THE DEVELOPMENT OF AN IMMUNOASSAY FOR THE DETECTION OF ARTEMISININ COMPOUNDS IN URINE

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Abstract. We have produced monoclonal antibodies against artelanic acid and investigated the reactivity with artemisinin drugs and metabolites. Antibody F170-10 is fairly specific for artelanic acid but does bind artemisinin and artemether (3−5% cross-reactivity). Dihydroartemisinin, artesunate, and metabolites of artemisinin showed less reactivity. With this antibody, an inhibition ELISA has been set up to detect artemisinin compounds in urine. In healthy subjects who received a single oral dose of artemisinin, artemether, artesunate or dihydroartemisinin, ELISA reactivity in urine was found. This reactivity in urine paralleled the plasma concentrations of artemether and dihydroartemisinin. The results show that this immunoassay for artelanic acid can be used to detect artemisinin compounds in urine for about 8 hr after intake. With a more sensitive test, this simple method as a urine dipstick may be become useful for drug use and compliance studies in malaria-endemic areas where the artemisinin derivatives are increasingly used.

Drugs-resistant Plasmodium falciparum is a still increasing problem in malaria control. In Southeast Asia, it has reached such proportions that use of the latest antimalarial drugs, e.g., artemisinin-derived drugs, is required to treat severe malaria. Fortunately the situation in Africa, which has the bulk of the world malaria cases, is less problematic with regard to the number of antimalarial drugs that still can be used. However, the impact of the still-increasing chloroquine resistance will be substantial as replacement of chloroquine by more expensive drugs will impose severe constraints on the health budgets of those countries with low economic wealth. Early treatment is important to decrease malaria mortality, since even the most effective drugs cannot prevent an unacceptable high mortality of 20% in severe malaria. Self-treatment is common in malaria-endemic areas where the amount of antimalarial drugs sold in shops and in the markets exceeds that used through the official health channels. People often have already taken antimalarial drugs before presenting themselves at a health center. Uncontrolled use of antimalarial drugs will favor the emergence of drug-resistant strains through selection of less susceptible parasites. There are indications that due to the increasing chloroquine resistance, malaria morbidity and hospitalizations are increasing.

Although only recently introduced, artemisinin compounds can already be freely purchased in many malaria-endemic areas, despite the fact that many of these drugs are still in the process of official registration. The artemisinin-derived agents have to be taken for at least 5 days to limit the recrudescence rate when they are not combined with other antimalarials. Since these drugs act quickly, it is likely that often the full course may not be taken.

No simple methods are available for detection of intake of artemisinin derivatives, which can be used to monitor compliance and to investigate self-treatment. Various methods have been described to measure artemisinin compounds in biologic fluids, but all of these methods require the use of sophisticated equipment. In this study, we have investigated whether monoclonal antibodies produced against artelanic acid could be used to demonstrate intake of artemisinin drugs by detecting these drugs or their metabolites in urine. For this purpose, single oral doses of artemisinin, artesunate, dihydroartemisinin, and artesunate were given to two volunteers. In an additional study, artemether was administered to another six volunteers in whom plasma levels of artemether and dihydroartemisinin were monitored and compared with the immunoassay results in urine over an 8-hr period after drug intake.

MATERIALS AND METHODS

Monoclonal antibodies against artelanic acid. Monoclonal antibodies against artelanic acid were produced by established procedures. The experiments were done in mice according to the guidelines of the Dutch Law for Animal Experiments. Briefly, BALB/c mice were immunized with a bovine serum albumin (BSA)−artelanic acid conjugate prepared by coupling artelanic acid to BSA using the carbodiimide method. A similar method was used to prepare a horseradish peroxidase−artelanic acid conjugate. Mice were immunized with 25 μg of immunogen intraperitoneally using Freund's complete adjuvant and were given a booster immunization intraperitoneally with Freund's incomplete adjuvant. The mice were then given a booster immunization intravenously with the immunogen dissolved in phosphate-buffered saline (PBS) 4 days before the mice were killed. Spleen cells were fused with NS1 cells and the fusion mixture was divided over ten 96-well culture plates. After 12 days, the hybridoma culture supernatants were screened by an ELISA for the presence of specific antibody. A polymeric coating antigen was used in the ELISA in which the allylamine amide of artelanic acid was copolymerized with acrylamide.

An inhibition assay was performed concurrently using a high concentration of artemisinin (50 μg/ml). Those hybridomas were selected that produced antibodies that showed good reactivity with the coating antigen and full inhibition in the presence of artemisinin. After three recloning steps, the hybridomas were injected intraperitoneally into the mice for production of ascites. Twelve F170 monoclonal antibodies against artelanic acid were obtained.

Cross-reactivity of monoclonal antibodies to artelanic acid. Test solutions of artemisinin derivatives and metabolites were made from stock solutions in methanol. From these stock solutions, 10-fold serial dilutions in PBS were...
made. Arteether and artelinic acid were provided by Artecef BV (Maarssen, The Netherlands). Artemether and dihydroartemisinin were obtained from Novartis (Basel, Switzerland). Dihydroartemisinin, deoxyartemisinin, 3-hydroxy-deoxyartemisinin, and the furano-acetate metabolite of artemisinin were derived from artemisinin using published procedures.\textsuperscript{12,13}

**Enzyme-linked immunosorbent assay procedure.** Polystyrene microtiter plates (Costar radioimmunoassay; Corning Costar Europe, Badhoevedorp, The Netherlands) were coated with 100 µl of a 1:10,000 dilution of monoclonal antibody F170-10 in PBS for 3 hr at room temperature. Plates were washed twice (2 min/wash) with PBS/0.05% Tween-20 (PBS/Tween). Plates were used directly or stored at -20°C. Fifty microliters of a series of 10-fold dilutions of the artemisinin compounds in PBS were added to the wells of a microtiter plate. Hereafter, 50 µl of a 1:20,000 dilution in 1% BSA/PBS/Tween-20 of a horseradish peroxidase conjugate of artelinic acid was added. The plate was then incubated for 1 hr at room temperature. After washing the plate with PBS/Tween, 100 µl of a substrate solution (0.4 mg/ml of tetramethylbenzidine in 0.1 M phosphate/acetate buffer, pH 4.0, containing 0.4 mg/ml of urea peroxide) was added to all wells of the plate. After incubation for 30 min in the dark, 50 µl of 1M sulfuric acid was added. The color (optical density) was measured at 450 and 540 nm.

**Studies in healthy subjects.** The study protocol was approved by the Ethical Review Board of the Academic Med-
ical Centre, Amsterdam and written informed consent was obtained from each subject before inclusion. Results of a physical examination and routine blood and urine examinations had to show no abnormalities before an individual could participate. Medications for the volunteer studies were obtained from the following sources. Artemisinin, 250 mg capsules, were obtained from Mekophar (Ho Chi Minh City, Vietnam). Artemether, 50 mg tablets (Artenam®), were obtained from Arenco (Geel, Belgium). Artesunate, 50 mg tablets, were obtained from the Atlantic Pharmaceutical Co., Ltd. (Bangkok, Thailand). Dihydroartemisinin, 20 mg tablets (Cotecxin®), were obtained from Cotec New Technology Corp. (Beijing, China). The chemical structures are shown in Figure 1.

Two healthy subjects (coauthors of this paper [TAE and MAvA]) received single oral doses of 500 mg of artemisinin, 100 mg of artemether, 120 mg of dihydroartemisinin, and 100 mg of artesunate on four different occasions. The various artemisinin drugs were taken at an interval of at least one week. Urine samples were collected at regular intervals between 0 and 12 hr after drug intake while adequate fluid intake was ensured.

Six other healthy subjects (all students) with a mean age of 26.8 years (range = 23–35), a mean weight of 75.2 kg (range = 65–90), and a mean height of 1.81 m (mean = 1.67–1.90) and a mean plasma creatinine concentration of 68.7 µmol/L (range = 62–81) were given a single oral dose of 100 mg of artemether. Blood samples were taken before drug intake and at 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 3.5, 4, 5, 6, 7, and 8 hr after drug intake for pharmacokinetic analysis of artemether and the metabolite dihydroartemisinin. Drug and metabolite concentrations were measured by high-performance liquid chromatography (HPLC) with electrochemical detection.14 Urine samples were collected as often as possible and hereafter tested using a similar ELISA procedure as described above for the cross-reactivity studies. Urine samples were diluted two-fold and four-fold with PBS. Duplicate test samples were mixed with 50 µl of a series of two-fold diluted calibration samples starting with 1 µg/ml of artemether and 10 µg/ml of dihydroartemisinin on each plate. Results were either expressed as the percentage extinction (percentage color) relative to the control (no drug present) or calculated as arbitrary units based on a calibration series of two-fold dilutions of a 1 µg/ml solution of artemether.

![Figure 3. Structures of the artemisinin metabolites deoxyartemisinin, 4-hydroxydeoxyartemisinin, and furano-acetate metabolite.](image_url)

![Figure 4. Cross-reactivity curves of monoclonal antibody F170-10 with artemisinin and artemisinin metabolites deoxyartemisinin, 4-hydroxydeoxyartemisinin and the furano-acetate metabolite of artemisinin. O.D. = optical density; Conc = concentration.](image_url)
FIGURE 5. ELISA reactivity of in urine samples of two volunteers after intake of artemisinin, dihydroartemisinin, artemether, and artesunate expressed as a color change in % optical density (O.D.) of the control (the uninhibited reaction when no drug was present).
RESULTS

Cross-reactivity studies. All monoclonal antibodies produced against artelimic acid were tested for reactivity with other artemisinin compounds. Monoclonal antibody F170-10 showed the most promising results and has therefore been used in further studies. This monoclonal antibody resulted in 50% inhibition under optimal conditions with concentrations artelimic acid of approximately 1 ng/ml. The results of the cross-reactivity studies of antibody F170-10 to artelimic acid with different artemisinin derivatives is shown in Figure 2. Antibody F170-10 is fairly specific for artelimic acid but reacts, be it to a lesser extent, with the other artemisinin compounds. Artemether, arteether (not shown in Figure 2), and artemisinin showed 3–5% cross-reactivity compared with artelimic acid. The reactivity with dihydroartemisinin and artesunate was much lower. The 50% inhibition concentration for artemether and dihydroartemisinin were on the order of 30 ng/ml and 300 ng/ml, respectively. Results of the testing of several known metabolites of artemisinin, deoxyartemisinin, 3-hydroxy-deoxyartemisinin, and the furano-acetate metabolite (chemical structures in Figure 3) are shown in Figure 4. These results show low cross-reactivity with artelimic acid compared with artemisinin.

Studies in healthy subjects. The results of the tested urine samples collected from the two coauthors after subsequent intake of artemisinin, artemether, dihydroartemisinin, and artesunate are shown in Figure 5. Despite the fact that 500 mg of artemisinin was given compared with 100 mg of the other drugs, less reactivity (inhibition) was seen in urine samples collected after intake of artemisinin than with the other drugs. When the same volunteer took a second dose of artemisinin after a wash-out period of two weeks, comparable results were obtained. Similar reactivity patterns were found upon testing the urine samples in both subjects after administration of the same series of artemisinin drugs.

The results obtained in the pharmacokinetic study in six healthy subjects to whom a single oral dose of 100 mg of artemether had been given are shown in Figures 6 and 7. These results are expressed as arbitrary units using a calibration series of artemether concentrations. Each result of the spectrophotometer was divided by the urinary creatinine concentration in that urine sample to correct for the variation in the time interval between urine collections and volume of each urine portion. This way the results were comparable within one subject and between subjects assuming a constant and similar creatinine clearance in the 6 subjects.

The results of the urine samples of all volunteers are shown in Figure 6. They show that a peak reactivity is reached after 2–5 hr and that the reactivity is almost negligible after 8 hr. The results of the urine reactivity together with the plasma artemether and dihydroartemisinin concentrations are shown for two of the volunteers in Figure 7. In this plot the urine results are placed at the time corresponding to the midpoint of the collection period. The reactivity found in the urine samples paralleled the course of the plasma concentrations of artemether and
dihydroartemisinin. As expected, a linear relationship was found between the urinary excretion rate and the plasma concentrations of the drug. The sharp increase and decrease versus the plateau in plasma concentrations of artemether and dihydroartemisinin that can be seen in volunteers A and B, respectively, are closely followed by the excretion in urine. A selected number of urine samples were also analyzed by HPLC. Artemether was not detected in any of the urine samples and only small amounts of dihydroartemisinin were detectable.
Assays in urine dipstick formats have been developed for a range of antimalarial drugs, e.g., chloroquine, quinine, mefloquine, pyrimethamine, and proguanil, based upon immunochemical techniques.\textsuperscript{15,16} We now extended this experience to the artemisinin drugs since there are no simple tests currently available for these group of antimalarial drugs. Such tests, preferably in a dipstick format, could be used in drug compliance and pharmaco-epidemiology studies.\textsuperscript{16}

Antibodies against artemisinin compounds have been produced by several groups using artesunate and the carboxymethyl derivative of dihydroartemisinin.\textsuperscript{9,17–19} These antisera showed high cross-reactivity with other artemisinin derivatives. In this study, we described the production of monoclonal antibodies produced against artelnic acid. Using these antibodies, we developed a highly sensitive test for artelnic acid. We have investigated whether these antibodies can also be used to detect artemisinin compounds (or metabolites) in urine after intake of artemisinin drugs. We found that the antibodies against artelnic acid do cross-react with other artemisinin drugs but not to a very high extent. Other investigators using polyclonal antibodies against other conjugates from dihydroartemisinin reported high cross-reactivity with other artemisinin derivatives.\textsuperscript{19} From cross-reactivity studies with known artemisinin metabolites as shown in Figure 4, it appeared that immune reactivity of the antibody requires the presence of an intact peroxide bridge. This confirms earlier observations by another group.\textsuperscript{19} On the other hand, the low reactivity of the antibody with dihydroartemisinin and artesunate in comparison with artemether and artemether is not readily explained. In our immunogen, we have a different linker (\(p\)-hydroxymethylbenzoate) at the 11-position of the dihydroartemisinin molecule, and part of this linker may be involved in optimal recognition.

We have attempted to enhance the sensitivity of the assay through affinity manipulation by using other peroxidase-artemisinin conjugates. Artesunate and the carboxymethyl derivative of dihydroartemisinin were coupled to peroxidase. However, little improvement of the sensitivity was obtained, and more importantly, these conjugates were vary labile in our hands. Using the artelnic acid-peroxidase conjugate, we showed that the reactivity with artemether and artemisinin was such that it was feasible to detect these drugs in urine. The structures of several metabolites of artemisinin have been elucidated. However, there are no reports about concentrations of these drugs and their metabolites in urine. Although it is thought that artemisinin drugs are extensively metabolized by the liver, this study shows that some degradation product of this group of drugs is excreted via the urine. The urine concentrations of artemisinin compounds (e.g., inhibition) that were found after intake of artemether followed a similar time-course as the plasma artemether and dihydroartemisinin concentrations. Simultaneous with a peak in the plasma drug concentration, a peak in urine reactivity was observed and after 8 hr, when hardly any artemether or dihydroartemisinin was detected in the blood, the reactivity in the ELISA was also very low. Based on HPLC analysis of selected urine samples with peak ELISA reactivity in our study, it was clear that no artemether is excreted into the urine and that only very low concentrations of dihydroartemisin (< 20 ng/ml) were present in the urine. Dihydroartemisinin is produced from all four investigated artemisinin compounds studied, although in case of artemisinin this may be only to a very minor extent. The concentrations dihydroartemisinin in urine after intake of artemether were such that these could never account for the reactivity (inhibition) seen in the ELISA. Therefore, the ELISA reactivity found in the urine samples cannot be explained on basis of the presence of artemether or dihydroartemisinin or other unchanged drugs in the urine. The reactivity is either caused by high concentrations of low cross-reacting compounds, such as metabolites without an intact peroxide bridge, or due to unknown metabolites.

In studies with mice, it has been shown that only a small amount (< 1\%) of radiolabeled dihydroartemisinin was excreted into the urine, but a considerable amount was excreted into the bile in the form of glucuronides.\textsuperscript{20} No mention has been made of glucuronide metabolites of dihydroartemisinin in humans. However, the presence of these metabolites may very well explain the results obtained in this study. The test for artelnic acid can be used to detect artemisinin compounds in urine for about 8 hr after intake. With a more sensitive test this may be extended and become more useful for drug use studies with artemisinin derivatives. When artelnic acid is introduced as a new antimalarial drug, being more stable with a more favorable pharmacokinetic and toxicologic profile than the current analogs, it is evident that the value of this assay will improve.\textsuperscript{21}

\textbf{REFERENCES}


