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

Ricardo Díaz de León-Ortega[a,1], Deirdre M. D'Arcy[b,1], A. Bolhuis[a], N. Fotaki[a,*]

[a] Department of Pharmacy and Pharmacology, University of Bath, Bath, United Kingdom
[b] School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin, Dublin 2, Ireland

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ABSTRACT

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

1. Introduction

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

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

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

Several parenteral formulations, that are not simple aqueous solutions, can be administered in the clinical setting to patients that have significant morbidities such as cancer or critically illness. Hypoalbuminaemia is common in the critical ill patients (affecting approximately 50% of patients), and while there is no reference value

* Corresponding author at: Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, United Kingdom.
E-mail address: n.fotaki@bath.ac.uk (N. Fotaki).
[1] Equal contribution.

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for hypoalbuminaemia, it can be considered when the plasma albumin levels are lower than 25 g/L [9]. Low levels of serum albumin may affect pharmacokinetics and pharmacodynamics of highly protein bound drugs. With a decrease in the protein levels in plasma there is more unbound drug in circulation which would lead to an increased pharmacological effect. On the other hand, the free drug can penetrate into tissues with a corresponding increase in the volume of distribution and a subsequent decrease in the maximum plasma concentration [9,10]. Release/dissolution of poorly soluble, highly protein bound drugs from parenteral formulations, and associated local drug concentrations are likely to be influenced by protein concentration at the site of release. Therefore, *in vitro* dissolution tests that simulate the *in vivo* environment are needed for parenteral formulations that are not aqueous solutions, which take into account the likely changes that arise in target patient groups, with particular reference to albumin concentration.

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

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

2. Materials and methods

2.1. Materials

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

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

2.2. Dissolution biorelevant media composition and characterization

The dissolution media employed were Krebs-Ringer Buffer (KRB), supplemented with BSA at different concentrations according to the experiment: 1.5, 2, 3 or 4% w/v. The pH was adjusted to 7.2–7.3 with 0.1 M HCl using a Seven Compact pH meter (Mettler Toledo, China). The osmolality of the media with 2 and 4% w/v BSA was measured via the freezing-point depression method with a Micro-Osmometer 3300 (Advanced Instruments, Massachusetts USA). Viscosity of all media was measured with a Bohlin Rheometer (Germany) with a shear rate 0.1–1.5 Pa (logarithmic scale), 20 integrations per measurement and with a delay time of 5 s and an integration time of 20 s. The geometry was a 4° and 40 mm diameter (CP 4/40) cone parallel to a plate and the experiments were conducted at 25 °C in triplicate. The measurement at the closest value to the steady state was recorded as the viscosity value.

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

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

2.4. Sample treatment of AmB in the biorelevant dissolution media

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

2.5. Liquid chromatography – Mass spectrometry (LCMS) studies

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