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Optimized and Validated Methods for The Evaluation of Artemisinin Derivatives in Pharmaceuticals Using Redox Reactions.

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ABSTRACT

The manufacture and distribution of counterfeit/fake artemisinin derivatives is assuming a dangerous dimension, putting in jeopardy the efforts to control malaria. Two simple sensitive reproducible methods are developed for the assay of artesunate (ART) and dihydroartemisinin (DHA) in commercial tablets procured from local Pharmacies in Uyo, South – South, Nigeria. The methods, titrimetric and spectrophotometric methods are based on the use of N-Bromosuccinimde (NBS) as the oxidimetric agent. In the titrimetric method the bromine released in the solution displaces iodine from potassium iodide and the liberated iodine is determined iodometrically. The titration method was found to be stoichiometric in the ratio of 1: 2 [Drug: NBS] and quantitative in the range of 1 – 10mg/mL for both ART and DHA. the spectrophotometric method was found to obey Beer's law in the range of 5 - 60.0µg/ml and 5 – 70 µg/ml for DHA and ART, respectively. The molar absorptivity and Sandell sensitivity are 4.0 x 10^3 L/mol/cm, 5.0 x 10^3 l/mol/cm and 0,071and 0.077 µg/cm^2 for DHA and ART, respectively. The limit of detection and quantification were 0.080, 0.090 and 0.20, 0.18 µg/ml for DHA and ART, respectively. The inter and intraday precision and accuracy were ≤ 2.9 in all cases. The proposed method was successfully used to assay commercial ART and DHA tablets procured locally and statistically compared with reference method via student t-test and F-test with the means showing no significant difference. The applicability and accuracy of the proposed method were confirmed by performing recovery studies via standard addition method with result showing excellent recoveries confirming that pharmaceutical excipients had no effect on the method.

Keywords: Artesunate, Counterfeit, Dihydroartemisinin, Iodometrically, Malaria

INTRODUCTION

The manufacture and distribution of fake and counterfeit antimalarial in sub Saharan Africa is assuming a dangerous dimension (Atemkeng *et al* 2007, Nayyar *et al* 2012). If this situation continues we may be having a serious public health crisis within this region as malaria remains the highest killer amongst parasitic diseases within the tropics. WHO defines counterfeit drug as one which is deliberately and fraudulently labelled.

Counterfeit and fake artemisinin derivatives were first reported in Southeast Asia (Rozendoal 2001, Newton *et al* 2003). Today there are reported cases of multidrug resistance malaria parasite emerging in southern Asia traceable to the manufacture and distribution of fake/counterfeit artemisinin derivatives (WHO 2013, WHO 2014). With this development the concerted integrated effort by WHO, health authorities in malaria endemic countries and donor nations seems to be in jeopardy. These criminally minded individuals take undue advantage of the weak legislations of Sub Saharan African countries to push fake artemisinin derivatives. Manufacturers and distributors of fake/counterfeit artemisinin derivatives are very sophisticated. Their products look absolutely genuine. They produce hologram, blister, packs, showing manufacturing and expiry dates, which can fool experts/Pharmacist and even government agents in the field (Ambroise-Thomas 2012). Cheap and substandard pharmaceutical excipients are commonly used in order to maximise profit. Some of the substandard pharmaceutical excipients used are very dangerous and some are known carcinogens. These include safrole, melamine and metamizole. Counterfeit/fake drugs are usually smuggled through the borders resulting in very huge profit for the criminal ring; since they do not pay taxes to the government. Bearing in mind the weak legislation and poor penal system of these poor malaria endemic countries.

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Criminal traders and organised criminal cartels are cashing in to make more money for which they use to execute their terrorist activities. To check this menace, highly technical laboratories with sophisticated equipment such as H.P.L.C, GC-MS are needed. Unfortunately, these poor countries can hardly afford these equipment. Those that can afford the equipment hardly generate enough power for their operations as the epileptic power situation cripple the equipment. Bearing in mind this problem the development of simple, sensitive method for the determination or assay of artemisinin derivatives is therefore a necessity, hence the development of this simple, sensitive titrimetric and spectrophotometric method. Dihydroartemisinin and artesunate are officially assayed by titrimetry, HPLC and spectrophotometry (IP, 2005). The two methods proposed here will complement the official methods. A careful search of the literature reveals that many methods have been developed for the assay of DHA and ART (Green *et al* 2001, Gabriels and plaizer-vercammen, 2004., Attih *et al* 2012, Attih *et al* 2015a, Attih *et al* 2015b, Attih *et al* 2016, Adegoke and Osoye 2011). Some of the developed methods are effective and reproducible but some of them have obvious analytical limitations such as tight pH control, low sensitivity, significant blank absorption, tedious time consuming extraction process using organic solvents that are harmful and hazardous to the analyst and the environment. The proposed methods are devoid of these short-comings. N-bromosuccinamide has been used by some workers for the determination of pharmaceuticals in bulk and tablet formulations (Basavaiah *et al* 2009, Vinay *et al* 2011, Prashanth *et al* 2013, Prashanth *et al 2014*). A careful search of literature also reveals that NBS has not been used to assay artemisinin derivatives to the best of our knowledge. The two methods proposed the use of NBS as the oxidimetric reagent. NBS releases low level of bromine which initiates a free radical reaction that attaches bromine at allylic position in the drug molecule (Allinger *et al* 1971, Morrison and Boyd 1983)

EXPERIMENTAL

Apparatus

All spectral measurements were recorded using Heylos β model of UV-VIS spectrophotometer from thermo electron corporation, USA, equipped with 1cm quartz cell to match.

Chemicals and Reagents

All chemicals and reagents used in this work were analytical grade with excellent shelf life.

N-Bromosuccinimde (NBS). A 0.01 NBS solution was prepared by dissolving about 1.78g of the chemical (sigma-Aldrich Germany) in warm distilled water and diluting to 1 litre with the same distilled water. The resulting solution was standardized iodometrically.

Potassium iodide (10%): A 10% solution of potassium iodide was prepared by dissolving 10g of the chemical (British Drug House England) in enough distilled water to make up to 100ml.

Sodium thiosulphate (0.04M). A 0.04M solution was prepared by dissolving about 6.34gram of the chemical (B.D.H. England).

Starch Indicator (1%) A 1% starch indicator was prepared by dissolving 1g of the substance (Merck Germany) in minimum cold distilled water and made into slurry, then boiling distilled water was added to make up to 100ml and allowed to cool at room temperature before use for the titrimetric method.

Hydrochloric acid (2M). Concentrated hydrochloric acid (BDH England sp.sg 1.18) was diluted approximately to obtain 2M solution with distilled water.

Indigo Carmine (1000 µg). A solution of 1000 µg/ml of indigo carmine was prepared by dissolving 115 of the dye (sigma, Germany) in 100ml of distilled water and further diluted to obtain 200µg/ml used in the spectrophotometric method.

Ethanol 98% - (Sigma, Germany)

Standard drug solutions

Pure dihydroartemisinin and pure artesunate powder were provided by the Director of Pharmaceutical Services of the University of Uyo Teaching Hospital, (UUTH) Uyo, Nigeria. The pure dihydroartemisinin powder was used to prepare standard stock solution of the drug. This was done by dissolving 100 mg of the DHA powder in 100ml of 98% ethanol to obtain 1mg/ml solution used for the titrimetric method. The remaining drug solution was diluted further to obtain 1mg/ml standard solution, which was used in the titrimetric method. The remaining was further diluted to a working concentration of 50ug/ml for the spectrophotometric method. All dilutions for the preparations were done with distilled water in place of 98% ethanol. Artesunate solution was similarly prepared but distilled water was used instead of ethanol.

General Procedure

Method A: Titrimetric Method

A 10ml aliquot of the drug (DHA or ART) containing 3-10ml was accurately transferred to a 100ml titration flask and acidified with 2ml of 2M HCl. Ten (10) ml of 0.01M NBS was added using a pipette, the content of the resulting mixture was shaken to mix well and kept aside for about 10 minutes. At the expiration of the 10 minutes 5ml of 10% potassium iodide was added and shaken well. The liberated iodine was titrated against 0.04M sodium thiosulphate, with the starch indicator added close to the end point. The end point is at the point where the blue colour is discharged. A blank titration was performed under exactly the same condition but without the drug.

The quantity of the drug per aliquot was calculated from the formula

Quantity of drug per aliquot (mg) = $V_B - V_D$ x Mwt x M

V_B = volume of sodium thiosulphate consumed in blank titration (ml)

V_D = volume of sodium thiosulphate solution consumed when the aliquot containing the drug was titrated.

Mwt = Relative molecular weight of the drug. (of either DHA or ART).

M = molarity of the NBS solution used.

Method B

Different aliquots (0.5 – 4ml) of the standard dihydroartemisinin solution (50µg/ml) or (ART 20µg) were carefully transferred into a series of 10ml calibrated volumetric flask using micro burette, the total volume of the flasks was rounded up to 5ml using 98% ethanol (water in the case of ART). To the content of each flask 1ml of 2M HCl and 2ml of 0.02M NBS and shaken to mix well and allowed to stand for 10 minutes, with occasional shaking within the 10 minutes duration. Finally, 1ml of 200µg/ml indigo carmine solution was added to each flask and the content in the flask was made up to the 10ml mark of the flask and shaken to mix well and allowed to stand for 1minute. The absorbance was then measured against reagent blank at 610nm. A calibration graph was generated by plotting absorbance versus drug concentration, the concentration of the unknown was determined from the graph or evaluated form the regression data derived from Beer's law.

Assay Procedure for Tablets

Twenty tablets of DHA (or ART) were randomly selected from popular local brand of dihydroartemisinin (or ART) procured from community pharmacies in Uyo and pulverised into fine powder using ceramic mortar and pestle. A portion of the powder equivalent to 100mg was carefully transferred into a 100ml calibrated volumetric flask containing 40ml of 98% ethanol (in case of DHA and water in case of ART). The mixture was sonicated for 10 minutes to dissolve the powder properly. Twenty (20) ml of 98% ethanol was further added (in case of ART distilled water) and shaken vigorously to extract the drug. Finally, the content was now made up to the 100ml mark with 98% ethanol (or water in case of ART). The content was shaken very well and filtered using Whatman filter paper no. 42. The first 10ml of the filtrate was discarded. The resulting drug solution with the concentration 1mg/ml was further diluted to a working concentration of 50µg/ml for DHA and 20µg/ml of ART from where a suitable aliquot was analysed.

Procedure for placebo blank analysis

A placebo blank was prepared using excipients commonly used in tablets formulations. A placebo composition of corn starch (10mg) sodium citrate (5mg) magnesium stearate (5mg), sodium alginate (5mg), talc (5mg), methyl cellulose (5mg), lactose (5mg), and acacia (5mg) were thoroughly mixed together and homogenised. A portion of the powder weighing 100mg was carefully weighed out and a solution of this was prepared as described in the procedure for tablets. This was then subjected to analysis using the proposed methods.

Procedure for determination of DHA or ART in synthetic mixture

A quantity of the pure DHA (or ART) powder equivalent to 100mg was added to the placebo blank composition as described above. The resulting mixture was homogenised and mixed thoroughly. The mixture was transferred to a 100ml calibrated volumetric flask and a solution made of the mixture same as described in the procedure for tablets. The resulting mixture was filtered using Whatman filter paper No. 42. The resulting mixture was assayed for 5 times by titrimetric method as described above. The synthetic mixture with the concentration of 1mg/ml was diluted to obtain 50µg/ml for DHA (or 20µg ART) using 98% ethanol (or water for ART). A suitable aliquot was then analysed using the spectrophotometric method described above.

RESULTS AND DISCUSSION

The Results of analytical and regression Parameters for the proposed methods; evaluation of Intra-day and inter-day Precision and Accuracy; Analysis of tablets using the proposed methods; and recovery

study via standard addition method are presented in NBS has been used by some researchers to assay many pharmaceuticals (Basavaiah and Kumar, 2006, Vinay et al 2011). To the best of our knowledge no method has been developed for the artemisinin derivatives using NBS. NBS is an organic oxidizing agent. It is involved in the free radical oxidative bromination of alkenes at allylic positions (Allinger et al, 1971., Morrison and Boyd 1983). The reaction of NBS and DHA or ART is a free radical reaction initiated by light and peroxides. The reaction is initiated by small quantity of bromine radical. The role played by NBS is to provide a constant supply of low concentration of molecular bromine. The mechanism of this reaction is most likely to be that in acid condition the oxygen centers in the endoperoxide bond of the artemisinin drugs are protonated leading to the cleavage of the carbon – oxygen bonds in the endoperoxide moiety. the Tables 1, 2, 3 and 4, respectively.
Hydrogen peroxide is generated in-situ which is the perfect initiator required to drive the reaction.

Table 1 Analytical and Regression Parameters for the proposed Methods.

Parameters	Values	
	DHA	ART
λ_{nm}	610nm	610m
Beers law range µg/ml	5 - 60µg/ml	5 - 70µg/ml
Molar absorptivity L/mol/com	4.0×10^3	5.0×10^3
Sandell sensitivity µg/cm^2	0.071	0.077
Limit of detection µg/ml	0.08	0.092
Limit of qualification	0.20	0.18
Regression equation	A=bc + B A = 0.01C + 0.06	A=bc + B A=0.0093C + 0.0023
Slope (b)	0.01	0.0093
Intercept	0.06	0.0023
Correlation coefficient	0.9998	0.9996

Table 2: Evaluation of Intra-day and inter-day Precision and Accuracy

Method	ART Taken (mg)	Intraday and inter-day Accuracy and precision			Inter-day accuracy			Intraday precision and accuracy				Inter-day precision and accuracy	
		ART found	RE%	RSD%	ART found	RE %	RSD %	DHA found	RE %	RSD %	DHA found	RE%	RSD%
Titrimetry	2.0	2.03	1.5	2.3	2.04	2.00	0.90	2.02	1.00	0.45	2.05	2.50	1.12
(µg/ml)	4.0	4.48	2.0	1.6	4.11	2.80	1.23	4.10	2.50	1.12	4.07	2.25	1.01
	6.0	6.13	2.2	1.0	6.15	2.50	1.11	6.11	0.80	0.80	6.11	2.83	1.26
Spectroph	30	30.79	2.63	1.18	30.65	2.17	1.06	30.68	2.27	1.01	30.66	2.20	0.98
otometry	60	61.60	2.66	1.20	61.63	2.70	1.21	61.70	2.83	1.27	61.20	2.00	0.89
(µg/ml)	90	92.20	2.44	1.10	92.10	2.33	1.04	92.30	2.56	1.14	92.28	2.53	1.13

Titrimetric Method

In this method the hydrogen peroxides generated in situ allows allylic centres to be created in the new artemisinin reaction product. Bromine is then attached. Excess bromine left within the reaction solution cause the liberation of iodine from the potassium iodide hence the iodometric determination. The reaction is quantitative with stoichiometry of 1:1 NBS: DHA or NBS: ART. In the case of artesunate apart from the cleaving of the endoperoxide bond, the succinate group in position 12 is hydrolysed converting artesunate (ART) to dihydroartemisinin (DHA).

Spectrophotometric method

Just as in the case of titrimetry, the hydrogen peroxide generated in situ in acid condition initiates the free radical reactions. In this reaction known excess of NBS is allowed to react with increasing drug concentration. The residual NBS is made to react with fixed indigo carmine. As increasing amounts of the drug react with NBS, some amount of NBS is consumed, and some amount of the fixed indigo carmine is also consumed. As this continues the residual amount of NBS left or available to oxidatively destroy the dye become increasingly smaller and hence the determination of the absorbance at 610nm.

Table 3: Result of Analysis of tablets using the proposed methods

Commercial Artesunate analysed	Label claim (mg)	Reference	Found (%of label claim ±SD	
			Method A: titrimetry	Method B: spectrophotometry
Lever Artesunate	50.00	99.80±1.44	102.10±1.74 F = 1.46 T = 2.26	1.01.62±1.10 F = 1.71 T =2.30
Artesunate Neros	50.00	99.74±1.38	101.30±1.04 F = 1.76 T =2.01	102.10±1.06 F = 1.69 T =2.00
DHA Coteсxin	60.00	99.82±1.52	101.35±1.60 F = 1.11 T =1.55	101.10±1.70 F = 1.21 T =1.25
Codecin	60.00	99.71±1.39	101.28±1.68 F = 1.46 T =1.61	102.20±1.44 F= T =2.77

Table 4: Results of Recovery Study via Standard Addition Method

Drug Formulations studied	Titrimetry				Spectrophotometry			
	Amount of drug in tablet (mg/ml)	Amount of pure drug added (mg/ml)	Total amount found (mg/ml)	% Recovery of pure drug ± SD	Amount of drug in tablet (µg/ml)	Amount of pure drug added (µg/ml)	Total amount found (µg/ml)	%recovery of pure drug ± SD
Artesunate Liver Artesunate	5.08 5.08 5.08	5.00 10.00 12.00	10.21 15.10 17.20	102.6±1.10 100.2±1.12 101.0±1.05	40.20 40.20 40.20	20.0 40.0 60.0	60.0 80.81 101.2	102.6±1.10 101.5±1.12 101.6±1.11
Artesunate (Neros)	6.03 6.03 6.03	4.00 8.00 12.00	10.03 14.07 18.08	100.0±1.60 100.5±1.56 100.4±1.50	41.00 41.00 41.00	20.0 40.0 60.0	61.4 80.9 102.2	102.0±1.08 9982±1.21 121.0±1.10
Dihydroartemisin (Cotecxin)	6.95 6.95 6.95	4.00 8.00 12.00	11.05 14.02 18.93	102.5±1.20 99.6±1.18 99.0±1.21	50.10 50.10 50.10	20.0 40.0 60.0	70.20 90.6 109.9	100.5±1.20 101.0±1.63 99.7±1.07
Codicin	7.05 7.05 7.05	4.00 8.00 12.0	11.06 15.03 19.07	100.3±1.40 99.6±1.37 100.2±1.18	50.10 50.10 50.10	20.0 40.0 60.0	70.20 90.20 110.0	100.3±1.64 100.2±1.10 199.8±1.08

*Mean value of 3 determinations

Optimization of experimental conditions

Acid type and concentration.

Three types of acid were used for this process, hydrochloric acid, sulphuric acid, and ethanoic acid. Nitric acid was not used because it is an oxidizing agent itself. Hydrochloric and sulphuric acid showed good result as the absorbance was proportional to the drug concentration. Ethanoic acid gave reasonable results initially but the colour species formed was not stable.

The concentration of HCl and H_2SO_4 were increased and the effect on the absorbance was monitored. The absorbance increased as the concentration of acid increased but to a maximum. Very high concentration gave very inconsistent results. Preliminary experiments performed showed that all other parameters being constant, 1ml of 2M HCl was quite suitable for a reaction volume of 10ml. The coloured species obtained was stable for up to 90 minutes.

Reaction time

This reaction was conducted at varying times. It was discovered that the release of active bromine which displaced equivalent amount of iodine was completed in about 10 minutes. Extension of time up to 20 minutes did not give much difference as per the stoichiometry of the reaction as the iodine liberation was probably concluded. The stoichiometry of this reaction was found to be 1:1:1 [DHA] or [ART]: [NBS]: [KI] that is [DHA] or [ART]: Br^-: I^-. Finally, it was discovered that in a 10 ml aliquot, 0.01M NBS solution was adequate for the active oxidative bromination of DHA or ART. The reaction was sharp in the range of 1-15ml. In spectrophotometric method the reaction between NBS and either DHA or ART proceeded well between 5-20 minutes. So, 10 minutes was chosen, as there was enough time for the oxidation of either DHA or ART and the oxidative destruction of the dye indigo carmine. The experiment was not greatly affected when 0.5ml to 2.0ml of 2M HCl was used so 1ml of 2M HCl was used. The coloured species obtained here was stable for about 12 hours. Sulphuric acid also showed very encouraging quantitative results with developed stable colour of the chromogen.

Effect of temperature

The effect of temperature on both titrimetric and spectrophotometric method for high and low temperature reactions was spontaneous and went on smoothly. The developed coloured species was very stable at room temperature (25˚C). Hence room temperature of 25±1˚C was chosen for both the titrimetric and spectrophotometric methods for both drugs (DHA and ART).

Methods Validation

Both the titrimetric and spectrophotometric methods were validated for linearity and sensitivity, precision, accuracy, selectivity and recovery.

Linearity and sensitivity

The linearity range in the titrimetric method was in the range of 5 – 10 mg/ml. Within this range the reaction was quantitative and the reaction stoichiometry was in the ratio of 1:2 for ART: NBS and the same for DHA: NBS. In the spectrophotometric method a linear relationship was observed when absorbance was plotted against drug concentration for both drugs. The calibration curve as generated was obtained via the least square method showed that Beer's law was obeyed in both cases of the two drugs. In the case of ART, the range was 5 – 70 µg/ml while for DHA the range was 5 – 60 µg/ml. The calibration graphs were described by typical straight-line equation: A= DC + F; Where A is absorbance, D is the slope, C the drug concentration and F the intercept. The values of correlation coefficient, slope, intercept as determined for calibration graphs are all recorded in Table 1. Parameters that determine the sensitivity of the proposed methods such as apparent molar absorptivity, Sandell sensitivity

were evaluated. The limits of detection (LOD) and limits of quantification (LOQ) were also calculated as per the current international committee on harmonisation guide lines.

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

Where σ is the standard deviation of 5 reagent determinations and S the slope of the calibration graphs. The values of the sensitivity parameters are also recorded in Table 2.

Precision and accuracy

The precision and accuracy of the developed methods were determined by performing six replicate analyses at 3 different concentration levels. This analysis was done the same day (intra-day and also performed for 5 consecutive days within a week (inter day). Percentage relative standard deviation (%RSD) was used in evaluation of precision while the percentage relative error (%RE) was for the evaluation of accuracy of the methods. The percentage relative error was calculated using the formula:

$$\% \text{ R.E.} = \frac{[\text{Amount of Drug found} - \text{Amount of Drug taken}]}{\text{Amount of Drug taken}} \times 100$$

The result of the intra-day and inter-day precision and accuracy are as recoded in Table 3.

Selectivity

The developed methods were evaluated for selectivity via placebo blank and synthetic mixture analysis as described earlier. A suitable aliquot of placebo blank was prepared and the synthetic mixture also prepared and both were analysed using the proposed methods as described. There was no reasonable interference from excipients used in the formulation of tablets. Analysis of the synthetic mixture via the proposed methods yielded excellent percentage recoveries of the pure drug within the range (98.5 – 101.9) with the standard deviation of 0.96 – 1.19 in all cases.

Application of the proposed Methods to Assay of tablets

The applicability of the proposed methods for the quantification of commercial brands of ART and DHA procured from pharmacies within Uyo metropolis, South-South Nigeria was successfully evaluated. The results obtained were compared with an official method (International Pharmacopoeia – titrimetric method) via students t-test (accuracy) and F (variance) test (precision at 95% confidence level and at 4 degrees of freedom). In all cases the calculated values were below the tabulated (critical) values. The results obtained showed excellent congruence with the official pharmacopoeial method.

Recovery studies

The validity and the practicability of the proposed methods were further tested by performing recovery studies via standard addition method. In this case pre-analysed drug tablet powder (ART or DHA) was spiked with pure drug (ART or DHA) at 3 different concentration levels and the resulting total being analysed using the proposed methods. The percentage recovery values of the pure drug ranged from 98.70 – 102.30% with standard deviation of 1.01 – 1.23 in all cases as recorded in Table 4. The results show excellent recoveries meaning that pharmaceutical excipients had no interference with the developed methods.

CONCLUSION

Two very simple, sensitive and accurate methods were developed for the assay of ART and DHA in tablets and bulk. These reproducible methods require no tedious extraction using hazardous organic solvents, no heat and tight pH control. The methods are strongly recommended for routine laboratories and field station analysis of ART and DHA. Finally, the analyst and the environment face no danger as the chemical/reagent used are relatively safe.

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