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Quantitative titrimetric and spectrophotometric determination of dihydroartemisinin based on the redox reaction with cerium ammonium sulphate

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ABSTRACT

Two titrimetric and one sensitive spectrophotometric methods are developed for the determination of Dihydroartemisinin (DHA) in bulk and in tablets formulations. Titrimetric method A is based on the generation of hydrogen peroxide from DHA in acid medium in situ; which is titrated against cerium ammonium sulphhate to a ferroin sky blue end point. The second titrimetric method B is based on the initial oxidation of the drug DHA with known excess of cerium ammonium sulphate and then back titrating the excess oxidant with ferrous ammonium sulphate using ferroin as an indicator. In the third method the drug was oxidized by cerium ammonium sulphate the oxidant in acid medium with the addition of ferroin indicator resulting in a sky blue chromogen measured spectrophotometrically at 510nm. The amount of cerium ammonium sulphate used was proportional to the drug concentration in all three methods proposed. The applicable range for both titrimetric method A and B were 2-20mg/ml and 1-15mg/ml respectively. The calibration graph generated in the spectrophotometric method was linear obeying Beer's law in the range of 1.0 - 10µg/ml. The method were validated for accuracy, precision, selectivity and successfully applied in the determination DHA tablets brands procured locally with no interference from pharmaceutical excipient. Statistically the proposed methods showed good congruence with official pharmacopoeial method.

Keywords: Counterfeit, Dihydroartemisinin, Malaria, Redox, Spectrophotometric, Titrimetric.

INTRODUCTION

Malaria remains the most deadly vector borne disease in the tropical region of the world. It continues to be massive global public health problem as it is prevalent in over 104 countries and nearly half of the world population (3.4 billion) is at risk (WHO malaria report [1] Though malaria is preventable and curable it is one of the most important cause of maternal and childhood morbidity and mortality in sub Sahara Africa [2]. The causative agent *Plasmodium* sp especially *Plasmodium falciparum* has developed resistance overtime to affordable and available drugs of the quinolines (chloroquine) and the antifolates (sulphadoxineand pyrimethamine). Artermisinin (qinghaosu), a sesquiterpene endoperoxide isolated from *Artermisia annua* and its derivatives as discovered in the 1970s became a remarkable life saving anti-malaria component effective against drug resistant *Plasmodium falciparum* and cerebral malaria [3,4]. These compounds combine potent, rapid anti malaria activity with a wide therapeutic index and an absence of clinically important resistance [5]. These excellent qualities of Artemisinin and it derivatives led to the directive by the WHO and health authorities in malaria endemic countries to recommend the use of Artemisinin combination therapy (ACT). That is the combination of an Artermisinin derivative with one other antimalaria. (Artesunnate plus Amodiaquin tablet). Currently the fight against multidrug resistant *Plasmodium*

falciparum malaria appears to be in jeopardy following the reported cases of manufacturing and distribution of substandard, sub therapeutic, adultratedand fake artermisinins in Asia and Africa [6,7,8,9,10].Producers of the fake and adulterated artemisinin derivatives are very sophisticated, they produce, identical holograms, batch number, expiry date and blister packs and tablet looking absolutely genuine making the detection of fake antimalarial very difficult. Their packaging is usually a perfect copy and their packaging cannot be assessed readily by lay persons or even experts of pharmaceutical industry without the aid of quality testing laboratory. These unique laboratories with high level of technical facilities are rarely available in developing countries [10] where the resistant problem is paramount. Simple and affordable quantitative method is therefore necessary to check this menace, within the supply chain at the field stations and point of entry. Officially Dihydroartemisinin is assayed using UV Spectrophotometry and HPLC. Artemisinin and it derivatives have been assayed using methods developed by many workers. These methods include HPLC [11], HTPLC [12] UV –Vis Spectrophotometry [13, 14,15,16]. Most of the developed methods are quite affordable and sensitive but a great number of them suffer from one shortcoming or the other. Some of the developed methods are expensive making the process in affordable in developing countries. This coupled with the fact that Artemisinin and its derivatives have no active chromophoric groups in the Uv - vis region; assay of dihydroartemisinin largely depend on derivatization reactions to develop the right assay. Dihydroartemisinin is the central metabolite and most potent of artemisinin and its derivatives this makes it a major target for counterfeiters. This method as developed is sensitive affordable and devoid of the usage of expensive and toxic organic reagents which could be hazardous to the analyst and the environment.

EXPERIMENTAL SECTION

All spectral determinations were made using spectrophotometers model heylos β. Thermo electron Corporation USA. With 1cm matched quartz cell.

Chemicals and Reagents

All chemicals used were analytical grade and solutions were prepared and diluted using distilled water and the pure drug solution was prepared using absolute ethanol.

Reagents

Ceric Ammonium sulphate: A 0.05N cerium ammonium sulpahte was prepared by dissolving 31.63g of the chemical (Merck, Germany) in 30ml 2N sulphuric acid. This was standardized using 0.05N solution of Analar grade ferrous ammonium sulphate (i.e. 19:61g per litre) using 2 drops of 0-phenanthioline –ferroin as indicator.

Ferroin indicator

0-Phenanthioline (ferroin indicator) procured from Merck Damstadt Germany and used as procured.

Sulphuric acid 2N: The concentrated sulphuric acid BDH, England sp.gr. 1.84 was diluted appropriately to obtain 2N.

Standard Dihydroartemisinin solution: A stock solution of pure dihydroarteminisin 2mg/ml was prepared by dissolving 500mg of the pure sample in 250ml of absolute ethanol. The pure sample of dihydroartemisinin wasa kind gift from the Directorate of Pharmaceutical Service, University of Uyo Teaching Hospital Uyo.

Ferrous Ammonium Sulphate (FAS): A 0.05N ferrous ammonium sulphate was prepared by weighing 19.61g of the chemical (Merck Germany and dissolved in 20ml of distilled water and made up to the 1litre mark with distilled water.

Pharmaceutical excipients

Glucose, lactose, sucrose, talc, magnessium stearate, corn starch, accassia gum.

Method A. (Cerimetric Titration).

Different 20ml aliquots of the standard solution of the reference drug containing 5-25mg of dihydroartemisinin was accurately transferred into a 100ml titration flask; using a burrette. Then 5ml of 2N sulphuric acid was added and titrated with 0.05N solution of Cerric ammonium sulpahte adding 2 drops of ferroin (1, 10-phenantholine) as an indicator until the first and permanent sky blue colour appears marking the end point. A blank titration was performed following exactly the same process as described but omitting the drug solution. The quantity of DHA in each aliquate is calculated using.

Formula

$$\%DHA\ (ie H_2O_2) = \frac{V_1 X N_1\ X\ Meq\ H_2O_2\ X\ F\ X\ 100}{M}$$

% DHA = concentration of H_2O_2 in weight % (generated *in situ*)
V_1 = volume of Cerium ammonium sulphate solution consumed by the H_2O_2 titration (ml).
N_1 = Normality of Cerium ammonium sulphate solution
Meq = Milliequivalent of DHA. This is the molecular weight of DHA divide by The number of electrons exchanged in the Redox reaction divided by 1000.
M = quantity of sample (g).
F = dilution factor.

Method B (indirect titratron)
Aliquots of the standard solution (20ml) containing 5-20mg of dihydroartemisinin was accurately measured and transferred into a 100ml titration flask and 5ml of 2N sulphuric acid was added to acidify the medium. Then 10ml of 0.05N Cerric Ammonium sulphate solution was added using the pipette. The mixture in the flask was shaken to mix well and allowed to stand for 15minutes and finally the unreacted cerium ammonium sulphate was then titrated against ferrous ammonium sulphate using 2 drops of ferroin as the indicator until the first permanent red colour appeared. A blank titration was carried out exactly as discussed here without the dihydroartemisinin.

The amount of DHA in each aliquot is calculated or determined using the formula.
Amount of DHA (mg) = H_2O_2 generated in situ

$$= \frac{(A-D)\ x\ Mwt\ x\ C}{n}$$

Where A is the volume of FAS consumed in the blank titration
D amount of FAS consumed in the analytic titration
Mwt = relative molecular mass of DHA
n = number of moles of Cerium ammonium sulphate reacting with 1 mole of DHA.

Method C

Different aliquots (0.25-5.0ml) of standard 10μg/ml of dihydroartemisinin solution were accurately measured and transferred to series of 10ml capacity volumetric flask. The content in the flask was acidified using 1ml of 5M sulphuric acid. The volume in the flask was adjusted to 5ml by adding absolute ethanol. Then 1ml of 0.05N cerium ammonium sulphate was added to the flasks and shaken to mix well and set aside for 15minutes. Finally 1ml of ferroin indicator reagent was added. The content of the flask was shaken to mix well and made up to the 10ml mark of the volumetric flask. The absorbance of the resulting solution was then measured at 510nm against reagent blank. A calibration curve was generated from where the unknown concentration was determined or calculated from Beer's law data.

ASSAY OF DHA IN TABLETS
Twenty (20) tablets of each of the local tablet brands were separately measured accurately and pulverized. A quantity of the powder equivalent to 100mg of the drug powder was weighed and placed in a 100ml volumetric flask containing 50ml of absolute ethanol and sonicated for 10minutes and shaken vigorously for another 10 minutes, then absolute ethanol was added was added to make up the 100ml mark and shaken vigorously for another 10 minutes and filtered using the whatman filter paper No42. The first 10 ml of the filtrate was discarded. The resulting concentration of the drug 1mg/ml was used for the titrimetric determinations in A and B; while fractions of the 1mg/ml solution was further diluted to 10µg/m from where convenient aliquots were analyzed using the spectrophotometric method.

Procedure for Placebo Blank
A placebo blank powder containing some pharmaceutical excipients and diluent such as talc 1mg, lactose 15mg, magnessium stearate 0.5mg, glucose 15mg, acacia 2mg, microcrystalline cellulose 12.8mg, sucrose 5mg and corn starch added to bulk up the mixture to 100mg. This mixture was agitated to mix properly and homogenized to a homogenous mixture and transferred into a 100ml capacity volumetric flask containing 40ml of distilled water. The resulting mixture was shaken vigorously and sonicated for 20minutes. A further 40ml of distilled water was added and shaken vigorously for another 10minutes. Finally the mixture was made up to the 100ml mark using distilled water and filtered using whatman filter paper number 42. Aliquots of the placebo blank were analyzed exactly by following the procedure for tablets dosage form as described above.

Procedure for analysis of synthetic mixture

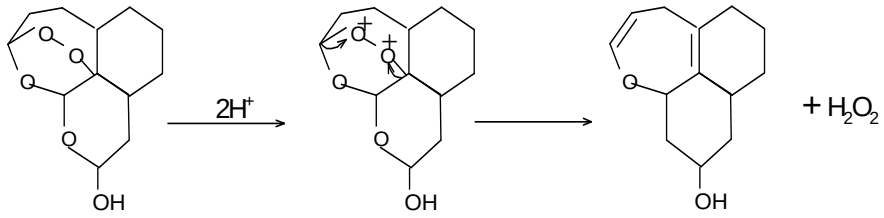
The synthetic mixture was prepared by carefully measuring 100mg of pure dilydroartemisinin powder and transferred into a beaker containing 100mg of the placebo blank powder as prepared above the resulting mixture of the two was homogenized and 100mg of the resulting mixture was carefully transferred to a 100ml calibrated volumetric flask containing 50ml of distilled water and sonicated for 10minutes. Thereafter two equal volumes of 25ml of distilled water was added and shaken at each time. The resulting synthetic mixture containing the drug was filtered using whatman filter paper number 42. The first 10ml of the filtrate was discarded. The resulting synthetic drug solution was diluted appropriately to a working concentration of 100µg/ml from where a suitable aliquot was analyzed as described in the procedure for tablets above.

RESULTS AND DISCUSSION

The proposed methods were possible because of the oxidizing capability of Cerium IV. In acid medium Cerium (IV) ion is a very strong oxidant comparable to $kMnO_4$. Its simplicity is based on its single-electron transfer redox reaction. Cerium IV is incidentally a single-electron acceptor $Ce^{4+} + e^- = Ce^{3+}$ in such reaction the equivalent weight is equal to the molecular weight. The first method involved the use of cerium (IV) solution as the titrant (cerimetry) against the hydrogen peroxide generated *in situ* by the cleavage of the endoperoxide bond of the dihydroartemisinin using ferroin indicator to a pale blue end point.

$2Ce^{4+} + H_2O_2 = 2Ce^{3+} + O_2 + 2H^+$

Step 1



Step 2

$2Ce^{4+} + H_2O_2 \longrightarrow 2Ce^{3+} + O_2 + 2H^+$

In acid medium the two oxygen centers of the endoperoxide bond of the dihydroartemisinin is protonated and cleaved to generate the needed hydrogen peroxide. The stoichiomety was evaluated to be 1: 1 for the dihydroartemisinin and cerium IV solution used. The second titrimetric method was indirect but was also based on the generation of the hydrogen peroxide from the endoperoxide bond of dihydroartemisinin in acid solution. In this method known amount of cerium IV solution was added to the DHA and allowed a reasonable time of 15 minutes for the reaction to be complete and then the excess or remaining oxidant in this case titrated against ferrous ammonium sulphate using 1 drop of ferroin indicator. The concentration of the DHA in each aliquot was calculated from the amount of cerium ammonium sulphate reacted. The stiochiometry was evaluated and discovered to be 1: 1 for the drug and the cerium IV.

Spectrophotometric method was also based on the reaction of the hydrogen peroxide generated *in situ* from the dihydroartemisinin's endoperoxide bond with the cerium IV solution being the oxidant. The ferroin indicator added lead to the sky blue chromogen observed which enabled the absorbance to be measured at 510nm. The increase in the absorbance corresponded with the increase in the concentration of drug in each aliquot. Hence Beer's law was obeyed.

Methods Optimization. Experimental conditions of the three methods were carefully studied and optimized. All other experimental parameters were kept constant while the particular parameter under study was varied and the effect on the method observed.

Contact time: In all the three proposed method the reaction time were studied in minutes up to 20minutes. The redox reaction between the oxidant (cerium IV) and the drug was complete in about 15 minutes. Hence at 15minutes the best result were obtained; further increase in the contact time gave no valuable result. In the spectrophotometric method the absorbance at a contact time of 15minutes were higher.

Temperature: Though increase in temperature results in increased rate of reaction. Reaction temperature of between 20 to 60^0C was studied. At higher temperature, the reaction time was too fast; and at above 600C very in consistent results was obtained. The reaction proceeded and got to completion at room temperature with good and reliable results. Hence the experiment was performed under room temperature.

Effect of Acidity on the Reaction: The redox reaction takes place in high acid medium. High acid medium aided the generation of the Hydrogen peroxide. The reaction of Ce IV solution and the generated hydrogen peroxide was clean and fast. High pH medium led to slow rate of reaction and possible precipitation of the cerium IV.

Effect of Acid Types on the Reaction: When all other parameters were kept constant while the H_2SO_4 concentration was increased, better results were obtained both for the titrimetric and the spectrophotometric method, H_2SO_4 strength of between 0.1N to 2N sulphuric acid was found to be suitable for the reactions. In the spectrophotometric method 1ml of 2N H_2SO4 in 10ml aliquot of drug solution was found to be adequate. Other acid types were tried HCl, HNO_3 and $HClO_3$. All gave very good and reasonable result but the HNO_3 and HCl were the least stable.

Method Validation

The accuracy and precision of both the titrimetric methods and the spectrophotometric method were evaluated. There were done by preparing the pure drug at three concentration levels and performing six replicate analyses for each method. Within three working days (intra day) and three consecutive days. The accuracy was evaluated as relative % Error while the precision was evaluated as the relative standard deviation percent (RSD %). The results as shown in table 1 show that the accuracy was < 2.08% while the precision ≤ 2.06%. Showing good accuracy, repeatability and reproducibility. The formular for accuracy as relative error %

$$RE\% = \frac{[\text{Amount found} - \text{Amount taken}]}{\text{Amount}} \times \frac{100}{1}$$

Table 2 Evaluation of Accuracy and Precision

S/N	AMOUNT OF DHA TAKEN	Intra-day Accuracy and Precision			Inter day Accuracy and Precision		
		AMOUNT OF DHA FOUND	R.E	RSD%	AMOUNT OF DHA FOUND	R.E%	RSD%
1	Titrimetry (method A) 5mg/m/ 10mg/m/ 20mg/m/	 5.11 10.26 20.35	 2.20 2.60 1.75	 0.90 1.05 1.71	 5.13mg/m 10.24mg/m 20.30mg/m	 2.60 2.40 1.50	 1.06 0.98 0.16
2	Titrimetry (method B 4mg/m/ 8mg/m/ 12mg/m/	 4.10 8.18 12.20	 2.5 2.25 1.66	 1.02 0.92 0.68	 4.11 8.23 12.25	 2.75 2.88 2.08	 1.12 1.17 0.85
3	spectrophotometry (method C) 3.0mg/m/ 6.0mg/m/ 9.0mg/m/	 3.08 6.15 9.19	 2.67 2.50 2.11	 1.08 1.02 0.87	 3.09 6.16 9.25	 3.00 2.66 2.78	 1.22 1.09 1.02

Table 1 Analytical parameters and optical characteristic of the purposed method

S/N	Parameter	Value
1	λmax. (Wavelength) (nm)	510
2	Beer's law linear range µg/ml	0.25-6.0
3	Molar absorptivity	3.20×10^4
4	Sandell sensitivity µgcm^{-2}	0.00884
5	Limit of detection µg/ml (LOD)	0.17
6	Limit of quantification µg/ml (LOQ)	0.32
7	Regression equation	A= 0.02820 c + 0.012
8	Slope	0.0282
9	Intercept	0.012
10	Correlation coefficient r^2 =	0.9986

Linearity: In the spectrophotometric method, it was observed that there is a linear relationship between when absorbance was plotted against concentration of the drug as shown in the calibration generated. Beer's law was obeyed in the region of 0.25 – 5µg/ml. The regression equation was in the form of A = bc +x. when A is the absorbance, b is the slope, c the drug concentration and x the intercept obtained by the least square method. The correlation coefficient, slope and the intercept are recorded in table 1. Sensitivity parameters for the spectrophotometric method such as molar absorptivity and sandell sensitivity were also determined and the values recorded in table 1. The limits of detection (LOD) and limits of qualification (LOQ) were evaluated based on the current ICH guidelines using the formulae.

LOD = $\frac{3.3\sigma}{S}$ and LOQ = $\frac{10\sigma}{S}$ where σ is

The standard deviation of 5 blank determination and S representing the slope of the calibration curve the values are also recorded in table 1.

Selectivity Interference

The reliability and feasibility of the results in the presence of interferences which is a measure of selectivity was studied using common excipient used in tablet formulation as discussed earlier in placebo blank and procedure for synthetic mixture. The percentage recovery of DHA by method A and B were 97.59±1.12 and 97.39±1.8 (n=7) respectively. For method C the percentage recovery 98.79±1.14 (n=7). Showing that the excipient had no effect of the proposed methods.

Robustness and Ruggedness

The robustness of the three methods was evaluated by small deliberate changes in the parameters that could affect the overall result obtained from the proposed methods. The parameter studied includes slight change in the acid concentration, reaction/contact time and diluting solvent. It was discovered that the capacity of the developed methods was not affected by these small but deliberate variation. The ruggedness of the spectrophotometric method was evaluated via RSD% on two instruments and by two different analysts for two consecutive days. There was no significant statistical difference from the RSD% result obtained by the two analysts using two different instrument conferring that the spectrophotometric method was very rugged.

Methods Application

The proposed method were successfully used to assay four commercially available brands of DHA in tables (cotecxin, codisin, santecxin and Alaxin), procured locally in Uyo, south/south Nigeria. The result obtained by the developed method were statistically compared with the UV spectrophotometric method in the international pharmacopoeia (IP 2003) via students T-test and for accuracy and F-test as a measure of precision the results are recorded in table 4. The t and F values at 95% confidence level and at 4 degrees of freedom showed that the values were lower than the critical (tabulated) value showing no significant difference between the developed methods and reference method.

Recovery Study

Recovery studies was performed for each of the developed methods via standard addition method just to further confirm the accuracy and practicability of the methods. A pre analyzed tablet powder was spiked with pure DHA at 3 different concentration levels and the total amount of the drug determined by the proposed methods. The percentage recovery of the added pure drug DHA were determined with the standard deviation and

Table 3 result of analysis of commercial branch of DHA tablet by the purposed method

Tablet analyzed	Label claim (mg)	Reference method	Results of the Develop Methods ± SD		
			Method A	Method B	Method C
Alaxin	60	110.0± 1.20	110.8± 1.15 F= 1.09 t=1.08	111.0± 1.0 F= 1.44 t=1.43	110.8 ± 1.95 F= 1.59 t=1.17
Cotecxin	60	110.0± 1.17	111.0± 1.15 F= 1.04 t=1.36	111.2 ± 1.21 F= 1.07 t=1.35	110.9 ± 1.08 F= 2.14 t=1.42
Sanctectin	60	110.0± 1.25	111.0± 1.20 F= 1.08 t=1.05	111.9 ± 1.08 F= 1.34 t=1.21	110.8 ± 1.10 F= 1.29 t=1.08
Codisin	60	110.0± 1.28	111.0± 1.10 F= 1.25 t=1.36	111.9 ± 1.0 F= 1.64 t=1.24	110.9 ± 1.13 F= 1.28 t=1.18

Mean of five determination. The value of t (tabulated at 95% confidence level and at four degrees of freedom is = 2.77. The value of F (tabulated at 95% confidence level at 4 degree of freedom = 6.37.

Table 6 Result of recovery study via standard addition method

	TITRIMETRIC METHOD A				TITRIMETRIC METHOD B				SPECTROPHOTOMETRIC METHOD			
Drug Formulation Studied	**Amount of DHA in Table (mg/m/)**	**Amount of Pure DHA Added**	**Total Found**	**% Recovery of Pure DHA**	**Amount of DHA in Table (mg/m/)**	**Amount of Pure DHA Added**	**Total Found**	**% Recovery**	**Amount of DHA in Table (mg/m/)**	**Amount of Pure DHA Added**	**Total Found**	**% Recovery**
ALAXIN®	6.04	3.0	9.07	101.1	5.04	2.0	7.03	99.6	10.0	5.0	15.01	100.3
	6.04	6.0	12.02	99.8	5.04	4.0	9.06	100.7	10.0	8.0	17.99	99.9
	6.04	8.0	14.15	101.4	5.04	6.0	11.11	101.2	10.0	9.0	19.1	101.1
COTECXIN®	5.10	3.0	8.09	99.7	4.10	2.0	6.13	10.15	10.2	5.0	15.01	99.8
	5.10	6.0	11.15	100.9	4.10	4.0	4.11	100.4	10.2	8.0	16.14	101.6
	5.10	8.0	13.08	99.8	4.10	6.0	10.20	101.6	10.2	9.0	19.11	101.00
SANTECTIN®	4.20	3.0	7.21	100.4	5.50	2.0	7.52	100.5	10.2	5.0	15.06	100.7
	4.20	6.0	10.19	99.8	5.50	4.0	9.54	101.3	10.2	8.0	18.23	100.4
	4.20	8.0	12.18	99.7	5.50	6.0	11.49	99.9	10.2	9.0	19.01	99.9
CODISIN®	4.70	3.0	7.75	101.7	4.60	2.0	6.59	99.6	10.50	5.0	15.48	99.6
	4.70	6.0	10.69	99.9	4.60	4.0	8.62	100.6	10.50	8.0	18.48	19.8
	4.70	8.0	12.71	100.1	4.60	6.0	10.65	100.8	10.50	9.0	19.6	101.1

Mean of 3 determinations

Recorded in table 5 the result ranged between 105.6± SD and 112.5%±SD. Showing a good recovery indicating little or no significant interference from pharmaceutical excipients.

CONCLUSION

Three new simple, sensitive and reproducible method are developed. Two titrimetric methods developed are very simple and can be used in field stations or at ports of entry to check the influx of counterfeit DHA that in daily being imported to Nigeria. The spectrophotometric method is also quite simple it can be used in routine quality control laboratories to check the sale and distribution of counterfeit and fake DHA currently in the local drug market by government agents (NAFDAC) very simple analytical reagents that are cheap and available easily in any laboratory were used. Fortunately the reagents are not dangerous and poses no hazard to the analyst or the environment and the equipment used are not sophisticated. Based on these advantages mentioned coupled with high accuracy, reliability and reproducibility. The methods are of great value for quality control laboratories of companies and government agents involved in checking imported drugs.

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