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Fluorometric determination of antimalarial efficacy of artemisinin and artemether against *Plasmodium falciparum in vitro*[1]

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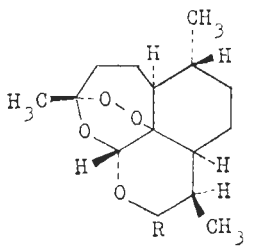
ABSTRACT A technique for assessment of the growth of *Plasmodium falciparum in vitro* by fluorometry was applied to evaluate the antimalarial efficacy of artemisinin, artemether and chloroquine. After cultivation in various concentrations of the above drugs for 40h, parasitized rbc were harvested, lysed, stained with ethidium bromide, solubilized with SDS, and measured by a fluorescence spectrophotometer. The IC_{50} of the 3 drugs determined by fluoroassay were closely related with those derived by visual counting of schizont-infected rbc in thin blood films under microscope. The currently used methods can be supplemented by this new technique with the following advantages; 1) more objective reading of the results than parasite-counting under microscope, 2) simpler and safer procedure than radiometric assay.

KEY WORDS *Plasmodium falciparum*: fluorescence spectrometry; antimalarials; ethidium; artemisinin; artemether; chloroquine

Microscopic counting of parasites for determination of drug efficacy takes much time and sometimes the results are rather variable among different examiners because of subjective reading and appearance of degenerated parasites caused by drugs. [^{3}H]hypoxanthine-uptake assay[1] recently introduced for evaluating drug action needs radioactive operation. For these reasons, the methods currently used for assessing antimalarial potency of compounds need to be innovated urgently.

A fluorescent dye, ethidium bromide, binds specifically to base-paired regions in nucleic acids (NA) and the amounts of bound NA can be determined quantitatively by measuring its fluorescence intensity[2]. This assay has been applied to the assessment of the growth of *P. falciparum in vitro* for detection of drug-resistant parasites[3]. In this paper, the antimalarial efficacy of artemisinin (qinghaosu) and artemether, recently developed in China, against FCC1/HN strain of *P. falciparum* are presented by the fluorometric assay.



R: =O —OCH$_3$

Artemisinin **Artemether**

MATERIALS AND METHODS

Parasite FCC1/HN strain of *P. falciparum* isolated from a patient in the Hainan Island in 1977[4] was used.

Cultivation Maintenance of the parasites and sensitivity tests were carried out by the candle jar method[5]. Experiments were

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conducted by quadruplicate assay in 24-well plastic plates (Nunc Multidish, Nunclon, Denmark). 500 μl of 5% parasitized rbc suspension, having 1.5% parasitemia at a stage of ring forms, were added to each well containing 20 μl of certain concentration of the tested drug. The RPMI 1640 medium was supplemented with HEPES 25 nmol/L, sodium bicarbonate 23.8 mmol/L and 15% rabbit serum[6]. An aliquot of 500 μl culture material was kept at −20℃ as a pre-culture control sample. The culture plates were shaken gently to mix the contents, and the parasites were cultured in a candle jar at 37℃ for 40 h.

Antimalarials Artemisinin and artemether, provided by The Kunming Pharmaceutical Factory, were dissolved in *N*, *N*-dimethylformamide (DMF), and chloroquine diphosphate (CQ) in water as stock solutions kept at −20℃. The stock solutions were given 5 serial dilutions with the above medium at ranges of 1 μmol/L to 10 nmol/L for artemisinin. 0.1 μmol/L to 1 nmol/L for artemether and 0.2 μmol/L to 12.5 nmol/L for CQ, respectively, before testing.

Fluoroassay Triplicate samples from each group were harvested into 10 ml plastic centrifuge tubes. The parasitized rbc were lysed by adding 4 ml of 0.1% saponin. After centrifugation (1500×*g*, 10 min), the packed cells containing free parasites were suspended thoroughly in 1 ml of ethidium bromide solution containing 10 μg of ethidium bromide in 0.15 mol/L phosphate buffered saline (PBS, pH 7.2) with the aid of a Whirlimixer (XW-80, Shanghai Medical University) and then incubated for 30 min at 25℃. After washing with 4 ml of PBS once (1500×*g*, 10 min), the stained parasites were solubilized by adding 1 ml of SDS solution containing 10 mg of sodium dodecyl sulfate in PBS. The base line scales for the pre-culture sample were adjusted to 100 in each fluoroassay and fluorescence intensity of the samples measured by a fluorescence spectrophotometer (HITACHI, Model 650-10) at excitation wave length 580 nm and at emission wave 604 nm.

Determination of parasitemia At the same time of harvesting parasites for fluoroassay, thin blood films were prepared from each of the remaining wells for parasite counting. The schizont-infected rbc were determined in the Giemsa-stained smears under microscope.

Calculation of IC_{50} The percentages of inhibition derived from comparing drugged samples with non-drugged control. Then, 50% inhibition concentrations (IC_{50}) were obtained by inputing percentages of inhibition and corresponding concentrations of drug to a computer (Model TRS-80, Radio Shack, *USA*) with the ED_{50} programme of Finney's probit analysis.

RESULTS AND DISCUSSION

In samples containing artemisinin, inhibitions of formation of schizonts were shown depending on the concentrations of artemisinin. High concentrations of artemisinin completely inhibited the maturation of schizonts and caused the degeneration of pre-existing parasites. As shown in Fig 1, dose-response curve between concentrations of artemisinin and inhibitions of fluorescence intensity is very close up to that between concentrations of artemisinin and inhibitions

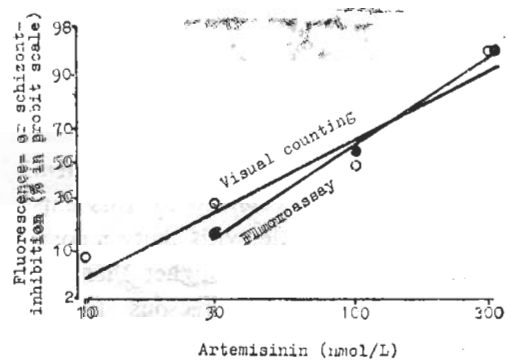

Fig 1. Efficacy of artemisinin against FCC1/HN strain of *P. falciparum in vitro* by schizont-counting and fluoroassay.

of schizont formation.

Sensitivity of *P falciparum* was compared among artemisinin, artemether and CQ by both visual counting of schizont-infected rbc and fluoroassay (Tab 1). The results showed that there were no significant differences between the IC_{50} value obtained by fluorometric determinations and that by visual counting of schizonts for the same drug. However, the fact that the 95% fiducial limits by fluorometric determination were usually broader than those by visual counting of schizonts, particularly in the case of CQ, indicated that this fluoroassay needs to be further improved. In our early study, there was a considerable correlation between the amounts of NA of *P falciparum* measured by fluoroassay and by [^{3}H] hypothanthine-uptake assay[7]. Thus, the fluoroassay system must be suitable as a supplementary method for both screening of antimalarials and determination of drug resistance of falciparum parasites in field surveys.

Tab 1. Antimalarial efficacy (IC_{50} and 95% fiducial limits in nmol/L) measured by schizont-counting and fluoroassay of artemisinin, artemether and chloroquine against FCC1/HN strain of *P falciparum in vitro*. *$p>0.01$ *vs* fluoroassay, ††$p<0.05$ *vs* artemisinin

	Artemisinin	Artemether	CQ
Schizont-counting	75.2*	29.4*††	43.2*
	64.2-88.2	23.8-36.3	38.9-47.8
Fluoro-assay	91.9	24.7††	21.4
	78.2-108	18.1-33.7	10.0-45.8

The effective concentrations of artemisinin and artemether obtained by this experiment were comparable with that reported by Thaithong[8], but were higher than that reported by Guan[9]. The reasons for the differences between ours and Guan's may be due to that: 1) the initial parasitemia in culture used by Guan was lower than that in this experiment; 2) different criterions in assessing antimalarial efficacy were adopted by each laboratory, eg we counted the schizont stage parasites only.

This *in vitro* test indicates that artemether has a higher level of schizont-killing action against *P. falciparum* than artemisinin, although both drugs had been considered to be highly active.

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荧光法测定青蒿素和蒿甲醚对体外培养的恶性疟原虫的抗疟效价

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提要　恶性疟原虫与青蒿素，蒿甲醚和氯喹一起培养 40 h 后，以溴化乙啶作荧光探针，再经 SDS 增溶后测定荧光强度，得到上述 3 个药物的 IC_{50} 分别为 75.2，29.4 和 43.2 nmol/L。这与光镜下用裂殖体计数法所得结果相当接近，相应值间无统计差异。而新的荧光测定法有下列优点：1）比形态计数法所得读数更客观，2）比放射性核素参入法简单。

关键词　恶性疟原虫；荧光光谱测定法；抗疟药；乙啶；青蒿素；蒿甲醚；氯喹

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