Direct, simultaneous measurement of liposome-encapsulated and released drugs in plasma by on-line SPE–SPE–HPLC

Eiichi Yamamoto [a,*], Kenji Hyodo [a], Naozumi Ohnishi [b], Takuya Suzuki [a], Hiroshi Ishihara [a], Hiroshi Kikuchi [a], Naoki Asakawa [c]

[a] DDS Research, Formulation Research Laboratories, Pharmaceutical Science & Technology, Eisai Co. Ltd., 5-1-3 Tokodai, Tsukuba, Ibaraki 300-2635, Japan
[b] Formulation Research Laboratories, Pharmaceutical Science & Technology, Eisai Co. Ltd., 1 Kawashimatakehaya, Kagamigahara, Gifu 501-6195, Japan
[c] Pharmaceutical Science & Technology, Eisai Co. Ltd., 5-1-3 Tokodai, Tsukuba, Ibaraki 300-2635, Japan

ARTICLE INFO

ABSTRACT

A method for the simultaneous measurement of liposome-encapsulated and released drugs in mouse plasma by on-line solid phase extraction (SPE)–SPE–HPLC with direct plasma injection was developed using a doxorubicin (DXR)-containing liposome formulation as the model drug. During SPE, the released DXR was extracted on the 1st restricted-access media (RAM) SPE column, whereas the liposomes were eluted. The eluted liposomes were collapsed on-line, and the released DXR was delivered to the 2nd RAM SPE column for extraction. The retained DXR on the SPE columns was analyzed via HPLC–fluorescent detector by switching the valves. The method was validated and applied to the pharmacokinetic study of DXR in mice after intravenous injection of DXR-containing liposomes.

1. Introduction

Liposomes, which are spherical, enclosed structures formed by one or several concentric lipid bilayers with aqueous phases inside and between the lipid bilayers, have received a great deal of attention as pharmaceutical carriers. Today, liposome drug delivery systems (DDSs) are an emerging technology for the rational delivery of chemotherapeutic drugs during the treatment of cancer. Liposomal surfaces can be modified with various ligands, e.g., protective polymers, such as synthetic polymers, peptides, antibodies, antibody fragments, or receptor ligands [1–7]. These surface-modified liposomes are classified as either passive targeting liposomes or active targeting liposomes, based on their capacity to recognize and bind to specific cells of interest [8].

PEG-modified liposomes (PEGylated liposomes), which are long-circulating passively targeted liposomes [1], rationally increase the drug delivery capacity based on the enhanced permeation effect [9]. The use of PEGylated liposomes offers improved pharmacokinetic (PK) properties, the controlled and sustained release of drugs, and lower systemic toxicity.

Today, ten liposome drug products are commercially available. Among these products, the DXR-containing PEGylated liposome formulation Doxil®, the daunorubicin-containing liposome formulation DaunoXome and the amphotericin B-incorporated liposome formulation AmBisome® have attracted much attention as research subjects or targets. The PK properties of these drugs have been intensively investigated. Most investigations deal with the total concentrations of encapsulated and released DXR [10–12], daunorubicin [13], or amphotericin B in plasma samples [14–16]. However, the processes involved in the delivery of these carriers and the release of the active agent, the variability of such processes, and the degree to which the active agent is released into the extracellular fluids surrounding tumor cells are still unknown [17].

To understand and predict the efficacy and/or toxicity of liposomal drugs in vivo, it is necessary to establish reliable methods to determine the amounts of liposomal and released drugs in biological fluids. Analytical methods for the measurement of released and liposomal drugs in plasma samples have previously been developed. These methods utilize ultracentrifugation [18], solid phase extraction (SPE) [19–22], gel filtration [19] and ultrafiltration in off-line sample preparation procedures [23]. However, most of these separation methods have limitations, which include difficulty in separating the large liposomes by ultracentrifugation, drug adsorption to ultrafiltration devices, high sample dilution during gel chromatography, and potential drug release from the

liposomes during sample preparation using reversed phase SPE [24]. Furthermore, these methods require large volumes of the plasma sample (~100 µL), and combined with complex sample pre-treatment, they cause physicochemical changes that may result in drug release from the liposomes during sample preparation [13,20].

Several reviews have described the role of analytical chemistry in liposomal drug delivery systems [25,26]. It is important to understand the properties of liposomes quantitatively *in vitro* and *in vivo* to allow comparisons between liposomes as drug carriers in pharmaceutical development [24,27]. Therefore, we attempted to develop simultaneous measurement of drugs and liposome-encapsulated drugs by taking into account the differences in physicochemical properties and sizes between the drugs and the liposomes. This report describes an effective and simple on-line SPE of released and liposomal drugs in the plasma using a column-switching HPLC system for DXR-containing liposome formulations as the model drug and a methylcellulose-immobilized octadecylsilylated silica (MC-ODS) SPE column [28], which is a restricted access media (RAM) column [29–31] capable of direct plasma injection [28,32].

2. Experimental

2.1. Chemicals and reagents

DOXIL® was purchased from Johnson & Johnson K.K. (Tokyo, Japan). DXR hydrochloride, ammonium acetate, acetonitrile, methanol, ammonium hydroxide, formic acid, ammonium sulfate and citric acid monohydrate were purchased from Wako Pure Chemicals (Osaka, Japan). Pentafluoropropionic acid was purchased from Tokyo Chemical Industry (Tokyo, Japan). Hydrogenated soy phosphatidylcholine (HSPC) was purchased from NOF Corporation (Tokyo, Japan). Cholesterol was purchased from Sigma–Aldrich (Tokyo, Japan). *N*-(Carbonyl-methoxypolyethyleneglycol-2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine, sodium salt (MPEG-DSPE) was purchased from Corden Pharma Switzerland LLC (Liestal, Switzerland). All other reagents were of analytical grade. Water was deionized and purified by a Milli-Q® TOC purification system from Millipore (Bedford, MA).

2.2. Preparation of liposomes

Blank liposomes were composed of MPEG-DSPE (3.19 mg/mL), HSPC (9.58 mg/mL) and cholesterol (3.19 mg/mL). The liposome interior solution contained 250 mM ammonium sulfate. The liposomes were suspended in 9% sucrose containing 10 mM L-histidine as the buffer. Hydrochloric acid and/or sodium hydroxide was added to adjust the pH to 7.5. The liposome size was limited to approximately 80 nm by several passages through a mini-extruder containing a polycarbonate filter with a pore size of 50 nm.

2.3. Sample preparation

Following anti-coagulation treatment with heparin, mouse plasma was obtained via centrifugation of mouse blood. The blank liposomes were added to the mouse plasma (10:90, v/v), and this suspension was used as the blank plasma.

A stock solution of DXR was prepared in 50% methanol at a concentration of 1.0 mg/mL. DOXIL® (DXR concentration; 20 mg/mL) was diluted in the blank liposome suspension to 1.0 mg/mL and was used as a stock solution for the DXR-containing liposome formulation. Mouse plasma containing DXR was prepared by adding DXR to the blank plasma at concentrations of 0.01, 0.1, 0.5, 1.0,

5.0, and 10.0 µg/mL. Mouse plasma with DXR-containing liposomes was prepared by adding the DXR-containing liposome formulation stock solution to the blank plasma at concentrations of 0.5, 1.25, 2.5, 5.0, 10.0, and 20.0 µg/mL.

2.4. Instrumentation

A column-switching HPLC system, illustrated in Fig. 1, was used. The system consisted of five LC-10ADvp pumps, an SIL-10ADvp auto-injector, two DGU-14 degassers, a CTO-10ADvp column oven, two FCV-12AH six-port valves, an RF-10AXL fluorescent detector, and an SCL-10Avp system controller. The LC Class VP software package ver. 6.12 was used for system control, data acquisition, and data analysis. All of the instruments used in this study were products of Shimadzu Corporation (Kyoto, Japan). Shimadzu MAYI-ODS (10 mm × 4.6 mm I.D. and 30 mm × 4.6 mm I.D., 50 µm particle, 12-nm pore size) (Kyoto, Japan), an MC-ODS column, was used as the SPE column. A GL Sciences InertSustain C18 (100 mm × 3.0 mm I.D., 3-µm particle) (Tokyo, Japan) was used as the analytical column for HPLC.

In the direct plasma injection methodology, the physical stability of the plasma samples during storage is a concern. When plasma proteins clog the SPE column or the system, the resulting pressure in the SPE system would result in instrumental problems. To avoid such a failure, two in-line filters were connected to the SPE system and the HPLC system to protect the SPE and HPLC columns, as shown in Fig. 1.

2.5. High performance liquid chromatography

The mobile phase for SPE was composed of 5 mM ammonium acetate (pH 7)–methanol (95:5, v/v). The washing mobile phase for the SPE system was methanol, which was delivered at 1 mL/min during the last 5 min of HPLC analysis.

The HPLC mobile phase A was 10 mM ammonium acetate (pH 7)–acetonitrile (95:5, v/v), and the HPLC mobile phase B was 10 mM ammonium acetate (pH 7)–acetonitrile (10:90, v/v). The injection needle of the auto-injector was rinsed with water–methanol (50:50, v/v).

A 10-µL portion of the plasma sample was directly injected and delivered to the SPE columns with the SPE mobile phase at a flow rate of 1.0 mL/min for 2 min. During the SPE procedure, a dilution mobile phase was delivered at 1.0 mL/min, and a 2-fold dilution was prepared for on-line SPE of the liposomal DXR for the 2nd SPE column. The 1st SPE column was maintained at ambient temperature, but the remaining columns were maintained at 45 °C. DXR extracted on SPE columns were transferred to HPLC and released DXR and liposomal DXR were assayed for fluorescence emission at 550 nm using an excitation wavelength of 470 nm.

2.6. Validation

The linearity of the released DXR was assessed at concentrations of 0.01, 0.05, 0.1, 0.5, 1.0, 5.0, and 10.0 µg/mL DXR. For liposomal DXR, concentrations of 0.5, 1.25, 2.5, 5.0, 10.0, and 20.0 µg/mL were selected. Intra- and inter-day accuracy and precision were evaluated for 0.1, 1.0 and 10.0 µg/mL released DXR. For liposomal DXR, intra- and inter-day accuracy and precision were evaluated at 0.5, 5.0, and 20.0 µg/mL. The lower limit of quantification (LLOQ) was defined based on validation results.

The accuracy (%) was determined by the following equation:

$$\text{Accuracy } (\%) = \frac{\text{Spiked concentration} - \text{Measured concentration}}{\text{Spiked concentration}} \times 100$$
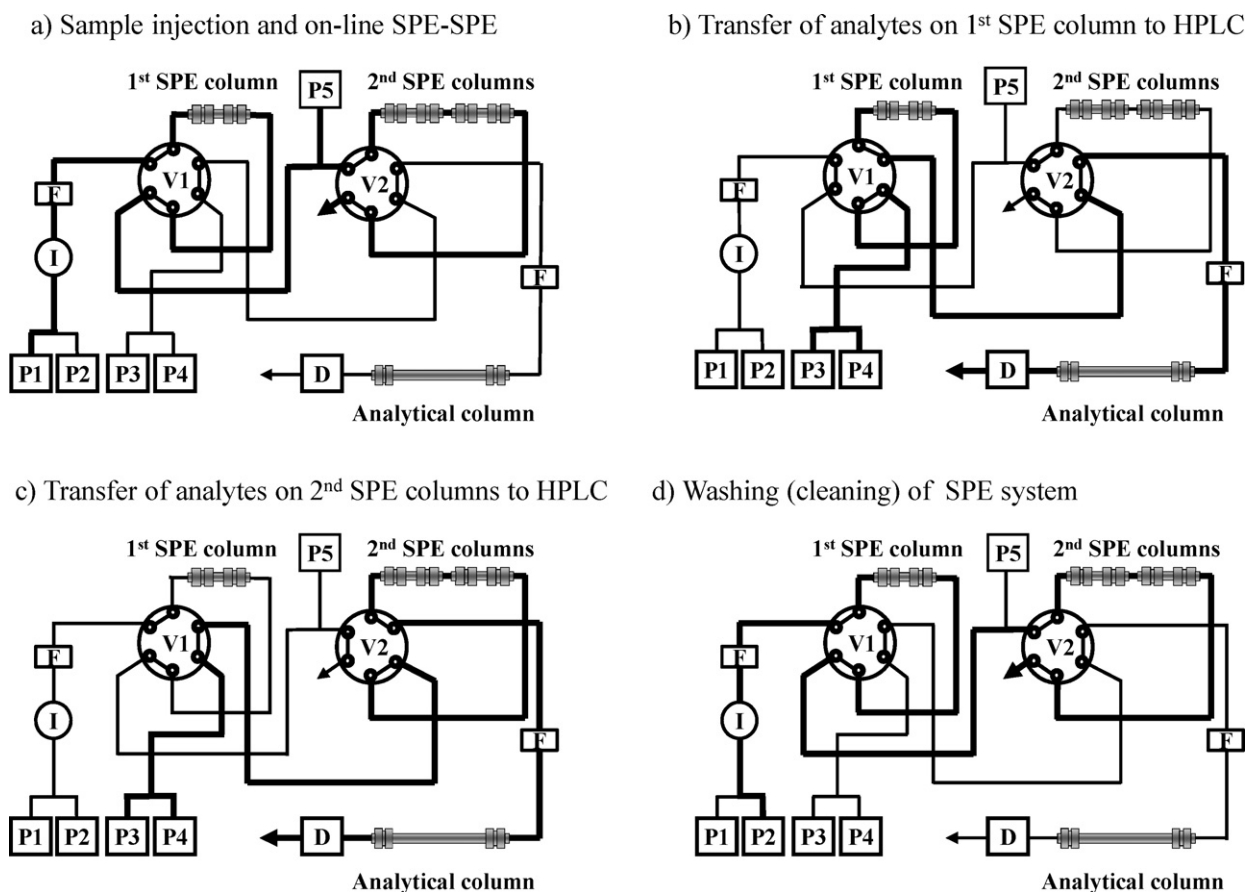
a) Sample injection and on-line SPE-SPE

b) Transfer of analytes on 1st SPE column to HPLC

c) Transfer of analytes on 2nd SPE columns to HPLC

d) Washing (cleaning) of SPE system

Fig. 1. Schematic diagram of the SPE–SPE–HPLC–FLD apparatus. (a) Sample injection and on-line SPE–SPE. (b) Transfer of analytes on 1st SPE column to HPLC column for analysis. (c) Transfer of analytes on 2nd SPE column to HPLC column for analysis. (d) Washing (cleaning) of SPE system after HPLC analysis for analytes. P1 and P2, pumps for SPE system; I, injector; F, in-line filter; V1 and V2, 6 port switching valve; P3 and P4, pumps for HPLC system, P5, dilution pump; D, fluorescence detector.

The precision was determined by the coefficient of variation (CV) for released DXR and liposomal DXR.

2.7. Stability

The stability of the plasma samples was investigated for plasma collected from mice 24 h after intravenous (IV) injection of the DXR-containing liposome formulation ($n = 3$). Aliquots of plasma samples (50 μL) were divided into polypropylene vials and stored at 4 °C for 48 h.

2.8. PK study of DXR in mice after IV injection of DXR-containing liposomes

Male BALB/c Slc-nu/nu mice weighing 20–26 g were obtained from Charles River Laboratories (Yokohama, Japan). DXR-containing liposomes were administered (1 mg/kg of DXR) in a total volume of 0.1 mL via tail-vein injections. At specific times (0.083, 0.5, 1, 3, 6, 24, and 48 h) after injection, three mice were anesthetized with isoflurane, and blood was collected from the inferior vena cava. The obtained blood samples were immediately placed on ice. Plasma samples were separated by centrifugation (13,300 × g) at 5 °C for 20 min and stored at 5 °C until analysis.

3. Results and discussion

3.1. Selection of the SPE column

Several reports in the literature have indicated that liposomes have an ability to pass through reversed-phase ODS silica gel SPE cartridges without being retained, whereas non-liposomal drugs are retained in the stationary phase of the SPE cartridges [19,20]. However, it has also been suggested that liposomal drugs are released and/or leaked from the liposomes during off-line SPE with the ODS SPE cartridges [13,20]. The release of the drugs from the liposomes would be caused by the hydrophobic interactions of the liposomes and the ODS stationary phase.

To establish a reliable sample preparation procedure without any physicochemical change to the liposomes, we attempted to develop a direct plasma injection methodology using RAM. RAM is an adsorption medium that has a sieving effect to exclude non-interacting macromolecules; it also allows the chromatographic retention of small molecules in the stationary phases. The outer surface employs both size exclusion and hydrophilic interactions to prevent large biomolecules from accessing the inner layer. However, small molecules can penetrate through to the inner surface and are retained in the stationary phases. The direct analysis of drugs and metabolites in biological fluids, such as plasma, using RAM with HPLC can rapidly yield sensitive chemical identifications [32,33].

The MC-ODS (MAYI-ODS) column is a RAM column with certain characteristic properties. The MC external surface of MC-ODS effectively elutes large molecules (>3 nm), such as proteins, whereas the internal surface of MC-ODS retains small molecules, such as drugs and drug metabolites in plasma, because of hydrophobic interactions. Thus, when plasma samples containing drugs and PEGylated liposomes (particle size 80–200 nm) were directly injected onto the column, the released drugs were extracted in the ODS stationary phase, whereas the PEGylated liposomes were eluted without any change in their characteristics under appropriate conditions.

For the SPE of the released DXR, the MAYI-ODS column (10 mm × 4.6 mm I.D.) was selected as the 1st SPE column. During the extraction of liposomal DXR, the liposome components, i.e., the phospholipids and cholesterol would also be retained on the SPE column in addition to DXR. Hence, two MAYI-ODS columns (30 mm × 4.6 mm I.D.) with large capacities were connected in tandem.

3.2. Selection of mobile phase additives in SPE

To extract the released drugs in plasma in the SPE with high selectivity, the mobile phase should be selected by considering that liposomes must stay intact without the leakage of the drug as well as that released drugs are retained in the stationary phase. Additionally, in the SPE of liposomal drugs, the efficient drug release from liposomes prior to or during SPE is required.

Hence, the effect of the mobile phase on the DXR release and leakage from the liposomes was investigated by examining the mobile phase pH and additives with regard to DXR release (%). In any drug–protein binding, hydrophobic interactions play an important role. Accordingly, the addition of a small amount (i.e., ≤10%) of organic modifier to the extraction mobile phase enhances the release of the drug from the binding protein [34,35]. This is an effective procedure to improve the extraction efficacy of drugs from plasma. However, the addition of an organic modifier may lead to the collapse of the liposomes. Because the liposome formulation was delivered to the SPE column with 5% methanol, about 5% of the total DXR was determined to be in the released form. In addition, ammonium acetate also released the DXR from the liposomes. In practice, however, it was found that the DXR-containing liposomes in the plasma sample did not release DXR during SPE when 5 mM ammonium acetate (pH 7):methanol (95:5, v/v) was employed. This phenomenon might indicate interactions between the PEG bound on the liposome surface and endogenous materials in the plasma [36], which would consequently physically stabilize the liposomes. The use of ammonium as a mobile phase additive has also been shown to improve the peak shape for basic compounds [37]. Thus, narrow peak bands of DXR in the SPE column were anticipated at pH 7, and the ammonium acetate–methanol mixture was selected as the mobile phase for the 1st SPE. Efficient and effective DXR release from the liposomes was required during the 2nd SPE.

The effect of the dilution mobile phase pH on the DXR release from liposomes during the 2nd SPE was investigated. As the SPE mobile phase pH decreased, DXR was more effectively released from liposomes in the pH range from 2 to 7. Actually, 1%, 19%, almost 100% of DXR was released from/or leaked liposomes with a dilution mobile phase at pH 7, pH 3.3 and pH 2.6, respectively. Consequently, an on-line dilution with a mobile phase at pH 2.6 was selected for the SPE of liposomal DXR.

DXR is a hydrophilic, basic compound with a pK_a value (of conjugated acid) of 8.3 [38] and a log P value of 1.85 [39] and the retention of DXR in the ODS stationary phase at acidic pH should be weak. To achieve sufficient retention of DXR at acidic pH, 0.1% pentafluoropropionic acid, an ion-pairing agent for basic compounds, was added to the dilution mobile phase. Furthermore, because drug leakage from liposomes is accelerated by the presence of ammonium in the external phase [24,40], ammonium hydroxide was added to the mobile phase. The pH of the mobile phase was adjusted by the addition of 1.6% (v/v) formic acid to pH 2.6. During the on-line SPE under the above conditions, pressure build-ups and decreased performance of the SPE columns were not observed, even after 100 analysis cycles.
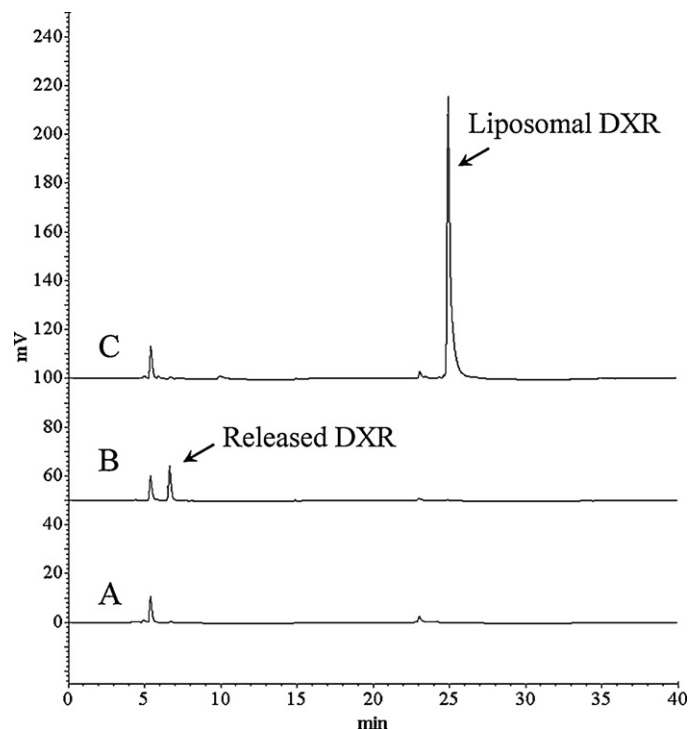

Fig. 2. Representative HPLC chromatograms of released DXR and liposomal DXR. Blank mouse plasma, (B) plasma spiked with 1 μg/mL DXR and (C) plasma spiked with DXR-containing liposomes (5 μg/mL of DXR).

3.3. Reduction of carryover

In column-switching HPLC analyses, the carryover of analytes in the system is often problematic [41] and a washing procedure for column-switching HPLC was proposed [42]. In the present SPE–SPE–HPLC analysis, released DXR and liposomal DXR on SPE columns were sequentially analyzed using HPLC. It has previously been reported that about 1% of the released DXR was present in plasma after IV injection of DOXIL® [43]. Therefore, DXR carryover in the SPE–SPE–HPLC that originates from liposomal DXR could significantly affect the analytical results for the concentration of the released DXR. To solve the problem of carry-over in the SPE–SPE–HPLC system, the tandem SPE system was washed at the end of each analysis with methanol delivered by a pump through the auto-sampler without complexion of the system. The programmed washing procedure effectively reduced the carry-over of DXR to less than 0.02%.

3.4. Validation

Representative HPLC–FLD chromatograms of blank plasma, plasma spiked with DXR (1 μg/mL) and liposomal DXR (5 μg/mL) are shown in Fig. 2. Significant interference peaks were not observed for released or liposomal DXR. The chromatogram of liposomal DXR demonstrated the absence of DXR released from liposomes during the SPE procedure. These data provided sufficient evidence for the identification and specificity for both DXR peaks. Calibration curves were obtained by plotting the peak area against the compound concentration. These curves were linear over concentrations of 0.01–10.0 μg/mL and 0.5–20.0 μg/mL for released DXR and liposomal DXR, respectively, with coefficients of determination (r^2) that exceeded 0.999. The equations for the calibration plots were $y = 36,7645x + 17,8878$ for the liposomal DXR and $y = 11,6451x - 5495$ for the released DXR. The slopes of the two equations were significantly different because the DXR

Table 1
Intra-and inter-day precision and accuracy for released DXR and liposomal DXR in plasma samples.

Analyte	Spiked concentration (μg/mL)	Found concentration (μg/mL)		Precision (%)		Accuracy (%)	
		Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
Released DXR	0.10	0.10 ± 0.00	0.09 ± 0.00	−5.0	−8.1	0.9	5.1
	1.00	1.02 ± 0.03	1.04 ± 0.09	1.8	3.5	3.3	8.5
	10.00	10.68 ± 0.17	11.05 ± 0.82	6.8	10.5	1.5	7.4
Liposomal DXR	0.50	0.51 ± 0.00	0.48 ± 0.01	1.3	−4.5	0.3	1.5
	5.00	5.08 ± 0.02	4.78 ± 0.25	1.6	−4.5	0.4	5.3
	20.00	19.80 ± 0.03	19.44 ± 0.11	−1.0	−2.8	0.2	0.6

fluorescence is dependent on the dielectric constant of the solution and pH; the fluorescence intensity decreases as the pH increases [44]. The DXR released in the plasma was extracted and analyzed by mobile phases with neutral pH values, whereas the liposomal DXR was extracted with an acidic mobile phase containing an ion-pair reagent with high ionic strength in SPE and was transferred to the HPLC column as a paired ion with pentafluoropropionic acid.

The intra- and inter-day precision and accuracy were evaluated by analyzing mouse plasma that had been supplemented with released DXR (0.1, 1.0 and 10.0 μg/mL) or liposomal DXR (0.5, 5.0 and 20.0 μg/mL). The intra- and inter-day accuracy and precision for the released DXR and liposomal DXR were satisfactory, as shown in Table 1. The intra-day precision ranged from −5.0% to 6.8%, and the intra-day accuracy ranged from 0.2% to 3.3% for both analytes. The inter-day precision ranged from −8.1% to 10.5%, and the inter-day accuracy ranged from 0.6% to 8.5% for both analytes. Therefore, the validation data met the established criteria for bioanalytical methods [45]. It was concluded that the on-line extraction of released DXR and liposomal DXR using the MC-ODS SPE column methodology could serve as a reproducible assay for the analysis of mouse plasma over a sufficient range. The data defined the LLOQ for the released DXR and liposomal DXR as 0.1 μg/mL and 0.5 μg/mL, respectively. These data confirmed our ability to prepare on-line samples that contain released and liposomal drugs in plasma using the MC-ODS SPE column in the context of the fully automated column-switching HPLC approach with plasma direct injection.

3.5. Stability of the sample

All of the experimental results were from frozen or fresh blank plasma; however, plasma samples containing liposomal DXR were from samples that had not been frozen according to a suggestion that the number of freeze/thaw steps should be minimized in the DXR liposome-handling process [46]. For long-term storage of plasma samples, freezing is preferable, and the literature explains that the freezing of plasma samples containing DaunoXome is only acceptable if 20% glycerol has been added [13]. Here, the stability of the plasma sample at 4 °C was investigated for plasma samples collected from mice 24 h after the IV injection of DXR-containing liposomes (n = 3). Plasma samples that contained 4.8 ± 1.0% released DXR were stored at 4 °C for 48 h. From the analytical data obtained after 24 and 48 h of storage, a total of 5.9 ± 1.8% and 6.3 ± 2.1% of DXR was released, respectively, without a decrease in the total DXR concentration.

3.6. PK study of DXR in mice after IV injection of DXR-containing liposomes

To confirm the applicability of the method to real-world samples, a PK study was performed in male BALB/c Slc-nu/nu mice following an IV injection of DXR-containing liposomes with 1 mg/kg of DXR. Representative HPLC chromatograms of mouse
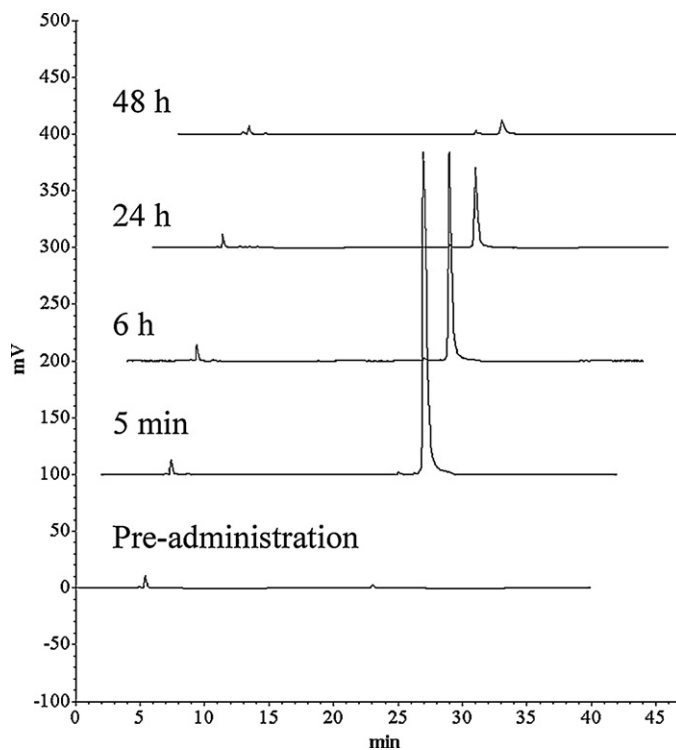

Fig. 3. Representative HPLC chromatograms of DXR after IV injection of DXR-containing liposomes in mice.
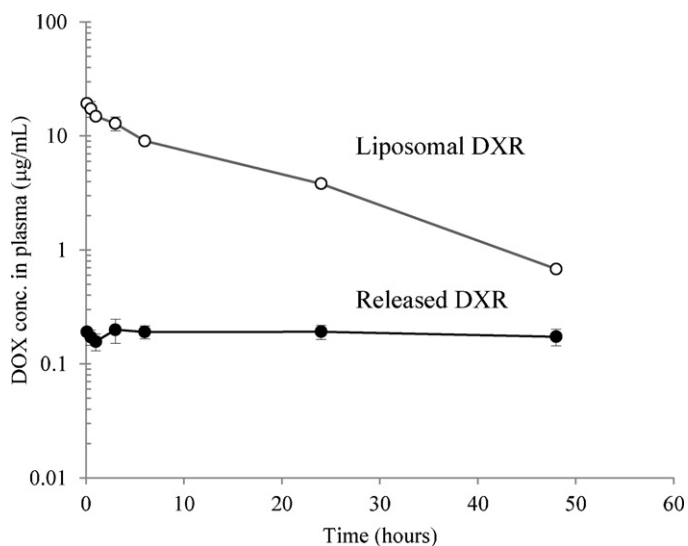

Fig. 4. Time course of released DXR and liposomal DXR in plasma after IV administration of DXR-containing liposomes (1 mg/kg of DXR, n = 3): (●) released DXR and (○) liposomal DXR.

plasma following IV injections of the DXR-containing liposomes for 5 min, 6 h, 24 h and 48 h are presented in Fig. 3. Fig. 4 shows the time course of the released and liposomal DXR in mice after the IV injection of the DXR-containing liposomes. The liposomal DXR was eliminated following a first-order exponential decay, whereas the released DXR concentrations barely reached 1% of the concentrations of the liposomal DXR. The obtained results were in agreement with the literature with regard to the clearance in the plasma and released drug levels [47]. It was concluded that the method was able to simultaneously measure the amount of the released and liposomal DXR in the mouse plasma.

The plasma concentration range of DXR in the investigation is equivalent to that of patients after IV injection of Doxil® (25 mg/m^2) [47] and it was indicated that the potential applicability of the method to determination of plasma concentration of DXR in clinic.

4. Conclusion

To develop a fully automated and reliable method for the measurement of released and liposomal drugs in plasma, we investigated the effectiveness of on-line extraction of released and liposomal drugs in plasma using an MC-ODS RAM SPE column in column-switching HPLC. Tandem SPE with direct plasma injection allowed the selective extraction of released drugs and liposomal drugs in plasma. The analytical method was validated, and the applicability to real-world samples was successfully demonstrated by the PK study of doxorubicin after IV injection of DXR-containing liposomes into mice. This direct plasma injection approach using the proposed SPE–SPE–HPLC system made it possible to simultaneously measure the liposomal and released drugs in plasma. The methodology enabled us to determine the *in vivo* properties of liposomes and contribute to the efficient development of liposomal drug products.

Acknowledgment

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