

Original Research Article

A Simple HPLC Bioanalytical Method for the Determination of Doxorubicin Hydrochloride in Rat Plasma: Application to Pharmacokinetic Studies

Senthil Rajan Dharmalingam*, Srinivasan Ramamurthy, Kumarappan Chidambaram and Shamala Nadaraju

School of Pharmacy, International Medical University, Bukit Jalil, Kuala Lumpur, Malaysia

*For correspondence: **Email:** senthil_rajana@imu.edu.my; **Tel:** +603 2731 7310

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Abstract

Purpose: To develop a simple, accurate, and precise high performance chromatography (HPLC) method with spectrophotometric detection for the determination of doxorubicin hydrochloride in rat plasma.

Methods: Doxorubicin hydrochloride and daunorubicin hydrochloride (internal standard, IS) were separated on a C₁₈ reversed-phase HPLC column. Following protein precipitation extraction, chromatographic separation was accomplished with a mobile phase consisting of acetonitrile: water at ratio of 30:70 (pH 3.0), and the drug was detected at 233 nm using a UV detector at flow rate of 1.0 ml/min and ambient temperature.

Results: Linearity was obtained over the range 1.0 – 50.0 µg/ml for doxorubicin hydrochloride with lower limit of quantitation of 1.0 µg/ml. For each level of quality control samples, inter- and intra-day precision (% CV) was < 9.6 and 5.1 %, respectively. Stability of doxorubicin hydrochloride in plasma was within the acceptance limit (± 15 %) with no evidence of degradation during sample processing and 30 days storage in a deep freezer at -70 ± 5 °C. Absolute extraction recovery of drug from plasma was ≥ 86 %.

Conclusion: The method is highly selective and rugged for the determination of doxorubicin hydrochloride in rat plasma and should be suitable for conducting pharmacokinetic studies and therapeutic drug monitoring.

Keywords: Doxorubicin, Daunorubicin, Validation, pharmacokinetics, rat plasma.

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INTRODUCTION

Doxorubicin hydrochloride, is a cytotoxic anthracycline antibiotic isolated from cultures of the fungus *Streptomyces peucetius* var. *caesius*. The most common uses of doxorubicin in cancer therapy is for various types of testicular cancer, leukemia, Ewing's sarcoma, Hodgkin's disease, and Kaposi's sarcoma [1]. Doxorubicin has been

shown to have mutagenic and carcinogenic properties in experimental models.

Doxorubicin hydrochloride is an anthracycline antibiotic that possesses broad spectrum antineoplastic activity, and is one of the most important anticancer agents in use [2,3]. However, clinical utility is hampered by cumulative, dose-limiting cardiotoxicity,

myelosuppression, and the development of drug resistance.

Several methods have previously been reported for quantification of doxorubicin in biological fluids and tissues. Techniques such as radioimmunoassay for determination of doxorubicin in plasma and urine [4], voltammetry for urine [5,6], high performance liquid chromatography (HPLC) for rat lymph and gall [7], rat plasma and tissues [8], rat serum, tissues [9] and bile [10], human plasma of cancer patients [11], and spectrofluorimetry for rabbit serum [12] and rat whole blood, plasma and tissues [13] were used.

Many of the reported HPLC methods used extraction procedures involving organic solvents such as chloroform or dichloromethane. Such procedures are tedious and involve likelihood of incomplete drug extraction, loss of drug during transfer of contents and also high volatility of the solvent used for extraction. Hence, a simple HPLC method, which avoids the above shortcomings, would be advantageous for the rapid determination of doxorubicin in biological samples. Furthermore, to the best of our knowledge, no simple HPLC method with UV detection has been reported for the determination of the drug in rat plasma, a medium that would best mimic pharmacokinetic analysis.

The objective of the present study, therefore, was to develop and validate a simple, rapid and sensitive HPLC method with UV detection for the determination of doxorubicin in rat plasma.

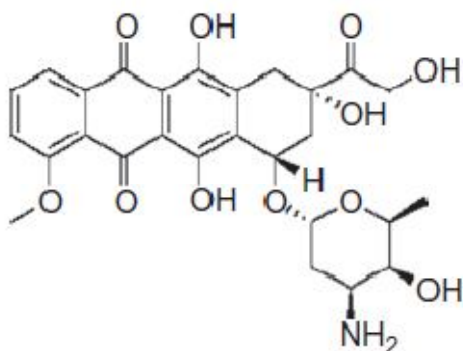


Figure 1: Structure of doxorubicin hydrochloride

EXPERIMENTAL

Materials

Doxorubicin hydrochloride and daunorubicin hydrochloride (EMD Chemicals, Inc, San Diego) and other chemicals of analytical grade purity were purchased from commercial sources. Water was purified using Ultrapure Water System

(Sartorius). All solvents including water used for extraction and in the mobile phase were of HPLC grade purity. Rat plasma doxorubicin samples were stored at -70 ± 0.5 °C prior to analysis and thawed, along with standards and quality controls, immediately before assay.

Apparatus and chromatographic conditions

HPLC system (1200 series, Agilent Technologies) consisted of a pump with a column from Merck C₁₈ (4.6 mm × 100 mm), a UV-detector with data processor (Chem Station software) was employed. UV detection for doxorubicin was set at 233 nm. The mobile phase of water: acetonitrile (30:70, pH 3.0, adjusted with 85 % phosphoric acid) was delivered at a flow rate of 1.0 ml/min and ambient temperature. The mobile phase was delivered by gradient method at acetonitrile (ACN) gradient of 15 % at 0 min, 25 % at 5 min, 40 % at 10 min, 70 % at 15 min and 100 % at 20 min. Peak identities were confirmed by retention time (RT) of doxorubicin hydrochloride at 7.8 min and daunorubicin hydrochloride (internal standard) at 8.6 min, respectively.

Preparation of stock solutions and quality control samples

The standard stock solution of doxorubicin (1.0 mg/mL) was prepared in acetonitrile. The working standard solution (10 µg/ml) was prepared by diluting the stock solution in mobile phase (30: 70, ACN: water) with pH adjusted to 3.0.

Direct precipitation method

A serial calibration line of sample concentrations of 1, 2.5, 5, 12.5, 25, 50 µg/ml of doxorubicin was prepared by diluting definite aliquots of working standard with rat plasma, and up to 90 µl of blank plasma with 5 µl each of doxorubicin hydrochloride and daunorubicin hydrochloride to give a final volume of 0.1 ml. It was then precipitated with 0.4 ml of acetonitrile, followed by centrifugation at 3000 rpm at room temperature for 15 min. The supernatant was filtered through a 0.45 µm filter.

Linearity

The linearity of the method was evaluated over the concentration ranges of 1.0 – 50.0 µg/ml. Calibration standards were freshly prepared in duplicate daily during the analysis. A stock solution of daunorubicin hydrochloride (1000 µg/ml) was prepared in methanol and used as internal standard (IS). For the quality control

(QC) sample, the concentrations of doxorubicin in plasma were 3.0 µg/ml as low quality control (LQC), 25 µg/ml as middle quality control (MQC), and 37.5 µg/ml as high quality control (HQC). All the stock and working solutions, and QC samples were protected from light and stored at 4 °C. Standard curves were obtained from the linear least square regression analysis of drug/IS. peak area ratio as a function of the theoretical concentration. The plasma used in the study was isolated from whole blood containing EDTA by centrifugation at 3000 rpm for 5 min.

Specificity

The specificity of the method was established using different concentrations of doxorubicin hydrochloride. In each plasma lot, blank samples and LLOQ (lower limit of quantitation) were processed and analysed as per assay procedure to determine any significant interference at the RT of analyte.

System suitability

System suitability test is used to verify that the resolution and reproducibility of the chromatographic systems are adequate for the analysis to be done. The tests are based on the fact that the equipment, electronics, samples to be analyzed constitute an integral system that can be evaluated as such. The limits for system suitability were set for theoretical plates, resolution, and asymmetry.

Sensitivity

The selectivity of the assay methodology was established using a minimum of six independent sources of the same matrix. There were no interferences from the endogenous material which was observed at retention time of both doxorubicin hydrochloride and daunorubicin hydrochloride. Selectivity was established by injecting six samples at the lower limit of quantification (LLOQ) level and each of the six blank plasma samples were tested for interference by comparing the mean peak response obtained by injecting blank plasma samples to that of mean peak response of LLOQ (1 µg/ml).

Extraction recovery

Recovery of doxorubicin hydrochloride and daunorubicin hydrochloride was evaluated by comparing the mean peak areas of LQC, MQC and HQC samples quality control samples with the mean peak areas of six reference solutions

containing the same amount of the test compound.

Precision and accuracy

Intra- and inter-day precision and accuracy of the developed method were evaluated in plasma samples spiked with doxorubicin hydrochloride at a concentration of 3.0, 25.0, and 37.5 µg/mL, and assayed. Inter-day precision and accuracy were evaluated on three consecutive days. The criteria for acceptability of the data include accuracy within $\pm 15\%$ of the coefficient of variation (CV) from the nominal values and a precision of within $\pm 15\%$ of the coefficient of variation (CV), except for LLOQ, where it should not exceed $\pm 20\%$ of accuracy as well as precision [14].

Stock solution stability

The working solution (1.0 mg/mL) of doxorubicin hydrochloride was repeatedly ($n = 3$) injected into the chromatograph immediately after preparation (0 h) and at 3, 6, and 9 h after bench top storage at room temperature and 4 °C. This injection protocol was repeated after 1, 8, 15 and 30 days storage of the stock solution between 4 – 8 °C.

Stability

Bench top stability was examined by keeping replicates of the low, medium and high plasma quality control samples at room temperature for 24 h. Freeze–thaw stability of the samples was obtained over three freeze–thaw cycles, by thawing at room temperature for 2 – 3 h and re-frozen for 12 – 24 h. Long-term stability of doxorubicin hydrochloride in rat plasma was tested after storage at approximately -70 ± 5 °C for 30 days. For each concentration and each storage condition, three replicates were analysed in one analytical batch. The concentration of doxorubicin hydrochloride after each storage period was related to the initial concentration as determined for the samples. The precision and accuracy for the stability samples is required to be within ≤ 15 and $\pm 15\%$, respectively, of their nominal concentrations.

Dilution integrity

Dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentration above the upper limit of quantification (ULOQ), which maybe encountered during real subject sample analysis. Dilution integrity was performed to extend the upper concentration limits with acceptable precision and accuracy. Six replicates each, at a concentration of double the uppermost

calibration standard, were diluted two-fold and four-fold with blank plasma. The diluted samples were processed and analyzed against calibration curve.

Haemolysis effect

Haemolysis is caused by the rupture of red blood cells, which releases haemoglobin into plasma. Haemolysis effect must be measured because it can impact quantitation by altering the sample matrix compared to the validated method matrix.

To evaluate the effect of haemoglobin in plasma samples on precision and accuracy, haemolyzed whole blood was spiked with non-haemolyzed plasma at 0 % (100 % normal plasma/serum) and 0.5 % haemolyzed whole blood in LQC and HQC plasma samples, and analysed in six replicates.

Data analysis

Data are reported as mean \pm standard deviation (SD) for replicates. Coefficient of variation (CV)

values and accuracy were calculated using Microsoft Excel 2003.

RESULTS

Specificity

Plasma samples of different lots were free from interfering substances at the retention time of doxorubicin, and there was no effect of haemolysis plasma on peak detection.

Typical chromatograms corresponding to blank plasma, doxorubicin hydrochloride, and daunorubicin hydrochloride in solution (without plasma) are given in Figures 2 and 3. No endogenous interference peaks were visible in blank plasma in the retention times of doxorubicin and daunorubicin, and throughout the chromatogram, thereby confirming the specificity of the analytical method as shown in Figure 4. Doxorubicin hydrochloride and the IS were well separated with retention times of 7.8 and 8.6 min, respectively.

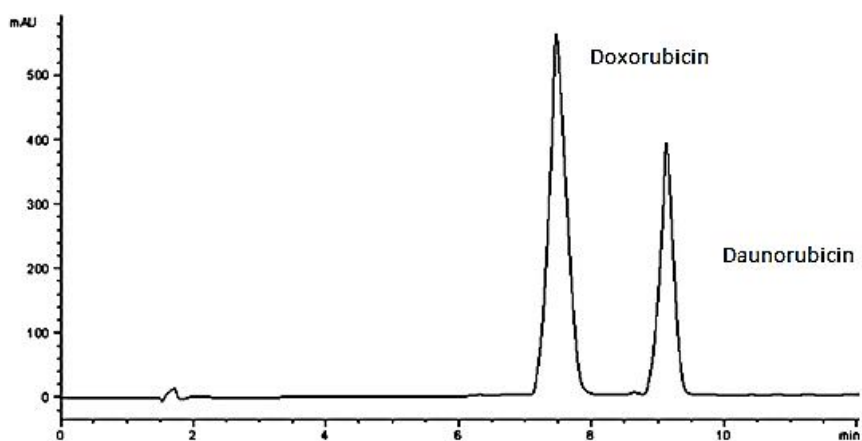


Figure 2: HPLC chromatograms of doxorubicin and daunorubicin (internal standard) in solution

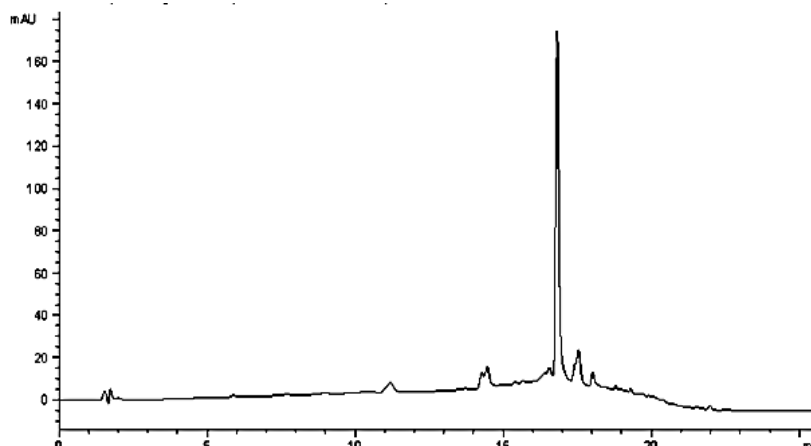


Figure 3: HPLC chromatogram of blank plasma

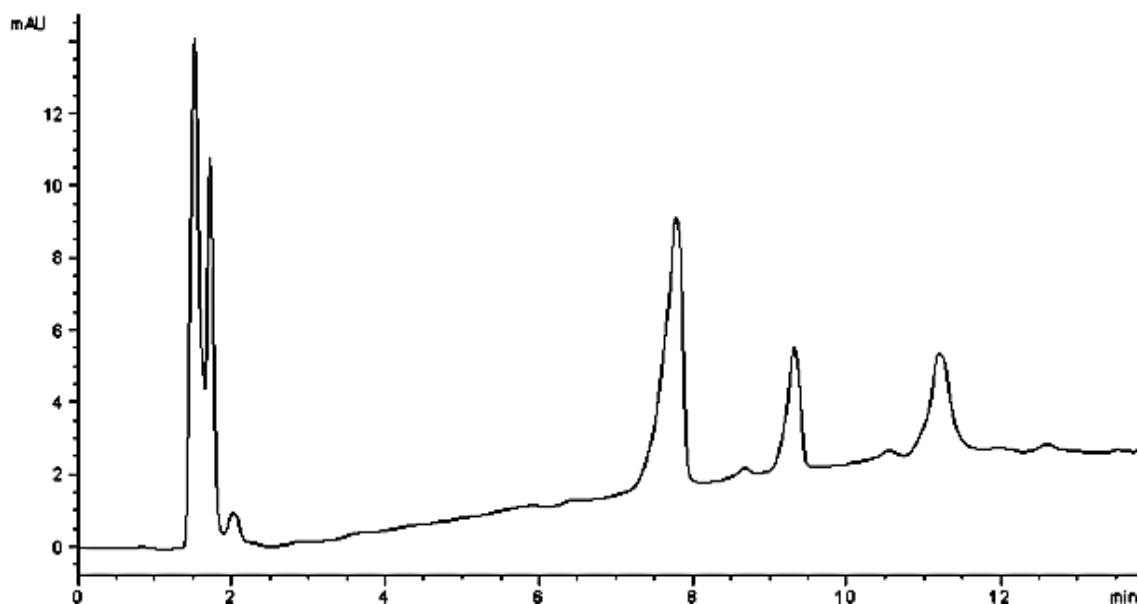


Figure 4: HPLC chromatograms of doxorubicin and daunorubicin (internal standard) in plasma

Table 1: Results of precision study (intra- and inter-day)

Nominal conc. ($\mu\text{g/ml}$)	Mean concentration of doxorubicin hydrochloride ($\mu\text{g/ml}$)					
	Intra-day			Inter-day		
	Mean \pm SD	%CV	% Accuracy	Mean \pm SD	%CV	% Accuracy
1.0 (LLOQ)	0.89 \pm 0.11	12.62	88.71	0.90 \pm 0.14	15.74	90.34
3 (LQC)	3.03 \pm 0.27	8.79	100.83	2.99 \pm 0.31	10.34	99.72
25(MQC)	24.10 \pm 1.13	4.68	96.40	24.43 \pm 1.75	7.18	97.70
37.5 (HQC)	37.70 \pm 0.85	2.24	100.53	36.96 \pm 1.89	5.10	98.56

Lower limit of quantification (LLOQ), LQC-Low quality control, MQC- middle quality control HQC- high quality control

Linearity, and limits of detection and quantification

The ratio of peak area of doxorubicin hydrochloride and IS (daunorubicin hydrochloride) multiplied by 1000 was used for the quantification of drug in plasma samples. The calibration curve was linear in the concentration range of 1.0 – 50 $\mu\text{g/ml}$.

The most variable regression equation of the calibration curve was $y = 2.78532x + 4.3692$ ($r^2 = 0.9972$) and the acceptable minimum criterion of the correlation coefficient (r) must not be < 0.98 ; hence, the correlation coefficient showed good linearity. The limit of detection (LOD) and quantitation (LOQ) of doxorubicin in plasma samples were determined to be 0.5 and 1 $\mu\text{g/ml}$, respectively. A standard curve of doxorubicin in plasma is shown in Figure 5.

Precision and accuracy

The coefficient of variation (%CV) of intra-day precision ranged from 2.24 to 12.62 %, whereas that of inter-day precision was from 5.10 to 15.74 %. Intra-day accuracy ranged between 88.71 and 100.83 %, while that of inter-day accuracy was between 90.34 and 99.72 %, which is within the acceptable criteria of coefficient of variation of ≤ 15 % for mean values. Coefficient of variation (%CV) for both intra- and inter-day precision was < 15 % for the lowest and 20 % for the highest concentration of US FDA criteria for biological fluids [14].

Extraction recovery

Protein precipitation was employed for the extraction of drug and IS from rat plasma. The precipitating method using acetonitrile as the precipitating solvent was able to give 86.86 to 91.76 % recovery and moreover, there were no

Table 2: Extraction recovery of doxorubicin hydrochloride from rat plasma (n = 6)

Analyte	Concentration added ($\mu\text{g/ml}$)	Mean recovery \pm SD (%)	CV%
Doxorubicin hydrochloride	3	86.86 \pm 4.76	54.48
	25	89.56 \pm 3.23	3.61
	37.5	91.76 \pm 1.99	2.17
Daunorubicin hydrochloride	10	76.86 \pm 5.57	7.24

Table 3: Stability data for doxorubicin hydrochloride

Nominal conc. ($\mu\text{g/ml}$)	Mean found concentration ($\mu\text{g/ml}$)	CV(%)	Bias (%)
Stock solution stability at 9 h (n=3)			
1.0	0.82 \pm 0.07	8.537	-0.180
3.0	2.80 \pm 0.04	1.485	-0.066
37.5	37.7 \pm 0.31	0.834	0.005
Bench top stability for 24 h (n=3) in plasma			
1.0	0.80 \pm 0.05	7.264	-0.170
3.0	2.75 \pm 0.09	3.272	-0.110
37.5	36.66 \pm 0.67	1.846	-0.018
Three freeze and thaw cycles (n=3)			
1.0	0.84 \pm 0.03	4.472	-0.153
3.0	2.82 \pm 0.04	1.636	-0.059
37.5	37.73 \pm 0.52	1.378	0.006
Long term 30-days stability at -70 ± 5 °C (n=3)			
1.0	0.86 \pm 0.03	3.566	-0.143
3.0	30.1 \pm 0.09	2.896	0.003
37.5	37.44 \pm 0.11	0.294	-0.002

Table 4: Haemolysis data for doxorubicin hydrochloride

Sample	Haemolysis	Concentration found (ng/mL , mean \pm SD)	CV (%)	% Accuracy
LLQC	1 $\mu\text{g/ml}$, 0.0% haemolysis	0.89 \pm 0.10	11.56	89.75
	1 $\mu\text{g/ml}$, 0.5% haemolysis	0.85 \pm 0.12	15.13	85.77
LQC	3 $\mu\text{g/ml}$, 0.0% haemolysis	2.98 \pm 0.26	8.91	99.41
	3 $\mu\text{g/ml}$, 0.5% haemolysis	2.88 \pm 0.27	9.61	96.00
MQC	25 $\mu\text{g/ml}$, 0.0% haemolysis	24.43 \pm 1.0	4.25	97.73
	25 $\mu\text{g/ml}$, 0.5% haemolysis	24.07 \pm 0.89	3.69	96.29
HQC	37.5 $\mu\text{g/ml}$, 0.0% haemolysis	37.57 \pm 0.67	1.78	100.19
	37.5 $\mu\text{g/ml}$, 0.5% haemolysis	36.90 \pm 0.62	1.69	98.41

interferences at the retention times of doxorubicin hydrochloride and IS.

Stability

The stability tests of the QC samples were designed to cover anticipated conditions that clinical samples may experience. Twelve-hour room temperature storage and freeze-thaw cycles for low, mid and high quality controls samples indicate that doxorubicin hydrochloride was stable in rat plasma under experimental condition. Stability test samples were stable for

at least 30 days when frozen at approximately -70 ± 5 °C.

The stability results from the current method demonstrated that spiked plasma samples were stable without significant degradability of drugs after three freeze-thaw cycles and long-term storage.

Twenty-four hour bench top stability and freeze-thaw cycles for LQC, MQC and HQC samples indicate that doxorubicin hydrochloride was stable in rat plasma under experimental conditions.

Dilution integrity

The precision for dilution integrity of doxorubicin hydrochloride were 2.3 % at 25 % dilution and 6.4 % at 50 % dilution. The accuracy for dilution integrity of doxorubicin was 99.3 % for 25 % dilution and 98.6 % for 50 % dilution.

Haemolysis effect

Three sets of spiked LLQC, LQC, MQC and HQC samples spiked in plasma separated from haemolysed blood were processed and analysed along with the calibration curve standards prepared in plain plasma. The results demonstrate absence of haemolysis effect. The precision ranged from 1.69 to 15.13 %. The accuracy ranged from 85.77 to 96.00 %.

REFERENCES

1. Stewart JT, Warren FW, King DT. Stability of Ondansetron Hydrochloride, Doxorubicin Hydrochloride, and Dacarbazine or Vincristine Sulfate in Elastomeric Portable Infusion Devices and Polyvinyl Chloride Bags. *Am J Health Syst Pharm* 1997; 54: 915–920.
2. Lown JW. Anthracycline and anthraquinone anticancer agents: current status and recent developments. *Pharmacol ther* 1993; 60(2): 185-214.
3. Licata S, Saponiero A, Mordente A. Doxorubicin metabolism and toxicity in human myocardium: role of cytoplasmic deglycosidation and carbonyl reduction. *Chem Res Toxicol* 2000; 13(5): 414-420.
4. Rahmani R, Gil P, Martin M, Durand A, Barbet J, Cano JP. Quantitation of Adriamycin doxorubicin in plasma and urine: comparative study of radio-immunoassay and HPLC methods. *J Pharm Biomed Anal* 1983; 1: 301–309.
5. Hu JB, Li QL. Voltammetric behaviour of adrimaycin and its determination at nickel ion-implanted electrode. *Anal Sci* 1999; 15: 1215–1218.
6. Chaney EN, Baldwin PR. Voltammetric determination of doxorubicin in urine by adsorptive pre-concentration and flow injection analysis *Anal Chim Acta* 1985; 10: 105–112.
7. Shinozawa S, Oda T. Determination of adriamycin (doxorubicin) and related fluorescent compounds in rat lymph and gall by HPLC. *J Chromatogr* 1981; 212: 323–330.
8. Alvarez Cedron L, Sayalero ML, Lanao JM. HPLC validated assay of doxorubicin in rat plasma and tissues. *J Chromatogr B: Biomed Appl* 1999; 721: 271–278.
9. Kummerle A, Krueger T, Dusmet M, Vallet C, Pan Y, Ris HB, Decosterd LA. A validated assay for measuring doxorubicin in biological fluids and tissues in an isolated lung perfusion model: matrix effect and heparin interference strongly influence doxorubicin measurements. *J Pharm Biomed Anal* 2003; 33: 475–494.
10. Zhou QY, Chowbay B. Determination of doxorubicin and its metabolites in rat serum and bile by LC: application to preclinical pharmacokinetic studies. *J Pharm Biomed Anal* 2002; 30: 1063–1074.
11. De Bruijn P, Verweiz J, Loos WJ, Kolker HJ, Planting AST, Nooter K, Stoter G, Sparreboom A. Determination of doxorubicin and doxorubicinol in plasma of cancer patients by high performance liquid chromatography. *Anal Biochem* 1999; 266: 216–221.
12. Shao Z, Zhang H, Zhang J. Determination of Adriamycin doxorubicin in serum by spectrofluorimetry and pharmacokinetic study on adriamycin in rabbits. *Zhongguo Yiyuan Yaoxue Zazhi* 1991; 11: 200–201.
13. Reddy LH, Murthy RSR. Fluorimetric estimation of doxorubicin hydrochloride in plasma, whole blood and tissues of rat. *ARS Pharm* 2004; 45: 157–174.
14. Food and drug Administration. Guidance for Industry: Bioanalytical method validation. Rockville, MD: US Department of Health and Human Services, Food and Drug Administration. Center for Drug Evaluation and Research, 2001.