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Review Article

A REVIEW ON PHYSICO-CHEMICAL PARAMETERS OF LIPOSOMAL DOXORUBICIN

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ABSTRACT

The development of a generic liposomal doxorubicin product requires the study of critical physicochemical properties of the formulation. Food and Drug Administration (FDA) draft guideline has suggested few parameters to be tested for *in vitro* bioequivalence study which include liposomal composition, state of encapsulated drug, internal environment, liposomal morphology and number of lamellae, lipid bilayer phase transition, liposomal size distribution, grafted Polyethylene Glycol (PEG) at liposomal surface, electric surface potential or charge and *in vitro* leakage under multiple conditions. Characteristic features of components of liposomal doxorubicin formulation and detail of parameters to be studied have been discussed. This review compile specific, current and historical research outcomes on *in vitro* analysis of liposomal doxorubicin and highlights the important features that have a critical impact on properties of liposomal doxorubicin formulation. It will provide a better insight to the generic manufacturers and will help them to identify the critical quality attributes during the formulation development phase.

Keywords: Bioequivalence, Doxorubicin, Liposome, Critical, Attributes

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INTRODUCTION

Doxorubicin is a member of the anthracycline class of antineoplastic drugs and is effective in a variety of solid tumours and hematological malignancies [1]. Doxorubicin (Dox) hydrochloride ($C_{27}H_{29}NO_4$) is a high molecular weight compound (579.98 gm/mol) and is isolated from *Streptomyces peucetius*. It was first introduced in the 1970s, and since then it has become one of the most commonly used drugs for the treatment of both hematological and solid tumors [2].

The therapy-limiting toxicity for this drug is cardiomyopathy, which may lead to congestive heart failure and death. Around 2% of patients who have received Dox experience of this condition. As the usage of Dox is limited to its dose-related cardiotoxicity and myelosuppression, the liposomal form of Dox is beneficial due to its superior efficacy and minimum cardiotoxicity [3, 4]. Some of the commonly available liposomal based doxorubicin products are DOXIL, CAELYX and MYOCET. DOXIL was one of the first drugs to be approved that is delivered using liposome as a carrier [5]. Maryam *et al.* (2019) have made attempts to reduce the toxicity of doxorubicin by co-administering it with PEGylated liposomal galbanic acid (PLGba) as it could improve the outcome of the chemotherapy even with a reduced dose of PEGylated liposomal doxorubicin (PLD) [6]. Suchismita *et al. (2019)* formulated controlled release doxorubicin in the nanocomposite form using graphene grafted with chitosan and polyaniline aiming to reduce the toxicity [7]. Srikanth *et al.* (2019) have formulated doxorubicin proniosomes to reduce the systemic toxicity of the drug [8].

DOXIL/Caelyx has a longer circulation lifetime which is due to the steric barrier provided by the surface-grafted PEG, which leads to large changes in bio-distribution and particularly increased amounts of drug being delivered to the skin. This has advantages for the treatment of skin localized cancers such as Kaposi's sarcoma but disadvantages in the observation of new dose-limiting toxicities such as hand and foot syndrome [9].

While designing liposomal formulations of Doxorubicin, the following criteria's are kept in mind: (a) Doxorubicin is released from the liposomes following intravenous administration, (b) The distribution of doxorubicin following administration of a liposomal formulation is dependent on the biodistribution characteristics of the liposomes, and (c) The biological activity of liposomal doxorubicin is dependent on when, where, and at what rate the drug is released. So a prerequisite knowledge of the chemical and physical properties and the mechanism (s) of action of this drug, is necessary before formulation [10]. Naozumi *et al.* (2008) has studied different *in vitro* parameters of liposomal formulations formed with egg phosphatidylcholine (EPC) as phospholipid and combination of EPC and hydrogenated soya phosphatidylcholine (HSPC) as lipid components and compared them with those of DOXIL [11].

Doxorubicin is an amphipathic molecule possessing a water-insoluble aglycone (adriamycinone: $C_{21}H_{18}O_9$) and a water-soluble, basic, reducing amino-sugar moiety (daunosamine: $C_6H_{13}NO_3$). Structure of doxorubicin is given in fig. 1.

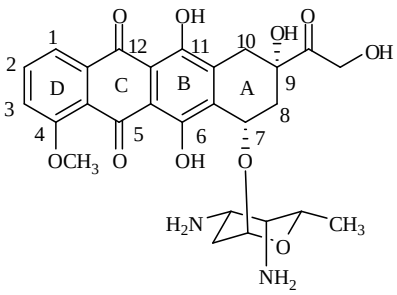


Fig. 1: Structure of doxorubicin

Doxorubicin has anti-mitotic and cytotoxic activity through the formation of complexes with DNA by intercalation between base pairs. It also inhibits topoisomerase II activity by stabilizing the DNA-topoisomerase II complex, preventing the religation portion of the ligation-religation reaction that topoisomerase II catalyzes [1].

Doxorubicin and its major metabolite, doxorubicinol, is 74-76% bound to plasma protein. The extent to binding is independent of plasma concentration up to 1.1 mcg/ml. Doxorubicin does not cross the blood-brain barrier.

Doxorubicin is capable of undergoing metabolism via 3 metabolic routes: one-electron reduction, two-electron reduction and deglycosylation.

However, approximately half of the dose is eliminated from the body unchanged. Two electron reduction is the primary metabolic pathway and it yields doxorubicinol, secondary alcohol [12].

FDA guideline for *in vitro* testing

FDA draft guideline gives recommendations regarding the *in vitro* bioequivalence study of injectable liposomal doxorubicin hydrochloride for product development and quality control of a generic formulation.

FDA's draft guidance for bioequivalence study mentions some of the following parameters which are to be studied. They are: liposomal composition, state of encapsulated drug, internal environment, liposomal morphology and number of lamellae, lipid bilayer phase transition, liposomal size distribution, grafted PEG at the liposomal surface, electric surface potential or charge and *in vitro* leakage under multiple conditions [13].

Liposomal composition

In liposomal composition following components are to be evaluated (a) Lipid content, (b) free and encapsulated drug, (c) internal and total sulfate and ammonium concentration, (d) histidine concentration and sucrose concentration [13]. Drug-to-lipid ratio and the percentage of drug encapsulation can be calculated from liposome composition values.

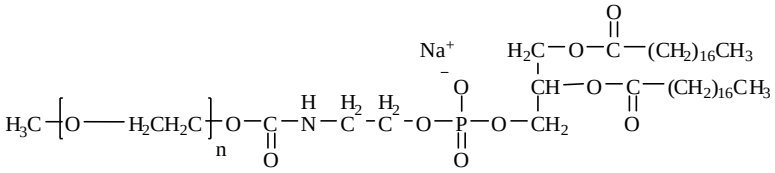
Reports show a strong association of liposomal composition and cytotoxicity and the key factor involved for this is the cellular uptake of the liposomes. PEGylation decreases the intracellular uptake of liposomes and consequently reduces cellular toxicity. Acyl chain length of lipid component influenced the release rate, cytotoxicity increases with short acyl chains as there is an increase in the release of doxorubicin with short acyl chains [14].

DOXIL/Caelyx liposomal formulation contains hydrogenated soya phosphatidylcholine (HSPC), cholesterol (Chol) and PEG-modified phosphatidylethanolamine in 55:40:5 molar ratio whereas Myocet has a different composition as it contains egg phosphatidylcholine (EPC) and Chol in 55:45 molar ratio [15].

Long and fully saturated acyl chains of HSPC confers tight packing of bilayer structure which helps liposomes for better retention due to very low permeability.

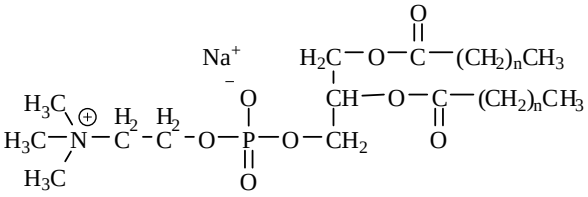
Cholesterol eliminates the phase transition of the phospholipids that helps to reduce the permeability of the liposome bilayer to ions and small polar molecules at a temperature range which is critical to the drug loading step during the manufacturing process. Concentration of cholesterol must be sufficient in the bilayer to achieve good drug loading and retention.

Microbubbles coated polyethylene glycol-di-stearoyl phosphatidyl-ethanolamine (MPEG-DSPE) chain confers the "stealth" property to the liposomes. Approximately 5% of MPEG-DSPE (on the basis of molar ratio) is present in DOXIL. Product with less than 2% MPEG-DSPE showed diminished "stealth" property hence, altered pharmacokinetics [12]. Structures of MPEG-DSPE and HSPC are given in fig. 2-3.



n ca. 45

Fig. 2: Structure of MPEG-DSPE (Microbubbles coated polyethylene glycol-distearoylphosphatidylethanolamine)



n, m = 14 or 16

Fig. 3: Structure of HSPC (Hydrogenated soybean phosphatidylcholine)

Mordente *et al.* (2009) studied and found that the presence of ammonium sulfate at optimal concentration inside the liposomes before the drug loading step is important to achieve high drug loading efficiency [12]. In addition, the sulfate inside of the liposomes complexes with doxorubicin to form a precipitate and is integral to drug retention and drug release.

Cersosimo *et al. (1989)* found that ion concentrations in a liposome helps to establish a stable and efficient drug encapsulation process. Ammonium ion controls not only the efficiency of the drug loading process, stability of the product and drug release profile of the product. Normal amounts of ammonia for a healthy adult range $0.5{\sim}1{\times}10^{-4}$ M, excess ammonia can potentially lead to severe neurodevelopmental and neurodegenerative complications [16-19].

***In vitro* leakage testing**

Doxorubicin liposomes are designed to retain doxorubicin during circulation and minimize clearance by the mononuclear phagocyte system and also limit uptake in healthy tissue. Table 1 shows the test conditions recommended by FDA to study the leakage from liposomal doxorubicin.

Factors affecting the release rate

There are several factors that can affect the release rate. Reported studies showed that only 10% drug is released in 50% plasma in normal saline at 37 °C after 48 h which could be due to insufficient temperature as the release rate is very low. Release in phosphate buffer solution (at all pH) was observed to be higher than the release rate in McLlvaine buffer. Release rate further increased with an increase in dilution. Effect of ultrasound radiation was due to temporary disruption of lipid layer causing rapid release of the drug but with an amplitude-dependent manner. Ultrasound also causes an increase in the particle size.

Shibata *et al.* (2015) reported that physical stability of bilayer, chemical stability of phospholipids, solubility of drug encapsulated in the liposomes affect the drug release from the liposomes. Increase in the concentration of lysophospholipids and free fatty acids was found to have a clear pH dependence but ultrasound irradiation does not cause an increase in the concentrations of lysophospholipids or free fatty acids. When the concentration of lysophospholipids and free fatty acids is 12.5% of total lipids or more permeability of lipid bilayer and hence release rates have been found to increase synergistically at pH 5.5 [20].

In a study, conducted by Luo *et al.* (2016), it was found that Near Infrared radiation (NIR) triggered the release of doxorubicin (Dox) was achieved by including 2 molar % porphyrin-phospholipid (PoP) from conventional sterically stabilized stealth liposomes [21].

Table 1: Different conditions along with reasons for testing of drug leakage

Conditions	Reasons	*In vivo* organ which it simulates	References
At 37 °C in 50% human plasma for 24 h.	Evaluate liposome stability in blood circulation.	Plasma mostly mimics blood conditions.	[13]
At 37 °C with pH values 5.5, 6.5, and 7.5 for 24 h in the buffer.	Mimic drug release in normal tissues, around cancer cells, or inside cancer cells.	Normal tissues: pH 7.3 Cancer tissues: pH 6.6 Insider cancer cells (endosomes and lysosomes): pH 5–6 (endosome and lysosomes of cancer cells may be involved in liposome uptake and induce drug release).	[13]
At a range of temperatures (43, 47, 52 and 57 °C) in pH 6.5 buffer for up to 12 h or until complete release.	Evaluate the lipid bilayer integrity.	The Tm of lipids is determined by lipid bilayer properties such as rigidity, stiffness and chemical composition. Differences in the release as a function of temperature (below or above Tm) will reflect small differences in lipid properties.	[13]
At 37 °C under low-frequency (20 kHz) ultrasound for 2 h or until complete release.	Evaluate the state of the encapsulated drug in the liposome.	Low-frequency ultrasound (20 kHz) disrupts the lipid bilayer via a transient introduction of pore-like defects and will render the release of doxorubicin controlled by the dissolution of the gel inside the liposome.	[13]

State of doxorubicin

Doxorubicin is commonly found as a salt form such as sulfate, citrate *etc.,* in the liposomes. Electron microscopy images showed that doxorubicin sulphate containing liposomes were ellipsoidal containing dark stripes. X ray diffraction pattern showed by Lasic *et al.* (1992) has a single sharp reflection at 27 Å which states that the DOX-sulphate interfibre spacing is 27 Å [22].

In another study on doxorubicin citrate, cryo-Transmission Electron Microscopy (TEM) images showed an interfibre spacing for DOX-citrate to be 30-35 Å. NMR studies on ^{13}C-citrate revealed that there exists a dynamic interaction between Dox-citrate with the rapid exchange of free and bound citrate.

Studies carried by Xingong *et al.* (1998) showed that interfibre space difference may be due to the fact that sulphate is a small ion than citrate. DOX-citrate showed different patterns were observed such as hexagonal arrangement, U-shaped arrangement, circular bundles and fibrous bundles with repeating units after 50 nm [23].

In contrast DOX-sulphate aggregates were noted as only straight bundles in DOX-sulphate.

Internal volume

The internal environment of a liposome mainly includes volume, pH, sulfate and ammonium concentration helps to maintain the precipitated doxorubicin [14].

Entrapment efficiency is directly related to the internal volume. Entrapment efficiency can be determined by using the following formula:

$$EE\% = [\frac{(C_i - C_f)}{C_i}] 100$$

It is an important parameter to measure the morphology of liposomes [24, 25]. In a study it that relatively low volume of entrapped aqueous space per mole of lipid was observed in the case of both MLV (multilamellar vesicles) and SUV which results in restricted ability to encapsulate large macromolecules. In MLV most of the lipid is participating in the internal lamellae, and there is a restriction of the internal water space due to the close apposition of the concentric adjacent bilayers. In case of SUV, which are single-compartment vesicles, due to large ratio of surface area to encapsulated volume only a small aqueous volume per mole of lipid can be attained [26]. Techniques such as reverse phase evaporation have been developed to prepare liposomes such that a large percentage of aqueous materials can be entrapped in the internal volume [27].

Internal pH

Liposomes are usually prepared by pH gradient method either by using citrate buffer or ammonium sulfate buffer. The internal pH was reported to be around 4 in the case of both citrate and ammonium sulfate buffers and was stable for at least 20 d [28]. Internal pH has an effect on the stability of the drug. Liposomal system loaded with curcumin was developed with an acidic internal microenvironment pH has an effect on chemical stability and anticancer activity [29].

Number lamellae and degree of lamellarity

PEGylated liposomal doxorubincin is an oval-shaped and unilamellar liposome [30]. It is important to study lamellarity as the extent the encapsulation efficiency, drug retention, the efflux rate of liposomally encapsulated material, and the fate of a drug after cellular uptake depends greatly on the lamellarity of liposomes [31, 13]. The degree of lamellarity refers to the number of lipid bilayers in a liposome. A liposome may have a single lipid bilayer, or multiple concentric lipid bilayers. Studies show that DOXIL has approximately 1 to 2% multilamellarity, the remaining 98 to 99% of the liposomes have only a single lipid bilayer. When multilamellar vesicles percentage is high, entrapped volume significantly decreases which results in lower drug-to lipid ratio. The net effect is that lower drug is loaded in the liposomes which leads to lower efficiency and higher toxicity. Lower drug release rate has been found in multi-lamellar vesicles as the drug has to pass larger number of lipid bilayer barriers before reaching to the external phase of the liposomes [32].

Particle size distribution

D50 and Polydispersity index (PDI) given as (D90–D10)/D50 is usually calculated using the population bioequivalence approach (PBE). It is also required to be measured according to the FDA draft guideline along with particle size distribution. To describe the particle size distribution of liposome [33].

The particle size and PDI of nanocarrier systems have a strong influence on the endocytosis-dependent cellular uptake.

Particles smaller than 50 nm can interact with hepatocytes, while particles larger than 1 µm are absorbed by mononuclear phagocytes as emboli [34].

It has been found that nanocarriers with particle size less than 150 nm are able to cross the fenestrated capillaries in the tumor microenvironment. Tumors have leaky vasculature which allows accumulation of high molecular weight therapeutics in them. This effect is known as the enhanced permeability and retention (EPR) effect. Through this, circulating nanocarriers with size less than around 150 nm extravasate from the circulation and accumulates within the tumour [35-38]. In some literature, the size below 200 nm has been mentioned. Reports have shown that by decreasing the diameter size of the liposomes to 50 nm or below reduced mononuclear phagocyte system (MPS)-mediated clearance in mice

models with comparable a plasma half-life as of long-circulating (PEGylated) vesicles with a diameter of 100–150 nm [39-40].

Lipid bilayer phase transition

The gel to the liquid-crystalline phase transition of the lipid bilayer can be determined by differential scanning calorimetery (DSC) and it provides information about bilayer fluidity and uniformity, drug interactions with the lipid bilayer and liposome formation [41]. The fluidity of lipid bilayer depends on the T_C which is the chain melting transition temperature. Phospholipid exists in different states above and below T_C.

Presence of cholesterol has been reported to enhance the mechanical stability of the membrane. Studies carried by Lorena *et al.* (2012) showed that low cholesterol contents lead to a phase-segregated system but high cholesterol contents gives a homogeneous bilayer [42].

PEG grafted

Standard liposome formulations such as Caelyx® (DOXIL® in the United States) are coated with polyethylene (PEG) which is a synthetic hydrophilic polymer. The bulky PEG head group serves as a barrier preventing interactions with plasma opsonins, hence, it retards recognition by the reticuloendothelial system and slowing elimination of the liposomes from circulation. These PEG-coated liposomes are known as sterically stabilized or STEALTH liposomes (fig. 4) [43].

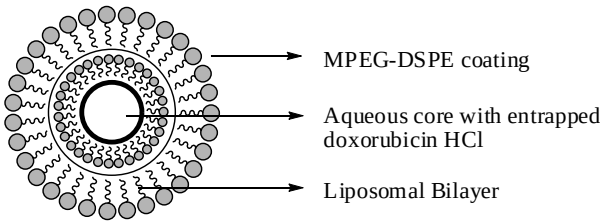


Fig. 4: Structure of stealth lipososme, *MPEG: Microbubbles coated Poly ethylene glycol, DSPE: Distearoyl phosphatidyl ethanolamine

Greater than 90% of the drug is encapsulated as STEALTH liposomes [23].

Jianwei *et al.* (2018) co-encapsulated doxorubicin and verapamil into gold liposomes to overcome the multi-drug resistance as verapamil inhibits multi drug resistance protein [44].

PEG grafts can be present in two different configurations: as a random coil grafted in one end ("mushroom") or as an approximately linear polymer ("brush").

Surface PEG conformation (mushroom or brush conformation) determination was based on the calculated ratio of the Flory dimension (R_f) to the average distance between adjacent PEG chains (D). PEG Flory dimension and the distance between surface grafted PEG chains were calculated using the given equation.

$$R_f = aN^{3/5}$$

Where a is PEG monomer size in Å (previously reported as 3.8 Å [45], N is the degree of polymerization,

$$D = \left(\frac{A}{M}\right)^{1/2}$$

A is the PEG area per lipid molecule in the bilayer (previously reported as 67 $Å^2$ [46]) and M is the mole fraction of PEG lipid determined experimentally as described above R_f/D values calculated below 1.0 indicates a mushroom regime, while those above 1.0 indicate brush [47].

Electric surface potential or charge

Surface charge (zeta potential) on liposomes has a significant effect on the clearance, tissue distribution and cellular uptake.

Cancer cell surfaces are usually charged negatively due to the translocation of negatively charged constituents of the inner layer of the cell membrane (e. g., phosphatidylserine, anionic phospholipids, glycoproteins and proteoglycans), the cationic liposomal doxorubicin (LPs-DOX) will have increased uptake due to better interaction between negatively charged cancer cells and positively charged (LPs-DOX) [48].

A strong linear relationship was noted between zeta potential values and the mole percentage of charged lipids within a liposome [49].

Negatively charged liposomes have been reported to be immunostimulant but zwitterionic ones were not. [50-51].

CONCLUSION

Parameters for *in vitro* bioequivalence study for generic product development of liposomal doxorubicin have been described in FDA draft guideline. The review helps to understand the importance of each physicochemical parameter critical for the formulation of a liposomal form of doxorubicin. Role of each component has been described which will be useful in formulation development scientist as well as to an analytical chemist.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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