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Rapid evaluation of artesunate quality with a specific monoclonal antibody-based lateral flow dipstick

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Abstract

Artesunate is a frontline antimalarial drug for treating *Plasmodium falciparum* malaria. To produce specific antibodies to artesunate, the carboxyl group of artesunate was directly conjugated to carrier protein as the immunogen. A specific monoclonal antibody (mAb) 3D₈2G₆ against artesunate was obtained by high-throughput screening of positive hybridoma clones. This monoclonal antibody had 4.0%, 0.5% and 0.9% cross reactivities with artemisinin, dihydroartemisinin and artemether, respectively. A dipstick immunoassay was developed and the indicator range for artesunate was 1,000–2,000 ng mL⁻¹. No interference was observed with artemisinin, dihydroartemisinin, artemether and other commonly used antimalarial drugs for up to 20,000 ng mL⁻¹. The dipsticks were used for determination of artesunate contents in commercial drugs, and the results were agreeable with those determined by high-performance liquid chromatography. This dipstick, with its specificity and sensitivity for artesunate and simplicity to use, makes it a potential point-of-care device for rapid quality evaluation of artesunate-containing antimalarial drugs.

Keywords

Artesunate; Dipstick; Malaria; Antibody

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Female Balb/c mice were purchased from the Laboratory Animal Center of the Institute of Genetics (Beijing, China). This study was performed strictly according to the standards described in the “Guide for the Care and Use of Laboratory Animals” (National Research Council Commission on Life Sciences, 1996 edition). All animal treatment procedures were approved by the Animal Care Committee of China Agricultural University (CAU) and all efforts were made to minimize suffering. Mice were housed under controlled temperature (22±2°C) and lighting (12 h light/12 h darkness) with food and water ad libitum in air-conditioned rooms. All experimental mice were killed by cervical dislocation.

Introduction

Artesunate is one of the main active ingredients in artemisinin-based combination therapies (ACTs) against *Plasmodium falciparum* malaria [1]. In some African countries, artesunate/amodiaquine is used as a first-line treatment for children with uncomplicated malaria [2]. A 3-day regimen of artesunate/mefloquine has been used in Thailand and Cambodia for over a decade [2]. However, the proliferation of counterfeit and substandard artesunate-containing antimalarial drugs is a serious problem in some malaria-endemic countries [3]. For example, a recent study showed that 25.8% (60/233) of the artesunate tablets in Cambodia were of poor quality with active pharmaceutical ingredient (API) lower than 85% or above 115% [4]. Of the 541 artesunate drugs collected from Tanzania's private sector, 6.1% were of poor quality (API < 85% or > 115%) [5]. Given the potential impact of counterfeit antimalarials on malaria control, there is a need for developing point-of-care methods for rapid assessment of the quality of ACTs.

Among reported analytical methods, high-performance liquid chromatography ultraviolet-visible spectroscopy (HPLC UV-Vis) [6], colorimetric method involves a diazonium salt fast red TR [7] and thin-layer chromatography (TLC) [8, 9] are the main assays used for quantifying artesunate in antimalarial drugs. However, none of these assays are suitable for rapid analysis of artesunate drugs under field settings in malaria-endemic areas. HPLC requires expensive instruments and highly trained personnel. Fast red TR is quick and simple, but it requires a reaction with poisonous reagent for sample pretreatment. Though TLC is simple, inexpensive and recommended by WHO for the detection of fake drugs in resource-limited regions, it is cumbersome and also uses toxic and flammable reagents. On the other hand, dipstick is an economic and one step assay with a simple sample preparation step. The test result can be read by naked eyes, and is highly suitable as a point-of-care diagnostic device. Besides, people in most malaria endemic areas are familiar with the dipstick format due to routine use of rapid diagnostic tests in malaria diagnosis.

To develop a dipstick assay for artesunate, a specific antibody against artesunate is required. Antibodies against small molecules normally require the conjugation of the small molecules to a carrier protein. In the case of artemisinins, the carboxyl group at position 12 of artesunate is most often used for direct conjugation to a carrier protein [10–14]. Yet, the reported monoclonal antibodies (mAbs) all had high cross reactivities with artemisinin and dihydroartemisinin [10–14]. In the present work, we identified a specific mAb 3D₈2G₆ against artesunate after large-scale screening of positive hybridoma clones against artesunate. Using this specific mAb, we developed an artesunate-specific lateral flow immunoassay and evaluated its suitability for qualitative and semi-quantitative analysis of artesunate content in antimalarial drugs.

Methods

Materials

Artemisinin, artesunate, dihydroartemisinin, and artemether were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Quinine and primaquine phosphate were purchased from J&K Chemical (Beijing,

China). Chloroquine diphosphate salt, pyrimethamine and lumefantrine were purchased from Sigma (St Louis, MO, USA). Traphasunat distributed by M/S Sandar Myaing Company Ltd. was purchased from Pathein, Ayeyawaddy, Myanmar. Artesunate, distributed by Liberty Group Trading Ltd., were purchased from pharmacies of different regions of Myanmar, including Lot No. 216214 from Ann, Raknine, Lot No. 212414 from Ngaputaw, Ayeyawaddy, Lot No. 264513 from Shwe Bo, Sagaing, Lot No. 210514 from Yatsauk, Shan, and an unknown Lot No from Thayetchaung, Tanintharyi.

Preparation of mAb against artesunate

Artesunate was conjugated to bovine serum albumin (BSA) and ovalbumin (OVA) via a previously described method (Fig. 1) [13]. Artesunate-BSA was used as immunogen to immunize mice as described previously [13].

The protocol for iELISA and icELISA was the same as that described previously [13, 15]. All the positive hybridomas were tested for their cross reactivity with artemisinin, dihydroartemisinin and artemether at the beginning of screening (cross reactivity = IC_{50} of artesunate/ IC_{50} of other compound $\times 100$). One artesunate- specific hybridoma was selected and cloned twice by limiting dilution, followed by expansion for large-scale production of mAbs. The specific mAb against artesunate was used for dipstick development.

Development of colloidal gold-based lateral-flow dipstick immunoassay

Colloidal gold with an average particle diameter of 30 nm (G30) was prepared according to the procedure described by Frens [16]. With gentle stirring, 10 μ L of aqueous solution of the mAb at a concentration of 3 mg mL⁻¹ was added to 1 mL of colloidal gold solution (pH 7.8). After 10 min, 40 μ L of 10% (w/v) BSA was added to the solution. Then the mixture was centrifuged at 9,600 $\times g$ for 20 min. The pellet was resuspended in 500 μ L of 0.01 M PB (pH 7.0), and stored at 4 °C. Finally, the conjugate pad was saturated with the gold-antibody conjugate, and dried at room temperature. Goat anti-mouse IgG was used as a control capture reagent. Artesunate-OVA was used as capture reagent immobilized at the test line on the membrane. Artesunate-OVA and goat anti- mouse IgG with the volume of 1 μ L/cm were immobilized to Nitrocellulose (NC) membrane as an important element of the test strip. The distance between the two lines was 5 mm. The dipstick was assembled as described by He et al. [14]. The NC membrane was pasted onto the center of the PVC plate which was served as the backing of the test strip. The colloidal gold-Ab conjugate pad was pasted on the plate by overlapping with the NC membrane. Absorption pad was pasted on the plate at the opposite end downstream from the control line and sample pad was pasted by overlapping with the conjugate pad. The card was cut to 3 mm width. Strips were then sealed in a plastic case with desiccant gel and stored at 4 °C.

Determination of the indicator range and cross reactivity of the dipsticks

Standard solutions at concentrations of 0, 62.5, 125, 250, 500, 1,000, 2,000, 4,000 and 8,000 ng mL⁻¹ were prepared by diluting artesunate stock solution (2 mg mL⁻¹ in ethanol) with distilled water. The indicator range, defined as the lowest concentration of the target analyte between which the test line was not visible, is used to indicate the sensitivity of the dipstick. To estimate the indicator range of the dipsticks, 80 μ L of standard solutions were added

dropwise into the sample well. The color of the test and control line was visually observed within 5 min. A number of commonly used antimalarial drugs including artemisinin derivatives, quinine, chloroquine diphosphate salt, primaquine phosphate, pyrimethamine, lumefantrine, amodiaquine, piperaquine tetraphosphate tetrahydrate and pyronaridine tetraphosphate were tested for specificity. The stock solutions of these drugs at 1 mg mL⁻¹ were prepared in absolute ethanol (quinine), water (chloroquine diphosphate salt, primaquine phosphate, amodiaquine, or pyronaridine tetraphosphate), acetonitrile (pyrimethamine or piperaquine tetraphosphate tetrahydrate), and chloroform (lumefantrine). A final water dilution of these stock solutions was used at 20,000 ng mL⁻¹. Each sample was analyzed in triplicates.

Testing of commercial artemisinin-based drugs

Artesunate tablets were grounded in a clean mortar to a fine powder. The powdered drugs were transferred into a 15 mL tube and extracted with 10 mL of ethanol. After thorough mixing, the extracts were stored at 4°C before analysis. For the commercial artesunate samples, the sample extracts were diluted into 2 mg mL⁻¹ with ethanol as stock solutions based on the labelled artesunate content of the drugs. All stocks were then diluted with distilled water to concentrations in the indicator range of the dipstick. Each sample was analyzed in triplicates.

To determine the efficiency of the extraction method, we performed a sample spike-in study. An artesunate tablet was weighed and grounded in a clean mortar to a fine powder. Every 1/25 tablet was spiked with 1, 2 and 3 mg standard artesunate, respectively. Sample with no artesunate added was used as the control. The samples were extracted as described above. The extracts were then analyzed with HPLC. Each spiked sample was repeated three times.

HPLC method

Drugs were analyzed by HPLC according to a procedure described previously [6]. The HPLC system (Shimadzu LC-20A) consisted of two LC-20AT solvent delivery units, an SIL-20A autosampler, an SPD-M20A photodiode array detector, a CBM-20A lite system controller (Kyoto, Japan). The mobile phase, standards and drug extracts obtained above were filtered through a 0.5 µm syringe filter prior to HPLC. A C18 column (250×4.6 mm, 5 µm particle size; Thermo, Vantaa, Finland) was used to resolve artesunate. A mobile phase for artesunate was acetonitrile-water (60:40, v/v) at a flow rate of 1 mL min⁻¹. The injection volume was 20 µL. Detection wavelength was 210 nm.

Results

Identification of a specific mAb against artesunate

To produce the specific mAbs against artesunate, mice with the highest antibody titer and specificity against artesunate were used for cell fusion. Fused cells were plated out on fifteen 96-well tissue culture plates. The screening process focused on specificity to artesunate over sensitivity. Approximately 200 positive hybridoma clones were first selected and then tested for specificity to artesunate. One of the artesunate-positive clones, 3D₈2G₆, showed low reactivities with dihydroartemisinin (0.5%), artemisinin (4.0%) and artemether (0.9%) (Table

1). The concentration causing 50% of inhibition (IC_{50}) and the working range in an icELISA for artesunate was 6.7 ng mL^{-1} , and $1.6\text{--}30.8 \text{ ng mL}^{-1}$ (Fig. 2).

Development of colloidal gold-based lateral-flow dipstick immunoassay

The mAb 3D₈2G₆ was selected to develop a colloidal gold-based lateral-flow dipstick immunoassay. The sensitivity of the dipstick was deeply affected by the concentration of gold-conjugated antibody and capture reagent. Since the API content of the ACT drugs (at the mg level) was much higher than the normal dipstick indicator range (at the ng level), we chose to use darker color for the test line to allow easier visual judgment albeit this would result in lower sensitivity of the dipstick for the analytes. This also required fewer sample dilution steps. The optimum concentrations of artesunate-OVA, goat anti-mouse antibody and colloidal gold-mAb 3D₈2G₆ were 1 mg mL^{-1} , 1 mg mL^{-1} and $30 \mu\text{g mL}^{-1}$, respectively. The concentration of colloidal gold-mAb was calculated based on the added concentration of antibody when preparing the colloidal gold-mAb conjugate. The concentration-dependent color intensity of the dipsticks for standard artesunate in distilled water was illustrated in Fig. 3. The indicator range of the dipstick was $1,000\text{--}2,000 \text{ ng mL}^{-1}$ for artesunate.

To determine the specificity of the dipstick for artesunate, several artemisinin derivatives and commonly used antimalarial drugs were used for the evaluation of cross reactivities. At $20,000 \text{ ng mL}^{-1}$, none of the tested drugs caused noticeable inhibition (Figs. 4, 5).

Semi-quantitative analysis of artesunate-based antimalarial drugs

We checked the efficiency of the extraction method using spike-in artesunate standard. The result showed that the average recovery rate of the spike-in artesunate ranged from 94% to 106% with an average of 101%. Concentrations of artesunate in the stock solutions of artesunate-based antimalarial drugs extracted using this method were determined using the dipsticks. The artesunate sample stocks were diluted in distilled water for 500, 1,000, 2,000, 4,000 and 8,000 times to give anticipated concentrations of artesunate at 4,000, 2,000, 1,000, 500 and 250 ng mL^{-1} . When analyzed using the dipsticks, no color development was observed on the test line for the 1,000- and 500-fold dilutions, whereas the color intensity of the test line for the 2,000-, 4,000-, 8,000-fold dilutions gradually increased. This suggested that the artesunate concentration in all 2,000-fold diluted samples was lower than the indicator range, which indicated that the artesunate concentration in the stock solution was between 2 and 4 mg mL^{-1} (100–200% to the actual content). This result agreed well with the result determined by HPLC (Table 2).

Discussion

Artesunate has the similar chemical structure with other artemisinin derivatives and differs only at position 12. To develop a specific mAb for artesunate, one way is to introduce a hydroxyl or a carbonyl group at positions opposite to position 12 and to derive a carboxyl group [17]. However, during hapten conjugation both the derived carboxyl group and the original carboxyl group could react with the free amino group of the carrier protein, and the resultant haptens may be heterogeneous and not useful for producing specific mAb. Therefore, we still used the carboxyl group on position 12 of artesunate for hapten

preparation. Although most antibodies derived this way are expected to be of low specificity to artesunate, we wanted to circumvent this problem by large-scale screening of positive hybridoma clones. This effort led to the identification of a mAb that was highly reactive with artesunate but showed very low cross reactivities with other artemisinin derivatives.

A mAb 3D₈2G₇ developed earlier in our lab showed strong reactivities with artesunate, dihydroartemisinin (70%) and artemisinin (22%), and a dipstick assay based on this mAb could detect artesunate, dihydroartemisinin and artemisinin. This dipstick was more sensitive for detecting artesunate and dihydroartemisinin with the indicator range of 100–200 and 200–500 ng mL⁻¹, respectively, whereas it was less sensitive for artemisinin (2,000–5,000 ng mL⁻¹). Although this dipstick is useful for rapid quality control of antimalarial drugs containing artesunate, dihydroartemisinin and artemisinin, the development of specific mAbs for individual derivatives offers us the ability to distinguish these derivatives and for pharmacokinetic studies. Consistent with the results from the icELISA, the dipstick based on the mAb 3D₈2G₆ in this study is sensitive (indicator range 1,000–2,000 ng mL⁻¹) and specific for artesunate with no cross reactivity to artemisinin, dihydroartemisinin, artemether or other commonly used antimalarials at 20,000 ng mL⁻¹. Besides, the partner drugs in ACTs did not affect the performance of the dipsticks for analyzing artesunate, indicating that the dipstick can be used for qualitative detection and semi-quantitative analysis of artesunate in unknown samples.

When we include multiple dilutions of a test sample, the dipstick may serve as a semi-quantitative device for artesunate. This has been validated with a number of artesunate-containing commercial drugs, and the results determined by the dipstick were agreeable with those determined by HPLC. Moreover, the use of ethanol instead of acetonitrile for sample preparation also enhances the suitability of this test for field settings. Thus, further evaluation of the artesunate dipstick as a point-of-care device for rapid quality check of artesunate-containing drugs used in malaria-endemic areas is warranted.

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Fig. 1.
Preparation of artesunate-protein conjugates.

Fig. 2.

A standard inhibition curve of artesunate in icELISA format. B_0 and B are absorbance in the absence and presence of a competitor, respectively. The concentration causing 50% inhibition (IC_{50}) by artesunate was 6.7 ng mL^{-1} . Each value represents the mean of three replicates.

Fig. 3.
Dipsticks showing color changes corresponding to concentrations of artemisinin in distilled water. The limit of detection was 1,000–2,000 ng mL⁻¹ of artesunate. C represents the control line, while T represents the test line

Fig. 4.

Dipstick specificity tests of artemisinin and its derivatives. Artesunate solution was diluted in distilled water at a concentration of $2,000 \text{ ng mL}^{-1}$, while the other standard solutions were diluted in distilled water at a concentration of $20,000 \text{ ng mL}^{-1}$. (1) blank; (2) artesunate; (3) artemisinin; (4) dihydroartemisinin; (5) artemether

Fig. 5.

Dipstick specificity tests of the partner drugs in the artesunate-based combinations.

Artesunate solution was diluted in distilled water at a concentration of $2,000 \text{ ng mL}^{-1}$, while the other standard solutions were diluted in distilled water at a concentration of $20,000 \text{ ng mL}^{-1}$. (1) blank; (2) artesunate; (3) amodiaquine; (4) pyronaridine tetraphosphate; (5) piperaquine tetraphosphate tetrahydrate; (6) primaquine phosphate; (7) pyrimethamine; (8) quinine; (9) chloroquine diphosphate salt; (10) lumefantrine

Table 1

Cross reactivity of the mAb with artemisinin and its derivatives

Analytes	IC ₅₀ (ng mL ⁻¹)	Cross reactivity ^a (%)
Artesunate	6.7±0.1 ^b	100.0±2.20
Artemisinin	169.6±25.3	4.0±0.60
Dihydroartemisinin	1392.5±27.4	0.5±0.02
Artemether	747.8±60.4	0.9±0.07

^aCross-reactivity (%) = (IC₅₀ of artesunate/IC₅₀ of other compound) × 100^bData represent means of triplicate ± standard deviation

Table 2

Comparison of the icELISA and HPLC method for the quantitation of artesunate in commercial drugs

Drug names ^a	Lot no.	Labeled content (mg/pill)	Content determined by dipsticks (mg mL ⁻¹)	Content determined by HPLC ^b (mg mL ⁻¹)
Artesunate	216214	50	2–4	1.87±0.06
	212414	50	2–4	1.96±0.09
	264513	50	2–4	1.73±0.01
	210514	50	2–4	1.94±0.11
	unknown	50	2–4	2.31±0.05
Traphasunat	02/090412	50	2–4	2.12±0.01

^aEach sample was analyzed in triplicate^bData represent mean ± SD. The theoretical content of all drugs was 2 mg mL⁻¹