Developed and Validated Spectrophotometric Methods for the Evaluation of Artesunate and Dihydroartemisinin in Tablets Using Potassium Permanganate

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ABSTRACT

The widespread importation and distribution of counterfeit antimalarials especially artemisinin derivatives into Sub Saharan Africa is becoming a serious problem as this may lead to the appearance of highly virulent multidrug resistant plasmodium species. Based on this, a simple, sensitive and reproducible method is developed for the determination of Artesunate (ART) and Dihydroartemisinin (DHA) in tablets as highly technical analytical equipment are lacking in this region. The method is based on redox reactions of ART and DHA with potassium permanganate in alkaline medium and the determination of the bluish-green coloured specie spectrophotometrically at 600 nm . The increase in absorbance in both cases were proportional to drug concentration obeying Beer's law in the range of 0.5 to 30 and 1.0 to 40 µg/ml with correlation coefficients of 0.9989 and 0.9995 respectively. The molar absorptivity were 2.10 x 10^4 and 3.02 x 10^4 l/mol/cm for ART and DHA respectively, Sandell sensitivity were 0.018 and 0.0127µg/cm^2 The limit of detection and quantification were 0.51 and 1.2 µg/ml for ART and 0.48 and 1;18 for DHA . The intraday and inter day precision evaluated as Relative standard deviation (RSD %) and accuracy evaluated as Relative error (RE%) were ≤ 3% in all cases. The method was statistically compared with an official method via t-test and F-test and thereafter used to assay artesunate and dihydroartemisinin tablets procured locally within Uyo Metropolis. The practicability of the method was confirmed by performing recovery study via standard addition method with excellent recoveries ranging between ≥99% and ≤104 in all cases showing no interference from excipients commonly used during tablets formulation.

Key Words: Artemisinin, Aretsunate, Dihydroartemisin, Potassium permanganate, Absorbance

INTRODUCTION

Artesunate and dihydroartemisinin are derivatives of artemisinin derived from Chinese plant *Artemisia* annua also known as Qinghaosu. These are frontline drugs for the treatment of uncomplicated malaria. They are often used in combination with other antimalarials as recommended by WHO as a typical artemisinin combination therapy such as amodiaquin for effective treatment of multidrug resistant plasmodium species. The rapid parasiticidal action and low clinical toxicity made these drugs to be major candidates for adulteration, faking and outright counterfeiting. Counterfeit artesunate has been reported in Southeast Asia (Rozendaal 2001, Newton *et al.,* 2001. Newton *et al.,* 2003, Newton *et al.*, 2006, Karunamoorthi 2014). These counterfeit artesunate and other artemisinin derivative have found their way into Sub Saharan Africa (Atemkeng *et al.,* 2007, Nayyar *et al*., 2012). Malaria is endemic in the Tropics mostly in Sub Saharan Africa where it is creating havoc in mostly in children under the age of five , pregnant women and other vulnerable people such as those living with HIV. Healthy adult are not spared while children of school age are out of school adult are completely incapacitated when under malaria attack. In fact the World Health Organization Reports reveal that the African region alone accounts for 85% of malaria cases and 90% malaria death worldwide (WHO 2012, 2014). The massive importation of these artemisinin derivatives into Sub Saharan Africa is a major source of concern. The manufacturers of counterfeit artesunnate are technically sophisticated, they actually chon out counterfeits that look absolutely genuine (Ambroise – Thomas, 2012). These copy cats have expiry and manufacturing dates properly fixed to fool government agents and pharmacist in the field, Apart from the imminent danger of effects of the counterfeits substandard pharmaceutical excipients which are dangerous are also used just to maximize profit. In this very scenario everyone is the loser except the counterfeiters. Governments of endemic countries will be losing revenue as counterfeit drugs don't pass through traditional routes where taxes are collected. If this nefarious trade is allowed to continue we may be facing a serious public health crises predicated by possible emergence of highly virulent and multidrug resistant plasmodium species which is beginning to appear in Southeast Asia (WHO, 2014).

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Officially artesunate is assayed by Uv-vis spectrophotometry and titrimetry (International Pharmacopoeia, 2005). A careful search of the literature revealed that some methods have been developed for the assay of atesunate and other artemisinin derivatives (Green *et al*., 2001, Gabriels and Plaizer-Vercammen, 2004, Adegoke and Osoye, 2011, Attih *et al*., 2016 , Attih *et al*., 2017). Some of the methods are simple, sensitive and reproducible but some have some obvious analytical limitations. A typical example of that is the method of Green *et al*., (2001). This method has a laudable innovation of scraping the surface of the tablet (About 1%) of the tablet for the assay and the colour generated being yellow. With the sophistication of the counterfeiters the outer surface of a chalk or starch core can be coated with pure artesunate in that case the effective pharmaceutical active ingredient (atesunate) in that tablet is 1%. The colour generated is specific for artesunate but some other drugs such as doxicycline could also give the same yellow colour. The proposed method is cost effective, sensitive and reproducible; the reagents used constitute no hazard to the environment and the analyst. This method can therefore be used in the field and routine analytical laboratory for the assay of artesunate and dihydroartemisinin.

MATIRIALS AND METHOD

All spectral measurements were made by Heλlos β model of Uv-vis Spectrophotometer by Thermo Electron Corporation Inc. USA.

Chemicals and Reagents

All reagents used were of Analytical grade with excellent shelf life. Sodium Hydroxide (1M) Solution: This was prepared by dissolving 4.0 g of sodium hydroxide (Merck. Darmstadt, Germany) in sufficient distilled water to make up to 100 ml. Potassium Permanganate (0.02M) Solution: Prepared by dissolving 3.2 g of the chemical (Merck, Darmstadt , Germany) in sufficient distilled water to make up to 1000 ml with distilled water and heating in the water bath at 30^{o} C for 1 hour and allowed to cool and filtered and standardized using analytical grade oxalic acid.

Standard Drug Solutions

Artesunate

A pure standard artesunate stock solution was prepared by weighing out 100 mg of pure artesunate powder a kind gift from director of pharmaceutical service, University of Uyo Teaching Hospital and dissolving in enough distilled water to make up to 100 ml in a calibrated volumetric flask. The resulting concentration of 1 mg / ml was further diluted to obtain 50 µ g / ml used for the method A.

Dihydroartemisinin

A pure dihydroartemisinin powder obtained from the Director of pharmaceutical services as a kind gift. From this 100 mg of the powder was weighed and carefully transferred into a 100 ml calibrated volumetric flask containing 20 ml absolute ethanol and shaken vigorously to dissolve then the volume in the flask was made up to the 100 ml mark. The resulting DHA solution with a concentration of 1 mg/ml was diluted further to obtain 60 µg / ml for the analysis.

General Procedure

Artesunate

Different aliquots (0.0 - 5.0 ml) of standard ART solution with concentration of 50 µg / ml were carefully transferred into a series of 10 ml capacity calibrated volumetric flasks using micro burette. The volumes in the flasks were raised to 5.0 ml using distilled water. One (1.0ml) of 1 M sodium hydroxide were added to each flask and shaken gently followed by the addition of 1 ml of 0.02 M potassium permanganate and shaken gently to mix well and allowed to stand for 15 minutes. The contents of the flask were swirled at an interval of 5 minutes within the 15 minute standing time. At the expiration of the 15 minutes the content of the flask were made up to the 10 ml mark using the distilled water and the absorbance measured at 600 nm against reagent blank prepared similarly without the drug. A calibration graph was generated by plotting the absorbance against drug concentration from where the unknown concentration was determined or evaluated from the regression equation derived from Beer's law data.

Dihydroartemisinin

Different aliquots (0.0 – 5.0) of standard stock solution of DHA having concentration of 60 µg/ml were carefully transferred into a series of 10 / ml capacity calibrated volumetric flask by means of a micro burette. The experimental procedure for DHA is exactly as stated above for ART only that ethanol was used in place of distilled water.

Procedure for the determination Tablets
Twenty (20) tablets of ART (or DHA) randomly selected from commercial brands of ART (or DHA) procured locally from community pharmacies within Uyo metropolis were weighed and pulverized into fine powder. An amount of the powder equivalent to 100 mg was weighed and transferred into a 100 ml capacity volumetric flask containing 40 ml of distilled water (40 ml of ethanol in the case of DHA) and shaken vigorously to extract the drug. Another 40 ml of distilled water (40 ml of ethanol in the case of DHA) was added also shaken vigorously for further drug extraction, the volume was made up to the 100 ml using distilled water (ethanol in the case of DHA). The content of the flask shaken finally and filtered using Whatman filter paper No 42. The first 10 ml of the filtrate was discarded. The resulting concentration of 1 mg/ml was further diluted to a working concentration of 60 µg/ml from where a suitable aliquot was assayed using the general procedure described above.

Procedure for Placebo Blank
A placebo blank was prepared using pharmaceutical excipients usually incorporated in the process of tablets formulation. The placebo blank was composed of 5 mg lactose, 5 mg Talc, 5 mg sodium citrate, 5 mg sodium alginate, 5 mg methyl cellulose. 5 mg acacia, 5 mg magnesium stearate and corn starch was used to bulk up the mixture to make up to 100 mg. The resulting mixture was shaken to mix well and homogenized to mix completely. A placebo solution was prepared exactly as described in the procedure for the assay of tablets. From this a suitable aliquot was analyzed using the general procedure described earlier.

Procedure for Synthetic mixture
The 100 mg of the synthetic mixture of either of the drug powder of either ART or DHA, was prepared by adding 100 mg of the placebo blank as prepared above and shaken to mix well and thereafter homogenized to have a completely mixed mass of the synthetic mixture. From the synthetic mixture 100 mg was carefully weighed out and transferred to a 100 ml calibrated volumetric flask containing 20 ml of distilled water (or ethanol in the case of DHA). The resulting mixture was sonicated for about 20 minutes to extract the drug. Then the resulting solution in the flask was made up to 100 ml mark of the volumetric flask with distilled water (ethanol in the case of DHA) and filtered using Whatman filter paper Number 42. The first 10 ml of the filtrate was discarded and the resulting solution with concentration of 1 mg / ml was further diluted to the working concentration 50µg/ ml (or 60 µg /ml in the case of DHA) from a suitable aliquot was analyzed using the general procedure as described above.

RESULTS AND DISCUSSION
Potassium permanganate is a versatile oxidizing agent. Oxidizing the Oxidizable pharmaceuticals in various pH media (in the acid, neutral or alkaline medium). Over the years it has been used in quantitative determination of pharmaceuticals, (Gouda *et al*., 2008, Harika *et al*., 2012, Rajendrapa and Basavaiah 2009, Prasanthi and venkateshWarlu, (2015) . The different colours of the potassium permanganate oxidative products in specific pH medium are the driving force behind the quantitative determination of pharmaceuticals. The oxidation of ART and DHA in alkaline medium results in a bluish-green manganate product which is measured at 600nm.The mechanism of this reaction is not very clear. In alkaline medium the lactone ring of the artemisinin derivative is opened. For artesunate the succinate moiety is first hydrolyzed and cleaved off to form dihydroartemisinin; with a strong alkaline medium the lactone ring is opened and with the $KMnO_4$, a hydroxy acid and a bluish green manganate ion is formed. In the present process potassium permanganate reacts with ART (or DHA) and gets reduced to bluish - green manganate specie measured spectrophotometrically at 600nm. The absorbance is proportional to the drug concentration; hence a calibration graph is plotted from where the concentration of unknown is determined.

Method Development
Optimization of experimental conditions leading to the development of best stable coloured specie were carefully studied and optimized. This was done by varying the experimental variables leading to the most stable coloured specie one at a time, while keeping other variables constant and then observing its effect on the absorbance of the coloured specie.

Effect of reaction time
The reaction of KMnO4 and ART (or DHA) in alkaline medium was spontaneous. The redox reaction was complete in 10 minutes which gave the highest absorbance, beyond this there was no further

increase in the absorbance and the developed colour was stable for about 3 hours (See figure 1).

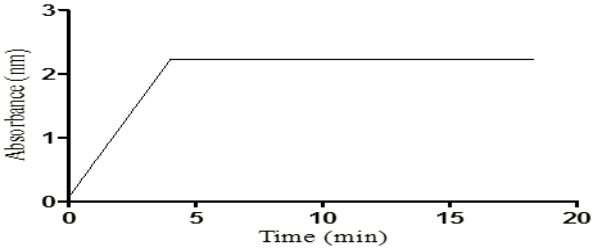


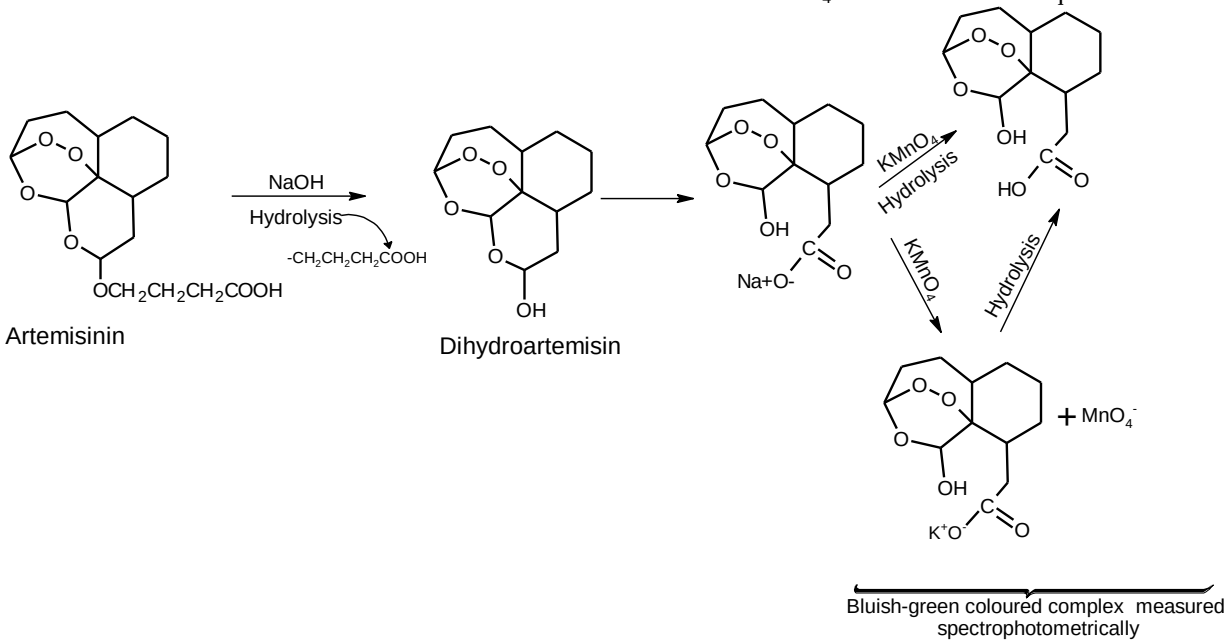
Figure 1: Absorbance – Time graph

Effect of NaOH

The absorbance of the bluish – green coloured manganate ion increased as the concentration of NaOH increased when other experimental parameters were kept constant in this redox reaction. The absorbance peaked at 1M NaOH. Hence 1ml of 1M NaOH was the maximum concentration needed for this redox reaction. Further increase in the concentration of NaOH resulted in very erratic absorbance and the coloured specie was no longer stable.

Effect of $KMnO_4$

The concentration of the oxidant ($KMnO_4$) was studied to evaluate its effect on the absorbance. This was done by varying the concentration of $KMnO_4$ while keeping the other experimental parameters constant. It was observed that 1 ml of 0.02 M KMnO4 solution gave the maximum absorbance and also the most stable bluish – green manganate coloured specie. Higher concentration gave erratic absorbances and less stable coloured specie formed. Therefore for a reaction volume of 10 ml, 1 ml of 0.02M $KMnO_4$ was found to be adequate.



Methods Validation

The proposed method was validated for linearity and sensitivity for the two drugs ART and DHA. Calibration curve generated by plotting absorbance Vs drug concentration. Beer's Law was obeyed in the range of 0.5 – 30 µg/ml and 1.0 - 40µg /ml for ART and DHA respectively. The regression equation

obtained as per least square method was a typical straight-line graph with the equation

$A = MC + K$

Where A is Absorbance, M is the slope of the calibration graph, C is the drug concentration and K the intercept of the calibration graph. The correlation coefficient for ART and DHA determinations were 0.9989 and 0.9996 respectively. The values of these parameters and that of the sensitivity parameters which include molar absorptivity, Sandell sensitivity, Limits of detection and quantification were evaluated as per the current ICH guidelines and recorded in table 1. The limit of detection (LOD) and limit of Quantification (LOQ) were evaluated using the formulae

$LOD = 3.3\sigma / S$ and $LOQ = 10\sigma / S$

Where σ is the standard deviation of five blank determination and S is the slope of the calibration Graph.

Precision and Accuracy

The precision and accuracy of the developed method were determined by preparing and analyzing six replicate (n=6) determinations at three different concentration levels. The relative standard deviation per cent (RSD%) as precision and relative error per cent (RE%) as accuracy the determination was done six times within the same day (intraday) and for five consecutive days (interlay). The precision and accuracy were in all cases $\leq$ 3% showing a good performance of the method.

Selectivity

The selectivity of the method was determined as per the placebo blank and the synthetic mixtures procedures discussed earlier. The result of the placebo blank analyses show that the excipients often used during tablet formulation had no interference with the proposed method. There was excellent recovery of the pure drug added as in the case of synthetic mixture also showing that excipients had no effect on the proposed method. This shows that the proposed method is highly selective.

Robustness and Ruggedness

The robustness of the proposed method was evaluated by making small and deliberate changes in the volume of 1M NaOH and 0.02 M $KMnO_4$ when analyzing three different concentrations of the drugs. It was discovered that minor and deliberate changes had no pronounced effect on the performance of the method. The ruggedness of the method was evaluated by performing analyses at three different concentration levels by 3 analysts and using three different spectrophotometers by a single analyst. The percentage relative standard deviation (RSD %) were $\leq$3%.

Application of the method to tablet analyses

Commercial ART and DHA tablets obtained locally from pharmacies in Uyo metropolis were analyzed using the proposed method. The result obtained from there was statistically compared with an official method (International Pharmacpoiea, 2005) via student's t-test and F-test (variance ratio test) at 95% confidence level at 4 degrees of freedom. The calculated t and F values were lower than the tabulated values showing good congruence between the two methods. The values are as recorded in table 4.

Recovery Study

Recovery study was performed via standard addition method just to test the applicability and accuracy of the proposed method. In this study are analyzed tablet powder of ART (or DHA) was spiked with pure ART (or DHA) powder and analyzed at different concentration levels using the proposed method. This was performed at 3 replicate determinations and the percentage recovery of the pure ART (or DHA) was evaluated and the values recorded in table 5. The results show that excipients had no effect on the performance of the proposed method.

Table 1: Sensitivity and Analytical Parameter of the proposed Method

Parameter	ART	DHA
λ_{max} (nm)	600	600
Linear range (µg/mL)	0.5 – 30	1.0 - 40
Molar absorptivity ($Lmol^{-1}cm^{-1}$)	2.0×10^4	3.02×10^{14}
Sandell sensitivity (µg/cm^2)	0.018	0.0127
Limits of detection (LOD) (µg/mL)	0.51	1.2
Limit of quantification (LOQ) (µg/mL)	0.48	1.18
Regression equation	A = mC +K (A=0.178c +0.006)	A = mC +K (A=0.01c +1.06)
Correlation coefficient	0.9989	0.9996

Table 2: Evaluation of Intraday and Interday Accuracy of ART

S/No	Amount of ART taken (µg/mL)	Intraday Precision and Accuracy			Interday Precision and Accuracy		
			RSD%	RE%		RSD%	RE%
1	5.00	5.10	1.09	2.00	5.13	1.83	2.60
2	10.00	10.28	1.97	2.80	10.24	1.69	2.40
3	15.00	15.43	2.12	2.86	15.44	2.07	2.93

Table 3: Evaluation of Intraday and Interday Accuracy of DHA

S/No	Amount of ART taken (µg/mL)	Intraday Precision and Accuracy			Interday Precision and Accuracy		
		Amt found	RSD%	RE%	Amt found	RSD%	RE%
1	5.00	5.30	1.09	2.60	5.14	1.83	2.80
2	10.00	10.30	1.97	3.00	10.30	1.69	3.00
3	15.00	15.45	2.12	3.00	15.42	2.07	2.80

Table 4: Results of Tablets procured locally using the Proposed Method

S/No	Tablet Brand analyzed	Label Claim	Reference Method	Amount found using the proposed method (Percentage of Label Claim ±SD
ART				
1	Lever Artesunate	50	110.00±1.10	110.35±1.09 F = 1.02, t = 2.30
	Articin	50	110.65±0.88	111.28±1.18 F = 1.80, t = 2.80
DHA				
2	Alaxin	60	110.00±1.25	110.14±1.10 F = 1.17, t = 1.02
	Cotecxin	60	110.00±0.87	110.11±1.16 F = 1.78, t = 1.00

Table 5: Further Assessment of Accuracy done by Recovery Studies via Standard Addition Method

S/No	Tablet studied	Amount of Drug (µg/mL)	Amount of Pure Drug (µg/mL)	Total Amount Found (µg/mL)	Recovery of pure Drug ± SD
ART	Lever artesuante	40.20	20.00	60.25	100.24±1.06
		40.20	40.00	80.60	101.00±1.13
		40.20	60.00	101.00	101.00±1.07
	Articin	41.00	20.00	61.30	101.50±1.42
		41.00	40.00	81.70	101.75±1.11
		41.00	60.00	107.20	102.00±1.16
DHA	Alaxin	45.00	20.00	65.40	102.00±0.76
		45.00	40.00	85.02	100.50±1.12
		45.00	60.00	104.80	99.70±1.10
	Cotecxin	50.00	20.00	70.20	110.00±1.13
		50.00	40.00	90.30	100.80±0.76
		50.00	60.00	109.95	99.90±1.01

Mean value of three determinations

CONCLUSION

A simple, sensitive, accurate and reproducible method is developed for the assay of ART and DHA in tablets . This method is cost effective. It is devoid of tight pH control, tedious extraction with organic solvents that could be hazardous to the analyst or the environment. The method is recommended for use in routine laboratories and field stations to check the influx of counterfeit ART and DHA into Sub-Saharan Africa especially where high precision analytical equipment are not available.

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