Journal of Pharmaceutical and Biomedical Analysis 162 (2019) 140–148

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Comparison of *in vitro*/*in vivo* blood distribution and pharmacokinetics of artemisinin, artemether and dihydroartemisinin in rats

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ARTICLE INFO

Article history:
Received 8 May 2018
Received in revised form 6 August 2018
Accepted 12 September 2018
Available online 13 September 2018

Keywords:
Blood distribution
Artemisinin
Artemether
Dihydroartemisinin
LC–MS/MS
Pharmacokinetics

ABSTRACT

Artemisinin and its derivatives have been widely used for treatment of malaria and the therapeutic targets are considered within the red blood cells. In the recent studies on the erythrocytes' uptake of artemisinin-derivatives *in vitro,* applying the radioisotope-labeled technology, it was trying to predict the *in vivo* disposition properties, but different distribution results were revealed from a preliminary study in one human. The pharmacokinetic differences among blood cells and plasma still remain unclear. To explore the therapeutic related pharmacokinetics and compare the *in vitro-in vivo* blood distribution in rats, an improving blood sample preparation and LC–MS/MS detection method was developed and successfully validated. The lower limit of quantification was smaller than the previous studies.

In the *in vitro* blood distribution studies, the content ratios from blood cells to plasma were compared in the concentrations from 20 ng/mL to 1000 ng/mL. Such ratios were determined to be 1.1–1.6 for artemisinin, 0.9–1.2 for artemether, and around 0.7 for dihydroartemisinin. In the oral administration pharmacokinetic studies in rats, the concentration ratios from blood cells to plasma were from high (2.6–3.6) to medium (1.3–2.5), and low (0.5–1.5) for artemisinin, artemether, and dihydroartemisinin respectively in all measuring time points, displaying the similar affinity order toward blood cells in artemisinin > artemether > dihydroartemisinin as the *in vitro* measurements.

The dosages of 10 mg/kg for intravenous administrations of artemisinin and 200 mg/kg for oral administrations of artemisinin or artemether were used for the pharmacokinetic study in rats. The geometric mean exposures ($AUC_{(0\text{-}t)}$) of artemisinin, artemether and dihydroartemisinin in blood cells were determined to be 2.6 folds, 1.7 folds, or 1.2 folds greater than those in plasma, respectively. Referring to the *in vitro* distribution, the $AUC_{(0\text{–}t)}$ ratios from the blood cells measurements to the plasma measurements of these three antimalarial drugs were also in a similar trend as the *in vitro* distribution measurements. Furthermore, the half-life ($t_{1/2}$) of artemether in blood cells was even longer than that in plasma, while the clearance of artemisinin, artemether, or dihydroartemisinin in blood cells was slower than that in plasma. Particularly, it was found that the concentrations of artemisinin and artemether were presented in blood cells over longer time period than in plasma above their antimalarial IC_{50}, which might result from both the affinity toward blood cells and the drugs clearance differences between blood cells and plasma.

These results were indicated that the exposures and pharmacokinetic properties in the whole blood or the blood cells should be taken into account for the drug candidates with higher distribution affinity toward blood cells especially for the antimalarial drugs.

1. Introduction

Artemisinin and its derivatives are currently recommended by the World Health Organization (WHO) for the treatment of malaria because it displayed rapid effect, low toxicity characteristics, and less reports for the drug resistance [1]. The therapeutic target was considered within the red blood cells (RBCs) [2,3]. The uptake and distribution of artemisinin and its derivatives in

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https://doi.org/10.1016/j.jpba.2018.09.024
0731-7085/

RBCs have caused much attention [4–9]. It has been demonstrated that radioisotope-labeled dihydroartemisinin and artemether were found to be accumulated from the culture medium into the infected erythrocytes, and the partitioning of artemisinin into parasitized RBCs was more than 50%. However, the actual disposition in blood and the pharmacokinetic differences of artemisinin or artemether in blood cells and plasma still remain unclear.

It has been reported that potassium dichromate was used to stabilize the endoperoxide group of artemisinin-derivatives in blood samples. Such method was applied to determinate the distribution for artesunate and its metabolite dihydroartemisinin in whole blood or plasma in a healthy person [8]. In addition to the protection of the endoperoxide group, measurements of artemisinin-derivatives in blood samples would face much challenge, including issues of the blood cells disruption and the extraction of the internal materials.

In the present studies, an improving method with the blood sample preparation and LC–MS/MS detection of artemisinin and artemether as well as artemether's metabolite dihydroartemisinin was carried out based on a previous study reported by Lindegardh et al. [8]. The significance of different pharmacokinetics determined from blood cells and/or whole blood, in addition to plasma, was investigated in rats. The elucidation of therapeutic related blood pharmacokinetics and further exploration of the consistency of *in vitro-in vivo* blood distribution would help for the preclinical research as well as the clinical dose-finding of such new candidates especially for the antimalarials.

2. Materials and methods

2.1. Chemicals, reagents and animals

Artemisinin, artemether, dihydroartemisinin and artesunate were purchased from Chengdu Pufei De Biotech Co., Ltd. (purity>99.0%, Chengdu, China). Artesunate was used as internal standard (IS) for artemisinin and artemisinin was internal standard for artemether and dihydroartemisinin [10,11]. HPLC grade methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). All other reagents were of analytical grade. The distilled water was purified by a Milli Q water purification system from Millipore Corporation (MA, USA).

Eighteen male pathogen-free Sprague-Dawley rats (220–250 g) were obtained from the Experimental Animal Center of Southern Medical University (Guangzhou, China Certificate No. SCXK 2013-0217). The animals were housed for 5 days on a 12-hours light-dark cycle, with room temperature of 22 ± 2 ° and humidity of 55% ± 5% before the experiments. They were fasted for 12 h before drug administration and for a further 2 h after dosing. Water was freely available to the rats during the experiments.

2.2. Instrumentation and LC–MS/MS method

The LC–MS/MS analysis was performed using a Shiseido NANOSPACE 1312 HPLC system (Tokyo, Japan) coupled with an AB Sciex 4000 Q Trap™ (Ontario, Canada). Data acquisition and quantification were conducted with Analyst 1.5 (Applied Biosystems, MA, USA).

Artemisinin, artemether, dihydroartemisinin and artesunate were LC–MS/MS analyzed using a LUBEX Ecosil ODS-3 column (50 × 2.1 mm, 5 μm, Guangzhou, China) at room temperature with a flow rate of 0.25 mL/min. The mobile phase A was composed of acetonitrile and water at ratio of (5:95, v/v) and B, acetonitrile and water, (95:5, v/v). Both phases contained of 10 mM ammonium acetate. In the LC gradient profile, the mobile phase B was 20% (v/v) for 0.4 min and linearly increased to 100% from 0.4 to 0.6 min, maintained at this composition from 0.6 to 2.6 min, and then returned to 20% from 2.8 to 3.0 min. The total running time was 4.0 min. The retention times for the compounds were 2.3, 2.6, 2.2 and 1.9 min for artemisinin, artemether, dihydroartemisinin and artesunate respectively.

MS/MS conditions were optimized as follows: source temperature, 25 °C; ion spray voltage, 5500 V; curtain gas, 10 psi; nebulizing-gas, 50 psi; turbo ion spray gas, 50 psi; collision gas, medium; entrance voltage, 4 V; and dwell time, 150 ms. The transitions for the multiple reaction monitoring (MRM) were *m/z* 300.3–209.4 for artemisinin, 316.4–267.3 for artemether, *m/z* 302.5–163.3 for dihydroartemisinin and *m/z* 402.4–267.2 for artesunate. The declustering potential was 25 V, 26 V, 28 V and 38 V for artemisinin, artemether, dihydroartemisinin and artesunate. The collision energy was set at 14 eV, 12 eV, 23 eV and 13 eV for artemisinin, artemether, dihydroartemisinin and artesunate, respectively.

2.3. Preparation of calibration solutions and quality control samples

Stock solutions (1 mg/mL) of artemisinin, artemether, dihydroartemisinin and artesunate were prepared in methanol, respectively. Working solutions were serially diluted with methanol to give concentrations from 1 to 5000 ng/mL for artemisinin and from 5 to 10,000 ng/mL for artemether and dihydroartemisinin. Calibration standards and quality control (QC) samples in whole blood, plasma or blood cells were prepared by diluting the corresponding working solutions with blank rat whole blood, plasma or blood cells. The final concentrations were 0.2, 0.5, 1, 5, 20, 100, 400 and 1000 ng/mL for artemisinin, and 1, 2, 5, 20, 100, 400, 1600 and 2000 ng/mL for artemether and dihydroartemisinin. QC samples were prepared at the concentrations of 0.5, 20 and 800 ng/mL for artemisinin, 2, 100 and 1600 ng/mL for artemether and dihydroartemisinin. All stock solutions, working solutions, calibration standards and QCs were immediately stored at 4 °.

2.4. Sample preparation

An aliquot of whole blood, plasma or blood cells (100 μL) with 20 μL methanol and 20 μL IS (1 μg mL^{-1}, artesunate was used as the IS for artemisinin, and artemisinin was used as the IS for artemether and dihydroartemisinin) were added into a 2.0 mL cryogenic vial and spiked with 20 μL potassium dichromate 0.4 M and 50 μL EDTA (3%, m/v) immediately [8]. The mixture was vortex-mixed for 1 min on ice. After froze in liquid nitrogen and thawed quickly in 37° for 3 froze-thawed cycles, the mixture was ultrasonicated for 5 min in ice water, and then extracted with 1 mL ethyl acetate by shaking for 15 min on ice. After centrifugation at 10,000 × g at 4 °C for 5 min, 950 μL of the supernatant was evaporated to dryness. The residue was reconstituted in 200 μL of the mobile phase and a volume of 20 μL of each sample was injected into the HPLC–MS/MS system for analysis. For *in vivo* rat samples, the IS and other solutions should be added proportionally into the plasma or cells samples according to their actual volume.

2.5. Method validation

According to the recent FDA Bioanalytical Method Validation Guidance [12], the methods for simultaneously detecting artemisinin, artemether, dihydroartemisinin and artesunate in whole blood, plasma or blood cells samples were evaluated through selectivity, linearity, lower limit of quantification, precision and accuracy, matrix effect, recovery, stability and dilution, respectively.