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Insights into accelerated liposomal release of topotecan in plasma monitored by a non-invasive fluorescence spectroscopic method

Kyle D. Fugit, Amar Jyoti, Meenakshi Upreti, and Bradley D. Anderson*

Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, KY 40536

Abstract

A non-invasive fluorescence method was developed to monitor liposomal release kinetics of the anticancer agent topotecan (TPT) in physiological fluids and subsequently used to explore the cause of accelerated release in plasma. Analyses of fluorescence excitation spectra confirmed that unencapsulated TPT exhibits a red shift in its spectrum as pH is increased. This property was used to monitor TPT release from actively loaded liposomal formulations having a low intravesicular pH. Mathematical release models were developed to extract reliable rate constants for TPT release in aqueous solutions monitored by fluorescence and release kinetics obtained by HPLC. Using the fluorescence method, accelerated TPT release was observed in plasma as previously reported in the literature. Simulations to estimate the intravesicular pH were conducted to demonstrate that accelerated release correlated with alterations in the low intravesicular pH. This was attributed to the presence of ammonia in plasma samples rather than proteins and other plasma components generally believed to alter release kinetics in physiological samples. These findings shed light on the critical role that ammonia may play in contributing to the preclinical/clinical variability and performance seen with actively-loaded liposomal formulations of TPT and other weakly-basic anticancer agents.

Keywords

Release kinetics; topotecan; liposomes; fluorescence spectroscopy; nanotechnology

1. Introduction

Many physiological factors (i.e. age, gender, dose regimen, type or location of cancer, mononuclear phagocyte system [1]) have been proposed to influence the pharmacokinetics (PK) and pharmacodynamics (PD) of nanoparticle formulations of anticancer agents.

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*Corresponding Author: Prof. Bradley D. Anderson, Department of Pharmaceutical Sciences, College of Pharmacy, A323A ASTeCC Bldg., University of Kentucky, Lexington, KY 40506, Telephone: 859-218-6536, Fax: 859-257-2489, bande2@uky.edu.

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Unfortunately, the correlation between these factors and nanoparticle efficacy remain largely unknown [2, 3]. For liposomal formulations, bilayer integrity may be compromised by the particles' interactions with proteins (e.g. vesicle binding and particle opsonization) [4–7] or osmotic stresses [8, 9] either in the circulation or at the tumor site. Actively-loaded liposomal formulations may also exhibit accelerated drug release due to *in vivo* factors that alter the intraliposomal pH [10]. Liposomal formulations that rely on pH gradients for active-loading of anticancer agents are numerous [11–16] and would share in these susceptibilities. Reports describing the effects of such physiological phenomena on *in vivo* release kinetics have been limited due to the lack of available *in-situ* methods to monitor and distinguish entrapped from free drug in physiologically relevant media. Methods to determine the release kinetics of drugs from circulating liposomes and/or at the tumor site are crucial to optimizing the efficacy of liposome-based drug delivery systems.

Ultimately, mathematical models will be needed to interpret release profiles and provide a mechanistic understanding of the *in vivo* factors that lead to variability in the performance of liposomal formulations in order to establish *in vitro-in vivo* correlations. Such models must distinguish between physicochemical release characteristics intrinsic to the drug/particle system and factors contributed by the release environment (i.e. kinetic or thermodynamic effects attributable to the particular medium within which release is determined) [10, 17–21].

Topotecan (TPT) is a camptothecin analogue known for its topoisomerase-I inhibitory activity and regulation of genes associated with angiogenesis [22]. Several preclinical studies have demonstrated increased anti-tumorigenic efficacy of liposomal formulations of TPT that have reduced systemic clearance, allowing greater uptake and extended tissue exposure in murine solid tumors [14, 23, 24]. Many of the liposomal formulations of TPT are actively loaded by establishing an acidic intravesicular compartment relative to the extravesicular pH of the loading solution. This process provides high drug loading efficiency while ensuring delivery of the pharmacologically active lactone form of TPT to the tumor. While actively loaded liposomal formulations have often shown prolonged retention in aqueous buffers [14, 25, 26], the same formulations may exhibit accelerated release in plasma [14, 26].

Although the low intravesicular pH has been shown to persist after active drug loading [14, 24, 25, 27, 28], to the authors' knowledge this is the first report of its use to differentiate between entrapped and free TPT during drug release. Realizing that the fluorescence of TPT is pH-dependent [13, 29], changes in TPT fluorescence in aqueous liposomal suspensions and in plasma were explored as a potential means of non-invasively monitoring liposomal release in real-time. Analyses of fluorescence spectra confirmed that free TPT exhibits a red shift in its excitation spectrum as pH is increased. Due to this red shift, release of TPT from actively loaded liposomal TPT (ALLT) formulations could be monitored using fluorescence at higher wavelengths (410–430nm) where entrapped drug at low intravesicular pH does not fluoresce.

The initial aim of this study was to validate a fluorescence method to non-invasively monitor liposomal release of TPT in a physiological environment. During the course of

comparing apparent liposomal release profiles in different media including PBS buffer, plasma, and plasma ultrafiltrate using either the fluorescence method or HPLC it became evident that: a) TPT release is dramatically accelerated in human plasma as initially reported by Liu et al. [14]; and b) similar release kinetics were obtained in plasma ultrafiltrates. Recognizing that a non-filterable plasma component must be responsible for the accelerated release and that normal human plasma contains low levels of ammonia [30, 31], additional studies were conducted to probe the concentrations of ammonia in the plasma samples and the effect of ammonia on TPT release. To mechanistically rationalize differences in release profiles using different analytical methods and media, mathematical models were developed to account for the effects of liposome concentration, intravesicular pH, TPT ionization, and ammonia concentration on release kinetics.

2. Materials and Methods

2.1 Materials

Powders of 1,2-distearoyl-sn-glycero-3-phosphatidylcholine (DSPC, >99% purity) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] (DSPE-PEG2K, MW = 2806, >99% purity) were purchased from Avanti Polar Lipids (Alabaster, AL). Topotecan hydrochloride was purchased from AK Scientific (Union City, CA). Heparinized human plasma samples from seven healthy donors of different ethnicity were purchased from Innovative Research (Novi, MI), aliquoted and stored at -20°C . An additional complement-protected plasma sample was also obtained from the same source by special request. Benzene sulfonic acid sodium salt (sodium besylate) was purchased from Spectrum Chemicals (New Brunswick, NJ). Millipore ultrafiltration cartridges (Amicon® Ultra 0.5 mL centrifugal filter device with 3,000 MWCO Ultracel® membrane), Nuclepore polycarbonate membranes (0.1 μm), Dowex 50Wx8-200 resin in the H^{+} form, solvents, and buffer salts were purchased from Fisher Scientific (Florence, KY). All solvents were HPLC grade.

2.2 Liposome preparation

Large unilamellar liposomes were prepared based on previously reported methods [10, 17, 19, 20, 32, 33]. Briefly, powders of DSPC and DSPE-PEG2K were dissolved in chloroform at a molar ratio of 95:5, then dried under nitrogen, and finally under vacuum (-30 in Hg) at 35°C for 6 hours. After drying, the films were hydrated in either 0.3 M ammonium besylate, 1 mM TPT in 50 mM pH 3.75 formate buffer, or pH 7.4 phosphate buffered saline (PBS) solutions to produce 30 mg/mL lipid suspensions. These suspensions were vortexed at 60°C , then extruded through two 100 nm polycarbonate membranes 10 times at 40 psig and 60°C to yield suspensions of ammonium besylate-loaded liposomes (ABLs), passively-loaded TPT-containing liposomes at pH 3.75, or blank liposomes, respectively.

The ammonium besylate solutions (0.3 M) used for liposome hydration were prepared in a manner similar to that previously used to make other amino-based salts [15, 16]. Solutions of sodium besylate (0.6 M) were passed through an ion exchange column loaded with Dowex 50Wx8-200 resin in the H^{+} form. The eluted solutions were subsequently titrated

with ammonium hydroxide (3.0 M) to the equivalence point and diluted to the desired concentration.

2.3 Active loading of TPT into ammonium besylate liposomes

Previous studies have shown that active-loading of weakly basic drugs results in high encapsulation efficiency and possibly longer drug retention *in vitro* and *in vivo* [14, 24]. Actively-loaded liposomal suspensions of TPT were prepared with the aim of evaluating a fluorescence method to analyze drug release *in vivo* or *ex vivo*. Active loading was performed by generating a low intravesicular pH via an ammonia gradient [12, 14]. This gradient was established when extravesicular ammonium besylate was removed by passing the suspension through a Sephadex G-25 column similar to previous reports [14]. In this case, 0.4 mL of the ABL suspension was passed through the column equilibrated with 100 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) pH 5.5 buffer and the first 5 mL of eluted suspension was collected for loading studies. Next, 1.5 mL of the eluted suspension was added to an equal volume of TPT dissolved in the same pH 5.5 buffer to achieve a total TPT suspension concentration of 60 or 180 μ M and a lipid concentration of 0.92 mg lipid/mL. Loading occurred over a 72 hour period within a 37 °C incubator.

ALLT suspensions were prepared for release studies by removing extravesicular buffer and any remaining unloaded drug by applying 0.5 mL of ALLT to a Sephadex G-25 column equilibrated with PBS similar to previous reports [10, 18]. The first 2.5 mL fraction eluted from the column was discarded. ALLT eluted in the next 2.5 mL fraction and was collected for use in release studies monitored by fluorescence or HPLC.

2.4 Liposome characterization

Particle size was determined for ALLT and PLLT using dynamic light scattering (DLS) using a Beckman DelsaTM Nano C Particle Sizer as previously reported [20, 21]. Lipid content was monitored by HPLC using an evaporative light scattering detector (ELSD). A Waters Alliance 2695 separations module equipped with an Allsphere (Alltech Associates, Inc., Deerfield, IL) silica column (4 × 150 mm, 5 μ m) and guard column (20 × 4.0 mm, 5 μ m) and a mobile phase consisting of 80% of solvent A (80% chloroform:19.5% methanol:0.5% (v/v) NH₄OH) and 20% of solvent B (80% methanol:19.5% water:0.5% (v/v) NH₄OH) flowing at 1 mL/min was used to quantify DSPC in conjunction with an ELSD (Sedere, Inc., Lawrenceville, NJ) operated at 40 psig and 40 °C. Standards of DSPC were dissolved in mobile phase A (0.05 – 0.3 mg DSPC/mL). Log-log plots of peak area versus concentration were linear over this concentration range. Samples (100 – 250 μ L) were dried at room temperature under N₂, then dissolved in chilled solvent A before analysis.

2.5 Fluorescence method development and validation

2.5.1 TPT Excitation Spectra—Samples and standards from validation and release studies were placed in 1 ml quartz cuvettes (NSG Precision Cells, Inc. Farmingdale, NY) for spectrometric analysis. Fluorescence excitation spectra (290–500 nm) were collected with a FluoroMax-3 spectrofluorometer (Jobin Yvon Inc., Edison, NJ) operating at a constant emission wavelength of 550 nm, slit width of 1.5 nm, and a 0.5 second integration time. The temperature of the sample chamber was maintained at 37 °C.

Excitation spectra of free TPT (2.5 μM) and PLLT (2.5 μM total suspension concentration of TPT after Sephadex removal of untrapped drug) in pH 3.75 formate buffer were analyzed to compare the excitation spectra of free and entrapped TPT under acidic conditions. These spectra were compared to excitation spectra of free TPT (2.5 μM) at pH 7.4 and ALLT suspensions in pH 7.4 PBS (2.5 μM suspension TPT, 37 μg lipid/mL) to determine if ALLT spectra were indicative of an acidic intravesicular environment and whether spectra of entrapped and untrapped drug were different.

2.5.2 TPT release studies by fluorescence—Release of liposomal TPT in the presence of extravesicular ammonia may be particularly important, as it is present in physiological fluids and tissues and may have an effect on intravesicular pH and subsequently on release kinetics. To observe these effects, release studies of ALLT were conducted at 37 °C in pH 7.4, phosphate buffered saline (PBS) solution and in PBS containing 60 μM of NH_4Cl .

For release studies monitored by fluorescence, 100 μL aliquots of the liposomal suspension collected after Sephadex purification were diluted to 1 mL with either PBS, human plasma, or plasma ultrafiltrate (obtained from the donors' plasma used in release studies) to achieve suspension concentrations of 19.2 $\mu\text{g/mL}$ lipid and 3.2 μM TPT (as determined by HPLC). Plasma ultrafiltrate was obtained by placing aliquots (500 μL) of human plasma in an Amicon® Ultra 0.5 mL semi-micro ultrafiltration cartridge (3,000 MWCO). The plasma was then centrifuged at 13,500 rpm at 4°C for 15 minutes to collect its ultrafiltrate. Excitation spectra were collected over time and compared to spectra for TPT standards (0.5–5 μM) in the same sample matrix analyzed at the same time to quantify the accumulation of free TPT released into the extravesicular solution.

TPT release was monitored by the increase of fluorescence intensity at an excitation wavelength of 410 nm for PBS and plasma ultrafiltrate while intensities at 420 nm were used for human plasma studies. TPT standard calibration curves were constructed using Equation 1 to adjust for fluctuations in lamp intensity at each sample time, $I_0(t)$, and TPT dimerization in solution [34, 35]:

$$I(t) = (i_1 T_1 + i_2 T_2) I_0(t) \quad (1)$$

where T_1 and T_2 are the solution concentrations of TPT monomer and dimer, respectively, and i_1 and i_2 are the corresponding response factors for these species. Using a mass balance equation for total TPT in solution ($T_0 = T_1 + 2T_2$), the TPT dimerization constant ($K_2 = T_2/T_1^2$), and Equation 1, fitted values for i_1 , i_2 , $I_0(t)$, and K_2 were obtained from these calibration curves and used to calculate the concentration of extravesicular TPT at each time point.

2.6 TPT release by HPLC

TPT release was monitored by HPLC in suspensions prepared by diluting 0.2 mL of the suspension collected after Sephadex to 4 mL with pH 7.4 PBS containing either no added ammonia, 12 μM , or 60 μM NH_4Cl . The resulting TPT and lipid suspension concentrations were 240 nM and 6.4 $\mu\text{g/mL}$, respectively. Aliquots (150 μL) withdrawn at various times

were diluted with chilled methanol (-20°C) to disrupt the liposomes and quench the lactone/carboxylate interconversion of TPT. Samples were immediately analyzed by HPLC to quantify both the lactone and carboxylate forms of TPT. A previously published HPLC method was employed with slight modifications [20]. Briefly, a Waters (Milford, MA) Alliance 2695 separation system with a Waters Symmetry® C18 column (3.9×150 mm, $5\ \mu\text{m}$) and guard column (3.9×20 mm) was used to separate lactone and carboxylate TPT using a mobile phase of 11.5% acetonitrile: 88.5% (v/v) of a 5% (pH = 5.5) triethylamine acetate, 50 mM tetrabutylammonium hydrogen sulfate (TBAHS) buffer at a flow rate of 1 mL/min. TPT lactone and carboxylate standards (20–200 nM) were prepared in chilled, acidified methanol (-20°C) and 10 mM sodium carbonate buffer (pH 10.1), respectively. Lactone and carboxylate retention times were 6.1 and 2.7 min, respectively. A Waters M474 fluorescence detector (operating at excitation and emission wavelengths of 380 and 560 nm, respectively) was used to analyze the fractions of lactone and carboxylate TPT after separation.

2.7 TPT degradation kinetics in the presence/absence of ammonia

Significant TPT degradation would affect the observed concentration of extravesicular TPT and must be incorporated into models describing liposomal TPT release. TPT ($0.5 - 5\ \mu\text{M}$) degradation was assessed in pH 7.4 PBS with or without $60\ \mu\text{M}$ NH_4Cl at 37°C . Degradation of TPT was measured in the presence of ammonia due to its presence in release studies and previous reports indicating that increasing concentrations of ammonia promote TPT degradation via formation of 9-amino methyl degradants [36]. Aliquots ($25 - 40\ \mu\text{L}$) of TPT solutions taken over a 5 day period were diluted to a final volume of 1 mL with acidified methanol ($0.001\ \text{N}$ HCl) to convert all TPT to its lactone form and analyzed by the HPLC method used to monitor release. TPT concentrations versus time (t) were fit to a first-order kinetic model as shown below in Equation 2 where k_d is the first-order degradation rate constant and X is the fraction of initial TPT remaining in solution.

$$X = e^{-k_d t} \quad (2)$$

2.8 Ammonia analyses

Potentiometric measurements of ammonia content in plasma were performed using an Orion ammonia electrode in conjunction with a Thermo Scientific Orion Star A214 pH, ISE, mV, temperature meter. Ammonia standards were prepared between 0.01–0.3 ppm in Milli-Q H₂O. Immediately before ammonia analysis, $100\ \mu\text{L}$ of NaOH reagent was added to 10 mL of standards to raise pH and convert any ammonium to ammonia. Solutions were allowed to equilibrate for 3–5 minutes under mild stirring and the final voltage was recorded. A Nernst relationship between ammonia concentrations and electric potential (mV) was observed and used to make a standard curve for the estimation of total ammonia in solution. Plasma samples ($100\ \mu\text{L}$) were analyzed after ultrafiltration and subjugation to the same dilution and addition of NaOH as standards to obtain ammonia concentrations within the sample.

2.9 General mathematical model for actively-loaded liposomal TPT release under non-sink conditions

While comparisons of spectrometric data can distinguish free from entrapped drug, quantitative analysis and determination of kinetic parameters requires a mathematical model able to interpret release kinetics from experimental data. This is described below.

Because of the low intravesicular pH established during the active loading process, encapsulated TPT exists solely in its lactone form [14, 37, 38]. At physiological pH, TPT undergoes pH-dependent conversion to its carboxylate counterpart after release (Scheme 1A) [14, 37]. TPT's ionization state also changes upon release as the unionized phenol dominates at low intravesicular pH ($pK_a = 6.56$) while the phenolate anion is the major species at physiological pH (Scheme 1B) [20, 34, 39]. By applying the appropriate mathematical model, it is possible to extract the critical release parameters from either the time-dependent profiles of TPT lactone and carboxylate generated by HPLC or changes in fluorescence excitation spectra.

A simple kinetic model describing drug release proceeding to equilibrium under non-sink conditions was used to quantify the release profiles obtained by both HPLC and fluorescence methods. Because previous studies have shown the lactone form of TPT to be the most permeable, this model assumes the intra- and extravesicular lactone species (L_i and L_o , respectively) govern the rates of change of total intra- and extra-vesicular TPT ($\frac{dT_i}{dt}$ and $\frac{dT_o}{dt}$, respectively).

$$\frac{dT_i}{dt} = -k_m L_i + k_m K L_o \quad (3a)$$

$$\frac{dT_o}{dt} = k_m L_i - k_m K L_o - k_d T_o \quad (3b)$$

where k_m is the rate constant for bidirectional TPT transport, K is the ratio L_i/L_o at equilibrium, and k_d is the first-order degradation constant for TPT released into the extravesicular solution.

Once released, the TPT lactone undergoes reversible, pH-dependent hydrolysis to form its ring-opened, carboxylate counterpart. This process may be assumed to be fast relative to release and thus at equilibrium. Assuming this pH-dependent equilibrium, an apparent acid dissociation constant (K_A') may be used to solve for the fraction of extravesicular TPT in the

lactone form ($f_L = \frac{H^+}{H^+ + K_A'}$). This expression allows L_o to be written in terms of T_o , and $L_o \approx T_i$ due to the low intravesicular pH resulting from active loading. Using this information, the rate equations can be rewritten as shown below.

$$\frac{dT_i}{dt} = -k_m (T_i - f_L K T_o) \quad (4a)$$

$$\frac{dT_o}{dt} = k_m(T_i - f_L K T_o) - k_d T_o \quad (4b)$$

T_o was directly monitored by fluorescence while the fractions of total drug remaining in the suspension in the lactone and carboxylate forms were monitored by HPLC. T_i and T_o could be obtained from the total lactone and carboxylate fractions ($L(t)$ and $C(t)$, respectively) and the total suspension concentration of TPT measured at each time point, $T(t)$:

$$L(t) = \frac{T_i + f_L T_o}{T(t)} \quad (5a)$$

$$C(t) = \frac{(1 - f_L) T_o}{T(t)} \quad (5b)$$

Initial conditions were required to accurately solve and fit the above differential equations to release data. In fluorescence studies, extravesicular drug present at the beginning of the release study (T_o^0) was directly analyzed by fluorescence; however, the initial intravesicular drug could not be determined directly from fluorescence. The initial concentration of intravesicular drug was determined after subtracting T_o^0 from the total initial suspension concentration T^0 obtained by HPLC analysis. These initial conditions are expressed by the equations below.

$$T_o(0) = T_o^0 \quad (6a)$$

$$T_i(0) = T^0 - T_o^0 \quad (6b)$$

HPLC studies had similar initial conditions. Assuming that any carboxylate in the suspensions was attributable to extravesicular drug, the initial fraction of carboxylate present in the release suspension (C_o) could be related to T_o^0 and subsequently be used in conjunction with the initial fraction of lactone (L_o) to solve for the initial intra- and extra-vesicular conditions as shown below.

$$T_o(0) = T_o^0 = \frac{C_o}{(1 - f_L)} \quad (7a)$$

$$T_i(0) = L_o - \frac{C_o}{(1 - f_L)} \quad (7b)$$

3. Results

3.1 Liposome characterization

Actively loaded liposomes had an average particle size diameter (using four independent determinations) of 101 ± 3 (95% CI) nm and a polydispersity index (PDI) of 0.10 ± 0.06 after TPT loading. The particles size and PDI of PLLTs was similar with 97.5 ± 4 nm and 0.09 ± 0.03 respectively. Loading efficiencies of the ammonium besylate liposomes used in release studies were 51% and 38% for those loaded with solutions containing 60 and 180 μ M TPT, respectively.

3.2 Differences in fluorescence spectra and quantitation of extravesicular TPT

Increases in pH result in a red shift in TPT excitation spectra in aqueous solution [20, 34]. Such a shift suggests TPT release from actively-loaded liposomes into a pH 7.4 buffer or plasma could be distinguished from entrapped drug. This hypothesis was confirmed by comparing the fluorescence excitation spectra obtained in various aqueous solutions and liposomal suspensions of TPT. In Figure 1, the excitation spectra of TPT under acidic conditions (either in solution or encapsulated) were nearly identical to the excitation spectrum obtained for ALLT suspended in pH 7.4 PBS with maximum excitation occurring at 380 nm. These results are indicative of a low intravesicular pH environment remaining after the active loading process [11, 12, 14–16, 25].

The red shift observed for free or extravesicular TPT in PBS at pH 7.4 resulting in maximum excitation at 410 nm is not altered in the presence of blank liposomes (Figure 1). Determination of extravesicular TPT is possible without significant interference from encapsulated drug because TPT under these more acidic conditions is not excitable at this higher wavelength.

Calibration curves for quantifying extravesicular TPT were constructed from excitation spectra at varying concentrations (0.2–5 μ M) of TPT in pH 7.4 PBS, human plasma, and plasma ultrafiltrate. Fluorescence intensity versus TPT concentration was nearly linear with slight quenching of fluorescence at higher concentrations ($\sim 5 \mu$ M). This quenching was due to TPT dimerization and accounted for in the calibration curve (see Methods) [34]. Quantitation of intravesicular TPT was not possible due to self-association and collisional quenching effects at the high intravesicular TPT concentrations (~ 15 mM) present as a consequence of the active loading process.

3.3 TPT degradation in the presence and absence of ammonia

TPT degradation was monitored by HPLC at pH 7.4 and 37 °C in PBS and PBS containing 60 μ M NH_4Cl (data not shown). The degradation was first-order and independent of the presence of ammonia. The rate constant for degradation was determined to be $1.15 \pm 0.08 \times 10^{-2} \text{ hr}^{-1}$ (95% CI). This value was incorporated into the models used to fit release data.

3.4 Comparison of fluorescence and HPLC methods to monitor release

Release studies were conducted in PBS with or without added ammonia and analyzed by HPLC and fluorescence methods to validate the use of fluorescence for determining release.

Due to the higher concentrations of extravesicular TPT needed to monitor release with the fluorescence method, ALLTs with higher drug loading were required (60 μM vs. 180 μM TPT loading solutions). Differences in loading did not alter release kinetics. Degradation of topotecan at pH 7.4 limited the time frame for release studies by fluorescence to ~ 24 h. Because longer times were necessary to establish equilibrium, however, both HPLC and fluorescence release data in PBS with and without ammonia were fit simultaneously to determine values for $K(T_i/f_L T_o)$ at equilibrium). The resulting fits indicated that K decreases with the addition of extravesicular ammonia to the release media.

While K was assumed to be independent of the method of analysis, separate k_m values were determined for each method and condition. The values obtained are shown in Table 1, and the resulting fits of the data from both methods are illustrated in Figure 2. The half-lives ($t_{1/2}$) applicable to sink conditions based on the release rate constant were also calculated for easier comparison. This $t_{1/2}$ is defined by the equation below.

$$t_{1/2} = \frac{\ln(2)}{k_m} \quad (8)$$

Figure 2c shows that both methods revealed similar trends in $t_{1/2}$, with faster release occurring at higher ammonia concentrations in the release media. The similarity of release kinetics determined by both methods is best illustrated by release conducted in PBS containing 60 μM NH_4Cl . The 95% confidence limits of the k_m values determined from both methods overlapped. However, in PBS without added ammonia k_m values differed significantly depending on the monitoring method, with TPT release monitored by fluorescence being faster than that obtained by HPLC. This was attributed to the lower concentration of liposomes in the experiments monitored by HPLC which resulted in more ammonia release prior to establishment of equilibrium in the ammonia concentrations. Reduction in the intravesicular concentration of ammonia lowered the intravesicular pH, thus slowing TPT release [20]. A detailed analysis of the differences in ammonia release and subsequent effects on intravesicular pH is provided in a later section.

3.5 Release experiments in human plasma and plasma ultrafiltrate

Red shifts in excitation spectra were also observed during release studies in plasma. These shifts were again used to monitor TPT release (Figure 3). Rate constants for release in plasma were ~ 30 -fold greater than in PBS (Figure 2b and Table 1) alone. To assess possible contributions of colloidal lipoprotein particles that might participate in lipid exchange with the lipid bilayer or protein effects such as opsonization [13, 40–43], plasma samples were ultrafiltered and the ultrafiltrates were then used in release experiments. TPT release profiles in plasma ultrafiltrates were indistinguishable from the plasma release profiles (see Figure 2b) and the release rate constants in both plasma and plasma ultrafiltrates were ~ 10 -fold greater than in PBS containing 60 μM NH_4Cl (Table 1). These observations provided motivation to measure ammonia concentrations in plasma to determine whether the accelerated release rates seen in plasma and plasma ultrafiltrates were related to higher ammonia concentrations in these samples. This appeared to be the case as shown by Figure 2c. The ammonia concentrations, analyzed using an ammonia selective electrode, were 180,

185, and 355 μM for these three plasma samples (each from a different donor) and their respective ultrafiltrates. These concentrations were much higher than those reported for normal human blood (15–60 μM) [30, 31]. Based on the trend in Figure 2c, there appears to be a significant correlation between extravascular ammonia concentration and TPT release rates. However, identifying the underlying mechanism affected by ammonia may provide a better correlation and explanation for the observed phenomenon.

Due to the elevated ammonia concentrations in the plasma samples employed for release experiments, additional human plasma samples were tested for ammonia concentration to determine if these elevated levels were due to the shipping process and/or the recommended storage conditions from the plasma supplier. Another five plasma samples from four different donors were analyzed in addition to the three plasma samples used in release studies. Figure 4a shows ammonia concentrations were above normal for all samples obtained via normal collection and shipping procedures. These plasma samples had an average basal ammonia level of $190 \pm 90 \mu\text{M}$ after immediately thawing the samples upon receipt from the supplier. Another complement-preserved plasma sample obtained upon request from the same supplier (illustrated by the open datum point in Figure 4a) was also analyzed. This sample contained a lower ammonia concentration and was the only sample analyzed that fell within the normal range for ammonia concentration. Complement protection also appeared to slow early production of ammonia at both 4 and 37 °C (see Figure 4b). By day seven, however, both complement protected and non-protected samples exhibit significant increases in ammonia concentrations. Elevated ammonia concentrations at –20 °C might have resulted from the freezing and thawing of the samples between two observations. Overall, significant production of ammonia in human plasma was observed under a variety of storage conditions. This observation in conjunction with the sensitivity of TPT release to ammonia further stresses the critical need to quantify ammonia levels at the onset of release studies for these actively-loaded liposomal formulations. These factors may also provide an explanation for previous instances of accelerated release of actively loaded liposomal TPT in plasma [14].

4. Discussion

4.1 Differences in liposome concentration led to changes in intravesicular ammonia, pH, and subsequent release kinetics

While attempts were made to keep the release media consistent between experiments analyzed by HPLC and fluorescence, the liposome suspension concentrations differed between the two methods. This was necessary to maintain TPT concentrations in an optimal range for quantification by each method. Simulations indicated that this seemingly minor difference could be important.

A preliminary estimate of the intravesicular pH under the different conditions in these experiments was obtained by simulating the effects of ammonia transport across the bilayer. The first-order rate constant for ammonia bilayer transport, k_{mm} , is related to the permeability coefficient for ammonia transport, $P_{\text{NH}_3}^m$, and liposome diameter, d [32]:

$$k_{m,n} = \frac{6 P_{NH_3}^m}{d} \quad (9)$$

The differential equations that govern ammonia transport are then:

$$\frac{dN_i}{dt} = -k_{m,n}(NH_{3,i} - NH_{3,o}) \quad (10a)$$

$$\frac{dN_o}{dt} = f_v k_{m,n}(NH_{3,i} - NH_{3,o}) \quad (10b)$$

Because the free base form of ammonia is the permeable species [44], the rates of change in the total concentration of ammonia in the intra- and extra-vesicular compartments (N_i and N_o respectively) are dependent on the concentration gradient between neutral ammonia in the intra- and extra-vesicular compartments ($NH_{3,i}$ and $NH_{3,o}$ respectively), the rate constant for neutral ammonia transport ($k_{m,n}$), and the ratio of liposomally-entrapped to unentrapped volume (f_v). The latter quantity, f_v , can be calculated from the particle size and lipid content in the liposome suspension with knowledge of the lipid surface density [32]. $NH_{3,i}$ and $NH_{3,o}$ may be written in terms of N_i and N_o by solving for the fractions of neutral ammonia in the intra- and extra-vesicular phases (f_i^N and f_o^N):

$$f_i^N = \frac{K_{AN}}{H_i^+ + K_{AN}} \quad (11a)$$

$$f_o^N = \frac{K_{AN}}{H_o^+ + K_{AN}} \quad (11b)$$

These fractions are dependent on the acid dissociation constant for ammonia, K_{AN} , and the acidity or hydrogen ion concentrations in the intra-or extra-vesicular compartments (H_i^+ and H_o^+ , respectively). Using these fractions, equations 10a and b can be rewritten to yield:

$$\frac{dN_i}{dt} = -k_{m,n}(f_i^N N_i - f_o^N N_o) \quad (12a)$$

$$\frac{dN_o}{dt} = f_v k_{m,n}(f_i^N N_i - f_o^N N_o) \quad (12b)$$

The pH in the intravesicular compartment decreases as ammonia release causes deprotonation of ammonium to replenish the released ammonia. This process governs the acidity of the intravesicular compartment by satisfying the charge balance equation:

$$H_i^+ = B^- + OH_i^- - (NH_{4,i}^+ + TPTH_i^+) \quad (13)$$

where B^- is the ammonium salt counterion (besylate) concentration and $TPTH_i^+$ is the concentration of the cationic form of topotecan. The ammonium ($NH_{4,i}^+$) and $TPTH_i^+$ concentrations can be expressed in terms of total intravesicular concentration of ammonia (N_i) and topotecan (T_i) while OH_i^+ can be rewritten in terms of H_i^+ and the ion product of water, K_w

$$H_i^+ = B^- + K_w / H_i^+ - [(1 - f_i^N)N_i + f_i^T T_i] \quad (14)$$

At low pH, the fraction of intravesicular TPT in its protonated form, f_i^T , is a function of H_i^+ and the TPT phenol acid dissociation constant, K_{A1} :

$$f_i^T = \frac{H_i^+}{H_i^+ + K_{A1}} \quad (15)$$

Simulations were performed using these equations and the values in Table 2 to calculate H_i^+ concentration versus time when the extravesicular solution initially contained either no ammonia (Figure 5A) or 60 μ M of NH_4Cl (Figure 5B) using the lipid concentrations measured in this study. From these simulations, it is apparent that the entrapped volume can have a significant impact on intravesicular pH depending on the concentration of extravesicular ammonia present. In solutions that initially contained no buffer, the higher lipid concentration (i.e. large entrapped volume) allows more ammonia release while the intravesicular ammonia is depleted to a lesser extent. Because of the resulting higher intravesicular ammonia concentration, the increase in H_i^+ is less for the liposome suspensions used in the fluorescence method. TPT release is pH-dependent and slower as H_i^+ increases [20]. The higher rate of TPT release determined by the fluorescence method compared to that observed by HPLC is consistent with this difference in H_i^+ .

This effect, however, is not apparent in the release studies conducted in PBS solutions which initially had ammonia present. At 60 μ M NH_4Cl , the extravesicular concentration of ammonia is sufficiently high and the volume entrapped low enough that the extravesicular concentration essentially remained constant. This normalized the ammonia concentration gradient to be the same and independent of the entrapped volume (Figure 4B). This results in nearly identical H_i^+ profiles for both methods and subsequently the same release kinetics for both methods.

For these simulations, the initial H_i^+ was calculated assuming a 1:1 exchange between ammonia and TPT during the active loading process (i.e. $N_o(0) = 0.3 - T_i$). While this can only be a rough estimation of the initial H_i^+ , simulations at a higher or lower initial H_i^+

($10^{-2.5}$ and $10^{-5.5}$ or pH of 2.5 and 5.5, respectively) also resulted in similar trends in the terminal H_i^+ simulated in Figure 4.

4.2 Effects of ammonia concentration in physiological samples and implications on liposomal TPT release

Initial simulations of intravesicular pH showed that the presence of extravesicular ammonia in the release media partially dissipated the pH gradient. Such an effect may also be possible in the release studies in plasma and plasma ultrafiltrates as relatively high levels of ammonia were detected in these samples. This was explored further in simulations of the intravesicular pH after accounting for the extravesicular ammonia present in the various release media studied (buffer, plasma, or plasma ultrafiltrate). These simulations, shown in Figure 6, indicate a negative correlation between the release half-life and the intravesicular pH. This relationship provides further evidence that the presence of extravesicular ammonia raises intravesicular pH, given the pH-sensitive release of liposomal TPT previously reported [20].

While further studies are necessary to fully understand the effect of ammonia transport on actively-loaded liposomal systems (e.g. in formulations with drug precipitation/complexation within the intravesicular environment), the potential implications are considerable. Many liposomal drug loading strategies rely on the generation of a pH gradient using ammonia [12, 14], an ionophore [25, 38, 47], or another highly permeable amine (e.g. di- or tri-methylamine) [15, 16]. In all of these strategies, the pH gradients generated to stabilize drug encapsulation are susceptible to the influx of ammonia or other highly permeable basic species present in physiological tissue or fluid. The intravesicular pH in these formulations should be calculable using an equation based on a charge balance similar to Equation 13 with appropriate modifications to account for precipitation, self-association, etc.

It is likely that ammonia is the primary basic-permeable species present in physiological fluids and tissues; however, other low molecular weight amines (e.g. di- and tri-methylamine) are also present at levels which vary from patient to patient [48–50]. Other effects have been suggested to account for variability of liposomal release kinetics in plasma such as destabilization of the bilayer due to protein interactions [5, 7, 40, 42, 51, 52]. However, these theories could not explain the effects seen here as release kinetics obtained in plasma would have been significantly different from release kinetics obtained in studies performed in an ultrafiltrate of the same plasma (which was not the case).

Lastly, the storage conditions and history of the plasma may also have a considerable effect on release rates from actively loaded liposomes. Previous reports on the production of ammonia under a wide variety of conditions typically encountered during the processing and storage of plasma are considerable [49, 53]. Furthermore, these studies indicate that ammonia production is significant at room temperature and even when samples have been frozen. This may account for the higher ammonia levels in these plasma studies than those reported in the literature for fresh plasma and blood samples [30, 31]. Such an issue could lead to overestimations of drug release *in vivo*. Characterization of the ammonia content and

possibly other protein degradants in release studies performed in plasma should be considered. Furthermore, ammonia generation during release studies may also affect release kinetics. In the present study, ammonia levels in plasma after a 48 hour release experiment were considerably higher (approximately two-fold) than the initial ammonia levels. This is yet another scenario that could lead to possible overestimation of drug release based on characterization studies in plasma, as renal excretion of ammonia would typically prevent such high levels in patients. In contrast, however, patients suffering from hyperammonemia could present much higher ammonia concentrations (~1 mM) [54, 55]. This condition may be quite relevant in cancer patients with diminished liver function,[56] either as a result of the cancer's pathophysiology, a side effect of a previous treatment,[56–59] or a preexisting condition (e.g. cirrhosis).[56] In such cases, further acceleration in liposomal drug release may be seen.

5. Conclusion

Reliable methods to monitor drug release in physiological fluids and tissues could improve predictions of *in vivo* performance of liposomal drug delivery systems. To this end, a non-invasive method was developed to monitor liposomal release kinetics of TPT. This method utilizes the pH-dependent shift in the excitation spectra of TPT to distinguish between drug entrapped at the low intravesicular pH in actively-loaded liposomal formulations from released drug. Release kinetics obtained by fluorescence were consistent with results using an HPLC method to monitor release.

Accelerated liposomal TPT release kinetics were observed in human plasma. Additional experiments in plasma that was ultrafiltered to remove protein and lipid components that have previously been theorized to alter release kinetics indicated similar accelerated release rates. When release studies were performed in PBS buffer at pH 7.4, the addition of ammonia to the buffer was also found to dramatically increase release rates. Analyses of ammonia concentrations in the plasma samples employed in release studies were therefore undertaken. Model-based simulations were used to estimate the intravesicular pH in the presence or absence of extravesicular ammonia. The intravesicular pH increased with increasing concentrations of extravesicular ammonia. A significant correlation was found between TPT release rates and intravesicular pH simulated based on the extravesicular ammonia present in the plasma, plasma ultrafiltrates, or PBS buffer in which release studies were conducted. These findings may partially account for the accelerated release rates typically experienced in physiological fluids and potentially some of the preclinical variability observed from ALLTs [26] and likely present for other actively-loaded, weakly basic drugs (e.g. doxorubicin, irinotecan, and vincristine) [11, 12, 16, 47, 60, 61].

Because extensive processing of sample is not required to analyze drug release, the non-invasive fluorescence method developed in this work has potential applications for analyzing release kinetics in real-time for physiological samples. One such application may include analysis of free and entrapped drug in blood samples taken for PK studies. This would allow for both particle clearance and liposomal release kinetics of drug in systemic circulation to be analyzed simultaneously. Currently, adaptation of this method is under investigation using two-photon fluorescence for intratumoral imaging of release kinetics in

mouse xenografts equipped with a dorsal window. This method may also be adaptable to other molecules that exhibit pH dependent fluorescence spectra or other spectroscopic techniques (e.g. absorbance, Raman, NMR, etc.) depending on the spectrometric properties of the particular drug and/or nanoparticle.

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Figure 1.

Illustration of differences in normalized excitation spectra between free and entrapped TPT at 37 °C. Excitation spectra of free TPT at pH 3.75, passively-loaded liposomal TPT (PLLT) at the same pH, and ALLT suspensions in pH 7.4 buffer have identical spectra, indicating an acidic intraliposomal pH within ALLT. At pH 7.4, spectra of free TPT solutions and suspensions of blank liposomes spiked with free TPT (i.e., spiked TPT pH 7.4) exhibit a red shift in the excitation spectrum (denoted by the arrow). The identical spectra of spiked and free TPT indicates that drug binding to the outer bilayer leaflet or particle scattering have no effect on the spectra of extravesicular TPT. All the spectra displayed contained total TPT concentrations of ~ 2.5 μ M. The lipid concentration in liposome suspensions was ~ 37 μ g lipid/mL.

Figure 2.

Comparison of release profiles obtained by HPLC and fluorescence methods. A) Changes in the fraction of TPT carboxylate versus time obtained by HPLC in release studies at 37 °C in pH 7.4 PBS with 60 μM NH_4Cl and without ammonia ($\diamond$ and $\blacksquare$, respectively) are shown along with fits of the carboxylate fraction to the release model (— and —). The open symbols in the inset reflect the change in the fraction of lactone over the same time frame with $\square$ and $\blacksquare$ reflecting their respective fits to the release model. B) The fraction of TPT in the extravascular compartment relative to the initial total suspension concentration of TPT (T_e/T^0) versus time determined by the fluorescence method in pH 7.4 PBS in the presence or absence of ammonia ($\diamond$ and $\blacksquare$, respectively). Solid lines (— and —) represent fits to the release model. The short-dashed lines (--- and ---) reflect simulated profiles using the parameters obtained from release data monitored by HPLC for comparison. Release rates were accelerated to a similar degree in plasma ($\bullet$) and plasma ultrafiltrates ($\circ$). The long-dashed line (---) is representative of the simultaneous fits of all six data sets (i.e. plasma and plasma ultrafiltrate from three separate donors) from which the parameters listed in Table 1 were obtained. C) A plot of release half-lives ($t_{1/2}$)

applicable to sink conditions determined from studies in various media (see legend) as a function of the ammonia concentration in the media.

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Figure 3. Fluorescence excitation spectra of ALLT in plasma over time. The change in fluorescence at 420 nm was used to monitor extravesicular TPT and subsequently liposomal release kinetics.

Figure 4.

Plots showing the ammonia concentrations in human plasma samples. The initial concentrations of ammonia obtained immediately after receipt from the supplier for eight separate donors are shown (A). The production of ammonia in plasma samples stored at several temperatures (see legend) is also shown (B). Each point represents the average of samples from three individual donors with error bars representing the standard deviation. Complement-protected plasma samples are represented by open symbols in both plots.

Figure 5.

The simulated profiles of $[H_i^+]$ and $[N_o]$ versus time in pH 7.4 PBS release media that initially contained no extravesicular ammonia (A) or 60 μM NH_4Cl (B). $[H_i^+]$ simulations shown are at the lipid concentrations at which release studies by HPLC (—) and fluorescence methods (—) were conducted. The dotted lines of corresponding color reflect the total extravesicular ammonia present over this time period for HPLC and fluorescence methods, respectively.

Figure 6.

The relationship between TPT release half-life and simulated intravesicular pH. Release studies performed in PBS (■), plasma (▲), and plasma ultrafiltrate (△) are shown. In some instances, the plasma ultrafiltrate data are difficult to observe due to overlap with data points from plasma studies. The resulting trend line along with its R^2 are shown to illustrate the negative correlation between TPT retention and intravesicular pH.

Scheme 1.

Physicochemical properties of TPT considered in modeling liposomal release kinetics. TPT undergoes pH-dependent interconversion between its lactone and ring-opened carboxylate forms which can be monitored by HPLC (A). Ionization of the A-ring phenol causes a shift in the fluorescence excitation spectrum of TPT when drug is exposed to a physiological pH upon liposomal release (B).

Table 1

Release parameters obtained from HPLC and fluorescence methods. *

Constant	PBS only				PBS w/ 60 μ M NH_4Cl				Plasma & Ultrafiltrate
	HPLC	Fluorescence	HPLC	Fluorescence	HPLC	Fluorescence	HPLC	Fluorescence	
$k_m(\text{hr}^{-1})$	0.037 ± 0.004	0.053 ± 0.008			0.15 ± 0.02	0.18 ± 0.04			1.5 ± 0.4
K	4.1 ± 0.6				0.5 ± 0.2				0
$t_{1/2}(\text{hr})$	18 ± 2	13 ± 1			4.6 ± 0.6	3.9 ± 0.8			0.54 ± 0.2
$k_d(\text{hr}^{-1})$	0.0115	0.0115			0.0115	0.0115			0.1**

* $\pm$ 95 % confidence intervals

** based on the fitted release data in plasma and plasma ultrafiltrate

Table 2Parameters used to simulate H_i^+ profiles at different lipid concentrations

Parameters	Values
$k_{m,n}$	$2.88 \times 10^4 s^{-1}{}^c$
K_{AI}	$2.8 \times 10^{-7}{}^d$
K_{AN}	$9.40 \times 10^{-10}{}^e$
K_w	$2.12 \times 10^{-14}{}^e$
H_o	$3.98 \times 10^{-8} M$
T_i	$1.45 \times 10^{-2} M$
B_i	$0.3 M$
f_v – HPLC conditions (6.4 μg lipid/mL)	$1.66 \times 10^{-5}{}^f$
f_v – Fluorescence conditions (19.2 μg lipid/mL)	$5.19 \times 10^{-5}{}^f$

^cCalculated from a previously reported ammonia permeability coefficient of $P_{NH_3}^m = 48 \times 10^{-3} cm/s$ [45]^dObtained from a previous study [20]^eValues adjusted to reflect conditions at 37 °C and 0.3 I ^fCalculated based on particle size, lipid content, and lipid surface density calculations previously reported [32, 46]