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A colorimetric field method to assess the authenticity of drugs sold as the antimalarial artesunate

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Abstract

Artesunate is the most widely used of the artemisinin derivatives. These drugs are being used increasingly throughout the tropical world, and are an essential component of the treatment of multi-drug resistant malaria. The recent and widespread appearance of counterfeit artesunate tablets in several countries in Southeast Asia poses a serious threat to health in this region. We have developed a simple, inexpensive colorimetric test to determine artesunate authenticity in tablets. The test is based on a reaction between an alkali decomposition product of artesunate and a diazonium salt, fast red TR (FRTR). The appearance of a yellow color indicates the presence of artesunate. The specificity of the test is dependent on the pH of the reaction. Among other antimalarials tested, (i.e. artemisinin, artemether, chloroquine, quinine, primaquine, sulfadoxine, and pyrimethamine) only artesunate produced a positive color reaction at pH 4. The assay requires only 1% of the total weight of a standard tablet containing 50 mg of artesunate and can be completed within 10 min. The method was tested on six genuine artesunate tablets and six counterfeit artesunate tablets obtained in Southeast Asia. The average amount of artesunate in the genuine tablets was determined to be 50.8 ± 2.9 mg while the counterfeit tablets were found to contain no artesunate. © Published by Elsevier Science B.V.

Keywords: Artesunate; Artesunic acid; Artemisinins; Colorimetric; Malaria; Fast red TR; Diazonium salt

1. Introduction

Artesunate or artesunic acid (ARTS) is the most widely available and widely used of the artemisinin derivatives. These drugs have become an essential component of the treatment of multi-drug resistant falciparum malaria. They are now used extensively in Southeast Asia, where resistance to many standard antimalarials has appeared [1]. Artemisinin (Qinghaosu) and its derivatives were developed in China, where the majority of the compounds used today are still

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manufactured. The rapidity of action and thus therapeutic response, the excellent tolerability, and the relatively high price (approximately US$ 1–2 per adult treatment) of the artemisinin compounds has stimulated the production of counterfeit ARTS preparations. In some areas the presence of these counterfeit drugs pose a major threat to health, approximately 90% of artesunate available in the market place in Cambodia today is fake (J. Rozendaal, personal communication).

ARTS is a semi-synthetic derivative of artemisinin, a naturally occurring sesquiterpene endoperoxide [2]. It is a particularly difficult compound to detect and identify by standard spectrophotometric methods, since it absorbs light only at low wavelengths, has a relatively low molar extinction coefficient, and has no distinct UV/vis spectra or fluorescent properties. The standard method used to determine the authenticity of ARTS tablets involves high performance liquid chromatography (HPLC). In many countries, resources to purchase and maintain such equipment are not always available, therefore, they are particularly vulnerable to the growing problem of counterfeit ARTS as well as other drugs. At the request of United States Agency for International Development, our laboratory employed HPLC separation and diode array detection to verify the presence of counterfeit ARTS tablets in Southeast Asia. In response to a need for a less sophisticated verification method, we have developed a simple, inexpensive, field-adapted colorimetric test to determine ARTS authenticity in tablets.

Antimalarials such as chloroquine and sulfadoxine have particular chemical groups that react with certain compounds to give color reactions [3,4]. Although ARTS does not possess these reactive groups, we discovered that an alkali decomposition product of ARTS readily reacts under mild conditions with diazonium salts to produce a visually distinct yellow product. This product absorbs visible light at 420 nm with a positive correlation between absorbance intensity and ARTS concentration.

2. Materials and methods[1]

2.1. Reagents and apparatus

All reagents were prepared with deionized water and were of analytical reagent grade. Fast red TR salt (FRTR), reagent grade, dye content ~ 20% was obtained from Aldrich (Milwaukee, WI, USA). ARTS, analytical grade was obtained from Mepha (Aesch-Basel, Switzerland). Buffers were prepared by adding a solution of 0.2 M boric acid/0.2 M acetic acid/0.2 M phosphoric acid or 0.37 M monopotassium phosphate to 1 N NaOH. All reactions took place at ambient temperature (22–25°C). Genuine artesunate tablets (50 mg ARTS) were manufactured by Guilin Pharmaceutical Factory, Guilin, China. The ARTS content of these tablets was verified in our laboratory by HPLC analysis using diode array detection. The counterfeit tablets (identical in appearance to the genuine tablets) showed no measurable content of ARTS using the analysis method described above.

Absorbance measurements were obtained with a Beckman DU-7 spectrophotometer (Beckman, Irvine, CA, USA). All reactions were conducted in 13 × 100 mm borosilicate glass tubes and absorbance measurements taken at 420 nm.

2.2. Optimization of method

2.2.1. Kinetics of artesunate alkali decomposition

The diazo-coupling reaction of FRTR, a diazonium salt, with ARTS depends on the formation of an alkali decomposition product of ARTS. This compound absorbs UV radiation at 290 nm [5]. Formation of the decomposition product (ARTS290) in 0.1, 1, and 5 N NaOH was observed by monitoring absorbance at 290 nm over time. At selected time intervals, 0.5 ml of ARTS290 in 1 N NaOH was removed and 1.5 ml of FRTR (10 mg ml^{-1} in 0.37 M KH_2PO_4) was added (final pH 7). After 5–10 min at room temperature the intensity of the yellow reaction product was measured by recording absorbance at

[1] The use of trade names is for identification only and does not imply endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

420 nm. From these data, optimum conditions for conversion of ARTS into ARTS290 were established.

2.2.2. Optimization of reaction pH

The effect of pH on the formation of the yellow reaction product (ARTS420) was observed. ARTS (1 mg) was incubated in 0.5 ml of 1 N NaOH for 20 min. The pH was adjusted to 4, 6, and 8 by the addition of 0.2 M boric acid/0.2 M acetic acid/0.2 M phosphoric acid solution. Water was added to maintain a consistent volume of 2.84 ml for each sample. One-half milliliter of FRTR (15 mg ml^{-1} in 0.2 M boric acid/0.2 M acetic acid/0.2 M phosphoric acid) was added and the solution allowed to react for 10 min. Corresponding blank solutions containing no ARTS were prepared and used to 0 the spectrophotometer. Absorbance at 420 nm was measured for each sample.

2.2.3. Assessment of fast red TR concentration on assay linearity

Quantitative analysis of ARTS content in a tablet relies on the correlation of the absorbance of the reaction product with ARTS concentration, therefore, the effects of FRTR concentration on the linearity of a standard curve were observed. Analytical grade ARTS (0.05, 0.125, 0.25, 0.5, and 1 mg) was added to 0.5 ml 1 N NaOH. These amounts corresponded to 0.10, 0.25, 0.50, 1.0, and 2.0% of the total weight of an ARTS tablet containing a standard dose of 50 mg. After 20 min, 1.5 ml of FRTR (1, 5, or 10 mg ml^{-1} in 0.37 M KH_2PO_4) was added. The final pH of the solution was 7. Absorbance measurements were taken at 5, 15, 30, and 60 min for each ARTS and FRTR concentration. The colored product was extracted into 2 ml of ethyl acetate and absorbance of the organic fraction was measured at 420 nm.

2.2.4. Method specificity

Specificity of the method for ARTS was evaluated at pH 4, 6, and 8 with other commonly used antimalarial drugs (i.e. artemisinin, artemether, chloroquine, quinine, primaquine, sulfadoxine, and pyrimethamine) using the procedure described in Section 2.2.2. The amounts of drug tested represent about 1% of the dosage typically found in tablets.

2.2.5. Quantitative determination of artesunate in genuine and counterfeit tablets

Approximately 1% of the total weight of each tablet was scraped from the tablet using a scalpel. The scrapings were weighed and transferred to 13×100 mm borosilicate glass tubes. A standard curve was prepared with samples containing 0 (blank), 0.25, 0.5, 1.0 mg of analytical grade artesunate. One-half milliliter of 1 N NaOH was added, the tubes gently swirled and the samples were allowed to sit at room temperature for 20 min. Then 1.34 ml of FRTR (5.6 mg ml^{-1} in 0.2 M boric acid/0.2 M acetic acid/0.2 M phosphoric acid) was added and the tubes were gently swirled. Final pH of the solution was 6. After 5 min, a distinctive yellow color appeared in the genuine artesunate tablet samples. For quantitative analysis, the yellow reaction product was extracted from the water insoluble tablet excipients by adding 2 ml of ethyl acetate and vigorously shaking the capped tubes. After phase separation, the upper organic phase was transferred to 13×100 mm borosilicate glass tubes and the absorbance measured at 420 nm. Absorbance measurements were 0 to the blank. Artesunate content for each tablet was determined from the standard curve. For comparison, a Fansidar tablet (500 mg sulfadoxine/25 mg pyrimethamine, Hoffman-LaRoche Inc., Nutely, NJ, USA) and a chloroquine phosphate tablet (150 mg chloroquine base, Winthrop Laboratories, New York, NY, USA) were also treated as described above.

The ARTS content for each tablet was verified using HPLC and diode array detection. A portion of material from each tablet was weighed and dissolved in 3.75% sodium bicarbonate. The sample solution was centrifuged and the supernatant was injected directly into the HPLC system. Chromatographic separation was accomplished using a 100×4.6 mm Polaris 3 μ C18-A column (Metachem Technologies Inc., Torrance, CA, USA) with a mobile phase consisting of acetonitrile–phosphate buffer (pH 3, 50 mM) (1:1 v/v). The flow rate was 1 ml min^{-1} and the column

temperature was 30°C. The diode array spectra of the ARTS chromatographic peak for the tablets were compared with an authentic artesunate standard to assess component purity.

3. Results and discussion

Batty et al. describes a HPLC method in which detection of ARTS is accomplished by post-column alkali decomposition [5]. ARTS is converted into a product that has strong absorbance at 290 nm. Derivatization methods involving exposure of artemisinin (a naturally occurring sesquiterpene endoperoxide similar to ARTS) to dilute sodium hydroxide have been used to transform artemisinin to compounds that are highly absorbant at 293 and 260 nm [6–9]. Zeng and Lan-na have described these compounds as an enolate/carboxylate [9]. Enolates readily couple to electrophilic dyes such as diazonium salts [10], therefore, it is possible that the alkali decomposition product of ARTS may be similar in structure.

Fig. 1 shows the kinetics of the formation of ARTS to ARTS290 at different concentrations of NaOH. Optimum yield of ARTS290 occurs after 20 min in 1 N NaOH. Even though the reaction time using 5 N NaOH solution is shorter, yield is lower possibly due to further decomposition of ARTS290. Formation of the colored reaction product (ARTS420) corresponds well with the appearance of ARTS290.

Increasing the pH of the reaction mixture containing ARTS290 resulted in precipitation of the colored reaction product. This yellow precipitant is extractable into ethyl acetate. Absorbance measurements of the ethyl acetate fraction show increased intensity with increased reaction pH (Fig. 2). Under the same assay conditions, other antimalarial compounds were compared in order to evaluate assay specificity (Fig. 2). At $pH > 6$, artemisinin develops a yellow color while primaquine develops an intense orange color. When pH is increased to 8, sulfadoxine gives a strong yellow color. Although less intense at lower pH, ARTS is the only drug from the compounds tested that develops a yellow color at pH 4.

Concentrations of FRTR above 9 mg ml^{-1} begin to exhibit a yellow color in blank samples. Therefore, FRTR concentrations of 7.5, 3.75, and

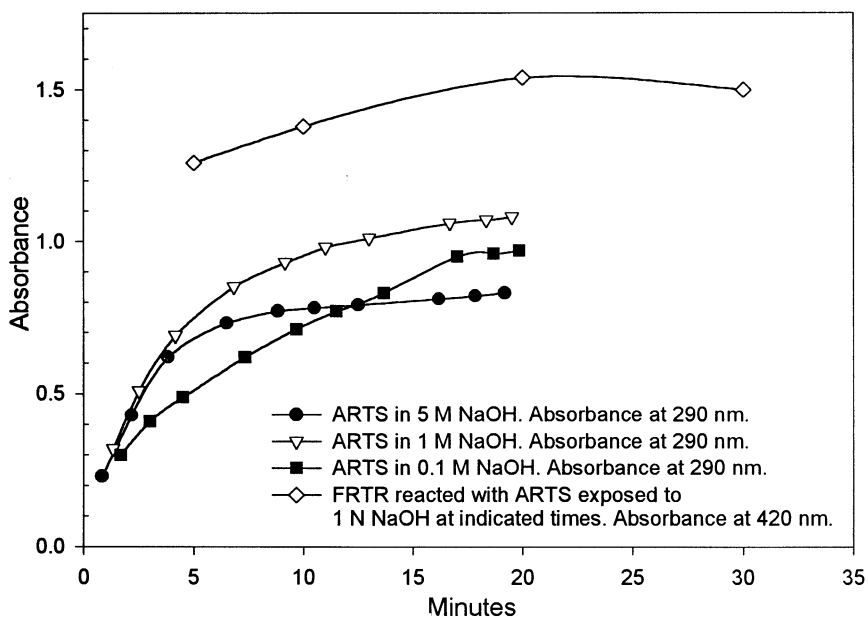

Fig. 1. Kinetics of the alkali decomposition product of 0.1 M artesunate exposed to NaOH.

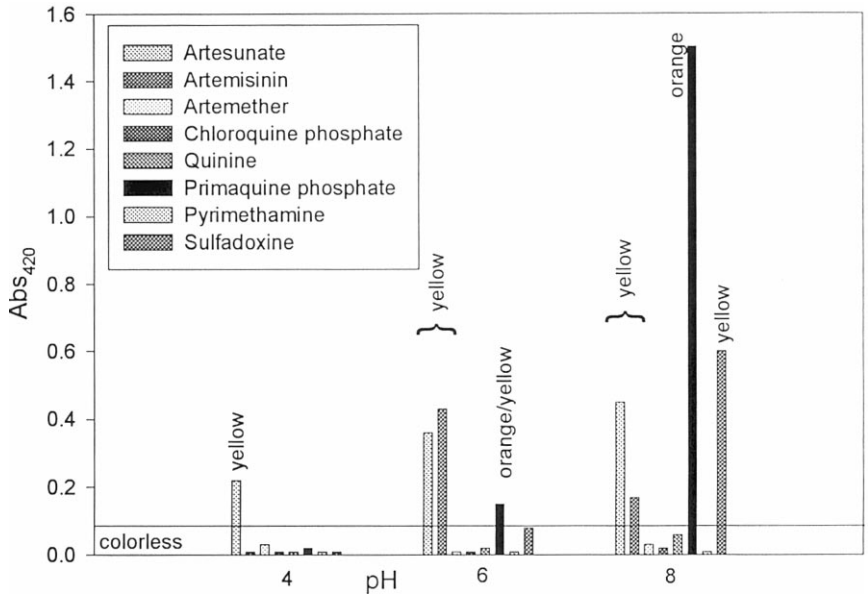

Fig. 2. Cross-reactivity of the colorimetric method with various antimalarial drugs. Absorbance was measured from the ethyl acetate extract.

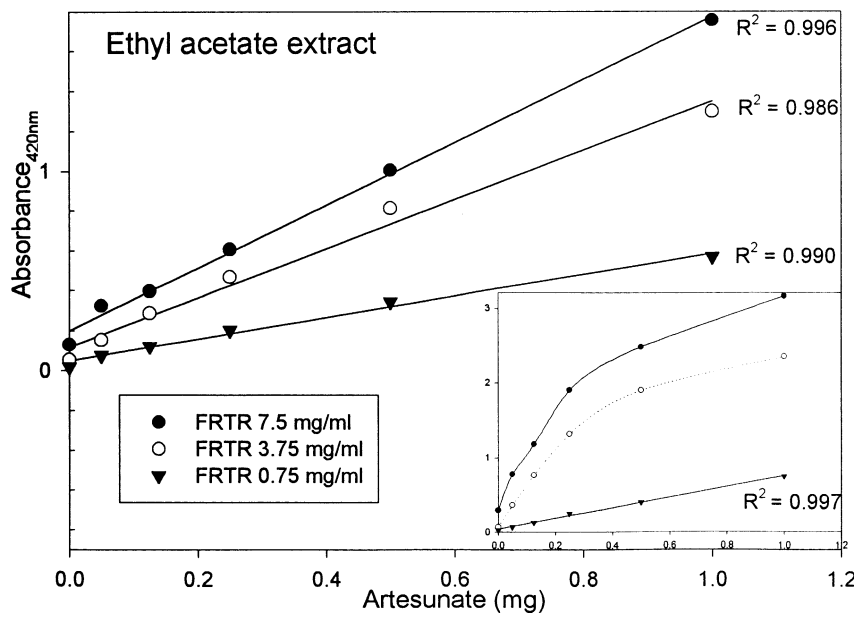

Fig. 3. Correlation of ARTS amount (mg) with the absorbance intensity of the colored reaction product in the ethyl acetate extract. The inset illustrates the loss of linearity due to formation of insoluble reaction product in the aqueous buffer (before extraction).

0.75 mg ml^{-1} were used to assess standard curve linearity (Fig. 3). The higher FRTR concentrations resulted in nonlinear standard curves, due to precipitation of the yellow reaction product in aqueous solution (Fig. 3 insert). Subsequent extraction into ethyl acetate improved curve linearity.

Using HPLC and diode array detection, each genuine tablet was verified to contain 50 ± 5 mg of ARTS while no ARTS was detected in the counterfeit tablets. The colorimetric assay method was then applied to these tablets to assess ARTS content. The method was also applied to sulfadoxine/pyrimethamine and chloroquine phosphate tablets since these drugs would be more likely substituted for ARTS. The average ARTS concentration for the six genuine ARTS tablets as determined by the colorimetric assay was 50.8 ± 2.9 mg per tablet. The quantitative analysis was performed with the ethyl acetate extract and is recommended in order to separate and dissolve the ARTS420 precipitant from the water insoluble tablet excipients. A pH of 6 was chosen because sulfadoxine does not react and the intensity of ARTS420 is stronger than observed at pH 4. A negative result (colorless, absorbance < 0.01) was observed when the method was applied to alleged counterfeit artesunate tablets, sulfadoxine/pyrimethamine and chloroquine phosphate tablets. For qualitative purposes, ethyl acetate extraction is not necessary.

4. Conclusions

This method provides a quick and simple way to identify counterfeit artesunate tablets. The color reaction takes place under a wide range of pH at room temperature. Although a quantitative assay requires a balance, a filter photometer and extraction with ethyl acetate, a qualitative assay can easily be accomplished in the field without the need for organic solvent extraction or the use of sophisticated equipment. While optimum incubation of ARTS in 1 N NaOH requires about 20 min at room temperature, a positive qualitative test can be performed after only a 5-min incubation in 1 N NaOH. After the addition of FRTR, the yellow-colored reaction product appears after 5 min, consequently the results of the test can be determined within 10 min. The method is sensitive enough to require only 1% of the total tablet (assuming 50 mg ARTS per tablet), therefore, the remaining tablet can still be used as an effective antimalarial. This derivatization method is also being investigated by our laboratory for the detection of ARTS and artemisinin in biological fluids using HPLC.

References

[1] P. Bloland, M. Ettling, Ann. Trop. Med. Parasitol. 93 (1999) 5–23.
[2] D. Klayman, Science 228 (1985) 1049–1055.
[3] D. Mount, B. Nahlen, L. Patchen, F. Churchill, Bull. Who 67 (1989) 295–300.
[4] M. Green, D. Mount, G. Todd, Analyst 120 (1995) 2623–2626.
[5] K. Batty, T. Davis, L. Thu, T. Binh, T. Ahn, K. Ilett, J. Chromatogr. B 677 (1996) 345–350.
[6] S. Zhao, Analyst 112 (1987) 661–664.
[7] H. ElSohy, E. Croom, M. ElSohy, Pharm. Res. 4 (1987) 258–260.
[8] S. Zhao, M. Zeng, Anal. Chem. 58 (1986) 289–292.
[9] M. Zeng, L. Lan-na, Tetrahedron 39 (1983) 2941–2946.
[10] H. Zollinger, Color Chemistry; Synthesis, Properties and Applications of Organic Dyes and Pigments, VCH, New York, 1991, p. 119.