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Determination of non-liposomal and liposomal doxorubicin in plasma by LC–MS/MS coupled with an effective solid phase extraction: In comparison with ultrafiltration technique and application to a pharmacokinetic study

Yaping Xie, Nan Shao, Yi Jin, Liang Zhang, Huan Jiang, Ningjie Xiong, Fangming Su, Haiyan Xu*

Pharmacy School, Shenyang Pharmaceutical University, Shenyang 110016, China

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ABSTRACT

Liposomal formulation of doxorubicin has been widely applied in clinic for treatment of various cancers. The separation and measurement of free drug (drug which is not entrapped in liposomes) and liposomal drug in the plasma after injection of liposomal doxorubicin is of prime importance due to toxicity and activity concerns. In this study, a rapid and convenient method was developed to isolate and determine the non-liposomal and liposomal drugs in plasma. Plasma samples were prepared by solid phase extraction (SPE) using Oasis HLB cartridges. Liposomal doxorubicin (L-DOX) was collected in the aqueous eluate with its internal standard (IS), metformin; and non-liposomal doxorubicin (NL-DOX) and its isotope labelling IS were eluted from the cartridge by methanol containing 0.5% formic acid. After SPE separation, L-DOX and NL-DOX were subsequently quantified by a validated sensitive LC–MS/MS method individually. The calibration curves were found to be linear for L-DOX in the range of 0.156–40.0 μg/mL and for NL-DOX in the range of 3.13–200 ng/mL. The extraction recovery was about 97% for L-DOX and about 65% for NL-DOX. This method was further applied to investigate the pharmacokinetics of doxorubicin in Beagle dogs after an intravenous dose of 1.0 mg/kg Doxil®. After injection of Doxil®, L-DOX was the predominant component circulating in plasma, whose amount was about 1000-fold higher than that of NL-DOX. The analytical method might be helpful in pharmacokinetics and toxicity assessment of liposomal formulation.

1. Introduction

Doxorubicin (DOX) is a highly active anthracycline antibiotic and displays a broad spectrum of anti-tumor activity. The drug is one of the most important anticancer agents in the treatment of breast cancer, ovarian cancer, sarcomas, leukemias and lymphomas [1,2]. However, the clinical application of DOX is restricted for dose-dependent myelosuppression, a significant incidence of irreversible cardiotoxicity, and the development of drug resistance [3–5]. Liposomal drug delivery systems, proposed initially by Gregoriadis in 1981 [6], have proven useful in alleviating toxicity while maintaining or increasing antitumor activity of certain chemotherapeutic agents. Doxil® in USA, known as Caelyx® in Europe, is a formulation of DOX encapsulated in pegylated (Stealth®) liposomes. It is the first clinical liposomal product approved by FDA in 1995. The Stealth liposomal DOX shows enhanced antitumor activity with decreased cardiotoxicity in comparison with free drug [7–10].

It is well known that liposomal drugs should be regarded as new entities different from free drugs because liposomal encapsulation dramatically changes drug pharmacokinetics and distribution compared to non-liposomal drugs [10–13]. The altered pharmacokinetics and distribution, playing an important role in therapeutic effects and toxicities, are closely related to the disposition of the three forms of liposomal formulations circulating *in vivo* after *i.v.* administration: liposome-entrapped, protein-bound and free drug. Unlike conventional dosage forms, the clearance of liposomal drugs occurs as a result of three processes with different elimination rates: (1) tissue uptake and elimination of entrapped drug, (2) leakage of drug from liposomes, and (3) metabolism and elimination of free drug. Usually, it is believed that the activity of liposomal drug depends on the circulating level of entrapped drug which was subsequently delivered to tumors by lipid carriers; while the toxicity is related to the plasma level of free drug which was released from liposomes. Hence, a pharmacokinetic study for conventional dosage forms limited to analysis of total drug plasma concentration is not suitable enough and may even be misleading for liposomal drugs. For pharmacokinetic investigation and safety evaluation of liposomal formulations, it is essential to trail the plasma profiles of both liposome-associated and free (or non-liposomal) drugs.

* Corresponding author at: Department of Pharmaceutical Analysis, Pharmacy School, Shenyang Pharmaceutical University, Shenyang 110016, China.
E-mail addresses: xhy1020@gmail.com, xhy411@163.com (H. Xu).

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In order to determine plasma levels of liposomal-entrapped and free drugs, the most direct approach is to isolate these two fractions from biological fluids individually. A satisfactory separation method for this purpose should (1) be fast and simple for high-throughput analysis, (2) provide immediate separation to avoid drug leakage from liposomes during sample preparation and storage, (3) result in efficient recovery of each fraction to assure the accuracy of quantification. Several methods have been proposed to isolate the free (or non-liposomal) and liposomal drugs, such as ion-exchange chromatography [14], size-exclusion chromatography [14,15], ultrafiltration [15–17], solid-phase extraction (SPE) [17–26] and capillary electrophoresis [27]. Nevertheless, almost all of these separation methods have obvious limitations, which include poor reproducibility with low recovery by ion-exchange chromatography, high sample dilution and slow separation during gel chromatography, drug adsorption to devices using ultrafiltration, potential drug release from the liposomes during SPE, and low-throughput analysis for capillary electrophoresis.

Basing on the literatures [14–27], the most common method used for separation of non-liposomal and liposome-entrapped drugs is SPE due to relative rapidness. However, the published SPE methods employed complex sample pretreatment and showed a high contamination (typically ≥1%) of the non-liposomal drug fraction from the liposomal fraction [17–26]. In view of the fact that total plasma drug concentrations are usually about 1000-fold higher for liposomal formulations than for comparable doses of free drugs, the high contamination of 1% indicated free drug concentration after liposome administration was as much as 10-fold higher than expected after injection of unentrapped agents. The overestimated free (or non-liposomal) drug concentration resulting from drug leakage during SPE might lead to confounding pharmacokinetic and pharmacodynamics comparisons between different formulations. In the present study, we developed an effective and convenient SPE method with controlled drug release to separate liposomal (L-DOX) and non-liposomal DOX (NL-DOX) from plasma. The drug concentration in each separated fraction was subsequently quantified by a validated, rapid and sensitive LC–MS/MS method. Compared with ultrafiltration technique resulting in distinctly underestimated free drug concentration caused by extensive adsorption on the device, the SPE method provided proper and reliable pharmacokinetic assessment of NL-DOX fraction. This method would be instructive for pharmacokinetic study of other liposome formulations, especially for the compounds with strong adsorption.

2. Material and methods

2.1. Chemicals and reagents

DOX hydrochloride (> 98% pure) and 13CD3-DOX trifluoroacetic acid (> 98% pure) were purchased from Dalian Meilun Biology Technology Co., Ltd (Dalian, China) and Shanghai ZZBio Co., Ltd (Shanghai, China), respectively. Metformin (MET) hydrochloride (> 98% pure) was obtained from Shanghai EfeBio Co., Ltd (Shanghai, China). Doxil®, a pegylated liposomal DOX formulation, was supplied by TTY Biopharm (Taipei, Taiwan). Blank liposomal formulation was prepared by Department of Pharmaceutics, Shenyang Pharmaceutical University (Shenyang, China). Glucose injection (5%) was bought from Cisen Pharmaceutical Co., Ltd. (Jining, China). HPLC-grade methanol and acetonitrile were obtained from Fisher Chemicals (NJ, USA). Human plasma and albumin from bovine serum (BSA) were supplied by Shenyang City Blood Centre (Shenyang, China) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Distilled water, prepared from demineralized water, was used throughout the study. All other chemicals and solvents were of analytical grade and used without further purification.

2.2. Sample preparation

2.2.1. SPE procedure for separation of L-DOX and NL-DOX

L-DOX and NL-DOX were separated from plasma using Oasis® HLB SPE columns (1cc/30 mg, Waters, MA, USA) attached to a Cerex 48 positive pressure SPE apparatus (Cerex, GA, USA). The separation protocol is summarized in Table 1. After conditioned with 2 × 1 mL of methanol and 2 × 1 mL of cold water (in ice-water bath), columns were protein-coated with 0.2 mL of human plasma to diminish the retention of L-DOX. Following addition of 10 μL internal standard (IS) solution (MET 16 mg/mL and 13CD3-DOX 6.4 μg/mL in 5% glucose) and 20 μL blank liposomes into 400 μL plasma sample, the sample was applied on the column and drawn through. The eluate was referred as Eluate A. Column was then washed with 0.2 mL of human plasma (Eluate B) followed by 1 mL of cold water (Eluate C). Eluate A, B and C containing L-DOX as well as MET (IS) were collected into a 2-mL centrifuge tube and mixed by vortex for 1 min (L-DOX fraction). After column was washed with another 1 mL of cold water (Eluate D), NL-DOX with 13CD3-DOX (IS) was eluted with 1 mL of methanol containing 0.5% formic acid (Eluate E, NL-DOX fraction).

Table 1
Protocol for separating liposomal and non-liposomal DOX from plasma by SPE.

Protocol		Collection of eluate
(1) Column preparation	(a) Methanol wash (2 × 1 mL) (b) Water wash (0 °C, 2 × 1 mL) (c) Human plasma wash (0 °C, 0.2 mL)	
(2) Separation and recovery of liposomal DOX	(a) Layer plasma sample (0 °C, 0.4 mL) onto column	Eluate A
	(b) Human plasma wash (0 °C, 0.2 mL)	Eluate B
	(c) Water wash (0 °C, 1 mL)	Eluate C
(3) Column wash	(a) Water wash (0 °C, 1 mL)	Eluate D
(4) Recovery of non-liposomal DOX	(a) Methanol containing 0.5% formic acid wash (1 mL)	Eluate E

Water, human plasma and plasma sample were stored in ice-water bath before adding onto column.

L-DOX and NL-DOX fractions were both kept at −70 °C until analysis. A 10 μL aliquot of non-liposomal fraction was injected for LC–MS/MS analysis without any further sample pretreatment after thawing at room temperature. For liposomal fraction, a 10 μL aliquot of the fraction was mixed with 500 μL methanol. The mixture was vortexed for 1 min and then sonicated for 20 min to disrupt the liposomes. After centrifugation at 12,000*g* for 5 min, a 10 μL aliquot of the supernatant was injected for LC–MS/MS analysis.

2.2.2. Ultrafiltration procedure for separation of liposomal and non-liposomal DOX

Separation of free DOX from liposomal and protein-bound drugs was performed using Amicon® Ultral (0.5 mL, Merck Millipore Ltd. Carrigtwohill, IRL) ultrafiltration device with a molecular weight cutoff of 30,000 Da. An aliquot of 200 μL plasma sample was transferred to Microcon-30 reservoirs and centrifuged at 4 °C for 15–30 min at 8000–12,000*g* in a microcentrifuge. A 10 μL aliquot of the ultrafiltrate was processed for LC–MS/MS analysis.

2.2.3. Extraction of total DOX (T-DOX) from plasma

To 10 μL of each plasma sample, 10 μL of IS solution (13CD3-DOX 32 μg/mL in methanol) was added before spiking with 500 μL of methanol for protein precipitation. The mixture was vortexed for 1 min and then sonicated for 20 min to release DOX from liposomes. After centrifugation at 12,000*g* for 5 min, 40 μL of the supernatant was diluted with 160 μL acetonitrile-water-formic acid (15:85:0.3, *v/v/v*). A 10 μL aliquot of the resulting solution was injected for LC–MS/MS analysis.