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

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25 **Abstract**

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

43

44 **Keywords:**

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

46

47 **1. Introduction**

48 The parenteral administration route is utilized when a quick or a depot effect is needed, when
49 the patient cannot take oral formulations for systemic therapy or when the physicochemical
50 properties of the drug make it impossible to be delivered by any other route [1]. Formulations
51 such as microspheres, liposomes, nanoparticles and emulsions (among others) have been
52 developed to be able to meet the requirements of a long or a sustained exposure. The dissolution
53 test is an *in vitro* test designed to characterize the dissolution/release of the drug from a
54 formulation and hopefully, predict the behaviour of the drug *in vivo*. There are 3 main methods
55 to assess dissolution/release from controlled release parenterals that have been described
56 extensively in the literature: Sample and separate, Continuous flow and Dialysis methods [2-
57 6].

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

64 For parenterals administered intravenously the release medium is blood, consisting of 2
65 fractions, the cellular fraction and plasma. Plasma is a fluid that contains ions and
66 biomolecules. Albumin is the major circulating protein in human plasma (up to the 60% of
67 plasma proteins). The normal reference value of plasma albumin for a healthy subject is 40 g/L
68 $\pm 10\%$ [9]. Albumin is the most relevant protein in terms of drug administration as it is a carrier
69 for metals, ions, fatty acids, amino acids, bilirubin, enzymes and drugs [9].

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

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

85 One drug that is administered to critically ill patients is Amphotericin B (AmB), which is still
86 one of the most effective therapies for systemic fungal infections. In clinical practice, AmB is
87 administered as an infusion using a multidose scheme usually lasting for several days [11].
88 AmB is highly bound ($>95\%$) to plasma proteins (Low Density Lipoproteins, albumin and $\alpha 1$
89 glycoproteins [12, 13]). The major drawback of AmB is its poor water solubility (reported
90 values: 0.09 $\mu\text{g/mL}$ [14], 1.38 $\mu\text{g/mL}$ [15] and 6 $\mu\text{g/mL}$ [16] at $\text{pH} = 7$). To tackle this problem,
91 several formulations have been developed, including Liposomal AmB (Ambisome®) and AmB
92 in a lipid complex (Abelcet®), where AmB is within the lipid structures. Furthermore, a
93 correlation has been observed between volume of distribution at steady state of total AmB
94 following administration of Abelcet® and albumin concentration, in critically ill patients [17],
95 illustrating the relevance of albumin concentration to AmB pharmacokinetics in the target
96 patient cohort.

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

102 **2. Materials and Methods**

103 **2.1 Materials**

104 AmB analytical standard (87.8%), Methanol (MeOH) High Performance Liquid
105 Chromatography (HPLC) grade, formic acid, Sabouraud Dextrose (SBD) Broth, NaOH,
106 MgCl₂, CaCl₂, NHCO₃ and NH₄HCO₂ were obtained from Sigma Aldrich (Germany); AmB
107 active pharmaceutical ingredient (API) powder (85%) from Cayman Chemical (USA); Bovine
108 Serum Albumin Protease Free Powder Fraction V (BSA), dimethyl sulfoxide (DMSO),
109 dextrose, Na₂HPO₄, NaH₂PO₄, NaCl and KCl were obtained from Fisher Scientific (USA);
110 Sabouraud dextrose (SBD) agar was obtained from Oxoid (UK), 25 mL sterile universal culture
111 tubes were obtained from Sterilin Thermo Scientific (UK); 10 µL plastic loops from Microspec
112 (UK); GF/D (pore size 2.7 µm, 25 mm diameter) and GF/F (pore size 0.7 µm, 25 mm diameter)
113 filters were obtained from Whatman (UK) and regenerated cellulose (RC) filters 0.45 µm 13
114 mm diameter from Cronus (UK).

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

116

117 **2.2. Dissolution biorelevant media composition and characterization**

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

121 The osmolality of the media with 2 and 4% w/v BSA was measured via the freezing-point

122 depression method with a Micro-Osmometer 3300 (Advanced Instruments, Massachusetts
123 USA). Viscosity of all media was measured with a Bohlin Rheometer (Germany) with a shear
124 rate 0.1 - 1.5 Pa (logarithmic scale), 20 integrations per measurement and with a delay time of
125 5 seconds and an integration time of 20 seconds. The geometry was a 4° and 40 mm diameter
126 (CP 4/40) cone parallel to a plate and the experiments were conducted at 25°C in triplicate.
127 The measurement at the closest value to the steady state was recorded as the viscosity value.

128

129 **2.3. Chromatographic conditions for the analysis of AmB from biorelevant dissolution** 130 **media samples**

131 The chromatographic method to quantify AmB was a modification of the method reported by
132 Nilsson-Ehle et al [19]. Briefly, AmB was quantified by HPLC analysis using a Hewlett
133 Packard Series 1100 equipped with an auto sampler, temperature regulated column
134 compartment, quaternary pump and diode array detector (DAD detector) (Agilent
135 Technologies). The column was a C18 Waters Sunfire Column (Ireland) 150 x 46 mm 5µm.
136 The temperature of the column compartment was set at 25°C. The mobile phase consisted of
137 formate buffer (50 mM; pH = 3.2): MeOH (25:75, v/v); the flow rate was 1 mL/min and
138 analysis was performed with the DAD detector at $\lambda = 406$ nm. The UV spectrum was recorded
139 from 300 to 450 nm (where necessary for detection of the degradant). Quantification of AmB
140 in samples was made based on calibration curves. Freshly prepared standard solutions (0.5 –
141 10 µg/mL) in the corresponding medium were prepared by appropriate dilution of a 500 µg/mL
142 stock solution of AmB analytical standard in 1:1 MeOH: DMSO v/v. The 5 µg/mL standard
143 solution in KRB – BSA 4% w/v was incubated at 37°C and was monitored every hour to check
144 the stability of the samples for up to 24 h. The limit of detection and the limit of quantification
145 were 0.12 and 0.37 µg/mL, respectively.

146

147 **2.4. Sample treatment of AmB in the biorelevant dissolution media**

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

153

154 **2.5. Liquid chromatography – Mass Spectrometry (LCMS) studies**

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

171

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

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

184

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

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

195

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

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

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

$$212 \quad AmB T_{undissolved} = AmB final - AmB dissolved at 24 h \quad Eq 1$$

$$213 \quad AmB T_{dissolved} = AmB initial - AmB T_{undissolved} \quad Eq 2$$

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

$$215 \quad AmB T_{degraded} = AmB T_{dissolved} - AmB dissolved at 24 h \quad Eq 3$$

216

217 **2.9. Antimicrobial activity assay of AmB in the biorelevant media: MIC and MFC** 218 **determination**

219 **2.9.1. Quantification of *C. albicans*.** The strain was maintained on SBD agar plates. A new
220 culture was started from a single colony in a culture tube with 5 mL of SBD broth and was
221 incubated at 37°C overnight in a shaking incubator (Innova 44, New Brunswick Scientific,

222 USA), after which the optical density (OD) was measured at 600 nm (OD₆₀₀). The Colony
223 Forming Units (CFU) of the culture was determined by preparing serial dilutions from 10⁻¹ to
224 10⁻⁶. 100 µL of the suspensions were plated on SBD agar plates, incubated overnight at 37°C,
225 the number of colonies were counted and the relationship with the OD₆₀₀ of the culture was
226 established.

227 **2.9.2 MIC and MFC studies:** The OD₆₀₀ of an overnight culture of *C. albicans* was measured,
228 and diluted to a final concentration of 10⁵ CFU/mL. Minimum Inhibitory Concentration (MIC)
229 studies were performed with the following concentrations of AmB: 0, 1.5, 3, 4.5, 6, 7.5, 9 and
230 10.5 µg/mL for the experiments with BSA 2% w/v, and 0, 3, 4.5, 6, 7.5, 9, 10.5 and 12 µg/mL
231 for the experiments with BSA 4% w/v. Culture medium without BSA was set as the control for
232 these experiments. The MIC was defined as the lowest concentration of AmB at which there
233 was no visual turbidity in the liquid broth. The Minimum Fungicidal Concentration (MFC)
234 experiments were performed by plating 10 µL of the yeast cultures from the MIC assays on
235 SBD agar plates and incubated for 24 h at 37°C. The MFC was defined as the lowest
236 concentration of AmB where there was no visible growth on the agar plates. The results are
237 expressed as the intervals where the MIC and MFC were found. The experiments were
238 performed in duplicate.

239

240 **2.10 Dissolution studies of AmB in biorelevant media:**

241 **2.10.1 Sample and separation method (bottle/stirrer)**

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

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

249 **2.10.2 Continuous Flow method (flow through cell apparatus)**

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

265

266 **2.11 Treatment of dissolution data**

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

$$270 \quad C_{corrected} = C_t + k_{deg} * AUC_{0-t} \quad \text{Eq 4.}$$

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

274 The corrected dissolution profiles were calculated based on $C_{corrected}$ and a first order curve
275 fitting (Eq 5) was performed in order to obtain the dissolution rate constant (GraphPad Prism
276 6, Graph Pad Software, Inc, USA).

$$277 X_{corrected} = X_{max} * (1 - e^{-k_{diss}t}) \text{ Eq 5.}$$

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

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

$$283 X_{simulated} = \frac{X_{max} * k_{diss}}{k_{diss} - k_{deg}} (e^{-k_{deg}t} - e^{-k_{diss}t}) \text{ Eq 6.}$$

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

286

287 **2.12 Statistical analysis**

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

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

292

293 **3. Results and Discussion**

294 **3.1 Biorelevant dissolution media simulating healthy and hypoalbuminaemic parenteral**
295 **conditions**

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

309

310 **3.2. Identification of the AmB degradation product**

311 During the development of the AmB quantification method, an extra peak in AmB
312 chromatograms with a shorter retention time was noted. When the sample was incubated at
313 37°C, the area of this unknown peak increased with time and the AmB peak area decreased.
314 The UV spectra of the two peaks are broadly similar with a main difference in the λ_{\max} [λ_{\max}
315 for AmB: 406 nm; λ_{\max} for unknown compound: 380 nm (Figure 1)], suggesting that the
316 unknown compound is related to AmB. LCMS experiments revealed that the mass spectrum
317 of the unknown compound has a difference in mass (corresponding to a chlorine adduct)
318 compared to the mass of AmB (Figure 2). Based on these studies, this unknown compound is

319 more polar than AmB (due to its shorter retention time) and it can be suggested that it is AmB's
320 degradation product (deg-AmB). It has been reported that AmB auto-oxidates in the presence
321 of oxygen with formation of free radicals and epoxidation is the most probable route of
322 degradation (but the epoxidation products have not yet been characterized) [27 - 29]. A
323 degradation product has not been reported previously in studies where AmB was quantified.
324 This could be due to the specific ion transition followed by the multiple reaction monitoring
325 [30-33] and the elution of the more polar AmB degradation product in the washing step of the
326 solid phase extraction used for the purification of the sample [31, 34-36] in these methods.
327 Furthermore, plasma components could potentially prevent/alter the rate of AmB degradation.

328

329 **3.3. Degradation studies of AmB in the biorelevant media**

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

338

339 **3.4. Solubility studies of AmB in the biorelevant media**

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

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

357

358 **3.5. Mass Balance studies of AmB in the biorelevant media**

359 The results of the mass balance studies of AmB in KRB with BSA 2% w/v and KRB with BSA
360 4% w/v are presented in Table 3. The *AmB dissolved at 24 h* is similar for both
361 concentrations of BSA (2% w/v and 4% w/v) in the medium, but the *AmB $T_{dissolved}$* in which
362 the degradation of AmB is taken into account is higher in the medium with BSA 4% w/v, as
363 the AmB degradation is higher in this BSA concentration. This supports the results in sections
364 3.3 and 3.4, implying that BSA has a critical effect on the degradation and solubility of AmB
365 in the medium, confirming the faster degradation and increased mass dissolved in medium with
366 higher BSA concentration.

367

368 **3.6. Antimicrobial activity of AmB in the biorelevant media**

369 In order to assess the effect of BSA in the activity of AmB against *Candida albicans*, MIC and
370 MFC values were determined (Table 4). A marked effect of BSA on the activity of AmB is
371 shown. The results for the control experiments are in agreement with what is reported in the
372 literature for the MIC of AmB against *C. albicans* (0.06 – 1 mg/L) [36-39]. When BSA was
373 added to the culture media, the MIC increased 10-fold in the medium with BSA 2% w/v and
374 approximately 20-fold in the medium with BSA 4% w/v. In the absence of BSA, the MFC was
375 fairly close to the MIC values, indicating that AmB is fungicidal. However, in the presence of
376 BSA, the MFC was higher than the highest concentration of AmB that was tested, suggesting
377 a fungistatic activity instead. The increase in the concentration of AmB to exert its antifungal
378 activity has been reported before in studies where the source of albumin was HSA (human
379 serum albumin) [40]. This could be explained by the fact that AmB is highly bound to proteins,
380 and only the free fraction can exert a pharmacological effect. If there is more albumin in the
381 medium, more drug will be bound to it and the concentration required to have the same efficacy
382 will be higher. *In vivo*, AmB can also be bound to α 1-acid glycoprotein [12, 13], but as this
383 protein's blood concentration is only 0.1% w/v (24 μ M) compared to the 2.0 - 4.0% w/v BSA
384 (300 – 600 μ M) (that is used to substitute HSA) its effect can be considered negligible for the
385 purposes of this study.

386

387 **3.7 Dissolution studies of AmB in biorelevant media**

388 **3.7.1 Sample and separation method (bottle/stirrer)**

389 The AmB dissolution profiles in biorelevant media (KRB with the addition of BSA 1.5 - 4
390 % w/v) obtained with the bottle/stirrer setup are presented in Figure 5. Dissolution studies were
391 performed in KRB without BSA, however neither the AmB dissolution nor the AmB
392 degradation could be quantified due to the very low solubility, and hence minimal dissolution,
393 of the drug in this medium. The AmB dissolution with its degradation occurring simultaneously

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

418 The simulated dissolution profiles of AmB in media with different levels of BSA and under
419 both agitation conditions in the bottle/stirrer setup are presented in Figure 6. The use of a
420 biexponential function in which the dissolution (k_{diss}) and degradation rate (k_{deg}) constants
421 were incorporated results in successful prediction of AmB dissolution. Dissolution modeling
422 could be a valuable tool to provide a mechanistic understanding of drug dissolution in cases
423 where other processes, such as degradation, occur simultaneously with dissolution.

424

425 **3.7.2 Continuous Flow method (flow through cell apparatus)**

426 AmB dissolution profiles in KRB with BSA 2% w/v and BSA 4% w/v under low and high
427 velocity conditions with the flow through cell apparatus are presented in Figure 7. The
428 dissolution of AmB was very low in all cases, with the maximum % dissolved being $3.10 \pm$
429 0.08 % dissolved in the medium with BSA 4% w/v under low velocity after 8h and the samples'
430 concentrations did not reach the corresponding AmB solubility in the medium (Figure 4). A
431 low % AmB dissolved was also observed in the case where a lower amount of AmB was used
432 (0.5mg; data not shown) revealing that the low dissolution does not relate to the AmB amount
433 in the cell. The AmB dissolution with the flow through set up was lower than the AmB
434 dissolution with the bottle/stirrer set up (Figure 5). The theoretical average linear velocities,
435 based on flow rate and cell diameter, employed in the flow through cell apparatus were 0.07
436 cm/s (low velocity) and 0.52 cm/s (high velocity) while the outer edge of the stirrer in the bottle
437 had a rotational linear velocity of 10.2 cm/s (low agitation) and 29.5 cm/s (high agitation). With
438 the lowest velocity in the bottle/stirrer setup being at least 20 times greater than the highest
439 velocity of the flow-through cell apparatus, the reduced AmB power dispersal leading to
440 aggregation in the flow through cell compared to the bottle/stirrer set up is evident. As the
441 AmB binding to BSA is a dynamic process, selection of appropriate hydrodynamics which

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

444

445 **4. Conclusions**

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

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

472

473 **Acknowledgments**

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587 **Tables**

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

Ion	Healthy Human Plasma	KRB
	Composition (Concentration (mM); [21 - 23])	
HCO ₃ ⁻	24.80	14.99
K ⁺	4.60	4.56
Cl ⁻	99.00	127.32
Na ⁺	150.00	136.17
Ca ²⁺	4.70	1.00
Mg ²⁺	1.60	0.49
Inorganic phosphorus	1.51	2.00
D-Glucose	5.60	10.00
Physicochemical properties		
pH	7.40 [21]	7.34 \pm 0.03 (+BSA 1.5% w/v) 7.35 \pm 0.03 (+BSA 2.0% w/v) 7.34 \pm 0.03 (+BSA 3.0% w/v) 7.36 \pm 0.01 (+BSA 4.0% w/v)
Osmolality (mOsm/L)	289.0 [21]	298.0 \pm 10.4 (+BSA 2.0% w/v) 308.7 \pm 2.5 (+BSA 4.0% w/v)
Viscosity (cps)	3.8 – 4.7 cP at 22°C [24]	Measurement at 25°C 3.70 \pm 0.03 (+BSA 1.5% w/v) 3.79 \pm 0.03 (+BSA 2.0% w/v) 3.88 \pm 0.02 (+BSA 3.0% w/v)

		3.98 ± 0.02 (+BSA 4.0% w/v)
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591

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

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

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

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

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607 Table 4. MIC and MFC ($\mu\text{g/mL}$) of AmB against *Candida albicans* in SBD broth and KRB
 608 supplemented with BSA (n = 2).

Condition	MIC ($\mu\text{g/mL}$)			
	SBD-BSA 2% w/v	SBD-BSA 4% w/v	KRB-BSA 2% w/v	KRB-BSA 4% w/v
Control	0.2 – 0.4			
AmB	3 – 4.5	6.4	4.5	7.5
Condition	MFC ($\mu\text{g/mL}$)			
	SBD-BSA 2% w/v	SBD-BSA 4% w/v	KRB-BSA 2% w/v	KRB-BSA 4% w/v
Control	0.8			
AmB	> 10.5	> 12.0	> 10.5	> 12.0

609 MIC: minimal inhibitory concentration, MFC: minimal fungicidal concentration

610

611

612 Table 5. Dissolution rate constants (k_{diss}), maximum corrected AmB % dissolved (X_{max}) and
 613 goodness of fit parameters (first order curve fitting; R^2 , AIC) for the dissolution studies with
 614 the bottle/stirrer setup (Mean \pm SD; n = 3).

615

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

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

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621 Figure 1. Representative UV spectra of AmB and deg-AmB.

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

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

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

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

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

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

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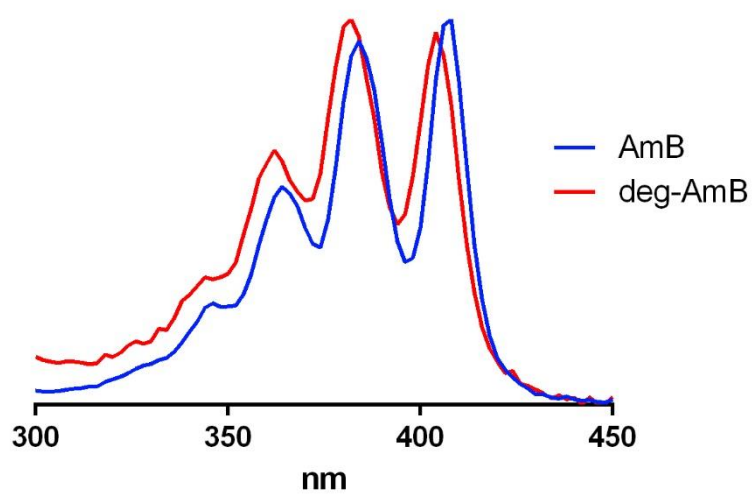
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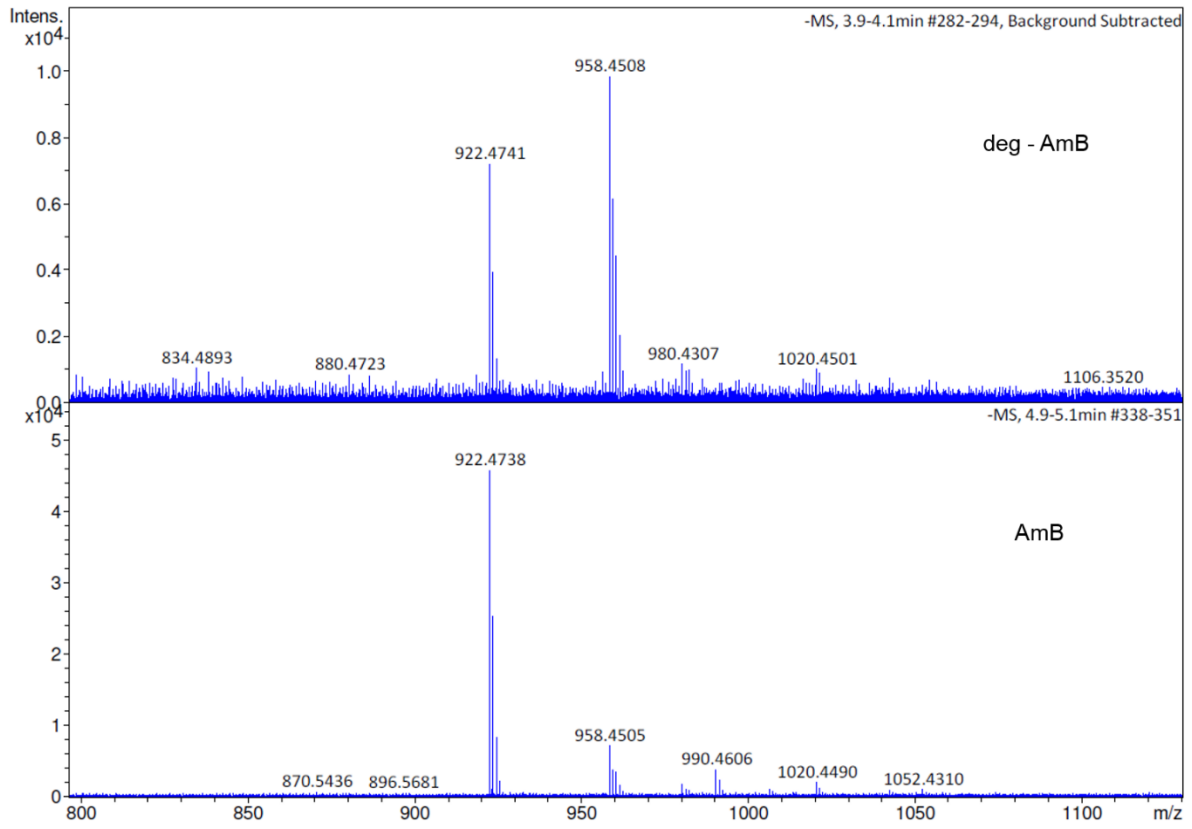


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644 Figure 1

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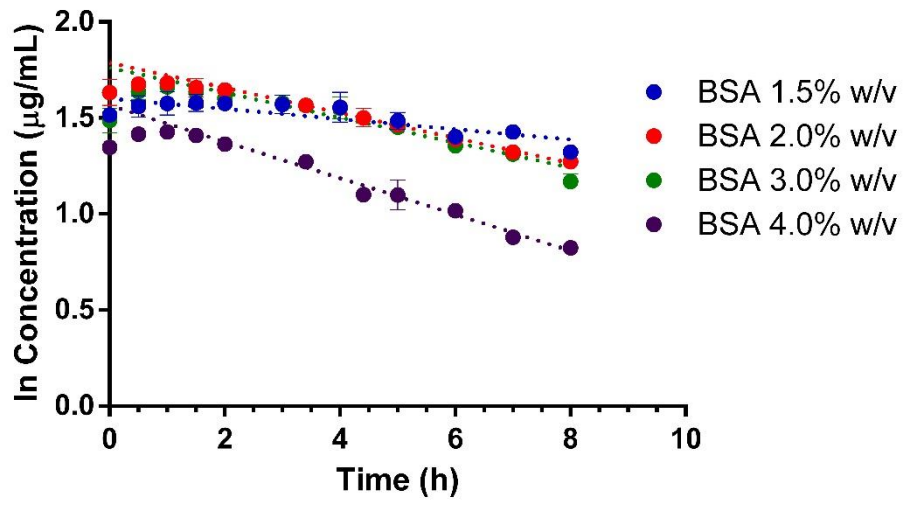


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648 Figure 2

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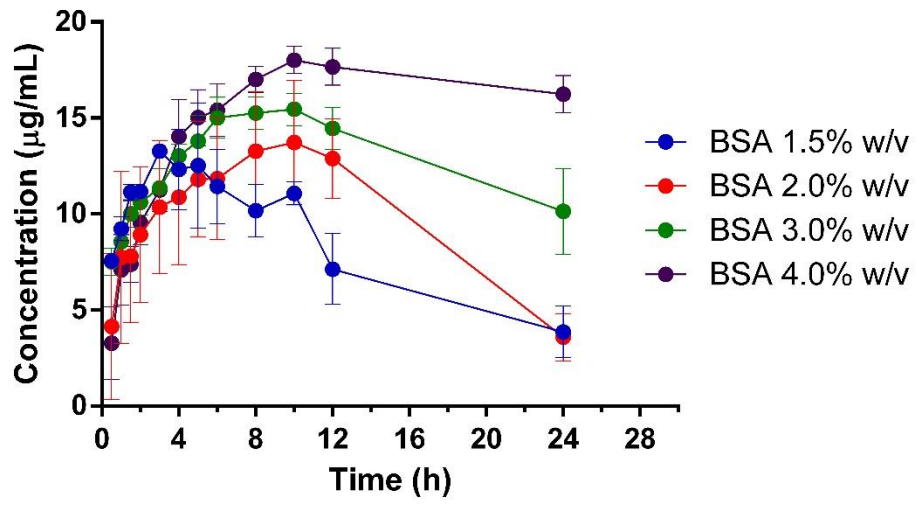


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652 Figure 3

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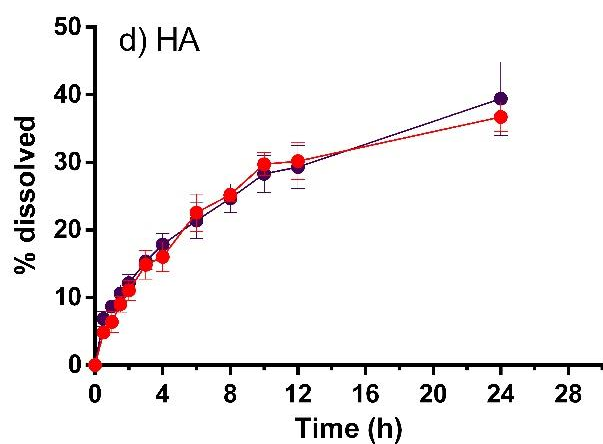
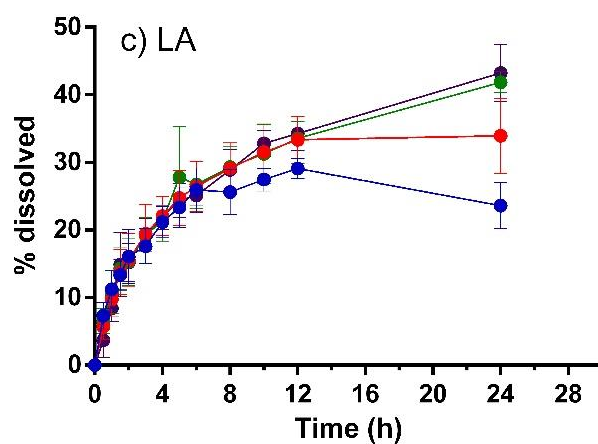
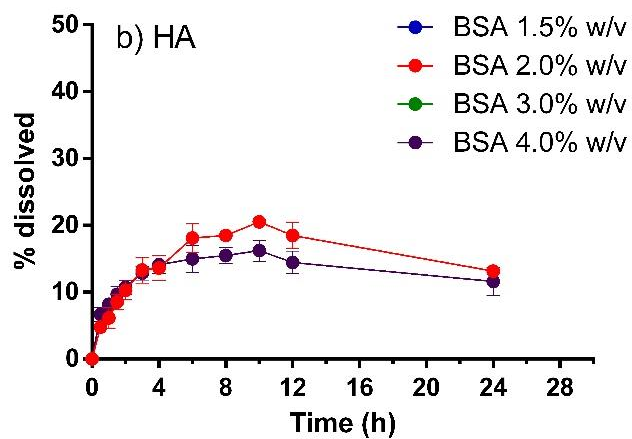
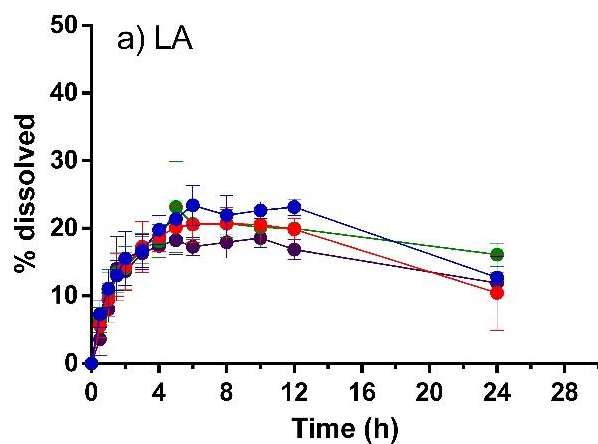


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656 Figure 4

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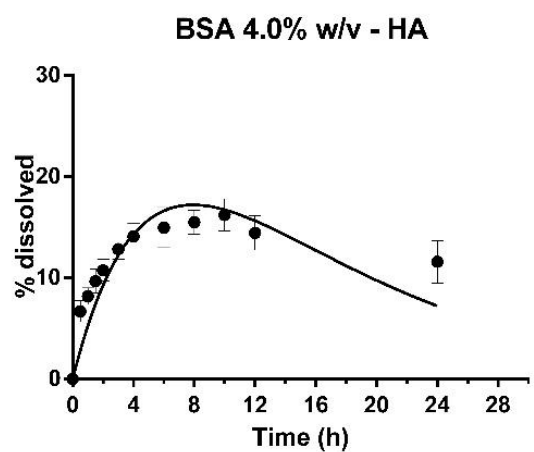
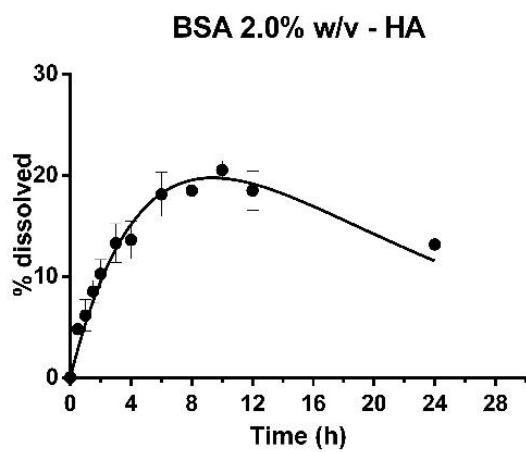
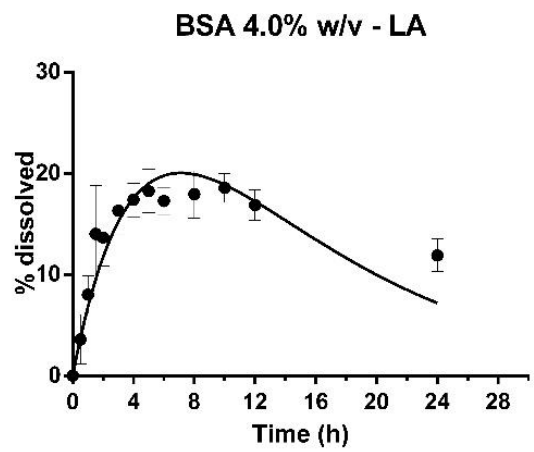
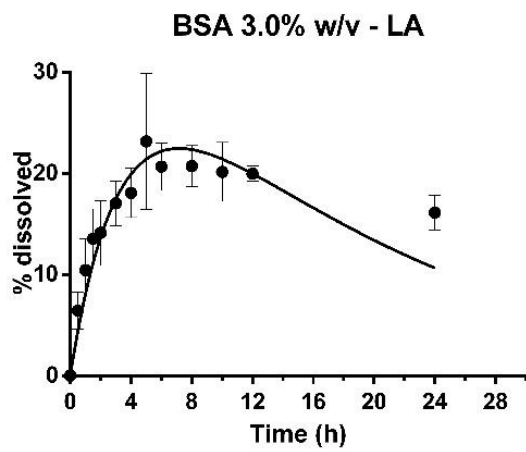
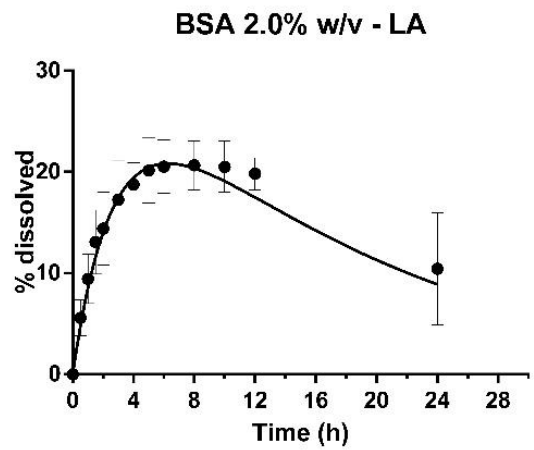
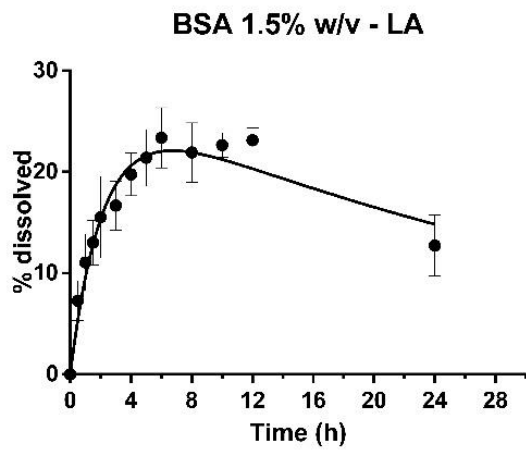


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660 Figure 5

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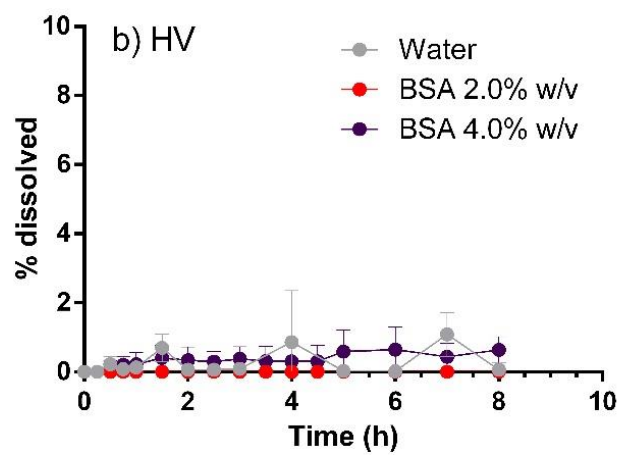
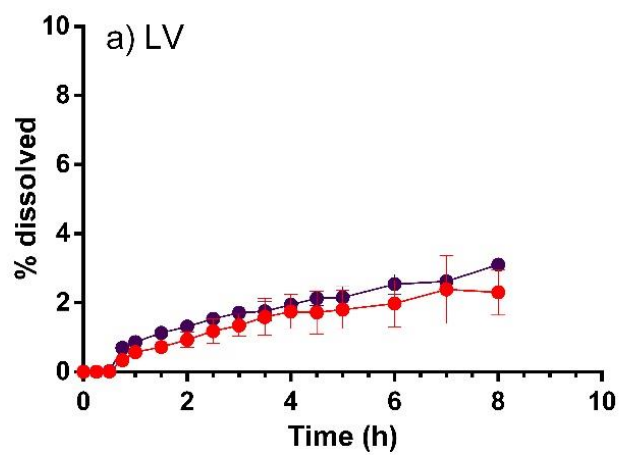


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664 Figure 6

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668 Figure 7