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Low-cost, high-speed identification of counterfeit antimalarial drugs on paper

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ABSTRACT

With the emergence of artesunate antimalarial counterfeiting in Southeast Asia and sub-Saharan Africa, we present the production of a rapid, inexpensive and simple colorimetric-based testing kit for the detection of counterfeit artesunate in order to preserve life and prevent the development of multi-drug resistant malaria. The kit works based on paper microfluidics which offer several advantages over conventional microfluidics, and has great potential to generate inexpensive, easy-to-use, rapid and disposable diagnostic devices. Here, we have developed a colorimetric assay that is specific to artesunate and turns yellow upon addition of the sample. The test can be done within minutes, and allows for a semi-quantitative analysis of the artesunate tablets by comparing the developed yellow color on the paper test to a color-coded key chart that comes with the kit. A more accurate and precise analysis is done by utilizing a color analyzer on an iPhone camera that measures the color intensity of the developed color on the paper chip. A digital image of the chip was taken and analyzed by measuring the average gray intensity of the color developed on the paper circle. A plot of the artesunate concentration versus the average gray scale intensity was generated. Results show that the intensity of the yellow color developed on the paper test was consistent and proportional to the amount of artesunate present in the sample. With artesunate concentrations ranging from 0.0 to 20 mg/mL, a linear calibration plot was obtained with a detection limit of 0.98 mg/mL.

1. Introduction

Artesunate, the most prevalent artemisinin derivative, is an anti-malarial drug that is critical in the treatment of severe malaria infections and multi-drug resistant infections caused by *Plasmodium falciparum*. The mortality rate of untreated severe malaria is reported to be 100%, though the mortality rate drops to 15–20% with proper treatment [1]. The rapid therapeutic response and high tolerability have made these artemisinin drugs essential in treating the infection, and have been used extensively in Southeast Asia. Thus, it is essential that those with severe malaria be treated rapidly with this life-saving artesunate drug. Owing to the emergence of artesunate anti-malarial drug counterfeiting in Southeast Asia and sub-Saharan Africa, we developed a rapid, inexpensive, simple colorimetric-based test kit for the detection of counterfeit artesunate. The artesunate detection kit is a novel product and well suited for resource-poor settings, and improves on the current lab-based testing practice with a portable, semi-quantitative, field-suitable test that removes the manual steps required during sample processing. The kit will bring an innovative approach to address a global health challenge facing the developing world, where counterfeiting of artesunate has become a significant threat to our ability to counter the spread of malaria.

In Southeast Asia and sub-Saharan Africa there is a growing problem of counterfeiting of artesunate drugs. At a cost of approximately US$ 1–2 per adult treatment, the relatively high priced artesunate drugs have stimulated the growth of counterfeit artesunate production. Such counterfeits contain sub-therapeutic quantities of artesunate or no active drug at all, and have resulted in loss of life due to inadequate treatment of malarial infection [2,3]. According to The World Health Organization, counterfeiting of anti-malarial drugs leads to an estimated 20% of the 1 million deaths a year from malaria [4]. It has been reported that artesunate counterfeits comprise 38–53% of the drugs in Cambodia, Laos, Myanmar, Thailand, and Vietnam [5]. Artesunate is also used to treat multi-drug resistant *P. falciparum* in locations where malaria has developed resistance to previously used drugs. While artesunate is still largely effective against multi-drug resistant malaria,

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sub-therapeutic treatment with such counterfeit drugs can promote the emergence of new multi-drug resistant malaria. Thus, counterfeiting artesunate not only leads directly to patient mortality by not obtaining adequate treatment, but it may exacerbate the problem further via the development of drug resistant malaria [6].

Identification of authentic artesunate is therefore essential to prevent fatalities. Detection of artesunate by the standard spectrophotometric method is proven to be challenging since it absorbs light only at low wavelengths, has a low molar extinction coefficient, and retains no distinct UV/vis spectra or fluorescent properties. Artesunate can be identified and quantified by High Performance Liquid Chromatography (HPLC); however, such instrumentation is not typically available in developing countries [7–9]. More laboratories are needed worldwide to test the authenticity of the drugs. Only one out of 47 countries in Africa where malaria is endemic is equipped to perform the test.

The US Centers for Disease Control and Prevention (CDC) has developed a colorimetric test for artesunate that is both rapid and inexpensive in the laboratory. The test involves reaction of an alkali decomposition product of artesunate with the diazonium salt solution of Fast Red TR [5]. In the presence of artesunate, a yellow color is produced [10,11]. We developed a kit that contains a paper-based test strip that carries out the colorimetric assay upon addition of the artesunate sample. Hence, the manual multi-step processes of adding and mixing of the chemicals involved in the currently used colorimetric artesunate test are removed, and the test can be rapidly performed in locations such as remote hospitals, pharmacies or in the field where resources are limited.

There have been several recent advancements in the fabrication of paper microfluidic chips. Fabrication of paper-based chips can be done by photolithography [12], wax printing [13,14], plasma etching [15], inkjet etching [16], and use of a cutting plotter [17]. Unlike traditional microfluidics, which often require pumps to move fluid through the microfluidic channels, paper microfluidics can be performed without such instrumentation due to the flow of fluid being driven by capillary action through the paper. Hence, paper-microfluidics are well suited for use in point-of-care diagnostics and developing countries where expensive instrumentation is not available.

The paper test is divided into layers with the appropriate dried reagents, and the easily prepared sample solution will flow through the regions via capillary action in order to carry out the artesunate detection test. The chemicals required to perform the assay cost approximately US$0.02 per test, which makes it a very practical solution to detect counterfeits [18]. Since the reagents for the colorimetric test are stored on paper in dry form, they are more stable and easier to transport, which provides advantages of easy handling and longer shelf life. Furthermore, while most counterfeiters are producing fake drugs that lack artesunate, some drugs are made with significantly lower active ingredients, which are incapable of killing all the parasites. This is an effort by the counterfeiters to misleadingly pass the test when a qualitative (positive/negative) evaluation is made to determine the presence of artesunate in the drug. Our test kit allows for quantitative analysis of artesunate tablets by providing a key that comes along with the kit and allows for comparison of the developed yellow color with the intensity of the yellow color corresponding to the approximate concentration of artesunate.

Our test device is capable of rapidly and inexpensively detecting artesunate in commercially available drugs. In this work, we developed a disposable paper-based microfluidic test equipped with a color indicator that turns yellow in the presence of artesunate. The chip is constructed from paper materials, with all the required chemicals and reagents to perform the test stored directly in the device. The artesunate sample can then be applied directly onto the test strip/device for measurement. The color will fully develop within minutes so that the user can determine whether artesunate is present in the drug formulation at a therapeutic dose or not. The kit provides a color-coded chart, similar to that accompanying pH paper, which is used to determine the relative concentration of artesunate in the tablet. The paper-based assay, together with the accompanying color chart provides a reliable semi-quantitative measurement of artesunate in a tablet. The rapid, simple, and inexpensive test is especially useful when used as a screening tool for counterfeits in remote areas.

For a more accurate quantitative measurement, we utilized a color analyzer on a camera phone to take a digital image of the

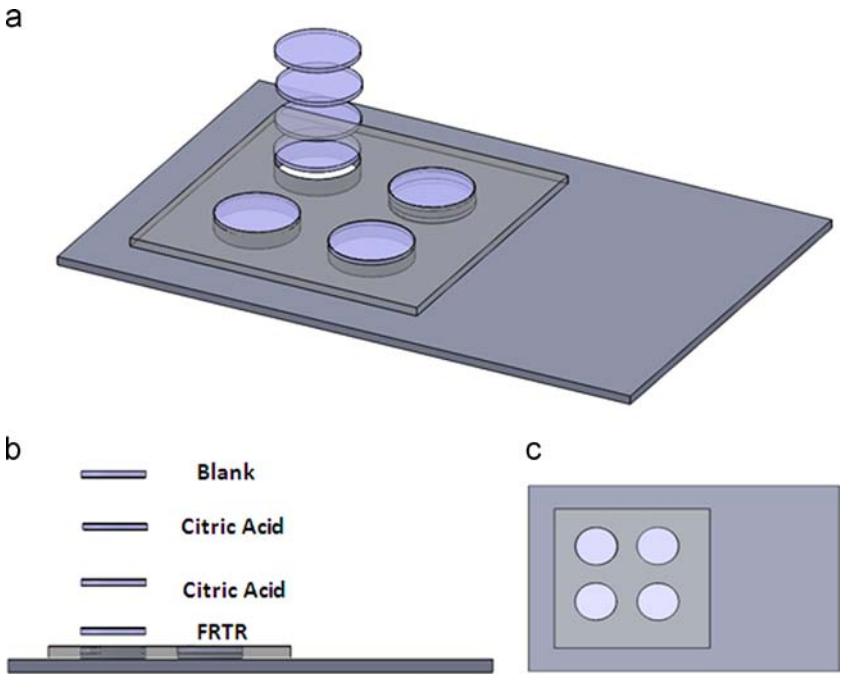

Fig. 1. Schematic diagram of the paper test kit.

chip and analyze the color developed on the paper test by measuring its gray intensity. To accomplish this, an iPhone camera equipped with a color analyzer app called ColorAssist is used to analyze the RGB value of the yellow color produced, and converts it into an average gray scale intensity. The intensity of the yellow color developed on the paper test was consistent and proportional to the amount of artesunate present in the sample. With artesunate concentrations ranging from 0.0 to 20 mg/mL, a linear calibration plot was obtained with a detection limit of 0.98 mg/mL.

2. Experimental

2.1. Paper microchip fabrication

The microchip was fabricated using Whatman filter paper (Grade 3 1003-055) or glass microfiber GF/B (both from Sigma Aldrich, St. Louis, MO 631778, USA). The filter paper is cut into circular test pads using a hole-punch (8-mm diameter). We designed the paper microchip to enable fluid transport vertically by stacking the paper test pads. Each of the paper cut out has been pretreated by spotting them with different reagents and allowed to dry. We assembled the pads as shown in Fig. 1, and secured the stack on a plastic holder made of polymethyl methacrylate (PMMA). Four holes of 8-mm diameter were drilled into the PMMA piece, and the pad-carrier was laminated with a plastic cover on one side to secure the test pads in place. The PMMA piece was then placed on the Artesunate Colorimetric Field Test coupon provided with a color chart guide (Fig. 1).

2.2. Reagent preparation

Sodium chloride and sodium citrate were purchased from Mallinckrodt (Phillipsburg, NJ, USA). Fast red TR salt, artesunate, artemisinin, NaOH, and citric acid were purchased from Sigma (St Louis, MO, USA). The reagents were prepared and loaded on the pad stack. We spotted 3 M citric acid buffer (25 μL) and 5 mg/mL fast red TR solution (25 μL) on the 8-mm paper pad stack. The spotted reagents were then allowed to air dry at room temperature.

2.3. Antimalarial drug testing

A series of artesunate solutions ranging from 1 to 20 mg/mL were prepared by diluting a 60 mg/mL stock solution in 1 M NaOH. We selected three commercially available drugs, one containing artesunate (Guilin Pharmaceutical, China), and two other anti-malarial drugs containing artemeter (Artem, Kunming Pharmaceutical Corp, China) and dihydroartemisinin (Arterakine, Pharbaco Central Pharmaceutical, Vietnam). The drugs were prepared at 5 mg/mL and tested against a standard.

2.4. Colorimetric assay with iPhone

Color measurement of each chip was obtained by using an iPhone app (ColorAssist). Sample was applied to the paper chip and allowed to incubate for 5 min for color development. Analysis of the image was performed by measuring the RGB value of each spot, and converted to average gray intensity. A plot of average gray intensity versus substrate concentration was generated using Excel.

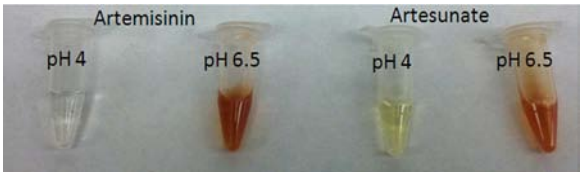

Fig. 2. pH dependence of FRTR colorimetric test in solution. Artemisinin turns an orange color at pH 6.5, while giving no color at a pH of 4. Artesunate turns an orange color at pH 6.5, while giving a yellow color at a pH of 4.

3. Results and discussion

Artesunate is the most widely used artemisinin derivative throughout the world for the treatment of severe malaria infections and multi-drug resistant infections caused by *P. falciparum*, yet more than one-third of the antimalarial drugs tested over the past decade in Southeast Asia and sub-Saharan Africa were either adulterated or of poor quality [19–22], containing the incorrect chemical formulation. Therefore, identification of authentic artesunate is essential to prevent needless fatalities. A colorimetric test has been developed by the CDC to determine the authenticity of artesunate in tablets. The test is based on the reaction of an alkali decomposition product of artesunate with a diazonium salt, fast red TR (FRTR), that results in an appearance of a yellow color in the presence of artesunate. The colored reaction product can be measured by spectrophotometry at 420 nm. The test is specific to artesunate when conducted at pH 4 [4].

In this work, we used Whatman filter paper to fabricate an effective, portable, low-cost paper-based test kit. The test kit consisted of four layers of paper, each containing different reagents, similar to what is used in lateral flow chip design. Each of the reagents was spotted on the paper and air-dried at room temperature. Since artesunate specificity is pH dependent, it was necessary to maintain the reaction at the proper buffer condition in each layer: from sample introduction to the final indicator layer. The test chip was assembled by stacking the paper circles to enable fluid transport vertically. We found that the vertical design yielded optimal pH control, and maintained pH 4 as the sample solution traveled down along the paper layers. Here, the sample flowed via capillary action through test zones pretreated with the appropriate reagents along the test path. Flow systems such as shown here are ideal platforms for sequential chemical processing. In addition, the vertical flow design resulted in reduced sample size and a more consistent yellow color product, in contrast to a more uneven band spreading found in the lateral flow design.

The specificity of FRTR colorimetric detection is dependent on the pH of the reaction. Among a list of other anti-malarials including artemisinin, artemether, chloroquine, quinine, primaquine, sulfadoxine and pyrimethamine, artesunate is the only drug that gives a yellow color at a pH of 4 [23]. Therefore, the reaction must be maintained at pH 4 in order to be specific to artesunate, and not give positive test results in the presence of certain other anti-malarials. We tested the specificity of the artesunate test on another anti-malarial drug, artemisinin. When the reaction was performed at pH 6.5, both the artesunate and artemisinin samples turned orange, indicating that the pH of the test was incorrect, resulting in invalid measurements. At pH 4, the artemisinin yielded no color product, while the artesunate generated a yellow color product. This test validated the specificity of the artesunate test at pH 4, as shown in Fig. 2.

The same reaction scheme was used to develop the paper microchip. In order to maintain the solution at pH 4 prior to mixing with FRTR, the volume ratio of the 1 M NaOH:3 M citric acid buffer was adjusted. For consistent test conditions, we used equal size of paper circles of 8-mm diameter, and spotted them with the citric acid and FRTR reagents. Each circle received equal volumes of the reagents (25 μL) and was allowed to dry at room temperature. When fully dried, the paper circles were assembled as shown in Fig. 1, and were ready for use. Next, 100 μL of the

Fig. 3. (a) pH dependence of FRTR colorimetric test on paper. (b) pH test of 1 M NaOH solution added on two layered paper chips that were preloaded with citric acid buffer (left) and a blank (right).

dissolved artesunate tablet was pipetted on the test device and allowed to react for 5 min for the color to fully develop.

The chip design was evaluated for its specificity to artesunate. Both artesunate and artemisinin were applied to paper. The far left image in Fig. 3 shows a test result for artemisinin as added to a single paper layer containing both the dried citric acid buffer and the FRTR reagent (equal volumes). An orange color developed, indicating that the test was not done at the proper pH of 4. The next image shows a test where artemisinin was added to a four-layered chip. The first layer was a blank serving as an absorbent pad, and was followed by two layers of dried citric acid, with a bottom layer containing dried FRTR reagent. No color developed as expected when the pH of the reaction occurs at the desired pH of 4. The four-layered chip allowed for the basic sample solution to react with the citric acid layers, and adjusted it to the appropriate pH of 4 as the analyte reached and the bottom layer containing the FRTR reagent. An orange color developed when artesunate was added to the one-layer chip (containing dried citric acid buffer and dried FRTR), indicating a pH above 4 when the reaction occurred. A yellow color developed as expected when artesunate was added to the four-layer chip at the desired pH of 4 as shown in the last picture. An additional pH test was also conducted by adding the 1 M NaOH solution onto a two-layered paper chip that was preloaded with the citric acid buffer in each layer. The pH of the solution was measured at the base of the paper chip, and was confirmed to give a pH reading between 3.5 and 4.0 (Fig. 3b).

We employed the fabricated paper chip for the detection and measurement of artesunate solutions of varying concentration. For this test, the four-layer paper device design, as shown in Fig. 1, was used. The first layer contained no reagent and served as an absorbent pad for addition of sample solution. The second and third layers contained the dry citric acid buffer reagent to adjust the pH of the basic sample solution (artesunate dissolved in 1 M NaOH) to the proper pH of 4. The final layer contained the dry FRTR reagent required for the colorimetric assay. The test showed that it was capable of detecting the presence (or absence) of artesunate in the sample as indicated by the color change. After a 5 min incubation time, the yellow color was developed. The determination of artesunate concentration was established using a calibrated color chart guide. The test showed no color development when blank was added on the paper chip indicating the absence of artesunate in the sample. A yellow color was produced when artesunate was present, with darker colors indicating higher artesunate concentration in the tablet. The increasing color intensity developed on the paper chip was consistent with the increased amount of artesunate in solution with 0.5 mg/mL being the lowest and 10 mg/mL the highest concentration tested. As can be seen from Fig. 4, the more concentrated artesunate solution resulted in a darker yellow color, with decreasing intensity as the concentration of artesunate is decreased. We were able to obtain reproducible color products for detection and measurement of artesunate using the test paper.

The performance of the paper test kit was assessed using commercially obtained anti-malarial drugs that are currently being used to treat patients. At the time of this study, we had no access to real counterfeit drugs to evaluate the paper test kit. Therefore, as proof of concept, the test was conducted on other

Fig. 4. Semi-quantitative data utilizing the designed paper test device. From left to right: decreasing concentrations of artesunate from 10 mg/mL to 0 mg/mL.

antimalarial drugs to demonstrate the specificity of the test kit in identifying artesunate and its accuracy in measuring artesunate concentration. We selected three commercially available drugs, one containing artesunate (Guilin Pharmaceutical), and two other anti-malarial drugs containing artemeter (Artem) and dihydroartemisinin (Arterakine). From the three drugs evaluated, only the sample containing artesunate from Guilin Pharmaceutical tested positive for artesunate while the other two drugs showed the absence of artesunate in both samples. All three antimalarial drugs were prepared at 5 mg/mL for testing. Using the paper test, we were able to determine the presence of artesunate in the Guilin drug at the correct concentration range based on the color chart reading (Fig. 5). The test was capable of successfully detecting the presence (or absence) of artesunate in real samples and their relative concentration based on a calibrated color chart.

The color chart assay proved to be a reliable method for detecting artesunate, especially when used as a field-test kit in remote areas. For a more accurate analysis, we utilized a color analyzer on a camera phone that measured the gray scale intensity of the yellow color developed on the test paper. After the reaction was complete, a digital photo was taken of the paper chip. The image was analyzed by measuring the intensity of yellow color developed on the paper circle. We used an iPhone camera equipped with the "ColorAssist" application to obtain red, green, and blue component data, and then convert to gray scale intensity. ColorAssist is an app that uses the iPhone or iPad camera to capture RGB values in real time. The iPhone camera was positioned at a set distance from the test paper to obtain reproducible and consistent pictures throughout the analysis. A plot of the artesunate concentration versus the average gray scale intensity was generated as shown in Fig. 6. The data showed that the intensity of the yellow color developed on the paper test coupon was consistent and proportional to the amount of artesunate present in the sample. With artesunate concentrations ranging from 0.0 to 20 mg/mL, a linear calibration plot was obtained with a detection limit of 0.98 mg/mL ($n=6$).

4. Conclusions

We have demonstrated proof-of-concept for detecting counterfeit drugs using a paper based colorimetric assay. This approach is suitable and can serve as a rapid, simple, and cost effective means that can be used by the end user to test the authenticity of the drug in question. In addition, the simple detection technique could benefit government officials and local pharmacies as a rapid screening method to combat the growing problem of drug counterfeiting today. Our future studies will include optimizing the assay to achieve lower limit of detection, and improved image analysis by

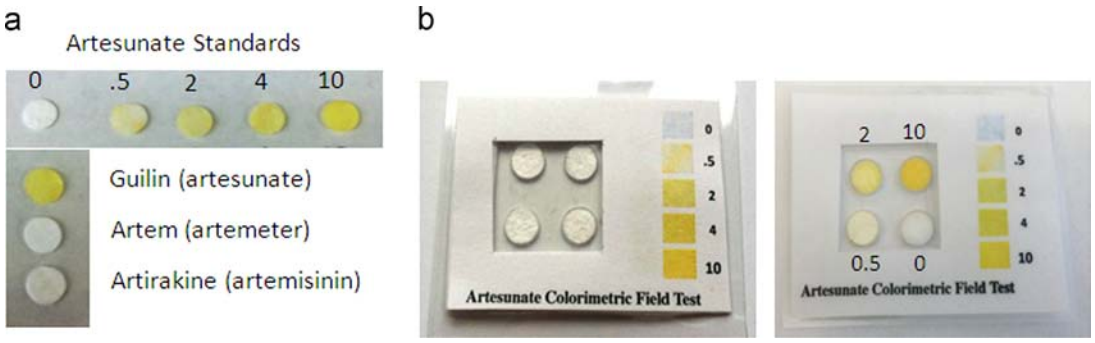

Fig. 5. (a) Semi-quantitative data utilizing the designed paper test device. From left to right is increasing concentrations of artesunate from 0 mg/mL to 10 mg/mL. The real anti-malarial drugs were prepared at 5 mg/mL, tested and compared with the standard. (b) The Artesunate Field Test Kit.

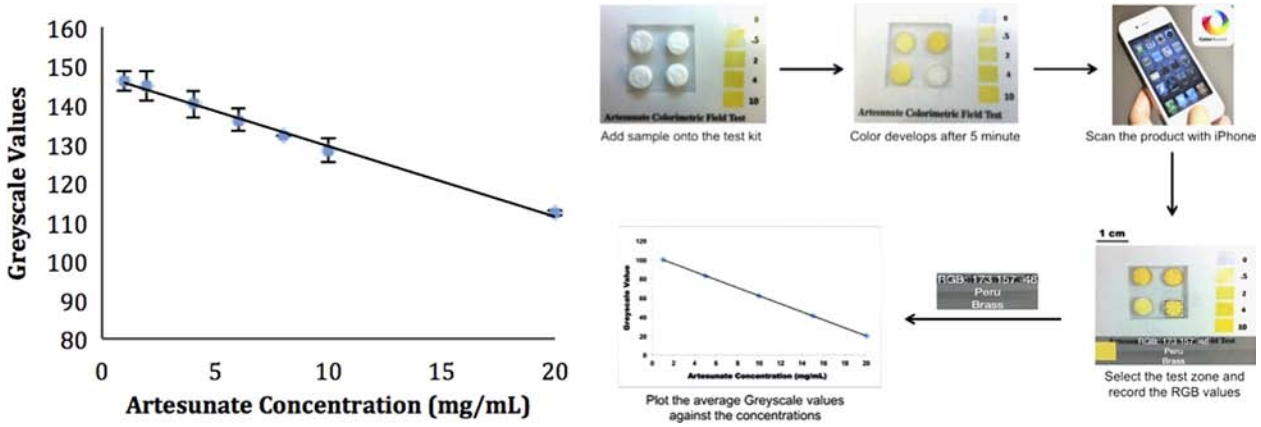

Fig. 6. Plot for the mean intensity of the color produced versus the artesunate concentration with iPhone ColorAssist app. The plot shows very good linearity (R^2 value = 0.9945) with detection limit of 0.98 mg/mL.

developing a paper device and a color sensor that further enhance sensitivity of the measurement.

Artesunate has been the most widely used artemisinin derivative throughout the world for the treatment of severe malaria infections and multi-drug resistant infections caused by *P. falciparum*. More than one-third of the antimalarial drugs tested over the past decade in Southeast Asia and sub-Saharan Africa have been shown to be counterfeit or of poor quality, containing the incorrect chemical formulation. This could undo decades of international effort to fight malaria, and will promote the spread of resistance against artemisin-based drugs. Therefore, identification of authentic artesunate is essential to prevent fatalities. We developed a paper-based colorimetric assay for the detection of antimalarial counterfeits. The test is capable of successfully detecting the presence (or absence) of artesunate in real samples and their relative concentration based on a calibrated color chart. This paper based assay, together with a calibrated color chart guide would be an extremely useful field test kit in providing a semi-quantitative analysis of the artesunate drug in question. Using a color analyzer on an iPhone, we were able to successfully perform a more quantitative colorimetric reading. A digital picture was taken and analyzed based on its gray intensity that linearly correlated proportional to its artesunate concentration. Future development of the device will include lower limit of detection of the assay and integration of a calibrated color analyzer that would enable a direct quantitative measurement by scanning the paper device.

Paper-based microfluidics assays have tremendous potential in the field of medical- and drug-diagnostics. We envision that this technology will open up a new approach and platform for detecting counterfeit drugs. Our future work will focus on improving the architecture and expanding the range of applications of the device proposed above to achieve faster and more accurate determination of counterfeit pharmaceuticals. In addition, a simple color reader application (app) that will allow users to determine the concentration values of the pictures taken is currently being developed in our lab. Because this is a platform development effort, once established, the technology can be utilized to address a wide range of applications. This would further demonstrate the advantage of this platform over current lab-based methods of drug detection, which require extensive sample preparation, manual processing steps and expensive instrumentation.

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