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Rapid determination of PEGylated liposomal doxorubicin and its major metabolite in human plasma by ultraviolet–visible high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic method was developed for the quantification of doxorubicin derived from PEGylated liposomal doxorubicin (Doxil) and its major metabolite in human plasma. This method utilizes Triton X-100 to disperse the liposome, followed by a protein precipitation step with 5-sulfosalicylic acid. Analytes in the resultant supernatant are separated on a Discovery RP amide C_{16} column (250×3 mm I.D., 5 μm) using an isocratic elution with a mobile phase consisting of 0.05 *M* sodium acetate (pH 4.0) and acetonitrile (72:28). The retention times for doxorubicin and the internal standard daunorubicin were 4.8 and 10.1 min, respectively. The column eluate was monitored by UV–visible detection at 487 nm. The determination of doxorubicin was found to be linear in the range of 1.0 ng/mL to 25 μg/mL, with intra-day and inter-day coefficients of variation and percent error ≤10%. The recovery of doxorubicin from plasma was >69.3%, with a liposomal dispersion efficiency of >95.7%. Our analytical method for free and PEGylated doxorubicin in human plasma is rapid, avoids organic extractions, and maintains sensitivity for the parent compound and its major metabolite, doxorubicinol.

Keywords: Doxorubicin; Doxorubicinol

1. Introduction

1.1. Doxorubicin

Doxorubicin (DOX) belongs to a family of anthracycline antibiotics first isolated from *Streptomyces peucetius*. The drug displays a broad spectrum of antitumor activity, including activity against acute leukemias, Hodgkin's and Non-Hodgkin's lymphomas, breast cancer, lung cancers, and sarcomas [1]. The mechanisms for the anticancer and toxic effects of DOX include DNA intercalation and strand breakage, inhibition of topoisomerase II, formation of metal complexes with iron or copper, and free radical formation [2].

In the clinical setting, commonly administered dose-schedules of DOX produce acute toxicities qualitatively similar to other antineoplastic agents, including nausea and vomiting, alopecia, stomatitis, and myelosuppression. Its long-term clinical use is

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limited by the development of a cumulative dose-related cardiotoxicity, characterized by myocyte damage and myofibrillar loss. It has been proposed that the cardiomyopathy occurs as a result of damage due to the drug-induced formation of intracellular oxygen radicals [2].

As many as ten DOX metabolites have been identified, resulting from carbonyl group reduction, cleavage of the aminosugar moiety, conjugation reactions, and demethylation [2]. The major metabolite in humans is the 13-hydroxyl derivative, doxorubicinol (DOXol) (Fig. 1). DOXol has been shown to have some pharmacologic activity, with approximately 10% of the antineoplastic activity of the parent compound [3,4]. The majority (~80%) of DOX is eliminated by hepatobiliary excretion with urinary excretion accounting for 5–10% of drug elimination [5].

1.2. Liposomal doxorubicin

Liposomal encapsulation is an area of growing interest and importance as a pharmaceutical drug delivery system [6–9]. Liposome-encapsulated doxorubicin was explored to improve the therapeutic index by alleviating acute toxicities and chronic cardiomyopathy associated with administration of conventional DOX, while maintaining or improving its antitumor effect [7]. The decreased toxicity and increased antitumor effect when compared to DOX was first observed in murine models [7,8,10]. Large liposomes (>1000 nm) were developed but were found to be rapidly cleared by the liver [6–8]. Compared to conventional DOX, smaller liposomes (70–100 nm) with a polyethylene glycol-coated lipid phase (LDOX, Doxil) have been shown to provide increased tumor exposure by producing a prolonged duration of circulation in the plasma and increased microvascular permeability of the tumors [6,11].

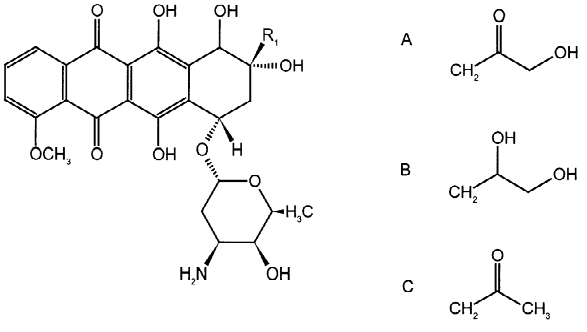

Fig. 1. Chemical structures of DOX, DOXol, and DNR. (A) R1, doxorubicin; (B) R1, doxorubicinol; (C) R1, daunorubicin.

1.3. Rationale for the development of a new LDOX assay method

We have developed a novel approach for the quantification of total DOX in the plasma of patients receiving free or PEGylated liposomal doxorubicin that possesses many advantages over previously published methods. The challenges in quantification of DOX encapsulated in liposomes have been threefold: (i) rapid extraction of encapsulated DOX from the liposome; (ii) preservation of the aminosugar moeity and other functional groups on DOX throughout the sample preparation; and (iii) extraction of non-liposomally-associated DOX from plasma. Because LDOX cannot be practically quantified while encapsulated, extraction of free DOX from the liposome is required. Previous published methods have required prolonged exposure to dilute acid, acidified organic solvents, or time-consuming isopropanol evaporations [7,12–15]. Acidification of DOX reduces the stability of the aminosugar group, increasing the conversion of DOX to its aglycone and aminosugar metabolites doxorubicinone and daunosamide, respectively [16]. We use a detergent to disperse the liposome. The sample can be directly injected onto the HPLC following a rapid protein precipitation. The advantages of this method are a more rapid drug extraction than previous methods, omission of toxic solvents or acids required for sample preparation, and equivalent or greater sensitivity compared to many previously published methods [17–20].

2. Experimental

2.1. Materials

PEGylated liposomal doxorubicin 2 mg/mL (LDOX) was purchased from Alza Pharmaceuticals (Palo Alto, CA, USA). Both doxorubicin (DOX) and daunorubicin (DNR) were ≥98% pure and were

purchased from Alexis Biochemicals (San Diego, CA, USA) and used without further purification. The sodium acetate trihydrate was purchased from J.T. Baker (Phillipsburg, NJ, USA). The Triton X-100 and 5-sulfosalicylic acid were purchased from Sigma (St. Louis, MO, USA). All solvents were purchased from Aldrich (Milwaukee, WI, USA). Human liver microsomes were purchased from Gentest (Woburn, MA, USA). They were generated from a pool of 11 donors who ranged from 21 to 59 years of age. Enzymatic activity of total P450 was found to be 553 pmol/(mg min). The glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PDH), and β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH) were purchased from Sigma.

2.2. Instrumentation and conditions

All HPLC was performed using a Hewlett-Packard/Agilent HP1100 system, equipped with a G1322A degasser, G1311A pump, G1329A/G1330 autosampler, G1316A column compartment, and a G1315A diode-array detector (Hewlett Packard, Palo Alto, CA, USA). Chemstation software (version 08.03) controlled all modules and was used for storage, analysis and reporting of chromatography. This software was implemented on a Windows NT platform on a Hewlett-Packard Kayak XA workstation (Palo Alto, CA, USA). A Discovery RP amide C_{16} 250×3 mm, 5 μm (Supelco, Bellafonte, PA, USA) analytical column was protected by a Sentry μBondapak C_{18}, 125 Å 3.9×20 mm 10 μm (Waters, Milford, MA, USA) guard column. The column eluate was monitored at 487 nm with a bandwidth of 16 nm. The reference wavelength was 623 nm with a bandwidth of 120 nm.

During analysis, the samples and column compartment were maintained at 4 and 40 °C, respectively. The mobile phase was comprised of 0.05 *M* sodium acetate (pH 4.0)–acetonitrile (72:28, v/v) delivered at a flow-rate of 1.0 mL/min. For LDOX quantification at clinical concentrations (0.5–25 μg/mL), the injection volume was 10 μL.

To quantify low concentrations of DOX (1.0–500 ng/mL), the injection volume was increased to 625 μL. Because of the 200 bar maximum pressure limitation of the large injection valve (required to accommodate injection volumes greater than 100 μL), the flow was decreased from 1.0 to 0.75 mL/min. During analysis of the major metabolite, the retention time was extended by altering the mobile phase of 0.05 *M* sodium acetate (pH 4.0)–acetonitrile (72:28, v/v) to 0.05 *M* sodium acetate (pH 4.0)–acetonitrile (74:26, v/v).

2.3. Reagent preparation

Sodium acetate buffer was prepared using analytical grade solid sodium acetate trihydrate in HPLC grade water and was titrated to pH 4.0 with concentrated HCl followed by vacuum filtration through a 0.45 μm nylon filter (Sigma). Triton X-100 was diluted to 3.0% (v/v) with HPLC grade water. Acid citrate dextrose human plasma (provided by the hospital transfusion service at Mills Peninsula Hospital, Burlingame, CA, USA) was thawed, centrifuged at 1000 *g* for 15 min and decanted. The supernatant was syringe-filtered through a C_{18} Sep-Pak SPE cartridge (Waters) and stored at −20 °C. Doxorubicin standards were prepared from a 250 μg/mL stock solution prepared in HPLC grade methanol. Concentrations were verified spectrophotometrically at 233 and 477 nm using the extinction coefficient ε_{233} and ε_{477} values of $38 \cdot 10^3$ and $13 \cdot 10^3$ L mol^{-1} cm^{-1}, respectively [21,22]. Liposomal doxorubicin solutions were prepared by serially diluting 2 mg/mL LDOX in filtered human plasma. Daunorubicin was used as the internal standard and was prepared as a 200 μg/mL solution in methanol.

2.4. Sample preparation

For known standard DOX concentrations, a 500 μL blank plasma sample was spiked with 50 μL of DOX standard and 50 μL of DNR internal standard. The mixture was pulse vortexed, then 50 μL 3.0% (v/v) Triton X-100 was added. The mixture was then vortexed for 10 s and 50 μL of 65% (w/v) 5-sulfosalicylic acid dihydrate was added [23]. The resulting mixture was vortexed again and then centrifuged at 20 000 *g* for 10 min. The resulting supernatant was decanted into silanized glass high recovery autosampler vials (Hewlett-Packard) and buffered with 15 μL of 3.0 *M* sodium acetate. For patient and blank control samples, 50 μL of metha-

nol was used to replace the addition of DOX standard. For analysis of clinical samples, 100 μL of plasma was used and one-fifth volume of reagents were used.

2.5. Human liver microsome studies

To identify the retention time of the major metabolite, DOXol was generated using human liver microsomes (HLM) by a protocol modified from a previously published method [24]. In a previous study using similar conditions, DOXol was the only metabolite identified using a standard reference [25]. We incubated 1.6 μg/mL LDOX with 20.6 mg/mL HLM in 0.3 *M* Tris–HCl (pH 7.4) in the presence of 4.0 m*M* glucose-6-phosphate, 0.1 U/mL glucose-6-phosphate dehydrogenase, and 1.0 m*M* β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH), in a total volume of 1500 μL. The negative control eliminated β-NADPH from the mixture. The HLM mixture was gently shaken and maintained at 37 °C for 1.5 h. The solution was centrifuged at 20 000 *g* for 5 min and the supernatant was directly injected.

2.6. Standard curve and analysis

DOX standards in methanol used for clinical analysis were prepared by serial dilution to concentrations of 5.0, 10, 25, 50, 100, and 250 μg/mL. Standards used for quantification of low concentrations of DOX were prepared at 10, 25, 50, 500, and 5000 ng/mL. All standards were stored at −80 °C. DOX, rather than LDOX, was used to prepare both standard curves since the concentration of DOX could be verified spectrophotometrically, and concentrations of clinical formulations of LDOX can vary by ±10% [26]. Quantification was determined by peak area ratio of DOX to DNR using a non-weighted least squares regression to the equation $y = mx$, where y is the area ratio, x is the amount ratio and m is the slope of the line.

2.7. Accuracy and precision

Inter-day and intra-day variation were measured in triplicate over 3 days both for low and high concentration curves (1.0–500 ng/mL and 0.50–25.0 μg/mL). Accuracy was evaluated by determining the percent error:

(concentration measured − theoretical concentration)/(theoretical concentration) · 100

Precision was evaluated by the determination of the coefficient of variation (C.V.):

(standard deviation/mean) · 100

The lower limit of quantification (LOQ) was defined as the lowest concentration of analyte that yielded a C.V. and percent error less than 15% [27].

2.8. Analyte recovery

The percent recovery of DOX from plasma was determined by comparing the peak area (mAU min) of dilute DOX extracted from plasma, to the peak area of dilute DOX in mobile phase:

(peak area DOX in plasma/peak area DOX in mobile phase) · 100

Recoveries (%) were determined at concentrations of 0.5, 1.0, 2.5, 5.0, 10, 25 μg/mL. To determine the dispersion efficacy of the LDOX analyte from plasma, we determined the relative recovery of free DOX to that of liposomal DOX in plasma. Dispersion efficiency was calculated by:

[(peak area LDOX analyte in plasma)/(peak area DOX in plasma)] · 100

This recovery was determined at LDOX concentrations of 1.0, 5.0, and 25 μg/mL.

2.9. Pharmacokinetics

Pharmacokinetic analyses were performed in seven adult patients receiving PEGylated liposomal doxorubicin (Doxil, Alza Pharmaceuticals) at a dose of 60 mg/m^2. The drug was administered as an intravenous infusion over 2 h as part of an ongoing phase I clinical trial in advanced solid tumors. Patient blood was serially sampled at baseline (0), 0.5, 1, 2, 3, 4, 6, 8, 12, 18, 24, 48, 72, 96 h following the beginning of the infusion. Pharmacokinetic parameters were derived using non-compartmental methods [28,29] using the XLPHARM M-IND pro-

gram (Dr. V.K. Piotrovski, VKPharmacokinetics, Turnout, Belgium) implemented in Microsoft Excel 2000 (Microsoft, Redmond, WA, USA).

3. Results

3.1. Retention time and chromatography

Under the conditions selected for analysis of clinical concentrations of LDOX, retention times of 4.8 and 10.1 min were observed for DOX and DNR, respectively (Fig. 2A and B). Under conditions selected for the determination of low concentrations of DOX or LDOX, retention times for DOX and DNR were 7.2 and 13.9 min, respectively (Fig. 3A and B). Conditions selected for analysis of the presumed major metabolite, DOXol, gave retention times of 8.8 and 12.9 min for DOXol and DOX, respectively (Fig. 4A and B). No endogenous substances in the HLM preparation produced interfering peaks under any of the selected conditions (Figs. 2B, 3B and 4B).

3.2. Linearity of calibration curves

Two calibration curves were used to separately measure low level DOX and its major metabolite (1.0–500 ng/mL) and clinically relevant levels of LDOX (0.50–25 μg/mL). The assay proved to be linear both in the LDOX ranges of 1.0–500 ng/mL and 0.50–25 μg/mL, giving values of $r^2 < 0.9992$ and $r^2 < 0.9996$, respectively. The regressions for the low and high concentrations gave slopes in the range of 0.033–0.038 and 6.30–7.74 with an intra-day and inter-day C.V. of <3.9 and 5.5, respectively.

3.3. Precision and accuracy for intra-day and inter-day assays

Assay reproducibility was determined in the concentration ranges 1.0–500 ng/mL and 0.50–25 μg/mL. The intra-day and inter-day precision and accuracy for the low and high curves are summarized in Tables 1 and 2, respectively. The C.V. determined for both low and high curves was <10 for all concentrations (Tables 1 and 2). The percent error determined was <10 for all values of both low and high curves.

3.4. Recovery of DOX from the liposome and from plasma

Doxorubicin was extracted from the liposomal encapsulation with a liposome dispersion efficiency of >95% and a standard deviation (SD) of <5.2% (Table 3). Both intra-day and inter-day variation gave a C.V. of <5. The mean recovery of DOX from plasma was also determined and was found to be >69% with a SD of <3.2% (Table 4). The intra-day and inter-day C.V. for recovery was <4.7%.

3.5. Pharmacokinetics in humans

Concentration–time profiles for LDOX for the seven patients studied are illustrated in Fig. 5. Concentrations were adequately quantified throughout the 96-h sampling period. Pharmacokinetic parameters determined in the seven patients are outlined in Table 5. These values are similar to those previously reported in patients receiving LDOX with concentrations determined by fluorescence detection in a similar patient population [30]. Similar to other studies of LDOX, DOXol was not detected in concentrations above the LOQ, and was not quantified [30].

4. Discussion

The use of liposomal preparations for the delivery of anticancer drugs has demonstrated the potential for an improved therapeutic index. The entrapment of doxorubicin in a PEGylated liposomal delivery system has led to successful treatment of AIDS-related Kaposis's sarcoma, and other tumors, with markedly reduced acute toxicities such as bone marrow suppression or nausea and reduced risk for cumulative dose-limiting cardiomyopathy [2].

Previous methods for the extraction of LDOX require time-consuming overnight incubation with organic solvents or acid [7,12–15]. Our method immediately disperses the liposome, yielding high recovery/reproducibility of DOX both from the liposome and plasma. In this assay, the supernatant

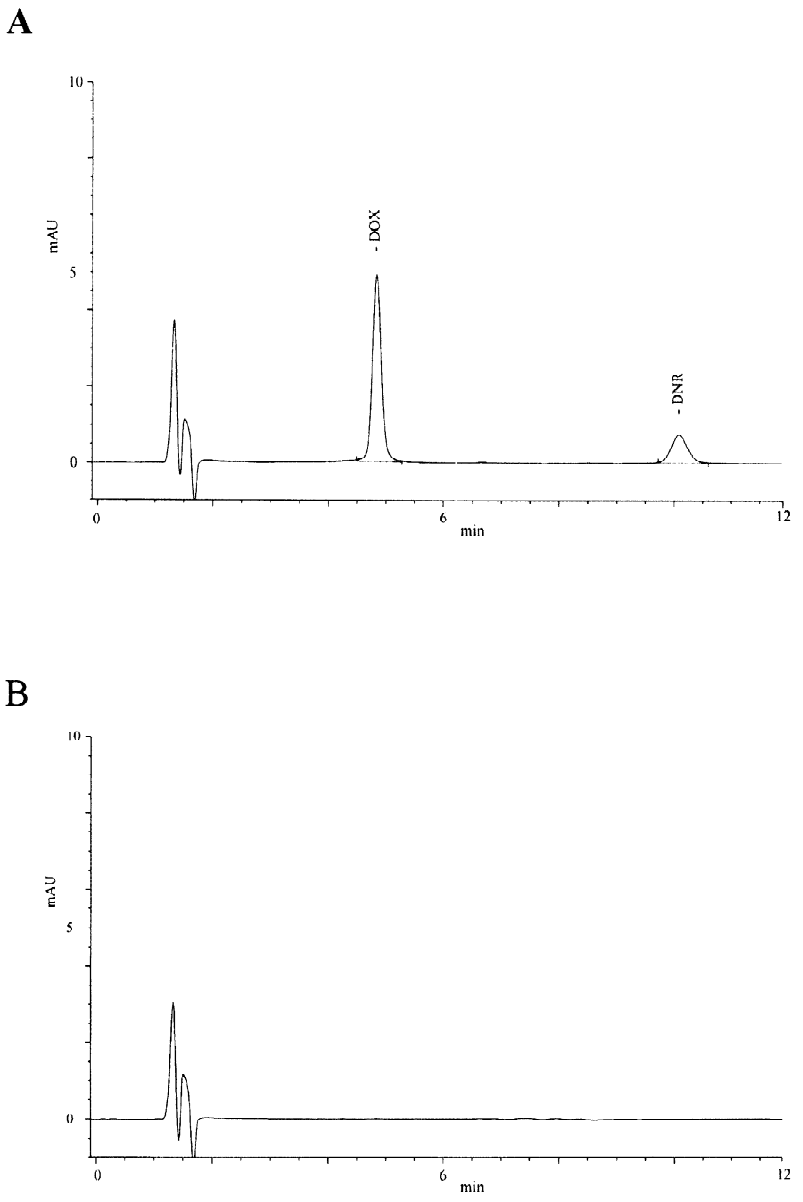

Fig. 2. Typical chromatogram following extraction of LDOX from human plasma. Conditions described in Section 2.2. (A) Retention times for LDOX and DNR are 4.8 and 10.1 min, respectively. (B) Blank plasma control injection.

prepared following a protein precipitation can be directly injected onto the HPLC for analysis without solid-phase extraction or a solvent evaporation step.

Our method has a recovery of ~70% for DOX, which is similar to previously reported methods [31–33]. The relative recovery of DOX from LDOX was almost identical (>96%) to that of free DOX from plasma, suggesting that complete dispersion of the liposome formulation occurred. In our HLM experiment, we adjusted the mobile phase in order to increase the analyte retention time in an attempt to resolve metabolites additional to DOXol. Because we did not resolve additional metabolites, for more rapid processing of clinical samples, we ran our

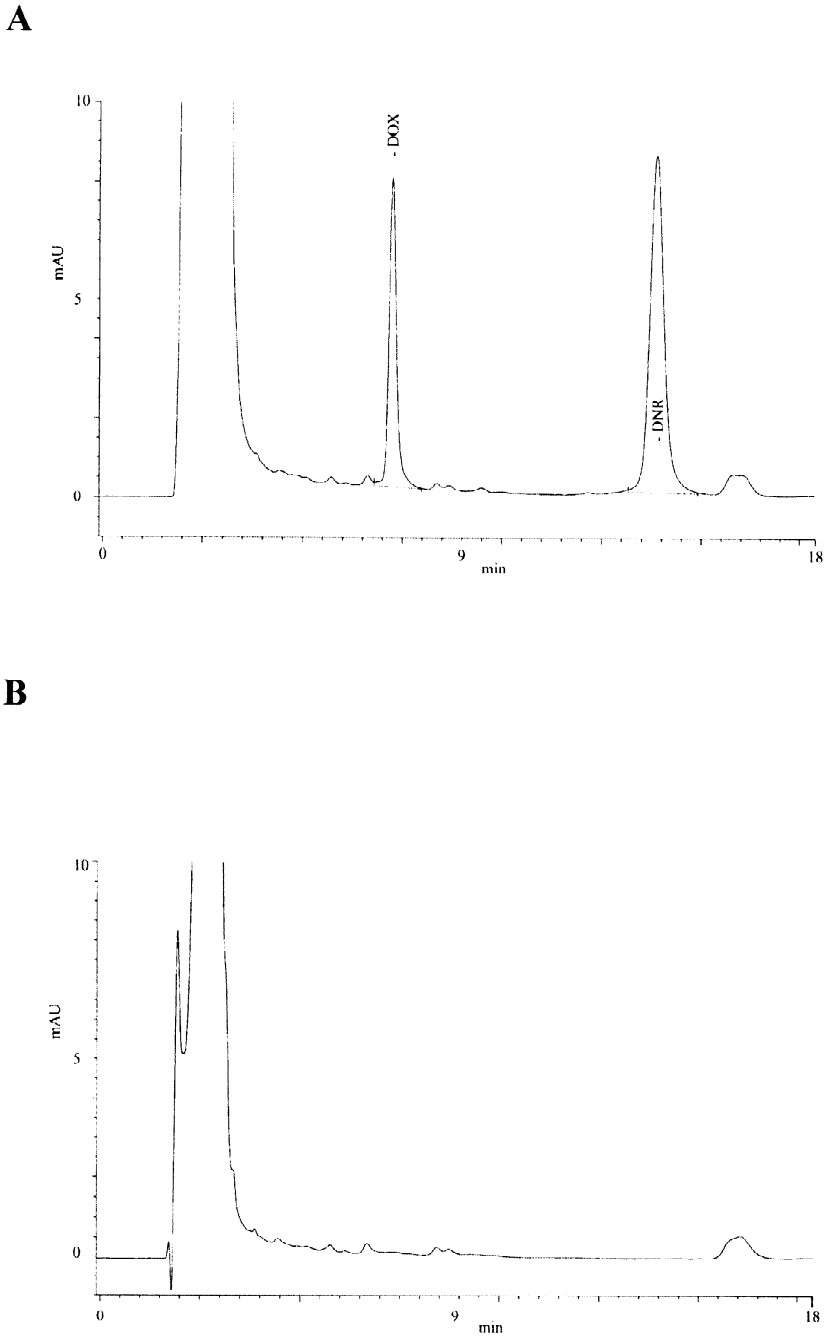

Fig. 3. Typical chromatogram following extraction of DOX from human plasma. Conditions described in Section 2.2. (A) Retention times for DOX and DNR are 7.2 and 13.9 min, respectively. (B) Blank plasma standard extracted for control.

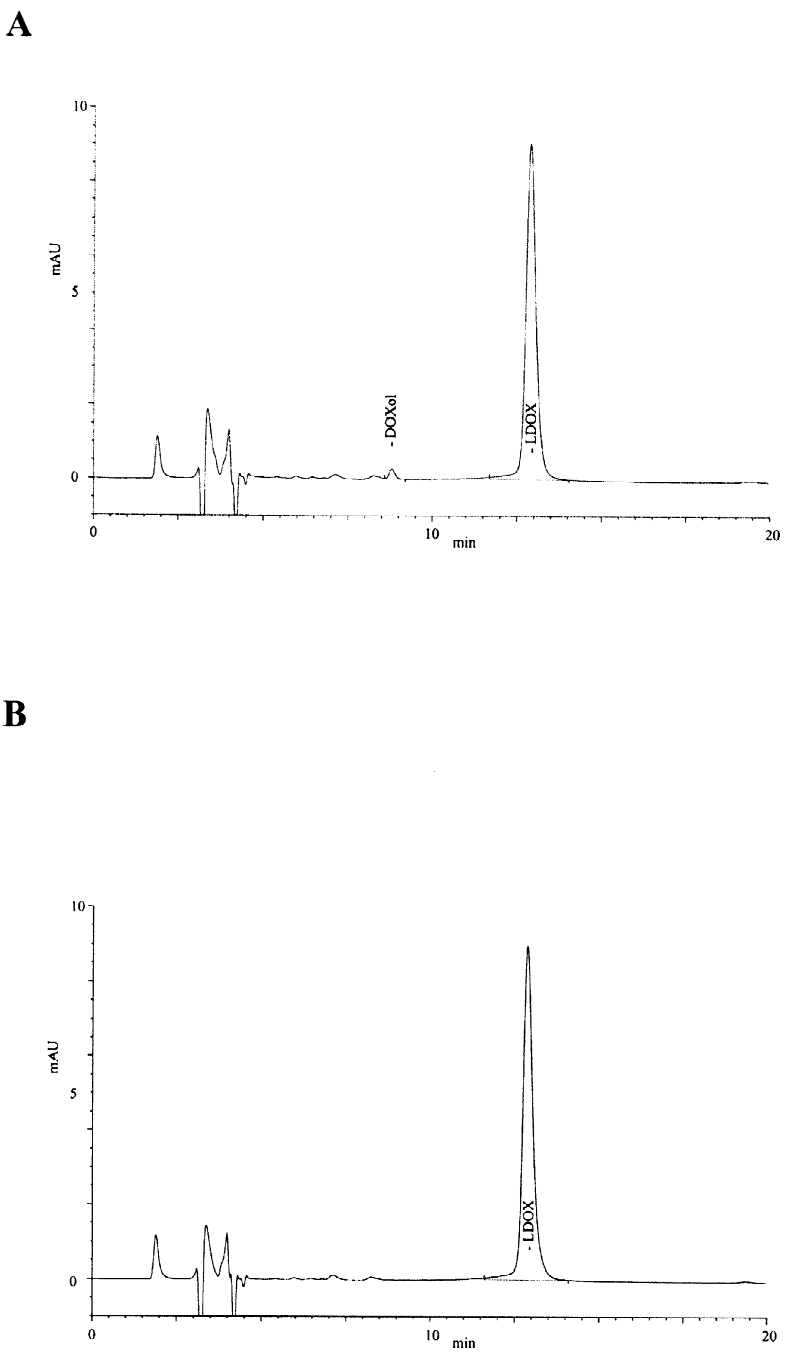

Fig. 4. Typical chromatogram following extraction of DOX and its major metabolite, DOXol, from human liver microsome metabolism studies. Conditions described in Section 2.2. (A) LDOX incubated with human liver microsomes in the presence of NADPH. Retention times for DOXol and LDOX are 8.8 and 12.9 min, respectively. (B) LDOX incubated under previous conditions without NADPH for control. LDOX retention time is 12.9 min.

Table 1
Intra- and inter-day assay precision and accuracy of the DOX assay method

Analyte	Conc. prepared (ng/mL)	Conc. found (mean±SD, ng/mL)	C.V.	Percent error
Intra-day assay				
DOX	1.0	1.1±0.1	10	10
	2.5	2.5±0.2	8.3	2.2
	5.0	5.22±0.4	7.3	4.4
	50	53.0±2.2	4.4	5.9
	500	547±14	2.9	9.4
Inter-day assay				
DOX	1.0	0.98±0.1	8.7	2.1
	2.5	2.50±0.03	1.1	1.2
	5.0	5.10±0.1	2.5	2.0
	50	50.7±2.0	3.9	1.3
	500	519±33	6.4	3.8

Intra- and inter-day assay experiments were performed in triplicate.

clinical samples under the LDOX assay conditions described previously and detected DOXol at a retention time of 3.2 min.

Most previous methods used fluorescent or electrochemical detection [15,17,19]. Our UV–visible detection method avoids many potential interfering species common when using either fluorescent or electrochemical detection. Further, our method offers the versatility to quantify samples containing DOX or LDOX at concentrations ranging over four orders of magnitude (1.0 ng/mL to 25 μg/mL) with high

Table 2
Intra- and inter-day assay precision and accuracy of LDOX assay method

Analyte	Conc. prepared (μg/mL)	Conc. found (mean±SD, μg/mL)	C.V.	Percent error
Intra-day assay				
DOX	1.0	1.1±0.1	8.0	8.5
	5.0	5.2±0.4	7.1	4.5
	25	25.5±1.6	6.7	2.1
Inter-day assay				
DOX	1.0	1.04±0.1	7.0	4.0
	5.0	5.09±0.1	2.4	1.7
	25	24.9±1.1	4.5	0.2

Intra- and inter-day assay experiments were performed in triplicate.

Table 3
Liposomal dispersion efficiency of the PEGylated liposome formulation

Analyte	Conc. (μg/mL)	Dispersion efficiency	C.V.
Intra-day assay			
	1.0	106.0±5.2	4.8
	5.0	98.6±3.4	3.4
	25	95.7±4.2	4.6
Inter-day assay			
	1.0	106.4±4.1	3.8
	5.0	98.5±3.0	3.0
	25	95.9±3.8	3.9

Intra- and inter-day assay experiments were performed in triplicate.

reproducibility and excellent linearity. This sensitivity and precision enables the determination of total doxorubicin in the plasma, and determination of the pharmacokinetic parameters of DOX or LDOX over a broad range of doses and sampling time periods.

A limitation of this assay is that it measures total doxorubicin in the plasma and not drug specifically entrapped within the liposome. This, however, may not be a clinically important factor in the determination of LDOX pharmacokinetics in plasma, since

Table 4
Analytical recovery of DOX and DNR from plasma

Analyte	Conc. (μg/mL)	Percent recovery	C.V.
Intra-day assay			
DOX	0.5	69.7±1.4	2.1
	1.0	72.9±2.7	3.7
	2.5	74.2±0.8	1.1
	5.0	72.5±0.3	0.5
	10	69.3±3.2	4.7
	25	70.8±1.7	2.3
DNR	10	66.8±2.3	3.4
Inter-day assay			
DOX	0.5	69.4± 1.1	1.5
	1.0	71.8±1.6	2.3
	2.5	71.7±2.5	3.5
	5.0	70.4±1.6	2.3
	10	70.6±2.8	4.0
	25	70.2±0.9	1.2
DNR	10	66.4±5.3	8.0

Intra- and inter-day assay experiments were performed in triplicate.

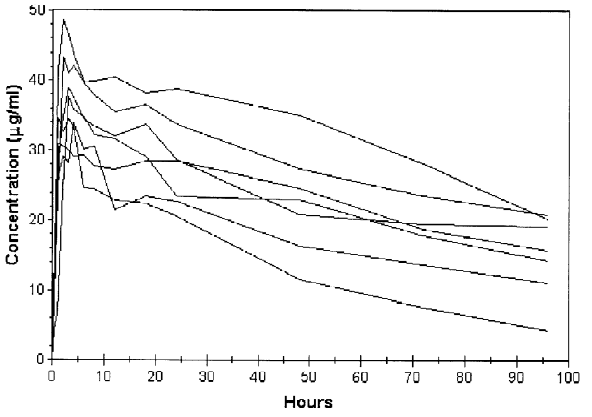

Fig. 5. Concentration–time profiles for seven patients receiving single agent LDOX therapy. All patients received 60 mg/m^2 LDOX intravenously as a 2-h infusion. Concentrations of DOXol were below the LOQ and are not reported.

the pharmacokinetics of total and encapsulated LDOX has been reported to be similar, suggesting that little of the drug is released from the liposome formulation following administration [30].

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Table 5
Pharmacokinetic parameters in seven representative patients receiving LDOX chemotherapy as a single agent[a]

Patient	Dose (mg/m^2)	C_{max} (μg/mL)	$T_{1/2}$ (h)	AUC (μg h/mL)	CL (mL/h/m^2)	V_{ss} (L/m^2)
1	60	40.7	133	5811	10.3	2.0
2	60	30.6	82.1	4103	14.6	1.8
3	60	37.0	69.1	3540	17.0	1.8
4	60	35.7	34.9	1563	38.4	1.9
5	60	44.2	106	5856	10.3	1.6
6	60	40.4	71.4	2858	21.0	2.2
7	60	48.7	60.4	4960	12.1	1.1
Mean	60	39.6	79.6	4099	17.7	1.8
SD	0	5.90	31.9	1583	9.92	0.3

[a] Units and abbreviations: C_{max}, maximum concentration; $T_{1/2}$, half-life of the terminal phase of the plasma concentration decay curve; AUC, area under the concentration–time curve, extrapolated to infinity; CL, clearance; V_{ss}, volume of distribution at steady state; m^2, body surface area in square meters.

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