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Research paper

Development of a flow-through USP 4 apparatus drug release assay for the evaluation of amphotericin B liposome

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ABSTRACT

AmBisome® is a liposomal formulation of amphotericin B (Amp B), a complex parenteral antifungal product with no US FDA approved generic version available to date. For generic Amp B liposomal product development, examination of the drug release profile is important for product quality control and analytical comparability evaluation with the reference listed drug. Yet, there is no standardized *in vitro* drug release (IVR) assay currently available for Amp B liposomes. In this study, we describe the development of a USP-4 apparatus-based IVR assay capable of discriminating liposomal Amp B formulations based on the drug release profile. The goal of the IVR assay development was to identify release media compositions and assay temperatures capable of facilitating 70–100% of drug release from AmBisome® in 24 h without Amp B precipitation or disruption of liposome structure. We found that an addition of 5% w/v of γ-cyclodextrin to the release media of 5% sucrose, 10 mM HEPES, and 0.01% NaN_3 (pH = 7.4) prevented Amp B precipitation and facilitated drug release. Increased IVR assay temperature led to increased drug release rate, and 55 °C was selected as the highest temperature that induced drug release close to our target without causing product precipitation. The developed IVR assay was used to discriminate between drug release rates from AmBisome® and micellar Amp B products like Fungizone® and Fungcosome. The IVR assay was also capable of discriminating between Amp B liposomes with the same composition as AmBisome® but prepared by either extrusion or homogenization processes, both of which resulted in measurable liposomal particle size heterogeneity and Amp B concentration differences. Finally, the USP-4 IVR assay was used to compare Amp B release profiles between AmBisome® and two generic products approved in India, Amphonex® (Bharat Serums and Vaccines Ltd.) (f2 = 66.3) and Phosome® (Cipla Ltd.) (f2 = 55.4). Taken together, the developed USP-4 IVR assay can be a useful tool for drug release profile characterization in generic liposomal Amp B formulation development.

1. Introduction

AmBisome®, a liposomal formulation of amphotericin B (Amp B), was first introduced into the U.S. market in 1997. Currently it is the most widely used treatment for numerous invasive fungal infections [1] and visceral leishmaniasis [2], or prophylaxis in patients with hematological malignancies undergoing chemotherapy and organ or allogeneic bone-marrow transplantation [3–5]. With annual product sales of over 350 million and U.S. patent expiration in 2016, AmBisome® is a prime target for generic production [6]. Yet, as for all complex parenteral drugs, the development of generic liposomal Amp B has been challenging. The difficulty comes from the fact that for drugs like AmBisome® not only product composition, but also process manufacturing steps significantly influence the overall product efficacy and

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safety [7,8]. The AmBisome® manufacturing process is rather elaborate due to Amp B's poor solubility in aqueous solution and the requirement of Amp B complexation with distearoyl phosphatidyl-glycerol (DSPG) within the liposome bilayer [9,10]. To assure Amp B liposomal stability, the lipid mixture selected for this product must have a high phase transition temperature overall; thus, many manufacturing steps have to be conducted at temperatures as high as 65 °C. In addition, the average liposomal particle size for AmBisome® is rather small (< 100 nm) with low polydispersity, requiring highly controlled particle size reduction to achieve matching of characteristics between generic and reference product.

Additionally, many physicochemical characteristics of the product define the rate of Amp B release *in vivo,* which directly correlate with toxicity in animals and humans. Some of these product quality attributes are the physical state of Amp B in the membrane, liposome particle size homogeneity, lipid phase transition temperature, and amount of unencapsulated drug [11,12]. To ensure that the desired product characteristics are achieved, several liposomal Amp B manufacturing steps are critically important. These process parameters include, among others, liposome component concentration in organic phase, organic phase composition, the order of component mixing, homogenization conditions, and product freeze-drying parameters [9,13,14].

While several generic versions of liposomal Amp B had been approved outside of the United States, many were subsequently withdrawn from the market due to product safety concerns [15–17]. Two of such products are Anfogen® (Genpharma S. A.), initially approved in Argentina, and Lambin® (Sun Pharmaceutical Industries), initially approved in India. When Anfogen® and Lambin® were compared with AmBisome®, the two generics were 2–10 times more toxic in mice based on LD-50 values and exhibited lower efficacy in pulmonary aspergillosis murine models [14,18]. The formulation compositions for two generic products were identical to AmBisome®, and the differences in the generic products' efficacies and safeties were attributed to more rapid release of Amp B [15]. Thus, development of an *in vitro* drug release method that is capable of discriminating between liposomal Amp B formulations is critical.

Recently, several product-specific USP apparatus 4-based IVR assays have been developed for characterization of suspensions, polymer microspheres, and liposomes [19–22]. These IVR assays have a few prominent advantages compared to conventional "dialysis sac" and "sample and separate" methods, including the use of a standard USP-4 dissolution apparatus, easily adjustable parameters for different release conditions, automatic drug detection without sampling, and a closed loop system without external disturbances [20,21,23]. In many instances, USP-4 dissolution testing for complex parenteral drugs is performed at accelerated temperatures and in the presence of solubilizers in the release media to facilitate release of active pharmaceutical ingredients (APIs) from highly stable liposomes, microspheres, or suspension formulations. All of these advantages would be highly conducive to the establishment of a robust and repeatable IVR assay for liposomal Amp B products.

Here we present the development of an Amp B IVR assay using a USP-4 dissolution apparatus that is capable of discriminating similar formulations of liposomal Amp B and liposomal products that have compositions identical to AmBisome® but exhibit different rates of Amp B release. Several solubilizers were added to the release media and screened for their capability of increasing Amp B solubility and release rate without instantly disrupting the liposomal structure. IVR assay conditions such as temperature, drug concentration, and solubilizer concentration in the release media were adjusted to ensure that over 70% of Amp B was released from AmBisome® in 24 h. The IVR assay was used to evaluate similarities of Amp B release rates between AmBisome® and two generic products, Amphonex® (Bharat Serums and Vaccines Ltd.) and Phosome® (Cilpa Ltd.) currently marketed in India. Other micelle-based formulations, such as Fungizone® (X-Gen Pharmaceuticals, approved by US FDA) and Fungcosome (Shanghai New Asia Pharmaceutical, approved in China), exhibited distinctly different release rates using our methodology. In addition, liposomal Amp B formulations prepared in-house using different methods from the AmBisome® manufacturing procedure, resulting in slightly different particle size distributions, also exhibited different release rates relative to AmBisome® in the established USP-4 IVR assay. We also evaluated whether the USP-4 IVR could give stability information by examining drug release from forced degradation of AmBisome® samples. Taken together, the established USP-4 IVR assay could be useful for quality control to guide development of generic liposomal Amp B products and for analytical comparability testing of generic drugs.

2. Materials and methods

2.1. Materials

AmBisome® (Astellas Pharma US, Inc.) and Fungizone® (X-Gen Pharmaceuticals, Inc.) were purchased from the University of Michigan Hospital Pharmacy. Amphotericin B (Amp B), γ-cyclodextrin (γ-CD), hydroxypropyl-cyclodextrin (HP-CD), and Fungcosome were purchased from SHJNJ Pharmatech (Shanghai, China). Amp B reference standard was purchase from USP (Rockville, MD). Amphonex® and Phosome® were kindly provided by Bharat Serums and Vaccines Ltd. (Mumbai, India). DSPG and HSPC were purchased from Lipoid (Newark, NJ). Cholesterol was purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama). Float-A-Lyzer® dialysis tubes with a 300 kDa molecular weight cut-off (MWCO) were purchased from Spectrum Laboratories (Rancho Dominguez, CA). Sucrose was purchased from Fluka. Sodium dodecyl sulfate (SDS), isopropanol (IPA), HEPES, alpha-tocopherol, NaN_3, and all other reagents were purchased from Sigma. To ensure the accuracy of quantitative analysis, all buffers used in this study were freshly prepared before use.

2.2. Methods

2.2.1. Preparation of Amp B liposomes

The preparation procedure for liposomes (35:20-8; 35:41-4; 35:48-7) was based on the published patent from NeXstar with some modifications [13]. First, 33.6 mg DSPG, 85.2 mg HSPC, 20.8 mg cholesterol, and 0.256 mg alpha tocopherol were added into 1 mL ethanol containing 14.0 μL water (2% v/v), heated to 65 °C, and vortexed to dissolve the lipids. 20 mg Amp B in 0.3 mL DMSO was preheated to 65 °C and added to the lipid solution. 2.6 mL of 70 °C pre-warmed buffer (10 mM sodium succinate, 10% (w/v) sucrose, pH 4.8) was added into Amp B/lipids solution, vortexed intermittently at 70 °C for 15 min, and cooled to room temperature. The suspension was dialyzed against buffer (10 mM sodium succinate, 10% (w/v) sucrose, pH 4.8) at 4 °C and subjected to two freeze-thaw cycles. The target concentration of Amp B was 0.7 mg/mL to avoid membrane pore clogging during extrusion. The suspension was extruded through a 200 nm polycarbonate membrane once and an 80 nm polycarbonate membrane seven times to obtain final liposomal Amp B formulation 35:20-8.

Formulation 35:41-4 was prepared by adding 252 mg DSPG and 150 mg Amp B into 7.5 mL MeOH/DMSO (7/2, v/v), heating to 65 °C, and adding 150 μL of H2O (2%, v/v) and 124 μL of 2.5 M HCl. The mixture was further incubated at 65 °C until all components were dissolved and a transparent orange DSPG/Amp B solution was seen. Additional liposome components, 639 mg HSPC, 156 mg cholesterol, and 1.92 mg alpha tocopherol, were dissolved in 7.5 mL MeOH/DMSO (7/2, v/v) at 65 °C for 5 min. The two prepared solutions were mixed and incubated for an additional 10 min at 65 °C, after which 73 μL of 2.5 M NaOH was added. The lipid solution was added to the rapidly stirred 135 mL of pre-warmed buffer (10 mM sodium succinate, 10% sucrose (w/v), pH 4.8) in a jacketed stirred vessel maintained at 65 °C. After stirring for 15 min, the formulation was cooled down, exchanged with 10 volumes of buffer (10 mM succinate, 10% sucrose (w/v), pH

5.5), and concentrated to a target 4 mg/mL Amp B by tangential flow filtration. The lipid suspension was homogenized using M-110P Microfluidizer® (Microfluidics Corporation, Westwood, MA) for 7 passes under 10,000 psi to form 35:48-7 and 4 passes under 20,000 psi to form 35:41-4.

2.2.2. Analysis of particle size of Amp B formulations

Before measurement, 0.1 mL Amp B liposomes were diluted with 0.9 mL release media or D.I. water to determine average particle size and polydispersity (PDI) at room temperature by dynamic light scattering using Malvern Instruments ZetaSizer 3000HSa (Westborough, MA). For Amp B micelle formulations, samples were analyzed without dilution. All samples were analyzed in triplicate.

2.2.3. Analysis of Amp B concentration by HPLC

The Amp B concentration in different formulations was measured by high performance liquid chromatography (HPLC) equipped with a UV detector. Chromatographic separation was carried out at room temperature using a Higgins PROTO 300 C18 column (250 × 4.6 mm, 5 μm, Higgins Analytical Inc., Mountain View, California). The mobile phase consisted of (A) methanol containing 0.1% v/v trifluoroacetic acid and (B) water containing 0.1% v/v trifluoroacetic acid with a gradient of 60–84% A over 9 min. The flow rate was 1 mL/min, and the detection wavelength was 390 nm. Amp B reference standard was dissolved in DMSO to form a stock solution (4 mg/mL), which was diluted to an 80 μg/mL solution with methanol. For all the Amp B formulations, 100 μL of the samples were dissolved in 1 mL DMSO and then diluted to 5 mL with methanol. All samples were centrifuged under 12000 rpm for 10 min before HPLC analysis, and the Amp B concentration in different formulations was calculated using an external standard method according to the peak area.

2.2.4. AmBisome® release condition screening in a single-unit vial-based IVR assay

For the selection of release media composition, release experiments were performed in a single-unit vial-based IVR assay by placing AmBisome® in a Float-A-Lyzer® (molecular weight cut-off 300 kDa) immersed in 38.4 mL of release media in a conical centrifuge tube. Briefly, 0.1 mL AmBisome® or stock solution of free Amp B (4 mg/mL) were diluted with 1.5 mL of release media and then placed in Float-A-Lyzer® (final Amp B concentration in the release media was 10 μg/mL). Solutions were incubated for 24 h at 45 °C with shaking at 180 rpm. In all cases, the release media consisted of 5% sucrose, 10 mM HEPES, and 0.01% NaN_3 (pH = 7.4). The effects of the addition of 5% γ -CD, 5% HP-CD, 0.25% SDS, or 10% IPA on Amp B release rate were examined. 0.1 mL of 4 mg/mL free Amp B stock solution directly added into 39.9 mL release media at 10 μg/mL served as a control. Dissolution media aliquots (300 μL) were removed at predetermined time intervals and replaced with fresh release media. The amount of Amp B released at various times was determined by UV absorbance at 414 nm using Synergy Neo HTS Multi-Mode Microplate Reader (BioTek Instruments, Inc., VT, USA).

The effects of release conditions in the single-unit vial-based IVR assay on Amp B release performance were examined. The release study was performed at temperatures of 45 °C, 55 °C, and 60 °C. To examine the impact of solubilizer concentration on Amp B release, concentrations of γ-CD 1, 2.5, 5 and 10% (w/v) were tested. The IVR assay was also performed with different Amp B initial concentrations 5, 10, and 30 μg/mL in total release media. Other release conditions and testing procedures were the same as described previously.

The average particle size and PDI of AmBisome® before and after release studies were compared using DLS analysis as described previously. Samples were diluted in the release media, and the mean particle size was obtained from three independent experiments.

2.2.5. Establishment of USP-4 apparatus IVR assay for discriminating AmBisome® from other Amp B formulations

Amp B release from AmBisome® or other Amp B formulations was examined using the USP-4 apparatus CE7 Smart (Sotax AG, Switzerland). Briefly, 1.6 mL AmBisome® or other Amp B formulations containing samples were placed in a Float-A-Lyzer® (Amp B concentration in tubes equals to 0.5 mg/mL) and then inserted into USP-4 flow-through cells. 78.4 mL release media was used for each cell (final concentration of Amp B was 10 μg/mL). The media was perfused in a closed-loop setting at 16 mL/min and kept at 55 °C. The released Amp B amount was monitored by UV absorption hourly. During the method development stage, an equal amount of free Amp B solution (10 μg/mL) was placed in the release media directly as a control to mimic the complete release of Amp B from formulations and monitor any changes in UV absorption of released Amp B over the release period. The cumulative release (%) was calculated by dividing the Amp B amount released from AmBisome® or other Amp B formulations by the measured amount of released Amp B control solution at each time point. The release was determined in triplicate for all conditions, and results were reported as the mean ± SEM.

The Amp B formulations tested by this method include: (a) Micelle Amp B formulation (Fungizone®, X-Gen Pharmaceuticals, Inc., USA); (b) Fungcosome, a mixed micelle formulation produced by Shanghai New Asia Pharmaceutical Co., Ltd, China; (c) generic liposomal Amp B formulations that have the same composition as AmBisome®, such as Amphonex® (Bharat Serums and Vaccines Ltd.) and Phosome® (Cilpa Ltd.); (d) Amp B liposomal formulations with the same composition as AmBisome® prepared by homogenization (35:41-4 and 35:48-7) or extrusion (35:20-8).

An f2 test was performed to compare whether a significant difference was observed between the cumulative release of AmBisome® and other Amp B formulations using the formula below.

$$f_2 = 50 \log \left[\frac{100}{\sqrt{1 + \frac{\sum_{i=1}^{n} (Rt - Tt)^2}{n}}} \right]$$

Variables are as follows: n is the number of time points, R_t is the cumulative release value of the reference product at time point t ($t > 0$), and T_t is the cumulative release value of the test batch at the same time point. The cumulative release values for all time points ($n = 24$) were used for the calculation of f2. A test formulation with a similarity factor $f2 \geq 50$ is considered similar to the reference formulation. An f2 value of 100 suggests that the test and reference profiles are identical and, as the value becomes smaller, the dissimilarity between release profiles increases [24–26].

2.2.6. Examining if USP-4 apparatus IVR assay is stability indicating

AmBisome® vials were hydrated according to manufacturing instructions and stored refrigerated for up to 7 months. The aged and freshly hydrated samples were analyzed by USP-4 IVR assay, and f2 values were calculated. In addition, hydrated AmBisome® aliquots were incubated at 60 °C for 7 h, 3 days, and 7 days. The IVR assay was conducted on stressed and unstressed samples, and f2 values were calculated.

3. Results and discussion

3.1. Effect of solubilizers on AmBisome® release by single-unit vial-based IVR assay

Due to the poor aqueous solubility of Amp B, solubilizers have to be added to the release media to ensure sink conditions. In addition, solubilizers are often added to the release media of complex parenteral drugs to facilitate active pharmaceutical ingredient (API) release. For example, hydroxyl propyl cyclodextrin was used to solubilize

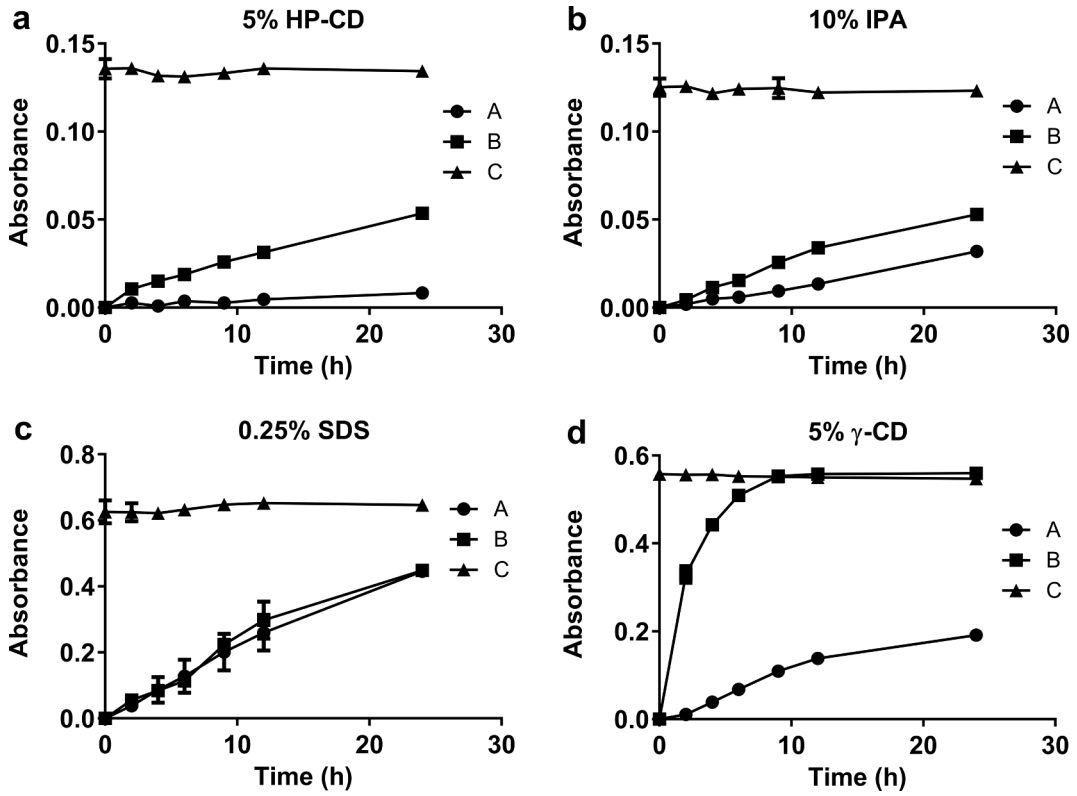

Fig. 1. The effect of solubilizer addition to the release media on Amp B release from AmBisome® in the single-unit vial-based IVR assay at 45 °C, including 5% HP-CD (a), 10% IPA (b), 0.25% SDS (c) or 5% γ-CD (d). Lines represent: A. AmBisome® in Float-A-Lyzer® (●); B. Free Amp B solution in Float-A-Lyzer® (■); C. Free Amp B in release medium (▲). The final Amp B concentration in the release media is 10 μg/mL for all the groups.

Table 1
Effect of different release conditions on the stability of AmBisome®.

	Release conditions			Size (nm)*	PDI	Precipitation
Before release				118.9 ± 2.3	0.19 ± 0.03	–
After release	10 μg/mL Amp B; 45 °C	Solubilizer type	5% HP-CD	135.6 ± 1.9	0.31 ± 0.06	√
			10% IPA	142 ± 2.4	0.24 ± 0.05	√
			0.25% SDS	22.8 ± 2.5	0.58 ± 0.09	–
			5% γ-CD	127.7 ± 1.8	0.27 ± 0.04	–
	10 μg/mL Amp B; 5% γ-CD	Temperature	45 °C	127.7 ± 1.8	0.27 ± 0.04	–
			55 °C	122.8 ± 3.9	0.25 ± 0.05	–
			60 °C	143.7 ± 7.1	0.34 ± 0.02	√
	10 μg/mL Amp B; 55 °C	γ-CD concentration	1%	107.8 ± 1.1	0.15 ± 0.02	–
			2.5%	112.5 ± 3.3	0.17 ± 0.03	–
			5%	122.8 ± 3.9	0.25 ± 0.05	–
			10%	264.1 ± 41.4	0.53 ± 0.16	√
	5% γ-CD; 55 °C	Amp B concentration (μg/mL)	5	122.1 ± 3.5	0.17 ± 0.03	
			10	122.8 ± 3.9	0.25 ± 0.05	–
			30	130.5 ± 4.9	0.32 ± 0.02	–

* The size was measured by DLS after dilution with the release media prior to and after the completion of release study.

doxorubicin and facilitate its release from Doxil® in a USP-4 IVR assay [21], while acetonitrile and ethanol were added to facilitate drug release for polymer microsphere formulations of everolimus and levonorgestrel, respectively [27–29]. To rapidly screen a number of solubilizers, AmBisome® release was performed in a single-unit vial-based IVR assay at 45 °C with shaking. To ensure that addition of solubilizers did not lead to liposome disruption, AmBisome® particle size was measured at the end of the release study. When 5% HP-CD or 10% IPA were used as solubilizers, precipitation of Amp B was observed in the release medium (Fig. 1a, b). Due to Amp B precipitation, the total absorbance of Amp B in the release media (line C) was significantly lower than the total absorbance of Amp B in media supplemented with 0.25% SDS (Fig. 1c) or 5% γ–CD (Fig. 1d). Addition of 0.25% SDS resulted in more rapid release of AmBisome® (line A), but the size of AmBisome® decreased from 119 nm to 23 nm (Table 1), indicating liposome structural disruption by the surfactant. In addition, the release of free Amp B from the Float-A-Lyzer® appeared to be slow and incomplete (line B) in the media with 0.25% SDS, indicating that Amp B is likely solubilized in SDS micelles that slowly permeate through the 300 kDa membrane. Similar structural disruption of liposomes accompanied by immediate release of liposomal content was observed by Bhardwaj et al. when 0.5% (w/v) SDS was added to the release media of dexamethasone-loaded liposomes [20]. In contrast to SDS, the addition of 5% γ-CD in the release media resulted in rapid and complete release of free Amp B solution from the Float-A-Lyzer® (Fig. 1d). The addition of 5% γ-CD caused only minor increases in AmBisome® particle size and allowed for

27.2% of drug to be released in 24 h at 45 °C (Fig. 1d). The average particle size of AmBisome® increased from 118 nm to 123 nm following IVR in 5% γ-CD media, suggesting that liposomes remained intact during drug release testing (Table 1). Based on this data, γ-CD was selected as the Amp B solubilizer, and additional IVR assay optimization studies were performed to increase drug release rate for the liposomes.

3.2. Effect of temperature, γ-CD concentration and Amp B concentration by AmBisome® release by single-unit vial-based IVR assay

The overall goal of this study was to establish an IVR assay that would allow for 70–100% drug release within 8–24 h. The time requirement comes from practical demands on the IVR assay to be conducted in an analytical laboratory for batch analysis. The need for 70–100% of the API release from liposomal formulation ensures the discriminative power of the IVR assay. With complete release, different rates of drug release between formulations are more likely to be identified. Assay conditions were further optimized to improve completeness of Amp B release using single-unit vial-based IVR assay. The phase transition temperature of AmBisome® is high at 56 °C, as determined by differential scanning calorimetry, because the liposome is composed of fully saturated phospholipids and cholesterol. To increase drug release rate from complex liposomal or polymeric microsphere products, the IVR assay is often performed at elevated temperatures either close to or above product phase transition temperature. For example, both Doxil® and Risperdal® Consta® USP-4 release assays are performed at 45 °C [21,23]. Thus, to increase Amp B release rates, the IVR assay was performed at 45 °C, 55 °C and 60 °C in release medium containing 5% γ-CD. As shown in Fig. 2a, the increase in temperature had significant effects on Amp B release from AmBisome® with 35%, 76% and 98% of cumulative drug release within 24 h at 45 °C, 55 °C and 60 °C, respectively. The increase in IVR assay temperature had no effect on the diffusion rate of free Amp B solution from the Float-A-Lyzer® (Fig. 2b). When the assay temperature was as high as 60 °C, particle size of AmBisome® increased moderately and product precipitation was observed (Table 1), indicating the instability of AmBisome® at such high temperatures. Thus, the 55 °C IVR assay temperature was selected for subsequent studies.

To examine the impact of the concentration of γ–CD in the release media on cumulative Amp B release from liposomes, we performed studies in the presence of 1%, 2.5%, 5%, or 10% γ–CD (w/v) in the release media (Fig. 3). All experiments were conducted at 55 °C. With the addition of 1% γ–CD, even release of free Amp B solution from the Float-A-Lyzer® appeared to be slow and incomplete, indicating limited solubility of drug in 1% γ–CD (Fig. 3b). For all other γ–CD concentrations, the release of free Amp B solution from the Float-A-Lyzer® was rapid and complete. We observed that the drug release rate from AmBisome® increased gradually as the concentration of γ-CD increased (Fig. 3a). This could be due to increased solubility of Amp B through γ-CD binding with cholesterol on the liposome surface. Cholesterol removal could effectively increase liposome bilayer fluidity and reduce effective phase transition temperature, thus leading to faster Amp B release and reduced liposome stability. We have observed this effect before during establishing the USP-4 based IVR assay for Doxil® [21]. This phenomenon is most noticeable with the addition of 10% γ–CD to the release media when AmBisome® size increased significantly from 118.9 nm to 264.1 nm after 24-hour release (Table 1), indicating possible liposome aggregation. Because of significant increase in AmBisome® particle size during release study, 10% γ–CD was not used in the subsequent studies. The 5% w/v γ–CD was selected for media composition in the final IVR assay to assure relatively complete drug release during the assay without liposome aggregation.

The effect of Amp B concentration on AmBisome® cumulative release was investigated by adjusting the volume of liposomes placed in a Float-A-Lyzer®. To achieve final theoretical Amp B concentrations in the release media of 5, 10 and 30 μg/mL (assuming 100% drug release), 0.05, 0.1 and 0.3 mL of AmBisome® (4 mg Amp B/mL) was added to the Float-A-Lyzer® and 80 mL of the release media was used. At the Amp B concentrations investigated, only small differences in the amount of drug released at 24 h were observed at all concentrations (Fig. 4a). Thus, the 10 μg/mL Amp B concentration was used for all subsequent studies as it provided sufficient sensitivity for the UV detection of released drug and minimal required amount of Amp B liposomes to perform the IVR assay.

3.3. Adaptation of AmBisome® IVR to USP-4 apparatus

Based on the experimental results, the optimized release conditions in single-unit vial-based IVR assay were adapted on the USP-4 apparatus CE7 Smart Sotax®. The IVR assay was performed at 55 °C in a closed-loop system with media circulating at 16 mL/min. The final release media consisted of 5% sucrose, 10 mM HEPES, 0.01% NaN_3 (pH = 7.4), and 5% γ-CD. The liposomes or micelles were placed in a Float-A-Lyzer® at the final concentration of 0.5 mg/mL by mixing 0.2 mL of AmBisome® (4 mg/mL) with 1.4 mL of the release media. Finally, 78.4 mL of media was added to the USP-4 cell to bring final Amp B concentration in the release media to 10 μg/mL assuming 100% drug release.

The release of Amp B from AmBisome® in the USP-4 IVR assay setup was similar to the single-unit vial-based IVR assay, with roughly 75% of drug release in 24 h (Fig. 5). While selected IVR conditions provided us with an opportunity to differentiate between various lipid formulations of Amp B, there are several obvious limitations of the assay. The assay is conducted at a temperature above normal physiological conditions and in the presence of 5% γ-CD acceptor; thus, the

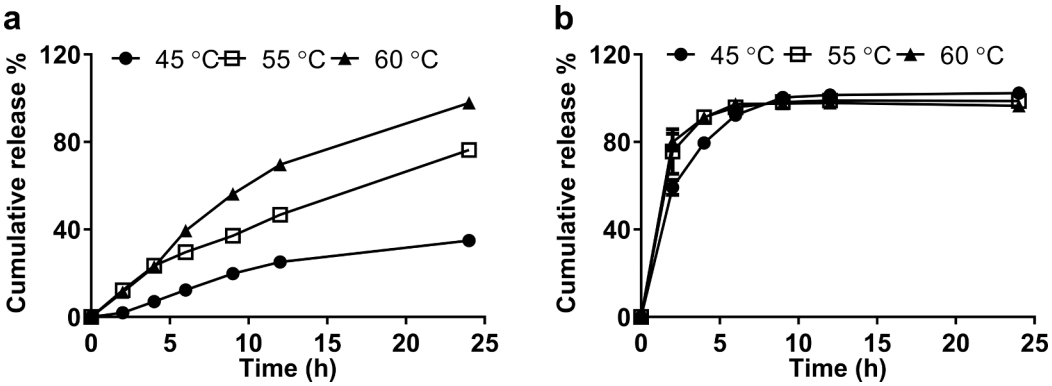

Fig. 2. The release of AmBisome® (a) and free Amp B (b) from Float-A-Lyzer® under different release temperature by the single-unit vial-based IVR assay. 5% γ-CD was added into media and total Amp B concentration is 10 μg/mL for all the groups.

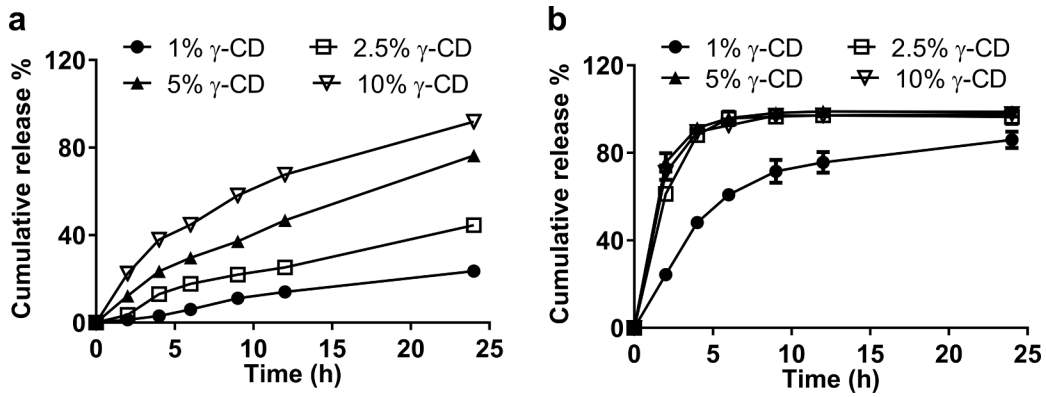

Fig. 3. The release of AmBisome® (a) and free Amp B (b) from Float-A-Lyzer® under different γ-CD concentrations by the single-unit vial-based IVR assay at 55 °C. Total Amp B concentration is 10 μg/mL for all the groups.

release rate measured by the IVR likely is different from Amp B release *in vivo*. In addition, AmBisome® is known to interact with endogenous plasma lipoproteins following *in vivo* administration and is taken up by the body's reticuloendothelial system [22,30,31]. It is important to point out that the Amp B concentration we measured following drug release from dialysis membranes is likely different from the concentration of Amp B released from the liposomes inside the Float-A-Lyzer®, as dialysis membranes represent a diffusion barrier for the drug [32]. The actual release rate of Amp B from the liposomes could be elucidated using a mathematical model correlating measured drug concentration outside the Float-A-Lyzer® with actual drug concentration inside the membrane as was done by Modi et al. [32].

To eliminate the possibility that slow release of Amp B from AmBisome® measured by the IVR is primarily due to surface binding of released Amp B to the liposome, the release of free Amp B from the Float-A-Lyzer® was measured in the presence of blank liposomes. Blank liposomes were prepared using the same ratios of HSPC, DPPG, and cholesterol used in the AmBisome® formulation. Blank liposomes and free Amp B were mixed at the same ratio as in AmBisome® composition (mimicking 100% of drug released scenario – high concentration) and at 10-fold lower amount of Amp B (mimicking 10% of drug release scenario – low concentration) (Supplemental Fig. 1). At the low drug concentration, no differences in Amp B release were observed in the absence or presence of blank liposomes. At a high drug concentration, Amp B release was slightly slower (~1 h delay) in the presence of blank liposomes but still more rapid than drug release measured for AmBisome®. Taken together, binding of released Amp B to the liposomal surface has a very limited impact on IVR test performance.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ejpb.2018.11.010.

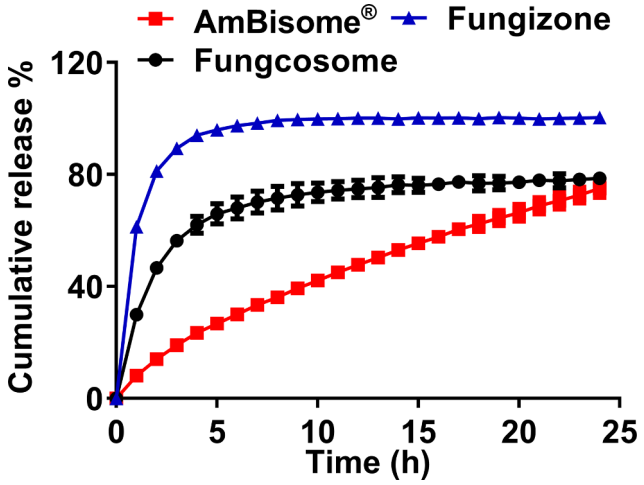

Fig. 5. The cumulative release of different commercial Amp B formulations on Sotax® at 55 °C. 5% γ-CD was added into media, and total Amp B concentration is 10 μg/mL for all the groups based on reported package insert drug concentrations.

3.4. Comparison of AmBisome® and commercial micellar Amp B formulations by USP-4 IVR assay

The IVR assay adapted to the USP-4 apparatus was tested for its ability to discriminate between liposomal and micelle-based Amp B products. Drug release from liposomal AmBisome® was compared with drug release from two other Amp B marketed products, Fungzione®, a

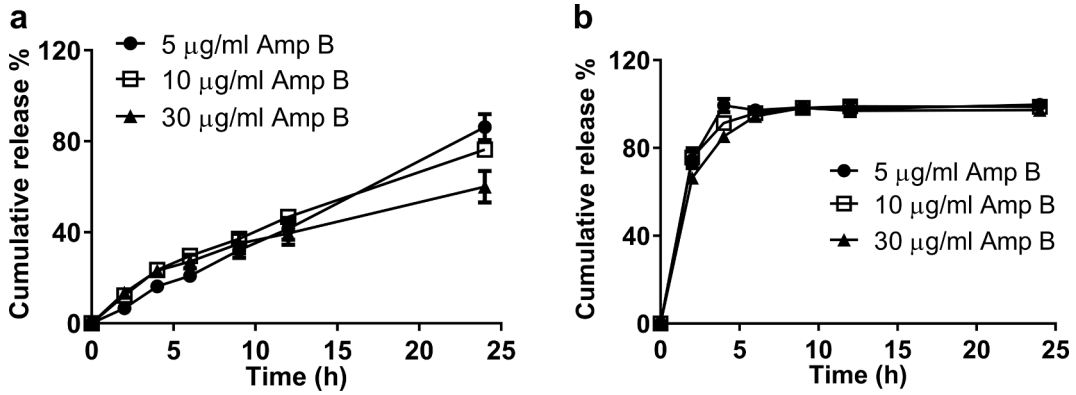

Fig. 4. The release of AmBisome® (a) and free Amp B (b) from Float-A-Lyzer® under different Amp B concentrations by the single-unit vial-based IVR assay at 55 °C, 5% γ-CD was added into media.

Table 2
The composition, characteristics and release f2 values of different Amp B formulations compared with AmBisome®.

Sample name	Manufacturer	Compositions (mg/vial)	Measured Amp B (mg/mL)	Size	PDI	f2 Values
AmBisome®	Astellas Pharma US, Inc.	Amp B/HSPC/DSPG/cholesterol (50/213/84/52)	4.15 ± 0.04	102.0 ± 1.0	0.167 ± 0.010	–
Fungizone®	X-Gen Pharmaceuticals, Inc.	Amp B/sodium desoxycholate (50/41)	4.12 ± 0.04	179.8 ± 4.0	0.560 ± 0.033	14.0
Fungcosome	Shanghai New Asia Pharmaceutical Co., Ltd.	AmpB (10 mg)/phosphatidylcholine (PC)/sodium desoxycholate	2.81 ± 0.05	653.2 ± 11	0.855 ± 0.032	28.6
35:20-8	Z1P	–	0.74 ± 0.01*	117.4 ± 0.2	0.116 ± 0.002	70.8
35:41-4	Z1P	–	3.54 ± 0.07	115.8 ± 0.9	0.256 ± 0.001	48.0
35:48-7	Z1P	–	3.90 ± 0.06	88.6 ± 0.4	0.190 ± 0.005	41.1
Phosome®	Cipla Ltd.	Same as AmBisome®	3.41 ± 0.06	100.0 ± 1.2	0.118 ± 0.010	55.4
Amphonex®	Bharat Serums & Vaccines Ltd.	Same as AmBisome®	3.82 ± 0.05	95.3 ± 0.1	0.089 ± 0.008	66.3

* This formulation was prepared at lower target Amp B concentration to aid the extrusion process. It was diluted accordingly to the target concentration of 10 μg/mL of Amp B in total release media for the USP-4 IVR assay.

micellar formulation produced by X-Gen Pharmaceuticals and marketed in US, and Fungcosome, a mixture of micelles and liposomes produced by Shanghai New Asia Pharmaceutical Co., Ltd and marketed in China. The compositions of all Amp B products used in this manuscript are summarized in Table 2 based on package inserts for these products. Fungizone®, a micelle composed of sodium deoxycholate and Amp B, exhibited very rapid drug release with nearly 100% cumulative drug release in the first 6 h (Fig. 5). Fungcosome has a different composition, containing a mixture of Amp B, sodium deoxycholate and phosphatidylcholine with an average particle size of 653 nm, much larger relative Fungizone® (180 nm) and AmBisome® (102 nm) (Table 2). Despite differences in formulation, drug release profiles of Fungcosome and Fungizone® were similar in the USP-4 IVR assay, characterized by a rapid release reaching a plateau in 6 h. This release profile indicated that Fungcosome behaves more like a mixed micellar formulation than a liposome [33–35]. Interestingly, Amp B release had not reached 100% release for Fungcosome, likely due to a discrepancy between a reported Amp B concentration of 4 mg/mL (10 mg/vial, dissolved with 2.5 mL D.I. water) used for the USP-4 test and the actual concentration of 2.8 mg/mL measured by us using HPLC. Taken together, these results indicate that the USP-4 method is capable of differentiating AmBisome® from other non-liposomal Amp B formulations.

3.5. The effect of manufacturing method of Amp B liposome on USP-4 IVR assay drug release drug release

For complex generic products, not only composition but also the manufacturing process determine drug release performance. Three different liposomal Amp B formulations were prepared by homogenization or extrusion processes to explore if the USP-4 IVR assay could distinguish between their drug release properties. All liposomal formulations were prepared using identical drug to lipid w/w ratios of Amp B:HSPC:DSPG:cholesterol at 50:213:84:52. The components were dissolved in acidified methanol-chloroform (50:50), mixed sequentially, and dried to powder by rotary evaporation. The powder was hydrated by lactose buffer and stirred at 65 °C, and the resulting suspension was either extruded or homogenized to reduce liposome particle size. The extrusion was performed by a Lipex extruder at 65 °C and resulted in a narrow size distribution of Amp B liposomes (35:20-8) with an average size of 117.4 nm and PDI of 0.116 (Table 2). To avoid clogging of the extrusion filters, diluted Amp B-lipid suspensions were used for extrusion at an Amp B concentration of 0.75 mg/mL, lower than the concentration of AmBisome® of 4.0 mg/mL. Thus, the final Amp B concentration in sample 35:20-8 was measured to be 0.74 mg/mL, and this formulation was diluted again for drug release and particle size analyses. Another two formulations were homogenized using a Microfluidics 110-PS homogenizer at 65 °C at either 20,000 psi (35:41-4) or 10,000 psi (35:48-7). When low pressure homogenization was used, more passes (7 times) through the M-110P Microfluidizer® were needed to reach the target liposome size and resulted in a heterogeneous size distribution with an average of 88.6 nm and the PDI of 0.190 (35:48-7). When liposomes were homogenized at high pressure, four passes were required to reach the target size and liposomes again had heterogeneous size distribution with an average size of 113.7 nm and the PDI of 0.26 (35:41-4).

The differences in liposome particle size distribution resulted in measurable differences in USP-4 Amp B release. The heterogeneous liposomes with the smallest particle size prepared by homogenization (35:48-7) exhibited the fastest release rate, while homogeneous liposomes with larger particle size prepared by extrusion exhibited slower Amp B release rate (35:20-8) (Fig. 6). Compared to AmBisome®, all formulations prepared by us released Amp B faster, but the f2 value calculated to assess the similarity of release profiles showed high similarity between extruded liposomes 35:20-8 and AmBisome® with an f2 of 70.8. In contrast, the Amp B liposomes prepared by homogenization had f2 values below 50 and were considered to be different from AmBisome®. In addition to the manufacturing process-influenced differences in liposome particle sizes, the existence of potential differences in the final ratios of formulation components could not be ruled out. Unlike Amp B concentration, the lipid component concentrations were not measured in the final products, somewhat limiting our drug release results interpretation. However, this data suggests that the USP-4 IVR assay is sensitive enough to discriminate Amp B release from liposomal formulations prepared by different processes.

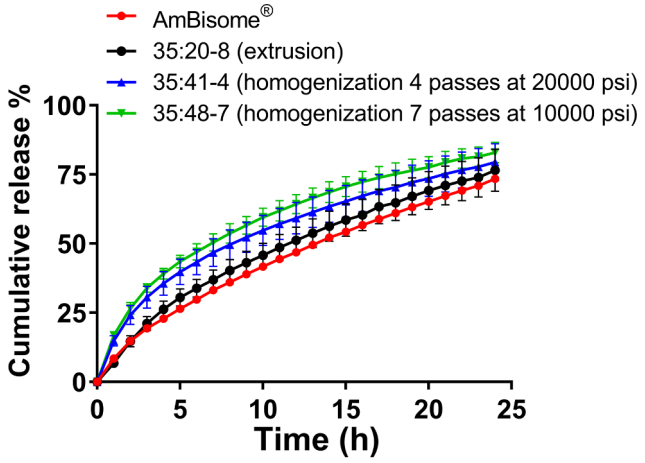

Fig. 6. The cumulative release of different liposomal Amp B formulations prepared by extrusion and homogenization from Z1P on Sotax® at 55 °C. 5% γ-CD was added into media, and total Amp B concentration is 10 μg/mL for all the groups.

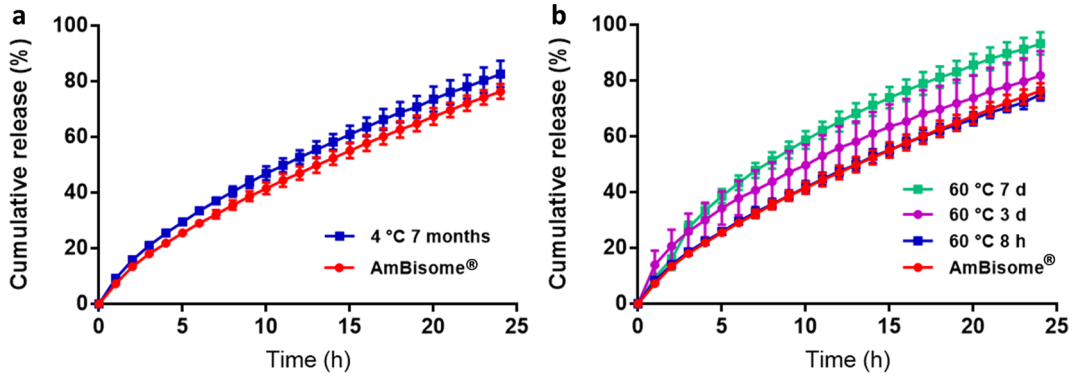

Fig. 7. The cumulative drug release from freshly hydrated AmBisome®, AmBisome® hydrated and stored at 2–8 °C for 7 months (a) and AmBisome® hydrated and incubated at 60 °C for the periods of 8 h, 3 and 7 days (b). The IVR test was performed on Sotax® at 55 °C. 5% γ-CD was added into media, and total Amp B concentration is 10 μg/mL for all the groups.

3.6. The effects of forced degradation of AmBisome® on Amp B release measured by USP-4 IVR assay

To evaluate if our IVR assay yields stability information, hydrated AmBisome® sample was stored refrigerated for 7 months and examined by the assay. Drug release was slightly faster from the stored product, but the difference was not statistically significant with the calculated f2 value of 63.4 (Fig. 7a). Additionally, hydrated AmBisome® samples were subjected to forced degradation at 60 °C for 8 h, 3 d, and 7 d. Amp B release from temperature-stressed AmBisome® samples was compared with drug release from freshly hydrated liposomes. The drug release was more rapid from samples incubated for 3 and 7 d relative to AmBisome® with calculated f2 values of 55.5 and 39.5, respectively (Fig. 7A). However, drug release from AmBisome® stressed for 8 h was similar to release from unstressed product with an f2 value of 94.5, indicating high stability of the AmBisome® product. Taken together, our IVR assay appears to be stability indicating, though mildly stressed AmBisome® samples do not exhibit statistically significant differences relative to unstressed AmBisome® as f2 values were often over 50. The lack of statistical significance could be due to the high stability of AmBisome® and the inability of the established IVR to detect small product differences.

In addition, to examine the ability of IVR assay to distinguish between drug release from disrupted liposomes, we have added 10% of SDS to AmBisome® samples resulting in conversion of turbid liposomal samples to clear solution of lipid-SDS-AmpB micelles with particles size of ~100 nm (Supplemental Fig. 2). AmBisome® was subsequently mixed with SDS disrupted liposomes at 100:0, 90:10, 80:20, 50:50 and 0:100 ratios. Drug release from disrupted liposomes and 50:50 mixtures was very fast, but Amp B release from 80:20, 90:10 mixtures was only slightly faster that from intact AmBisome®. The lack of difference could be due to formation of lipid-SDS-Amp B micelles that also exhibit slow release of Amp B. Overall, the results of stress study and SDS addition studies highlight some shortcoming in the discriminative power of the developed IVR assay as well as overall difficulty in development of discriminative IVR assays foe liposomal products.

3.7. Comparison of AmBisome® and generic liposome Amp B products approved in India by USP-4 IVR assay

To further examine the utility of the USP-4 IVR assay, we compared Amp B release from AmBisome® and Indian approved generic versions of liposomal Amp B. While there is no generic liposomal Amp B currently approved by the US FDA, there are multiple products approved around the world. Several years ago, multiple generic versions of AmBisome® were approved in India, some with identical compositions and others with slightly different compositions [15–17]. In 2016, Indian Regulatory Authorities met to discuss safety concerns associated with the approved liposomal Amp B products and issued recommendations for additional 14-day toxicology and single low dose pharmacokinetic evaluation in healthy subjects [36]. To the best of our knowledge, this new human pharmacokinetics comparability requirement was met only for Amphonex® manufactured by Bharat Serums and Vaccines Ltd. and Phosome®, manufactured by Cipla Ltd. Thus, these two products remained available on the Indian market, and samples of both products were procured for this study. The compositions of Amphonex® and Phosome® are both identical to AmBisome® according to package inserts [37–39]. We analyzed the particle size distribution and drug concentration in all products and observed that both products had average particle sizes and PDIs, 100.0/0.118 (Phosome®) and 95.3/0.089 (Amphonex®), both close to that of AmBisome® (102.0/0.167) (Table 2). The Amp B concentrations in hydrated liposomal solutions were 3.41 mg/mL for Phosome® and 3.82 mg/mL for Amphonex®, and the label concentration for both products was the same as for AmBisome®, 4 mg/mL. While there were some measurable differences between liposomal Amp B products, only a single vial or single batch was analyzed per product due to limited availability. The release of Amp B from AmBisome®, Phosome®, and Amphonex® using the USP-4 IVR assay indicated similarity between all products. Both generic products showed similarity to AmBisome® with f2 values greater than 50; however, drug release from both products was slightly slower relative to AmBisome® (Fig. 8). The calculated f2 values showed slightly higher similarity between Amphonex® and AmBisome® (f2 = 66.3) than

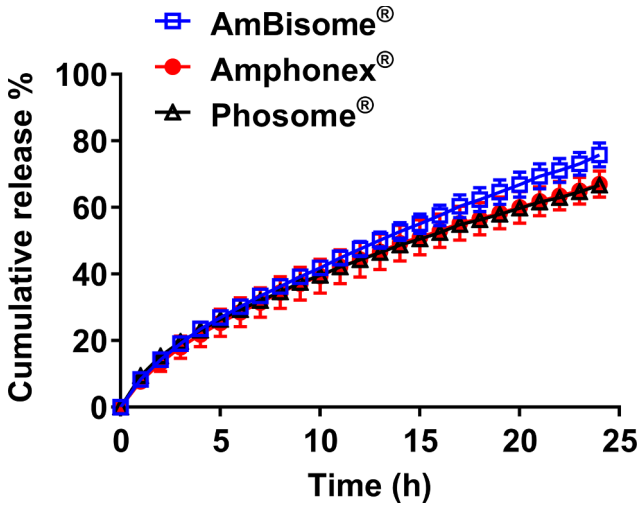

Fig. 8. The cumulative release of different generic products on Sotax® at 55 °C. 5% γ-CD was added into media, and total Amp B concentration is 10 μg/mL for all the groups.

between Phosome® and AmBisome® (f2 = 55.4). Importantly, these values are of limited statistical power as only one lot was analyzed for each product, and understanding lot-to-lot variability is critical for the assessment of generic product similarity to the innovator drug. In addition, it would be of great value to show that the USP-4 IVR assay is capable of discriminating between AmBisome® and a generic Amp B liposomal product that was removed from the market by Indian regulatory authorities like Anfogen® (Genpharma) or Lambin® (Sun Pharmaceutical Industries). Yet, we were not able to obtain these samples and only show that the IVR assay discriminates between AmBisome® and fast drug releasing Amp B liposomes prepared by high-pressure homogenization. Taken together, the USP-4 IVR assay is a useful tool to assess similarities and differences in Amp B release between AmBisome® and its generic versions.

While the established IVR assay could be a useful tool for quality control testing of liposomal Amp B formulations, the assay is not designed to mimic drug release *in vivo*. Moreover, we cannot claim that more rapid drug release in the IVR assay will correlate with more rapid drug release *in vivo* and higher toxicity of the product. No *in vitro-in vivo* correlation (IVIVC) between product performance in the IVR and drug release *in vivo* has been established. It would be very helpful to design Q1/Q2 identical formulations of liposomal Amp B that have various drug release rates *in vitro* or obtain commercial formulations with slightly different drug release profiles [40]. By examining pharmacokinetics of these formulations and correlating *in vivo* performance with the *in vitro* drug release, an IVIVC could be established. We will attempt to establish IVIVC in subsequent studies pending sourcing of fast- and slow-releasing formulations and obtaining reliable pharmacokinetic data from these formulations.

4. Conclusions

The USP-4 apparatus IVR assay was established for evaluation of liposomal Amp B formulations. While the conditions of the IVR assay (temperature of 55 °C and 5% w/v of γ-CD acceptor in release media) were far from physiological conditions, they were optimized to facilitate drug release within 24 h without disrupting liposome structure. Under these IVR assay conditions, the drug release was accelerated, and initial Amp B burst release differences between formulations were amplified. This IVR assay was successfully used to discriminate between AmBisome® liposomes and sodium deoxycholate-containing micellar formulations of Amp B like Fungizone® and Fungcosome. The IVR assay was capable of distinguishing between AmBisome® and liposomal Amp B formulations prepared in-house using different methodologies. The relative sameness between drug release profiles of two different generic Amp B liposomes and AmBisome® was evaluated, but analysis of additional lots will be necessary before making a conclusion regarding the quality of these Indian generic products. In summary, we developed a USP-4 IVR assay that is useful for the generic industry in rapidly assessing drug release from AmBisome® and similar liposomal Amp B products being developed. In addition, the principles applied during IVR assay development could be applied by others to set up USP-4 based IVR assays for other complex products such as liposomes, nanoparticles, microspheres, gels, or suspensions.

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Disclaimer

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