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Validation of high-performance liquid chromatography method for the determination of doxorubicin in proliposomal drug delivery system formulation

Mine Diril*, Mehmet Ali Ege, H. Yeşim Karasulu

Ege University, Faculty of Pharmacy, Department of Pharmaceutical Technology, 35100, İzmir, Türkiye

Abstract

The objective of this study to develop a novel proliposome formulation containing Doxorubicin (Dox) and was to validate a sensitive and selective reversed-phase high-performance liquid chromatographic (HPLC) method for the evaluation of Dox concentrations of proliposome formulation. The samples were chromatographed on C18 column (Zorbax Eclipse Plus 5µm 4.6 x 250 mm) using a mobile phase with Sodium Lauryl Sulphate solution:Acetonitrile (50%:50%) at 254 nm. Linearity was confirmed in the concentration range of 10.0–75.0 µg/mL. Specificity, linearity, working range, LOD, LOQ, accuracy, precision, robustness, and system suitability studies were done from HPLC validation parameters. Liposome formulation containing Dox was developed by pH gradient method then proliposome formulation was developed with lyophilisation technique. In the developed HPLC method, the encapsulation capacity (EE%) was found to be 90% ± 0.5 and the drug loading capacity (DL%) was found to be 100.0% ± 0.3. In vitro release studies and stability study results were evaluated with, validated HPLC method. It was observed that developed Dox-proliposome formulation increased Dox release at pH 5.5, pH 6.5, and pH 7.5 by 23.9%, 30.2%, and 14.8%, respectively, compared to commercial products. The result of F2 test performed in pH 7.5 media was 51.4%. According to the results of the physicochemical tests performed within the stability studies, it was observed that there was no significant change at the end of 12 months. These results show that the HPLC method developed, and validation study performed are important and applicable in the development, characterization, in vitro release, and stability studies of the novel proliposome formulation.

Keywords: Doxorubicin; proliposomal drug delivery systems; high-performance liquid chromatography; validation; in vitro release tests

1. Introduction

Doxorubicin (Dox) (Fig. 1) is a chemotherapeutic agent belonging to the anthracycline group [1,2]. Its primary mechanism of action is that Dox intervenes in DNA base pairs, causing DNA breakage, inhibiting both DNA and RNA synthesis. Dox inhibits topoisomerase II enzyme, causing DNA damage and induction of apoptosis and it is usually administered intravenously at 21-day intervals [3]. Side effects such as fatigue, hair loss, nausea and vomiting, and mouth sores are common after Dox administration, also associated with noteworthily cardiac toxicity so this limiting the long-term use of the drug [4,5]. Therefore, it is very important to develop formulations that will reduce the toxicity of Dox.

Lipids and fatty acids considered as the primary component of liposomes; They are structures that are considered biocompatible and biodegradable because they are found in the natural structure of cell membranes. Liposomes have become very interesting in recent years as they are versatile drug delivery systems suitable for the encapsulation of both hydrophilic and hydrophobic substances. Moreover, these vesicular structures allow the encapsulation of both small ion-sized molecules and large molecules of several hundred thousand Daltons [6]. Liposomal encapsulation of drug molecules; it reduces systemic toxicity and improves tolerable dosing regimens for anticancer drugs. However, liposomes exhibit poor chemical and physical stability due to oxidation and hydrolysis of the lipids in their structure, which limits their shelf life.

Physical instability of the aqueous dispersion occurs due to vesicle aggregation and fusion of liposomes, leading to a change in vesicle size and leakage of the active substance. Chemical instability is associated with the tendency for hydrolysis and oxidation of phospholipids in liposomal drug delivery systems. Oxidation and hydrolysis of lipids can lead to the formation of short-chain lipids and subsequently less hydrophobic derivatives in the bilayers, resulting in the formation of liposomes with altered physicochemical

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Author of correspondence: mine.diril@gmail.com
Tel: +90 (232) 311 39 81
Fax: +90 (232) 388 52 58

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properties or disruption of the liposome structure and and alteration of the drug release profile [7–9]. Therefore, removal of water in their structure can reduce or completely eliminate both hydrolysis and oxidation reactions [7]. Proliposome drug delivery system formulations have been developed to overcome the stability problems associated with the liposome [8]. Proliposomes are dry, free-flowing granular products that form liposomal dispersion on hydration or contact with biological fluids in the body and were discovered in 1986. They consist of water-soluble porous powder and phospholipids [9–11]. Proliposomes can be obtained by various methods such as fluidized bed method, supercritical anti-solvent method, lyophilization method [12].

The development and validation of analytical methods play important roles in the discovery, development, production, and stability monitoring of pharmaceuticals. The main purpose of the analytical method development and validation is to prove that proposed analytical method is accurate, specific, precise, and robust in the pharmaceutical industry for drugs. Therefore, analytical methodology development and validation has become essential activity for the development of new drug delivery systems [13,14]. Studies involving methods developed in recent years for the determination of Dox from drug delivery systems are listed in Table 1.

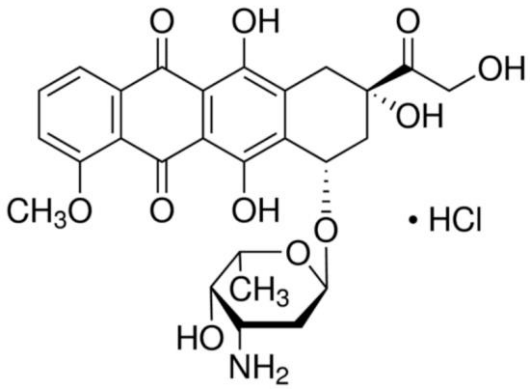

Figure 1. Doxorubicin-HCl chemical structure [15]

In this study, HPLC method was developed and validated to determine drug loading capacity, encapsulation capacity, evaluated in vitro release studies for different pH (pH 5.5, pH 6.5, and pH 7.5) and determined stability studies of Dox in the developed novel proliposome formulation. While HPLC methods were recommended for the determination of Doxorubicin in the developed liposome formulation containing Dox, no study was found in which Dox validation parameters were applied to the proliposome formulation and the results were shared in detail. Our study will contribute to the literature in this respect.

Table 1. List of quantification methods for Dox in pharmaceutical drug delivery systems last years

	Method	Purpose	Year	Ref.
Rus et al.	UV-Vis	Dox Evaluation From Drug Delıvery Systems	2021	[16]
Laxmi et al.	HPLC	Dox Determination Co-crystal	2019	[17]
Scheeren et al.	HPLC	Determine Dox in pH-sensitive chitosan nanoparticles	2018	[18]
Du et al.	HPLC	Dox Evaluation From Gold Nanoparticles	2018	[19]
Gowda et al.	RP-HPLC	Determination of Dox in Pure and Pharmaceutical Dosage Forms	2017	[20]

2. Experimental

2.1. Chemical and reagents

Doxorubicin hydrochloride (Dox) (99.8% purity) was selected as the active pharmaceutical ingredient donated from by DEVA Holding (Istanbul, Türkiye). Hydrogenated Soy L-α-phosphatidylcholine (HSPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPG), Cholesterol and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol) (DSPE-PEG(2000) Carboxylic Acid) were obtained from Avanti Polar Lipids (Birmingham, United States). Mannitol was obtained from Roquette (Lestrem, France). Sodium Lauryl Sulphate and ortho-phosphoric acid were purchased from Sigma (Burlington, United States). HPLC-grade acetonitrile was obtained from Carlo Erba (Val-de-Reuil, France). All chemicals used throughout the study were pharmaceutical grade or special analytical grade. Ultrapure water for all analyses was purified using the Millipore Direct-Q® 3 Water Purification System.

2.2. Instrumentation and analytical conditions

The HPLC-PDA system consisted of Shimadzu model of LC-20AT and PDA detector in series connected to a computer loaded with, LC Solution post-run programme (Duisburg, F.R. Germany). The chromatographic separation was performed on an Agilent Zorbax Eclipse Plus -C18 analytical column (150 mm × 4.6 mm, 5 µm particle size, and 100 Å pore size). In addition, PDA detector was set at 254 nm. For the mobile phase, 2.88 grams of Sodium Lauryl Sulphate was weighed accurately and dissolved by adding approximately 990 mL of ultrapure water. The pH was adjusted to 2.5 by adding ortho-phosphoric acid and completed with water to a volume of 1000 mL. Acetonitrile was mixed with 50%-50% (v/v) of this solution and degassed in an ultrasonic bath for 10 minutes. The solution was filtered through a 0.2 µm membrane filter. For the analysis, isocratic solvent elution was performed at a flow-rate of 1 mL/min. The column temperature was maintained at 25 °C and the injection volume was 5 µL. On each day of

analysis, mobile phase flow was allowed from the column to equilibrate for 30 minutes.

2.3. Preparation and characterization of proliposome formulation

Liposome formulation was developed by a lipid hydration method and proliposome formulation was developed by lyophilisation technique. Because of Dox showed weak basic properties, pH gradient method was used to highly encapsulate the Dox in the liposome formulation [21,22]. Citric acid-sodium citrate buffer and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer systems (HEPES) were used to create the pH gradient method. Details of the liposome formulation developed by the lipid hydration method by creating a pH gradient are stated in the publication of the study by Mine et al [23]. Mannitol as lyoprotectant was added to the liposome dispersion and dissolved. Proliposome formulation was obtained by applying the lyophilisation. The liposome formulation samples were frozen at -65 °C. They were then subjected to gradual heating to 20 °C at 0.01 mbar pressure for 18 hours for primary drying in a lyophilization device. Secondary drying was carried out for 3 hours at 25 degrees Celsius under 0.01 mbar pressure. Characterization studies such as pH, viscosity, particle size (PS), polydispersity index (PDI), zeta potential (ZP), water content % and reconstitution time of proliposome formulation were performed. All analyses were carried out by dispersing, except water content, developed proliposome formulation in 5% dextrose solution (C_{Dox}=2 mg/mL). The pH of Dox-proliposome formulation was measured using pH meter (Mettler Toledo, Switzerland) and viscosity was measured at 37 ± 1 °C by using Ula spindle in a viscometer (Brookfield DVII + Pro, USA). The PS, PDI, and ZP values of the Dox-proliposome formulation were determined by using zetasizer (Malvern Nano ZS, England) device at room temperature (25 ± 2 °C). The water content of formulation was evaluated by Karl Fischer titration instrument (SI Analytics 7500, Germany). For reconstitution time analysis, the time taken for the developed Dox-proliposome formulation to be dispersed in 5% dextrose solution was observed with a stopwatch.

2.4. Preparation of Standard solutions and samples

2.4.1. Stock standard solution

20 mg of Dox working standard was weighed accurately and transferred to a 100 mL flask, made up to volume with mobile phase, and mixed by shaking (main stock solution). 2.5 mL of this solution was taken and transferred to a 10.0 mL flask. For encapsulation efficiency and drug loading capacity, it was made up to volume with the same solvent and mixed by shaking. To evaluate in vitro release studies, it was made up to volume with pH 5.5, pH 6.5, and pH 7.5 phosphate buffer, separately. They were filtered through a 0.20 µm PTFE membrane filter (Sartorius Minisart SRD, Germany) then injected into the HPLC ($C_{Dox\ standard}$ = 50 µg/mL).

2.4.2. Sample Proliposome solution preparation

To extract the drug from the proliposomal matrix, formulations were first mixed with 5 mL mobile phase then sonicated for 10 min. Then the solution was made up to 10 mL with mobile phase to a concentration of 50 µg/mL. The sample was filtered through a 0.20 µm PTFE membrane filter and injected HPLC. For the specificity parameter, placebo samples were prepared. Dox-free proliposome formulations were transferred to a 10 mL flask. Dox-free proliposome formulations were first mixed with 5 mL mobile phase and then sonicated for 10 min. The final solution was made up to volume with the mobile phase to a concentration of 50 µg/mL. They were filtered through a 0.20 µm PTFE filter and injected into the HPLC.

2.5. Validation of the HPLC method

The method has validated the requirements of the International Council for Harmonisation Q2(R2) guidelines. For this purpose, specificity, linearity, working range, accuracy, precision, robustness, and system suitability parameters were studied [24]. For the specificity parameter; Dox standard solution, unloaded proliposome formulation (placebo) solution, and doxorubicin-loaded proliposome (Dox-proliposome) formulation solution were injected. All peaks purity was analysed by PDA detector.

The linearity analysis was established through preparation of six concentration levels of standard linearity curve in the concentration range of 10.0–75.0 µg/mL (n=3). The calibration curve was plotted using the average of area versus known concentration. In order to evaluate in vitro release studies and to observe the effect of buffer solutions (pH 5.5, pH 6.5, and pH 7.5) to be used in the release medium, these buffer solutions were used as dilution solutions, and linearity, LOD, LOQ, and working range parameters were validated. Linearity assessment was done by the analysis of relative standard deviation of the slope (Sb%), y-residuals and correlation coefficient (r^2) at a confidence level of 95%.

The LOD and LOQ were evaluated from the signal-to noise ratio of chromatograms for blank samples (S/N=3 for LOD and S/N=10 for LOQ). Then it was expressed in concentration of via the relation with the signal-to-noise ratio of a 10.0 µg/mL spiked blank [25]. For the working range analysis, six injections were studied with lowest concentration 10.0 µg/mL and as highest concentration

of 75.0 µg/mL (for mobile phase, pH 5.5, pH 6.5, pH 7.5, separately). In addition, RSD% was evaluated for solvents.

Accuracy is the nearness of a measured value to the true or accepted value. Accuracy was evaluated by the standard addition method with placebo solutions. For the accuracy analysis; Dox-proliposome formulation solution samples were prepared at 80%, 100%, and 120% levels of assay test concentration and calculated recovery. A total of 9 samples were prepared, 3 for each level. The recovery% calculation is given in Equation 1.

$$Recovery, \% = \frac{Concentration\ of\ analysis\ results}{Theoretical\ concentration} \times 100 \quad (1)$$

The precision of the method was determined by intra-day and interday studies. After the system suitability check with six standard solution injections, repeatability was performed by analyzing six samples of Dox-proliposomal formulation at the same concentration (50 µg/mL). The intermediate precision was evaluated by performing the analysis on two different days (interday) and also by different operators performing the analysis (interanalyst). For precision analysis, the standard deviation must be less than 2.0%.

The robustness of an analytical method indicates its ability to remain unaffected by small changes made in the parameters of the analytical method. For robustness study, one standard and two sample solutions were prepared. For each changed condition, six injections of the standard solution and two injections of the sample solutions were analyzed. These minor changes for method were mobile phase buffer pH (± 0.2), flow rate (± 0.1 mL/ min), and column temperature (± 5 °C). Theoretical plate number, retention time, and tailing factor were observed for each condition. RSD% of Dox assay between normal and modified working conditions were evaluated.

System suitability was determined from six replicate injections of standard solution containing 50 µg/mL of Dox. For the acceptance criteria; relative standard deviation (RSD%) was found less than 2% for peak area and retention time, greater than 2000 theoretical plates number, and tailing factor of less than 2.0 [26].

2.6. Studies with validated analytical method

2.6.1. *Drug loading capacity.*

Extraction of Dox encapsulated in proliposomal vesicles is important for the determination of drug loading capacity. For this purpose, an aliquot of the Dox-proliposom formulation was diluted in 5 mL mobile phase and sonicated for 10 min to extract the drug. The final solution was made up to 10 mL with the mobile phase at a concentration of 50 µg/mL. It was calculated relative to a reference solution of the same concentration and represents the total drug content in the formulations.

2.6.2. *Encapsulation efficiency*

Encapsulation efficiency (EE%) of proliposome formulations was found by ultracentrifugation technique with Amicon Ultra 100 kDa EMD-Millipore (Billerica, MA). The unencapsulated drug was separated by ultrafiltration technique (Sigma 2-16P Centrifuge, Sigma, Germany) at 5000 rpm for 40 min. EE% was evaluated as the ratio of the analyzed drug amount to the initial drug amount using the HPLC method [27].

2.6.3. *In vitro release study*

The release conditions recommended by FDA in liposomal formulations containing Dox are pH 5.5 as endosomes and lysosomes of cancer cells, pH 6.5 as cancer tissues and pH 7.5 as normal tissues [28]. In vitro release studies were performed at different pH phosphate buffers (pH 5.5, pH 6.5, and pH 7.5) conditions using the dialysis membrane method. Proliposomal formulation was dispersed 5% dextrose solution (C_{Dox}= 2 mg/mL) put in dialysis bag (Sigma-Aldrich, 14,000 Da molecular weight cut-off) and the dialysis bags were put into 100 mL of pH 5.5, pH 6.5 and pH 7.5 phosphate buffer solution, respectively. The systems were maintained at 47 °C in a stirred water bath at 100 rpm. 2 mL of samples were taken at predetermined time intervals (0, 0.5, 1, 2, 3, 6, 8, 12, 20, and 24 h) and then added with 2 mL of fresh buffer to maintain constant volume and sink conditions. Studies were also performed for commercial products. Calculation of the cumulative quantification of Dox was performed by HPLC method. To evaluate the effect and release results of the different release media used, the method was co-validated for linearity and working range using release media (PBS pH 5.5, 6.5, and 7.5) as diluent [18,29]. In addition, F2 similarity analysis was performed between the commercial product and the Dox-proliposome formulation.

2.6.4. *Stability study*

The stability of Dox-proliposomal formulation was investigated at 5 ± 3 °C, 25 ± 2 C, 60% ± 5 relative humidity, 30 ± 2 C, 65% ± 5 relative humidity, and 40 ± 2 °C, 75% ± 5 °C relative humidity. The sample was evaluated for physicochemical properties (pH, viscosity, PS, PDI, ZP, EE%, DL%, water content % and reconstitution time) under these stability conditions.

2.7. Statistical analysis

Student-t test or ANOVA variance analysis for parametric tests and Krusger Wallis analysis for nonparametric tests were preferred. *$p < 0.05$ and ***$p < 0.001$ were considered significant and highly significant, respectively.

3. Results and Discussion

3.1. Preparation and characterization of proliposome formulation

The ratios of lipids, cholesterol, and pegylating agent were determined according to the pegylated liposomal commercial formulation containing of the active ingredient Dox (mol ratios phospholipid%:cholesterol%:pegylating agent%; 56:38:5) [30]. The physicochemical properties of Dox-proliposome formulation were done dispersed 5% dextrose solution, except the water content, and the results of physicochemical properties are shown Table 2. PDI is a measure of the heterogeneity of a sample based on size and distribution [31]. In addition, less than 0.5 PDI value indicates homogenous and stable particles [32]. The freeze thaw cycle enables the conversion of liposomal vesicles from MLVs to SUVs (Small unilamellar Vesicles) and Large Unilamellar Vesicles (LUVs), thus affecting PS and PDI results, while the extrusion process affects PS results [33]. As seen from the physicochemical results, low PS and acceptable PDI results were obtained, indicating that the extrusion and freeze thaw cycle processes applied to the Dox-proliposome formulation were successful. By lyophilizing liposomal formulations; hydrolysis, oxidation, drug leakage, and aggregation can be prevented by removing aqueous structures from formulations [34].

In lyophilization, substances that protect formulations from stress conditions during freezing and drying are known as lyoprotectants. During the lyophilization phase of liposomal drug delivery systems, lyoprotectants maintain the stability of liposomes by protecting the lateral spaces of the head groups of phospholipids [35]. Mannitol, a lyoprotectant, is one of the excipients frequently used in liposomal drug carrier systems, which reduces redistribution time by providing cake formation after drying [36]. For this purpose, effective drying studies were carried out using Mannitol as a lyoprotectant in the study.

Effective drying accelerates the dispersion of particles and reduces the reconstitution time. Also, since the water contained in liposomes causes hydrolysis and oxidation, effective drying is very important to ensure stability [37]. In addition, water content analysis is very important to predict whether efficient drying is achieved by lyophilisation. The results of low water content and fast reconstitution time indicate that efficient drying was achieved by lyophilization in Dox-proliposome formulation.

Table 2. Physicochemical properties of Dox-proliposome formulation

Physicochemical properties	Results ± SD
pH	6.50 ± 0.95
Viscosity (cP)	12.63 ± 1.25
PS (nm)	120.6 ± 2.50
PDI	0.35 ± 0.21
ZP (mV)	-8.45 ± 1.25
Water Content (%)	0.053 ± 0.03
Reconstitution time (sec)	11.5 ± 1.1

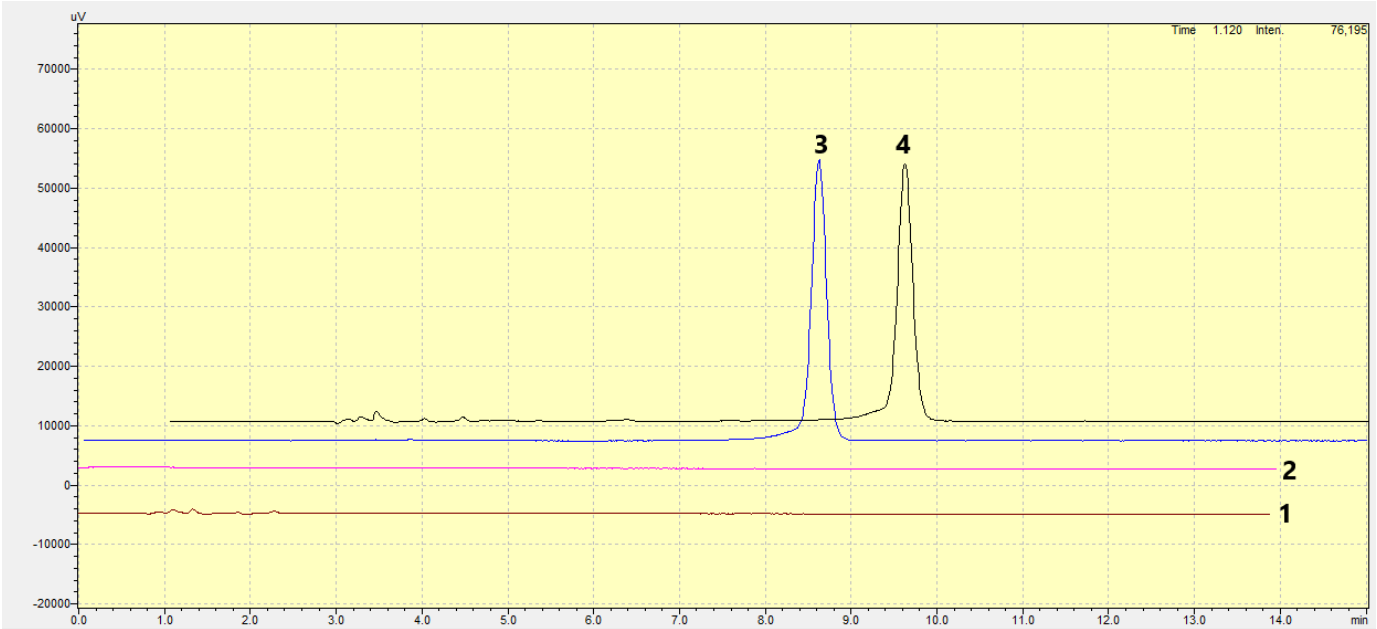

Figure 2. HPLC chromatograms from specificity parameter injection on HPLC where trace (1) represents unloaded proliposome formulation solution, (2) represents mobile phase, (3) represents Dox standard solution (10 µg/mL), (4) Dox-proliposome formulation solution

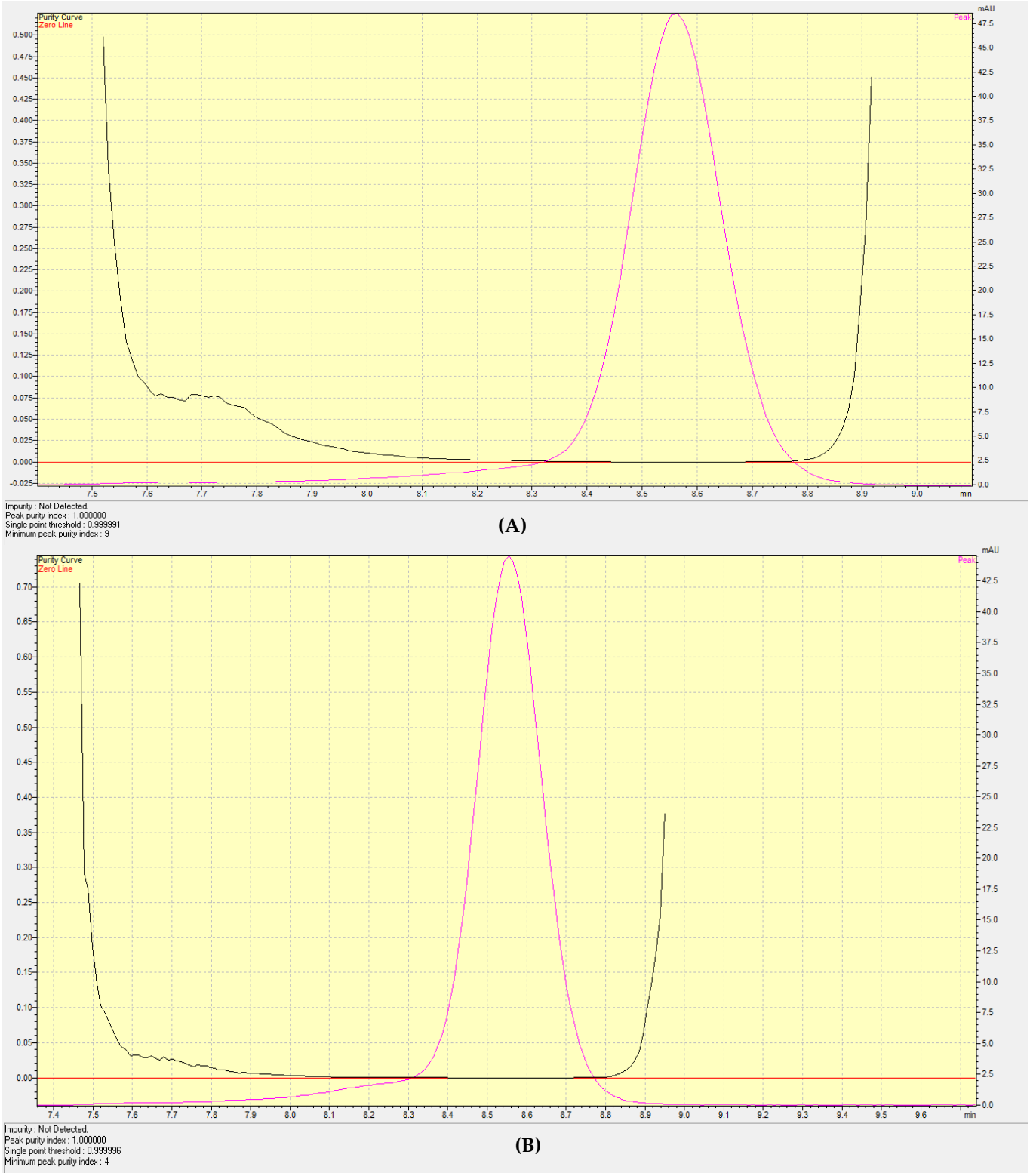

Figure 3. Peak purity of **(A)** Dox in standard solution, **(B)** Dox-proliposome formulation solution

3.2. Validation of the method

The specificity of the method for the quantitation of Dox was successfully achieved by demonstrating that there was no interference from the matrix components. The chromatograms (Fig. 2) showed that no signal was detected for the mobile phase and unloaded proliposome formulation solution. In order to show no interference in the HPLC method, peak purity testing was performed using PDA detector.

The peak purity index of chromatograms was evaluated for Dox standard and Dox-proliposome solutions and was higher than 0.9999. This indicates that there is no interference to the Dox peak from excipients or impurities. All these findings indicate that the method is specific (Fig. 3).

The data for the analytical curves constructed (n=3) suggest acceptable linearity parameter of concentration range of 10 – 75 μg/mL for mobile phase, pH 5.5, pH 6.5,

and pH 7.5, respectively. The linear regression equation was determined by the least squares method and thus the correlation coefficients were calculated, these results are shown in Table 3. LOD and LOQ were calculated using mobile phase, pH 5.5, pH 6.5 and pH 7.5 as solvent and the results are given in Table 3.

For the operating range analysis, the RSD% results of six injections at 10 µg/mL and 75 µg/mL in each solvent medium were calculated and the results are given in Table 4. Acceptance criterion of RSD% <2% was achieved.

Values between 98% and 102% are acceptable for accuracy analysis [38]. The recovery results for the accuracy parameter were between 98% and 102% for each concentration, with an RSD% of <2%, achieving the acceptance criterion (80%, 100%, and 120%) (Table 5).

For the precision parameter six different Dox-proliposome formulations batches, containing 50 µg/mL Dox, were extracted as part of the repeatability study and analysed. In the intermediate precision study performed as part of the precision parameter, six separate proliposome formulation samples were studied on two separate days and by two separate analysts. The results are shown in Table 5 and the RSD values obtained are lower than the 2.0% acceptance criterion.

The robustness test demonstrates the reliability of the analytical method against certain changes in parameters [39]. The method was considered robust because RSD% values for Dox assay were below 2% as seen in Table 6. System suitability tests represent an integral part used to ensure adequate performance of the analytical procedure and the chromatographic system for resolution and reproducibility [40]. For system suitability analyses, RSD% values calculated for the peak area, retention time and tailing factor were found 1.28, 0.11, 0.43, and 0.02%, respectively. The number of theoretical plates was found 16601.68 and RSD% 0.43%.

The data of all parameters performed in method validation studies were found within the acceptance criteria. This shows that the method can be applied successfully and is suitable for the analyses to be carried out. The advantages of these developed methods compared to the method in this study are evaluated in Table 7.

3.3. Studies with validated analytical method

As with all drug delivery systems, it is very important to achieve high drug loading capacity and encapsulation capacity in liposomal drug delivery systems during formulation development. To achieve optimum efficacy in a drug delivery system, it is necessary to encapsulate the maximum possible amount of toxic active substances, especially as Dox.

Table 3. Linearity, LOD, and LOQ results (Concentration range: 10–75 µg/mL)

Solutions	Equations	R^2	LOD (µg/mL)	LOQ (µg/mL)
Mobile phase	$y = 12180037.469x + 7767.114$	0.9996	1.5	4.6
pH 5.5	$y = 12240914.436x - 3262.502$	0.9999	0.7	2.2
pH 6.5	$y = 12016875.912x + 629.902$	0.9994	1.9	5.6
pH 7.5	$y = 9785246.7153x - 10345.3163$	0.9996	0.8	2.3

Table 4. Working range analysis RSD% results for solvents

Concentration	RSD%			
(µg/mL)	Mobile phase	pH 5.5	pH 6.5	pH 7.5
10	0.7	0.7	0.2	0.8
75	0.1	0.2	0.1	0.5

Table 5. Repeatability, accuracy, and intermediate precision data of Dox-proliposome formulation solution

Validation parameters	Recovery ± RSD (%)
Accuracy (n= 3)	
40 µg/mL	100.33 ± 0.85
50 µg/mL	100.73 ± 0.76
60 µg/mL	99.57 ± 0.35
Precision	
Intraday (n= 6)	101.00 ± 1.2
Interday	
Day 1 (n=6)	99.60 ± 0.56
Day 2 (n=6)	99.30 ± 1.21
Mean (n= 12)	99.45 ± 0.21
Between-analysts	
Analyst 1 (n=6)	100.23 ± 0.74
Analyst 2 (n=6)	99.56 ± 0.96
Mean (n= 12)	99.90 ± 0.47

Table 6. Results of robustness parameter for Dox-proliposome formulation solution

Chromatographic parameter	Theoretical plates	Retention time (min)	Tailing Factor	Dox Assay (%)
Normal working condition	16459	8.2	0.94	99.7
pH 2.3	14599	7.9	0.96	98.3
pH 2.7	15340	8.2	0.93	99.4
Flow rate 0.9 mL/min	16229	8.4	0.94	99.1
Flow rate 1.1 mL/min	15322	8.1	0.96	99.2
Column temperature 20° C	16521	8.3	0.95	98.8
Column temperature 30° C	16673	8.1	0.96	98.6
Mean				99.0
RSD(%)				0.49

Table 7. Advantages of the method developed in this study compared to the literature

	Advantages
Scheeren et al. [18]	They showed similar retention times and peak shapes, but were studied with a higher injection volume (20 µl) than our method.Long use may cause peak shape distortion and low theoretical plate count.
Gowda et al. [20]	Retention time is 3 minutes. This is very close to the dead time and may increase the possibility of overlapping with possible peaks over time in stability studies.

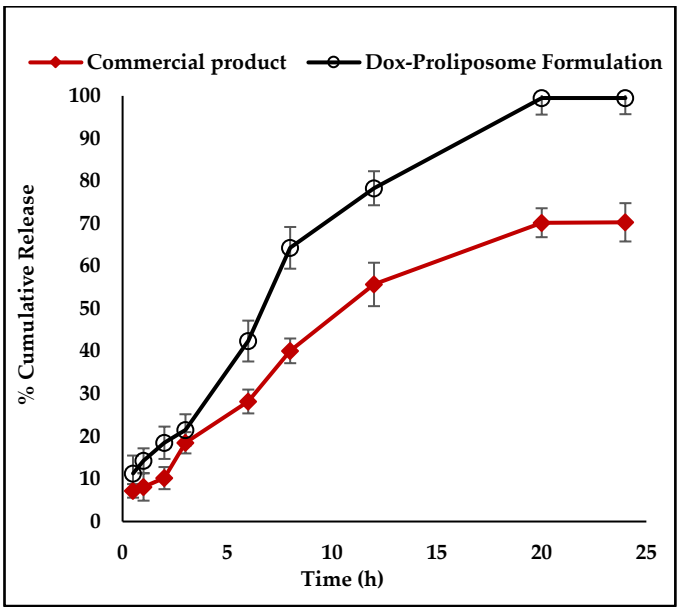

Figure 4. In vitro release of Dox from commercial product and Dox-proliposome formulation at pH:5.5

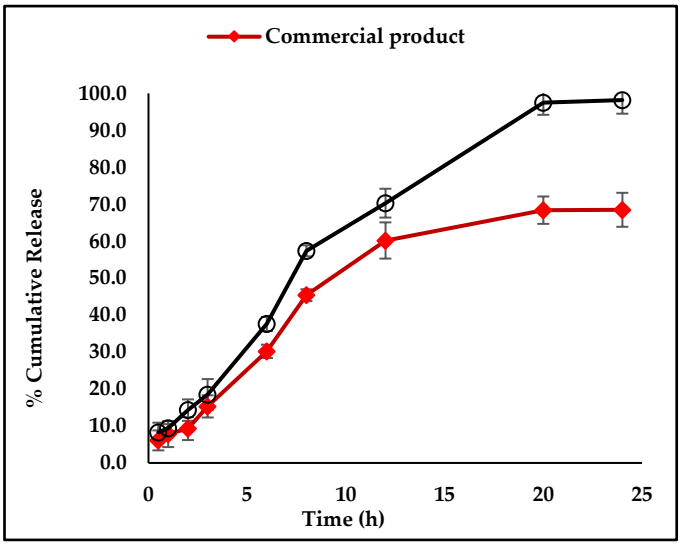

Figure 5. In vitro release of Dox from commercial product and Dox-proliposome formulation at pH:6.5

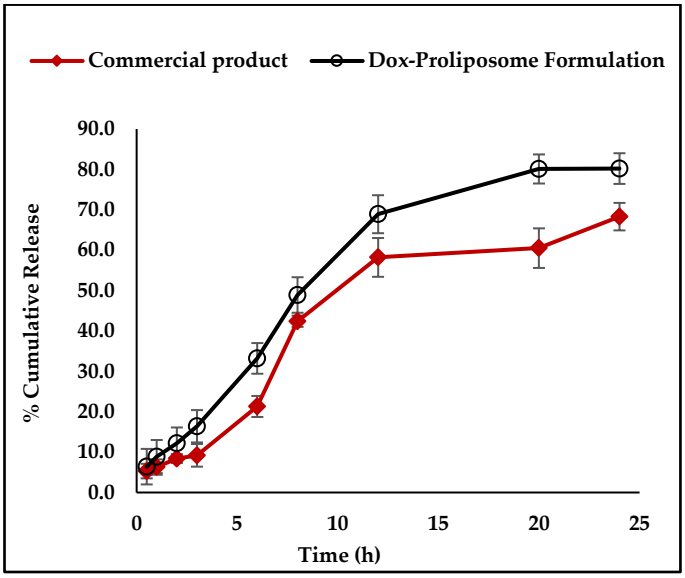

Figure 6. In vitro release of Dox from commercial product and Dox-proliposome formulation at pH:7.5

Dox encapsulated in liposome vesicles with high encapsulation efficiency provides high antitumor efficacy by altering tissue distribution and pharmacokinetics while reducing toxicity [41]. The drug loading capacity and encapsulation capacity of the proliposome formulation were determined during the development of the proliposome formulation with the validated HPLC method. The drug loading and encapsulation capacity of the developed optimal proliposome formulation were 90% ± 0.5 and 100.0% ± 0.3, respectively (n=6). RSD% were lower than 2.0% of each sample. In the study presented by Aghdam et al., which achieved high encapsulation into liposomal drug carrier systems with the pH gradient method, it was shown that clearance from the body could be reduced by 2.5 times with Dox [42]. This shows that liposomal Dox formulations, which can provide high encapsulation for in vitro studies, may be promising drug delivery systems in the in vivo environment. In addition, linearity and working range analyses were carried out to observe the effect of pH change in the in vitro release studies to be performed with the HPLC method developed. Dox assays of in vitro release studies performed in pH 5.5, pH 6.5 and pH 7.5 media were evaluated with linearity equations performed with these media for commercial product and Dox-proliposome formulation (Table 3). The release graphs of pH 5.5, pH 6.5, and pH 7.5 are given in Fig. 4, Fig. 5, and Fig. 6, respectively. It was observed that drug release increased because low pH values increase the hydrophilicity of Dox and its solubility in the liposome [43]. Similar to our study, in the study conducted by Mohammadi et al., higher release was observed at pH 5.5 compared to pH 7.4 for the developed liposomal formulations [44]. Therefore, Dox showed the highest release at pH 5.5 at the end of the 24th hour in the commercial product and the developed Dox-proliposome formulation. With the developed Dox-proliposome formulation, the release at the end of 24 hours was increased by 23.9%, 30.2%, and 14.8% at pH 5.5, pH 6.5, and pH 7.5, respectively, compared to the commercial product. The difference in vitro release values of the developed Dox-proliposome formulation compared to the commercial product may be due to the different lipids used in the developed proliposome formulation [45]. The lipid differentiation used can result in a change in the glass transition temperature, resulting in different release rates and kinetics [46]. In addition, due to the membrane-induced barrier effect of the vesicles in liposomal drug delivery systems, the release of the active substance occurs more slowly and in a controlled than the release of the active substance [47]. Obtained in vitro release results; the commercial product and the developed proliposomal drug delivery system formulation have shown that

controlled drug release is similar to the literature data [48,49].

In addition, according to the F2 similarity test between the commercial product and the developed Dox-proliposome formulation, the results in pH 5.5, pH 6.5, and pH 7.5 environments were found to be 37.5, 42.0, and 51.4, respectively. The fact that the F2 value between the two release profiles is between 50-100 indicates similarity by the FDA. [50]. F2 similarity ratio of Dox-proliposome formulation and commercial product in pH 7.5 medium was found to be 51.4% and it was found to comply with FDA similarity requirement. At 5.5 and 6.5, in vitro release profiles were not found to be similar to the commercial formulation, however, it was shown in Fig. 4 and Fig. 5 that the developed proliposome formulation was released more rapidly and in a controlled release than the commercial product in environments mimicking endosome and lysosomes of cancer cells (pH 5.5), cancer tissues (pH 6.5).

Moreover, there was no significant change in the results of the physicochemical analyses performed at all stability conditions to evaluate the stability for 12 months. This shows that the stability problem observed in liposomal drug delivery systems can be avoided with the developed proliposome formulations.

4. Conclusion

The results of the study show that the proliposome formulation containing Dox was successfully developed and validation of Dox by HPLC was performed by the acceptance criteria. Altogether, the results showed that the validated HPLC method for the developed novel Dox-proliposome formulation is a suitable tool for the determination of drug loading capacity, determination of encapsulation capacity, measurement of release properties in different pH media, and evaluation of stability studies. In conclusion, the developed assay method and its validation have shown that promising results can be achieved at every stage of the development of the novel proliposome formulation and have greatly contributed to the study.

References

[1] A.F. Yu, A.T. Chan, Steingart RM, Cardiac Magnetic Resonance and Cardio-Oncology: Does T 2 Signal the End of Anthracycline Cardiotoxicity, J Am Coll Cardiol 73(7), 2019, 792–4.

[2] G. Marcq, E. Jarry, I. Ouzaid, J. Hermieu, F. Henon, J.C. Fantoni and E. Xylinas, Contemporary best practice in the use of neoadjuvant chemotherapy in muscle-invasive bladder cancer, Therapeutic Advances in Urology, 11, 2019, 1–8.

[3] M. Kciuk, A. Gielecinska, S. Mujwar, D. Kołat, Z. Kałuzinska-Kołat, I. Celik, R. Kontek, Doxorubicin—An Agent with Multiple Mechanisms of Anticancer Activity, Cells, 659 (12), 2023, 1–30.

[4] A.Z. Luu, B. Chowdhury, M. Al-Omran, H. Teoh, D.A. Hess, S. Verma, Role of Endothelium in Doxorubicin-Induced Cardiomyopathy, JACC Basic to Transl Sci, 3(6), 2018, 861–70.

[5] M. Pipicz, V. Demján, M. Sárközy, T. Csont, Effects of cardiovascular risk factors on cardiac STAT3, Int J Mol Sci, 19(11), 2018, 1–29.

[6] J. Shaji, V. Bhatia, Proliposomes: A brief overview of novel delivery system, Int J Pharma Bio Sci, 4(1), 2013, 150–60.

[7] N.I. Payne, C.V. Ambrose, P. Timmins, M.D. Ward, F. Ridgway, Proliposomes: A novel solution to an old problem, J Phar m Sci, 75(4),1986, 325–9.

[8] D. Chordiya, S. Shilpi, D. Choudhary, Proliposomes: a potential colloidal carrier for drug delivery applications, Advances in Pharmaceutical Product Development and Research, 2020, 581–608.

[9] K.Y. Janga, R. Jukanti, A. Velpula, S. Sunkavalli, S. Bandari, P. Kandadi, P.R. Veerareddy, Bioavailability enhancement of zaleplon via proliposomes: Role of surface charge. Eur J Pharm Biopharm, 80(2), 2012, 347–57.

[10] C. Chu, S.S. Tong, Y. Xu, L. Wang, M. Fu, Y.R. Ge, J.N. Yu, X.M. Xu, Proliposomes for oral delivery of dehydrosilymarin: Preparation and evaluation in vitro and in vivo, Acta Pharmacol Sin, 32(7), 2011, 973–80.

[11] N. Singh, P. Kushwaha, U. Ahmad, M. Abdullah, Proliposomes : An Approach for the Development of Stable Liposome Proliposomas: Una aproximación para el desarrollo de liposoma estables, Ars Pharm, 60(4), 2019, 231–40.

[12] S. Muneer, Z. Masood, t S. But, S. Anjum, H. Zainab, N. Anwar, et al., Proliposomes as Pharmaceutical Drug Delivery System : A Brief Review Nanomedicine & Nanotechnology Proliposomes as Pharmaceutical Drug Delivery System : A Brief Review, 2017.

[13] M. Doltade, R. Saudagar, Analytical Method Development and Validation: a Review, Int J Pharm Biol Sci Arch, 9(3), 2019, 563–70.

[14] S. Sharma, S. Goyal, K. Chauhan, A review on analytical method development and validation, Int J Appl Pharm, 10(6), 2018, 8–15.

[15] A. Rahdar, Hajinezhad, H. Hamishekar, A. Ghamkhari, G.Z. Kyzas, Copolymer/graphene oxide nanocomposites as potential anticancer agents, Polym Bull, 78(9), 2021,4877–98.

[16] I. Rus, M. Tertiș, V. Paşcalău, C. Pavel, B. Melean, M. Suciu, C. Moldovan, T. Topală, C. Popa, R. Săndulescu, C. Cristea, Simple and fast analytical method for the evaluation of the encapsulation and release profile of doxorubicin from drug delivery systems, Farmacia, 69(4), 2021,670–81.

[17] P. Laxmi, A. Varma, A. Pai , M.B. Sathyanarayana, Experimental data of fabricated co-crystals of doxorubicin HCl with flavonoids, Indian J Pharm Educ Res, 53(3), 2019, S225–30.

[18] L.E. Scheeren, D.R. Nogueira-Librelotto, J.R. Fernandes, L.B. Macedo, A.I.P. Marcolino, M.P. Vinardell, C.M.B. Rolim, Comparative Study of Reversed-Phase High-Performance Liquid Chromatography and Ultraviolet–Visible Spectrophotometry to Determine Doxorubicin in pH-Sensitive Nanoparticles, Anal Lett, 51(10), 2018, 1445–63.

[19] Y. Du, L. Xia, A. Jo, R.M. Davis, P. Bissel, M.F. Ehrich, et al, Synthesis and Evaluation of Doxorubicin-Loaded Gold Nanoparticles for Tumor-Targeted Drug Delivery, Bioconjug Chem, 29(2), 2018, 420–30.

[20] Gowda BG, Hanumanthanaik D. Development and Validation of RP-HPLC Method for the Determination of Doxorubicin Hydrochloride in Pure and Pharmaceutical Dosage Forms, Int J Adv Technol Eng Sci, 2017, 5(6), 323–9.

[21] J. Lee, M.K. Choi, I.S. Song, Recent Advances in Doxorubicin Formulation to Enhance Pharmacokinetics and Tumor Targeting, Pharmaceuticals, 16(6), 2023, 802.

[22] M. Alyane, G. Barratt, M. Lahouel, Remote loading of doxorubicin into liposomes by transmembrane pH gradient to reduce toxicity toward H9c2 cells, Saudi Pharm J, 24(2), 2016, 165–75.

[23] M. Diril, K.V. Özdokur, Y. Yıldırım, H.Y. Karasulu, In vitro evaluation and in vivo efficacy studies of a liposomal

doxorubicin-loaded glycyrretinic acid formulation for the treatment of hepatocellular carcinoma, Pharm Dev Technol, 28(10), 2023, 915–27.
[24] European Medicines Agency, ICH guideline Q2(R2) on validation of analytical procedures, Vol. 2, 2022.
[25] R. Rodriguez, E. Castillo, D. Sinuco, Validation of an HPLC method for determination of bisphenol-A migration from baby feeding bottles, J Anal Methods Chem, 2019.
[26] A.K. De, P.P. Chowdhury, S. Chattapadhyay, Simultaneous Quantification of Dexpanthenol and Resorcinol from Hair Care Formulation Using Liquid Chromatography: Method Development and Validation, Scientifica (Cairo), 2016.
[27] L. Zou, F. Chen, J. Bao, S. Wang, L. Wang, M. Chen, C. He, Y. Wang, Preparation, characterization, and anticancer efficacy of evodiamine-loaded PLGA nanoparticles, Drug Deliv, 23(3), 2016, 908–16.
[28] FDA, Draft Guidance on Doxorubicin Hydrochloride, Vol. 1, Draft Guidance on Doxorubicin Hydrochloride, 2018.
[29] D.R. Nogueira, L.B. Macedo, L.E. Scheeren, M. Mitjans, Infante MR, Rolim CMB, et al., Determination of Methotrexate in pH-Sensitive Chitosan Nanoparticles by Validated RP-LC and UV Spectrophotometric Methods, J Appl Biopharm Pharmacokinet, 2, 2014,59–67.
[30] L.E. Scheeren, D.R. Nogueira-Librelotto, J.R. Fernandes, L.B. Macedo, A.I.P Marcolino, M.P. Vinardell, et al., Comparative Study of Reversed-Phase High-Performance Liquid Chromatography and Ultraviolet–Visible Spectrophotometry to Determine Doxorubicin in pH-Sensitive Nanoparticles, Anal Lett, 51(10), 2018, 1445–63.
[31] S. Rao, Y. Song, F. Peddie, A.M. Evans, Particle size reduction to the nanometer range: a promising approach to improve buccal absorption of poorly water-soluble drugs, Int J Nanomedicine, 6, 2011,1245–51.
[32] R.C. Lino, S.M. de Carvalho, C.M. Noronha, W.G. Sganzerla, M.R. Nunes, C.G. da Rosa, et al., Development and Characterization of Poly-ε- caprolactone Nanocapsules Containing carotene Using the Nanoprecipitation Method and Optimized by Response Surface Methodology, Brazilian Arch Biol Technol, 2020, 63.
[33] J.D. Castile, K.M.G. Taylor, Factors affecting the size distribution of liposomes produced by freeze-thaw extrusion, Int J Pharm, 188(1), 1999, 87–95.
[34] I. Sopyan, I. Sunan, D. Gozali, A Review: A Novel of Efforts to Enhance Liposome Stability as Drug Delivery Approach, Sys Rev Pharm, 2020, 11(6), 555 562.
[35] Y. Wang, D.W., Grainger, Lyophilized liposome-based parenteral drug development: Reviewing complex product design strategies and current regulatory environments, Adv Drug Deliv Rev, 2019,151–152.
[36] S.S. Kulkarni, R. Suryanarayanan, J.V. Rinella, Bogner RH, Mechanisms by which crystalline mannitol improves the reconstitution time of high concentration lyophilized protein formulations, Eur J Pharm Biopharm, 131, 2018, 70–81.
[37] M.R. Toh, G.N.C. Chiu, Liposomes as sterile preparations and limitations of sterilisation techniques in liposomal manufacturing, Asian J Pharm Sci, 8(2), 2013, 88–95.
[38] J. Ermer, Validation in pharmaceutical analysis, Part I: An integrated approach, J Pharm Biomed Anal, 24, 2001, 755–67.
[39] N.N. Ferreira, F.I. Boni, F. Baltazar, M.P.D. Gremião, Validation of an innovative analytical method for simultaneous quantification of alpha-cyano-4-hydroxycinnamic acid and the monoclonal antibody cetuximab using HPLC from PLGA-based nanoparticles, J Pharm Biomed Anal, 2020, 190.
[40] U.S. Department of Health and Human Services Food and Drug Administration, Guidance for Industry, 2015, 1-18.
[41] S.M. Rafiyath, M. Rasul, B. Lee, G. Wei, G. Lamba, D. Liu, Comparison of safety and toxicity of liposomal doxorubicin vs. conventional anthracyclines: a meta-analysis, Exp Hematol Oncol, 1(1), 2012,1–9.
[42] M.A. Abri, R. Bagheri, J. Mosafer, B. Baradaran, M. Hashemzaei, A. Baghbanzadeh, M. Guardia, A. Mokhtarzadeh, Recent advances on thermosensitive and pH-sensitive liposomes employed in controlled release, J Control Release, 315, 2019, 1–22.
[43] F. Haghiralsadat, G. Amoabediny, M.N. Helder, M.H. Sheikhha, T. Forouzanfar, B. Zandieh-doulabi, A comprehensive mathematical model of drug release kinetics from nano-liposomes, derived from optimization studies of cationic PEGylated liposomal doxorubicin formulations for drug-gene delivery, Artif Cells, Nanomedicine, Biotechnol, 2017.
[44] Z.A. Mohammadi, S.F. Aghamiri, A. Zarrabi, M. Reza Talaie, Liposomal Doxorubicin Delivery Systems: Effects of Formulation and Processing Parameters on Drug Loading and Release Behavior, Curr Drug Deliv, 13(7), 2016, 1065–70.
[45] M. Anderson, A. Omri, The Effect of Different Lipid Components on the In Vitro Stability and Release Kinetics of Liposome Formulations, Drug Delivery, 11, 2004, 33–9.
[46] G. Liu, K. McEnnis, Glass Transition Temperature of PLGA Particles and the Influence on Drug Delivery Applications, Polymers (Basel), 14(5), 2022.
[47] L. Peng, C. Guiliang, Jingchen Z., A Review of Liposomes as a Drug Delivery System: Current, Molecules, 27(1372), 2022, 1–23.
[48] M. Fazel, M. Daeihamed, M. Osouli, A. Almasi, A. Haeri, S. Dadashzadeh, Preparation, in-vitro characterization and pharmacokinetic evaluation of brij decorated doxorubicin liposomes as a potential nanocarrier for cancer therapy, Iran J Pharm Res, 17, 2018, 33–43.
[49] E. Csuhai, S. Kangarlou, T.X. Xiang, A. Ponta, P. Bummer, D. Choi, et al., Determination of key parameters for a mechanism-based model to predict doxorubicin release from actively loaded liposomes, J Pharm Sci, 104(3), 2015, 1087–98.
[50] U.S. Food and Drug Administration (FDA), FDA guidance for industry dissolution testing of immediate release solid oral dosage forms, In: Dissolution Testing of Immediate Release Solid Oral Dosage Forms US, 1997.