma-Aldrich, 2019); this might be counterproductive to AmB solubilisation, as the components need to be in solution in order to dissolve AmB. When CH or BS-PL are present in the media without BSA the AmB solubility values are the lowest of all the values measured in all the tested media (Figure 2). In the media where BSA is present along with BS-PL or BS-PL CH, regardless of its concentration, AmB solubility is slightly higher than in these media without BSA, but lower than the solubility values measured in media with only BSA (Figure 2). It has been reported that BSA is capable of binding bile salts (Farruggia and Picó, 1999; Green et al., 1971; Pico and Houssier, 1989), cholesterol (Sankaranarayanan et al., 2013; Zhao and Marcel, 1996) and phospholipids (Jonas, 1976). The results suggest that, rather than CH or BS-PL having an additive effect to BSA on the solubility of AmB, BSA is hindering solubilisation by the biorelevant components added to the medium, while at the same time the biorelevant components are reducing the solubilising effect of BSA on AmB. Only in the medium with the highest BSA concentration (KRB BS PL BSA 8.0% w/v) AmB solubility was increased, but still it was lower than in the medium with only BSA 4.0% w/v (category 1

biorelevant medium). As category 2 media failed to produce better solubility values than category 1 media, no further studies were performed with these media.

3.2.3.1. Category 3a media

The concentrations of BS and PL in the medium were the only significant factors identified in the analysis of AmB solubility for the development of category 3a media (Figure 3).

Equation 5 was obtained from the DoE analysis and was used to predict the concentrations of BS and PL in both KRB and PBS to produce AmB solubility values similar to those observed in plasma (clinically relevant).

$$Solubility\ (\mu g/mL) = 5.56 - 2.09 * Buffer + 0.33 * BS(mM) + 0.04 * PL(mM) + 0.29 * Buffer * BS(mM) + 0.81 * Buffer * PL(mM) + 0.18 * BS(mM) * PL(mM) \quad \text{(Eq 5)}$$

The predicted concentrations of BS and PL were BS 20.0 mM PL 4.0 mM for KRB BSA 4.0% w/v and BS 19.7 mM PL 7.9 mM for PBS BSA 4.0% w/v. The media characterization showed that the presence of BSA increases the buffer capacity, the osmolality and the pH (in KRB); similar to the effects noted in category 2 media (Table 2). Osmolality was similar to that of plasma only for the media prepared without BSA (the concentration of BS-PL had a high impact on the osmolality and when BSA was added, osmolality increased up to 425 mOsm/L). The pH value was only similar to that of plasma for the medium prepared in PBS, with the buffer capacity being only statistically similar to the plasma value in PBS BS 19.8 mM PL 7.9 mM BSA 4.0% w/v. Degradation rate constants were not statistically similar to that observed in plasma except in the KRB BS 20.0 mM PL 4.0 mM medium (Table 1). In both buffers, the presence of BSA resulted in faster degradation of AmB than in the media without BSA. The higher AmB degradation rate constant values in these media could potentially be due to the absence of plasma components which could reduce the AmB degradation.

AmB solubility values in the media with BSA were lower than those observed in plasma, but in the media without BSA, AmB solubility values were similar to those observed in plasma (clinically relevant) (Figure 4).

The predicted BS and PL concentrations to attain clinically relevant solubility were much higher than the initial exploration ranges. Thus the effects of BS - PL on the solubilising potential of BSA and vice versa were not captured in the predicted media compositions as the equation was not validated outside of these exploration ranges. However, the effect of BSA in the first place seems to have been immaterial in equation 3.5, as clinically relevant solubility was achieved without inclusion of BSA in the media.

3.2.3.2. Category 3b: clinically relevant media

The CMC of SLS and CTAB in water is 8.1 mM and 1.0 mM, respectively (Attwood and Florence, 1983). The CMC of charged surfactants tends to decrease in a medium where the ionic strength is high, such as in saline buffers like PBS and KRB (Thongngam and McClements, 2005). The CMC of SLS in PBS and KRB was 1.3 ± 0.0 mM and 1.4 ± 0.2 mM, respectively and the CMC of CTAB in PBS and KRB was 0.9 ± 0.0 mM and 0.2 ± 0.0 mM, respectively. The surfactant concentrations selected for the solubility studies were above their CMC. CTAB was not soluble at 100.0 mM and KRB CTAB 50.0 mM BSA 4.0% w/v medium gelatinized, thus, AmB solubility could not be measured in these media. Figure 5 shows the AmB solubility in the various category 3b media with and without BSA.

The apparent AmB concentration, even with the highest surfactant concentration, was limited by the AmB amount added ~ 1.0 mg, hence, the concentration could not be higher than ~100.0 µg/mL .The charged surfactants (anionic and cationic) solubilised more AmB than the non-ionic surfactant despite the fact that AmB is a hydrophobic molecule (log P 0.8 (Sigma-Aldrich, 2015)). AmB, as an amphoteric molecule with two pKa values (pKa_1 = 5.5, pKa_2 = 10.0) will be charged at pH 7, thus, the interaction between its charges and those of the surfactant would

promote contact of the molecules leading to solubilisation of the drug. Similar to category 2 and 3a media observations, BSA presence in the media decreased AmB solubility. It has been reported that BSA binds to SLS, CTAB and Triton X-100 (non-ionic surfactant) with with the weakest binding observed for Triton X-100 (De et al., 2005; Gull et al., 2009; Valstar et al., 2000). Figure 6 shows the effect of surfactants (in both category 3a and 3b media) and BSA on AmB solubility.

The surfactant presence has a statistically significant positive effect on AmB solubility, whereas BSA and the interaction between BSA and surfactant have a negative effect. The results suggest that the presence of BSA and the surfactant alone in the medium, solubilise AmB (Diaz de Leon-Ortega et al., 2018), but when both are in the medium, they interfere with each other preventing AmB solubilisation and leading to a decrease in the solubility compared to the solubility reached in the medium with the surfactant alone. The ability of BSA to interfere with several types of molecules (BS, PL, CH, SLS, CTAB, Tween 80, as found in this study) has to be taken into account when a biorelevant or clinically relevant medium, incorporating BSA, is developed. The required concentration of other components with surfactant/solublising activity could be under-estimated due to protein-surfactant interactions, leading to an unintended reduction in API solubility. Among the media with the different surfactants tested, only media which included SLS resulted in clinically relevant solubility values of AmB in KRB and PBS either with or without BSA.

The compositions of media based on either KRB or PBS with BSA were calculated to target an AmB clinically relevant solubility value (based on the single point solubility studies). The calculated compositions of the media to reach AmB solubility values similar to the observed plasma value were KRB SLS 30.0 mM BSA 4.0% w/v and PBS 60.0 mM BSA 4.0% w/v. The media composition without BSA could not be obtained directly by just removing BSA from the media as AmB solubility would be extremely high (Figure 5). Therefore, lower

concentrations of SLS (0.1 – 4.0 mM) were tested in PBS and KRB to find the appropriate concentration for clinically relevant AmB solubility values without BSA. The SLS concentrations where the AmB solubility was similar to that observed in plasma were 1.5 mM for KRB and 1.4 mM for PBS. In the clinically relevant media developed with SLS, the presence of BSA did not affect the viscosity but increased the buffer capacity and the osmolality as in category 3a media (Table 2). AmB degradation rate constants were not statistically similar to that observed in plasma, and were lowest in media composed of SLS alone in buffer solution, which can be considered minimal (Table 1). The AmB degradation rate constants in media with BSA were significantly higher than in the media without BSA. This effect could be related to observations found in category 1 media (Table 1) where an increasing BSA concentration had a positive effect on the degradation rate constant. The results of the solubility studies in the developed category 3b media with and without BSA are presented in Figure 7. It can be observed that the AmB concentration profiles in the category 3b media are similar to that observed in plasma.

3.2.3.3. Category 3c media

As solubility data in plasma was only available from plasma obtained from healthy subjects, a target solubility value for hypoalbuminaemic plasma was not available. This section therefore represents characterisation of media evolved from category 3b as potential candidates for further patient-centric media development. The composition of the media was as follows: a) PBS SLS 60.0 mM BSA 2.0% w/v, b) PBS SLS 30.0 mM BSA 2.0% w/v to reflect a reduction of the inhibition of BSA on SLS solubilisation of AmB (observed during category 2 and 3a and 3b media development) when the lower BSA concentration was used and c) PBS SLS 30.0 mM BSA 4.0% w/v to explore the reduced potential for SLS solubilisation which might better reflect a potentially lower solubility in hypoalbuminaemic plasma. These 3 media had characteristics similar to healthy plasma (Table 2) apart from a reduced buffer capacity ($p <$

0.05) and slightly lower pH. The AmB degradation rate constants in media with BSA 2.0% w/v are apparently lower than those obtained in media with BSA 4.0% w/v (category 3b and 3c) but the difference was not statistically significant. Only the degradation rate constant in PBS SLS 60.0 mM BSA 2.0% w/v was statistically similar to that observed in plasma. The results of the single point (12h) AmB solubility studies in these media were: a) PBS SLS 60.0 mM BSA 2.0% w/v = 65.57 ± 4.98 µg/mL, b) PBS SLS 30.0 mM BSA 2.0% w/v = 25.84 ± 0.97 µg/mL, c) PBS SLS 30.0 mM BSA 4.0% w/v = 19.77 ± 0.29 µg/mL. This supports the hypothesis that BSA interferes with the action of the surfactants, in this case SLS, with the 12 h concentrations highest and lowest in the media with SLS 60.0 mM and BSA 2.0% w/v and SLS 30.0 mM and BSA 4.0% w/v, respectively.

3.2.4. Summary and evaluation of the test media

Table 3 presents a summary and an evaluation of solubility, degradation rate constant and media characterization in comparison with the parameters obtained/reported in plasma. Although category 1 media (Diaz de Leon-Ortega et al., 2018) was not clinically relevant for solubility or degradation, this media could be useful to investigate the impact of albumin concentration on poorly soluble and highly – protein bound drugs as demonstrated for AmB in terms of solubility, degradation and pharmacological activity (Diaz de Leon-Ortega et al., 2018). Clinically relevant AmB solubility values are achieved by using biorelevant surfactants in category 3a media without BSA. These media could be used for *in vitro* release tests of AmB lipid-based formulations and also, these media with BSA could be used in order to explore effects of BSA on release from the formulation. Clinically relevant AmB solubility values were achieved in all the category 3b media developed. Category 3b media without BSA could be proposed as a basis for the development of appropriate media for compendial release tests, as they easier to prepare and the degradation of the drug is minimal (Table 1). For category 3c media, there were no reference values for solubility, degradation and media characterization

due to the lack of availability of hypoalbuminaemic plasma. Assuming that AmB solubility will be lower in hypoalbuminaemic plasma compared to healthy plasma, PBS SLS 30.0 mM BSA 4.0% w/v is a good candidate for the development of simulated hypoalbuminaemic plasma as the AmB solubility at 12 h in this medium was lower than the clinically relevant value.

The multiple linear regression analysis showed that neither the solubility nor the degradation rate constant could be related to any of the properties of media characterization shown in Table 2 ($p > 0.05$).

3.4. Dissolution studies of AmB with the flow through cell apparatus in clinically relevant media

Dissolution studies were performed in media with clinically relevant AmB solubility from category 3a and 3b, and in each of the media explored in category 3c (Figure 8).

Clinically relevant AmB solubility values were obtained in PBS and in KRB, but the media with PBS were selected for dissolution studies due to their reduced medium complexity (compared to KRB). First order equation parameters of AmB, for dissolution profiles (corrected for degradation) fitted to Eq 3, are listed in Table 4.

Dissolution profiles in PBS BS 19.8 mM PL 7.9 mM could not be modeled using Eq 3 in either low or high velocity conditions. In the low velocity environment, the %AmB dissolved reached a maximum value of 44.93 ± 2.89% at 3.0 h and then started to decrease until the end of the experiment (8 h). This could indicate that mixed micelles of BS – PL need a stronger agitation (as in the bottle/stirrer setup used in the solubility and degradation studies) for a notable, durable effect on AmB dissolution. There was no reduction over time of the AmB percent dissolved when the test was conducted at high velocity, however in this situation there was no discernible plateau and rather a very gradual increase in % dissolved over time, prohibiting

514 fitting of Eq 3 to the data. In summary, AmB dissolution is greater under the high velocity
515 conditions in the clinically relevant category 3a medium (Figure 8).
516 There was a statistically significant negative effect of velocity on the dissolution rate constant
517 for the 0.5 mg dose and a positive effect on maximum % dissolved on the 5 mg dose; however,
518 these differences were of low practical significance and possibly reflect process variability.
519 The %AmB dissolved in the experiment with the high dose of AmB reached a plateau value of
520 around 20%, due to the solubility of AmB in this medium (~30 µg/mL), (Figure 8). In category
521 3b medium with BSA, with respect to velocity, the low and high velocity conditions had little
522 effect on AmB dissolution in comparison to medium composition.
523 The velocity regimes in the flow through apparatus (average velocities: low velocity: 0.07 cm/s,
524 high velocity: 0.52 cm/s), are low overall in comparison to the bottle/stirrer set up. A faster
525 dissolution and a higher %AmB dissolved is observed in the category 3b medium with BSA
526 compared to that observed in the medium without BSA. This could be attributed to increased
527 wetting/dispersion from either the BSA or the SLS. PBS SLS 1.4 mM is therefore suggested
528 as a simplified medium that could be used in compendial dissolution testing of AmB parenteral
529 formulations, as clinically relevant AmB solubility values were obtained in this medium.
530 In category 3c media, the %AmB dissolved in PBS SLS 60.0 mM BSA 2.0% w/v started to
531 decrease before the 1.5 h sample in the low velocity conditions. The results suggest that the
532 agitation required to keep SLS, BSA and AmB in solution at these concentrations was higher
533 as the decrease was not observed when the high velocity conditions were used. The highest
534 %AmB dissolved was observed in PBS SLS 60.0 mM BSA 2.0% w/v and the lowest in PBS
535 SLS 30 mM BSA 4.0% w/v, again supporting the hypothesis that BSA binds the surfactants
536 and prevents their interaction with AmB. The AmB dissolution profiles in PBS SLS 30.0 mM
537 BSA 2.0% w/v and PBS SLS 60 mM BSA 4.0% w/v were similar, probably due to the same
538 ratio of SLS/BSA in these two media. Velocity had a negative effect only on the dissolution

rate of AmB in PBS SLS 30.0 mM BSA 2.0% w/v whereas this was not observed in the AmB dissolution in the other category 3c media. Based on the presumption of lower AmB solubility in hypoalbuminaemic plasma, the results suggest that the most suitable clinically relevant medium to simulate hypoalbuminaemic conditions is PBS SLS 30 mM BSA 4.0% w/v; confirmation with solubility studies in plasma from hypoalbuminaemic patients would be needed. As the dissolution profiles were calculated based on concentrations corrected accounting for AmB degradation, the degradation rate constant was not a factor to be considered in the analysis, as it was already taken into account in the profiles.

The dissolution studies provided information on which test media would be suitable for formulation release studies. For example, some test media were not able to keep AmB solubilized for the duration of an 8 h test, even after correcting for degradation, despite being designed to produce clinically relevant solubility values. Furthermore, there is scope for further exploration of dissolution test conditions in terms of apparatus and hydrodynamic environment to determine suitably discriminating conditions for dissolution/release testing of parenteral formulations.

Summarizing, in order to develop a clinically relevant test for a poorly soluble, degradation-susceptible and highly protein-bound drug for parenteral administration, based on the current work focussed on AmB, the following steps are proposed:

1. Determine API solubility in the target fluid, in this case plasma
2. If the API is highly protein-bound, a suitable protein should be incorporated in the medium or a surfactant able to match the desired solubility; and if both are going to be incorporated, investigate the effect of any interaction between them on the solubility of the drug
3. Determine the degradation rate of the API in the media investigated for further correction of dissolution/release profiles

4. Test the API dissolution/solubility behaviour in the test media and hydrodynamic conditions that will be used in the formulation dissolution/release testing, as stability/solubility problems will be easier to be identified using the API alone before the drug dissolution/ release testing from the formulation.

4. Conclusions

AmB is an antifungal drug that is highly bound to plasma proteins, including albumin and lipoproteins. It is administered to patients intravenously as lipid-based formulations. Therefore, a test medium to assess the release profile of the drug from its lipid-based parenteral formulations that can provide clinically relevant results is desirable.

In this study we have developed biorelevant media based on plasma composition and clinically relevant dissolution media based on the solubility of AmB in plasma. This was achieved by using saline buffers, surfactants (biorelevant and synthetic) and BSA. Clinically relevant AmB solubility was not achieved in the biorelevant media. It was shown that addition of BSA in the medium generally induces a faster degradation of AmB. Another role of BSA in the media is the capability to interfere with the solubilizing activity of almost all of the components that were added to the media. Instead of resulting in an improved AmB solubility by combining surfactants with BSA, the opposite effect was observed. This has to be taken into consideration for development of *in vitro* test media where the protein binding is an important feature. Based on the dissolution study results, medium complexity and minimal degradation, PBS SLS 1.4 mM is suggested as an option for development of release tests for AmB formulations. For the simulated hypoalbuminaemic media, PBS SLS 30 mM BSA 4.0% w/v is suggested as a potential candidate medium for further (patient centric) medium development, although future experiments with hypoalbuminaemic plasma are needed to confirm the effect of hypoalbuminaemia on solubility. Guidance is given for the initial stages of the development of

a clinically relevant *in vitro* dissolution/release test for parenteral formulations. Development of clinically relevant media is a first step for developing clinically relevant dissolution/ release tests with a view to obtaining *in vitro* data predictive of the *in vivo* behaviour of the formulation and the drug.

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712 **Tables**

713 Table 1. Degradation rate constants of AmB in plasma, category 1 and 3 media. (Mean ± SD;

714 n = 3).

Type of medium		Medium	k_{deg} (h^{-1})
Plasma		Plasma	0.033 ± 0.002
Category 1		KRB BSA 1.5% w/v	0.026 ± 0.000 (Diaz de Leon-Ortega et al., 2018)
		KRB BSA 2.0% w/v	0.065 ± 0.005 (Diaz de Leon-Ortega et al., 2018)
		KRB BSA 3.0% w/v	0.065 ± 0.021 (Diaz de Leon-Ortega et al., 2018)
		KRB BSA 4.0% w/v	0.095 ± 0.003 (Diaz de Leon-Ortega et al., 2018)
Category 3	3a	KRB BS 20.0 mM PL 4.0mM	0.023 ± 0.010
		KRB BS 20.0 mM PL 4.0 mM BSA 4.0% w/v	0.060 ± 0.008
		PBS BS 19.8 mM PL 7.9 mM	0.048 ± 0.006
		PBS BS 19.8 mM PL 7.9 mM BSA 4.0% w/v	0.097 ± 0.021

	3b	KRB SLS 1.5mM	0.009 ± 0.007
		KRB SLS 30.0mM BSA 4.0% w/v	0.038 ± 0.001
		PBS SLS 1.4 mM	0.005 ± 0.003
		PBS SLS 60.0 mM BSA 4.0% w/v	0.052 ± 0.015
	3c	PBS SLS 30.0 mM BSA 2.0% w/v	0.062 ± 0.014
		PBS SLS 30.0 mM BSA 4.0% w/v	0.082 ± 0.015
		PBS SLS 60.0 mM BSA 2.0% w/v	0.041 ± 0.013

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717 Table 2. Media composition and media characterization. Plasma characterization is included (Mean ± SD; n = 3 for Vis and BC HCl).

Category	Medium	Osm (mOsm/L)	Vis (cPs)	pH	BC HCl (mEq/L/pH)
Plasma	Plasma	275 - 300 (Dasgupta and Wahed, 2014)	3.5 (Rand et al., 1964)	7.34 ± 0.04 (Shepherd, 1979)	16.1 ± 0.9 (Ellison et al., 1958)
Category 1	KBR BSA 4.0% w/v	308 (Diaz de Leon-Ortega et al., 2018)	4.0 ± 0.0 (Diaz de Leon-Ortega et al., 2018)	7.36 (Diaz de Leon-Ortega et al., 2018)	12.0 ± 1.2
Category 2	KRB BS 12.0 μM PL 2.5 mM	278	5.0 ± 0.1	8.64	1.5 ± 0.0
	KRB BS 12.0 μM PL 2.5 mM CH 4.5 mM	273	5.0 ± 0.1	8.79	1.5 ± 0.1
	KRB BS 12.0 μM PL 2.5 mM BSA 2.0% w/v	314	5.2 ± 0.1	7.79	7.8 ± 0.0
	KRB BS 12.0 μM PL 2.5 mM CH 4.5 mM BSA 4.0% w/v	306	5.0 ± 0.1	7.80	7.5 ± 0.2

		KRB BS 12.0 μM PL 2.5 mM BSA 8.0% w/v	351	5.2 ± 0.0	7.65	7.7 ± 0.0
Category 3	3a	KRB BS 20.0 mM PL 4.0 mM	310	4.8 ± 0.1	8.25	1.7 ± 0.0
		PBS BS 19.8 mM PL 7.9 mM	330	4.7 ± 0.1	7.36	6.1 ± 0.1
		KRB BS 20.0 mM PL 4.0 mM BSA 4.0% w/v	415	5.0 ± 0.1	8.50	5.2 ± 0.1
		PBS BS 19.8 mM PL 7.9 mM BSA 4.0% w/v	425	4.9 ± 0.1	7.18	14.2 ± 0.8
	3b	KRB SLS 1.5 mM	289	4.6 ± 0.1	8.54	1.2 ± 0.0
		PBS SLS 1.4 mM	280	4.4 ± 0.1	7.43	5.6 ± 0.1
		KRB SLS 30.0 mM BSA 4.0% w/v	350	5.0 ± 0.1	7.84	7.9 ± 0.1
		PBS SLS 60.0 mM BSA 4.0% w/v	325	5.2 ± 0.1	7.56	10.3 ± 0.2
	3c	PBS SLS 30.0 mM BSA 2.0% w/v	300	4.8 ± 0.0	7.10	9.3 ± 0.2
		PBS SLS 30.0 mM BSA 4.0% w/v	297	4.6 ± 0.0	7.18	9.6 ± 0.6
		PBS SLS 60.0 mM BSA 2.0% w/v	302	4.9 ± 0.1	7.04	11.6 ± 0.3

718 Osm: Osmolality, Vis: Viscosity, BC HCl: buffer capacity determined with HCl.

719 Table 3. Evaluation of solubility, degradation rate constant and media characterization in comparison with the parameters obtained/reported in

720 plasma. ND = not determined due to data unavailable for comparison, ✗ = not biorelevant, ✓ = biorelevant.

Category	Medium	CRS	CRD	Osm	Vis	pH	BC HCl	Medium Complexity
Category 1	KRB BSA 4.0% w/v (Diaz de Leon-Ortega et al., 2018)	No	No	✗	✗	✓	✗	1
Category 3a	KRB BS 20.0 mM PL 4.0 mM	Yes	Yes	✗	✗	✗	✗	4
	PBS BS 19.8 mM PL 7.9 mM	Yes	No	✗	✗	✓	✗	3
	KRB BS 20.0 mM PL 4.0 mM BSA 4.0% w/v	No	No	✗	✗	✗	✗	4
	PBS BS 19.8 mM PL 7.9 mM BSA 4.0% w/v	No	No	✗	✗	✗	✓	3
Category 3b	KRB SLS 1.5 mM	Yes	No	✓	✗	✗	✗	2
	PBS SLS 1.4 mM	Yes	No	✓	✗	✓	✗	1
	KRB SLS 30.0 mM BSA 4.0% w/v	Yes	No	✗	✗	✗	✗	2
	PBS SLS 60.0 mM BSA 4.0% w/v	Yes	No	✗	✗	✗	✗	1
Category 3c	PBS SLS 30.0 mM BSA 2.0% w/v	ND	ND	ND	ND	ND	ND	1
	PBS SLS 30.0 mM BSA 4.0% w/v	ND	ND	ND	ND	ND	ND	1

	PBS SLS 60.0 mM BSA 2.0% w/v	ND	ND	ND	ND	ND	ND	1

721 CRS: AmB clinically relevant solubility, CRD: AmB clinically relevant degradation rate constant, Osm: osmolality, Vis: viscosity, BC HCl: buffer

722 capacity determined with HCl, MC: medium characterisation score

723

724 Table 4. Parameters obtained after fitting (Eq 3) of %AmB dissolution profiles in category 3b clinically relevant media and 3c media with the flow

725 through cell apparatus (Mean ± SD, n = 3).

Type of medium	Medium	Flow/velocity	k_{diss} (h^{-1})	X_{max} (%)	R^2	AIC
Category 3b	PBS SLS 1.4 mM Low dose	Low	2.39 ± 0.13	36.28 ± 0.59	0.94 ± 0.00	50.03 ± 0.29
	PBS SLS 1.4 mM High dose	Low	2.98 ± 0.74	16.75 ± 1.16	0.91 ± 0.01	38.77 ± 2.28
	PBS SLS 60.0 mM BSA 4.0% w/v	Low	3.65 ± 1.59	52.79 ± 5.52	0.91 ± 0.05	63.05 ± 3.77
	PBS SLS 1.4 mM Low dose	High	1.88 ± 0.26	38.66 ± 2.08	0.93 ± 0.02	53.94 ± 4.06
	PBS SLS 1.4 mM High dose	High	2.50 ± 0.40	19.47 ± 0.81	0.92 ± 0.01	40.40 ± 1.32
	PBS SLS 60.0 mM BSA 4.0% w/v	High	5.60 ± 1.30	62.86 ± 5.22	0.91 ± 0.10	63.03 ± 12.28

Category 3c	PBS SLS 30.0 mM BSA 2.0% w/v	Low	4.21 ± 0.96	53.38 ± 9.94	0.96 ± 0.05	50.47 ± 9.28
	PBS SLS 30.0 mM BSA 2.0% w/v	High	2.33 ± 0.35	63.07 ± 3.48	0.94 ± 0.01	63.20 ± 1.21
	PBS SLS 30.0 mM BSA 4.0% w/v	High	7.66 ± 5.39	37.22 ± 8.20	0.89 ± 0.04	57.06 ± 3.45
	PBS SLS 60.0 mM BSA 2.0% w/v	High	6.43 ± 2.87	78.38 ± 0.50	0.96 ± 0.01	63.08 ± 3.31

726

727 R^2: correlation coefficient, AIC: Akaike Information Criterion.

728 **Figures' captions**

729

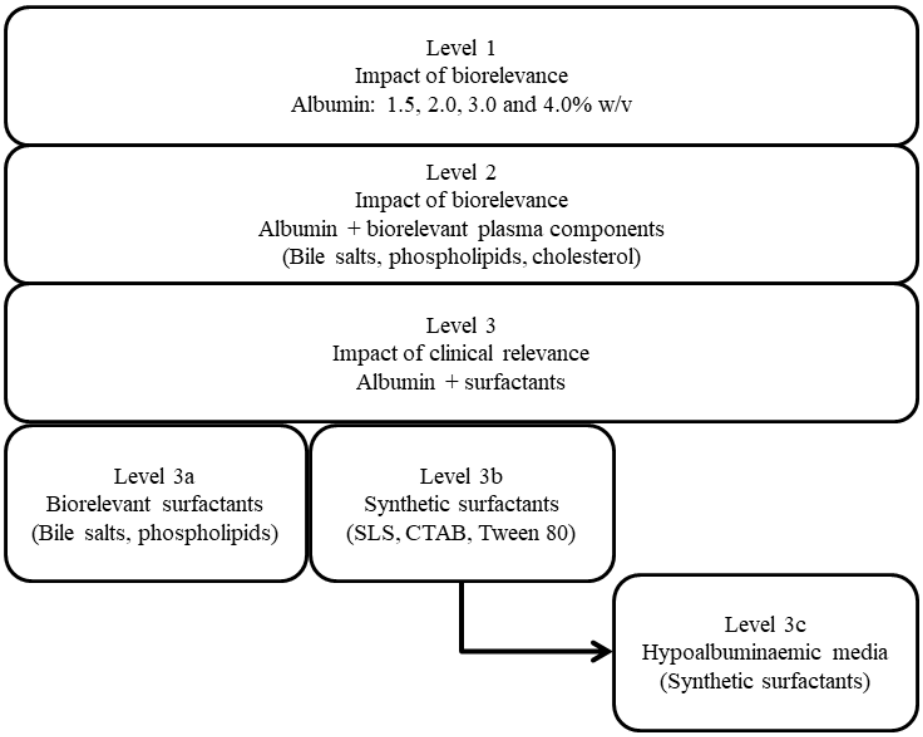
730 **Figure 1**. Categories of media development for evaluation of performance of parenteral drug
731 products.

732 **Figure 2.** Solubility study of AmB in category 2 media: AmB concentration as a function of
733 time in plasma and category 2 media (Mean ± SD; n= 3). KRB BSA 4.0% w/v solubility
734 (category 1 medium) and KRB BS 12.0 μM PL 2.5 mM BSA 8.0% w/v were added for
735 comparison purposes (Diaz de Leon-Ortega et al., 2018). BSA concentration is in % w/v units.
736 **Figure 3.** Pareto chart for the standardized effects of the main factors and 2 level interactions
737 of the analysis of AmB solubility in media with BS, PL and different types of buffer. The black
738 horizontal line represents the significance threshold for the effects. The factors with an effect
739 (bar) larger than the threshold are statistically significant.
740 **Figure 4.** Solubility study of AmB in category 3a media: AmB concentration as a function of
741 time in category 3a media with and without BSA (Mean ± SD; n= 3). BSA concentration is in
742 % w/v units. AmB plasma solubility was added for comparison purposes [solid lines represent
743 the media with BSA 4.0% w/v; dashed lines represent the media without BSA].
744 **Figure 5.** Solubility study of AmB in media with surfactants for the development of category
745 3b media: AmB concentration in PBS or KRB with 5.0, 50.0 and 100.0 mM concentrations of
746 SLS, CTAB or Tween with and without BSA 4.0% w/v at 12 h (Mean ± SD; n= 3). Black
747 horizontal line represents the AmB solubility value in human plasma from healthy subjects.
748 **Figure 6.** Pareto charts for the standardized effects of the main factors and 2 level interactions
749 of the analysis of AmB solubility in media with surfactants and BSA 4.0% w/v. The black
750 horizontal line represents the significance threshold for the effects. The factors with an effect
751 (bar) larger than the threshold are statistically significant.

Figure 7. Solubility study of AmB in category 3b media: AmB concentration as a function of time in the category 3b clinically relevant media with and without BSA (mean ± SD; n= 3). AmB plasma solubility was added for comparison purposes. The BSA concentration is in % w/v units.

Figure 8. %AmB dissolved as a function of time in the category 3a and 3b clinically relevant media and in category 3c media with the flow through cell apparatus at 37°C (mean ± SD; n= 3). High dose = 5 mg of AmB. BSA concentration is in % w/v units. Points represent observed data and the lines the first order (Eq 3) equation fittings.

762



763

764 **Figure 1**

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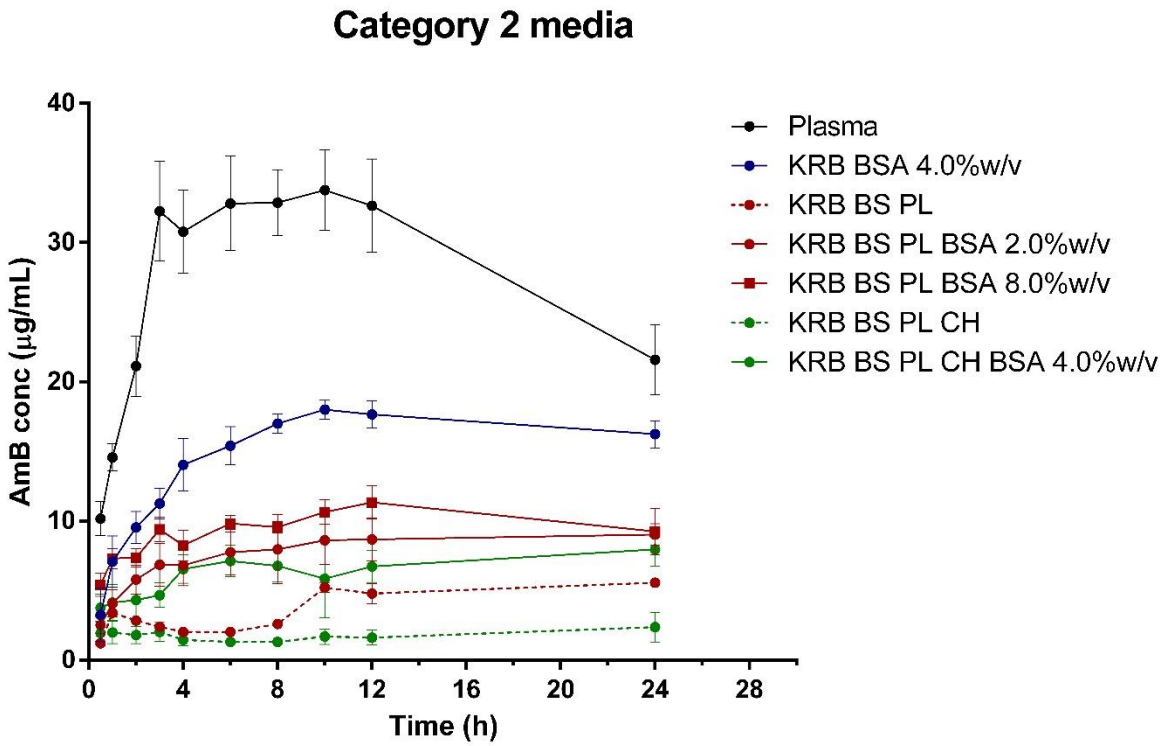

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770 **Figure 2**

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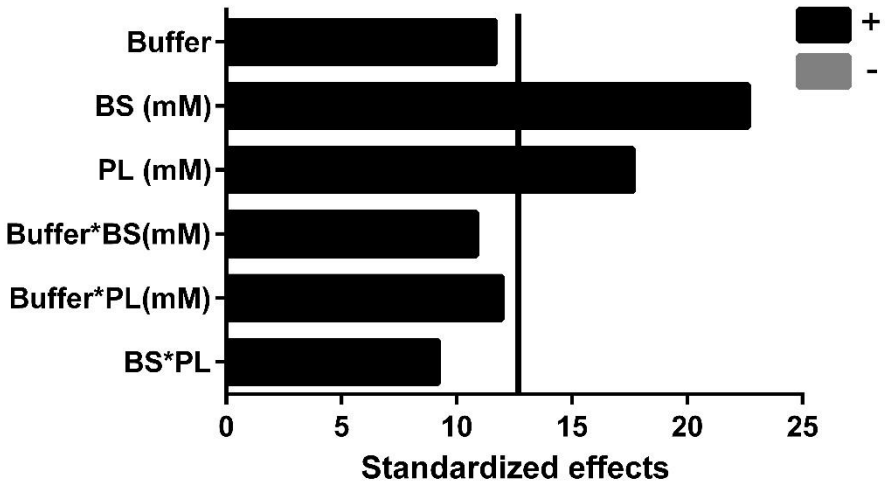


773

774 **Figure 3**

775

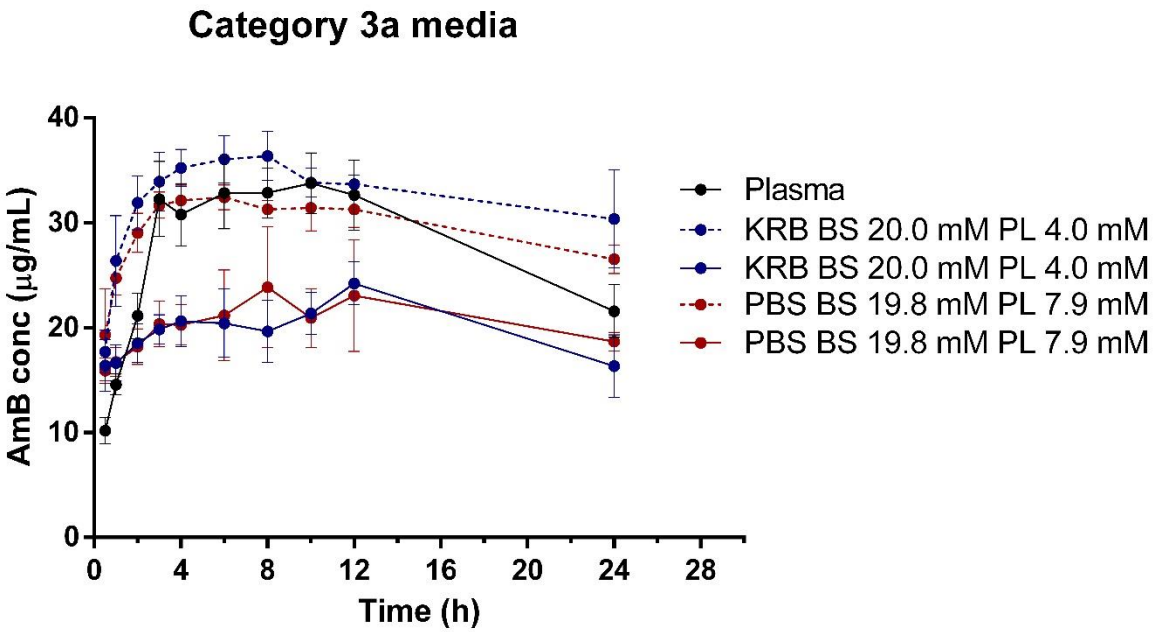
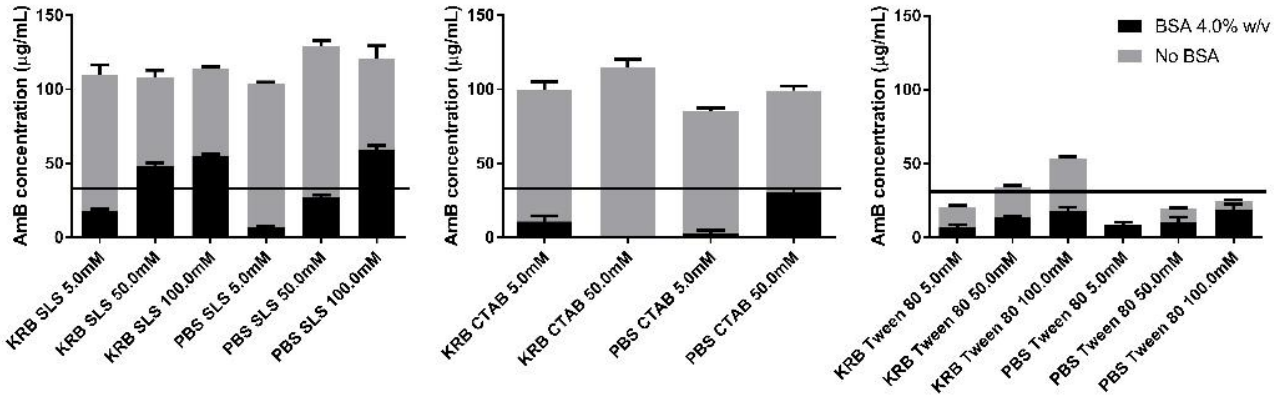

Category 3a media
AmB conc (µg/mL)
Time (h)
0
10
20
30
40
0
4
8
12
16
20
24
28
Plasma
KRB BS 20.0 mM PL 4.0 mM
KRB BS 20.0 mM PL 4.0 mM
PBS BS 19.8 mM PL 7.9 mM
PBS BS 19.8 mM PL 7.9 mM

Figure 4

781



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784 **Figure 5**

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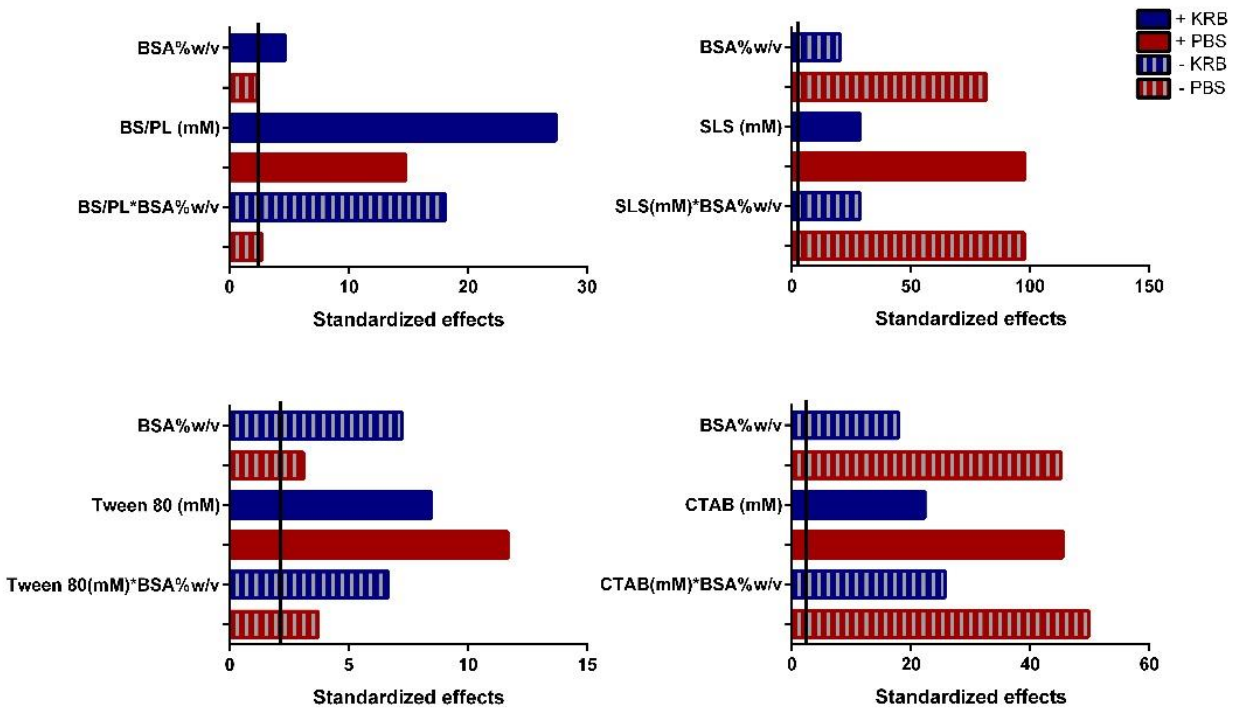

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788 **Figure 6**

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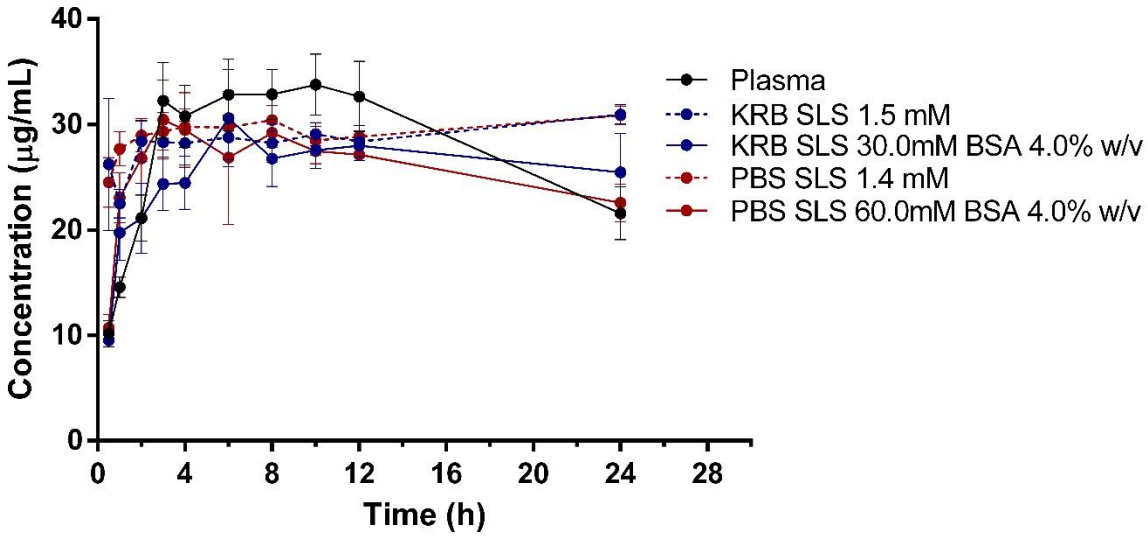

791

792 **Figure 7**

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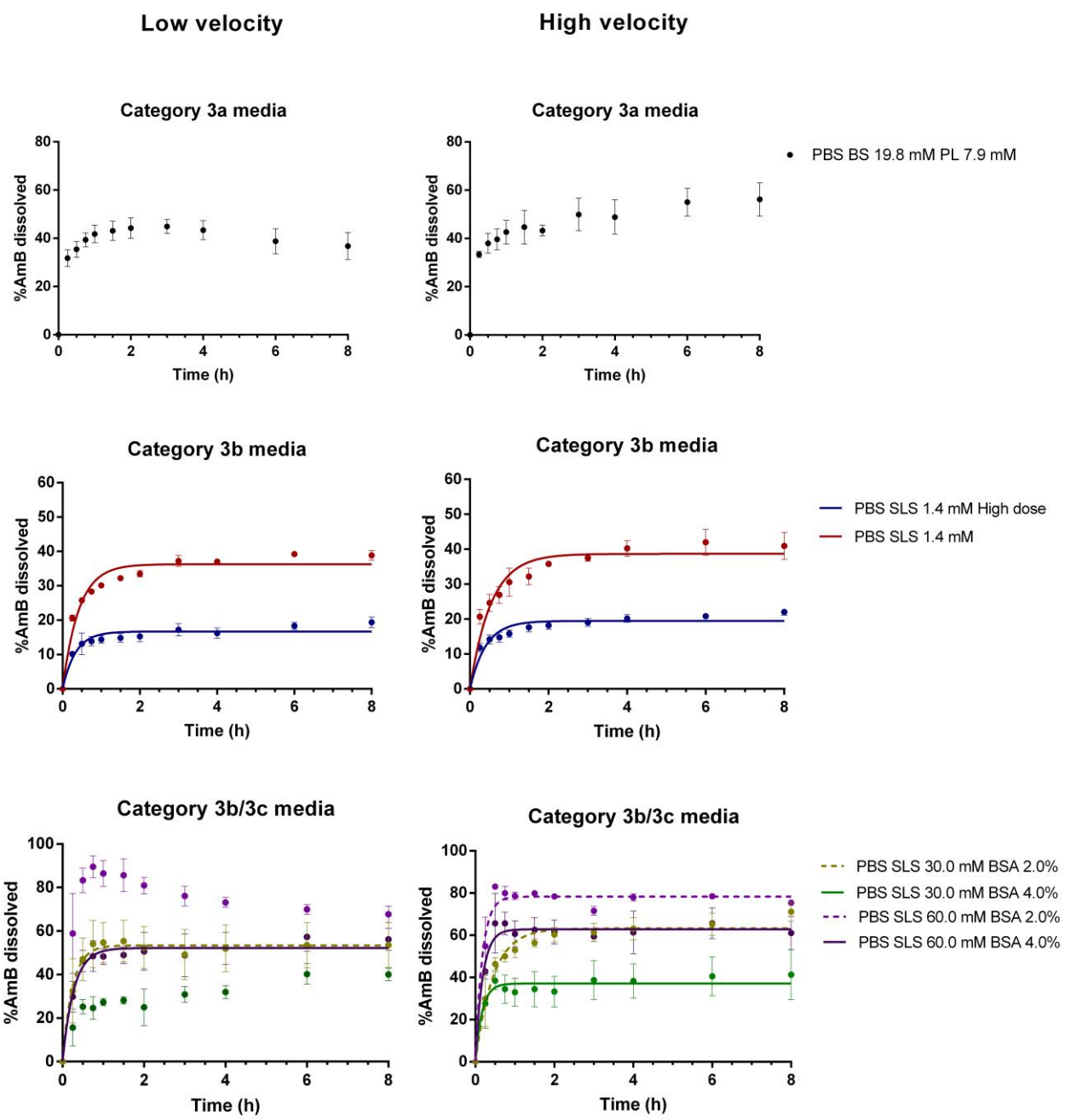

795

796 **Figure 8**