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A spectrophotometric assay for quantification of artemisinin

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ABSTRACT

A sensitive and rapid spectrophotometric method for determination of artemisinin concentration is described. The method is based on the measurement of a reaction product of the drug in strong alkali solution. The interaction produces a homogenous electronic transition band from 250 to 330 nm with maximum transition at around 291 nm. The absorption curve shows Gaussian distribution with identical half bandwidth, thus providing information for formation of a possible mono-type reaction product. The 291 nm absorption intensity increases with increasing concentration of artemisinin and obeys Beer's law in the range of 0.44–172 nmol (ml^{-1}). The optimum reaction conditions and other analytical parameters were evaluated including its recovery from human plasma and erythrocyte samples.

1. Introduction

Artemisinin (Fig. 1), an endoperoxide-containing sesquiterpene lactone [3R,5aS,6R,8aS,9R,12S,12aR)-octahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4.3-j]-1,2-benzodioxepin-10(3H)-one], derived from sweet wormwood, *Artemisia annua*, is currently the mainstay of anti-malarial chemotherapy. The World Health Organization (WHO) has recommended that artemisinin-based combination therapy (ACT) should be the norm for the treatment of *falciparum* malaria in most endemic countries [1]. Artemisinin is the starting compound for a class of highly active anti-malarials, such as artesunate, artemether, arteether and dihydroartemisinin, which are produced by chemical modification of artemisinin. In addition to their anti-malarial activity, artemisinins are effective against a number of other parasitic diseases, particularly schistosomiasis, and there is now growing experimental evidence that they may potentially be useful against cancer, especially as their mechanism of action is so different from that of other anti-cancer drugs [2].

Sensitive chromatographic methods using thin-layer chromatography, high pressure liquid chromatography (HPLC), HPLC–Mass spectroscopy, gas chromatography, etc. [3, and references therein] have been developed in last several years to identify and quantify the artemisinins. A spectrofluorimetric method using cytochrome-c catalyzed reaction has also been developed [4]. However, most of these methods are cumbersome, time consuming, and require expensive instrumentation. No suitable UV–visible spectrophotometric methodology has yet been developed to quantify the drug in low enough concentration, since the compound with its limited number of solubilizing solvents does not register any discernible electronic transition all through the visible to UV wavelengths. However, attempts were made to study the electronic transition of artemisinin in alkali solution [5] yielding a large number of transient absorption peaks that have never been explored further to suggest a definite stable spectrophotometric protocol that can used to determine the artemisinin concentration. Although HPLC-UV method for detection of underivatized artemisinin has been developed [6], UV detection is considered not sensitive enough to quantify artemisinin from blood sample of patients treated with artemisinin.

In this communication, we describe a UV light sensitive electronic transition of an artemisinin–alkali reaction product (?) that can identify and quantitate artemisinin concentration. The method is highly sensitive, specific, and precise. The method is cheap, simple, requires only a sensitive spectrophotometer, and thus overcomes the shortcomings of existing methods.

2. Materials and methods

2.1. Standards and reagents

Purified artemisinin standard was obtained as gift from Dr. A. Pareek, IPCA Laboratories (Pharmaceuticals) and all other reagents used were of analytical grade. Double distilled water was used in all experiments.

A stock solution of artemisinin was prepared in dimethyl sulfoxide (DMSO), and was stored at room temperature (25 °C). Stock preparation of artemisinin in DMSO stored at room temperature is stable at least for 6 months. Working solution of artemisinin with various concentrations was prepared by diluting the stock solution

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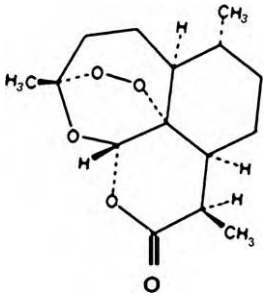


Fig. 1. Structure of artemisinin. The compound is an endoperoxide-containing sesquiterpene lactone [3R,5aS,6R,8aS,9R,12S,12aR)-octahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4.3-j]-1,2-benzodioxepin-10(3H)-one] having chemical formula as $C_{15}H_{22}O_5$ and molecular mass 282.332 g mol^{-1}.

using DMSO as solvent. Stock solutions of artemisinin were also made in organic solvents like methanol, ethanol, acetonitrile and ethyl acetate to examine the solvent effect on sensitivity of measurement.

2.2. Instrumentation

Measurements were made in an UV–visible double beam spectrophotometer (Varian Bio-300) with 1 cm matched quartz cuvettes. Spectral measurements were made at desired temperature (see Section 3) using Peltier assembly that remains attached to the spectrophotometer. The wavelength accuracy of the spectrophotometer was validated using software supplied by the manufacturer (Varian).

2.3. Quantitative recovery of artemisinin from human plasma and erythrocytes

Quantitative recovery of artemisinin from plasma and erythrocytes supplemented with known concentration of artemisinin was examined. Two milliters of heparinized blood was collected by phlebotomy from human volunteers. After centrifugation of the blood at 1500 × *g* for 15 min, the plasma was collected and stored at ice temperature till use. The buffy coat was discarded and the erythrocyte pellet was washed twice with PBS and used for experiments.

Artemisinin (354 nmol ml^{-1}) was incubated with plasma (plasma equivalent to 150 μg protein) and 3 μl of erythrocytes in 1 ml of PBS buffer for 12 h at 4 °C and 37 °C. The samples were deproteinized with trichloroacetic acid (TCA, 3% final concentration) and then centrifuged at 20,000 × *g* for 10 min. The concentration of artemisinin was determined in both supernatant and the pellet of the plasma and erythrocyte samples. A measured volume of supernatant and the whole pellet was allowed to react with 0.05N NaOH for 30 min and the samples were read for their absorption intensity at 291 nm. The absorbance value was translated into nmol quantity of artemisinin using the molar absorptivity determined during this investigation.

3. Results and discussion

3.1. Optimization of analytical conditions

Artemisinin can react with alkalis but the highest reactivity is noticed with NaOH and KOH than with $Ca(OH)_2$, $Na_2(CO_3)_2$ and $NaHCO_3$ (supplementary 1). The NaOH–artemisinin reaction product shows as a clear spectral resolution from 250 to 330 nm with maximum electronic transition around 291 nm (Fig. 2). The peak transition varied with changing concentrations of alkali, highest transition was seen with 0.05N NaOH (Fig. 3). The reaction was saturated within 15 min and the product remains stable up to 5 h after which it declines (Fig. 4A and B). Therefore, a 15-min incubation time or as mentioned was followed for all subsequent experiments. Artemisinin dissolved in the solvents like methanol, ethanol and ethyl acetate showed similar spectral resolution but with varied peak intensity (Fig. 5). Temperature ranging from 20 to 42 °C did not influence the 291 nm transition of artemisinin in the concentration range of 0.44–17.7 nmol ml^{-1} (Fig. 6). In all subsequent experiments we used 25 °C as incubation temperature.

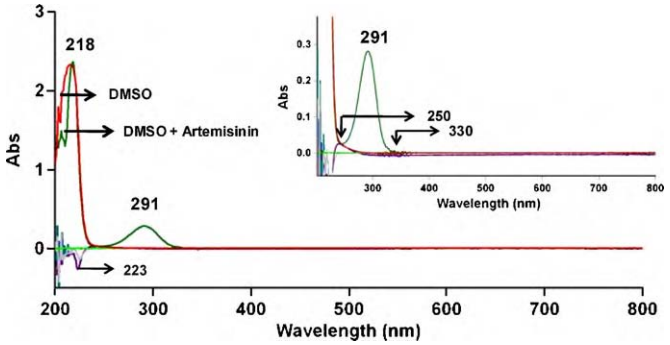


Fig. 2. Room temperature (25 °C) UV electronic transition spectra of artemisinin in 0.05N NaOH. DMSO solubilized artemisinin (17.7 nmol ml^{-1}) in 0.05N NaOH showed the presence of two prominent UV electronic transition peaks; one at 218 and another at 291 nm when scanned against 0.05N NaOH as reference. The 218 nm absorption was reproduced with DMSO alone in NaOH. Further, the 218 nm absorption peak was missing with appearance of a prominent negative transition at 223 nm upon inclusion of identical concentration of DMSO in the reference cuvette without any alteration in spectral quality, peak intensity and peak position at 291 nm. Inset shows the spectral resolution of artemisinin in an extended scale for clarity. These results suggest that the 218 nm electronic transition is related to DMSO while, 250–330 nm transition band having peak at 291 nm refers to the reaction product of artemisinin with NaOH. All measurements here and other places were made at room temperature (25 °C).

3.2. Determination of sensitivity and precision of the measurement

The working standard of artemisinin ranging from 0.44 to 354 nmol ml^{-1} was obtained by serial dilution of the stock in DMSO. The reaction mixture (artemisinin and NaOH) was incubated at

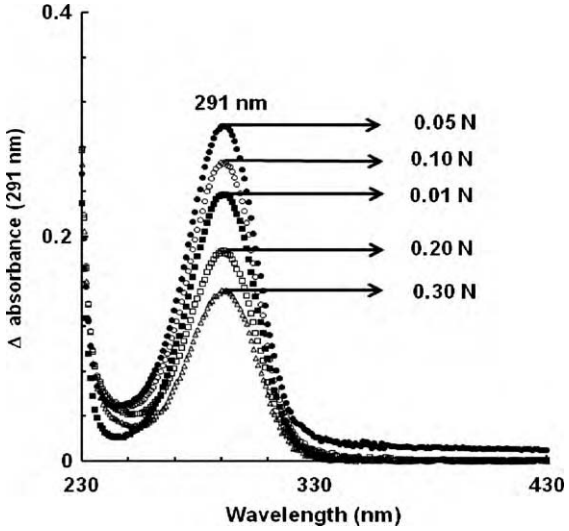


Fig. 3. Effect of NaOH concentration on 291 nm absorption intensity of artemisinin. The relative concentration of artemisinin (17.7 nmol ml^{-1}) and NaOH reaction product was measured as alteration in intensity of 291 nm absorption with changing molar strength of NaOH.

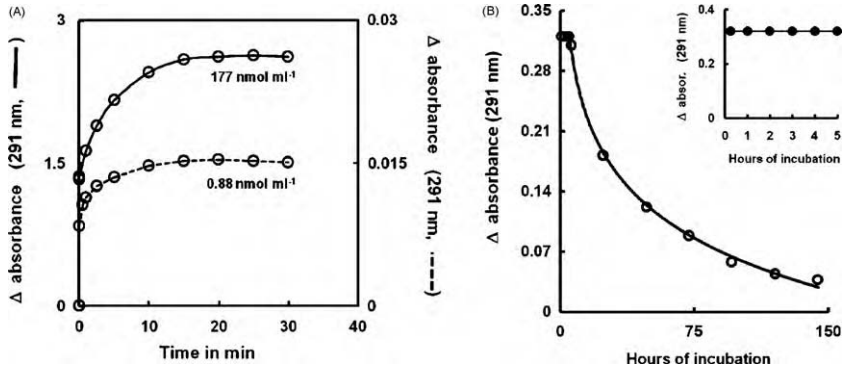


Fig. 4. Incubation time dependent alteration in 291 nm absorption intensity of artemisinin–NaOH reaction product. (A) The increase in absorbance for initial 30 min of incubation at two different concentrations of the drug dissolved in DMSO. (B) The stability of reaction product for longer hour of incubation. The inset in figure B shows the absorbance of artemisinin–NaOH product during 1–5 h of incubation.

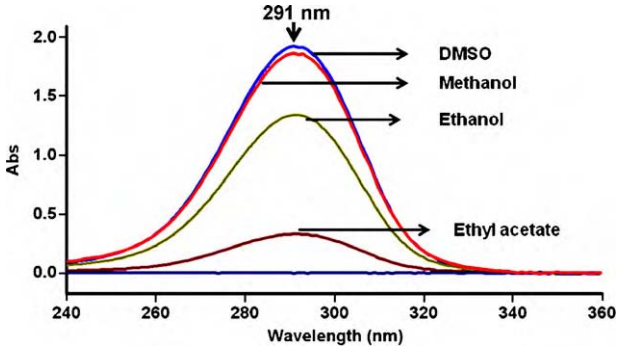


Fig. 5. Effect of solvent on the 291 nm absorption intensity of artemisinin–NaOH reaction product. The stock and working standard of artemisinin was prepared separately in DMSO (100%), methanol (100%), ethanol (100%) and ethyl acetate (100%), and an equal quantity (100 nmol) of the compound was allowed to react with 1 ml of 0.05N NaOH for 15 min, and submitted for spectroscopic analysis. Artemisinin dissolved in ethyl acetate and ethanol showed nearly 20 and 85% lower 291 nm intensity, respectively, as compared to DMSO or methanol.

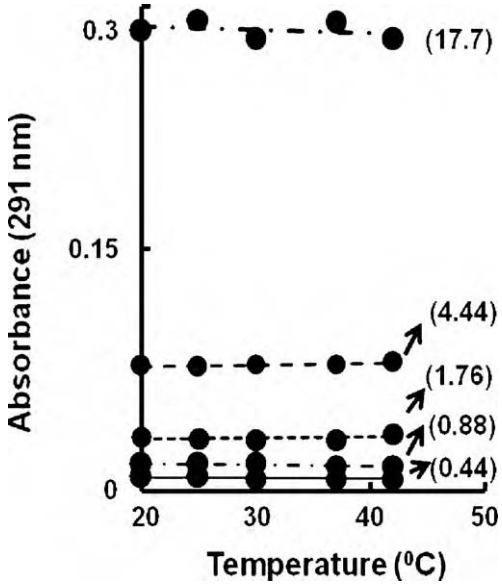
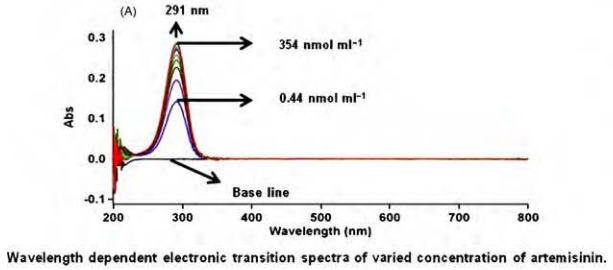


Fig. 6. Effect of incubation temperature on artemisinin–NaOH reaction product. Varied concentrations of artemisinin (nmol ml^{-1}, shown against the arrows in brackets) were allowed to react with alkali for 30 min. The measurements were performed at respective temperatures.



Wavelength dependent electronic transition spectra of varied concentration of artemisinin.

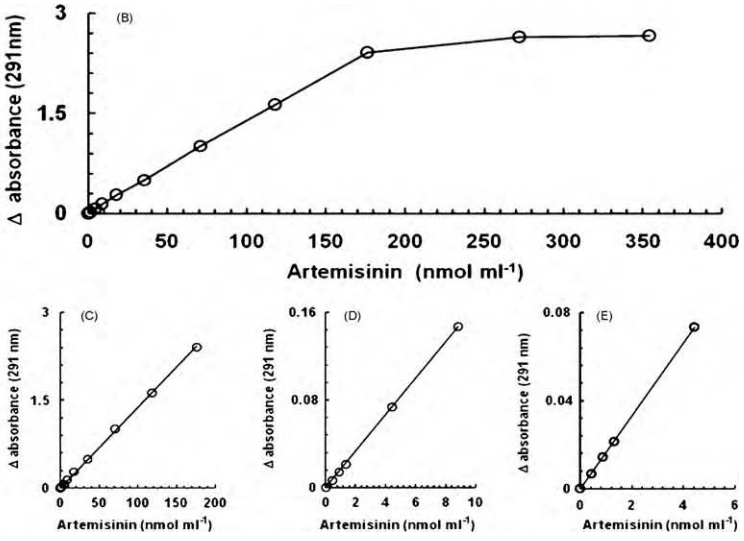


Fig. 7. Determination of linear concentration range of artemisinin that obeys Beer's law. The reaction mixture consisted of 1 μl of the artemisinin standard and 999 μl of 0.05N NaOH. (A) Direct read out from spectrophotometer showing the relative increase in 291 nm absorption peak with increasing concentration of artemisinin. (B) The concentration dependent respective absorption maximum (OD) is plotted as *X*–*Y* graph to verify the linear response of the reaction product. For clarity, (B) has been shown in three different extended abscissa and ordinate scales (C–E).

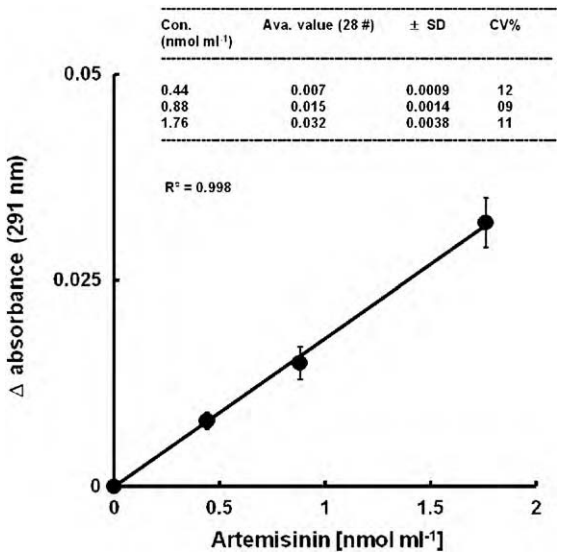


Fig. 8. Intra-batch variation in determination of artemisinin concentration. The standard deviation (±SD) was calculated from the optical density obtained from 28 numbers of samples at three concentrations of artemisinin (see the inset for numerical data points) that was further manipulated for determination of CV.

25 °C for 15 min and the absorbance was measured at 291 nm. The reference cuvette contained identical concentration of DMSO (Fig. 7A–E). The analysis showed that the absorbance obeys Beer's law in the range of 0.44–176 nmol (ml^{-1}) (Fig. 7B–E). The molar absorptivity under our measuring conditions was calculated as 0.0164 $nmol^{-1}$ cm^{-1}, which was determined form the linear part of the drug concentration spanning from 0.45 to 170 nmol. The method was found to be highly precise. Intra-batch coefficient of variation at 0.44, 0.88, and 1.76 nmol ml^{-1} ($n = 28$) was noted to vary between 9 and 12% (Fig. 8).

3.3. Recovery of artemisinin from human plasma

The absorption characteristics of plasma protein suspended in PBS and in 0.05N NaOH were evaluated to optimize the estimation of artemisinin supplemented in the plasma sample. Plasma in buffer showed a characteristic 280 nm peak transition and a high absorption below 210 nm. However, in NaOH solution three well discernible absorption maxima; 290 nm (a peak), 246 nm (as a hump) and a 219 nm (peak) become apparent (supplementary document 2A). These transitions become negligible in acetonitrile and TCA mediated de-proteinized plasma samples (supplementary document 2B and C). However, TCA was found to be a better de-proteinizer compared to acetonitrile. Therefore, we estimated the artemisinin concentration in plasma samples following its de-proteinization with TCA.

In 4 °C incubated plasma samples the recovery of artemisinin in supernatant following TCA precipitation was found to be only 61%. We therefore, washed the TCA precipitated pellet thrice with PBS and the washings were pooled together. The washing was assayed for the presence of artemisinin. However, no artemisinin could be detected in the washing. The washed pellet was than dissolved in 0.05N NaOH and incubated for 30 min and the amount of artemisinin was determined against TCA precipitated plasma protein without added artemisinin as blank. The artemisinin concentration was found to be in the tune of 34% in the TCA pelleted sample, making the total recovery to 95% (Fig. 9A, open bar). The procedure was repeated for 37 °C incubated samples and the corresponding recovery were 73, 19 and 92%, respectively (Fig. 9B, open bar).

3.4. Recovery of artemisinin from human erythrocyte

Spectral sensitivity of the erythrocyte was first evaluated in saline buffer (PBS), distilled water, and also in 0.05N NaOH (supplementary document 3). The major difference between the saline buffer and water or NaOH suspended erythrocyte samples was the induction of a higher resolution of heme transition (415 nm) with a significant lower or none in scattering properties in water or alkali suspended samples (see the amplitude of 750 nm absorption). Disruption of erythrocyte membrane by water or alkali treatments also induced a large transition in 210 regions. The other characteristic absorption peaks of erythrocyte like 577, 542, 343, 375 nm (typical oxy-hemoglobin spectra [7]), however, remain unchanged.

Identical protocol that was used for estimation of artemisinin in plasma was also employed for erythrocyte samples. Under 4 °C incubation, the total recovery of artemisinin from erythrocyte was fairly comparable to plasma, i.e., 52% in the supernatant and 36% in pelleted sample amounting to total recovery of 88% (Fig. 9A, solid bar). However, the total recovery in 37 °C incubated erythrocyte was of lower percentage as compared to plasma samples, i.e., 51, 25, and 76%, respectively (Fig. 9B, solid bar).

The failure in complete recovery of artemisinin both from plasma and erythrocyte suggests that the compound may have an

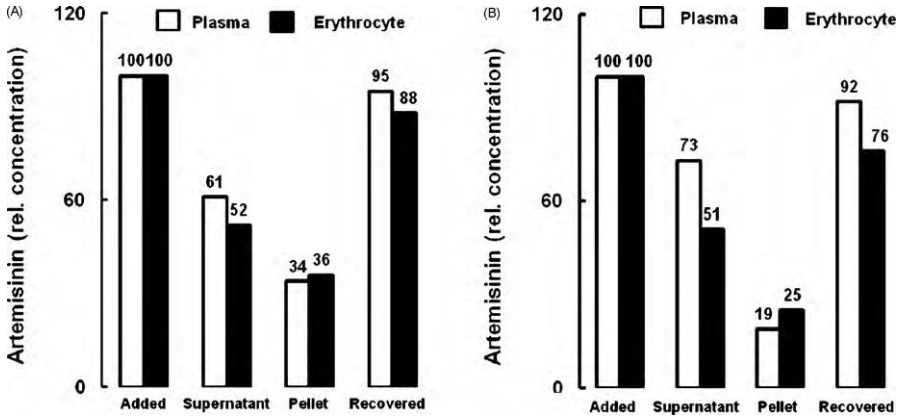


Fig. 9. Relative recovery of artemisinin from plasma and erythrocyte samples. (A) 150 μg protein equivalent plasma (open bar) and 3 μl of erythrocyte (solid bar) were incubated with known concentration of artemisinin for 12-h at 4 °C followed by de-proteinization with 3% TCA. The mixture was centrifuged at 20,000 × *g* for 10 min and the supernatant and pellet were assayed for the presence of artemisinin in 0.05N NaOH. (B) Similar conditions were maintained only with a change in incubation temperature from 4 to 37 °C. The 100% refers to 354 nmol artemisinin ml^{-1} of incubation medium.

interaction with the protein factor(s), which might have induced a change in structure of artemisinin which is no more detectable by the present protocol. The physico-chemical nature of sequestration of artemisinin in plasma and erythrocyte sample remains to be investigated.

4. Conclusion

The proposed method for quantification of artemisinin as an alkali reaction product is simple, stable and highly precise and does not involve use of costlier instrumentation. The method obeys Beer's law in the concentration range of 0.44–172 nmol (ml^{-1}). The precision check indicated an acceptable low CV between the ranges of 9–12%. The present protocol that yielded a single spectral resolution having maxima at 291 nm is highly specific to artemisinin, since the protocol does not respond to other derivatives of the drug like, dihydro artemisinin (obtained from Sigma) and artesunate (Falcigo, obtained from open market) even though they bear similar structural backbone (data not shown). We are trying to overcome this limitation of the method so that it can be effectively used both for industrial as well as clinical purposes. Additionally, as the spectral resolution of artemisinin–NaOH reaction product (most likely a alkali salt of [3R,5aS,6R,8aS,9R,12S,12aR)-octahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4.3-j]-1,2-benzodioxepin-10(3H)-one]) represents a bell shaped curve close to Gaussian distribution, it is suggested that the product formed is of mono-type. We have not been able to characterize the complete chemical identity of the product.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2010.06.015.

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