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BROMOMETRIC EVALUATION OF ARTEMISININ DERIVATIVES IN PHARMACEUTICALS BASED ON REDOX REACTION AND COMPLEX FORMATION

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ABSTRACT

The widespread distribution of counterfeit artemisinin antimalerials is becoming a serious public health problem in Africa and South-East Asia. Based on this two spectrophotometric methods are developed for the quantification of dihydroartemisinin (DHA) and artesunate (ART). In the methods, excess bromine generated in the solution by the action of acid on bromated-bromide mixture reacts with the drug and the residual bromine in method A reacts oxidatively with fixed amount of methyl orange producing a chromogen that absorbs maximally at 510 nm. In method B the residual bromine displaces iodine fromm potassium iodide which forms a complex with starch indicator which absorbs maximally at 570 nm. In both methods, the concentrations of the drugs increase proportionally with absorbance obeying Beer's law in the range of 0.5 – 7.0 and 0.3 – 6.0 in methods A and B, respectively. The correlation coefficient were r =0.9989, 0.9998 and r = 0.9992, 0.9996 for methods A and B, respectively for DHA and ART. The methods were validated as per the currently ICH guidelines. The molar absorptivity were 1.59 x 10^4, 3,0 x 10^4 and 2.7 x 10^4 ,2.6 x 10^4 L/mol/cm and Sandell sensitivity of 0.018,0.013 and 0.011,0.015µg/cm^2 respectively for methods A and B. The limit of detection and limit of qualification were within the range of 0.12, 0.30, 0.36, 0.11 µg/ml and 0.42, 0.35, 0.78, 0.32 µg/ml for both methods, respectively. The methods were evaluated for intra and inter-day accuracy (R.E. %) and precision (RSD %) and found to be ≤ 3.16 % and ≤ 2.15 %, respectively. The methods were successfully used to assay DHA and ART tablets procured locally and the results were statistically compared with official methods. The accuracy and practicability of the methods were ascertained by performing recovery experiment via standard addition method showing no interference from pharmaceutical excipient.

KEYWORDS: dihydroartemisinin (DHA), artesunate (ART), artemisinin antimalerials.

INTRODUCTION

Being endemic in 109 countries in the world, with over 3.3 million people at risk and 1 – 2 million people dying every year from *Falciparum* malaria[1,2,3] makes it about the highest killer of all parasitic diseases in the world. In terms of morbidity and mortality in Africa, malaria has become a very serious public health problem especially in children below the age of five and pregnant women and poor and susceptible population.

In terms of morbidity and mortality, its scourge is paying a heavy toll on susceptible people especially pregnant women and children under the age of five. In fact, the burden of malaria is causing a very serious health problem in Sub-Saharan Africa as 90% of all malaria death occurs here.[4] The combination of tools and methods to combat malaria especially the use of long lasting insecticidal nets, indoor residual spraying of insecticide and the use of artemisinin combination therapy recorded a remarkable progress in the fight oriented towards the elimination of malaria as reported in WHO, Global malaria Programme, 2014. For example, reduction up to 64% in the case of malaria and 79% reduction in deaths since 2000 in the Americas, 4 endemic countries in the Eastern Mediterranean (UAE, Morocco, Syria and Iraq) are remaining malaria free since 2000. Significant reduction in both the malaria incidence (31%) and mortality rate (49%) between 2000 and 2012 in Africa is quite commendable. Unfortunately, these novel achievements could be in jeopardy as there are reported cases of multi drug resistant *Plasmodium falciparum* in South-East Asia – Cambodia, Vietnam.[5,6,7] The emergence could be linked to the manufacture and distribution of substandard/counterfeit artemisinin antimalarial in South-East Asia.[8,9,10,11] There are also reported cases of fake/counterfeit artemisinin derivatives in Africa.[12, 13]Artemisinin and its derivatives were selected by the WHO because of their rapid malaria parasite clearance rate and largely no significant clinical side effect. To reduce the probability of resistance, WHO recommended the use of artemisinin combination therapy (ACT); that is adding a second antimalarial to artemisinin derivative for effective treatment of malaria. With the distribution of counterfeit/sub-standard artemisinin there is likely going to be development of resistance because sub-therapeutic doses are daily delivered to the patients. The sub-therapeutic doses result in delayed parasite clearance. This delayed parasite clearance after the treatment with ACT is of paramount concern to WHO: failure to rapidly clear parasite will compromise the use of artemisinin for the treatment of severe malaria. Slow parasite clearance in patients treated with ACT causes more parasites to be exposed to the partner medicine alone, increasing the risk of resistance developing to the partner medicine. If this occurs, treatment failures are likely to increase.[15] Dihydroartemisinin and artesunate are assayed officially by uv-vis spectrophotometry, titrimetry and HPLC.[16]Many workers have developed methods for the assay of these artemisinin derivatives. These include HPLC, UV-vis , LCMS, colourimetry, titrimetry, etc. some of these methods are reproducible [17,18,19,20,21,22,23,24] Some also have obvious defects and faults; still some assay use very expensive equipment such as HPLC and LCMS mentioned earlier. These equipment are not affordable by local authorities in these poor endemic areas. Since the counterfeiters are very sophisticated producing holograms blister packs that can deceive even qualified experts in the field. There is the need for the development of methods that are affordable and sensitive that can complement the official method. Hence this work is designed to use the oxidizing capacity of the bromate-bromide mixture for the assay of dihydroartemisinin and artesunate. In this work, bromine generated in situ in acid condition is allowed to react with DHA, ART in the presence of methyl orange. The resulting chromogen is determined spectrophotometrically.

EXPERIMENTAL

Equipment: All spectral measurement were made using Heλ los β model of Uv-vis spectrophotometer from Thermo Electron Corporation, USA.

Reagents and materials: All reagents used in this work were analytical grade with excellent shelf life. All solutions were freshly prepared daily.

Bromate-bromide mixture: To obtain a 0.05 M bromine solution, 0.274 g of dried KBrO (Merck, Germany) and 1.3 g of KBr (fivefold excess) were dissolved in sufficient distilled water to produce 100 ml. this solution was further diluted to obtain 100 µg/ml for the two spectrophotometric methods.

Hydrochloric acid: A 2 mol/L solution of the concentrated acid (Merck, Germany, specific gravity 1.18) was prepared by diluting appropriately with distilled water.

Potassium iodide (2 %): A 2 % potassium iodide solution was prepared by dissolving 2 g of the chemical (Merck, Darmstadt Germany) in sufficient distilled water to make 100 ml in the 100 ml calibrated volumetric flask.

Starch indicated solution (1.0 %): 1 g of starch (BDH, England) was made into paste with 10 ml of water and with constant stirring sufficiently boiled water was added to make 100 ml. This was allowed to cool before use.

STANDARD DRUG SOLUTIONS

Dihydroartemisinin: Pure dihydroartemisinin powder was provided by the Director of Pharmaceutical Services, University of Uyo Teaching Hospital and used as received. 100 mg was dissolved in 100 ml of absolute ethanol and further diluted to obtain 100 µg/ml using the same ethanol.

Artesunate: Pure artesunate powder was provided by the Director of Pharmaceutical Services, University of Uyo Teaching Hospital as a kind gift and used as provided. An amount equivalent to 100 mg was dissolved in distilled water to make up to 100 ml. This was further diluted to obtain a working concentration of 40 µg/ml.

Method A: Different aliquots of the pure drug (0.25 – 5.0 ml) containing 100 µg of dihydroartemisinin was accurately measured and transferred into a 10 ml calibrated volumetric flask. The drug solutions in the flasks were diluted to 5 ml using absolute ethanol. The resulting solution was acidified using 1 ml of 2 M HCl. Then 2 ml of the bromate-bromide mixture (100 µg/mi in $KBrO_3$) was added, shaken well to mix and allowed to stand for 10 minutes in a dark cupboard. Then 1 ml of 50µg/ml methyl orange solution was added and mixed well. Finally the contents in the flasks were made up to the 10 ml mark using absolute ethanol. The absorbance was then measured at 520 nm against reagent blank prepared exactly without the drug.

For artesunate, the same method was used only that 40 µg/ml of artesunate was used and distilled water was used in the place of absolute ethanol. The absorbance was measured at 525 nm.

Method B: Aliquots of the standard drug (DHA) solutions (0.25 – 5.0 ml) were measured and transferred into a series of 10 ml calibrated volumetric flask using micro burette. The volumes in the flasks were adjusted to 5.0 ml with absolute ethanol and acidified using 1 ml of 2 M HCl. Then 1 ml of the bromate-bromide mixture (100 µg/mi in $KBrO_3$) was added and shaken well to mix. The resulting solution was placed in a dark cupboard. At the expiration of 10 minutes 2 ml of 2 % potassium iodide was added followed by the addition of 1 ml of starch indicator. The resulting mixture in the flask was made up to the 10 ml mark and shaken. The absorbance of the blue chromogen was measured at 570 nm against reagent blank prepared exactly but without the drug.

Artesunate - The same method was applied to artesunate except distilled water was used for dilution.

Methods for tablets: Twenty tablets each of dihydroartemisinin and artesunate were separately weighed and pulverized using ceramic mortar and pestle. An amount of the powder equivalent to 100 mg was transferred into a beaker containing 20 ml of ethanol (in the case of DHA and 20 ml of distilled water in the case of ART). The mixture was sonicated for 20 minutes then 20 ml of absolute ethanol was added and shaken vigorously to extract the drug. The content of the flask was made up to 100 ml mark, shaken further and filtered using Whatman filter paper No. 41. The first 10 ml of the filtrate was discarded. The resulting drug solution with the concentration of 1 µg/ml was diluted further to obtain a working concentration of 100 µg/ml and 60 µg/ml for DHA and ART respectively. From here a suitable aliquot was analysed using the proposed method.

Preparation of placebo blank and synthetic mixture

A. A placebo blank: This was prepared using some pharmaceutical excipients including magnesium stearate (50 mg), talc (60 mg), lactose (70 mg), acacia (10 mg), sodium alginate (5 mg), calcium gluconate (30 mg) and starch (80 mg). these excipients were weighed out and homogenized. An amount of the mixture equivalent to 100 mg was weighed out and transferred to a 100 ml volumetric flask containing 50 ml distilled water and shaken vigorously to dissolve. The content in the flask was made up to 100 ml mark using distilled water. The solution was filtered using Whatman filter paper No. 41, discarding the first 10 ml of the filtrate. The resulting solution was prepared and analysed according to the procedure for tablets discussed above.

B. Synthetic mixture: A synthetic mixture with the composition shown above (outlined in A above) was prepared and 100 mg was measured out and added to 100 mg of the pure drug substance. Both were homogenized and mixed well. Then 100 mg of the resulting mixture was measured out and prepared as per the method for tablets discussed above. Suitable aliquots were analyzed.

RESULTS AND DISCUSSION

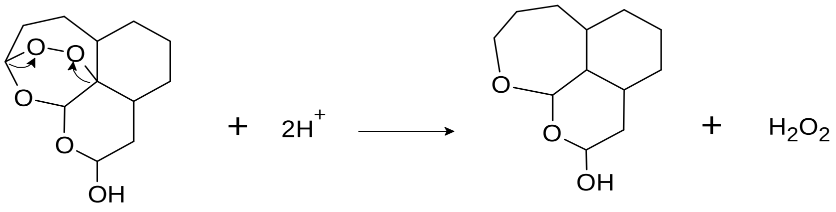
Many pharmaceuticals have been assayed based on the use of bromate-bromide mixture by many workers.[25, 26] dihydroartemisinin and artesunate are the derivatives of artemisinin molecule derived from the plant *Artemisia annua*;whose antimalarial activity is linked to the endoperoxide bond. In this reaction, the endoperoxide bond is cleaved in acid condition. The two oxygen centers in the endoperoxide moiety in the molecule are protonated as a result hydrogen peroxide is generated in situ by the action of acid on the bromine-bromide mixture (see equation 1). A second mechanism is also being proposed here; in this case the concentrated acid cause the cleaving of the OH-group in position 12 of the DHA molecule. On the whole the active bromine generated in situ is the driving force for the redox reaction between DHA or ART and the bromate-bromide mixture.

In method 1, the active free bromine generated oxidatively destroy the dye (methyl orange). Fixed amount of the active bromine generated is made to react with increasing amounts of DHA or ART in acid condition. When this happens proportionately increasing amount the of oxidant (bromine) is used up by the drug (DHA or ART); leading to a concomitant decrease in the bromine (oxidant) available for the reaction (oxidative destruction) with fixed amount of dye (methyl orange). As a result of this, there is a concomitant increase in the absorbance of the chromogen formed which is proportional to the concentration of the drug.

In method 2, the free and active bromine generated in situ reacts with the drug (DHA or ART). The residual or unreacted bromine displace iodine from the potassium iodide. The iodine liberated forms a blue iodine-starch complex which absorbs at 570 nm. The amount of the iodine liberated is proportional to the drug concentration. The absorbance of the iodine-starch complex formed is proportional to the drug concentration. The stoichiometry of the reaction is in the ratio of 1:1:1, [Bromine]:[Iodine]:[Drug] concentration.

Mechanism of Reaction

$$BrO_3^- + 5Br^- + 6H \longrightarrow 3Br + 3H_2O$$

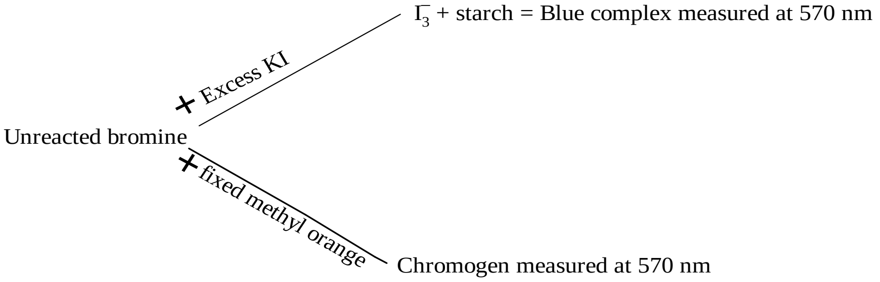


$$H_2O_2 + Br_2 + H_3O^+ \longrightarrow 2H_2O + 2HBr$$

$$2HOBr + 2H_2O$$

$$\mathrm{HOBr} + \underset{\text{coloured}}{\mathrm{Dye}} \longrightarrow \mathrm{HBr} + (\underset{\text{colourless}}{\mathrm{Dye} + \mathrm{O}})$$

Scheme:



Optimization of experimental conditions
The experimental variables were observed, studied and optimized. One variable was varied at a time while other conditions were fixed and the effect of this particular variable on the results was studied.

1. Acid type and concentration: The redox reactions were carried out in HCl, H_2SO_4 and CH_3COOH. The stable coloured species were developed when HCl was used. H_2SO_4 and CH_3COOH gave some results that were not as stable as that of HCl. Nitric acid was not used because it is also an oxidizing agent on its own. One of the different concentrations of HCl (1.0 M, 2.0 M and 5.0 M) was used to study the effect of acid concentration on the redox reaction. All other conditions were kept constant, and the absorbance of coloured species formed was measured. It was observed that the absorbance remains constant when 1 ml of 2.0 M HCl in a total of 10.0 ml reacting solution was used.

2. Order od Addition: Formation of the coloured species that was stable did not follow any particular order of addition of reagents; but this sequence gave the best results: Drug + Acid + Bromate + Bromide mixture + Dye for method 1; for method 2 the order was Drug + Acid + Bromate +Bromide mixture + Potassium iodide + Starch indicator.

3. Temperature and time: Even though temperature is a major determinant in most chemical reactions, slight increase in temperature from room temperature (25^oC + 5) gave very erratic results. The reaction became so fast so the experiment was carried out at room temperature and coloured species formed under this condition was stable for about 90 minutes. The reaction was spontaneous for both methods. The absorbance of the species formed occurred in about 10 – 15 minutes. Extension of the time to 30 minutes still gave reproducible results.

4. Bromate-bromide mixture – oxidant: The effects of bromate and bromide on the sensitivity of the redox reactions were independently studied and optimized. Though bromate is a good oxidant, some bromine was liberated to drive the reaction but the addition of bromide increased the sensitivity of the reaction because enough free and active bromine was liberated in situ for the complete oxidative process. For both methods 1 and 2, the oxidation of the drug was accelerated when bromide was added in large quantity. The oxidation of DHA or ART required 1.0 ml of 100 µg and was found to be adequate for a reacting volume of 10 ml. It was observed that a mixture in the ratio of 1:5 to 1:10 $KBrO_3$:KBr was found to be most suitable for the generation of free and active bromine to drive the process.

Validation of Proposed Methods
The proposed methods were validated for linearity, accuracy and precision, robustness and ruggedness, and selectivity.

1. Linearity: For both methods, standard calibration curves were generated by plotting absorbance vs drug concentration (DHA or ART) under optimum experimental conditions. Beer's law was obeyed which is shown by a linear graph plotted between absorbance and concentration of the drugs. The graphs were typical linear equation graphs with the equation of $A = bc + B$;

where A is the absorbance, b is the gradient, c is the concentration and B the intercept obtained using the least square method. The regression parameters including slope (b), intercept (B) and the correlation coefficient for the both methods are recorded in table 1. The molar absorptivities and Sandell sensitivities for both methods are also recorded in table 1. The limit of detection (LOD) and limit of quantification were determined as per the current International Committee on hamonisation (ICH) using the formula:

$$\mathrm{LOD} = \frac{3.36}{s} \quad \text{and} \quad \mathrm{LOQ} = \frac{106}{s}$$

Where 6 is the standard deviation of five reagent blank and s is the slope of the calibration graph. The parameters are all recorded in table 1.

Table 1: Analytical and Regression Parameters

S/N	Parameter	Dihydroartemisinin		Artesunate	
		Method A	Method B	Method A	Method B
1	λmax nm	520	570	530	570
2	Beer's law limit (µg/ml)	0.4 – 7.0	0.3 – 6.0	0.3 – 8.0	0.5 – 8.0
3	Molar absorptivity (L/mol/cm)	1.59×10^4	2.7×10^4	3.0×10^4	2.6×10^4
4	Sandell sensitivity (µg/cm^2)	0.018	0.011	0.013	0.015
5	Limit of detection (µg/ml)	0.12	0.11	0.30	0.36
6	Limit of quantification	0.42	0.35	0.78	0.32
7	Regression equation	A=0.01336c+0.08	A=0.163c+ 0.028	A=0.0117c +0.0001	A=0.012c + 0.0100
8	Slope	0.0133	0.0163	0.0117	0.012
9	Intercept	0.0083 0.9989	0.0280 0.9992	0.0001 0.9998	0.0100 0.9996

2. Accuracy and Precision: The accuracy and precision of the proposed methods were evaluated using solutions at three different concentrations of the pure drug and analysed in six replicates (n=6) within the same day (intra-day). The same determinations were carried out for five consecutive days (inter-day) accuracy and precision. The accuracy was determined as the relative error (% R.E) derived from the formula.

$$\%\ \text{R.E.} = \frac{\text{Amount found - Amount taken}}{\text{Amount taken}} \ \text{X}\ \frac{100}{1}$$

The precision was determined as the relative standard deviation (% RSD). The result of the accuracy and precision are recorded in table 2.

Table 2: Evaluation of inter-day and intra-day accuracy and precision of the methods

Methods	Amount of DHA taken (µg/ml)	Intra-day accuracy and precision			Inter-day accuracy and precision		
		Amount found	RE%	RSD%	Amount found	RE%	RSD%
A	40	41.05	2.63	1.15	41.08	2.70	1.19
	80	81.70	2.13	2.15	81.68	2.10	0.93
	120	123.80	3.16	1.56	123.74	3.12	1.37
B	40	41.00	2.50	1.23	41.08	2.70	1.33
	80	81.74	2.18	1.07	81.68	2.10	0.93
	120	122.50	2.08	1.03	122.60	2.16	1.07
Methods	**Amount of ART taken (µg/ml)**	**Intra-day accuracy and precision**			**Inter-day accuracy and precision**		
		Amount found	**RE%**	**RSD%**	**Amount found**	**RE%**	**RSD%**
A	40	41.08	2.70	1.32	41.12	2.80	1.38
	80	82.28	2.85	1.39	82.21	2.76	1.36
	120	123.21	2.68	1.31	123.15	2.63	1.29
B	40	41.02	2.55	1.98	41.05	2.63	1.28
	80	82.15	2.69	1.32	82.12	2.65	1.31
	120	122.85	2.36	1.05	122.92	2.43	1.20

3. Robustness and Ruggedness: To evaluate the robustness of the method, small but deliberate incremental changes were done as regards the volume of acid and the reaction time. The minor incremental changes had no observable changes in the absorbance of the formed coloured specie. The results in terms of precision (%RSD) were ≤ 2.10 % for both drugs. The ruggedness of the proposed methods were determined in terms of RSD % and were found to be ≤ 2.16 % for both drugs when the methods were performed by two other analysts on two different machines with all other experimental conditions remaining constant.

4. Selectivity: Placebo blank and synthetic mixture analyses were used to evaluate the selectivity of the proposed methods. As described earlier, suitable aliquots from the placebo blank were analysed using the proposed methods. The results showed that these excipients had no effect on the developed methods. A suitable aliquot from the synthetic mixture prepared as described earlier was analysed using both methods separately. The percentage recovery of DHA and ART were 102.0 ± 1.36 and 101.0 ± 1.62 respectively for method A and 102.0 ± 1.37 and 102.0 ± 1.66 respectively for method B. this result showed the high selectivity of the proposed methods in the presence of pharmaceutical excipients.

Application of the methods to tablet formulations

Two brands of DHA and ART locally procured in pharmacies in Uyo, South-South Nigeria were successfully analysed using the two proposed methods. The results presented in table 3 showed that the values obtained were in close agreement with the label claims of the drugs. The methods were statistically compared with a reference method in the international pharmacopeia by applying student's t-test for accuracy and F-test for precision at 95% confidence level and at 4 degree of freedom. The calculated t and F values were below the tabulated values as shown in table 3.

Table 3: Result of analysis of the tablets by the proposed methods

S/N	Tablets studied	Label claims (mg)	Reference method	Results for method 1	Results for method 2
1	Alaxin	60	110.0 ± 1.20	111.1 ± 1.12 F = 1.15 t = 1.28	111.3 ± 1.46 F = 1.48 t = 1.41
2	Cotecxin	60	110.0 ± 1.11	111.5 ± 1.80 F = 2.63 t = 1.55	112.0 ± 1.85 F = 2.78 t = 2.06
3	Lever Artesunate	50	110.0 ± 1.20	111.2 ± 1.78 F = 2.20 t = 1.22	111.5 ± 1.82 F = 2.30 t = 1.53
4	Articin (Evans)	50	110.0 ± 1.20	111.6 ± 1.92 F = 2.56 t = 1.60	111.3 ± 1.87 F = 2.43 t = 1.31

Recovery study

The accuracy and that applicability of these methods were confirmed by performing recovery studies using standard addition method. Pure DHA and ART powder at three different concentration levels were added to a pre-analysed (fixed) tablet powder and the resulting mixture was analysed using the proposed methods; each for three times. The percentage recovery values were between 99.0 and 103.4 with relative standard deviation ranging between 1.01 to 2.45%. These results as recorded in table 5 showed that excipients had no effects on the proposed methods.

Table 4 – Results for recovery study via standard addition method

S/N	Formulation studied	Spectrophotometric method A			Recovery of pure drug % ±S.D.	Spectrophotometric method B			Recovery of pure drug % ±S.D.
		Amount of drug (µg)	Amount of pure drug added (µg)	Total amount found (µg)		Amount of drug (µg)	Amount of pre drug added (µg)	Amount of pure drug found (µg)	
1	Alaxin (DHA)	40.20	20.00	60.50	101.5 ± 0.75	40.20	20.00	61.80	104.15 ± 2.0
		40.20	40.00	81.25	102.5 ± 1.25	40.20	40.00	81.35	102.5 ± 1.76
		40.20	60.00	101.93	102.9 ± 1.44	40.20	60.00	102.20	103.3 ± 2.36
2	Cotecxin (DAH)	41.00	20.00	61.65	103.23 ± 1.68	41.00	40.20	61.25	101.30 ± 0.88
		41.00	40.00	82.10	102.80 ± 1.38	41.00	40.20	82.20	103.00 ± 1.50
		41.00	60.00	101.80	103.30 ± 0.67	41.00	40.20	107.00	103.00 ± 1.18
3.	Lever Artesunate (ART)	40.20	20.00	61.00	104.00 ± 2.0	40.20	40.20	61.66	102.10 ± 1.63
		40.20	40.00	81.25	102.60 ± 1.31	40.20	40.20	82.50	103.30 ± 1.88
		40.20	60.00	99.99	99.50 ± 0.58	40.20	40.20	101.80	103.10 ± 0.67
4	Articin (ART)	41.00	20.00	61.65	103.30 ± 1.63	41.00	40.20	61.55	102.00 ± 1.75
		41.00	40.00	82.20	102.50 ± 1.25	41.00	40.20	81.40	101.20 ± 0.50
		41.00	60.00	101.20	100.30 ± 0.70	41.00	40.20	102.51	103.10 ± 1.25

* S.D. – Standard Deviation
Mean value of 3 determination.

CONCLUSION

Two simple, reproducible, sensitive and highly selective methods have been developed for the determination of dihydroartemisinin and artesunate in both bulk and pharmaceutical formulations. The methods were devoid of exhaustive and tedious extractive procedures with organic solvents that could be hazardous to the analyst and the environment. The methods are quite versatile and are recommended for use in field stations and entry point to check the current influx of adulterated and substandard artemisinin antimalarials currently imported massively into sub-Saharan Africa.

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