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Nonclinical Study of the Active Components of Doxorubicin Hydrochloride Liposome Injection *in Vivo*

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Abstract

Objectives: A non-clinical study was performed to establish a LC-MS/MS method to determine the *in vivo* active components of doxorubicin hydrochloride liposome injection in the plasma of Sprague-Dawley rats. **Methods:** Ten male SD rats were administered tail vein with a single dose of 10 mg/kg, and the concentrations of doxorubicin hydrochloride in plasma, heart, liver, spleen, lung, and kidney were determined by liquid chromatography-tandem mass spectrometry, and the pharmacokinetic parameters were calculated. **Results:** The final concentration of doxorubicin hydrochloride ranged from 500 ng/mL to 250,000 ng/mL, and the lower limit of quantification was 500 ng/mL; the main pharmacokinetic parameters: $T_{1/2}$ was (19.282 ± 10.305) h, C_{max} was (118514.828 ± 26155.134) ng/mL, AUC_{0-24} and $AUC_{0-\infty}$ were (1216659.205 ± 192706.268) ng/mL·h and (2082244.523 ± 860139.487) ng/mL·h, MRT_{0-24} and $MRT_{0-\infty}$ were (9.237 ± 0.423) h and (26.52 ± 14.015) h, respectively, and clearance (CL) was (0.005 ± 0.002) mL/h·ng. **Conclusions:** The method is simple, rapid, and sensitive, which can be used for the determination of doxorubicin hydrochloride concentration in the plasma of SD rats and pharmacokinetic non-clinical studies.

Keywords

Doxorubicin Hydrochloride Liposomes, Pharmacokinetics, LC-MS/MS

1. Introduction

Doxorubicin hydrochloride (DOX-HCl) is a widely used first-line chemothera-

peutic agent in clinical antitumor therapy. Doxorubicin hydrochloride has a molecular formula of $C_{27}H_{30}ClNO_{11}$ and a molecular weight of 579.98 g/mL. Doxorubicin (DOX) was isolated in 1969 from a light-gray strain of Streptococcus pineus (Str. peucetius var. caesius). Doxorubicin is an anthracycline antibiotic isolated from Str. peucetius var. caesius in 1969, which has strong anticancer activity and a high chemotherapeutic index and can be used alone or in combination with other anticancer drugs to effectively treat a variety of malignant tumors in clinical practice. Liposome is a kind of artificially prepared phospholipid bilayer very similar to the structure of biological membranes, which was first discovered by British scientists Bangham and Standish [1]. Since the 1970s, some scholars have proposed the use of liposomes as drug carriers [2]. In recent years, liposomes, as a new type of targeted drug carriers, can increase drug efficacy and reduce toxic side effects and are highly valued in tumor drug development [3]. A large number of studies have shown that liposome technology is particularly effective in overcoming doxorubicin hydrochloride cardiotoxicity, doxorubicin hydrochloride liposomes have therefore rapidly become a hot spot for the development of major pharmaceutical companies, and several drugs have been listed on the market and entered into the clinical application, of which doxorubicin liposomes are the most numerous. The clinical application of doxorubicin liposomes has various advantages, including increasing the targeting of tissues and organs [4], reducing adverse reactions such as cardiotoxicity [5], improving the therapeutic index, increasing the solubility of the drug, preserving the stability of the drug *in vivo* or improving the patient's compliance [6] and having the ability to change the pharmacokinetics [7] and so on, so that it has better therapeutic efficacy and tolerance than the parent drug. Doxorubicin hydrochloride liposome injection was first approved for marketing in November 1995, and subsequently received approval for importation into China in 2002. However, differences in prescription and manufacturing processes between generic and reference formulations can potentially affect the pharmacokinetic behavior and consequently the efficacy and safety of the drug. Therefore, it is important to conduct pharmacokinetic studies comparing generic drugs with their reference formulations prior to clinical trials.

Based on the advantages of liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods, such as high sensitivity, high selectivity, wide application range, faster analysis speed and structural information [8]. In this regard, a method using liquid chromatography-tandem mass spectrometry has been established for the detection of doxorubicin hydrochloride liposomes in plasma. Non-clinical pharmacokinetic studies have been conducted using this method to better understand the pharmacokinetics of doxorubicin hydrochloride liposome injection.

2. Materials and Methods

2.1. Materials

Drugs: Doxorubicin hydrochloride liposome injection (Changzhou Jinyuan

Pharmaceutical Manufacturing Co., Ltd.), Specification: 20 mg/10mL, Batch No. 2206151, doxorubicin hydrochloride, Shanghai Aladdin Biochemical Technology Co.

CAS No.: 23541-50-6.

Reagents: acetonitrile: Merck, Germany, CAS No.: 75-05-8, ≥99.9% (GC); methanol: Merck, Germany, CAS No.: 67-56-1, ≥99.9% (GC); acetic acid: Shanghai Lingfeng Chemical Reagent Co.

Instruments: QTRAP™ 4500 mass spectrometer with ion spray source and Analyst 1.7.1 data processing system, Sciex, USA; XR LC-30AD Prominence™ ultra-high performance liquid chromatography system, Shimadzu, Japan; MS 205 DU 100,000 electronic balance, Mettler, Switzerland Toledo; ML503/02 electronic balance, Mettler Toledo, Switzerland; BDA-1002 ultrasonic cleaner Ltd.; Eppendorf pipette gun (2.5 μL, 20 μL, 100 μL, 200 μL, 1000 μL), Eppendorf, Germany; MS-3digital digital vortex mixer, IKA, Germany; FRESCO 21 microcentrifuge, Thermo Scientific; N-EVAP 111 nitrogen blowing instrument, Organomation, USA.

Animals: The animal experiments were carried out in accordance with the approved agreement by the Institutional Animal Ethics Committee of China Pharmaceutical University. The laboratory animal procedures were conducted in accordance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals, as specified by the Ministry of Science and Technology of China. The study utilized male Sprague-Dawley (SD) rats obtained from Yangzhou University, with a specific pathogen free (SPF) grade laboratory environment. The rats weighed between 180 - 220 g and were kept in controlled settings, including a 12-hour light/dark cycle, constant temperature of 24˚C - 26˚C, and humidity levels of 40% - 50%.

2.2. Analysis Conditions

Chromatographic conditions

Chromatographic column: ACQUITY UPLC®BEH C18 column (1.7 μm, 2.1 × 50 mm column).

Mobile phase: 0.1% acetic acid aqueous solution (A) - acetonitrile (B), gradient elution.

Flow rate: 0.3 mL/min.

Column temperature: 25˚C.

Injection volume: 10 μL.

Autosampler temperature: 10˚C.

The gradient elution procedure was as follows:

Time (min)	A %	B %
0.01	95	5
2.00	60	40
7.00	25	75

Continued

8.00	5	95
10.00	95	5

Mass Spectrometry Conditions

Scanning mode: multi-reaction ion monitoring (MRM); ion source: ESI source (AB Sciex, USA); scanning mode: positive ion mode; ion spray voltage: 5500 V; ion source temperature: 400˚C; flow rate of source gas 1 and source gas 2 (GS_2, N_2) 35 psi, respectively; roll-off gas (N_2) pressure: 35 psi; data acquisition software: Analyst (AB Sciex, USA). The ion reactions used for quantitative analysis were m/z 544.2 → 397.3 (doxorubicin hydrochloride) and m/z 528.1 → 321.0 (daunorubicin hydrochloride, internal standard), respectively. The MRM parameters of the test article and the internal standard are shown in **Table 1**. The chemical structures of the test article and the two internal standards are shown in **Figure 1**.

Selection of internal standard substances

The internal standard should have similar chemical structure and physicochemical properties as the analyte, not interfere with the analyte, and have a good mass spectrometric response. Doxorubicin hydrochloride is an anthracycline cytotoxic antitumor drug with structural similarity to the broad-spectrum antitumor drug daunorubicin hydrochloride [9]. The chances of combining daunorubicin hydrochloride and doxorubicin hydrochloride at the same time in

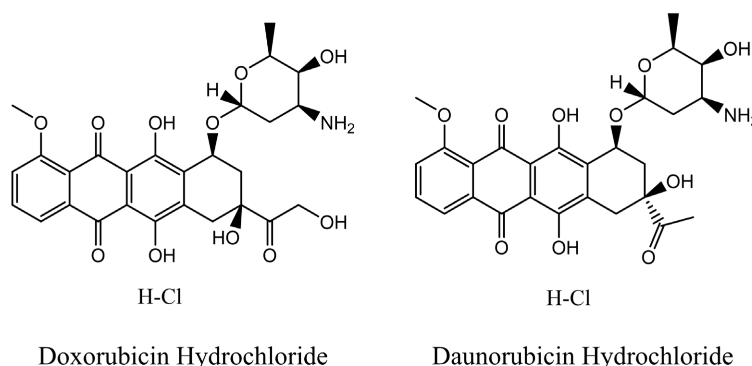

Figure 1. Chemical structures of component and internal standard.

Table 1. MRM parameters of declustering potential (DP), entrance potential (EP), collision energy (CE), and cell exit potential (CXP) for compounds, daunorubicin hydrochloride.

compounds	parameters					
	Q1 Mass (Da)	Q3 Mass (Da)	DP (V)	EP (V)	CE (V)	CXP (V)
Doxorubicin hydrochloride	544.2	397.3	80	9	16	6
Daunorubicin hydrochloride	528.1	321.0	71	12	36	11

clinical treatment are small, so the selection of daunorubicin hydrochloride as an internal standard has important theoretical and practical application value.

2.3. Preparation of Solutions

The accurate measurement of doxorubicin hydrochloride was conducted to determine the proper amount, which was subsequently made as a stock solution using methanol. The resulting concentration of the stock solution was determined to be 1.02 mg/mL. The internal standard, daunorubicin hydrochloride (IS), was prepared in methanol as a reserve solution with a concentration of 1.09 mg/mL. Each stock solution was mixed in the appropriate volume, and then the standard working solution was diluted stepwise with methanol. All solutions were stored at 4˚C.

2.4. Dosing Regimen and Sample Collection

A group of ten male Sprague-Dawley rats, with weights ranging from 180 - 220 g, were subjected to a period of acclimatization. This phase of acclimatization lasted for a total of seven days and was carried out in conditions of controlled environmental conditions throughout the entire procedure. These conditions included a 12-hour photoperiod, a temperature maintained at 25˚C ± 5˚C, and a humidity level of 50% ± 5%. A single dose of 10 mg/kg was delivered via the tail vein.

Plasma sample collection

Prior to administration, a group of six rats underwent a fasting period of 12 hours. Following this, a volume of 0.3 mL of blood was collected from the orbital region of each rat at specific time intervals (5 min, 10 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, and 24 h) after tail vein administration. The blood samples were collected in centrifuge tubes containing heparin, thoroughly mixed, and then subjected to centrifugation at a speed of 3000 rpm for a duration of 10 min. The resulting supernatant was carefully removed to obtain plasma samples, which were subsequently stored at a temperature of −20˚C.

Tissue distribution

Following a 24-hour period of blood sampling, tissues from the liver, heart, spleen, lung, and kidney of rats were collected and weighed. A portion of 0.25 g of each tissue was obtained, to which 0.5 mL of saline solution was added. The amount of saline added was lower than the predetermined ratio of 0.5 g/mL. The tissues were then homogenized, and a solution derived from the tissues was extracted and subsequently stored at a temperature of −20˚C.

Furthermore, a total of four rats were selected following a 12-h fasting period. Blood samples were collected from the orbital region of each rat to the maximum extent feasible. After that, these blood samples were placed in centrifuge tubes that contained heparin, given a thorough mixing, and then centrifuged at a speed of 3000 rpm for a duration of 10 min. The resulting supernatant was carefully extracted and stored at a temperature of −20˚C to serve as blank plasma for future use.

2.5. Plasma Sample Pre-Treatment Method

Using the methanol precipitation method, proteins were extracted from plasma samples. 50 μL of plasma sample was mixed with 10 μL of mixed internal standard stock solution and 400 μL of methanol, shaken well, vortexed, and centrifuged at 14,000 rpm for 10 min. The supernatant was blown dry with nitrogen at room temperature. Add 100 μL of methanol, re-dissolve, vortex, and centrifuge at 14,000 rpm for 10 min. 80 μL of supernatant was removed. 10 μL of sample was injected into Q-Q-Q for analysis.

2.6. Data Processing

The collected plasma samples were pretreated according to "2.5", and analysed and determined by chromatography and mass spectrometry according to "2.2". Quality control (QC) samples of low, medium, and high concentrations were measured simultaneously, and the number of QC samples was not less than 5% of the total number of samples in the batch. The QC samples were utilized to assess the trade-offs associated with the batch data. The DAS 3.2.8 software was used to calculate the main pharmacokinetic parameters, including area under the concentration-time curve (AUC_{0-t}, $AUC_{0-\infty}$), maximum concentration (C_{max}), time to reach maximum concentration (T_{max}), elimination half time ($T_{1/2}$), mean residence time (MRT_{0-t}, and $MRT_{0-\infty}$). Among them, T_{max} and C_{max} were measured; AUC was calculated by the trapezoidal method. The pharmacokinetic parameters obtained were analyzed using an independent sample t-test to assess if there were any notable differences between the pharmacokinetic parameters of doxorubicin hydrochloride and doxorubicin hydrochloride liposomes after administering the injection to both blank rats and single tail vein rats. All data were expressed as mean ± SD (n = 6).

3. Results

3.1. Methodological Evaluation

3.1.1. Selectivity

Chromatograms of six control rat serum samples were collected and analyzed from various sources without the addition of methane and internal standard solutions according to the plasma sample pretreatment method under "2.5", and the chromatograms of blank blood samples were obtained (A); standard solvents and internal standard solutions were added to the blank blood pressure samples at a certain concentration; and the chromatograms were analyzed according to the blood sample pretreatment method under "2.5". The plasma sample was analyzed, and the chromatogram of the simulated plasma sample was obtained (B); the plasma sample of mouse 2 was injected with doxorubicin hydrochloride liposome injection for 2 h in one tail, and the plasma sample was analyzed according to the method of pretreatment of the plasma sample under "2.5". The actual plasma sample chromatograms were obtained according to the method of pretreatment of plasma samples under "2.5" (C). The results are shown in **Fig-**

ure 2. The retention times of doxorubicin hydrochloride and IS were about 2.48 and 2.70 min, respectively, without endogenous interference.

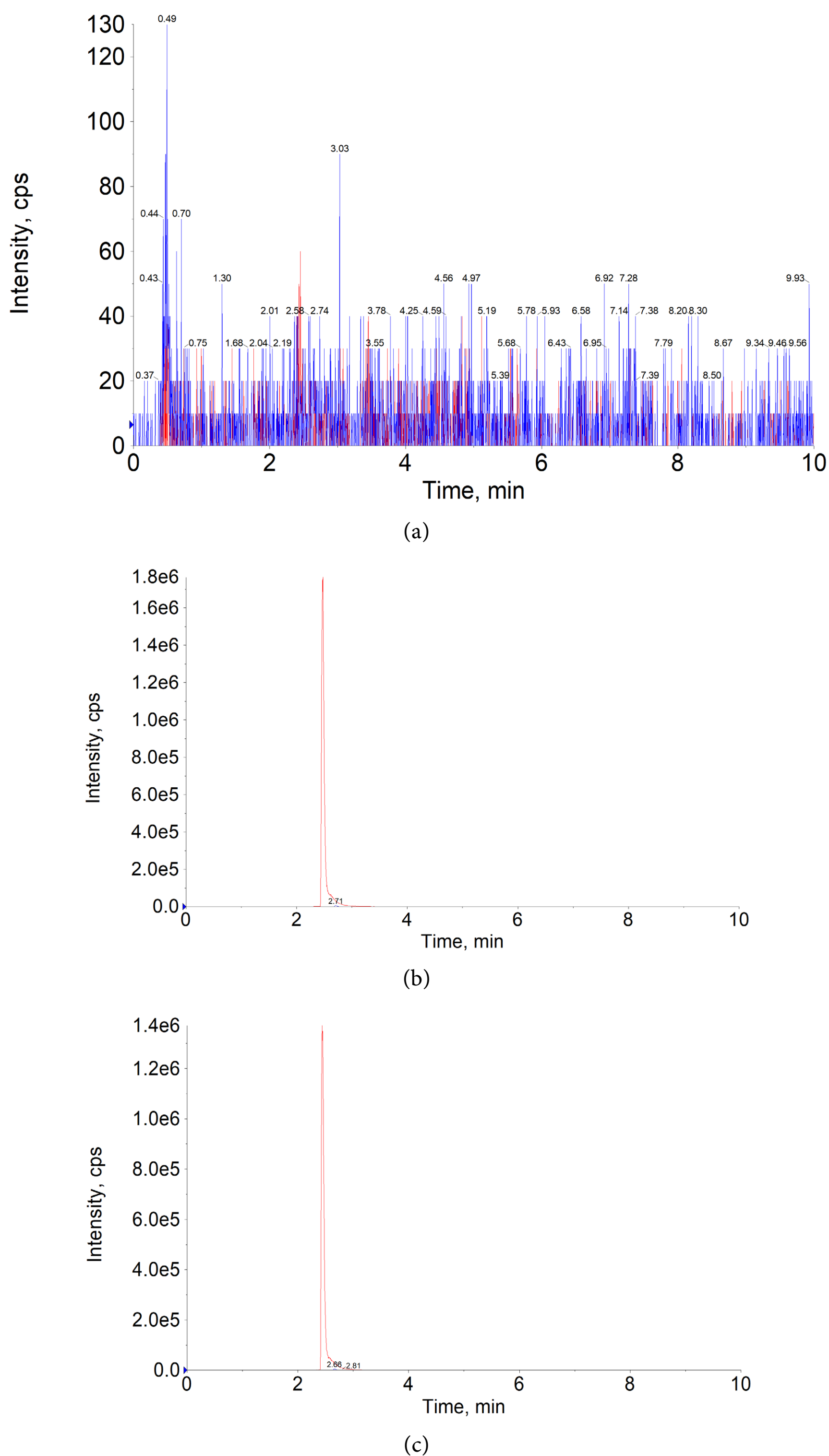

Figure 2. The chromatogram of (a) blank plasma, (b) blank plasma spiked into analyte and IS and (c) Plasma samples from rats after single tail injection of doxorubicin hydrochloride liposome injection.

3.1.2. Linearity and Sensitivity

A total of six plasma samples from control rats were combined, and a volume of 50 μL was extracted. Subsequently, 10 μL of a standard series solution containing doxorubicin hydrochloride was introduced, resulting in a final concentration range of doxorubicin hydrochloride spanning from 500 ng/mL to 250,000 ng/mL. The chromatograms were obtained following the plasma sample pretreatment technique outlined in section 2.5. The calibration curve was determined by evaluating the ratio between the peak area of the test substance and the internal standard. This ratio was used as the vertical coordinate (y) for constructing the standard curve, while the concentration of the test substance served as the horizontal coordinate (x). Table 2 displays the regression equation, linear range, and lower limit of quantification for the analytes. The findings indicated that the correlation coefficient of the analytes within the linear range exceeded 0.99. The quantitative lower limit of doxorubicin hydrochloride was found to be less than 500 ng/mL. The findings of the study demonstrated that the analytes exhibited a high degree of linearity within the tested concentration range using the established method.

3.1.3. Precision and Accuracy

The preparation of the samples followed the standard curve method and lower limit of quantification as outlined in Section 3.1.2. Three concentrations were used: low, medium, and high. A total of six samples for each concentration were subjected to analysis over a period of three consecutive days. The calibration curve, which was established on the same day, was assessed to ascertain the precision and accuracy within the same day (intra-day) as well as across different days (inter-day). Accuracy refers to the comparison between the true concentration of a QC sample and the standard concentration. The precision of the measurements fell within the interval of 85% to 115%, while the relative standard deviation (RSD) was within the range of ±15.0%. Table 3 displays the precision and accuracy outcomes for the intra-day and inter-day QC samples across the

Table 2. The regression equation, linear ranger, and lower limit of quantification (LLOQ).

compounds	regression equation	R	linearity range	LLOQ (ng/mL)
Doxorubicin Hydrochloride	$y = 0.0037x - 2.3718$	0.9965	500.0 - 250000.0	2

Table 3. The precision and accuracy of compound in rat plasma (n = 6).

compound	concentration (ng/mL)	intra-day		inter-day	
		accuracy (%)	*RSD* (%)	accuracy (%)	*RSD* (%)
Doxorubicin hydrochloride	500	103.1	4.6	99.8	7.6
	10,000	88.9	5.8	87.5	4.7
	100,000	94.2	3.4	92.6	3.2

three concentration levels. The RSD for intra-day and inter-day precision were found to be 3.4% - 5.6% and 3.2% - 7.6%, respectively. The observed accuracy values spanned a range of 87.5% to 103.1%. The findings suggest that the methodology exhibits a high level of dependability and can be effectively employed for the quantification of constituents in rat plasma.

3.1.4. Recovery Rate and Matrix Effects

The QC samples were prepared according to the method of standard curve and lower limit of quantification under "3.1.2." for three concentrations: low, medium, and high, and each concentration was repeated six times, and the peak areas were recorded. The recoveries were determined by calculating the peak area ratio of each compound before and after extraction. Matrix effects were determined by comparing the peak area ratios before and after the addition of blank plasma to the QC samples. The accuracy of the recoveries and matrix effects should be in the range of 85% - 115% and the RSD should be less than 15.0%. The recoveries and matrix effects of the analytes to be measured are shown in **Table 4**. The recoveries were 102.9% - 114.9% with an RSD of 5.0% - 8.6%, and the matrix effects were 86.5% - 95.9% with an RSD of 2.6% - 6.4%.

3.1.5. Stability

Prepare 50 μL of rat control plasma as the standard curve and the lower limit of quantification under "3.1.2." to form QC samples at three concentrations: low, medium, and high. Repeat each concentration six times and record the peak area. Stability includes 24-hour room temperature stability, three freeze-thaw cycles (freezing at −20˚C and thawing at room temperature), and long-term stability (samples stored at −80˚C for two weeks). The precision of stability should be between 85% and 115%, and the RSD should be less than 15.0%. The results are shown in **Table 5**. The composition was stable at three quality control levels, indicating that the method is stable.

Table 4. Recoveries and matrix effect of components in rat plasma (n = 6).

compound	concentration (ng/mL)	recovery		matrix effect	
		accuracy (%)	*RSD* (%)	accuracy (%)	*RSD* (%)
Doxorubicin hydrochloride	500	105.0	8.6	87.2	2.6
	10,000	102.9	5.9	95.9	6.4
	100,000	114.9	5.0	86.5	3.3

Table 5. The stability of components in rat plasma (n = 6).

compound	concentration (ng/mL)	autosampler for 24 h		freeze-thaw cycle		at −80˚C for 2 w	
		accuracy (%)	*RSD* (%)	accuracy (%)	*RSD* (%)	accuracy (%)	*RSD* (%)
Doxorubicin hydrochloride	500	98.9	4.9	102.9	3.3	99.6	8.4
	10,000	87.9	5.0	104.5	6.5	90.8	5.0
	100,000	92.3	2.3	105.4	3.3	104.5	7.5

3.2. Pharmacokinetic Study

3.2.1. Blood Concentration-Time Curve

The plasma doxorubicin hydrochloride concentration increased rapidly and peaked at 15 min with a peak concentration of 110584.471 ng/mL, which showed a long residence time in the blood circulation and showed the characteristics of long circulating liposomes. The results are shown in Figure 3.

3.2.2. Tissue Distribution

The results of tissue distribution showed that there was a significant difference in doxorubicin content in all tissues of rats after administration of the drug. The results are shown in Figure 4.

3.3. Pharmacokinetic Parameters

Doxorubicin hydrochloride blood concentration-time curves were fitted and the pharmacokinetic parameters are shown in Table 6.

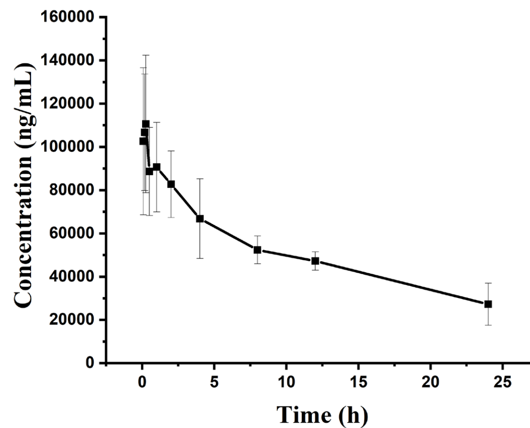

Figure 3. Plasma concentration-time curve of adriamycin liposome injected by single tail vein in rats.

Table 6. Main pharmacokinetic parameters of single tail vein injection of doxorubicin hydrochloride (10 mg·kg^{-1}).

parameter	value
AUC_{0-24} (ng/mL·h)	1216659.205 ± 192706.268
$AUC_{0-\infty}$ (ng/mL·h)	2082244.523 ± 860139.487
MRT_{0-24} (h)	9.237 ± 0.423
$MRT_{0-\infty}$ (h)	26.52 ± 14.015
CL	0.005 ± 0.002
Vz	0.13 ± 0.017
T_{max} (h)	0.333 ± 0.333
$T_{1/2}$ (h)	19.282 ± 10.305
C_{max} (ng/mL)	118514.828 ± 26155.134

n = 6, mean ± SD.

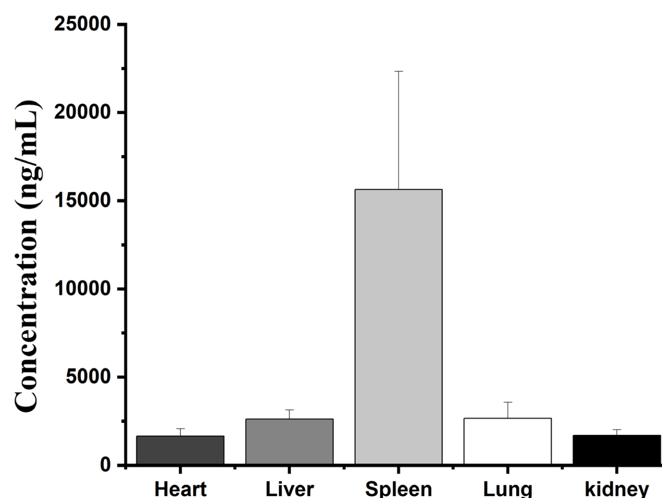

Figure 4. Histogram of tissue distribution concentration of doxorubicin hydrochloride liposomes in rats after 24 h single tail vein injection.

4. Discussion

The amount of plasma sample used in this study was only 50 μL, which was a highly sensitive assay without interfering peaks.

The objective of this work was to create a quantification test with enhanced sensitivity and efficiency for measuring the plasma amounts of doxorubicin hydrochloride. A plasma sample size of 50 μL was employed in our study, and the LC-MS/MS analytical methodology was utilized. It is important to note that this approach was validated according to established methodologies. Daunorubicin was selected as an internal standard due to its favorable physicochemical stability and ability to be effectively separated from endogenous chemicals. This choice was made based on its established use as an anticancer medication.

Sample treatment was streamlined by employing the protein precipitation method using methanol. Its simplicity and satisfactory recovery made it an ideal choice for processing the biological samples. Optimization of mass spectrometry conditions, including cone well voltage, ion source temperature, and capillary voltage, was carried out by consulting relevant literature [10] [11] and tailoring the parameters for each ion of interest.

The developed method showcased exceptional performance in terms of analysis time, achieving results within a mere 10 minutes per plasma sample. The 0.1% acetic acid aqueous solution (A) and acetonitrile (B) in the mobile phase kept sample components from breaking down, so measurements were accurate.

Our results indicated that the plasma concentration of doxorubicin hydrochloride reached its peak at 15 minutes, revealing a prolonged half-life of approximately 19 hours. This observation suggests that doxorubicin hydrochloride liposomes exhibit a controlled and sustained release effect *in vivo* [12], resulting in a prolonged high concentration. The specificity of the method was shown by the fact that there were no endogenous impurities in the plasma that could have gotten in the way of doxorubicin hydrochloride detection.

The precision of our method was demonstrated by controlling the RSD of intra-day and inter-day precision for doxorubicin hydrochloride within 8%. Furthermore, the recoveries fell within the pharmacokinetic criteria of 85% to 115%, confirming the method's accordance with the established standards. Therefore, our study fulfils the requirements for pharmacokinetic analysis and is suitable for pharmacokinetic non-clinical studies, facilitating future clinical research.

In conclusion, the LC-MS/MS technology employed in our study offers a swift, uncomplicated, and very responsive approach for precisely quantifying the amounts of doxorubicin hydrochloride in plasma samples obtained from SD rats. This methodology exhibits potential in promoting the progress of clinical research pertaining to the administration of doxorubicin hydrochloride, as it is strategically designed to facilitate pharmacokinetic studies.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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