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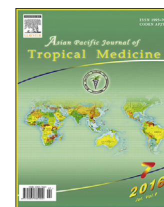

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Effects of arteannuin B, arteannuic acid and scopoletin on pharmacokinetics of artemisinin in mice

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ABSTRACT

Objective: To explore the effects of arteannuin B, arteannuic acid and scopoletin on the pharmacokinetics of artemisinin in mice.

Methods: Artemisinin and a combination of artemisinin, arteannuin B, arteannuic acid and scopoletin were administered together to mice via oral administration. Blood samples were collected at different time intervals and pretreated by liquid–liquid extraction. The contents of four compounds in mouse plasma were determined by a validated HPLC-MS/MS method.

Results: Compared to single artemisinin group, the C_{max} values from the combination group rose from 947 ng/mL to 1 254 ng/mL. $AUC_{(0\text{-}t)}$ (2 371 h ng/mL) was significantly higher than that from single artemisinin group (747 h ng/mL). The peak time lag and the CL values reduced at a proportion of 66%.

Conclutions: Arteannuin B, arteannuic acid and scopoletin can markedly affect the pharmacokinetics of artemisinin.

EDITOR'S NOTE

This research project of article is conducted by the Nobel Laureate Professor Youyou Tu's first Ph.D student, Associate Professor Man-Yuan Wang together with his student. Their three generations have been studying on artemisinin and made great achievements, significantly contributing to defeating malaria.

1. Introduction

Artemisinin-based drugs are now the first line drugs for malaria treatment. Since 2001, WHO has recommended combination therapies of artemisinin-based combination therapies (ACTs) [1–3] to treat malaria in order to avoid drug resistance. Generally, combination therapies of shorter half-life artemisinin-based drugs with longer half-life ones can avoid the emergence of low plasma concentration and delay development of drug resistance due to different anti-malarial mechanism [4,5]. However, the current ACTs almost refer to artemisinins in combination with amino-quinoline drugs [6]. WHO pointed out in 2005 that people may seriously face reduced susceptibility of anti-malarial drugs, and warned likely occurrence of drug resistance in the relevant reports. Emergence of drug resistance of *Plasmodium falciparum* to artemisinins has been confirmed at border areas of Cambodia and Thailand in 2009. The same situation was found in Myanmar and Vietnam later. Once malaria parasites were resistant to current ACTs in a wide range, it would lead to a difficult situation of no cure for malaria. Complex life cycle of *Plasmodium* objectively increase the difficulty of vaccine development. So far, there is still no effective vaccine for malaria prophylaxis. According to World Malaria Report in 2015 from WHO [7], about 3.2 billion people worldwide were faced with the risk of malaria. In 2015, there were still about 214 million new cases of malaria, with 438 000 deaths. It would be a disaster without effective anti-malarial drugs to achieve global malaria control goal. Therefore, research of looking for new anti-malarial drugs has practically been an urgency.

In 1970s, research team led by Youyou Tu discovered that the effectiveness of traditional Chinese medicine *Aretemisia annua* L. (*A. annua*) to treat malaria is a result of artemisinin-based multicomponent interaction. We conducted multicomponent compatibility test of anti-malaria effectiveness in our early studies, and found antimalarial activity of artemisinin is significantly improved when combination of arteannuin B, arteannuic acid and scopoletin extracted from *A. annua* was

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used [8,9]. On the other hand, without artemisinin, the significant antimalarial activity was not observed when arteannuin B, arteannuic acid and scopoletin neither alone or combined. Experiments confirmed that combination use of artemisinin, arteannuin B, arteannuic acid and scopoletin in a mass ratio of 1:1:1:1 showed better *Plasmodium* inhibition in malaria mouse model, achieving the same anti-malarial effect of four times dose of artemisinin. This study aims to further explore synergistic mechanism of artemisinin, arteannuin B, arteannuic acid and scopoletin on malaria mouse model which is considered more convenient and reliable generally than other model. Studies of single artemisinin therapy and four-component combination therapy on malaria mouse model were carried out to explore the their pharmacokinetic characteristics, especially to explore the potential interaction and rules of arteannuin B, arteannuic acid and scopoletin on artemisinin so that mechanism of four components combination can be further explained, and the foundation for further development of new ACTs can be laid.

2. Experimental and methods

2.1. Chemicals and reagents

Artemisinin, arteannuin B and arteannuic acid (purities >98.4%) were provided by our laboratory. Scopoletin (purity of 98%) was purchased from Beijing Lark Technology Co., Ltd. (Beijing, China). Buspirone (purity of 99.3%) was purchased from Sigma Co., Ltd. (St. Louis, Mo, USA). Methanol and acetonitrile were HPLC grade and purchased from Fisher (Massachusetts, USA). Formic acid (analytical grade) was purchased from Beijing Yili Fine Chemicals Co., Ltd. (Beijing, China). Methyl tert-butyl ether (analytical grade) was purchased from Tianjin Fuchen Chemical Reagent (Tianjin, China). Sodium carboxymethylcellulose was purchased from Beijing Fengli Jingqiu Commerce and Trade Co., Ltd. (Beijing, China).

2.2. Animal

ICR mice were obtained from Beijing Vitalriver Experimental Animal Technical Co., Ltd. (Beijing, China). Ninety-six male mice (20 ± 2) g were kept in an environmentally controlled breeding room for one week before starting the experiments and fed with standard laboratory food and water. They were randomly divided into sixteen groups (six animals per group) and were fasted overnight with free access to water before administration.

2.3. LC–MS/MS instruments and conditions

The liquid chromatography (LC) was performed on an Agilent 1200 series LC (Agilent Technologies, Palo, Alto, CA, USA), which included an Agilent 1200 binary pump (model G1316B), vacuum degasser (model G1322A), Agilent 1200 autosampler (model G1367C), and temperature controlled column compartment (model G1330B). Chromatographic separation was carried on an Agilent Zorbax XDB-C18 analytical column (50 mm × 2.1 mm, 3.5 μm, Agilent Technologies, Santa Clara, CA, USA) maintained at 30 °C. The mobile phase was consisted of 0.1% formic acid solution (A) and acetonitrile (B). The solvent gradient was as follows: 0–1 min with 5% B, 1–1.1 min with 5%–10% B, 1.1–5 min with 10%–95% B, 5–6 min with 95% B, 6–6.1 min with 95%–5% B and 6.1–10 min with 5% B. The flow rate was 0.3 mL/min and the injection volume was 10 μL.

The LC system was coupled to an Agilent 6410 triple quadrupole mass spectrometer (USA) equipped with an ESI source. The mass spectrometer was operated in the positive (artemisinin, arteannuin B and scopoletin) and negative (arteannuic acid) ESI mode with the drying gas temperature of 300 °C with N_2 gas flow at 10 L/min, nebulizer pressure of 30 psi, and capillary voltage of 4 000 V. The multiple reaction monitoring (MRM) transitions were chosen to be *m/z* 283.2 → 247.1 for artemisinin, *m/z* 249.1 → 189.1 for arteannuin B, *m/z* 232.8 → 232.8 for arteannuic acid, *m/z* 193.0 → 133.0 for scopoletin and *m/z* 386.3 → 122.1 for buspirone. The fragmentor voltage values set for artemisinin, arteannuin B, arteannuic acid, scopoletin and buspirone were 100 V, 120 V, 130 V, 120 V and 100 V, respectively. The collision energies were set to be 9 eV, 10 eV, 3 eV, 25 eV and 40 eV, respectively.

2.4. Design of pharmacokinetic experiments

Ninety-six animals were divided into two groups. The group of single artemisinin mice (n = 48) were orally administered with artemisinin of 100 mg/kg; while the mice of the combination group (n = 48), were orally administered with a dosage of 100 mg/kg of artemisinin, arteannuin B, arteannuic acid and scopoletin. Each group was randomly divided into 8 groups corresponding to a total number of 8 sampling points at 0.08, 0.25, 0.5, 1, 2, 5, 8, 12 h after dosing and each sampling points has 6 mice. Aliquots of 300 μL of blood was collected into heparinized syringes from eyeball of mice under anesthesia at 0.08, 0.25, 0.5, 1, 2, 5, 8, 12 h after oral administration. Blood samples were centrifuged at 3 000 r/min for 10 min. Then, all the plasma samples were stored at −80 °C until analysis.

2.5. Plasma sample preparation

All samples were stored in a freezer at −80 °C and allowed to thaw at room temperature before processing. An aliquot of 50 μL mouse plasma was mixed with 10 μL 20% methanol aqueous solution, 10 μL buspirone solution (100 ng/mL) and 10 μL 0.1% formic acid solution. Then, 400 μL of the extraction solution, methyl tert-butyl ether, was added. After mixing in automatic shakers for 1 min, samples were submitted to centrifugation at 3 500 r/min for 10 min. Then, 300 μL of the supernatant were transferred to a clean tube and evaporated under nitrogen flow. The residue was reconstituted in 100 μL 50% acetonitrile aqueous solution and vortexed for 1 min. Then aliquots of 10 μL solution were injected into the HPLC–MS/MS system for analysis.

2.6. Pharmacokinetic calculation and statistical analysis

The pharmacokinetic parameters including C_{max}, T_{max}, AUC, and $t_{1/2}$ *etc.*, were calculated by noncompartmental analysis, using pharmacokinetic analysis package Winnonlin software. Statistical analyses were performed using SPSS software version. $P < 0.05$ was considered statistically significant difference.

Table 1

Correlation coefficient, linear range and LLOQ of artemisinin, arteannuin B, arteannuic acid and scopoletin in mice plasma tested by LC-MS/MS.

Analyte	Calibration curves	Correlation coefficient (R)	Range (ng/mL)	LLOQ (ng/mL)
Artemisinin	y = 0.002 070x + 0.001 960	0.999	2.00–1 000.00	2.00
Arteannuin B	y = 0.006 370x + 0.008 780	0.998	2.00–1 000.00	2.00
Arteannuic acid	y = 0.000 139x + 0.001 180	0.995	2.00–1 000.00	2.00
Scopoletin	y = 0.004 620x + 0.006 530	0.999	2.00–1 000.00	2.00

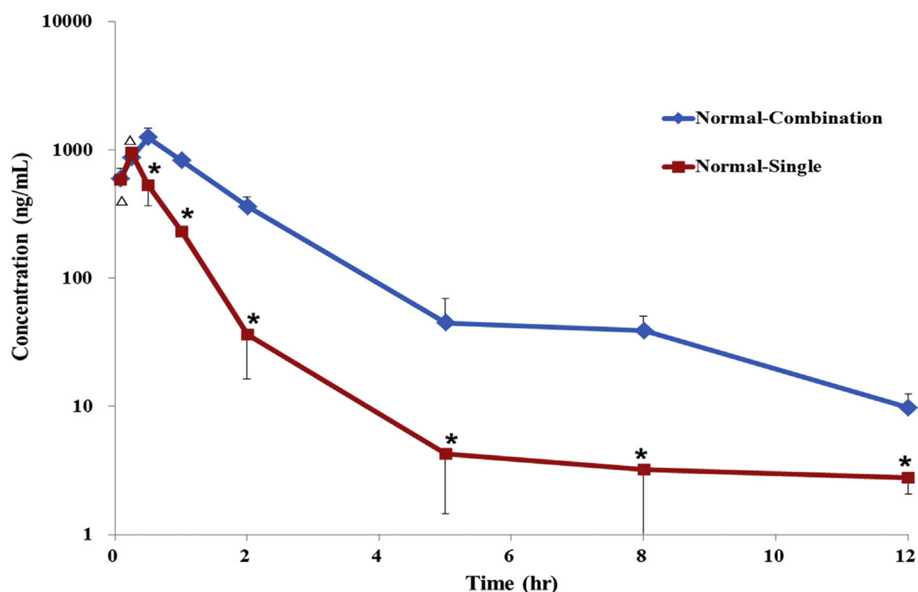

Figure 1. Mean plasma concentration–time curve of artemisinin in mouse plasma after oral administration of single artemisinin or the combination of artemisinin, arteannuin B, arteannuic acid and scopoletin (n = 6). Compared with normal single artemisinin, $^{\Delta}P > 0.05$; $^{**}P < 0.01$.

3. Results

3.1. Assay validation

The blank plasma sample spiked with standard and a plasma sample from a mouse after an oral administration of drugs were tested. All samples were found to have no interference from endogenous substances affecting the retention times of artemisinin, arteannuin B, arteannuic acid, scopoletin and buspirone. No cross-talk peaks were observed among the analytes. The run time of each sample was 10.00 min, and the respective retention times of artemisinin, arteannuin B, arteannuic acid, scopoletin and buspirone were 6.91 min, 6.68 min, 7.59 min, 5.20 min and 5.32 min. The calibration curves were linear over the concentration ranges of 2–1 000 ng/mL in mouse plasma with good reproducibility and linearity. It was assessed by analyzing the calibration curves using the peak area ratios of analyte/buspirone versus the nominal concentrations of the analyte calibration standard with a weighting factor ($1/x^2$). Obtained correlation coefficients (r) were higher than 0.99, indicating that all calibration curves met the acceptance criteria (Table 1).

Precision and accuracy of the developed method were validated by assaying the QC samples at three concentration levels in plasma. In this assay, the intra- and inter-day precisions were measured to be below 8.55% and 9.20%, respectively. These values were within the acceptable range, and the method was thus judged to be suitably accurate and precise. The stability of the analytes during the sample preparation procedures and storage was evaluated by analysis of three levels of QC samples. The results indicated the stability of artemisinin, arteannuin B, arteannuic acid and scopoletin in plasma after storage at room temperature for 24 h, at −80 °C for 30 d and after three freeze–thaw cycles at −20 °C. The results indicated that four components were stable under different storage conditions. The

Table 2

Main Pharmacokinetic parameters of single artemisinin, arteannuin B, arteannuic acid and scopoletin in mice plasma after oral administration of the combination of artemisininn *(n* = 6).

PK Parameters	Unit	Single	Combination	Arteannuin B	Arteannuic acid	Scopoletin
$AUC_{(0\text{-}t)}$	h·ng/mL	747.00	2 371.00	461.00	550 477.00	1 242.00
$AUC_{(0\text{-}\infty)}$	h·ng/mL	793.00	2 396.00	477.00	556 782.00	1 452.00
C_{max}	ng/mL	947.00	1 254.00	278.00	195 570.00	716.00
T_{max}	h	0.25	0.50	0.25	0.50	0.25
$MRT_{(0\text{-}t)}$	h	0.93	1.84	2.46	2.12	2.82
$MRT_{(0\text{-}\infty)}$	h	2.55	1.98	2.93	2.26	5.50
$t_{1/2}$	h	11.50	1.81	2.84	1.74	6.49
Ke	1/h	0.06	0.38	0.24	0.40	0.11
Vd	mL/kg	2 099 106.00	109 228.00	859 071.00	450.00	645 168.00
CL	mL/h/kg	126 111.00	41 732.00	209 636.00	180.00	68 871.00

recovery experiments were performed at three QC levels to demonstrate that there was no concentration bias. The mean recovery was 89.5%–99.3% for artemisinin, 86.9%–99.0% for arteannuin B, 93.4%–99.3% for arteannuic acid and 85.2%–91.6% for scopoletin. These available data demonstrated that the proposed method was accurate, reliable and reproducible for the quantitation of artemisinin, arteannuin B, arteannuic acid and scopoletin in mouse plasma.

3.2. Pharmacokinetic study

The validated LC–MS/MS method was successfully applied to the pharmacokinetic study of artemisinin, arteannuin B, arteannuic acid and scopoletin in mice after an oral administration. The mean plasma concentration–time curves of artemisinin in mouse plasma after oral administration of single artemisinin or the combination of artemisininn are shown in Figure 1 and main pharmacokinetic parameters are presented in Table 2. The main pharmacokinetic parameters of arteannuin B, arteannuic acid and scopoletin in mouse plasma after oral administration of the combination of artemisinin are presented in Table 2.

4. Discussion

During LC–MS/MS assay development, the sensitivity, selectivity and robustness of the assay had been taken into the consideration. Artemisinin is sesquiterpene lactones containing peroxide bridges, missing from the molecular structure of the conjugated system; using conventional UV detector is difficult to achieve the purpose of the quantitative analysis of biological samples. The experiment used liquid chromatography tandem mass spectrometry (LC-MS/MS) to establish artemisinin, arteannuin B, arteannuic acid and scopoletin determination of drug concentration in mouse plasma. In order to improve the accuracy of the method and reduce the experimental error, buspirone was selected as an internal standard, to avoid the errors caused in sample handling and injection process. The negative ion mode and the positive ion mode were tested, the test results showed that response intensity obtained was high of arteannuic acid by negative ion mode, but other three components adapted to be detected in positive ion mode. At the same time, the test results also showed that given a large collision energy, arteannuic acid precursor ion all became fragmented, and there was no characteristic ions, while a smaller energy collision was used, there was only the parent ion, and no fragment ions, so final collision with a smaller energy only detect the parent ion concentration.

An efficient method for bio-sample cleanup to remove protein and potential interference from endogenous substances prior to LC–MS/MS analysis is important in the method development. At present, the common biological sample preparation includes protein precipitation (PPT), solid-phase extraction (SPE) and liquid–liquid extractions (LLE). Because of using SPE need high cost, in our study, different extraction procedures like PPT and LLE were tested. The test results showed that artemisinin was not detected with PPT, however, four components could be detected by liquid–liquid extraction method. Based on this, two kinds of organic solvents, methyl tert-butyl ether and dichloromethane with different volumes were tested as the LLE solvent in order to obtain higher extraction recovery. The test results showed that the recovery of dichloromethane was lower than methyl tert-butyl ether. Finally, LLE with eight volumes of methyl tert-butyl ether was adopted for sample preparation. The supernatant after centrifugation was dried using nitrogen prior to the LC–MS/MS assay for enhancing the sensitivity.

Artemisinin can be absorbed quickly after oral administration, the T_{max} (time to reach maximum plasma concentration) is 1–2 h in blood. But it cannot be completely absorbed and the bioavailability is low [10]. The radioactivity in mouse blood rapid rose after oral administration of ^{3}H- artemisinin, the T_{max} was 1 h, then plasma concentrations of artemisinin declined rapidly with terminal elimination half-life ($t_{1/2}$) was 4 h [11]. Weathers *et al.* [12] investigated and found the T_{max} was 1 h and the C_{max} (the maximum plasma concentration) was 74 ng/mL in mouse plasma after oral administration of 70 mg/kg dose of artemisinin; at the same time, T_{max} was much more short after oral administration the leaves of *A. annua*. In the study, the artemisinin was rapidly absorbed by mice with 0.25 h to reach C_{max} of 947 ng/mL after oral administration artemisinin (100 mg/kg).

Melillo de Magalhaes *et al.* [13] reported rosemary acid and chlorogenic acid can increase bioavailability of artemisinin by inhibiting the activity of CYP3A4 and CYP1A1. Compared with the oral administration of single artemisinin, the C_{max} of the artemisinin after the oral administration of the combination of artemisinin was significantly increased from 947 ng/mL to 1 254 ng/mL and area under the plasma concentration–time curve (AUC) from time 0–12 h (AUC_{0-12}) of artemisininn was significantly increased by 3.17 times in combination group. The reasons for these results, on the one hand, may be due to the enzymatic interaction of arteannuin B, arteannuic acid and scopoletin with artemisinin, which can inhibit the metabolic enzymes. On the other hand, three components may affect the binding of artemisinin with serum albumin, and then the protein storage and transport of artemisinin *in vivo* was changed. Preliminary studies showed that the quenching constant and binding constants of artemisinin with bovine serum albumin in four-components combination group were increased, compared to the single artemisinin group, which can affect the pharmacokinetic behavior of artemisinin [14].

Since ACTs was considered as first-line antimalarial drugs recommended by WHO, in Africa and other places, the general public is difficult to accept such a high price of drugs and the clinical use of these drugs were hindered. On the other hand, although there are no clear clinical teratogenic and mutagenic effect reports of artemisinin-based drugs, some patients during pregnancy fear the toxicity rather than treatment of malaria using ACTs, because it has been observed that artemisinin-based drugs can lead to fetal resorption toxicity in animal models of rats and rabbits. These factors lead to the application of the herb *A. annua* for treating malaria being more common in some parts of Africa [15]. The research team of Youyou Tu has confirmed the acidic parts of ether extract has strong toxicity [16]. At the same time, the WHO made it clear that due to the fear that the use of the herbal medicine of *A. annua* for treating malaria patients is difficult to ensure adequate therapeutic drug plasma concentration, malaria parasites cannot be killed effectively and the patients received a dose of artemisinin insufficient to eliminate the parasites. Considering such sub-curative doses could promote the emergence of *Plasmodium* resistance to artemisinins, the herb *A. annua* is not recommended to treat malaria [17].

In recent years, in order to seek cheap and effective plant anti-malarial drugs, some foreign researchers has developed and paid more attention to the artemisinin-based natural components in *A. annua*. De Donnoa *et al.* [18] found that the antimalarial activity of *A. annua* tea is higher than purified artemisinin. Suberu *et al.* [19] found artemisinic acid and arteannuin B showed additive interaction while rosmarinic acid showed synergistic interaction with artemisinin in the chloroquine sensitive strain; in the chloroquine resistant parasite, arteannuin B was synergistic. Elfawal *et al.* [20] found that a single dose of the dried leaves of whole plant *A. annua* (pACT) (containing 24 mg/kg artemisinin) reduces parasites more effectively than a comparable dose of purified artemisinin. The bioavailability of artemisinin in the blood of mice fed the pACT increased 40-fold. The above results suggest that *A. annua* can be developed into the possibility of multicomponent plant antimalarial agents. Elfawal *et al.* [21] showed that pACT overcomes existing resistance to pure artemisinin in the rodent malaria infected by *Plasmodium yoelii*, with the ability to reverse the drug resistance of *Plasmodium*. The pACT has the potential for further development of nonpharmaceutical forms to treat human malaria.

This study suggests the pharmacokinetics of artemisinin can be changed by other components in *A. annua* to enhance the antimalarial effect. Further studies about the optimal ratio and synergistic mechanism of the multi-components combination therapies from *A. annua* are very significant for the development of new ACTs and new antimalarial strategies.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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