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Determination of doxorubicin and its metabolites in rat serum and bile by LC: application to preclinical pharmacokinetic studies

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Abstract

A simple, accurate and precise high-performance liquid chromatographic method was developed and validated for the simultaneous determination of doxorubicin and its three metabolites, including doxorubicinol, doxorubicinolone and doxorubicinone, in rat serum and bile. Following a single protein precipitation step, chromatographic separation was accomplished using a C-18 column with a mobile phase consisting of 50 mM sodium phosphate buffer–acetonitrile–1-propanol (65:25:2, v/v), pH 2.0. The analytes were measured by fluorescence detection with excitation wavelength of 480 nm and emission wavelength of 560 nm. The lower limits of quantitation were 10 ng/ml for doxorubicin, and 5 ng/ml for the three metabolites. The calibration curves were linear over a concentration range of 10–2500 ng/ml for doxorubicin, and 5–1250 ng/ml for its three metabolites. The average recoveries were greater than 89% for all analytes. The within-day and between-day coefficients of variation were generally less than 13%. Doxorubicin and its metabolites were stable in the precipitated serum and bile samples at room temperature in darkness for at least 48 h. This method permitted the analysis of samples without the presence of the anticoagulant sodium citrate and thus was applied to serum and bile samples collected from rats that were administered doxorubicin intravenously in a pharmacokinetic study.

Keywords: Doxorubicin; Doxorubicinol; Doxorubicinone; Doxorubicinolone; Pharmacokinetics

1. Introduction

Doxorubicin (Adriamycin), an anthracycline glycoside antibiotic originally produced by *Streptomyces peucetius* var. *caesius*, has been used in the treatment of a wide range of malignant tumours for almost three decades [1]. However, the clinical use of doxorubicin is limited by a cumulative dose-dependent irreversible chronic cardiomyopathy, which can subsequently lead to congestive heart failure, with an ultimate mortality rate of 20–40% [2,3].

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Various mechanisms have been proposed for the development of doxorubicin-induced cardiotoxicity [4]. More recently, the relationship between the doxorubicin-induced cardiotoxicity and doxorubicin metabolism has been highlighted in several studies [5–7]. Doxorubicin is rapidly metabolised by the cytoplasmic NADPH-dependent aldo-keto reductase to the secondary alcohol metabolite, doxorubicinol, and by the NADPH-dependent cytochrome P450 reductase to a broad panel of hydroxy- or deoxy-aglycones [5] (Fig. 1). The predominant excretion route of doxorubicin and its metabolites is through the bile [8]. Of these metabolites, doxorubicinol has been demonstrated to be cardiotoxic [9]. The role of doxorubicin aglycones in the cardiotoxicity remains unclear although an in vitro study of Licata et al. [10] has shown that doxorubicinol and doxorubicin aglycones are formed by mutually exclusive mechanism and a switch from deglycosidation to carbonyl reduction possibly exists in human myocardium.

Till now, a number of methods for the simultaneous quantification of doxorubicin and its metabolites in the biological matrix have been described utilising high-performance liquid chromatography (HPLC) with fluorescence detection [11–17]. Sample pre-treatment procedures vary greatly. Some of the published methods include time-consuming and tedious solid-phase extraction processes [11,12], or liquid–liquid extraction procedures [13,14]. From a practical point of view, a simple and rapid sample pre-treatment procedure is achieved in a single protein precipitation step. In two of the published methods for the determina-

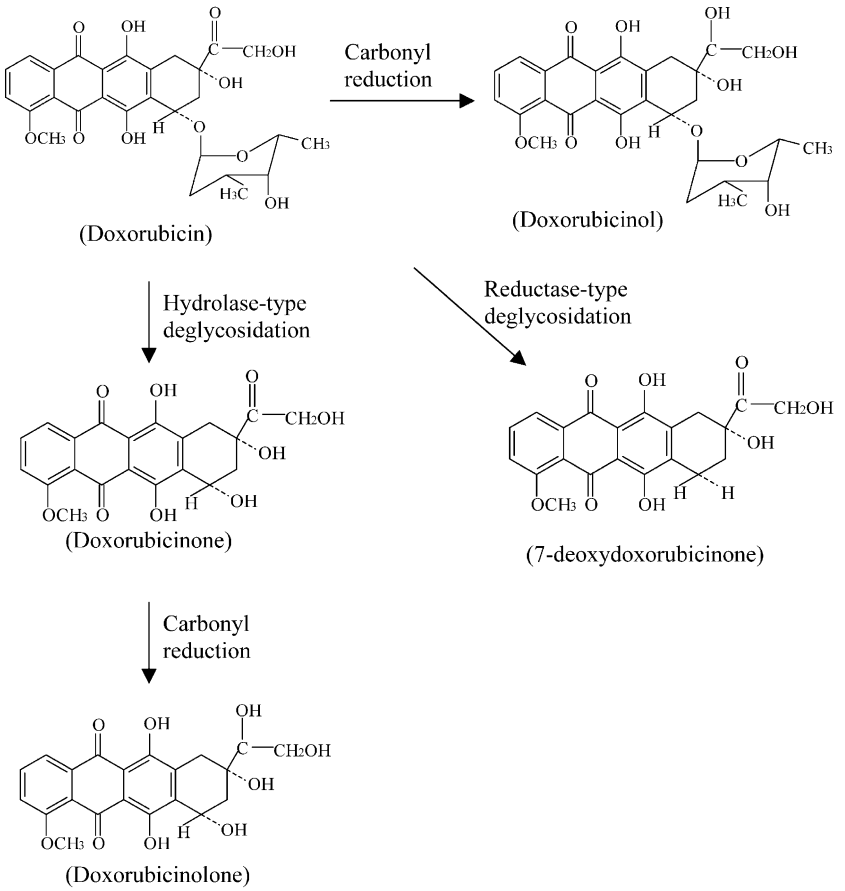

Fig. 1. Metabolic pathway of doxorubicin.

tion of doxorubicin and its metabolites in plasma, the single protein precipitation step was carried out in the presence of zinc sulphate [15,16]. The application of this method to other biological samples is limited because protein precipitation with zinc sulphate is dependent on the presence of acid citrate dextrose or sodium citrate that is present in the plasma as anticoagulant [15].

In order to facilitate our ongoing preclinical studies, we developed a relative simple and rapid HPLC method allowing the simultaneous determination of doxorubicin and its metabolites in the biological samples that are not citrated, e.g. serum and bile samples. With this method a sample size of 50 μl was sufficient, and the lower limits of quantitation achieved for doxorubicin and its metabolites were comparable or even better than the published methods [11–16]. This method was thoroughly validated and then applied to determine pharmacokinetic profiles and biliary excretion of doxorubicin and its metabolites in rats to which an intravenous bolus injection of 5 mg/kg doxorubicin was given.

2. Experimental

2.1. Chemicals and reagents

Doxorubicin (LOT 0011BS47F) and daunorubicin were provided by Pharmacia Upjohn S.p.A. (Milan, Italy). The metabolites doxorubicinol (LOT AS 15116-09), doxorubicinone (LOT GF 10821-60) and doxorubicinolone (13-dihydroadriamycinone) (LOT AS 15116-10) were gifts from Dr Antonio Suarato (Pharmacia Upjohn S.p.A.). AnalaR grade sodium dihydrogen phosphate and phosphoric acid were from Merck (Schuchardt, Germany). The HPLC grade methanol and acetonitrile were obtained from Labscan Limited Co. (Dublin, Ireland). Water was purified on a Milli-Q system (Millipore Corporation, Bedford, MA).

2.2. Experimental animals

Male Sprague-Dawley rats (220–250 g) were purchased from Laboratory Animal Centre, Singapore. Animal experiments were approved by the Institutional Animal Care and Use Committee of National Cancer Centre (Singapore). Animals were housed separately in a metabolic cage and kept on a 12-h light/dark cycle for a minimum of 3 days before being used in experiments. Food and water were available *ad libitum*.

2.3. Instrumentation and chromatographic conditions

Analyses were performed on a reverse-phase HPLC system consisting of a Waters 2690 separations module (Waters Assoc. Milford, MA) coupled to a Waters 474 scanning fluorescence detector (Waters Assoc.). The excitation wavelength was set at 480 nm and emission at 560 nm, and the gain was set at $\times 100$. Chromatogram was acquired, stored, and processed using the Millennium software program (version 3.20, Waters Assoc.). Chromatographic separations were carried out using an Xterra® C-18 analytical column (particle size 5 μm, 150 mm long $\times$ 4.6 mm i.d.; Waters). The mobile phase consisted of 50 mM sodium phosphate buffer (pH 2.0), acetonitrile and 1-propanol (65:25:2, v/v/v). The flow rate was kept constant at 1.0 ml/min.

2.4. Standard stock solution, calibration and quality control samples

Stock solutions of doxorubicin, doxorubicinol, doxorubicinone, and doxorubicinolone were prepared by dissolving weighed amounts of the respective reference materials in methanol to obtain concentrations of 40 000, 20 000, 20 000, and 20 000 ng/ml, respectively. A working solution containing doxorubicin and its three metabolites was prepared by mixing the stock solutions to obtain final concentrations of 10 000 ng/ml for doxorubicin and 5000 ng/ml for each metabolite. The working solution was then successively diluted with methanol. A stock solution of daunorubicin (4000 ng/ml) was prepared in Milli-Q water and used as the internal standard (I.S.). For the standard curve, the final concentrations of doxorubicin and its metabolites in rat serum and bile were as follows: 2500, 1000, 400, 160, 64, 26 and 10

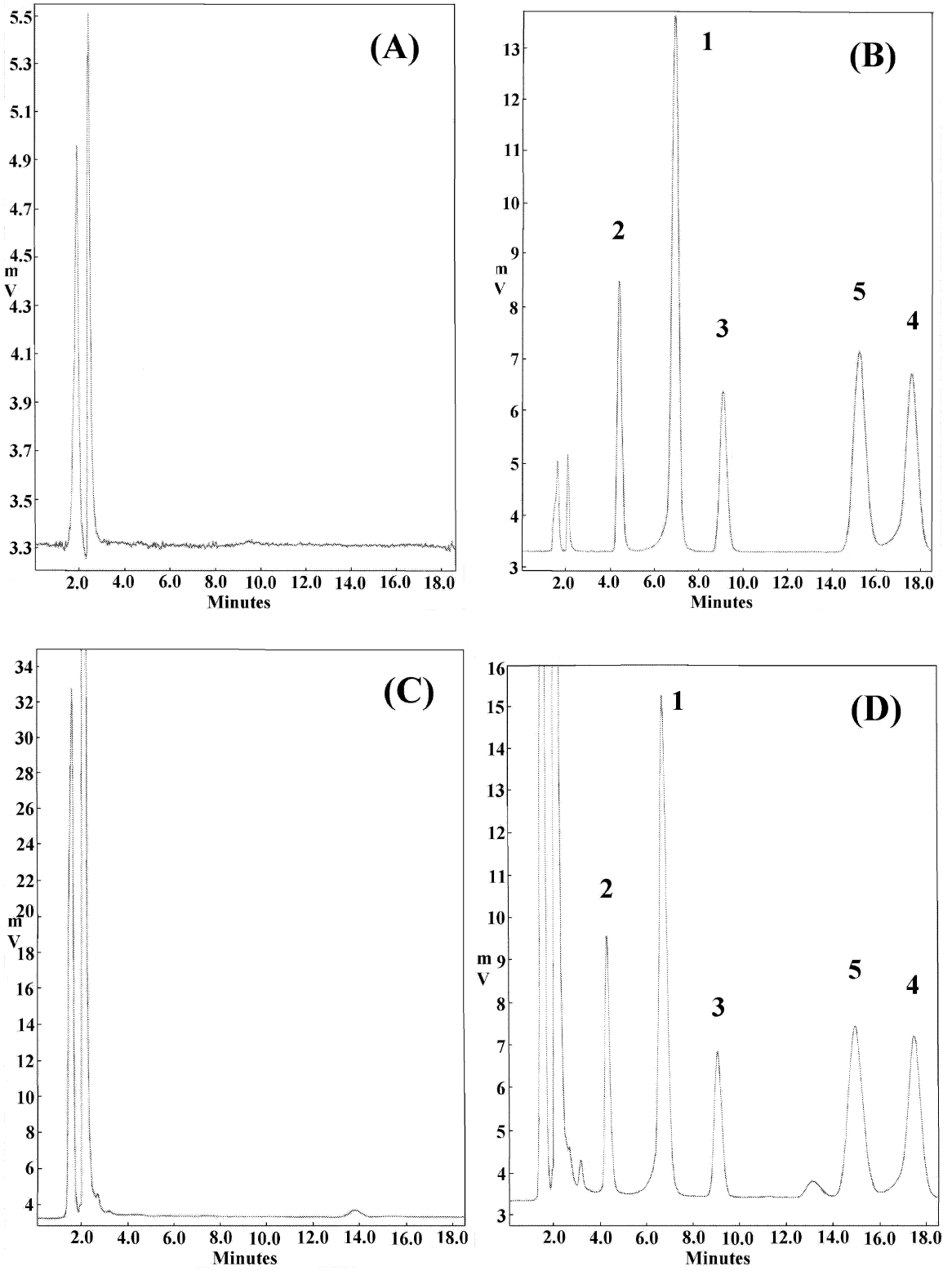

Fig. 2

ng/ml for doxorubicin, and 1250, 500, 200, 80, 32, 13 and 5 ng/ml for each metabolite. The calibration standards were freshly prepared on each analysis day by adding 80 μl aliquot of the spiking working solution, 60 μl of methanol, and 10 μl 4000 ng/ml daunorubicin to 50 μl of blank rat serum or bile. For the quality control (QC) sample, the concentrations for doxorubicin and its metabolites in rat serum and bile were 2500, 160 and 10 ng/ml for doxorubicin, and 1250, 80 and 5 ng/ml for each metabolite. All the stock solutions, working solutions and QC samples were protected from light and stored at −20 °C.

2.5. Sample processing

Sample preparation involved a single protein precipitation step. A 50 μl aliquot of serum or bile sample were pipetted into a 1.5-ml polypropylene Eppendorf micro test tube (Hamburg, Germany), followed by 10 μl of I.S. solution (daunorubicin, 4000 ng/ml) and 140 μl of ice-cold methanol. The tube was vigorously mixed for 30 s and centrifuged for 10 min at $9000 \times g$. The supernatant was transferred into a sample vial of the autosampler, and an aliquot (50 μl) was injected into the HPLC system.

2.6. Assay validation

2.6.1. Linearity

The linearity of the method was evaluated over the concentration ranges of 10–2500 and 5–1250 ng/ml for doxorubicin and its metabolites, respectively. Calibration standards were freshly prepared in duplicate every day during ongoing analysis. Standard curves were obtained from the linear least square regression analysis of drug/I.S. peak area ratio as a function of the theoretical concentration.

2.6.2. Within-day and between-day accuracy and precision

Within-day and between-day accuracy and precision of the assay were assessed by performing replicate analyses of three QC sample concentrations. The procedure was repeated on 5 different days on the same spiked standards to determine between-day repeatability. Within-day repeatability was assessed by 5 replicate assays of the QC samples on the same day.

The accuracy was evaluated as relative mean error (R.M.E.), i.e. ((mean of measured-mean of added)/mean of added) × 100. The precision was given by the relative standard deviation (R.S.D.), i.e. (S.D./mean) × 100, where S.D. represents the standard deviation of the mean. The acceptance criteria for precision and accuracy are not more than 15% coefficient of variation for precision and not more than 15% deviation from the nominal value for accuracy [18]. The lower limit of quantitation (LLOQ) for each analyte was set as the lowest standard concentration that had both R.S.D and R.M.E $< 20\%$ [18].

2.6.3. Recovery

The absolute recovery was determined by comparing peak areas from drug-free serum and bile spiked with known amounts of drugs, with peak areas of the same concentrations prepared in methanol and injected directly onto the analytical column. The recoveries were examined at concentrations of 10, 160 and 2500 ng/ml for doxorubicin and 5, 80 and 1250 ng/ml for its metabolites. Each sample was determined in triplicate.

2.6.4. Freeze-thaw stability

The freeze-thaw stability of doxorubicin and its metabolites was investigated by using LLOQ, medium and high QC samples. Serum and bile samples were subjected to three freeze-thaw cycles

Fig. 2. Chromatograms of extracted (A) drug-free rat serum, (B) drug-free rat serum spiked with 250 ng/ml of doxorubicin, 125 ng/ml of doxorubicinol, doxorubicinolone and doxorubicinone, and 20 ng/ml of daunorubicin, (C) drug-free rat bile, and drug-free rat bile spiked with 250 ng/ml of doxorubicin, 125 ng/ml of doxorubicinol, doxorubicinolone and doxorubicinone, and 20 ng/ml of daunorubicin. Peaks: 1 = doxorubicin; 2 = doxorubicinol; 3 = doxorubicinolone; 4 = doxorubicinone; 5 = daunorubicin (I.S.). For chromatographic conditions, see Section 2.5.

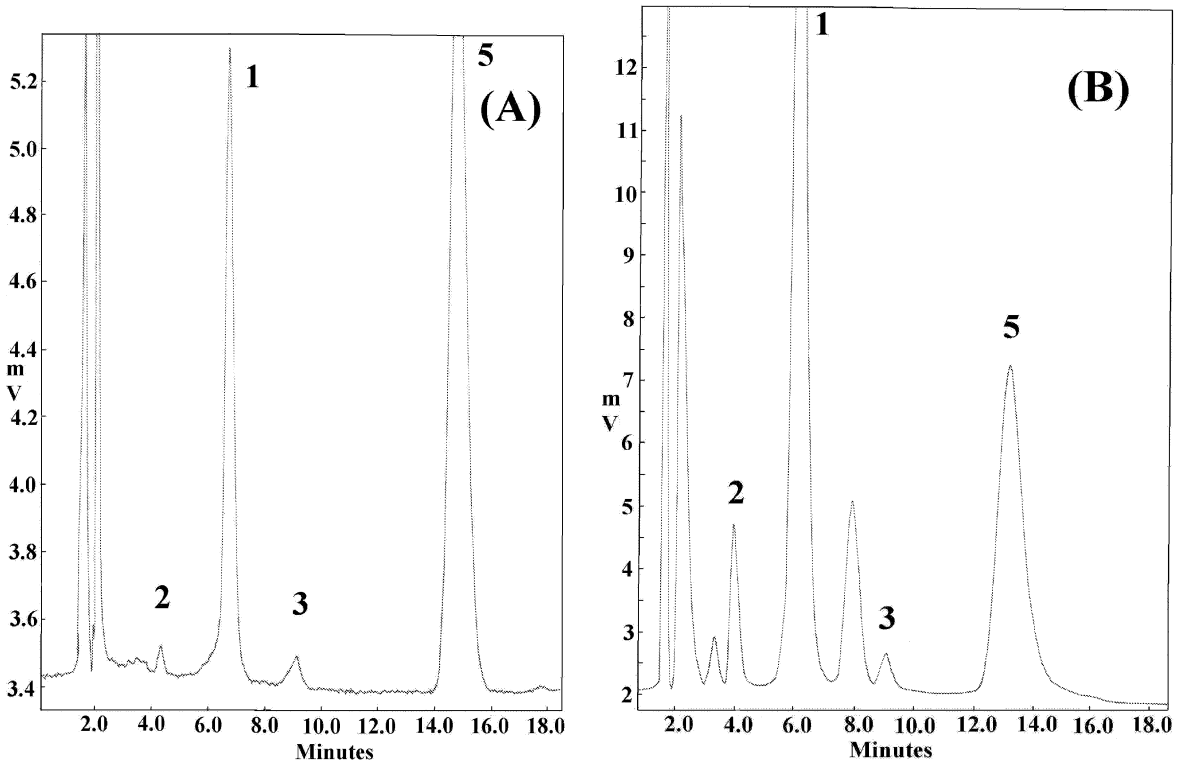

Fig. 3. Typical chromatograms of (A) the serum sample and (B) the bile sample. The serum sample was taken at 2 h after an intravenous (i.v.) injection of 5 mg/kg doxorubicin to a rat, and the bile sample was collected during 2–4 h after the i.v. injection of 5 mg/kg doxorubicin to a rat. Peaks: 1 = doxorubicin; 2 = doxorubicinol; 3 = doxorubicinolone; and 5 = daunorubicin (I.S.).

Table 1
Calibration curves for the HPLC assay of doxorubicin, doxorubicinol, doxorubicinone and doxorubicinolone in rat serum and bile samples ($n = 5$)

	Concentration range (ng/ml)	Linear regression equation ($y = ax + b$)		
		Slope (S.D.)	Intercept (S.D.)	r^2
Serum				
Doxorubicin	10–2500	0.001950 (0.000088)	0.001184 (0.001318)	0.9966
Doxorubicinol	5–1250	0.001133 (0.000018)	0.001403 (0.000641)	0.9998
Doxorubicinolone	5–1250	0.001024 (0.000024)	0.002582 (0.000967)	0.9994
Doxorubicinone	5–1250	0.001973 (0.000030)	0.000024 (0.000967)	0.9998
Bile				
Doxorubicin	10–2500	0.001953 (0.000067)	−0.001720 (0.007582)	0.9984
Doxorubicinol	5–1250	0.001179 (0.000021)	0.000951 (0.000782)	0.9995
Doxorubicinolone	5–1250	0.001057 (0.000023)	0.002894 (0.000927)	0.9998
Doxorubicinone	5–1250	0.001875 (0.000039)	0.000008 (0.001233)	0.9992

Table 2
Within-day reproducibility of the HPLC determination of doxorubicin, doxorubicinol, doxorubicinone and doxorubicinolone in rat serum and bile samples ($n = 5$)

	Concentration (ng/ml)	R.S.D. (%)		R.M.E. (%)	
		Serum	Bile	Serum	Bile
Doxorubicin	10	2.6	4.6	−11.3	−13.5
	160	3.9	7.2	9.1	9.2
	2500	4.6	3.2	12.3	11.8
Doxorubicinol	5	9.6	3.5	−12.2	−13.9
	80	1.6	2.5	10.2	7.3
	1250	1.1	0.4	12.4	11.8
Doxorubicinone	5	4.3	5.7	14.8	12.6
	80	4.7	3.9	12.0	11.5
	1250	2.1	3.5	7.1	12.7
Doxorubicinolone	5	4.4	6.5	−12.1	−11.7
	80	2.6	2.9	7.9	5.1
	1250	1.4	0.7	10.0	13.5

R.S.D., relative standard deviation. R.M.E., relative mean error.

and subsequently analysed in triplicate. These samples were stored at −20 °C overnight and then thawed unassisted at room temperature.

2.6.5. Stability

The stability experiments of doxorubicin and its metabolites were carried out under three conditions: after storing at room temperature for 48 h, and at 4 °C for 1 week (e.g. 7 days), and at −20 °C for 3 weeks (e.g. 21 days). The stability of the four compounds in rat serum and bile was investigated in triplicate at the concentrations of 160 and 80 ng/ml for doxorubicin and its metabolites, respectively.

Table 3
Between-day reproducibility of the HPLC assay of doxorubicin, doxorubicinol, doxorubicinone and doxorubicinolone in rat serum and bile samples ($n = 5$)

	Concentration (ng/ml)	R.S.D. (%)		R.M.E. (%)	
		Serum	Bile	Serum	Bile
Doxorubicin	10	7.2	11.7	−12.6	−9.7
	160	8.0	4.9	8.9	9.4
	2500	6.9	2.4	8.5	11.2
Doxorubicinol	5	7.0	12.8	−12.4	−7.6
	80	8.9	6.8	7.7	2.3
	1250	5.5	7.3	9.8	9.9
Doxorubicinone	5	8.4	6.4	5.6	−2.0
	80	3.8	5.5	8.9	8.2
	1250	3.6	3.6	6.1	3.6
Doxorubicinolone	5	1.7	12.0	−12.0	−14.1
	80	7.7	9.5	−0.9	−0.9
	1250	5.5	6.5	9.8	9.3

Table 4
Recovery (Mean ± S.D.) of doxorubicin, doxorubicinol, doxorubicinone and doxorubicinolone in rat serum and bile samples ($n = 3$)

	Concentration (ng/ml)	Absolute recovery (%)	
		Serum	Bile
Doxorubicin	10	94.0 ± 2.7	93.4 ± 4.1
	160	89.6 ± 3.5	89.1 ± 6.6
	2500	91.3 ± 4.7	93.2 ± 3.3
Doxorubicinol	5	93.4 ± 8.6	88.5 ± 3.3
	80	91.6 ± 1.6	91.9 ± 2.4
	1250	92.4 ± 2.1	92.8 ± 1.1
Doxorubicinone	5	103.1 ± 4.0	91.6 ± 5.1
	80	89.7 ± 4.5	90.3 ± 3.5
	1250	91.4 ± 3.6	92.1 ± 4.1
Doxorubicinolone	5	95.4 ± 3.5	89.8 ± 5.6
	80	93.5 ± 2.2	91.9 ± 2.5
	1250	90.9 ± 1.2	88.8 ± 1.4

2.7. Animal study

In order to test the ability of the method to measure doxorubicin and its metabolites in the course of pharmacokinetic studies, doxorubicin was administered intravenously to a group of six male Sprague-Dawley rats at a dose of 5 mg/kg. Blood samples (200 μl) were collected through indwelling femoral venous catheters prior to, and at 5, 10, 20, and 40 min, 1, 1.5, 2, 4, 6, 8, 10, 12, 24, 36, and 48 h after the administration of doxorubicin. Blood samples obtained were centrifuged (10 min at $3000 \times g$) to separate serum. Bile was collected through bile duct catheters over 0–0.5, 0.5–1, and 1–2 h intervals, and then at 2-h intervals up to 12 h, and then at 12-h intervals up to 48 h after doxorubicin administration. Both serum and bile samples were stored at −20 °C before HPLC analysis.

3. Results and discussion

3.1. Chromatograms

Since doxorubicin and its metabolites show native fluorescence, they can be sensitively analysed by the detection of their fluorescence. Chromatograms resulting from the analysis of doxorubicin, doxorubicinol, doxorubicinolone, doxorubicinone and the I.S. (daunorubicin), are presented in Fig. 2. No interfering peaks were observed in the chromatograms of blank serum and bile samples. Doxorubicin, doxorubicinol, doxorubicinolone, doxorubicinone and daunorubicin were separated by the HPLC condition described above, and their retention times were 6.5, 4.2, 8.9, 17.1, and 14.2. Fig. 3 shows the typical chromatograms of the serum and bile samples obtained after an intravenous administration of 5 mg/kg of doxorubicin to a rat. Doxorubicin and two of its metabolites, doxorubicinol and doxorubicinolone, were detectable in both rat serum and bile samples, and their peaks were well separated from each other.

3.2. Calibration

The calibration plots for doxorubicin, doxorubicinol, doxorubicinolone, and doxorubicinone were linear over their respective concentration ranges sufficing for the pharmacokinetic studies in small laboratory animals, with coefficient of determination (r^2) of 0.9966–0.9998. The results of the linear regression analysis are shown in Table 1. For all the linear regression equations, y is the area ratio of the peak area of each tested analyte to that of the I.S. (daunorubicin), and x is the concentrations of the corresponding tested analyte.

3.3. Precision and accuracy

The within-day and between-day precision (R.S.D.) and accuracy (R.S.M.) for the HPLC assay are shown in Table 2 and Table 3, respectively. The LLOQ determined for doxorubicin was 10 ng/ml and that for its metabolites was 5 ng/ml. The results indicate that precision and accuracy of this method are acceptable.

3.4. Extraction recovery

The absolute recoveries of doxorubicin, doxorubicinol, doxorubicinolone, and doxorubicinone

Table 5
Summary of results obtained from freeze-thaw stability studies for doxorubicin, doxorubicinol, doxorubicinone and doxorubicinolone in rat serum and bile samples ($n = 3$)

	Concentration (ng/ml)	R.S.D. (%)		R.M.E. (%)	
		Serum	Bile	Serum	Bile
Doxorubicin	10	2.3	0.4	−9.6	−13.8
	160	3.9	6.2	−5.8	2.6
	2500	3.3	0.6	5.1	6.8
Doxorubicinol	5	2.8	1.9	−8.8	6.8
	80	1.3	2.7	−2.8	7.5
	1250	3.2	2.9	−0.3	−0.8
Doxorubicinone	5	6.3	6.8	−2.3	−0.9
	80	3.6	3.0	6.6	9.6
	1250	1.9	4.5	−1.1	−3.4
Doxorubicinolone	5	13.4	6.2	−13.6	9.3
	80	2.8	5.0	−0.3	5.3
	1250	2.1	3.6	9.4	−1.6

are listed in Table 4. The mean ± S.D. absolute recoveries of the I.S. extracted from spiked serum and bile samples were 91.7 ± 1.8% and 95.9 ± 0.8%, respectively ($n = 9$).

3.5. Freeze-thaw and storage stability

The freeze-thaw stability of doxorubicin and its metabolites in the samples prepared with rat serum and bile is summarised in Table 5. The variation of doxorubicin, doxorubicinol, doxorubicinolone, and doxorubicinone in rat serum and bile in the freeze-thaw operations was within 14 and 7%, respectively, at any concentrations, suggesting that these compounds are stable during the three freeze-thaw cycles. The storage stability of doxorubicin and its metabolites in rat serum and bile at different temperatures is depicted in Fig. 4. In both serum and bile samples, all compounds were stable for at least up to 1 and 3 weeks when the samples were stored at 4 and −20 °C, respectively. At room temperature (22 °C), the concentration of doxorubicin in rat serum and bile declined to 68 and 60% of the initial values, respectively, after 48 h of storage, whereas the concentrations of the three metabolites of doxorubicin remained stable during the same period of time. The instability of doxorubicin in the biological samples at 22 °C suggests that doxorubicin samples should be stored in the cold as soon as possible after collection, which is consistent with the earlier study [15]. In addition, there were no indications of instability of doxorubicin and its metabolites the samples precipitated with methanol and stored at room temperature in darkness for at least 48 h (doxorubicin: 98 ± 3% and 94 ± 3% of the initial values in bile and serum, respectively; doxorubicinol: 99 ± 3% and 101 ± 2% of the initial values in bile and serum, respectively; doxorubicinone: 93 ± 2% and 96 ± 3% of the initial values in bile and serum, respectively; doxorubicinonol: 101 ± 2% and 100 ± 4% of the initial values in bile and serum, respectively ($n = 3$)).

3.6. Application

The validated HPLC assay method was used for the simultaneous determination of doxorubicin and its metabolites in rat serum and bile after the intravenous administration of 5 mg/kg doxorubicin to rats. The smaller amount of serum required for the determination enabled many sampling points. Doxorubicin and two of its metabolites, doxorubicinol and doxorubicinolone, were detected in rat serum and bile above the LLOQ up to 24 h after the administration of doxorubicin.

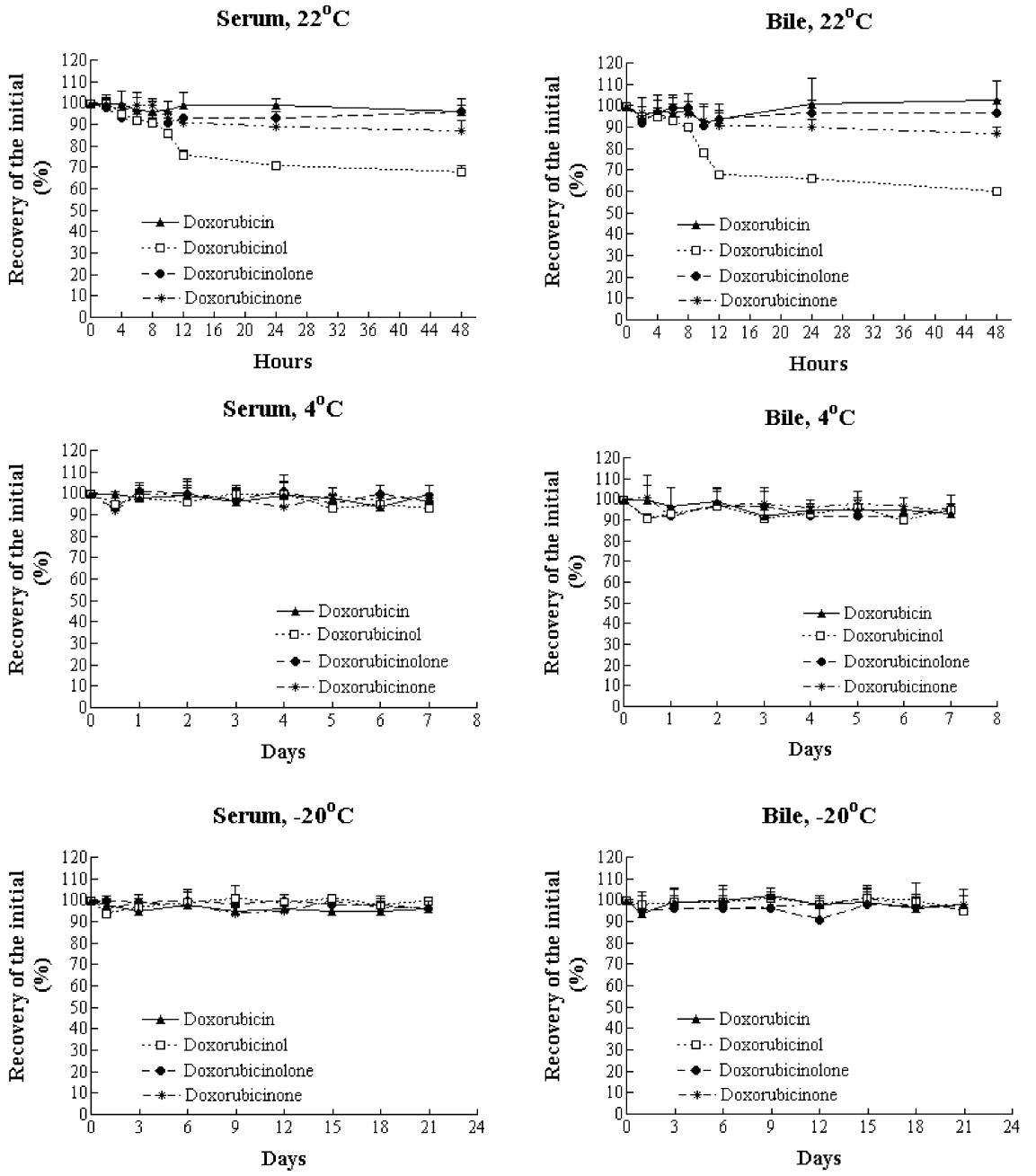

Fig. 4. Stability of doxorubicin and its metabolites in rat serum and bile samples. Drug-free rat serum and bile samples were spiked with 160 ng/ml of doxorubicin and 80 ng/ml of doxorubicinol, doxorubicinolone and doxorubicinone, and stored at different temperatures. Symbols and bars represent the means and S.D. from 3 observations.

Table 6
Comparison of the new HPLC assay method for doxorubicin and its metabolites with the conventional methods

	van Asperen et al. [13]	Andersen et al. [15]	New method
Sample preparation	Liquid–liquid extraction, evaporation of organic layer, and re-dissolution	Deproteinisation with methanol and $ZnSO_4$	Deproteinisation with Methanol
Analytical time	17 min	10 min	18 min
Limit of quantitation	Doxorubicin: 1.3 ng/ml; doxorubicinol: 1.0 ng/ml; doxorubicinone: 1.0 ng/ml; doxorubicinolone: 0.8 ng/ml	Doxorubicin: 19 ng/ml; doxorubicinol: 19 ng/ml; doxorubicinone: 15 ng/ml; doxorubicinolone: 15 ng/ml	Doxorubicin: 10 ng/ml; doxorubicinol: 5 ng/ml; doxorubicinone: 5 ng/ml; doxorubicinolone: 5 ng/ml
Sample volume	200 μl (plasma)	200 μl (citrated plasma)	50 μl (serum or bile)

The mean serum concentration-time profiles and the biliary excretion profiles of doxorubicin and its metabolites for the rats are presented in Fig. 5. The results obtained demonstrate that our modified HPLC method is suitable for the pharmacokinetic studies of doxorubicin and its metabolites in rats.

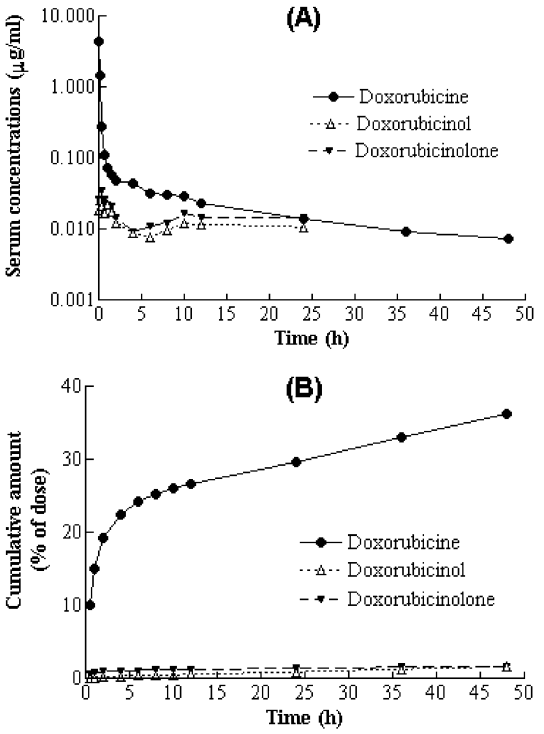

Fig. 5. Time courses of mean serum concentrations (A) and cumulative amount of biliary excretion (B) of doxorubicin, doxorubicinol, and doxorubicinolone after an intravenous bolus injection of doxorubicin at a dose of 5 mg/kg to rats.

4. Conclusion

A sensitive and reliable HPLC method has been developed and validated for the simultaneous quantification of doxorubicin and its metabolites in rat serum and bile. Compared with the conventional methods (Table 6[13,15]), this method has the advantage of simplicity and rapidity because the sample processing procedure involves a single protein precipitation step, which is independent upon the presence of the anticoagulant sodium citrate. As a result, reliable quantitation of doxorubicin and its metabolites can be achieved over the range of 10–2500 and 5–1250 ng/ml, respectively. This method has been successfully used to study the pharmacokinetic profiles and biliary excretion of doxorubicin and its metabolites in rats after a single dose administration of doxorubicin.

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