Tropical Journal of Pharmaceutical Research November 2019; 18 (11): 2415-2424
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v18i11.27

Original Research Article

Simple and sensitive chromatographic method development for *in-vitro* and *in-vivo* analysis of doxorubicin-loaded poly lactic-co-glycolic acid nanoparticles

Sumaira Irum Khan, Zafar Iqbal*, Fazli Nasir, Muhammad Ismail, Naila Shahbaz, Asma Khan, Muzna Ali Khattak, Mirina Sakhi
Department of Pharmacy, University of Peshawar, Peshawar 25120, Pakistan

For correspondence:* ***Email:* *zafar_iqbal@uop.edu.pk;* ***Tel:*** *+92-91-9239619;* ***Fax:*** *+92-91-9218131*

Sent for review: 5 March 2019 *Revised accepted: 19 October 2019*

Abstract

Purpose: *To develop a bioanalytical high performance liquid chromatography (HPLC) method for the quantification of doxorubicin in biological fluids and polymeric nano-formulations.*
Methods: *Analysis of doxorubicin in polymeric nanoparticles and biological samples was carried out at 252 nm using Purospher® RP-18 end-capped column (250mm×4.6mm,5µm) secured with a guard column cartridge RP18 (30mm×4.6mm,10µm). The mobile phase used was 0.025M phosphate buffer and acetonitrile (ACN) (65:35, v:v) in isocratic mode at a flow rate of 0.9 ml/min, run time of 10 min, column oven temperature of 30 °C, and injection volume of 40 µL.*
Results: *The standard curve for doxorubicin was linear (0.999) in the concentration range of 0.022 - 1.00 µg/mL in human and albino mice plasma. Nominal retention times of doxorubicin and IS were 3.5 and 5.5 min, respectively. Mean recovery was within acceptable limits (100 ± 2 %). The limit of detection (LOD) and limit of quantification (LOQ) were 0.012 and 0.022 µg/mL, respectively.*
Conclusion: *A reliable HPLC method has been successfully developed, validated and applied for the in vitro analysis of doxorubicin released from polymeric nanoparticles and in vivo pharmacokinetic studies in albino mice. The method may also be applicable to the analysis of doxorubicin in human fluids.*

Keywords: *RP-HPLC, Doxorubicin, Polymeric nanoparticles, Pharmacokinetics*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Doxorubicin is an antineoplastic drug in the anthracycline class which was identified in 1969 from cultures of *Streptomyces peucetius var. caesius*. It exerts antimitotic and cytotoxic effects through the formation of complexes with DNA by their base pair intercalation. Moreover, it represses topoisomerase II by stabilization of DNA-topoisomerase II complex, inhibition of polymerase activity, regulation of gene expression and free radical damage to DNA [1]. Doxorubicin produces antitumor effects against a wide range of tumors. It is ordinarily used to treat certain leukemia's, Hodgkin's lymphoma, and malignancies of the bladder, breast, stomach, lung, ovaries, thyroid, as well as delicate tissue

sarcoma and multiple myeloma [2,3]. The degree of binding of doxorubicin to plasma protein is approximately 75%; the terminal half-life of the drug is 20-48 h, while its relative bioavailability following oral administration is about 5% [3,4].

Doxorubicin is available in liposome-encapsulated forms as Doxil® (pegylated form), Myocet® (non-pegylated form), and Caelyx®, although these forms must be given via intravenous injection. Doxorubicin is known to be a fluorescent compound, a quality which has frequently been utilized to determine its concentration, which indicates the possibility of using the molecule as a theranostic agent [5].
A number of methods are accessible to determine doxorubicin and its metabolites in commercially available dosage forms and bio-fluids. The commonly used analytical methods for doxorubicin and its metabolites are as follows: capillary electrophoresis-UV detection [6], LC-MS/MS [7], chemiluminescence [8], RP-HPLC-fluorescence (490/590 nm) [9,10] and RP-HPLC-UV (237 and 254 nm) [9,11].

Poly (lactic-co-glycolic acid) is synthetic co-polymer approved by Food and Drug Administration (FDA) and European Medicines Agency (EMA). It is biocompatible and biodegradable polymer safe for parenteral administration. It is used to improve surface properties so as to enhance interaction with biological materials and drug targeting [12,13]. It has been reported that the leaky vasculature of the endothelial tissues in tumors is responsible for enhanced concentration of PLGA nanoparticles inside the tumors through a process called "*enhanced permeation and retention"* (EPR) mechanism [14]. Therefore, PLGA is most commonly used in drug delivery systems and especially in cancer drug delivery systems [15].

In the present study a reverse-phase High Performance Liquid Chromatographic method was successfully developed, validated and applied to find out the encapsulation efficiency, drug loading of polymeric nanoparticles, analysis of doxorubicin in biological samples, measurement of invitro release of doxorubicin and pharmacokinetics studies.

EXPERIMENTAL

Chemicals

Doxorubicin (purity 98.9 %) was purchased from Shanghai Soyoung Biotech. Inc., China The internal standard (IS) prednisolone was a kind gift from Sanofi-Aventis Karachi Pakistan. Poly lactide-co-glycolide (PLGA: 75:25, Mwt: 66,000-107,000 Da) and Pluronic® F-127 were purchased from Sigma–Aldrich. Sodium lauryl sulphate (Mwt. 288.38) acetonitrile (ACN), dichloromethane (DCM), methanol (MeOH), ethanol (EtOH), diethyl ether, phosphate buffered saline, and phosphoric acid (H_3PO_4) were product of Scharlau Chemie Spain. Analytical grade and HPLC chemicals and solvents were used throughout the experiment. Distillation apparatus used for the purification of water was Ultrapure Millipore (Milford, USA). Dialysis membrane (MWCO: 12,000 Da; Dia 1.0" – 25mm; size 6 Inf. 30M) was purchased from Sigma – Aldrich.

Instrumentation

HPLC apparatus used in present study was Perkin Elmer (Norwalk, USA) with a Network Chromatography Interface (NCI) 900, peltier column oven, pump, auto-sampler and vacuum degasser (series 200). This system is connected to UV/VIS detector with variable wavelength. Chromatographic data was processed and analyzed by Perkin-Elmer (6.3.1) Totalchrom Software.

Preparation of stock solutions

Stock solutions of doxorubicin and IS prednisolone (0.1 mg/mL) were prepared in acetonitrile and kept in amber glass bottles at -20 °C until analysed. Dilutions of doxorubicin *(0.01-1* µg/mL*) with IS (0.5* µg/mL*)* were prepared in Eppendorf tubes (2 mL) using mobile phase. All dilutions and stock *solution* was kept at -20 °C for one month.

Sample preparation

Spiked plasma

Following informed consent, blood sample was taken in EDTA glass tube from a human volunteer, and centrifuged for 5min at -4 °C and 10,000 rpm. The separated plasma was kept at -20 °C till analysis. For the preparation of spiked plasma sample, 200 µL of plasma warmed at room temperature was spiked with doxorubicin solutions to obtain different concentrations in the range of 0.01-1 µg/mL. Equal volume (5 µL) of IS was added to each sample (final concentration 0.5 µg/mL) and vortexed for 5 min. Extraction of doxorubicin from plasma samples was performed by precipitation method. In this method, methanol was used as a protein precipitating agent, while mobile phase served as an extraction solvent. Injected volume of reconstituted sample into HPLC system was 10-50µL.

Mice plasma samples

Samples were collected at designated time intervals from albino mice, into heparinized glass tubes and doxorubicin was extracted from the plasma using the procedure earlier described.

Preparation of formulations

Doxorubicin loaded PLGA nanosuspensions were prepared with double emulsion method using pluronic F-127 and sodium lauryl sulphate (SLS) as stabilizers. Nano-formulations were prepared by keeping the concentration of PLGA and SLS constant, while varying the concentrations of the emulsifier (pluronic F-127) and drug (doxorubicin). The formulations were prepared with five different amounts of drug (1, 2, 3, 4 and 5 mg) and 4 different concentrations of pluronic F-127 (0.5, 1.0, 1.5 and 2 %). The following formulations were prepared: PLGA = 5 mg, pluronic acid = 0.5 %, SLS= 0.05 %, and doxorubicin = 1, 2, 3, 4 or 5mg (DNF-136, DNF-137, DNF-138, DNF-139, and DNF-152, respectively);PLGA = 5 mg, pluronic F-127 = 1.0% , SLS = 0.05% and doxorubicin = 1, 2, 3, 4 or 5mg (DNF-140, DNF-141, DNF-142, DNF-143 and DNF-153, respectively); PLGA = 5 mg, pluronic = 1.5% , SLS = 0.05% and doxorubicin = 1, 2, 3, 4 and 5mg (DNF-144, DNF-145, DNF-146, DNF-146 and DNF-154, respectively); and PLGA = 5 mg, pluronic F-127 = 2.0% , SLS = 0.05% and doxorubicin = 1, 2, 3, 4 and 5mg (DNF-147, DNF-148, DNF-149,DNF-150 and DNF-155, respectively). Initial selection criteria for these formulations were made on drug loading, entrapment efficiency, particle size, zeta potential, polydispersity index (PDI) and stability.

Optimization of chromatographic conditions

Stationary phase

Isolation of doxorubicin was tried with different stationary phase such as Discovery HS C18 column (250 mm × 4.6 mm, 5 µm), Supelcosil ™ LC-CN (250 mm × 3 mm, 5 µm), Purospher® RP-18 endcapped column (150 mm × 3.9 mm, 5 µm) and Purospher® RP-18 endcapped column (250 mm × 4.6 mm, 5 µm), with guard-column cartridge RP-18 (30mm × 4.6 mm, 10 µm; Norwalk, USA).

Mobile phase compositions

Organic solvents i.e acetonitrile, methanol, tetrahydrofuran (THF; 0.05 and 0.1%), trifluoroacetic acid (TFA 0.05 – 1% at different pH), and phosphate buffers of varying pH (0.025 to 1M) were used for the analysis of doxorubicin.

Flow rate

Different flow rates ranging from 0.8 – 1.2 mL/min was used to analyze doxorubicin (with a difference of 0.1 ml/min), to optimize the chromatographic conditions. The flow rate was chosen and optimized on the basis of peak resolution and sensitivity.

Column oven temperature

The temperature was varied in the range of 25–50°C (with a difference of 5 °C) in the study of the effect of temperature on the doxorubicin chromatogram. The temperature that showed the best sensitivity, peak resolution and retention time was chosen and optimized.

Internal standard

Different compounds (teniposide, etoposide, 5-flurouracil, temoxifen, paclitaxel, dexamethasone, metoclopramide and prednisolone) were assessed for compatibility, good instrumental response and peak resolution with doxorubicin for use as an IS.

Injection volume

The sample loop size was evaluated in the range of 10–50µL, with a difference of 10µL. The adjustment of sample loop size was important so as to avoid column loading problem and lack of sensitivity for doxorubicin.

Detector wavelength

The wavelength was varied from 240-261 nm, with a difference of 3nm was checked and optimized. The wavelength that gave the best sensitivity and good peak resolution was selected.

Method validation

The following parameters were validated and optimized as per FDA guidelines for Bioanalytical Method Validation.

Linearity

The linearity of the method was checked in 0.01-1µg/ml concentration range in spiked plasma samples. Calibration curves were plotted for standard drug, spiked human/mice plasma. Calibration curves of the ratio of peak areas of doxorubicin to IS (peak response ratios) against doxorubicin concentration was plotted for the

drug in the mobile phase, spiked human and albino mice plasma. Correlation coefficient (R), intercept (b) and slope (m), were calculated with the help of linear regression-least squares analysis.

Sensitivity

Sensitivity was assessed by evaluating the lowest drug concentration which can be accurately and precisely measured. The limit of detection (LOD) of a drug is "the concentration at which signal-to-noise ratio (S/N) is 3:1", while limit of quantification (LOQ) of a drug is find out at signal-to-noise ratio (S/N) of 10:1.

Precision

Method precision was investigated using injection and analysis repeatability techniques. In injection repeatability technique, 1 µg/mL of drug and 0.5 µg/mL of IS in spiked plasma were injected six times repeatedly into the HPLC. The samples were analyzed at different times on the same day, and the same analyses were performed over different days for determination of intra-day and inter-day precision. The recoveries and their respective %RSD (co-variance) were applied to quantify the precision of this method.

Accuracy

Accuracy was determined as the percentage of the difference between the measured concentration and the spiked concentration. Recovery (R) was evaluated by injecting each sample in triplicate and calculated as in Eq 2.

$$R (\%) = (A/B)100 \quad \ldots\ldots\ldots (2)$$

where A is the response ratio of the drug with reference to IS in the mobile phase and B the response ratio of the drug with reference to IS in spiked plasma.

Stability of samples

Stability of the doxorubicin and IS was assessed in the stock, working dilutions and in spiked samples at different temperature, during processing after three freeze-thaw cycles. For long-term stability, samples were kept at -20 °C for 30 days; and for freeze-thaw stability, 3-freezing (-20 °C) and 3-thawing cycles (25 °C) were used to assess stability (S) in triplicate, and calculating it by Equation 3.

$$S (\%) = (S_t/S_o)100 \quad \ldots\ldots\ldots\ldots (3)$$

where S_t is the sample stability at time, t, and S_o is the sample stability at zero time.

Application of the developed method

The developed method was applied to doxorubicin-loaded PLGA nano-formulations for *in-vitro* release kinetics and *in-vivo* pharmacokinetic studies in animal models. The animal study of this experiment was approved by the "Committee for Research Ethics", Pharmacy department, Peshawar University, having reference number "08/EC-18/Phar". The study was performed in line with the guidelines of the "National Institutes of Health, Guide for the care and use of laboratory animals" [16].

Evaluation of *in-vitro* release kinetics

The *in-vitro* release kinetics of doxorubicin-loaded PLGA nano-formulations were performed using dialysis membrane. Blank nanoformulations (doxorubicin free PLGA nanoformulation) were used as reference. In dialysis membrane 1 mL of each nano-formulation was individually added and the membranes were stirred at 100rpm and dialyzed against 50 mL of PBS at 37 °C. At specific time intervals of 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216 h, sample (1 ml) was withdrawn and analyzed for doxorubicin contents using HPLC-UV. All samples were analyzed in triplicate, and their mean ± standard deviation (SD) was calculated.

Assessment of *in-vivo* pharmacokinetics

In-vivo experiments were performed using adult 6-8 weeks old albino mice (weighing 30 ± 5 g) obtained from National Institute of Health (NIH) Islamabad, Pakistan. The mice were housed in ventilated cages in groups of 15, and acclimatized for one week prior to the study. They were maintained on standard laboratory feed.

Selected nano-formulations of doxorubicin and commercially available formulation ROBOL was injected intravenously into the tail vein of the albino mice. Blood samples of 0.5–1.0 mL were collected in EDTA tubes at designated periods and centrifuged at 10,000 rpm, -4 °C for 5 min. Deproteination of plasma samples was performed using methanol (3-times the plasma volume. Aliquot of plasma (200 µL) and IS were taken into an Eppendorf tube, and MeOH (600 µL) was added, vortexed for 2 min and centrifuged at 6000 rpm for 3 min. The supernatant was transferred to a different Eppendorf tube. Mobile phase was added to bring the volume up to 1 mL. It was then

vortexed for 2 min and kept at -20 °C prior to further analysis. Before analysis on HPLC, the frozen plasma samples were allowed to warm at room temperature, then extracted by following same procedure as adopted for spiked plasma and analyzed in triplicate. Pharmacokinetic parameters such as time to reach maximum concentration (T_{max}), maximum concentration (C_{max}), half-life (t½), clearance, maximum area under the curve (AUC_{max}), mean residence time (MRT), area under curve (AUC) and area under the first moment curve (AUMC) were calculated for each subject using PK® Solutions software.

RESULTS

Optimized chromatographic conditions

Chromatographic conditions were optimized for detector wavelength, stationary phase, column oven temperature, mobile phase, mobile phase flow rate, IS and sample volume size.

The stationary phase that resulted in better resolution and separation of doxorubicin was determined using Purospher® RP-18 endcapped column (250 mm × 4.6 mm, 5 µm) among the tested columns.

The mobile phase comprising ACN and 0.025M phosphate buffer at a volume ratio of 35:65 adjusted to pH (2.5) was selected for the analysis of doxorubicin. The retention time of the drug decreased with increasing the proportion of the organic portion of mobile phase up to 40:60 (v/v), beyond which the peak of the selected compound decreased. Various concentrations of the drug at constant concentration of IS, were run to check the stability and recovery of the method (Figure 1). Better peak shape and sensitivity was obtained at 252 nm as shown in Figure 2. Sample loop volume was assessed in the range of 10–50 µL, and an optimized loop volume of 40 µl was chosen. The effect of column oven temperature on the resolution and sensitivity of peak was also investigated. Sharp and better peaks were obtained at 30 °C for the drug and IS as shown in Figure 3.

An optimized flow rate of 0.9 ml/min was chosen as shown in Figure 4, for the analysis of doxorubicin because mobile phase flow rate has great effect on the analysis of the studied drug. Although run time decreased significantly at higher flow rates with better resolution of the peak, the sensitivity of method was decreased.
Various internal standards such as teniposide, etoposide, 5-flurouracil, temoxifen, paclitaxel, dexamethasone, metoclopramide and prednisolone were evaluated. Prednisolone had good resolution, better compatibility and relatively good recovery with doxorubicin. Thus it was selected as an IS. Doxorubicin and IS prednisolone were separated applying this method in standard mixture, spiked human plasma and mice plasma as shown in Figure 5.

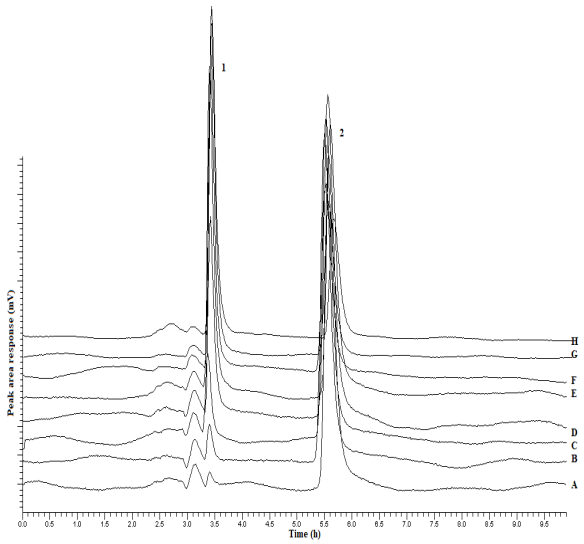

Figure 1: Overlay chromatogram showing various concentrations of doxorubicin (1) with fixed concentration (0.005 µg/ml) of IS (2). (Chromatograms A = 0.001 µg/mL, B = 0.005 µg/mL, C = 0.1 µg/mL, D = 0.2 µg/mL, E = 0.4 µg/mL, F = 0.6 µg/mL and G = 0.8 H 1 µg/mL)

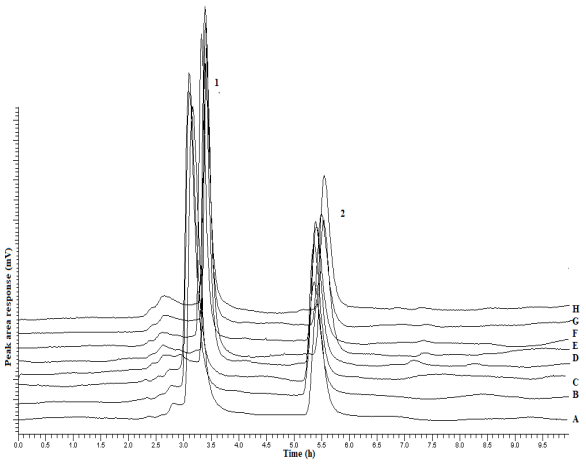

Figure 2: Overlay chromatogram showing various wavelength of detector at fixed concentrations of doxorubicin (1µg/mL) and IS (0.005 µg/mL). (Chromatograms A = 240nm, B = 243nm, C = 246nm; D = 249nm, E = 252nm, F = 255nm, G = 258nm and H = 261nm)

Optimized sample preparation method

Different organic solvents were evaluated for use in the preparation of stock solutions and extraction of drug from spiked human and albino mice plasma. Stock solution of doxorubicin and

IS were prepared in ACN, extraction/deproteination was done using methanol. The concentrations of the stock solution of doxorubicin and IS were 1 mg/ 10mL respectively. Stock solutions were used to prepare daily working solutions in the mobile phase. Drug recovery (%w/w) was better in methanol than in the other solvents, in both spiked human and mice plasma samples.

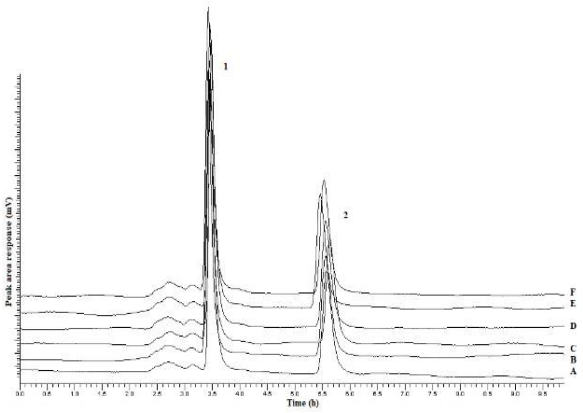

Figure 3: Overlay chromatograms showing various column oven temperatures at fixed concentration (1 µg/ml) of doxorubicin (1) and (0.005 µg/ml) of IS (2). (Chromatograms A= 25 ^{0}C, B = 30 ^{0}C, C = 35 ^{0}C, D = 40 ^{0}C, E = 45 ^{0}C, and F = 50 ^{0}C)

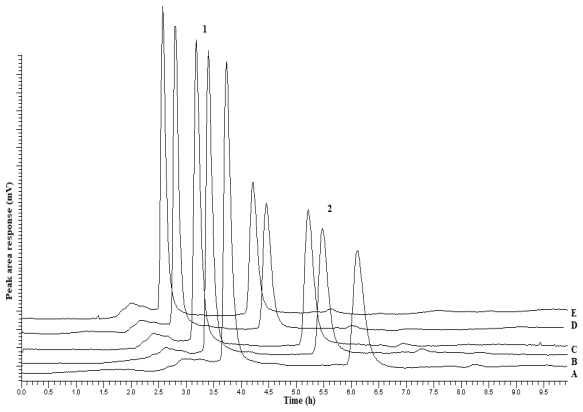

Figure 4: Overlay chromatogram showing various mobile phase flow rates of doxorubicin (1) at fixed concentration (0.005 µg/mL) of IS. (2) (Chromatograms A = 0.8µL/min, B = 0.9 µL/min, C = 1 µL/min, D = 1.1 µL/min and E = 1.2 µL/min)

Optimized formulation characteristics

Five out of twenty polymeric nano-formulations of doxorubicin (DNF-136, DNF-137, DNF-138, DNF-141 and DNF-145) were selected and optimized based on initial characterizations as shown in Table 1. The particle size and poly dispersity index of nano-formulation increased with increasing concentration of drug from 1 to 5mg. Nano-formulations having encapsulation efficiencies greater than 68 % were selected for *in vitro* and *in vivo* studies. High encapsulation efficiency shows large amount of drug encapsulated in the polymer, which is a measure of its *in vitro* and *in vivo* fates.

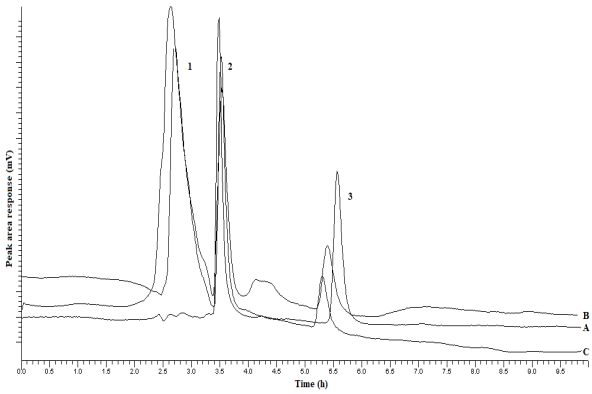

Figure 5: Overlay chromatogram of spiked plasma (1) doxorubicin (2) at fixed concentration (0.005 µg/ml) of IS (3). (Chromatograms A = standard solution, B = spiked human plasma and C = spiked albino mice plasma)

Validation of method

The proposed HPLC method was validated in terms of linearity, sensitivity, intra-day and inter-day precision, accuracy and stability in line with standards of FDA's approved Bioanalytical method validation [17].

Linearity

Calibration curves were found linear (as shown in Figure 6) in the range of 0.01 to 1µg/mL with co-efficient correlation (R^2) 0.998 and 0.996 in human and albino mice plasma respectively. The slope of the calibration curves and regression equations were shown in Table 2.

Table 1: Characteristics of doxorubicin-loaded poly lactic-co-glycolic acid nano-formulations

Formulation	Particle size	PDI	Zeta potential	Drug loading	Encapsulation efficiency
DNF-136	360±6.8	0.21±0.02	-29±0.6	69%	0.94
DNF-137	369±5.5	0.23±0.01	-24±0.5	71%	1.49
DNF-138	381±10	0.26±0.03	-19±0.8	78%	2.34
DNF-141	322±2.3	0.23±0.01	-13±1.0	73%	1.46
DNF-145	348±7.5	0.26±0.01	-10±0.2	81%	1.62

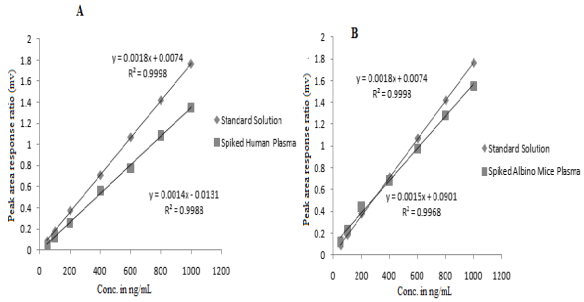

Figure 6: (A) Human plasma, R^2= 0.9983; (B) Albino mice plasma, R^2= 0.9968

Table 2: Linearity and calibration range

Parameter	Doxorubicin (mean ± SD; % RSD)
Linearity	0.01 -1µg/ml
Standard mixture	
Regression equation	y=0.0018x + 0.0074
R^2(Correlation co-efficient)	0.9998
Spiked human plasma samples	
Regression equation	y=0.0014x – 0.0131
R^2(Correlation co-efficient)	0.9988
Spiked albino mice plasma samples	
Regression equation	y =0.0015x + 0.0901
R^2(Correlation co-efficient)	0.9968

Accuracy

The accuracy of this method was determined on the basis of amount of doxorubicin recovered, and percent recoveries at 0.25, 0.5 and 1 µg of drug as shown in Table 3. More than 90% of the drug was recovered using the proposed method.

Sensitivity

The sensitivity of the proposed method was determined based on limit of detection (LOD) and limit of quantification (LOQ) of the drug in spiked plasma samples as shown in Table 3. The LOD and LOQ values in human spiked plasma were 0.014 and 0.030 µg/mL, respectively, and 0.012 and 0.022 µg/mL in mice spiked plasma, respectively.

Table 3: Percentage recovery, amount recovered of doxorubicin and sensitivity of the method

Parameter	Doxorubicin (mean ± SD; % RSD)
Accuracy (% recovery) (spiked human plasma sample) (n=3)	
0.25 µg/mL	92.70 ± 0.50; 0.64
0.5 µg/mL	94.54 ± 0.54; 0.75
1 µg/mL	98.18 ± 0.56; 0.73
Accuracy (amount recovered) (spiked Human plasma sample) (n=3)	
0.25 µg/mL	0.23 ± 0.006; 2.74
0.5 µg/mL	0.47 ± 0.003; 0.70
1 µg/mL	0.98 ± 0.007; 0.81
Accuracy (% recovery) (spiked albino mice plasma sample, n=3)	
0.25 µg/mL	93.9 ± 0.29; 0.33
0.5 µg/mL	98.4 ± 0.11; 0.12
1 µg/mL	101.1 ± 0.56; 0.73
Accuracy (amount recovered) (spiked albino mice plasma sample) (n=3)	
0.25 µg/mL	0.23 ± 0.002; 0.85
0.5 µg/mL	0.49 ± 0.009; 1.66
1 µg/mL	1.01 ± 0.003; 0.37
Repeatability (n=6) Injection repeatability	
Spiked human plasma sample (1 µg/mL)	3.50 ± 0.016 ;0.46 [a] 93078.3 ± 1555.7 ;1.67 [b]
Spiked albino mice sample (1 µg/mL)	3.48 ± 0.033 ;0.97 [a] 87111.7 ± 79401; 0.91 [b]
Analysis repeatability (% recovery) (n=6)	
Spiked human plasma sample (1 µg/mL)	87.89 ± 0.096 ;0.001[c] 91.82±0.045; 0.0005[c]
Spiked albino mice sample (1 µg/mL)	
Sensitivity in human plasma samples	
LOD (Limit of detection)	0.014 µg/mL
LOQ (Limit of Quantification)	0.030 µg/mL
Sensitivity in albino mice plasma samples	
LOD (Limit of detection)	0.012 µg/mL
LOQ (Limit of Quantification)	0.022µg/mL

[a] Retention time (minutes); [b] peak area; [c] % recovery

Inter-day and intra-day precision

The results of inter-day and intra-day precisions of the proposed method were shown in Table 4. The RSD values of repeatability and intermediate precision obtained were all less than 2.0% as shown in Table 4 and mean recovered amount of samples were satisfactory.

Stability of drug in plasma samples

When plasma samples were kept at 4 °C or below temperature, no significant changes were observer in the % recovery of the drug concentrations, indicates that the samples were stable as shown in Table 5. When the samples were kept at room temperature for long, the drug was degraded to a significant level. This indicates that the samples should be kept at

lower temperature (-20 °C to 4 °C) to get optimum result and evade stability issues.

Application of the method

The method was successfully applied for the evaluation of *in vitro* release kinetics and *in vivo* evaluations of selected nano-formulations of doxorubicin.

***In-vitro* release kinetics**

Various kinetic models such as zero order, 1st order, Higuchi, Hixon-Crowell and Korsmeyer Pappas were applied to find out the doxorubicin release and mechanism of release from the nanoparticles. All five (05) formulations displayed a bi-phasic pattern of release, with approximately 30-40% release within 24 h (burst release) and approximately 70-80% released after 120 h (sustained release) as shown in Figure 7.The characteristics release profile of PLGA is initial burst phase followed by a near zero-order phase as reported in previous studies [18].

The coefficient of regression (R^2) extracted from above mentioned mathematical models are given in Table 6. In comparison, the release data of the nano-formulations were best fitted to the Higuchi model because the values of R^2were high [19].

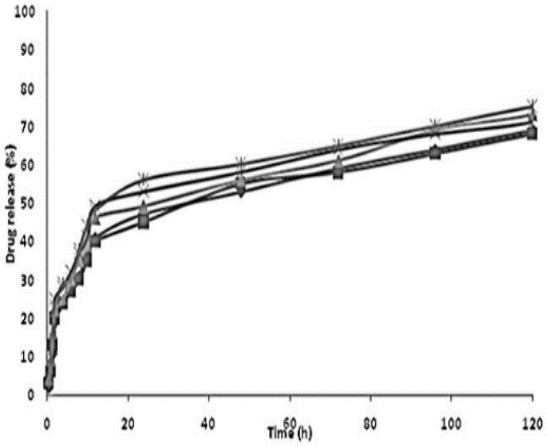

Figure 7: *Invitro* release profile of doxorubicin from nano-formulations

Table 4: Inter-day and intra-day precision

Spiked concentration of doxorubicin (µg/ml, n=3)	Concentration recovered (µg/ml)	
	Inter-day (mean ± SD); %RSD	***Intra-day (mean ± SD); %RSD***
Spiked human plasma		
0.25	0.24 ± 0.002; 0.249	0.23 ± 0.003; 0.121
0.5	0.49 ± 0.003; 0.701	0.47 ± 0.002; 0.503
1	0.98 ± 0.007; 0.813	0.98 ± 0.0002 0.019
Spiked albino mice plasma		
0.25	0.26 ± 0.003; 0.701	0.25 ± 0.003; 0.701
0.5	0.54 ± 0.009; 1.662	0.51 ± 0.0004; 0.101
1	0.99 ± 0.003; 0.366	0.98 ± 0.003; 0.041

Table 5: Stability of doxorubicin in plasma

Storage temperature	Storage time (h)	Recovery (mean ± SD; % RSD)		
		0.25 µg/ml	***0.5 µg/ml***	***1 µg/ml***
4°C	0	89.34 ± 0.54; 0.74	94.17 ± 0.47 ;0.44	99.22 ± 0.31 ;0.18
	6	90.40 ± 0.52; 0.73	93.58 ± 0.44 ;0.81	99.99 ± 0.57 ;0.08
	24	90.38 ± 0.50; 0.71	93.71 ± 0.61 ;0.32	99.83 ± 0.61 ;0.41
	72	91.2 9± 0.57; 0.64	93.62 ± 0.64 ;0.57	99.77 ± 0.53 ;0.39
25 °C	6	88.41 ± 0.55; 0.70	92.51 ± 0.62 ;0.54	96.80 ± 0.81 ;0.57
	24	88.54 ± 0.57; 0.44	92.29 ± 0.72 ;0.63	98.76 ± 0.77 ;0.59
	72	88.84 ± 0.50; 0.68	92.33± 0.69 ;0.46	97.63 ± 0.72 ;0.37
Freeze and thaw 3x		87.87 ± 0.61; 0.09	93.51 ± 0.55 ;0.35	95.52 ± 0.73 ;0.71

N = 3 for all sample storage conditions, except time = 0 where n = 6

Table 6: *In vitro* release kinetics of doxorubicin polymeric nano-formulations

Formulation	1st order (R^2)	Zero order (R^2)	Higuchi (R^2)	Korsemyer (R^2)	Hixon-Crowell (R^2)
F 136	0.814	0.956	0.989	0.982	0.968
F 137	0.761	0.913	0.965	0.955	0.923
F 138	0.825	0.965	0.984	0.977	0.971
F 141	0.753	0.950	0.975	0.949	0.956
F 145	0.773	0.943	0.981	0.964	0.951

Table 7: Pharmacokinetic parameters of reference standard and polymeric nano-formulations

Parameter	Polymeric nanoformulation					
	ROBOL	***F136***	***F 137***	***F 138***	***F 141***	***F 145***
Half-life (h^{-1})	14.83	44.58	88.75	266.37	49.37	85.81
AUC∞[d] (expo)[e] (µg-h/L)	206.0	10860.1	14331.1	14670.5	12613.7	12435.1
AUMC∞(area)(µg h*h/L)	31070.9	41114.9	45651.7	75747.7	47827.3	34302.3
MRT (expo) h	212.1	254.2	360.7	411.6	323.4	247.6
Vd (obsarea)[f] L	32.8	3.1	3.7	6.3	2.1	2.1
CL (expo) L/h	0.039	0.001	0.024	0.001	0.003	0.001

[d]Infinity, [e]extrapolated area, [f]observed area

In-vivo pharmacokinetics

The pharmacokinetics parameters of polymeric nano-formulations and reference drug (ROBOL®) were evaluated by intravenous route via the mice tail vein. The results are shown in Table 7.

DISCUSSION

Most of the methods developed previously were based on fluorescence or radioactivity measurement; they are poorly validated, and their sensitivity is low [20]. The method developed in the present study for the determination of doxorubicin in nano-formulations, human plasma and albino mice plasma using prednisolone as an IS is simple, sensitive, and rapid. Doxorubicin and prednisolone were completely separated at a short run time of 10 min, with good instrumental response. Various chromatographic parameters were optimized in line with standard guidelines [21]. The optimized column oven temperature was 30 °C using acetonitrile and 0.025M di-sodium hydrogen phosphate buffer (35:65, v/v) in isocratic mode and flow rate of mobile phase 0.9 mL/min.

This method had high sensitivity, with LLOD of 0.012 µg/mL, and 99 % drug recovery which is higher than values previously reported. The linearity range of this method was 0.022 – 1 µg/mL, whereas linearly ranges of available methods are 1 – 100 µg/mL.

In vitro release studies showed that the doxorubicin was released in a biphasic manner i.e. initial burst release followed by sustained release, consistent with reports in the literature [18]. The results obtained from different mathematical models showed that Higuchi model best fitted the release data, was clear from the R^2 values. This means that diffusion occurred is the main mechanism of the drug release from the nanoparticles thus avoiding the erosion during the process.

Half-life, AUC, AUMC and MRT were extensively increased, when compared to the standard, showing that the circulation half-life was increased for polymeric nano-formulations. Vd and Cl decreased for the nano-formulations, relative to standard, an indication that the nano-formulations remained for longer time in the body.

CONCLUSION

An easy sample extraction method has been developed and found to give sharp recovery for doxorubicin as well as IS. Preparation of mobile phase is straightforward and gives well separated peaks for doxorubicin. In order to determine targeted drug in polymeric nanoformulations, spiked human and spiked mice plasma, experimental conditions (mobile phase, column oven temperature, UV wavelength and mobile phase flow rate) have been optimized for a novel RP-HPLC system.

DECLARATIONS

Acknowledgement

The authors are grateful to the Higher Education Commission and University of Peshawar Pakistan, for providing financial support and research facilities respectively.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Iqbal Z. is corresponding author on the study. Sumaira I.K. was first author and responsible for all research activities and writing of the article. Nasir F. helped in experimental design. Ismail M.,

Shahbaz N. and Khan A. edited in the article chromatograms. Khattak M.A. and Sakhi M. assessed the article and made recommendations. All authors read and approved the final manuscript.

REFERENCES

1. *Jackson TL. Intracellular accumulation and mechanism of action of doxorubicin in a spatio-temporal tumor model. J TheorBiol 2003; 220: 201-213.*
2. *Weiss RB. The anthracyclines: will we ever find a better doxorubicin. Semin Oncol 1992; 19: 670-686.*
3. *Speth P, Van Hoesel Q, Haanen C. Clinical pharmacokinetics of doxorubicin. ClinPharmacokinet 1988; 15: 15-31.*
4. *Sparreboom A, Planting AS, Jewell RC, Loos W, Nooter K, Chandler L, Wissel PS, Verweij J. Clinical pharmacokinetics of doxorubicin in combination with GF120918, a potent inhibitor of MDR1 P-glycoprotein. J. Anti-cancer drugs 1999; 10: 719-728.*
5. *Mosafer J, Abnous K, Tafaghodi M, Mokhtarzadeh A, Ramezani M. In vitro and in vivo evaluation of anti-nucleolin-targeted magnetic PLGA nanoparticles loaded with doxorubicin as a theranostic agent for enhanced targeted cancer imaging and therapy. Eur J Pharm Biopharm2017; 113: 60-74.*
6. *Yang X, Gao H, Qian F, Zhao C, Liao X. Internal standard method for the measurement of doxorubicin and daunorubicin by capillary electrophoresis with in-column double optical-fiber LED-induced fluorescence detection. J Pharm Biomed Anal 2016; 117: 118-124.*
7. *He W, Martin JH, Shaw PN, Lu X, Walpole ET, Dimeski G. A Simple and Sensitive LC-MS/MS Method for the Simultaneous Determination of Cyclophosphamide and Doxorubicin Concentrations in Human Plasma. Curr Pharm Anal2018; 141: 53-59.*
8. *Zhou J, Chen S, Sun F, Luo P, Du Q, Zhao S. Indirect chiral separation of tryptophan enantiomers by high performance liquid chromatography with indirect chemiluminiscence detection. J ChromatogrB Biomed SciAppl2015; 1006: 65-70.*
9. *Chen CL, Thoen KK, Uckun FM. High-performance liquid chromatographic methods for the determination of topoisomerase II inhibitors. J ChromatogrB Biomed SciAppl2001; 764: 81-119.*
10. *Lucas AT, O'Neal SK, Santos CM, White TF, Zamboni WC. A sensitive high performance liquid chromatography assay for the quantification of doxorubicin associated with DNA in tumor and tissues. J Pharm Biomed Anal 2016; 119: 122-129.*
11. *Rodrigues AS, Lopes AR, Leão A, Couceiro A, Ribeiro ABS, Ramos F, Noronha da Silveira MI, Resende de Oliveira C. Development of an Analytical Methodology for Simultaneous Determination of Vincristine and Doxorubicin in Pharmaceutical Preparations for Oncology by HPLC-UV. J ChromatogrSci 2009; 47: 387-391.*
12. *Danhier F, Ansorena E, Silva JM, Coco R, Le Breton A, Préat V. PLGA-based nanoparticles: an overview of biomedical applications. J Control Release 2012; 161: 505-522.*
13. *Sharma S, Parmar A, Kori S, Sandhir R. PLGA-based nanoparticles: a new paradigm in biomedical applications. Trends AnalytChem2016; 80: 30-40.*
14. *Yallapu MM, Gupta BK, Jaggi M, Chauhan SC. Fabrication of curcumin encapsulated PLGA nanoparticles for improved therapeutic effects in metastatic cancer cells. J Colloid Interface Sci 2010; 351: 19-29.*
15. *Acharya S, Sahoo SK. PLGA nanoparticles containing various anticancer agents and tumour delivery by EPR effect. AdvDrug Deliv Rev2011; 63: 170-183.*
16. *National Institutes of Health. Guide for the care and use of laboratory animals. Washington: National Academies; 2011. Available from: https://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-use-of-laboratory-animals.pdf*
17. *Food and Drug Administration, Guidance for industry: Bioanalytical method validation 2018. Available from:https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf*
18. *Costa P, Lobo JMS. Modeling and comparison of dissolution profiles. Eur J Pharm Sci 2001; 13: 123-133.*
19. *Dash S, Murthy PN, Nath L, Chowdhury P. Kinetic modeling on drug release from controlled drug delivery systems. Acta Pol Pharm 2010; 67: 217-23.*
20. *Yen MJ, Chen SH, Yen CM, Chung TC. Simultaneous determination of doxorubicin and active metabolite doxorubicinol in plasma by capillary electrophoresis and field-amplified sample stacking. 2015.*
21. *Epshtein N. Validation of HPLC techniques for pharmaceutical analysis. Pharm Chem J 2004; 38: 212-228.*