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Research Article

Development of a Flow-Through USP-4 Apparatus Drug Release Assay to Evaluate Doxorubicin Liposomes

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Abstract. Doxil® is a complex parenteral doxorubicin (DOX) liposome formulation approved by the FDA. For generic doxorubicin liposomes, analyzing the release profile of DOX is important for quality control and comparability studies. However, there is no robust standard drug release assay available for doxorubicin liposomes. In this study, we describe a USP-4 apparatus assay capable of discriminating DOX liposomal formulations based on release profile. Establishment of the assay was hindered by limited DOX release from liposomes in physiological conditions at 37°C. The addition of NH_4HCO_3 to the release media facilitated DOX release proportionally to the salt concentration added but caused precipitation of released drug in USP-4 apparatus. Precipitation of DOX was avoided by adding hydroxypropyl-cyclodextrin (HP-CD) to the release medium. We optimized conditions for DOX release by varying a number of parameters such as: concentration of HP-CD, testing temperature, and concentration of tested samples. The optimized release medium contained: 100 mM NH_4HCO_3, 75 mM 2-(N-morpholino) ethanesulfonic acid (MES) and 5% *w/v* HP-CD, 5% *w/v* sucrose, 0.02% *w/v* NaN_3 (pH 6). The drug release assay was performed at 45°C. The optimized release assay can discriminate between DOX liposomal formulations of different compositions, physicochemical properties, and prepared by different manufacturing methods. This indicates that the assay could be used to compare DOX release from generic DOX formulations to the innovator product Doxil®.

KEY WORDS: Doxil®; doxorubicin liposomes; release assay; USP-4 apparatus.

INTRODUCTION

Doxil® is a doxorubicin liposome formulation initially approved by the FDA in 1995 and is used for the treatment of different malignancies, such as AIDS-related Kaposi's sarcoma, recurrent ovarian cancer, metastatic breast cancer, and multiple myeloma (1). Doxil® is sold by Johnson and Johnson (J&J) and lost patent protection in 2010 (2). Doxil® manufacturing is highly complex and involves multiple steps including formation of multi-lamellar vehicles, extrusion to form unilamellar liposomes, remote loading of the doxorubicin into liposomes, buffer exchange/purification and sterile filtration and vialing (3). Due to product complexity, development of generic versions of the product has been limited. In 2013, the first generic doxorubicin HCl liposome injection, produced by Sun Pharma (India) referencing Doxil®, was imported approved by the US FDA (4). A number of analytical tests are recommended to assess the physicochemical equivalence of Doxil® and its generic version. The FDA product-specific recommendations for generic development of doxorubicin liposome injection emphasizes *in vitro* leakage testing "to support a lack of uncontrolled leakage

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under a range of physiological conditions and equivalent drug delivery to the tumor cells" (5). Unlike conventional dosage forms, for which standard pharmacopoeial release tests are available, there is no compendial release assay available for complex liposomal products, such as Doxil®, making the inter-laboratory comparisons and generic liposome development difficult. Therefore, there is a great need to develop a standard release assay for testing drug leakage from complex liposomal formulations like Doxil®.

A USP-4 apparatus has been recently used for the release studies of microparticle and liposomal formulations with encouraging results (6–9). Thus, our objective is to develop a USP-4 release assay for doxorubicin liposomes. Compared to conventional release assays, such as "dialysis sac" and "sample and separate" methods (10–14), the advantages of the USP-4 release assay for doxorubicin liposomes include the following: (a) the use of a standard USP-4 dissolution apparatus; (b) the ability to easily adjust different parameters of the release assay, such as temperature, flow rate, and detection wavelength; and (c) the continuous and automatic detection of doxorubicin release by the online UV detector without sampling. Collectively, these advantages contribute to the establishment of a robust release assay that is less affected by operational error and could be easily transferred among manufacturers of liposomal doxorubicin products.

In the current study, we developed a USP-4 release assay for doxorubicin liposomes capable of discriminating between formulations of different liposomal compositions as well as those of the same composition but prepared by different processes. The established USP-4 release assay assesses doxorubicin leakage under accelerated conditions when over 70% of drug is released within 24 h. The assay was established by optimizing the sample-to-release media ratio, temperature, and composition such as buffer components, addition of ammonia ions, and addition of cyclodextrin acceptors for doxorubicin. This assay could be useful to assess lot-to-lot product variability, test for consistent product quality after manufacturing process or facility changes, and provide useful feedback for generic product design. It could be used in the future to potentially establish a correlation between *in vitro* release behaviors of these formulations with their *in vivo* pharmacokinetics.

MATERIALS AND METHODS

Chemicals and Reagents

Doxorubicin hydrochloride was purchased from SHJNJ Pharmatech (Shanghai, China) and LC Laboratories (Woburn, MA). The lipid components, hydrogenated soybean phosphatidylcholine (HSPC); 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC); and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] ammonium salt (DSPE-PEG2000) were purchased form Lipoid (Newark, NJ), and cholesterol was purchased from Avanti Polar Lipids (Alabaster, AL). Doxil® was purchased from the University of Michigan Hospital Pharmacy. β-cyclodextrin (β-CD) and hydroxypropyl-cyclodextrin (HP-CD) were gifts from Roquette Pharma. In addition, HP-CD, β-CD, and γ-CD was purchased from SHJNJ Pharmatech (Shanghai, China). NH_4HCO_3 (Catalog no. A6141) was purchased from Sigma. Float-a-lyzer® dialysis tubes with a 10–300 kDa molecular weight cut–off (MWCO) were purchased from Spectrum Laboratories (Rancho Dominguez, CA). Sucrose was purchased from Fluka. All other reagents were of analytical grade and purchased from Sigma. To assure the accuracy of the quantitative analysis and release assay, all buffers used in this study were freshly prepared immediately before use.

METHODS

Preparation of DOX-Loaded Liposomes

A lipid solution containing HSPC, cholesterol, and DSPE-PEG2000 at a weight ratio of 3:1:1 (Table I) was prepared by dissolving these components in 1 mL ethanol (EtOH). For the POPC-liposome preparation, HSPC was substituted by equivalent weight amount of POPC (Table I). The mixture was heated to 65°C until all the solids were completely dissolved. Multi-lamellar liposomes (MLVs) were formed through the addition of the lipid to the ammonium sulfate solution (lipid addition method). Specifically, the lipid/

Table I. Abbreviations and Characteristics of Formulations Used in the Release Study

Formulation	Formulation description	Size nm	PDI	Lysolipid % of HSPC	DOX mg/mL	Phospholipid mg/mL	Cholesterol mg/mL	Total lipids mg/mL
L-DOXp	Replicate of Doxil® except MLV prepared by *pouring lipids*	91.0	0.042	ND	2	12.65	3.06	15.71
L-DOXi	Replicate of Doxil® except MLV prepared by *high pressure injection of lipids*	86.2	0.081	<0.2	2	12.26	3.43	15.69
H-DOX	Replicate of Doxil® except homogenized, 2 passes	79.4	0.274	ND	2	13.22[a]	3.31	16.52[a]
POPC L-DOX	Replicate of Doxil® except HSPC is replaced with POPC	84.2	0.067	ND	2	13.10	3.31	16.40
Doxil®	Doxil® obtained at the pharmacy (lot# 1211158)	87.0	0.102	<0.2	2	12.77	3.27	16.00

PDI polydispersity index, *DOX d*oxorubicin, *HSPC* hydrogenated soybean phosphatidylcholine, *ND* no difference, *POPC* 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine

[a] Phosphate assay was not performed on H-DOX. Total lipids concentration was calculated using the cholesterol analysis and ratio of PL/cholesterol used when formulating the liposomes

EtOH solution was *poured* into a stirring 0.25 M ammonium sulfate solution (L-DOXp) at 65°C or was *injected* under high pressure into a stirring 0.25 M ammonium sulfate solution (L-DOXi) at 65°C and stirred for 10 min at the same temperature before extrusion. The MLVs were subsequently processed in an identical manner to make L-DOX. Briefly, the Lipex extruder (Northern Lipids, Burnaby, BC, CA) was heated to 65°C and rinsed with 0.25 M ammonium sulfate solution before the addition of the MLVs. After the MLVs were heated in the extruder for 1 min, the pressure was increased until a constant flow was maintained from the outlet tube. The extrusion was repeated for approximately six passes until the mean liposome diameter was between 85 and 90 nm and polydispersity (PDI) less than 0.1 as measured by dynamic light scattering (DLS, ZetaSizer 3000HSa) at room temperature. H-DOX was prepared by forming MLVs in the same manner as L-DOXp, followed by high-pressure homogenization using a microfluidizer (Microfluidics M-110P, Microfluidics, Westwood, MA), specifically two passes at 10,000–15,000 psi. Liposomes were separated from the external ammonium sulfate by dialysis (4°C) against 5 mM HEPES, 10% *w/v* sucrose pH 6.5.

To load doxorubicin (DOX) into liposomes, doxorubicin hydrochloride (DOX•HCl) powder was dissolved in 5 mM HEPES, 10% *w/v* sucrose, pH 6.5 at a concentration of 10 mg DOX•HCl/mL and then added to the liposomes at a ratio of 0.125 g DOX/g lipid. The suspension was mixed and heated to 60°C for 1 h, followed by cooling in ice water for at least 15 min. Unloaded doxorubicin (DOX) was separated from L-DOX by dialysis (4°C) against histidine 1.55 g/L, sucrose 94 g/L pH 6.5. The resulting liposome formulation was filtered through a 0.2-μm syringe filter (polyethersulfone), diluted to 2.0 mg DOX•HCl/mL and stored at 4°C until use.

Analysis of Doxorubicin-Loaded Liposomes

DOX final concentrations in various liposomal formulations were measured by HPLC. For HPLC sample preparation, 10 μL prepared doxorubicin liposome was diluted with 990 μL methanol in HPLC vial. The mixture was vortexed to disrupt the liposome and dissolve liposomal components. HPLC was performed with an isocratic pump (Agilent 1100) and UV-visible detector. A Zorbax eclipse XDB-C8 column (4.6 Å~150 mm, i.d., 5-μm particle size) equipped with Phenomenex holder (KJO-4282) and cartridges C-8 (AJO-4290) was used for separation. DOX was detected at 254 nm. The mobile phase consisted of 0.1% *v/v* trifluoroacetic acid (TFA) in water (*A*) and 0.1% *v/v* TFA in methanol (*B*). The mobile phase was delivered at a flow rate of 1.0 mL/min using a linear 40% to 100% *B* over 12 min. The column temperature was 30°C; and the injection volume of 10 μL.

Analyses of cholesterol, phospholipids, and lyso-PC concentrations were performed by HPLC with an isocratic pump (Agilent 1100) and UV-visible detector and evaporative light scattering detector (ELSD). A Zorbax eclipse XDB-C8 column (4.6 × 150 mm, i.d., 5-μm particle sizes) was used and equipped with Phenomenex holder (KJO-4282) and cartridges C-8 (AJO-4290). Cholesterol was detected at 205 nm by the UV–Vis detector. Phospholipids were analyzed by ELSD which was set to the following conditions: evaporator temperature of 50°C, nebulizer temperature at 88°C, nitrogen gas flow rate of 1.0 L/min, photomultiplier tube at 10, and smoothing at 5. The HPLC pump program consisted of the following conditions: the mobile phase consisted of 0.1% TFA in water (*A*) and 0.1% TFA in methanol (*B*) with isocratic elution at 91% *B* and flow rate of 1.0 mL/min; injection volume = 20 μL, column temperature = 50°C; run time = 30 min. The lyso-PC content is reported as mol% of HSPC with a limit of quantitation of 0.2 mol% of HSPC. HSPC and PEG-DSPE content in the liposomes was analyzed using a standard phosphate assay (15).

Average particle size and polydispersity (PDI) was determined at room temperature by dynamic light scattering using Malvern Instruments ZetaSizer 3000HSa (Westborough, MA). Z-averages are reported in Table I.

Selection of Dialysis Device Membranes

The effect of the dialysis tube on free doxorubicin release was examined using Float-a-lyzer® with 8–300 kDa molecular weight cut–off (MWCO) on the USP-4 apparatus CE7-smart (SOTAX®). Briefly, 1.6 mL of 2 mg/mL free doxorubicin stock solution (in 10% *w/v* sucrose and 10 mM hisitidine/HCl, pH 6.5) were placed in dialysis tubes and inserted into USP-4 flow-through cells using 78.4 mL of 10 mM histidine-hydrocloride (His/HCl), 5% *w/v* sucrose and 0.02% *w/v* NaN_3 (pH 6.5) as a release medium (the total volume of release media was 80 mL, and the final doxorubicin concentration (C_{DOX}) in the release media was equivalent to 40 μg/mL). The flow rate and running temperature of the release medium were set at 16 mL/min and 37°C, respectively. The release was determined in triplicate and the results were reported as the mean ± SEM.

Establishment of USP-4 Apparatus Release Assay for DOX Liposomes

Doxorubicin (DOX) release was examined using the USP-4 apparatus CE7-smart (SOTAX®). Briefly, 1.6 mL DOX containing liposome samples (0.5, 1, and 2 mg/mL concentration of L-DOX) were placed in Float-a-lyzer® and then inserted into USP-4 flow-through cells. The 78.4 mL release medium was used for each cell. The medium was perfused in a closed-loop setting at 16 mL/min.

The effects of release media temperature, DOX concentration, and release media composition on the performance of release assay were examined. The release study was performed at 37°C, 45°C, and 55°C during assay establishment phase and a temperature of 45°C was used to select other assay parameters. The release assay was performed at 10, 20, and 40 μg/mL final concentration of DOX at the total release media which corresponds to adding 1.6 mL of 0.5, 1, and 2 mg/mL solution of DOX liposomes to the dialysis tubes. The effects of addition of HP-CD (1.5%, 5%, and 20% *w/v*); β-CD (1.5% *w/v*); and γ-CD (1.5% and 5% *w/v*) to the release media on DOX release were examined. The NH_4^+ concentration in release media was varied at 0, 25, 50, and 100 mM. Unless otherwise noted, release media contained 75 mM of 2-(N-morpholino) ethanesulfonic acid (MES) to control pH and 0.02% *w/v* NaN_3 as a preservative. The initial pH of the release media was 6, as pH 6 was found to induce ~50% cumulative release of DOX, which is high enough to

distinguish different formulations based on the pilot study (supplementary Fig. 1). The release media were freshly prepared prior to release experiments.

During the method development stage, an equal amount of free doxorubicin solution was placed in the release medium directly as a control to mimic the complete release of doxorubicin from liposome formulations and to monitor any changes in UV absorption of released doxorubicin over the release period. The cumulative percent of release (cumulative release%) of doxorubicin from the liposome formulations at different time points was calculated using the following equation:

$$\text{Cumulative percent DOX release} = \frac{C_{DOX} \text{ of liposome formulations}}{C_{DOX} \text{ of free doxorubicin control}} \times 100\%,$$

where C_{DOX} of liposome formulations is the detected concentration of doxorubicin released from liposomes in the release medium at a certain time point, and C_{DOX} of free doxorubicin control is the detected concentration of free doxorubicin at the same time point. The release was determined in triplicate for all conditions and the results were reported as the mean ± SEM.

Conventional Release Assay

The conventional release assay was used to investigate DOX release in the absence of Teflon flow-through cell caps and tubings on SOTAX®. Briefly, 0.8 mL liposome formulations ($C_{DOX} = 2$ mg/mL) were placed in 300 kDa Float-a-lyzers and subsequently inserted into 50-mL centrifuge tubes containing 39.2 mL release media. Total release media volume was 40 mL, and final C_{DOX} in total media was 40 μg/mL. The centrifuge tubes were placed on an orbital shaker operating at 320 rpm speed and incubated at 37°C. An equal amount of free doxorubicin solution was used as a control. At predetermined time points, 150 μL of release media sample was drawn for UV detection at 480 nm on a plate reader (Synergy NEO HTS Multi-Mode Microplate Reader (Bio-Tek)) and replaced with an equal amount of fresh release media. The cumulative percent DOX release was calculated using the earlier equation.

Limited Qualification of Final Assay and Analysis of DOX Release for Various Liposomal Formulations

The final optimized USP-4 release assay condition required as follows: (1) UV detection at 480 nm; (2) Float-a-lyzer® dialysis tubes with 300 kDa cut–off; (3) In each assay, 0.4 mL of 2 mg/mL DOX liposomal formulation was mixed with 1.2 mL of release medium to obtain a total of 1.6 mL of diluted liposome solution (0.5 mg/mL) in the dialysis tube; (4) 78.4 mL of external release media (10 ug/mL DOX in total media concentration); (5) release media composed of 5% *w/v* HP-CD, 100 mM NH_4HCO_3, 75 mM MES, 0.02% NaN_3, and 5% *w/v* sucrose (pH 6) abbreviated as 5% HP-CD ammonium bicarbonate, MES, NaN_3, Sucrose (AMNS)) media; (6) flow rate of 16 mL/min; and (7) 45°C temperature.

Various liposomal DOX formulations were analyzed in triplicate and average cumulative release and SEM were plotted. The similarity or differences between DOX release profiles from various formulations was assessed by *f*2 test using the following equation (16, 17):

$$f2 = 50 \log \left[\frac{100}{\sqrt{1 + \frac{\sum_{i=1}^{n} (R_t - T_t)^2}{n}}} \right],$$

where n is the number of time points, R_t is the cumulative release value of the reference batch at time t ($t > 0$), and T_t is the cumulative release value of the test batch at time t ($t > 0$). The cumulative release values for all time points ($n = 24$) were used for the calculation of *f*2. A test formulation with a similarity factor $f2 \geq 50$ is considered similar to the reference formulation (18).

To determine the day-to-day variability, the same sample was analyzed on three different days; three repeats were performed on each day. The average values, SEM, and variance for 7- and 24-h cumulative DOX release are reported. The *f*2 was calculated and reported as well.

RESULTS

Preparation and Characterization of Doxorubicin Liposomes

Liposomal formulations of identical composition to Doxil® were prepared and characterized for use in USP-4 drug release assay development. The differences in compositions, the processes used to prepare liposomes and their analytical characteristics are summarized in Table I. The liposome average size and polydispersity index (PDI) were measured by DLS and z – averages are reported in Table I. The DOX, lyso-PC, and cholesterol contents of the liposomes were determined by different HPLC methods. The phospholipid content or the sum of HSPC and DSPE-PEG2000 were measured by a standard phosphate assay. The total lipid is an arithmetic sum of phospholipid and cholesterol contents (Table I).

There is a difference in how MLVs are prepared for L-DOXp and L-DOXi. The lipids solution in ethanol is poured into stirring aqueous ammonium sulfate solution for L-DOXp and injected under high pressure for L-DOXi. While the average size and polydispersity are very similar for L-DOXi and L-DOXp, L-DOXp releases DOX more slowly, as discussed later in the manuscript. In order to further investigate effects of the manufacturing process on DOX release, we prepared liposomes by homogenization. The resulting liposomes had a slightly smaller average size and higher PDI when compared to liposomes prepared by extrusion. The liposomes prepared by homogenization released DOX faster relative to extrusion-made liposomes. To investigate if the USP-4 assay could distinguish between slow and rapidly releasing formulations, we prepared liposomes by substitution of HSPC by the same amount of POPC. POPC has lower melting temperature of −2°C (19) relative to HSPC 53°C (20), and thus is expected to release DOX more rapidly.

Although substitution of HSPC to POPC slightly altered the molar ratios of lipids to DOX, the weight ratios were kept the same.

Selection of Dialysis Device Membranes

The dialysis bag/tube serves as a barrier that should enable the rapid diffusion of released doxorubicin into the release medium while retaining all doxorubicin still encapsulated in liposomes. To find a specific dialysis bag/tube membrane that can meet this requirement, membranes with different MWCO and materials were tested by directly adding a solution of free doxorubicin into the dialysis bags to mimic the condition in which all drug molecules have been released from liposomes.

The results showed that Float-a-lyzer® CE membranes with 100–300 kDa MWCO led to a fast doxorubicin release, with almost 100% DOX release within 7 h at 37°C, while only 33.9% ± 2.2% cumulative release% was observed within 24 h for membranes with 8–10 kDa MWCO (see Fig. 1). There was no significant difference in DOX transport rate for 100 and 300 kDa MWCO membranes, thus membranes with 300 kDa MWCO membranes were used for all subsequent studies. The pore sizes of membranes with 100–300 kDa MWCO ranged from 5–8 nm—much smaller than the size of our liposomes. Thus, doxorubicin still encapsulated in liposomes should not cross the membrane. In addition, after 24 h of release, no liposomes were detected in the release media, as evidenced by the absence of phospholipids by HPLC detection.

DOX Release Assay Primary Development

Solving DOX Precipitation in the Release Media

After identifying suitable dialysis device membranes for free doxorubicin, the release of doxorubicin from liposomes was performed on a USP-4 apparatus, SOTAX®. Standard curves were established for free DOX solution in various release media including 10 mM His/HCl and 5% sucrose (pH = 6.5), PBS (pH = 7.4) and 100 mM NH_4HCO_3/5% *w*/*v* sucrose (pH 6.0) in the range of 0–45 μg/mL. DOX was detected by UV 480 nm using the Evolution Array UV-visible Spectrophotometer (Thermo) spectrophotometer and excellent linearity was observed for all media at 0–45 μg/mL concentration range. The preliminary drug release

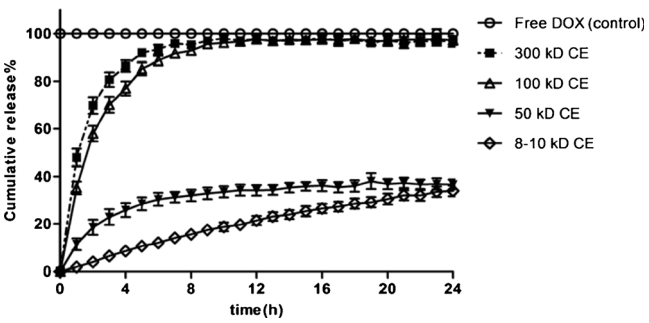

Fig. 1. Effect of dialysis tube molecular weight cut–off on the release profile for free doxorubicin solution in 5% *w*/*v* sucrose/10 mM histidine/HCl (pH 6.5) at 37°C and 40 μg/mL DOX-total media concentration ($n = 3$, mean ± SEM)

experiments were performed at 40 μg/mL total DOX concentration in the media 100 mM NH_4HCO_3 (5% *w*/*v* sucrose, pH 6) (Fig. 2a) or PBS (pH 7.4) (data was not shown). Unexpectedly, precipitation of DOX was observed in SOTAX® in both media. Further studies showed that small red crystals formed on the Teflon surface of SOTAX® which increased in size over time and eventually induced precipitation of the doxorubicin. To explore whether SOTAX®'s Teflon surfaces or limited DOX solubility in the release media was the reason for drug precipitation, the release was performed in polypropylene centrifuge tubes (see "Conventional Release Assay"). In the Teflon-free condition, DOX precipitation also occurred albeit, to a lesser extent. When the Teflon stirring bar was purposely introduced in the centrifuge tubes, the formation of red precipitate on the Teflon surface was observed, even at DOX concentration as low as 3 μg/mL. To avoid precipitation during release in the USP-4 system, we added hydroxypropyl-cyclodextrin (HP-CD) to the release media as a DOX solubility enhancer.

HP-CD can increase DOX solubility in the release media when added at 1.5%, 5%, and 20% (*w*/*v*). The release study was performed using the conventional assay both before and after addition of HP-CD to the release medium. No precipitation of DOX was observed during the 48-h incubation. The addition of a Teflon surface to the conventional assay along with HP-CD in the medium did not cause DOX precipitation (Fig. 2b). The initial release experiments were performed at 37°C in PBS, 10 mM his/HCl buffer, or 100 mM NH_4HCO_3; however, DOX release from liposomes was only observed in NH_4HCO_3 buffer. In addition, the DOX percent release was highly dependent on NH_4HCO_3 solution pH between pH levels 5 and 7 (supplementary Fig. 1). The pH 6 media was found to induce ~50% cumulative release of DOX within 24 h, which is high enough to distinguish different formulations based on the pilot study. However, during the release of DOX at 37°C, the pH of 100 mM NH_4HCO_3/5% *w*/*v* sucrose media increased from 6 to 8, resulting in a decrease in the UV absorption of DOX. This reduced the assay accuracy. Furthermore, bacteria growth was observed in the sucrose containing media. In order to avoid pH increase during release, 75 mM MES buffered to pH 6.0 was added to the release media. After adding 75 mM MES to the release medium, the pH change was very modest: the pH 6 increased to pH 6.2 over 48 h at 37°C and DOX UV absorption remained constant. Addition of preservative, 0.02% *w*/*v* NaN_3, to the release media prevented bacterial growth. Hence, this NH_4HCO_3, MES, and NaN_3 containing media was selected for further evaluation. We have abbreviated this media as 'AMNS' (Ammonium bicarbonate, MES, NaN_3, Sucrose). However, even in presence of 100 mM NH_4HCO_3, the extent of DOX release was limited to about ~7% released within 24 h at 37°C by conventional release assay (Fig. 2b) so additional measures were explored to increase the release rate. The subsequent experiments were performed using SOTAX® USP-4 dissolution system.

Effect of temperature on DOX release on SOTAX®

The release of doxorubicin from L-DOXi was highly dependent on temperature, with 91.0% ± 1.6%, 42.4% ± 0.6%. and 4.5% ± 0.1% of cumulative percent DOX release

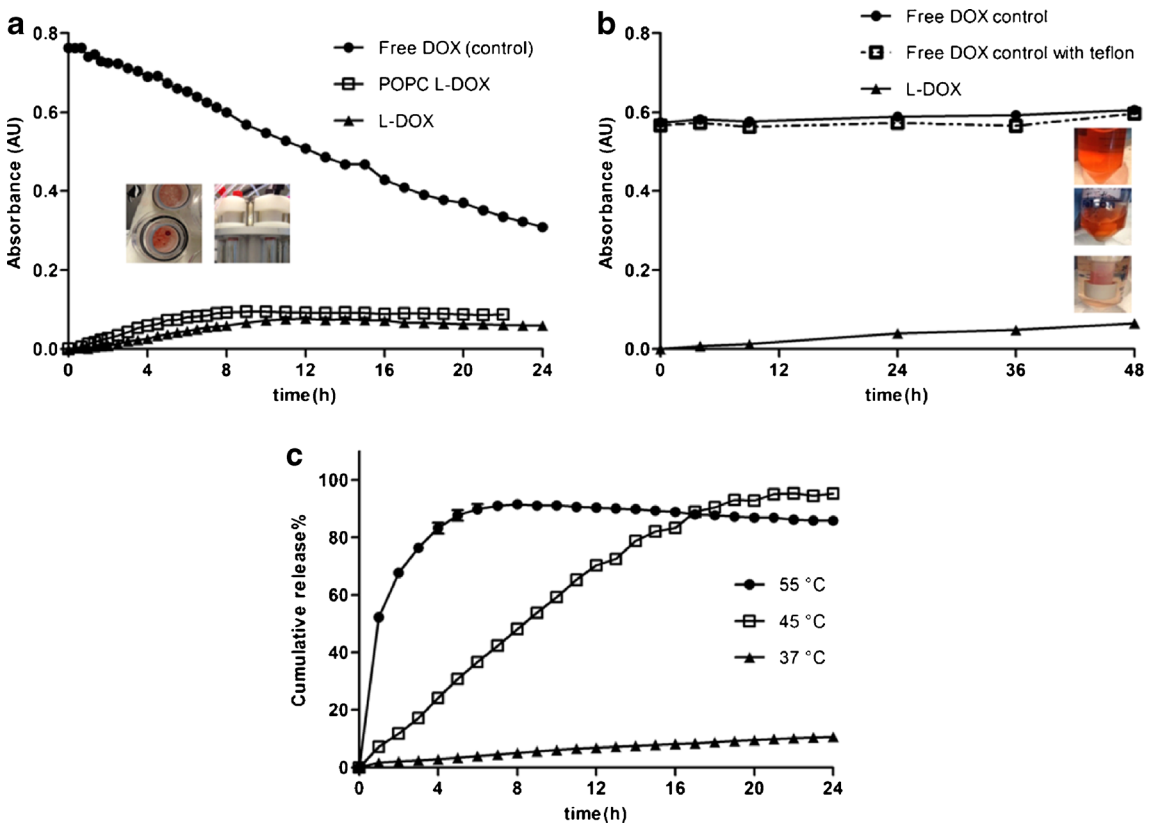

Fig. 2. **a** Precipitation of doxorubicin on Teflon surface of SOTAX® at 37°C in 100 mM NH_4HCO_3 at 40 μg/mL DOX-media concentration. **b** The precipitation avoided by addition of 20% *w/v* HP-CD to AMNS media at 40 μg/mL DOX-media concentration. **c** Effect of temperature on DOX release from L-DOXi on SOTAX® in 5% *w/v* HP-CD AMNS media and 10 μg/mL DOX-media concentration. ($n = 3$, mean ± SEM)

within 7 h at 55°C, 45°C, and 37°C, respectively (Fig. 2c). The gel-liquid crystal transition temperature (Tm) of HSPC, the main component of DOX liposome is 53°C. Not surprisingly, the release increased dramatically as temperature was increased above Tm. The 45°C temperature was selected for subsequent studies since the cumulative percent DOX release from L-DOXi was nearly complete within 24 h (95.3% ± 1.2%) yet the release rate was slow enough allowing to distinguish the difference between formulations.

Optimization of the USP-4 Release Assay

Effect of different concentrations of L-DOX on DOX release. The effect of the volume ratio of L-DOX formulation to the total release media on the rate of DOX release in USP-4 assay was investigated. L-DOX has a standard DOX concentration of 2 mg/mL. L-DOX was diluted to 0.5, 1, and 2 mg/mL DOX concentrations and 1.6 mL of diluted L-DOX was placed in Float-a-lyzer®. The Float-a-lyzer® tube was inserted in SOTAX® cell and 78.4 mL of release media was added into each release line. Hence, 100% of DOX release would result in 10, 20, and 40 μg/mL drug concentration in total release media. The lower the initial L-DOX concentration, the higher the cumulative DOX release: 77.0% ± 2.7%, 60.9% ± 0.6%, and 48.3 ± 0.7% DOX released within 24 h for 10, 20, and 40 μg/mL groups, respectively (Fig. 3a). Since the DOX detection was accurate at the lowest concentration and the risk of DOX precipitation in the SOTAX® is lower at the lowest concentration, 10 μg/mL DOX concentration in total media (0.5 mg/mL DOX concentration in Float-a-lyzer®) was used for all subsequent studies.

Effect of Cyclodextrin Type and Concentration on DOX Release. The effect of HP-CD concentration in the release media on DOX release was investigated. No significant difference in cumulative drug release was observed for 1.5%, 5%, and 20% HP-CD (*w/v*) containing release media ($f2 = 53.1$ for 1.5% and 5% HP-CD, $f2 = 52.7$ for 1.5% and 20% HP-CD, and $f2 = 68.7$ for 5% and 20% HP-CD) (Fig. 3b). While no DOX precipitation was detected in 5% *w/v* and 20% *w/v* HP-CD containing release media, a trace precipitation occurred when 1.5% *w/v* HP-CD was used.

To compare the effects of cyclodextrin type on DOX release 1.5% *w/v* γ-CD, β-CD, or HP-CD was added to the release media containing 100 mM NH_4HCO_3, 75 mM MES, 5% *w/v* sucrose, and 0.02% *w/v* NaN_3. The experiment was performed at 1.5% *w/v* cyclodextrin media concentration due to a limited aqueous solubility of β-CD. The DOX release was faster for γ-CD relative to HP-CD and β-CD (Fig. 3c). Unfortunately, trace precipitation of DOX was observed for all three cyclodextrin types when they were used at only 1.5% *w/v* concentration. In order to circumvent DOX precipitation drug release was compared for media containing 5% *w/v* γ-

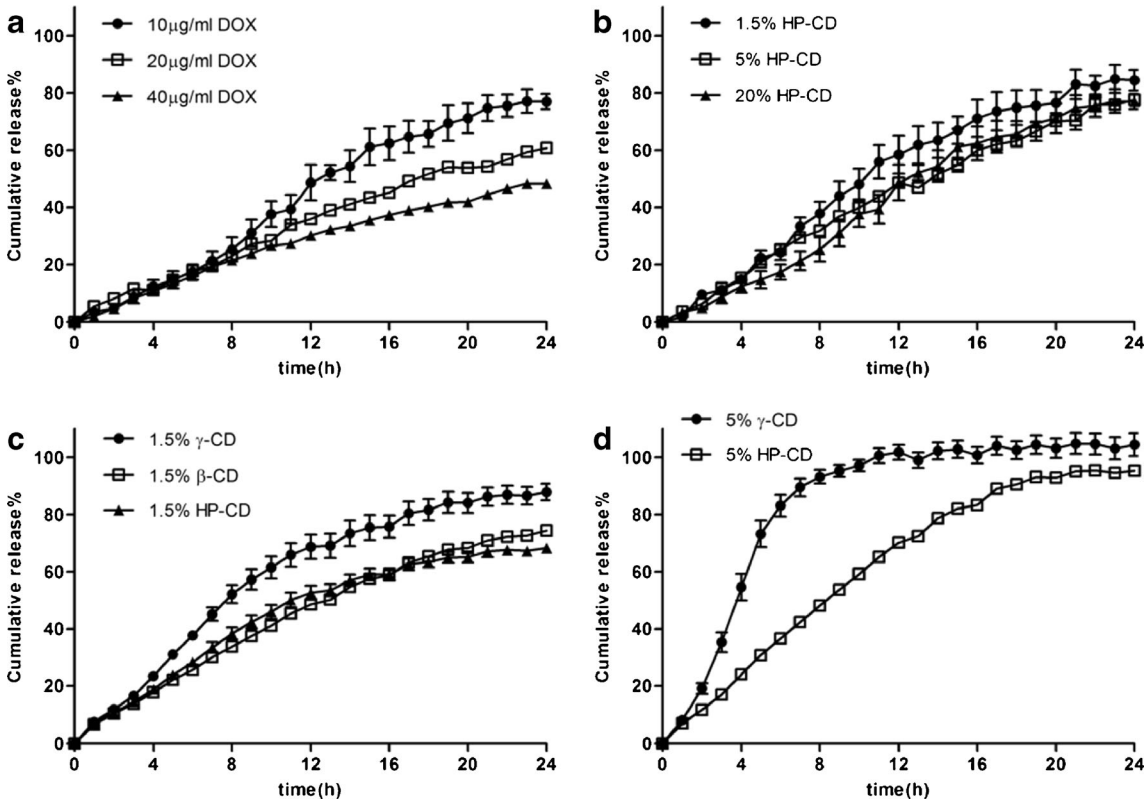

Fig. 3. **a** Effect of DOX concentration on DOX release at 10, 20, and 40 μg/mL of L-DOXp in total 20% *w/v* HP-CD-AMNS release media. **b** Release profile of L-DOXp in release media containing different concentrations (*w/v*) of HP-CD in AMNS media at 10 μg/mL DOX-media concentration. **c** Release profile of L-DOXi in media containing 1.5% *w/v* γ-CD, β-CD, or HP-CD in AMNS media at 10 μg/mL DOX-media concentration. **d** Release profile of L-DOXi in release media containing 5% *w/v* γ-CD or HP-CD in AMNS media at 10 μg/mL DOX-media concentration. All release were performed on SOTAX® at 45°C ($n = 3$, mean ± SEM)

CD and HP-CD. The DOX release in 5% *w/v* γ-CD containing media with 89.5% ± 3.1% cumulative release at 7 h relative to 42.4% ± 0.6% cumulative release for 5% *w/v* HP-CD containing media (Fig. 3d). The DOX release was too rapid for 5% *w/v* γ-CD media potentially decreasing the ability of the assay to discriminate between different L-DOX formulations. In addition, γ-CD is significantly more expensive excipient relative to HP-CD, thus, making the use of it in a release assay impractical. Hence, 5% *w/v* HP-CD was used in the optimized USP-4 release assay.

Effect of NH_4^+ on Doxorubicin Release. The effect of release media's NH_4^+ concentration on DOX release rate was examined in SOTAX®. The release media concentration of NH_4HCO_3 had profound effect on DOX release (Fig. 4). Very limited DOX release was observed without NH_4HCO_3 and the release rate increased with increase in NH_4HCO_3 concentration. Thus, 100 mM NH_4HCO_3 containing medium was used for the subsequent studies.

Based on experimental results, the USP-4 apparatus release assay for DOX liposome was finalized. The optimized release medium composition was 100 mM NH_4HCO_3, 75 mM MES, and 5% *w/v* HP-CD, 5% *w/v* sucrose, and 0.02% *w/v* NaN_3 (pH 6). The release media was circulated 16 mL/min at 45°C and DOX release was detected for 24 h. The release media volume was 80 mL, and the final concentration of DOX in total release media was 10 μg/mL, which corresponds to adding 1.6 mL of 0.5 mg/mL DOX concentration of DOX liposomes to the dialysis tubes (Float-a-lyzer®).

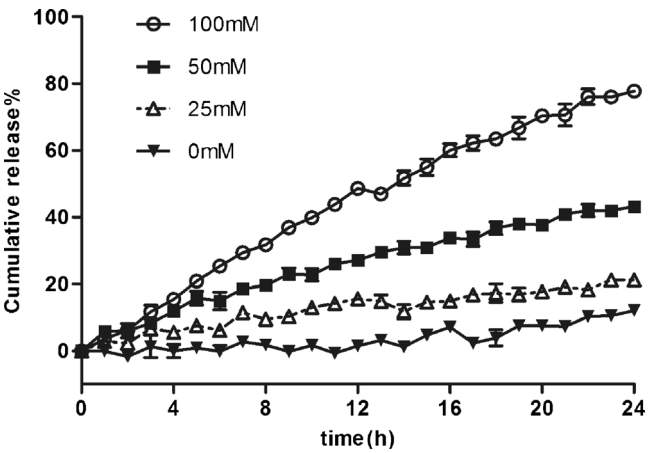

Fig. 4. Release profile of doxorubicin from L-DOXp in different concentrations of NH_4HCO_3. The experiment was performed in 5% *w/v* HP-CD AMNS media at various NH_4HCO_3 concentrations at 45°C and 10 μg/mL DOX-media concentration ($n = 3$, mean ± SEM)

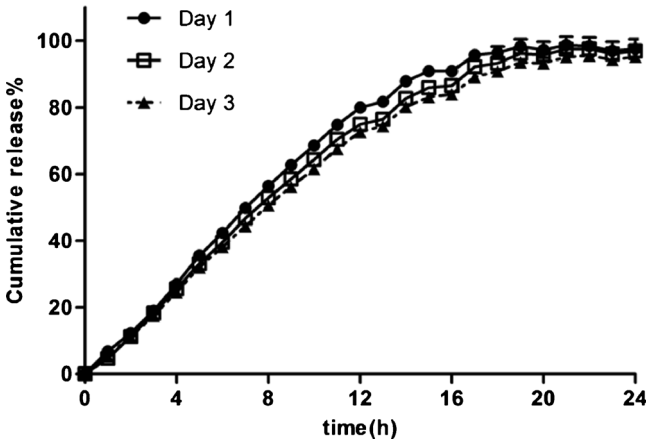

Fig. 5. Limited qualification of the release assay. The release experiments were performed using the same L-DOXi sample by an optimized USP-4 assay on three different days; three repeats were performed on each day

Limited Qualification of the Release Assay. The day-to-day variability was analyzed by running the release assay on three different days using the same L-DOXi formulation by the optimized USP-4 apparatus release assay mentioned earlier. The cumulative release at 7 and 24 h was 47.0% ± 1.6% and 96.5% ± 0.7% indicating limited assay variance. No significant difference in the release profile was observed ($f2 = 73.4$ for days 1 and 2, $f2 = 63.4$ for days 1 and 3, and $f2 = 81.0$ for days 2 and 3) (Fig. 5). The changes in UV intensity of the control DOX solution were evaluated on four different days (supplementary Fig. 2).

The Effect of Liposome Composition and Method of Preparation on DOX Release. The ability of the established USP-4 release assay to distinguish DOX release differences for various liposomal formulations was examined (Fig. 6). Commercially available Doxil® was analyzed as a control. The substitution of HSPC for POPC resulted in rapid and complete DOX release within 10 h (Fig. 6a). All other liposomes had the same compositions as Doxil® but present slightly different physicochemical properties. The differences in DOX release among these formulations were quantitatively assessed by $f2$ calculations (Table II). When the $f2$ value is greater than 50, two formulations are considered to be similar in terms of the release profiles (18). In the multiple comparisons listed in Table II, the comparisons had $f2$ values less than 50 and were considered to be different in their DOX release behavior compared to each other.

The DOX release rate from all formulations prepared by us, were slower than that of commercially available Doxil®. In the prepared liposomes, those made using high-pressure homogenization (H-DOX) exhibited more rapid DOX release compared to L-DOXp with the 24-h cumulative release% of 82.9% ± 0.3% and 77.7% ± 1.3%, respectively (Fig. 6a). H-DOX formulations also appear to have a smaller average size and broader size distribution, 79.4 ± 0.3 nm, relative to L-DOXp with a size of 91.0 ± 0.1 nm. The heterogeneity in size of H-DOX may explain the faster DOX release.

The method of MLV preparation prior to liposome extrusion also had an effect on DOX release. When MLVs were prepared by pouring lipid solution in aqueous ammonium sulfate solution (L-DOXp) the DOX release was slower than when MLV were prepared by high-pressure injection of lipid solution into aqueous solution (L-DOXi). In addition, the presence of cyclodextrin and NH_4HCO_3 in the release media did not cause liposome disintegration characterized by drastic change in particle size. The liposomes remained intact during release and underwent only a limited increase in particle size (Table II).

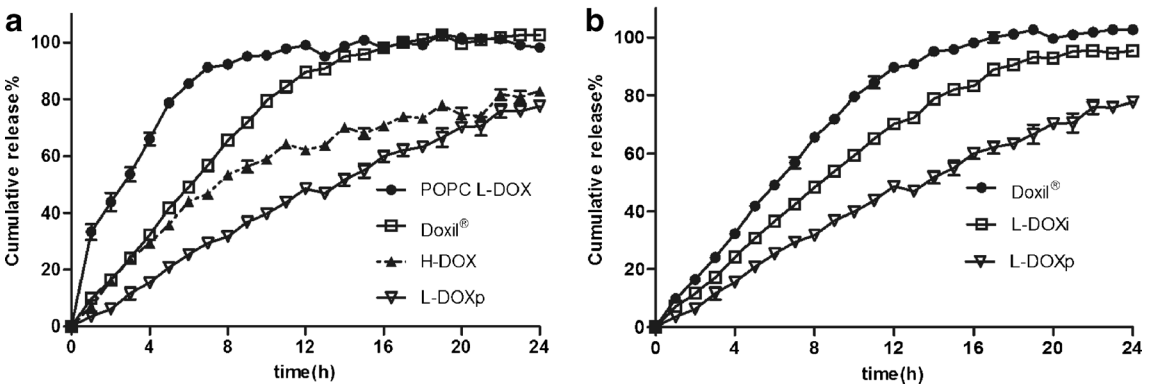

Fig. 6. Release profiles of different formulations under the optimized release condition. **a** Effect of composition and liposome preparation conditions on DOX release. **b** Effect MLV preparation method during liposome manufacturing on DOX release. Doxil® was used as control and release was performed using optimized USP-4 assay conditions. ($n = 3$, mean ± SEM)

Table II. Summary of *f*2 Values for the Release of Different DOX Liposome Formulations and Particle Size Change During Release

*f*2 Release Similarity Comparison						Size (nm)	
DOX liposome	L-DOXp	L-DOXi	H-DOX	POPC L-DOX	Doxil®	Before release	After release
L-DOXp		35.2	43.0	17.3	24.8	91.0	111.7
L-DOXi	35.2		48.1	26.6	44.3	86.2	107.2
H-DOX	43.0	48.1		25.0	34.8	79.4	98.7
POPC L-DOX	17.3	26.6	25.0		35.5	84.2	114.0
Doxil®	24.8	44.3	34.8	35.5		87.0	116.6

DOX doxorubicin, *POPC* 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine

DISCUSSION

The choice of release medium is crucial for the successful development of the USP-4 release assay. Although the low ionic strength medium, 10% *w*/*v* sucrose and 10 mM his/HCl (pH 6.5), could well dissolve free doxorubicin without causing any significant precipitation, this release medium could not induce the release of doxorubicin from DOX liposomes. Similarly, the use of PBS alone did not induce doxorubicin release either. Recently, it was reported that tumor cells can produce ammonia by glutaminolysis and induce doxorubicin release from liposomes (21). Our preliminary study also found the cumulative release of doxorubicin was dramatically increased when NH_4^+ was added to the release medium. Moreover, the DOX release from liposomes was highly dependent on pH and NH_4^+ concentration of the release medium. At the same NH_4^+ concentration of 100 mM a 24-h cumulative percent of DOX release from the liposomes increased from 0% to 80% when the pH of the release medium was increased from 4.5 to 7.0 (supplementary Fig. 1). Similarly, the cumulative percent of DOX release also increased when the NH_4^+ concentration in the release medium was increased from 0 to 100 mM (Fig. 4). This is not surprising because doxorubicin is loaded into the liposomes by the ammonium sulfate gradient method (22), and doxorubicin can form crystals in the presence of sulfate inside liposomes. Doxorubicin, as a free organic base, has high permeability for the lipid bilayers of liposomes, but low permeability when it is protonated and thus trapped inside liposomes (23). Upon pH-dependent decomposition of ammonia salts in the release media, NH_3 is able to penetrate liposome membranes, de-protonate doxorubicin, and facilitate its transport across lipid membrane. The ammonia in the assay release media becomes more membrane permeable when it is neutralized at increasing pH. Therefore, higher pH and higher NH_4^+ concentration can induce more doxorubicin release.

However, when NH_4^+ containing release medium was used for the USP-4 release assay, doxorubicin precipitated along the Teflon flow-through cell caps and tubings. The precipitation was induced by the presence of Teflon even at DOX concentrations as low as 3 μg/mL. To avoid the precipitation of released doxorubicin, HP-CD, which can solubilize guest molecules by encapsulating them in the cavity, was added to the release medium (24). As a result, no precipitation occurred after adding HP-CD. However, use of cyclodextrins in the release media enhanced DOX release by a different mechanism. Cyclodextrins are capable of extracting cholesterol from the liposome membrane and, thus, increase membrane's fluidity and permeability (25). The HPLC analysis of cholesterol content remaining in the liposomes after the completion of 24-h release study as well as analysis of the release media confirmed that 30% of cholesterol leaves the liposomes and is found in the 5% *w*/*v* HP-CD release media. In the plasma similar events could take place as circulating lipoproteins could extract cholesterol from the injected liposomes (25).

After addressing the precipitation problem, the USP-4 release assay was further optimized to allow for a nearly complete DOX release within 24 h in order to increase the assay's discriminative ability. The DOX concentration in release media, release media composition, and temperature effects were evaluated. It is not surprising that higher temperature lead to a faster DOX release since the lipid membrane is more fluid at higher temperature. The fluidity of membrane facilitates ammonium penetration into liposome, diffusion of DOX out of liposome, and cholesterol extraction by cyclodextrin. In addition, at higher temperature, the rate of NH_3 formation rate is higher, effective DOX solubility, diffusion and dissociation constants are higher, resulting in faster transport and reactivity of molecules. However, an excessively high release rate (at 55°C) or an overly low release rate (37°C) may mask any slight differences among different formulations, so a medium release rate (at 45°C), which allows one a better opportunity to distinguish different formulations, was used as the temperature for the release assay.

The main limitation of the USP-4 assay established here is that the assay conditions themselves define the driving force for DOX release from the liposomes. Thus, the assay could be primarily used to determine relative differences between liposomal DOX formulations, rather than define *in vivo* relevant release behaviors. It appears that the presence of NH_4HCO_3 and pH-dependent formation of NH_3 is a critical driving force for dissolution and release of liposomal DOX. There, practically almost no DOX release

was observed at <25 mM NH_4HCO_3 (Fig. 4) even when all other parameters, such as HP-CD and high temperature, remained constant. Faster DOX release with decreased L-DOX concentrations of 10, 20, and 40 μg/mL could also be attributed to a higher NH_4HCO_3 to liposomal DOX ratio and transient pH changes inside the dialysis tube during DOX-sulfate dissolution. While the same amount of NH_3 is available to dissolve liposomal DOX, later on, it dissolves a higher fraction of the total DOX, resulting in a more complete drug release.

There is also a clear influence of temperature on release rate (Fig. 2c). In all the same media containing HP-CD, MES, and 100 mM NH_4HCO_3, almost no DOX release was observed at 37°C, rapid and almost compete drug release reaching plateau in 6 h at 55°C, in contrast to a continuous DOX release at 45°C. At 55°C, the liposome membrane is likely fluid and, thus, DOX could rapidly diffuse through the membrane. Presence of HP-CD in the release media facilitates cholesterol removal from the lipid membrane, reduces effective transition of the membrane, and increase DOX permeability. It is clear that the assay temperature significantly impacts the effective DOX release rate and the final selected USP-4 method parameters are far from physiological conditions. However, the accelerated release conditions have been used by other investigators to set up USP-4 based methods for evaluation of polymer microspheres (26, 27). Extreme release media pH of 2.4, addition of 10% *v*/*v* ethanol and elevated temperatures of 45°C, 50°C, 53°C, and 60°C have been used to discriminate between polymer microsphere formulations in USP-4 assay (26).

Apart from release media composition and temperature, set-up of the USP-4 release apparatus itself influences DOX release. To examine drug release from the liposome, we used a dialysis insert set-up of USP-4. We identified that the dialysis membrane represents a diffusion barrier for DOX from the Float-a-lyzer® into the USP-4 flow-through cells. It is important to note that there is a lag time in release from the highly permeable 300 kD dialysis bag, which delayed our effective observation of release kinetics by roughly 1 h (the time to 50% release in Fig. 1 was about 1 h). Therefore, since release from the liposomal preparation was observed over a much longer time scale (e.g., half time of ~7 h), we are viewing a valuable representation of the release kinetics of drug from the liposomes (Fig. 5). However, use of other less permeable membranes of 50 and 10 kD resulted in significant slow down and incomplete release of DOX solutions from the Float-a-lyzer®. This effect is likely dependent on drug size and hydrophobicity and, thus, could be potentially more profound for poorly soluble, larger molecular weight drugs.

The final conditions selected for the assay are far from physiological release conditions for L-DOX. The primary optimization criteria for USP-4 assay conditions was to see over 80% of drug release from Doxil® within 12 h, in order to be able to discriminate liposomal formulations with faster or slower DOX release behavior within 24 h of assay duration. Indeed, when the optimized release assay was used to determine the release profiles of different L-DOX formulations, it was able to distinguish slow and fast-releasing liposome formulations. For example, POPC L-DOX had a faster release rate than any other formulations, with almost 100% release of doxorubicin within 10 h. This is not surprising because the transition temperature of POPC is much lower than that of other liposome formulations. Phospholipids with lower transition temperature have higher fluidity, which allows for an easier transport of molecules across the lipid bilayer. We also found that formulations of identical compositions but prepared by different method and, thus, having slightly different physico-chemical properties could exhibit very different release *in vitro*. Homogenized liposomes exhibited broader size distribution (Table I) and faster DOX release relative to liposomes made by extrusion. The process of downsizing MLVs by homogenization does not allow for the same size control as extrusion. The greater distribution in liposome diameters results in some liposomes having faster release rates. The MLV preparation methodology appeared to have a large impact on the DOX release rate, as L-DOXi exhibited faster drug release relative to L-DOXp. Perhaps there is a difference in the lamellar arrangement of lipids or ammonium sulfate is some population of the liposomes or presence of small size heterogeneities that are below DLS detection capabilities. This difference will need to be further explained by microscopy analyses of different liposomes.

While no formal assay validation was performed, a limited qualification showed low variability of analysis of the same L-DOX formulation performed on different days (Fig. 5) and low day-to-day variability in signal intensity of DOX solution control (supplementary Fig. 2). However, we found that the rate of DOX release strongly depends on ionic strength of NH_4HCO_3 solution and initial pH of the media. This may present a potential issue with good laboratory practice (GLP) validation of the assay; thus, further optimization of ammonia salt concentration and pH might be needed to increase assay robustness. In addition, using an L-DOX standard formulation as a control in each USP-4 run and presenting the dissolution results in terms of percent difference from the control could be implemented in GLP assay validation.

The design philosophy and approach to the selection of appropriate release conditions employed in our study could be a useful paradigm for the development of assays for other generic complex liposomal formulations.

CONCLUSION

A USP-4 apparatus release assay for DOX liposome was established using a release medium containing 100 mM NH_4HCO_3, 5% *w*/*v* sucrose, 75 mM MES, 0.02% *w*/*v* NaN_3 and 5% *w*/*v* HP-CD at 45°C for 24 h on SOTAX®. This release assay was used to discriminate differences of similar DOX liposomes prepared with different processes. The established USP-4 can be used to distinguish possible differences between generic and innovator DOX liposomes, provide useful feedback on designing better formulations and, potentially, bridge the *in vitro* release behaviors of these formulations with the *in vivo* pharmacokinetics.

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REFERENCES

1. Gabizon AA. Pegylated liposomal doxorubicin: metamorphosis of an old drug into a new form of chemotherapy. Cancer Investig. 2001;19(4):424–36.
2. Barenholz Y. Doxil®—the first FDA-approved nano-drug: lessons learned. J Control Release. 2012;160(2):117–34.
3. Niu G, Cogburn B, Hughes J. Preparation and characterization of doxorubicin liposomes. Methods Mol Biol. 2010;624:211–9.
4. FDA approval of generic version of cancer drug Doxil is expected to help resolve shortage. 2013; Available from: http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm337872.htm.
5. Draft guidance on doxorubicin hydrochloride. 2014; Available from: http://www.fda.gov/downloads/Drugs/…/Guidances/UCM199635.pdf.
6. Bhardwaj U, Burgess DJ. A novel USP apparatus 4 based release testing method for dispersed systems. Int J Pharm. 2010;388(1–2):287–94.
7. Rawat A, Bhardwaj U, Burgess DJ. Comparison of in vitro-in vivo release of Risperdal(®) Consta(®) microspheres. Int J Pharm. 2012;434(1–2):115–21.
8. Rawat A, Burgess DJ. USP apparatus 4 method for in vitro release testing of protein loaded microspheres. Int J Pharm. 2011;409(1–2):178–84.
9. Rawat A, Stipper E, Shah VP, Burgess DJ. Validation of USP apparatus 4 method for microsphere in vitro release testing using Risperdal ® Consta ®. Int J Pharm. 2011;420(2):198–205.
10. Glavas-Dodov M, Goracinova K, Mladenovska K, Fredro-Kumbaradzi E. Release profile of lidocaine HCl from topical liposomal gel formulation. Int J Pharm. 2002;242(1–2):381–4.
11. Ruozi B, Tosi G, Forni F, Angela Vandelli M. Ketorolac tromethamine liposomes: encapsulation and release studies. J Liposome Res. 2005;15(3–4):175–85.
12. Vemuri S, Yu CD, Pushpala S, Roosdorp N. Drug release rate method for a liposome preparation. Drug Dev Ind Pharm. 1991;17(2):183–92.
13. Xiao C, Qi X, Maitani Y, Nagai T. Sustained release of cisplatin from multivesicular liposomes: potentiation of antitumor efficacy against S180 murine carcinoma. J Pharm Sci. 2004;93(7):1718–24.
14. Hitzman CJ, Wiedmann TS, Dai H, Elmquist WF. Measurement of drug release from microcarriers by microdialysis. J Pharm Sci. 2005;94(7):1456–66.
15. Bartlett GR. Phosphorus assay in column chromatography. J Biol Chem. 1959;234(3):466–8.
16. Stevens RE, Gray V, Dorantes A, Gold L, Pham L. Scientific and regulatory standards for assessing product performance using the similarity factor, f2. AAPS J. 2015;17(2):301–6.
17. Waiver of in vivo bioavailability and bioequivalence studies for immediate-release solid oral dosage forms based on a biopharmaceutics classification system. 2015; Available from: http://www.fda.gov/downloads/Drugs/Guidances/ucm070246.pdf.
18. Saranadasa H, Krishnamoorthy K. A multivariate test for similarity of two dissolution profiles. J Biopharm Stat. 2005;15(2):265–78.
19. Koynova R, Caffrey M. Phases and phase transitions of the phosphatidylcholines. Biochim Biophys Acta. 1998;1376(1):91–145.
20. Lichtenberg D, Barenholz Y. Liposomes: preparation, characterization, and preservation. Methods Biochem Anal. 1988;33:337–462.
21. Silverman L, Barenholz Y. In vitro experiments showing enhanced release of doxorubicin from Doxil® in the presence of ammonia may explain drug release at tumor site. Nanomed Nanotechnol. 2015;11(7):1841–50.
22. Haran G, Cohen R, Bar LK, Barenholz Y. Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases. Biochim Biophys Acta. 1993;1151(2):201–15.
23. Barenolz Y, Haran G; Yissum. Method of amphiphatic drug loading in liposomes by pH gradient. United States Patent US5192549 A. 1993.
24. Gould S, Scott RC. 2-Hydroxypropyl-beta-cyclodextrin (HP-beta-CD): a toxicology review. Food Chem Toxicol. 2005;43(10):1451–9.
25. Guo LS, Hamilton RL, Goerke J, Weinstein JN, Havel RJ. Interaction of unilamellar liposomes with serum lipoproteins and apolipoproteins. J Lipid Res. 1980;21(8):993–1003.
26. Shen J, Burgess DJ. Accelerated in vitro release testing of implantable PLGA microsphere/PVA hydrogel composite coatings. Int J Pharm. 2012;422(1–2):341–8.
27. Shen J, Lee K, Choi S, Qu W, Wang Y, Burgess DJ. A reproducible accelerated in vitro release testing method for PLGA microspheres. Int J Pharm. 2016;498(1–2):274–82.