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Original Article

Simultaneous determination of doxorubicin and its dipeptide prodrug in mice plasma by HPLC with fluorescence detection ☆

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ABSTRACT

A simple and sensitive high performance liquid chromatography with fluorescence detection (HPLC–FD) has been developed for simultaneous quantification of doxorubicin (DOX) and its dipeptide conjugate prodrug (PDOX) in mice plasma. The chromatographic separation was carried out on an Amethyst C_{18}–H column with gradient mobile phase of 0.1% formic acid and 0.1% formic acid in acetonitrile at a flow rate of 1.0 mL/min. The excitation and emission wavelengths were set at 490 and 550 nm, respectively. The method was comprehensively validated. The limits of detection were low up to 5.0 ng/mL for DOX and 25.0 ng/mL for PDOX. And the limits of quantification were low up to 12.5 ng/mL for DOX and 50 ng/mL for PDOX, which were lower than those for most of the current methods. The calibration curves showed good linearity ($R^2 > 0.999$) over the concentration ranges. The extraction recoveries ranged from 84.0% to 88.2% for DOX and from 85.4% to 89.2% for PDOX. Satisfactory intra-day and inter-day precisions were achieved with RSDs less than 9.1%. The results show that the developed HPLC–FD method is accurate, reliable and will be helpful for preclinical pharmacokinetic study of DOX and PDOX.

1. Introduction

Doxorubicin (DOX) is one of the most efficacious drugs used in cancer chemotherapy [1] for the treatment of leukemia and a broad range of solid tumors [2]. However, its applications suffer from serious adverse effects such as heart damage [3], bone marrow toxicity [4], gastrointestinal disorders [5], and stomatitis [6]. Our collaborator, Dr. Yan Li group, developed a prodrug of DOX (PDOX) [7]. As cathepsin B can effectively recognize Phe-Lys-Phe-Lys and covalently link target peptides with DOX, it can improve the specificity of drug and reduce adverse effects [7,8]. The structures of DOX and PDOX are shown in Fig. 1. The peptide linker served as a substrate for the tumor-associated protease, cathepsin B, which is overexpressed in several solid tumors [9,10]. Hence, DOX will be largely released in tumor sites but rarely in normal tissues.

It is necessary to establish a sensitive bioanalytical method for simultaneously monitoring the parent compound and its active metabolites in mice plasma [11,12]. High performance liquid chromatography–mass spectrometry/mass spectrometry (HPLC–MS/MS) [13] and HPLC with ultraviolet detection (HPLC–UV) [14] have been applied to the simultaneous determination of DOX and its some prodrugs. HPLC–MS/MS is highly sensitive and reliable [15], but the instrument used is expensive, which limits its availability [16]. HPLC–UV is easy to access, but its sensitivity is limited. It is well known that fluorescence detector (FD) is sensitive and selective. Since DOX and PDOX possess native fluorescence [17], we aimed to develop a sensitive HPLC–FD method for the determination of DOX and PDOX in biological matrices in the present study. The fluorescence properties of the analytes were investigated and the applications of the proposed method were evaluated. In the experiment, a single-step protein precipitation by mixing methanol with blood sample was adopted to eliminate interference of protein. To improve the accuracy and precision, daunorubicin was selected as the internal standard (IS) [18].

2. Experimental

2.1. Chemicals and reagents

IS, DOX and PDOX were provided by Dr. Yan Li, Wuhan University Zhongnan Hospital. Methanol and acetonitrile (HPLC

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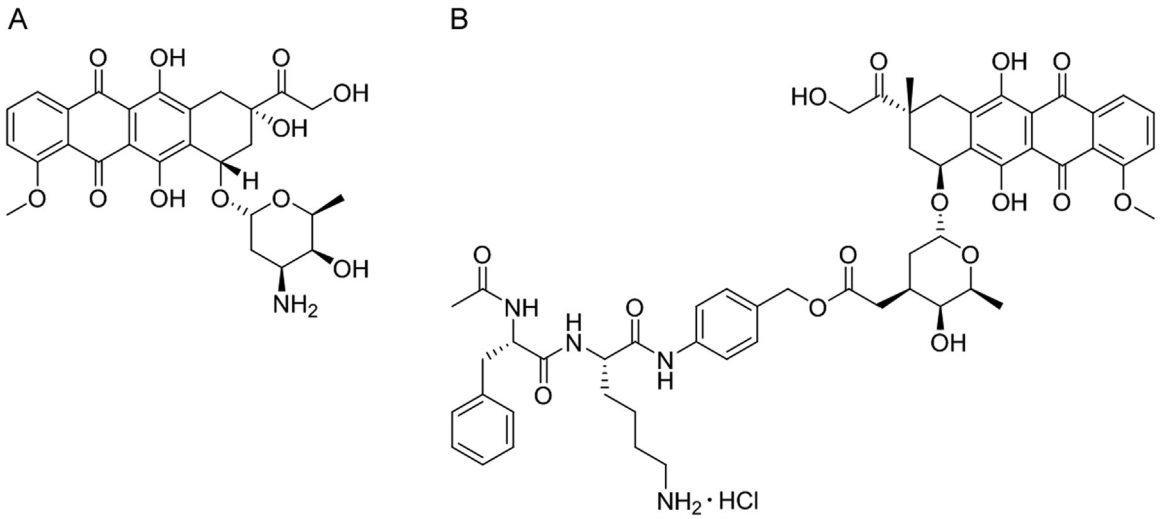

Fig. 1. The structures of (A) DOX and (B) PDOX.

grade) were supplied by Tedia Chemical (Fairfield, OH, USA). Deionized water (18.25 MΩ cm, QYSW-20A, Chongqing Qianyan Water Treatment Equipment Co., LTD) was used throughout the experiment. All other reagents were of analytical grade and were commercially available unless otherwise stated.

2.2. Measurement of fluorescence properties

In order to record fluorescence spectra, Luminescence LS55 spectrometer (PerkinElmer, USA) was used. The fluorescence spectra of DOX and PDOX in methanol were measured. The stock solutions of these two compounds were diluted to 0.5 μg/mL with methanol.

2.3. HPLC–FD conditions

The HPLC separation was conducted on an LC-20AD HPLC system (Shimadzu, Japan) with an RF-10A_{XL} fluorescence detector (Shimadzu, Japan), and a manual injector matched up with a 20 μL sample loop. The separation was conducted on a Sepax Technologies Amethyst C_{18}-H column (4.6 mm × 250 mm, 5 μm). The mobile phase consisted of 0.1% formic acid (aqueous) and 0.1% formic acid in acetonitrile (organic solvent). A gradient elution was used with a 1.0 mL/min flow rate, where initially 5% organic solvents (acetonitrile contained formic acid) was increased linearly to 65% over 20 min, and finally decreased to 5% in 20.1 min, where it was held until the end of the 30 min run. The fluorescence detector was set for excitation at 490 nm and emission at 550 nm for detection of DOX and PDOX. All analyses were performed at 30 °C. The mobile phase was filtered through 0.45 μm nylon filter membranes (Millipore, Milford, USA) and degassed in an ultrasonic bath before use.

2.4. Animals

All animals involved in the experiments were BALB/c mice, weighing around 20 g and aged 6–8 weeks. SGC-7901 cells (5×10^6/0.2 mL per mouse) were injected intraperitoneally into nude mice on day 0. The mice were fed with PDOX (28.8 mg/kg) every seven days. All the mice were executed on day 28, and had the blood collected by removing eyeball. All the mice were kept under standard conditions with normal access to water and food.

2.5. Standard and quality control samples preparation

Appropriate amount of IS, DOX and PDOX were respectively dissolved in methanol to prepare a stock solution of 1.0 mg/mL. Then stock solutions were diluted with methanol to the concentration of 50 μg/mL as working standard solutions. All solutions were kept at 4 °C before use.

Plasma calibration standards and quality controls (QCs) were prepared by adding blank plasma with the appropriate amount of working standard solutions and 20 μL of working IS solution. Calibration standards of DOX were prepared at eight concentrations ranging from 12.5 ng/mL to 2000 ng/mL, and calibration standards of PDOX were prepared at seven concentrations ranging from 50 ng/mL to 4000 ng/mL. Promptly after preparation, all solutions were transferred into amber colored volumetric flasks and kept at 4 °C. Standards calibration samples and QCs were stored at −20 °C until analysis.

2.6. Samples pretreatment

As for plasma samples, 20 μL of working IS solution and 800 μL of methanol were added to 200 μL of plasma samples. After vortex-mix for 2 min, the samples were centrifuged at 10,000 rpm for 15 min. The supernatant was transferred into another tube and evaporated under a stream of nitrogen at 30 °C. The residue was reconstituted with 200 μL of mobile phase and centrifuged again. 20 μL of the supernatant was injected into the HPLC–FD system for analysis.

2.7. Method validation

The method was validated for selectivity, linearity, accuracy and precision and extraction recovery according to the US Food and Drug Administration (US FDA) guidelines for the bioanalytical method.

2.7.1. Specificity

Specificity was assessed by analyzing blank matrices, blank matrices spiked with IS, DOX and PDOX, and plasma.

2.7.2. Linearity of calibration curves and lower limits of quantification (LLOQ)

Standard curves were measured by plotting the peak area ratios (analyte/IS) against the theoretical concentration (x) using a $1/x^2$

weighting. The LLOQ was defined as the lowest drug concentration that could be detected with a relative error and precision (relative standard deviation, RSD) no more than 20%.

2.7.3. Accuracy and precision

QC samples at three concentrations were analyzed within one day for intra-day assessment and five continuous days for inter-day assessment. A certain amount of DOX (PDOX) was spiked into blank plasma to obtain 25 ng/mL, 500 ng/mL, 2000 ng/mL of DOX (PDOX) solutions ($n=5$). The accuracy was determined as the percentage of deviation (relative error, RE%) between the measured and true concentrations. The precision was evaluated according to the RSD. Intra- and inter-day accuracies and precisions for QC concentrations of less than or equal to 15% were regarded to be acceptable.

2.7.4. Recovery

Extraction recovery for DOX and PDOX was determined at the three levels of QC and calculated as the ratio of analyte peak area of extracted QC samples to that of extracted blank matrices spiked with DOX and PDOX standard solution.

3. Results and discussion

3.1. Characteristics of fluorescence spectrometry for DOX and PDOX

In order to achieve highly sensitive and simultaneous determination of DOX and PDOX, the fluorescence spectra of these two compounds in methanol were first investigated to estimate the feasibility of the method. All analytes showed typical excitation and emission spectra, as shown in Fig. 2. The emission curves of DOX and PDOX show a mirror image of their excitation curves. The maximum excitation wavelengths were both at 490 nm and the maximum emission wavelengths were obtained at 550 nm and 600 nm. After some pre-experiments, 550 nm was chosen for detection. All compounds provide sufficient and much better fluorescence to be detected and quantified. As a result, in the study, the native fluorescence of the analytes could be directly detected without the need of any fluorescence derivatization.

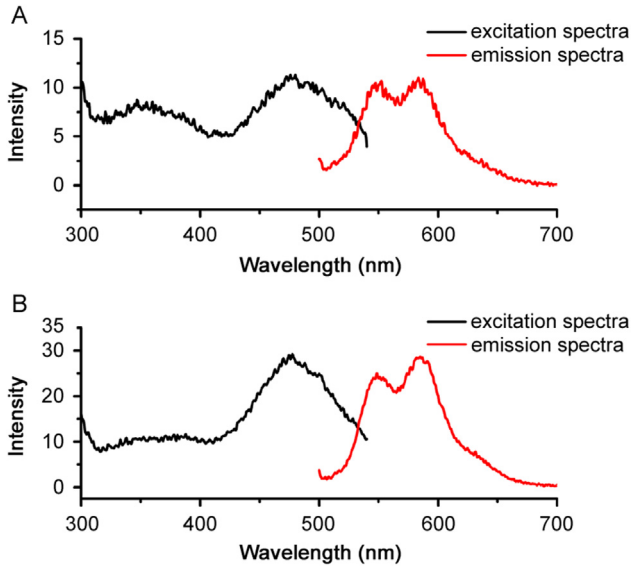

Fig. 2. Fluorescence spectra of (A) PDOX and (B) DOX.

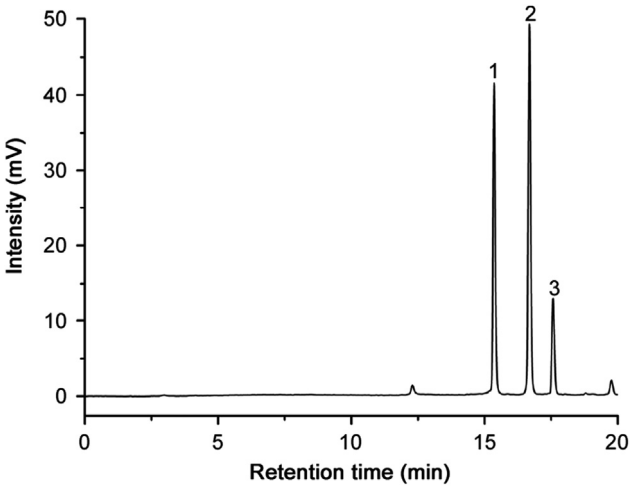

Fig. 3. Typical chromatogram of DOX (1), daunorubicin (2) and PDOX (3).

3.2. Optimization of HPLC conditions

The present study chose different columns, mobile phases and fluorescence detection wavelengths to optimize HPLC system.

Acetonitrile and methanol, as the most common mobile phase solvents, were compared for their performance in separation. It was found that the fluorescence intensities of the analytes were higher when using acetonitrile as the mobile phase. When 0.1% formic acid was added into the water, the peak became much sharper. As a result, acetonitrile and water (containing 0.1% formic acid) were selected for the further study. However, the problem is that the retention time of PDOX is long under isocratic elution. It was solved by appropriate gradient elution, which can be achieved by increasing acetonitrile from 5% to 65% linearly within 20 min. After finishing the run, the gradient was set back to 5% acetonitrile and the system was allowed to equilibrate. The typical chromatogram is shown in Fig. 3.

3.3. Method validation

3.3.1. Linearity and LLOQ

The calibration curves, correlation coefficients and linear ranges of DOX and PDOX are listed in Table 1. The calibration curves were linear over the concentration ranges of 12.5–2000 ng/mL for DOX and 50–4000 ng/mL for PDOX with correlation coefficients higher than 0.999. The LLOQ was 12.5 ng/mL for DOX and 50 ng/mL for PDOX.

3.3.2. Accuracy and precision

Intra- and inter-day precisions and accuracies for DOX and PDOX are exhibited in Table 2. All results for the samples tested ranged from 1.9% to 9.1% within the acceptable criteria of 15%, which suggested that the method was accurate and reproducible for the determination of DOX and PDOX in mice plasma.

Table 1
Standard curves, linear ranges, correlation coefficients, lower limit of detection and lower limit of quantification of DOX and PDOX in plasma samples.

Analyte	Regression equation	Linear range (ng/mL)	R^2	LLOD (ng/mL)	LLOQ (ng/mL)
DOX	$y=1.1565x+0.0015$	12.5–2000	0.9992	5.0	12.5
PDOX	$y=0.3995x+0.0007$	50.0–4000	0.9999	25.0	50.0

Table 2
Accuracy and precision of DOX and PDOX in mice plasma ($n=5$).

Analyte	Spiked conc. (ng/mL)	Precision (RSD%)		Accuracy (RE%)	
		Intra-day	Inter-day	Intra-day	Inter-day
DOX	25	6.2	7.5	5.6	8.5
	500	3.4	4.0	3.1	5.6
	2000	2.8	6.8	2.8	4.7
PDOX	50	7.4	9.1	2.1	7.4
	500	3.6	5.0	1.9	2.3
	2000	2.1	4.5	2.5	4.4

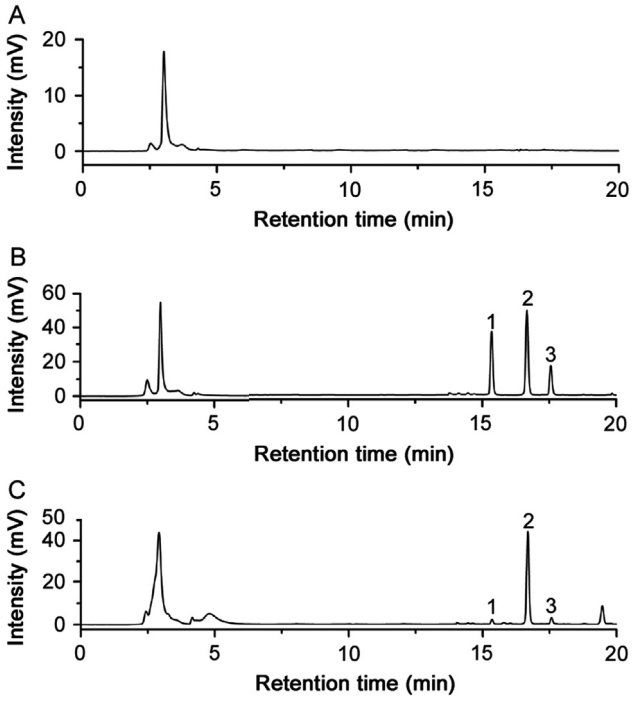

Fig. 4. (A) Chromatograms of the analytes in blank plasma; (B) chromatograms of blank plasma spiked with DOX, IS and PDOX; (C) chromatograms of plasma sample obtained after mice were fed with PDOX. Peak identification: DOX (1), IS (2) and PDOX (3).

3.3.3. *Extraction recovery*

The extraction recoveries ranged from 84.0% to 88.2% for DOX and from 85.4% to 89.2% for PDOX, which demonstrated that recoveries were consistent, precise and reproducible at different concentrations.

3.4. *Application in mice plasma*

It is necessary to analyze drugs in biological samples in pharmacological and clinical studies. The proposed HPLC–FD method can be further applied to analyze biological samples with high sensitivity. The mice plasma samples were injected into the HPLC system for HPLC–FD analysis. The typical chromatograms of blank plasma sample, the blank matrices spiked with IS, DOX and PDOX, and the plasma sample after oral administration of PDOX which can convert into DOX in vivo are shown in Fig. 4. The retention time was 15.3 min for DOX, 16.6 min for IS and 17.5 min for PDOX. Due to the high selectivity, no significant endogenous components can interfere with the analytes and IS which can be well detected and quantified in the plasma samples.

4. Conclusion

A simple and rapid HPLC–FD method for simultaneous determination of DOX and PDOX presented in plasma has been developed and validated. The method has a good linearity, precision, accuracy and recovery, and can be used for quantitative analysis of DOX and PDOX at the same time. The proposed HPLC–FD method has further been successfully applied to the analysis of rat plasma sample after oral administration of PDOX. The method is rapid, accurate and fully validated, and can be instructive for content determination in real samples.

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