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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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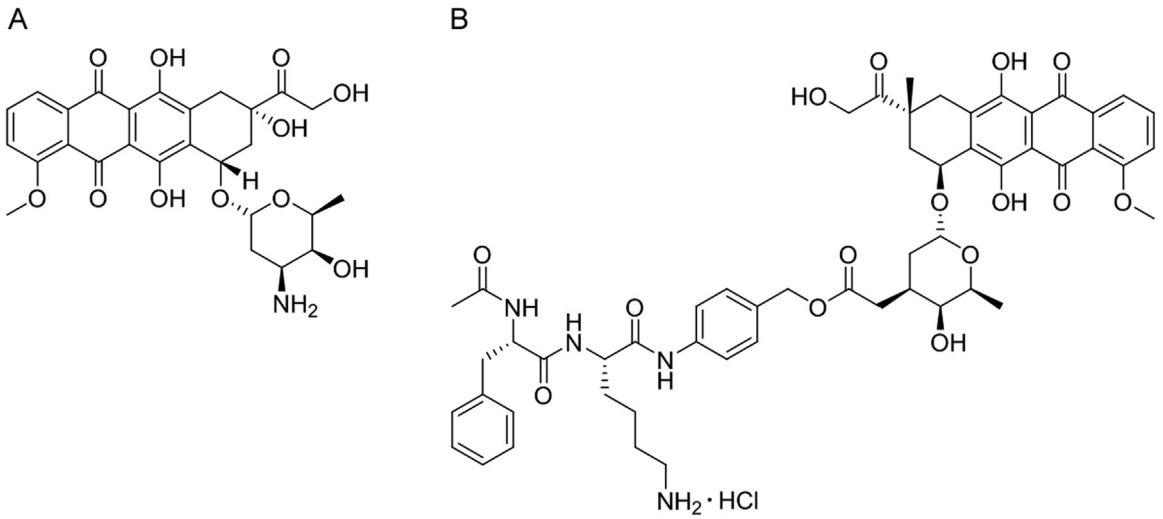

Fig. 1. The structures of (A) DOX and (B) PDOX.

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 $1/x^2$