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Determination of doxorubicin and metabolites in murine specimens by high-performance liquid chromatography

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Abstract

A sensitive and selective reversed-phase high-performance liquid chromatographic method for the quantification of doxorubicin and its metabolites doxorubicinol, 7-deoxydoxorubicinone and 7-deoxydoxorubicinolone was developed and validated for a variety of murine specimens. Daunorubicin was used as internal standard. Sample pretreatment involved liquid–liquid extraction of 200 μl sample with 1 ml of chloroform–1-propanol (4:1, v/v). Chromatographic separation was achieved isocratically on a LiChrosorb RP-8 analytical column at ambient temperature. The mobile phase consisted of acidified water (pH 2.05)–acetonitrile–tetrahydrofuran (80:30:1, v/v/v). The column effluent was monitored fluorimetrically at an excitation wavelength of 460 nm and an emission wavelength of 550 nm. The lower limits of quantitation were in the range 1.8–2.4 n*M*. Spiked murine specimens and samples from treated mice were subjected to stability studies. The results demonstrated the importance of validation in all relevant specimens, since the accuracy and precision were highly matrix-dependent. Accuracies and precisions of measured drug concentrations in liver, spleen, muscle, gastrointestinal tissues, diluted bile, feces and urine were lower than in the other matrices. Doxorubicin was unstable in diluted bile, but not in the other specimens. The method is suitable for studying the pharmacokinetics of doxorubicin and its metabolites in mice.

Keywords: Doxorubicin; Doxorubicinol; 7-Deoxydoxorubicinone; 7-Deoxydoxorubicinolone

1. Introduction

Doxorubicin is an anthracycline antibiotic which is widely used in the treatment of human malignancies such as leukemia, lymphoma and a number of solid tumors, particularly breast cancer. It is produced by the fungus *Streptomyces peucetius* (var. caesius). Since its discovery in 1969 by Arcamone et al. [1] several biochemical effects of doxorubicin have been described which may contribute to its therapeutic action. The compound can intercalate between DNA bases or generate free radicals, but also interaction with cellular membranes and inhibition of the nuclear enzyme topoisomerase II have been reported (reviewed in [2]). Drug resistance, however, is a major impediment to successful chemotherapy. The presence or upregulation of a large transmembrane protein, called P-glycoprotein, is one of the factors which has been associated with resistance to anthracyclines [3]. P-glycoprotein is not only found in various malignant tissues but also in many normal tissues. To get more insight into the physiological

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and pharmacological role of P-glycoprotein we study the pharmacokinetics of anticancer agents, including doxorubicin, in mice with a genetic disruption of this protein [4–6].

Two important routes of metabolic transformation of doxorubicin in animals and man are the reduction of the side chain carbonyl group to the secondary alcohol doxorubicinol (I) and the reductive deglycosidation with the formation of 7-deoxydoxorubicinone (II) and 7-deoxydoxorubicinolone (III) (Fig. 1, reviewed in [7]).

To study the pharmacokinetics of doxorubicin in small laboratory animals such as mice a sensitive analytical assay is a prerequisite. Although numerous analytical methods for the determination of doxorubicin and its metabolites have been described, including radiolabeled assays [8], fluorescence assays [9–11], and methods based on thin-layer chromatography [8,11] or high-performance liquid chromatography (HPLC) [12–16], an assay suited for the individual quantification of doxorubicin and metabolites in many different murine specimens has not been reported to date. We have previously described a sensitive and selective analytical reversed-phase HPLC method for doxorubicin in plasma samples of HIV-infected patients [16]. However, it is important to investigate the accuracy and precision of an analytical assay for each relevant specimen, since the results may be strongly dependent on the matrix. Therefore, we have now designed and thoroughly validated an HPLC assay for doxorubicin and metabolites in all relevant murine specimens. The validation program followed the guidelines as given in [17].

2. Experimental

2.1. Chemicals

Doxorubicin·HCl and daunorubicin·HCl (Fig. 1) were purchased as powder for injection from Pharmacia-Farmitalia-Carlo Erba (Milan, Italy) and Rhône-Poulenc Rorer (Cedex, France), respectively. The metabolites doxorubicinol (I), 7-deoxydoxorubicinone (II) and 7-deoxydoxorubicinolone (III) were kindly provided by Pharmacia-Farmitalia-Carlo Erba. Bovine serum albumin (BSA) was obtained from Organon (Boxtel, The Netherlands). Hypnorm originated from Janssen (Tilburg, The Netherlands) and Dormicum from Roche (Mijdrecht, The Netherlands). Saline was purchased from NPBI (Emmer-Compascuum, The Netherlands). Other chemicals and solvents were of analytical or LiChrosolv® gradient grade and purchased from Merck (Darmstadt, Germany). Water was purified by the Milli-Q system (Millipore, Milford, USA) and acidified water (pH 2.05) was prepared by the addition of perchloric acid. Blank human plasma was supplied by healthy donors.

2.2. Preparation of stock solutions

Stock solutions of doxorubicin (86.2 μM) and I (72.7 μM) were prepared by dissolving the appropriate amount of drug, accurately weighed, in 25 ml saline. Metabolite II and III were dissolved in dimethylacetamide at 4°C according to a similar procedure, resulting in stock solutions of 95.8 μM and 75.4 μM, respectively. Assuming similar molar extinction coefficients (the compounds have overlapping UV spectra), the concentrations of the stock solutions of the metabolites were calibrated relative to doxorubicin by HPLC with UV detection at the absorption maximum of 490 nm.

A 4 mg/ml solution of the internal standard daunorubicin was prepared in acidified water (pH 2.05). This solution was purified by HPLC with UV detection at 460 nm using a stainless steel analytical column (125×4 mm I.D.) packed with 5 μm LiChrosorb RP-8 material (Merck). The mobile phase consisted of acidified water (pH 2.05)–acetonitrile–tetrahydrofuran (80:30:1, v/v/v). Sample volumes of 50 μl were injected. The column eluent containing the central part of the daunorubicin peak was collected for further use as internal standard (I.S.) stock solution. All stock solutions were stored in polypropylene tubes at −20°C.

2.3. Collection of murine specimens

Male and female FVB mice were anesthetized with diethylether and exsanguinated. The blood samples were collected in heparinized tubes, which were centrifuged (10 min, 2000 *g*, 4°C) to separate the plasma fraction. Blank plasma was stored at

−20°C. Furthermore, the following tissues were dissected: brain, muscle, colon, cecum, small intestine, stomach, liver, gall bladder, kidney, lung, spleen, heart, ovary, uterus, breast, testis, epididymis and eye. The blank tissues were homogenized with a Polytron tissue homogenizer (Kinematica, Littau, Switzerland) in 4% (w/v) BSA in water, resulting in final concentrations of approximately 0.05–0.2 g tissue/ml. Urine and feces were obtained from mice housed in metabolic cages. Feces was homogenized in 4% (w/v) BSA in water (0.03–0.1 g feces/ml) according to the procedure described for tissue specimens. For the collection of blank bile, mice were anesthetized with 7 ml/kg body weight (intraperitoneally) of a mixture containing Hypnorm (fentanyl 0.2 mg/ml, fluanisone 10 mg/ml), Dormicum (midazolam 5 mg/ml) and water (1:1:2, v/v/v). A detailed description of the surgical procedures has been published previously [6]. In brief, after opening of the abdominal cavity and distal ligation of the common bile duct, bile was collected for a period of 1.5–2 h via a polyethylene cannula, which was directly inserted into the gall bladder. The bile was diluted in blank human plasma (1:19, v/v). All biological specimens were stored at −20°C.

2.4. Preparation of calibration standards and quality controls

For each analytical run all stock solutions were sonicated for at least 30 min at room temperature. A mixed working solution of doxorubicin, I, II and III was freshly prepared by diluting the stock solutions in blank human plasma to final concentrations of 5388 n*M*, 4544 n*M*, 5988 n*M* and 4713 n*M*, respectively. Calibration standards at seven different concentration levels were obtained by serial dilution of the mixed working solution in blank human plasma. The calibration curves ranged from 2.2 to 2155 n*M*, 1.8 to 1818 n*M*, 2.4 to 2395 n*M* and 1.9 to 1885 n*M* for doxorubicin, I, II and III, respectively. A working solution of the I.S. was obtained by dilution of the I.S. stock solution in acidified water (1:39, v/v).

Quality control samples containing doxorubicin and all metabolites were prepared at three levels (low, medium and high) by spiking blank human plasma with doxorubicin (10.8 n*M*, 107.8 n*M* and 1078 n*M*), I (9.1 n*M*, 90.9 n*M* and 908.8 n*M*), II (12.0 n*M*, 119.8 n*M* and 1198 n*M*) and III (9.4 n*M*, 94.3 n*M* and 942.7 n*M*) and stored at −20°C.

2.5. Sample pretreatment

Frozen samples were thawed at room temperature and thoroughly homogenized by vortexing. Dilutions of feces homogenate (20-fold) and urine (100-fold) were prepared in blank human plasma, whereas the other specimens were used without further dilution. Of each sample a 200-μl aliquot was pipetted into a 2-ml polypropylene tube (Eppendorf, Hamburg, Germany). After the addition of 200 μl of a 6% (w/v) borate buffer (pH 9.5) and 100 μl I.S. working solution, the tubes were vortexed. The analytes were extracted from the samples with 1 ml of chloroform–1-propanol (4:1, v/v) by mixing for 5 min, followed by centrifugation for 10 min at 4°C (3000 *g*). The aqueous layer and the pellet were removed by suction. The organic layer was decanted into a clean polypropylene tube and evaporated by vacuum in a Speed-Vac Plus SC210A system (Savant, Farmingdale, USA) at 43°C. The residue was reconstituted in 100 μl of acetonitrile–tetrahydrofuran (40:1, v/v), vortexed for 20 s and then sonicated for 5 min. After adding 300 μl acidified water (pH 2.05) and vortexing, a 50-μl aliquot was injected into the HPLC system.

2.6. Chromatographic equipment and conditions

The HPLC system consisted of a SpectroFlow 400 solvent delivery system (Kratos, Ramsey, USA), a Basic Marathon autosampler provided with a cooled (4°C) sample tray (Spark Holland, Emmen, The Netherlands) and a Model FP920 fluorescence detector (Jasco, Hachioji City, Japan). The Chromsep glass analytical column (100×3 mm I.D.) packed with 7 μm Lichrosorb RP-8 material (Chrompack, Middelburg, The Netherlands) was protected by a guard column (10×2 mm I.D.) packed with pellicular reversed-phase material (Chrompack). The mobile phase consisted of acidified water (pH 2.05)–acetonitrile–tetrahydrofuran (80:30:1, v/v/v) and was degassed by ultrasonication. A flow-rate of 0.4 ml/min was used. The column eluent was monitored fluorimetrically at an excitation wavelength of 460

Table 1
Accuracy [deviation (%)], within-run precision (WRP) and between-run precision (BRP) of doxorubicin in murine specimens

Specimen	Nominal concentration (n*M*)	Deviation (%)	WRP (%)	BRP (%)	*N*[a]
4% BSA	10.1	−1.7	6.2	10.0	12
	101.3	1.2	4.5	8.0	12
	1013	−8.9	4.0	14.9	12
Plasma	10.1	−13.0	5.8	8.6	8
	101.3	1.6	4.1	17.3	7
	1013	−13.6	12.8	7.9	9
Brain	10.1	4.2	5.6	12.2	10
	101.3	10.8	3.3	10.4	10
	1013	1.7	3.8	3.4	10
Muscle	10.1	−17.0	12.7	25.3	10
	101.3	−12.0	18.1	27.4	10
	1013	−8.3	8.3	10.5	10
Colon	10.1	−23.3	10.8	26.0	10
	101.3	−3.5	15.3	18.9	10
	1013	−1.5	8.4	12.6	10
Cecum	10.1	−16.8	11.7	17.4	10
	101.3	−7.3	10.3	23.0	10
	1013	−7.5	6.6	8.2	10
Small intestine	10.1	−34.9	14.2	9.8	10
	101.3	−18.7	5.1	10.7	10
	1013	−25.3	7.5	6.5	10
Stomach	10.1	−13.0	4.5	23.9	10
	101.3	−7.3	7.3	16.7	10
	1013	−13.2	7.5	5.0	10
Liver	10.1	−35.9	14.9	13.4	10
	101.3	−18.1	6.9	18.9	10
	1013	−21.0	9.6	6.0	10
Gall bladder	10.1	−0.6	6.4	19.0	8
	101.3	2.6	3.4	14.4	10
	1013	1.4	5.7	6.9	10
Kidney	10.1	−13.8	6.5	18.6	10
	101.3	−7.8	7.9	12.2	10
	1013	−4.0	5.1	13.9	10
Lung	10.1	−19.4	10.5	15.8	10
	101.3	−13.3	3.2	8.2	10
	1013	−10.8	14.7	8.1	10
Spleen	10.1	−16.1	3.7	19.2	10
	101.3	−13.0	5.8	8.8	10
	1013	−12.8	7.9	11.6	10
Heart	10.1	−12.0	6.8	20.8	10
	101.3	−4.7	7.2	15.2	10
	1013	−4.6	8.6	9.1	10
Ovary	10.1	−12.3	5.8	21.6	10
	101.3	−2.3	7.7	20.2	10
	1013	−3.5	5.9	12.0	10
Uterus	10.1	−5.8	5.4	16.4	12
	101.3	2.2	8.8	10.4	12
	1013	−6.1	5.9	4.7	12
Breast	10.1	−15.3	5.4	19.1	12
	101.3	−12.0	4.9	10.2	12
	1013	−25.3	7.1	13.3	12

Table 1 (continued)

Specimen	Nominal concentration (n*M*)	Deviation (%)	WRP (%)	BRP (%)	*N*[a]
Testis	10.1	−11.8	18.7	[b]	10
	101.3	7.6	6.9	13.1	6
	1013	−10.0	2.5	4.7	6
Epididymis	10.1	−10.1	21.2	8.8	10
	101.3	−6.0	10.1	16.9	8
	1013	−9.3	1.4	8.2	6
Eye	10.1	11.3	9.1	[b]	6
	101.3	15.2	3.9	11.4	6
	1013	1.9	3.8	18.8	6
Bile (diluted)	10.1	−27.2	2.3	20.6	6
	101.3	−25.5	4.6	5.9	6
	1013	−44.7	3.6	43.6	6
Feces	101.4	−8.5	7.2	19.1	18
	1014	−10.5	7.6	11.3	18
	5069	−20.2	9.0	16.0	18
Urine	506.9	63.2	7.3	25.7	12
	1014	36.6	5.6	21.1	12
	2027	25.8	4.8	16.8	12
	5069	20.4	2.3	12.7	12

[a] Total number of observations at each concentration.
[b] No additional variation was observed as a result of performing the assay in different runs.

nm and an emission wavelength of 550 nm, with a bandwidth of 40 nm.

Peak recording and integration was performed with a SP4600 DataJet integrator connected to a WINner/286 data station (Spectra Physics, San Jose, USA). Calibration curves were calculated by weighted ($1/y^2$) least squares linear-regression analysis of the nominal concentration (abscissa) versus the ratio (y) of the peak area of doxorubicin, I, II or III and the internal standard (ordinate).

2.7. Precision and accuracy

All blank murine specimens, except urine, were spiked at three levels (low, medium and high, exact concentrations are given in Tables 1–4) with a mixture of doxorubicin, I, II and III for evaluation of accuracy and precision. Feces was spiked in triplicate using three different homogenates. Two different batches of urine were each spiked at four levels. Control samples of blank 4% (w/v) BSA in water were spiked at all concentration levels. The (spiked) specimens were stored at −20°C until analysis. A validation run included calibration samples, quality control samples and spiked murine samples, all in duplicate. Each specimen was analyzed in two or three different analytical runs by repeated freezing and thawing. After a period of approximately 2 years, new batches of all specimens, except eyes, diluted bile, urine and feces, were spiked and evaluated again for accuracy and precision according to the procedures described above. The stabilities of doxorubicin and metabolites in diluted bile, feces and urine were examined by analyzing freshly spiked specimens, specimens stored for 1 month at −20°C and specimens subjected to up to three repeated freeze–thaw cycles. The precision of the assays was assessed by the between-run and within-run precision. Estimates of the between-run precision were obtained by one-way analysis of variance (ANOVA) using the run day as classification variable. The between-groups mean square (MS_{bet}), the within-groups mean square (MS_{wit}) and the grand mean (GM) of the observed concentrations across runs were calculated using the NCSS package (Version 5.0, J.L. Hinze, East Kaysville, USA, 1991). The between-run precision was defined as:

$$\text{Between-run precision} = (\text{S.D.}_{run}/\text{GM}) \cdot 100\% \quad (1)$$

where $\text{S.D.}_{run} = [(MS_{bet} - MS_{wit})/n]^{0.5}$ and n repre-

Table 2
Accuracy [deviation (%)], within-run precision (WRP) and between-run precision (BRP) of I in murine specimens

Specimen	Nominal concentration (n*M*)	Deviation (%)	WRP (%)	BRP (%)	*N*[a]
4% BSA	7.7	−5.4	15.9	6.8	12
	77.3	−6.0	4.7	8.0	12
	772.7	−10.8	2.3	15.2	12
Plasma	7.7	−20.1	9.0	5.5	8
	77.3	−9.6	2.6	7.3	7
	772.7	−18.3	8.2	8.9	9
Brain	7.7	−15.0	8.8	3.1	10
	77.3	−4.8	4.4	1.0	10
	772.7	−6.9	3.5	7.1	10
Muscle	7.7	−19.1	9.0	7.9	10
	77.3	−9.6	7.0	15.7	10
	772.7	−1.4	15.8	12.8	10
Colon	7.7	−24.7	15.9	15.1	10
	77.3	−1.4	18.8	[b]	10
	772.7	−2.1	7.0	21.1	10
Cecum	7.7	−4.5	18.4	4.9	10
	77.3	−13.0	15.2	15.6	10
	772.7	−3.4	12.0	11.3	10
Small intestine	7.7	−29.9	7.8	13.0	10
	77.3	−15.0	1.9	3.9	10
	772.7	−18.1	5.6	5.8	10
Stomach	7.7	−10.3	8.1	22.1	10
	77.3	−11.4	7.6	7.8	10
	772.7	−5.0	9.1	[b]	10
Liver	7.7	−15.1	5.1	19.1	10
	77.3	−17.7	5.8	9.5	10
	772.7	−15.6	7.5	7.2	10
Gall bladder	7.7	2.0	6.4	11.9	8
	77.3	−2.3	4.4	7.4	10
	772.7	−3.3	4.6	8.5	10
Kidney	7.7	−12.6	12.3	12.5	10
	77.3	−9.4	4.2	5.6	10
	772.7	−9.2	6.7	6.7	10
Lung	7.7	−17.9	7.0	9.9	10
	77.3	−12.6	8.4	5.8	10
	772.7	−11.4	16.4	[b]	10
Spleen	7.7	−28.3	19.2	[b]	10
	77.3	−19.4	4.4	5.4	10
	772.7	−20.9	5.2	[b]	10
Heart	7.7	−12.0	7.3	23.5	10
	77.3	−10.3	6.8	6.7	10
	772.7	−11.2	9.2	13.6	10
Ovary	7.7	−8.0	11.6	11.5	10
	77.3	−0.3	11.3	12.1	10
	772.7	−7.2	8.2	12.4	10
Uterus	7.7	−8.6	14.5	9.9	12
	77.3	−6.0	15.6	[b]	12
	772.7	−12.2	8.3	10.5	12
Breast	7.7	−0.2	7.4	9.6	12
	77.3	−10.3	7.0	5.9	12
	772.7	−11.5	8.4	8.1	12

Table 2 (continued)

Specimen	Nominal concentration (n*M*)	Deviation (%)	WRP (%)	BRP (%)	*N*[a]
Testis	7.7	−7.7	5.4	9.5	10
	77.3	−7.6	5.5	5.0	6
	772.7	−16.8	1.5	3.9	6
Epididymis	7.7	12.8	11.1	12.7	10
	77.3	2.9	11.1	14.8	8
	772.7	−8.9	8.6	18.0	6
Eye	7.7	0.7	7.9	7.5	6
	77.3	−4.0	4.2	8.4	6
	772.7	−14.8	2.8	7.2	6
Bile (diluted)	7.7	2.5	3.6	11.9	6
	77.3	−11.7	4.9	7.6	6
	772.7	−28.0	3.4	13.0	6
Feces	76.8	13.6	9.0	23.7	18
	767.7	21.3	6.5	15.1	18
	3839	9.5	7.8	22.3	18
Urine	383.9	170.1	5.9	8.4	12
	767.7	80.9	4.8	7.4	12
	1535	43.3	4.8	7.2	12
	3839	15.7	0.9	6.5	12

[a] Total number of observations at each concentration.
[b] No additional variation was observed as a result of performing the assay in different runs.

sents the number of replicates within each run. In cases where MS_{wit} is greater than MS_{bet}, the resulting variance estimate is negative, implying that no significant additional variation was observed as a result of performing the assay in different runs.For each concentration, the estimate of the within-run precision was calculated as:

$$\text{Within-run precision} = [(MS_{wit})^{0.5}/GM] \cdot 100\% \quad (2)$$

The accuracy was expressed as the mean percentage deviation [dev (%)] from the nominal concentration (nominal conc.):

$$\text{Dev}(\%) = [(\text{mean observed concentration} - \text{nominal conc.})/\text{nominal conc.}] \cdot 100 \quad (3)$$

Values within ±20% for precision and accuracy were considered acceptable.

2.8. Lower limit of quantitation

Blank human plasma spiked at seven concentration levels with doxorubicin (0.2–5.4 n*M*), I (0.2–4.5 n*M*), II (0.2–6.0 n*M*) and III (0.2–4.7 n*M*) was analyzed in six-fold to determine the lower limit of quantitation (LLQ). The LLQ was defined as the lowest concentration at which the deviation of all the samples was within ±25%.

2.9. Extraction recovery

To estimate the recovery of the extraction procedure for doxorubicin, I, II, III and the internal standard, nonprocessed samples were prepared in mobile phase solution at concentrations corresponding with those used for the calibration curves in plasma. The recovery was determined in three analytical runs by calculating the ratio of the slopes of the processed versus unprocessed calibration curves.

2.10. Specificity

The specificity of the assay was assessed by duplicate analysis of pooled blank murine specimens obtained from 2 to 6 animals, blank human plasma (three batches) and 4% (w/v) BSA in water.

Table 3
Accuracy [deviation (%)], within-run precision (WRP) and between-run precision (BRP) of II in murine specimens

Specimen	Nominal concentration. (n*M*)	Deviation (%)	WRP (%)	BRP (%)	*N*[a]
4% BSA	9.13	3.1	9.4	17.7	12
	93.4	−1.7	4.7	4.8	12
	934.2	−2.5	3.7	12.1	12
Plasma	9.3	−14.9	8.3	0.6	8
	93.4	−3.5	5.8	13.0	7
	934.2	−14.1	6.3	7.4	9
Brain	9.3	−5.1	13.0	[b]	10
	93.4	2.7	3.7	15.1	10
	934.2	−1.0	3.3	14.4	10
Muscle	9.3	−9.3	4.8	12.6	10
	93.4	−0.6	6.6	22.9	10
	934.2	−4.2	4.3	11.4	10
Colon	9.3	−11.4	5.2	21.4	10
	93.4	−7.9	8.2	19.3	10
	934.2	−10.0	4.7	12.7	10
Cecum	9.3	−6.0	3.2	37.9	10
	93.4	−12.9	6.1	23.4	10
	934.2	−14.7	4.5	14.8	10
Small intestine	9.3	−13.2	6.4	12.7	10
	93.4	−14.5	4.7	16.6	10
	934.2	−20.0	8.5	7.2	10
Stomach	9.3	−22.2	6.0	13.3	10
	93.4	−6.3	8.1	10.1	10
	934.2	−18.6	6.9	11.7	10
Liver	9.3	−8.8	25.9	[b]	10
	93.4	−19.3	11.1	14.8	10
	934.2	−20.1	10.8	4.6	10
Gall bladder	9.3	−2.8	1.3	7.2	8
	93.4	4.0	5.4	12.3	10
	934.2	−0.9	4.6	12.9	10
Kidney	9.3	−16.7	12.5	[b]	10
	93.4	−10.7	5.8	11.9	10
	934.2	−15.6	14.7	[b]	10
Lung	9.3	−18.2	7.8	11.2	10
	93.4	−10.5	10.9	7.7	10
	934.2	−14.7	8.0	[b]	10
Spleen	9.3	−9.3	3.9	19.7	10
	93.4	−9.0	5.0	11.9	10
	934.2	−12.1	3.7	10.0	10
Heart	9.3	−2.3	8.0	22.6	10
	93.4	−6.0	4.5	5.5	10
	934.2	−10.6	4.9	9.7	10
Ovary	9.3	−4.9	3.9	26.1	10
	93.4	−1.7	3.3	19.3	10
	934.2	−9.3	5.8	10.8	10
Uterus	9.3	−5.5	5.7	10.0	12
	93.4	−10.3	5.5	4.3	12
	934.2	−13.2	1.8	9.1	12
Breast	9.3	9.4	4.8	23.8	12
	93.4	2.1	4.4	14.1	12
	934.2	−12.2	2.4	10.9	12

Table 3 (continued)

Specimen	Nominal concentration. (n*M*)	Deviation (%)	WRP (%)	BRP (%)	*N*[a]
Testis	9.3	−1.6	4.6	18.1	10
	93.4	−8.6	3.1	[b]	6
	934.2	−16.5	2.2	9.7	6
Epididymis	9.3	6.4	3.3	13.8	10
	93.4	−0.6	5.7	10.3	8
	934.2	−11.0	5.0	12.9	6
Eye	9.3	22.3	4.8	14.1	6
	93.4	0.3	3.6	5.3	6
	934.2	−5.0	2.4	17.9	6
Bile (diluted)	9.3	33.0	4.6	15.0	6
	93.4	−7.0	5.6	6.4	6
	934.2	−16.7	4.2	16.4	6
Feces	93.5	39.3	12.3	29.4	18
	935.0	−6.1	6.4	24.3	18
	4675	−23.2	12.1	22.4	18
Urine	467.5	11.3	13.0	[b]	12
	935	2.6	5.8	2.6	12
	1870	−0.5	6.4	3.4	12
	4675	−5.0	3.3	4.2	12

[a] Total number of observations at each concentration.
[b] No additional variation was observed as a result of performing the assay in different runs.

2.11. Reproducibility

The quality control samples which were assayed in duplicate within each analytical run were also used to evaluate the reproducibility of the assay. The deviation (%), the within-run precision and the between-run precision were calculated for these samples, using the results of more than 60 analytical runs.

2.12. Applicability of the assay and stability of samples

The suitability of the assay for the investigation of the pharmacokinetics of doxorubicin in murine specimens has been studied extensively. Plasma, tissues, bile, urine and feces of female FVB mice were collected (Section 2.3) after intravenous administration of 5 mg/kg doxorubicin. The samples were stored at −20°C and analyzed in at least two separate analytical runs. After approximately 1 year, a selection of specimens was reanalyzed to examine the stability of doxorubicin and metabolites in these samples.

3. Results and discussion

The metabolism of doxorubicin to the secondary alcohol doxorubicinol (I) and the formation of 7-deoxydoxorubicinone (II) and 7-deoxydoxorubicinolone (III) is shown in Fig. 1. However, two additional metabolites of doxorubicin have been found in man, namely the aglycones doxorubicinone and doxorubicinolone [14]. To separate doxorubicin and all its metabolites, including these 7-hydroxy-aglycones, chromatography was initially performed using two coupled LiChrosorb RP-8 analytical columns. A preliminary pharmacokinetic study in mice revealed, however, that doxorubicin was not metabolized into doxorubicinone and doxorubicinolone, which is in agreement with results of previous studies performed in this species [18–20]. Since sufficient separation of doxorubicin, I, II and III could be obtained with a single LiChrosorb RP-8 analytical column, the initial chromatographic system was modified. This resulted in a substantial reduction of the total run time. Slight modifications of the analytical procedures described by Beijnen et al. [16] were also required to optimize the assay for murine specimens.

Table 4
Accuracy [deviation (%)], within-run precision (WRP) and between-run precision (BRP) of III in murine specimens

Specimen	Nominal concentration (n*M*)	Deviation (%)	WRP (%)	BRP (%)	*N*[a]
4% BSA	5.6	−1.0	7.5	9.4	12
	55.6	3.1	4.2	1.9	12
	556.2	−1.1	2.5	10.5	12
Plasma	5.6	−2.4	7.2	10.5	8
	55.6	−1.2	5.1	7.7	7
	556.2	−3.4	4.7	6.7	9
Brain	5.6	−2.5	7.7	14.2	10
	55.6	1.5	3.6	8.2	10
	556.2	1.8	3.0	4.0	10
Muscle	5.6	11.1	4.6	13.8	10
	55.6	9.7	7.1	8.0	10
	556.2	9.4	4.2	2.4	10
Colon	5.6	−8.7	10.3	16.7	10
	55.6	3.6	7.6	12.2	10
	556.2	2.5	2.3	6.0	10
Cecum	5.6	0.3	6.1	10.1	10
	55.6	3.7	4.9	8.6	10
	556.2	−0.3	5.1	1.8	10
Small intestine	5.6	−11.4	4.2	11.1	10
	55.6	0.4	1.9	9.1	10
	556.2	−0.2	5.2	3.7	10
Stomach	5.6	−2.2	10.6	18.5	10
	55.6	0.8	2.4	11.8	10
	556.2	2.2	7.1	[b]	10
Liver	5.6	−18.4	14.9	9.8	10
	55.6	−8.6	7.5	6.5	10
	556.2	−6.2	9.1	6.7	10
Gall bladder	5.6	8.6	5.9	12.3	8
	55.6	11.3	4.5	6.5	10
	556.2	3.4	9.1	2.7	10
Kidney	5.6	−7.3	5.8	15.6	10
	55.6	−0.8	3.2	9.8	10
	556.2	−3.1	7.3	4.1	10
Lung	5.6	−4.1	10.4	13.8	10
	55.6	4.1	5.4	9.7	10
	556.2	−1.7	8.9	[b]	10
Spleen	5.6	−1.5	3.9	14.4	10
	55.6	1.9	5.0	5.7	10
	556.2	0.6	4.2	3.9	10
Heart	5.6	0.6	10.6	13.4	10
	55.6	0.6	4.6	8.6	10
	556.2	−4.5	1.9	7.0	10
Ovary	5.6	7.8	4.8	15.4	10
	55.6	8.7	2.3	7.9	10
	556.2	0.0	5.7	5.6	10
Uterus	5.6	−2.3	5.7	10.6	12
	55.6	−3.2	4.5	6.8	12
	556.2	−6.2	4.6	10.7	12
Breast	5.6	15.0	6.4	4.4	12
	55.6	9.2	4.6	4.7	12
	556.2	2.2	3.2	9.4	12

Table 4 (continued)

Specimen	Nominal concentration (n*M*)	Deviation (%)	WRP (%)	BRP (%)	*N*[a]
Testis	5.6	−1.4	6.2	6.4	10
	55.6	−3.0	4.8	1.5	6
	556.2	−10.7	1.8	13.1	6
Epididymis	5.6	14.0	6.1	8.6	10
	55.6	9.2	5.6	12.5	8
	556.2	−7.3	3.6	19.9	6
Eye	5.6	0.8	7.7	9.8	6
	55.6	0.6	3.5	2.9	6
	556.2	−5.7	3.4	14.1	6
Bile (diluted)	5.6	−7.8	1.9	5.8	6
	55.6	−6.6	4.1	0.6	6
	556.2	−9.8	2.7	8.1	6
Feces	56.1	44.5	19.9	23.6	18
	561.0	20.7	6.7	14.1	18
	2805	7.3	11.2	7.6	18
Urine	280.5	14.3	7.2	11.6	12
	561.0	4.5	6.4	7.0	12
	1122	1.5	3.4	6.9	12
	2805	−1.0	1.6	6.8	12

[a] Total number of observations at each concentration.
[b] No additional variation was observed as a result of performing the assay in different runs.

Blank chromatograms of human plasma, murine plasma and murine liver are shown in Fig. 2A and B and Fig. 3A. All other specimens were also free of interfering peaks, except urine. In this matrix an endogenous component was present, which eluted at the same retention time as I. Representative chromatograms of spiked murine plasma and murine liver sampled at 24 h after intravenous bolus administration of 5 mg/kg doxorubicin are depicted in Fig. 2C and Fig. 3B, respectively. Baseline separation was obtained for all components. The retention times of doxorubicin, I, II, III and the internal standard were 6.5, 4.3, 16.6, 9.6 and 12.7 min, respectively. The overall chromatographic run time was 22 min.

An acceptable accuracy (within ±25%) was achieved in human plasma spiked at 2.2 n*M*, 1.8 n*M*, 2.4 n*M* and 1.9 n*M* with doxorubicin, I, II and III, respectively. Consequently, these concentrations were established as the LLQ and were used as the lowest concentrations in the calibration curves. The calibration curves were linear over the concentration range tested for all compounds and calculated by weighted $(1/y^2)$ linear regression analysis.

The results of the validation in terms of accuracy and precision are listed in Tables 1–4. The within-run precision of each compound was in general acceptable for all tissues. However, not all the results obtained in tissues of the gastrointestinal tract, liver, spleen, muscle, urine, feces and diluted bile met the requirements for between-run precision and accuracy. Factors which might contribute to this are: extraction recovery, adsorption, interference of endogenous components, homogeneity of samples, metabolic activity and stability upon prolonged storage or repeated freeze–thawing. The potential role of each factor in the accuracy and precision of the assay will be discussed successively. (a) Differences in the extraction recovery of an analyte between human plasma and murine specimens might have caused systematic deviations from the observed concentration, since the calibration samples were prepared in human plasma. The extraction recovery of doxorubicin from urine and of II from diluted bile is probably higher than their recoveries from human plasma. The same holds true for the extraction recovery of all compounds from feces although it is not apparent from the accuracy, but this will be explained below. The results obtained for I and/or doxorubicin in small intestine and liver, respectively, might indicate lower extraction recoveries of these

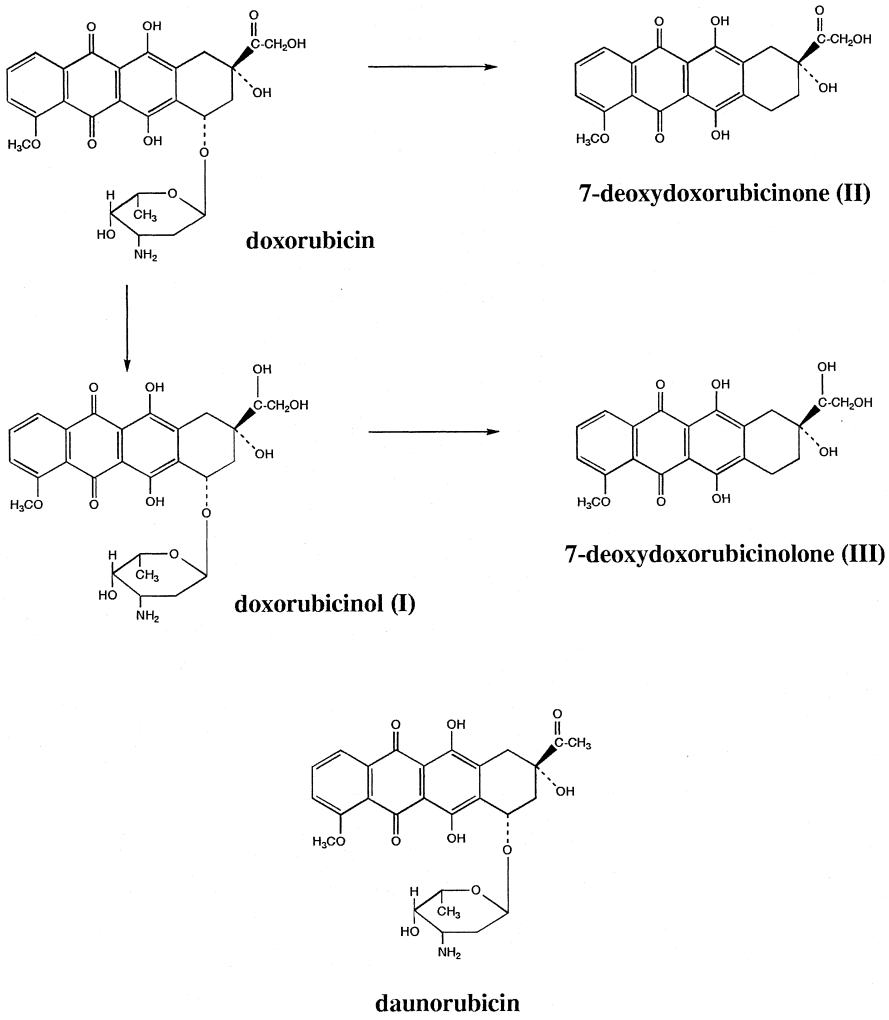

Fig. 1. Chemical structures of doxorubicin, doxorubicinol (I), 7-deoxydoxorubicinone (II), 7-deoxydoxorubicinolone (III) and daunorubicin (internal standard).

compounds (relative to the internal standard) in comparison to human plasma. (b) Adsorption is characterized by negative values of the accuracy ($< -20\%$), which are predominant at the lowest concentration level. Adsorption processes were possibly of importance for one or more compounds in colon, small intestine, liver, spleen, and stomach. (c) The observation of systematically higher concentrations of I in urine are explained by a coeluting interference of an endogenous component. (d) The homogeneity of samples plays principally a role in the precision. Samples of tough tissues like muscles, stomach and intestines were possibly not completely homogeneous. (e) Metabolic activity in specimens like liver, small intestines, feces and diluted bile might have contributed to systematic deviations from the observed concentrations. (f) There were no indications that the repeated freeze–thaw cycles in combination with prolonged storage affected the stability of the compounds in spiked specimens, except in diluted bile and feces. A stability study revealed, that doxorubicin was unstable in diluted bile after storage for 1 month at -20°C as well as after repeated freeze–thaw cycles. The results of the

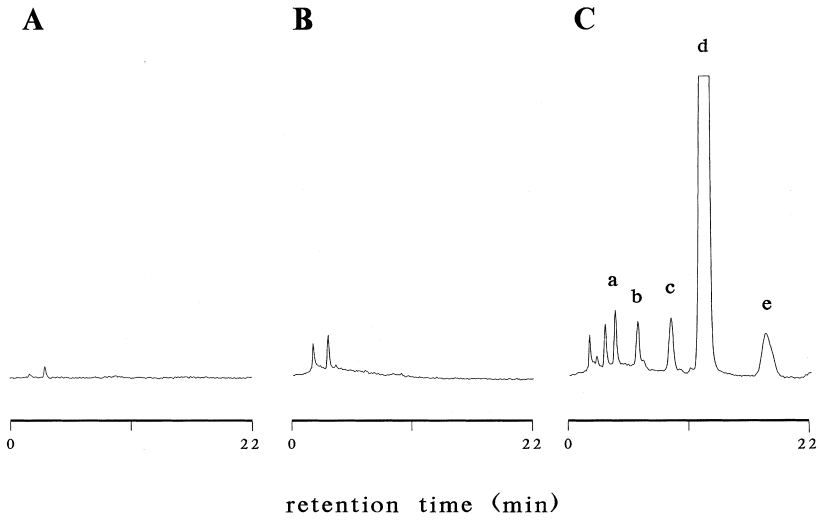

Fig. 2. Chromatograms of (A) blank human plasma; (B) blank murine plasma; (C) spiked murine plasma. Peaks labeled a, b, c, d and e correspond with I (7.7 n*M*), doxorubicin (10.1 n*M*), III (5.6 n*M*), internal standard and II (9.3 n*M*), respectively.

stability study in feces are summarized in Table 5. In comparison to the initially observed concentrations significantly lower concentrations of all compounds were observed after three freeze–thaw cycles. Doxorubicin and II were the most unstable compounds with significant reductions of their concentrations already present after one freeze–thaw cycle. Instability in feces or diluted bile was not accompanied by the appearance of extra chromatographic peaks. The stability problems in feces were not clearly reflected by the accuracy. The initially observed concentrations in feces were substantially higher than the nominal concentrations for all compounds (Table 5), which may have counterbalanced the impact of the stability problems on the accuracy.

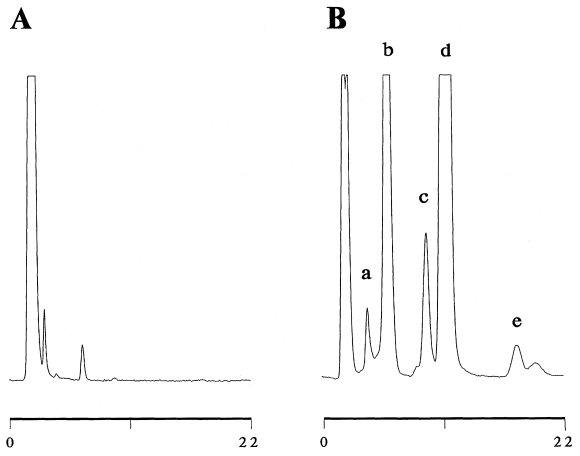

Fig. 3. Chromatograms of (A) blank murine liver; (B) liver of a female mouse, 24 h after intravenous bolus administration of 5 mg/kg doxorubicin. Peaks labeled a, b, c, d and e correspond with I, doxorubicin, III, internal standard and II, respectively.

The extraction recoveries for doxorubicin, I, II, III and the internal standard were 64.0±1.5%, 66.1±1.1%, 60.8±7.6%, 76.7±3.8% and 77.0±2.4%, respectively (results expressed as mean±S.D.). An interfering peak eluting at the same retention time as III was observed when processed samples which contained high concentrations of doxorubicin were kept at room temperature until analysis. This problem was not encountered in processed samples stored up to at least 48 h at 4°C, indicating the necessity of a thermostated autosampler tray.

Quality control samples of more than 60 analytical runs have been used to determine the reproducibility of the assay over a period of 2 years (Table 6). For each compound the method was shown to be accurate ([deviation (%)] was between −2.2 and +5.0%) and precise (within-run and between-run variation were within 15%) at all concentration levels.

The described method has been applied successfully in several pharmacokinetic studies. Reproduc-

Table 5
Stability of doxorubicin, I, II and III in spiked feces homogenate as percentage of the initially observed concentration

Compound	Nominal concentration (n*M*)	Initially observed concentration (n*M*)	Storage for 1 month at −20°C	Freeze–thawing		
				1 Cycle	2 Cycles	3 Cycles
Doxorubicin	101.4	129.6±0.4	105.0±0.7	83.6±5.4	59.6±8.2*	65.1±27.0
	1014	1188±13.9	87.8±0.8*	83.5±6.2	77.8±4.4*	71.7±0.2**
	5069	6047±415	n.d.	82.6±3.1*	68.3±0.6**	56.3±3.1**
I	76.8	112.9±5.5	96.2±1.5	91.2±1.5	80.4±12.0	52.9±20.5*
	767.7	973.8±19.3	95.0±9.1	110.0±4.1	94.5±5.6	80.1±0.8**
	3839	4959±408	n.d.	107.0±0.6	91.4±1.2	73.3±7.2**
II	93.5	166.1±0.6	91.9±6.2	80.2±11.4	76.1±2.3	47.8±3.0**
	935.0	1346±5.7	74.5±8.1*	79.7±13.2*	72.4±5.3**	52.3±3.7**
	4675	5891±516	n.d.	81.5±5.7*	63.3±3.5**	44.9±4.1**
III	56.1	87.2±0.8	107.0±6.9	85.8±8.5	96.2±28.8	88.0±34.4
	561.0	783.3±32.2	97.6±14.5	93.4±19.9	93.1±14.6	78.2±6.7*
	2805	3691±393	n.d.	95.0±4.9	84.4±6.7*	74.2±8.0*

Results are expressed as means±S.D. Two batches spiked feces homogenate were used at each concentration. The initial value was obtained by analyzing freshly spiked feces homogenates. The differences between the initially observed concentration and the observed concentration after 1 month storage at −20°C or after one, two or three freeze–thaw cycles, were tested on significance by the paired Student's *t*-test. * $P<0.05$; ** $P<0.01$; n.d.=not determined.

ible data were obtained for doxorubicin, I, II and III. All compounds were shown to be stable in the intestinal contents of treated mice, also after repeated freeze–thaw cycles, which is in contrast with the results observed in spiked feces homogenate. In addition, no stability problems were encountered in the other samples originating from in vivo studies, except in diluted bile. It was possible to quantify doxorubicin in this specimen reproducibly up to at least 5 months after sample collection. Substantially reduced concentrations of doxorubicin were observed in bile samples which were reanalyzed after 1.5 years.

In conclusion, the analytical method for determination of doxorubicin and three major metabolites previously described by Beijnen et al. [16] has been successfully modified for murine specimens. An analytical method for doxorubicin and metabolites validated in many different murine specimens, according to the current requirements for validation of

Table 6
Quality control data of the assay for doxorubicin and metabolites represented by the accuracy [deviation (%)], the within-run precision (WRP) and the between-run precision (BRP)

Compound	Nominal concentration (n*M*)	Deviation (%)	WRP (%)	BRP (%)	*N*[a]
Doxorubicin	10.8	−2.2	11.8	14.0	137
	107.8	3.5	6.3	14.1	133
	1078	0.4	5.9	14.4	128
I	9.1	0.8	10.0	9.8	138
	90.9	2.1	4.6	12.5	133
	908.8	−0.2	5.9	12.1	120
II	12.0	5.0	13.0	11.3	136
	119.8	2.4	9.0	10.6	132
	1198	−0.4	6.2	9.6	119
III	9.4	3.1	7.4	10.2	137
	94.3	2.3	4.9	9.4	133
	942.7	−0.8	7.7	10.8	120

[a] Total number of observations at each concentration.

bioanalytical methods [17], has not been reported earlier. Analysis of the compounds in murine plasma, brain, gall bladder, kidney, lung, heart, ovary, uterus, breast, testis, epididymis and eye meets these international standards. The validation data clearly illustrate the importance of analyzing all relevant specimens, since the accuracy and precision were highly dependent on the matrix. Relative inaccuracies and imprecisions of one or more compounds have to be taken into account when analyzing samples of liver, spleen, muscle, gastrointestinal tissues, bile, feces and urine. Bile samples have to be analyzed shortly after collection, as doxorubicin was shown to be unstable in this matrix. The method will be implemented in future studies to investigate the role of P-glycoprotein in the pharmacokinetics of doxorubicin and metabolites in a mouse model.

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