SPECTROPHOTOMETRIC DETERMINATION OF DIHYDROARTEMISININ USING THE FERRIC-THIOCYANATE COMPLEX FORMATION

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ATTIH, EMMANUEL[1], USIFOH, C. O.[2] AND EBONG, ANIEKAN[1]
[1]*Department of Pharmaceutical and Medicinal Chemistry, University of Uyo, Uyo, Nigeria*
[2]*Department of Pharmaceutical and Medicinal Chemistry, University of Benin, Benin City, Nigeria*
emmanuelattih@uniuyo.edu.ng

ABSTRACT

A simple and sensitive spectrophotometric method is developed for the determination of Dihydroartemisinin (DHA) in pure and pharmaceutical formulation. The method is based on the oxidation of Fe(II) to Fe(III) and the subsequent formation of red ferric-thiocyanate complex measured at 472 nm. The experimental conditions for the assay were studied and optimized. The absorbance was found to increase linearly with the drug concentration to give a calibration curve which obeys Beer's law in the range of 50-70 μgml^{-1} with a linear regression coefficient of 0.9979. The molar absorptivity was 2.18 x 104 $Lmol^{-1}cm^{-1}$, and Sandell sensitivity was 0.013 μgcm^{-2}. The Limit of Detection (LOD) and Limit of Quantification (LOQ) were found to be 0.37 μgml^{-1} and 1.22 μgml^{-1} respectively. The intra-day and inter day accuracy expressed as percentage relative error was <3% and the corresponding precision (%RSD) was >2.5. The proposed method was validated and applied in the determination of DHA in tablets procured commercially and the result obtained was statistically compared with official international pharmacopoeial method. The results of both showed good congruence. The accuracy and applicability of the method was further ascertained by performing recovery studies via standard addition method with the results showing no interference from pharmaceutical excipients.

INTRODUCTION

Malaria, a typical tropical disease, remains the most important source of concern in terms of morbidity and mortality. Though endemic within the tropics, it is a source of very great concern as it has extended to over 40% of the world's population (Robert et al 2001). Over 200 million people live in Sub-Saharan Africa and WHO in 1998, estimated there were 273 million cases of malaria worldwide and more than 1 million deaths were due to it. Current WHO (2012) report reveals that the African region accounts for 85% of malaria cases and 90% of malaria death worldwide. The treatment of malaria is becoming a problem because of the emergence of multidrug resistant *Plasmodium falciparum* species (WHO 11). They are no longer susceptible to the quinolines (chloroquine) and anti folates (sulfadoxine-pyrimethamine). The rapid emergence of this multidrug resistant parasite in Nigeria and West Africa raises the prospect that untreatable malaria may soon be encountered. To make matters worse, there are reported cases of the manufacture and distribution of fake and adulterated artemisinin and its derivatives in Southeast Asia and some parts of Africa (Amboise-Thomas 2012, Nayyar *et al* 2012, Zakeri *et al* 2012, Atemkeng *et al* 2007, Newton *et al* 2003) There are also confirmed cases of reduced susceptibility of the parasite in some known foci in Southeast Asia including Cambodia-Myanmar and the Vietnam (Rozendaal 2001). The main source of genuine artemisinin and its derivatives into West Africa is China and Asian countries. Artemisinin and its derivatives in combination with other antimalerials (Artemisinin Combination Therapy – ACT) remains the treatment of choice for multidrug resistant

falciparum malaria. DHA is the major metabolite and the most potent antimalarial of the artemisinin derivatives and it is the best candidate for adulteration or outright faking. To cope with this potential threat, simple assay methods for the determination of DHA and other artemisinin derivatives will be of immense benefit to all. In the International Pharmacopeia, DHA is assayed using two methods; the first is by HPLC and the second is by UV-spectrophotometry. Many primary health institutions in malaria edermic region lack the capacity to procure and operate the HPLC because of its cost. The UV-spectrophotometric method which is of moderate cost is highly recommended and hence the development of the proposed method which could be complementary to the pharmacopeial method. From the literature, some methods have been developed for the determination of DHA and other artemisinin derivatives. The methods, HPLC (Gabriel and Plaizer-Vercammen 2004, Na-Bangchang *et al*1998,Na-Bangchang *et al* 1997, Karburange *et al* 1997,Barth *et al* 1996) HTPLC,(Argawal *et al* 2009) UV-vis spectrophotometry(Adegoke and Osoye 2011 ,Green *et al* 2001,developed were mostly for metabolites in the body fluid and were hardly applicable for assay of DHA in tablet formulations. The proposed method is based on the oxidation of iron II to iron III by hydrogen peroxide generated from the DHA *in situ* and the subsequent interaction of the ferric iron with thiocyanate ion to give a blood-red coloured complex measured spectrophotometrically at 472 nm.

All spectral determinations were made using spectrophotometer Model HeyLos β from Thermoelectron Corporation, USA with 1-cm matched quartz cell.

Reagents and Chemicals

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

Reagents

1. Ferrous ammonium sulphate (FAS) (Marck, Damstadt, Germany). A 0.05 M solution of FAS was prepared by dissolving 19.6 g of the substance in 5 ml of dilute sulphuric acid and made up to 1 liter with distilled water.
2. Potassium thiocyanate (Sigma). A 2 M solution of potassium thiocyanate was prepared by dissolving 19 g of the chemical in distilled water and made up to 100 ml.
3. Hydrochloric acid (Sigma). A 2 M solution of the acid was prepared by diluting the concentrated acid appropriately with distilled water.

Pure sample

Pharmaceutical grade DHA was donated by Directorate of Pharmaceutical Services of University of Uyo Teaching Hospital, Uyo, as a kind gift and was used as received. A standard stock solution of 200 µg/ml Dihydroartemisinin was prepared by weighing and accurately dissolving 20 mg of DHA in 100 ml of absolute ethanol. This stock solution was further diluted to 50 µg/ml for the assay.

General Procedure

Different aliquot (0.25, 0.50, and 5.00 ml) containing 50 µg/ml of standard DHA solution were accurately transferred into a series of 10 ml calibrated volumetric flask using a micro burette. The total volume of the content in the flask was adjusted to 5 ml using absolute ethanol. The content of the flask was acidified with 1 ml of 2 M hydrochloric acid and shaken gently, followed by the addition of 1 ml of 0.05 M solution of ferrous ammonium sulphate (FAS). The resulting mixture was swirled gently and allowed to stand for 10 minutes, then 1 ml of 2 M potassium thiocyanate was added and made up to the mark using absolute ethanol. The absorbance of the resulting solution was measured at 472 nm against absolute ethanol as blank. A calibration curve was generated by plotting the absorbance as a function of drug concentration. The concentration of the unknown was deduced from the calibration curve or computed from the regression equation derived by evaluating Beer's law data.

Procedure for tablets

Twenty tablets of Alaxin® (DHA) were weighed and pulverized using a ceramic mortar and pestle. A quantity of the powder equivalent to 100 mg of the drug (DHA) was weighed and placed in a 100 ml volumetric flask containing 20 ml of absolute ethanol and sonicated for 10 minutes. A further 50 ml of absolute ethanol was added and shaken vigorously to extract the drug. Finally, the content was then made up to 100 ml mark and filtered using Whatmann filter paper No. 42. The first 10 ml portion of the filtrate was discarded. The resulting concentration of the drug (DHA) was 1 mg/ml which was diluted stepwise to obtain a working concentration of 100 µg/ml from where a convenient aliquot was analyzed using the general procedure discussed above. The method was validated for precision, accuracy, specificity, robustness and ruggedness. Recovery method was performed via standard addition method. Other brands of DHA, i.e. Cotecxin®, Santecxin® and Codisin® were evaluated using the proposed method discussed above in the procedure for tablet.

RESULTS AND DISCUSSION

The presence of peroxide is indicated by the formation of red colour when a solution is shaken with an aqueous solution of ferrous ammonium sulphate and potassium thiocyanate. The peroxide oxidizes ferrous ion into ferric ion, which reacts with thiocyanate ion to give the characteristic blood red colour of the complex (Morrison and Boyd 1985). The formation of a blood red colour is a confirmatory test for peroxide and hence forms the basis for this determination showing maximum absorbance at 472 nm.

Mechanism of Reaction

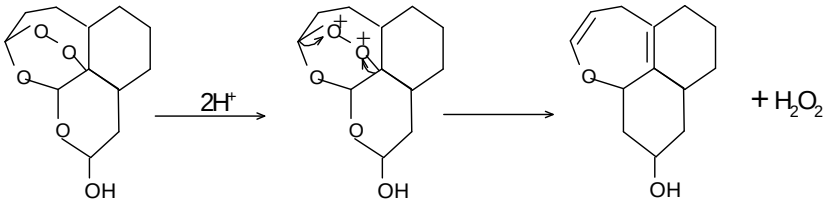
The mechanism of reaction is not very clear but it may likely follow the scheme below:

$$\text{Peroxide} + Fe^{2+} \longrightarrow Fe^{3+} \xrightarrow{SCN} Fe(SCN)_n$$

red complex where n = 1 to 6.

Equation 1

The 2 oxygen centers in DHA (the endoperoxide bond) is protonated in the presence of acid leading to the cleavage of the bonds hence the generation of hydrogen peroxide *in situ* as shown in figure 1 (equation 1).



Equation 2

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} \xrightarrow{SCN} Fe(SCN)$$

blood red complex

Equation 3

The hydrogen peroxide generated *in situ* then oxidizes Fe^{2+} to Fe^{3+} ion which finally reacts with the thiocyanate ion to give the characteristic blood red complex as shown in Figure 1 (equation 2).

Optimization

Optimum experimental conditions for this reaction was carefully studied and optimized. This was done by varying one parameter at a time, while keeping others constant and then observing the effect produced on the absorbance of the blood red complex formed.

Effect of pH

The variation of pH with absorbance was studied, solutions of 0.1 M HCl and 0.1 M NaOH were chosen for the initial observation. It was observed that the acidic medium resulted in the formation of better colour for the complex. Therefore the absorbance was observed by varying

the concentration of the acid used. It was observed that 1 ml of 2 M HCl gave the best absorbance for the coloured complex, higher concentrations gave erratic absorbance readings Figure 1.

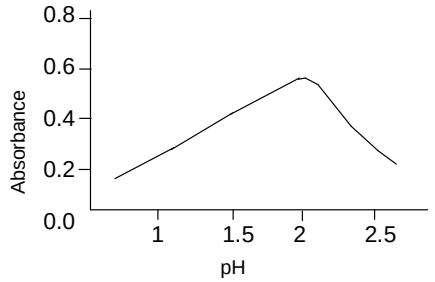


Figure 1: Effect of pH on absorbance

The maximum absorbance was observed at pH value of 2; this could be as a result of maximum protonation of the endoperoxide bond of DHA leading to the easy generation of hydrogen peroxide *in situ*. In alkaline medium, hydrogen peroxide is not generated *in situ* instead the lactone ring of DHA is opened.

Effect of ferrous ammonium sulphate concentration (Iron II concentration)

To study the effect of FAS concentration on the absorbance of the formed complex, the concentration of FAS was varied between 0.01 – 0.10 M while holding other parameters constant. It was discovered that the concentration of 0.05 M FAS was the most suitable as it gave the best absorbance. Further increase gave erratic values of absorbances.

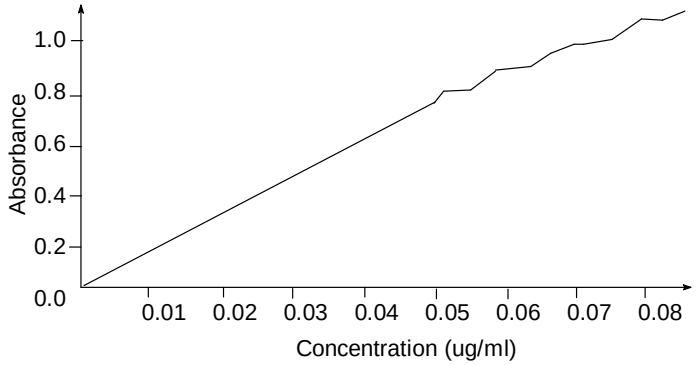


Figure 2: Effect of concentration of FAS on the absorbance of formed complex

Effect of thiocyanate ion concentration

Other parameters were kept constant while the volume and concentration of thiocyanate ion were varied. It was found that 1 ml of 2 M potassium thiocyanate was adequate for the rapid oxidation of iron II to iron III as well as the optimum colour formation. Higher values and the concentration of the thiocyanate ion gave higher unreliable absorbance readings (Figure 3).

Effect of the type of acid

The reaction was carried out using sulphuric acid, phosphoric acid and acetic acid in the place of hydrochloric acid. It was observed that hydrochloric acid and sulphuric acid gave the best absorbances and 1 ml of 2 M hydrochloric acid was adequate in a total volume of 10 ml of reactants. Further increase in volume had no effect on the absorbance (Figure 4).

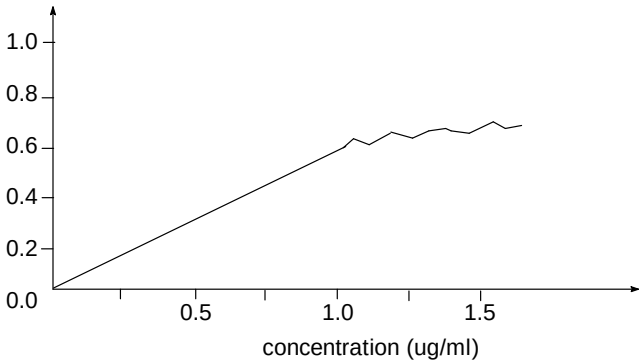


Figure 3: Effect of concentration of thiocyanate on the absorbance of the formed complex

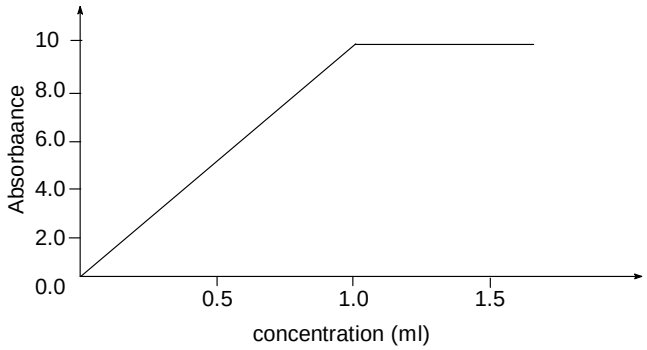


Figure 4: Effect of concentration on the absorbance of formed complex

Order of addition

The absorbance of the coloured complex formed was observed by varying the order of addition of the reagent as shown and the effect on absorbance monitored.

1. Drug + HCl + FAS + Thiocyanate
2. Drug + FAS +HCl + Thiocyanate
3. Drug + Thiocyanate + FAS + HCl
4. Drug + Thiocyanate + HCl + FAS

It was observed that the order that produced the best absorbance was Drug + HCl + FAS + Thiocyanate ion

Stability

The stability of the complex formed was studied under normal laboratory conditions at 25^{o}C and normal day light. Under optimum condition at the specific concentration of the reagents measured above, the absorbance of the complex formed was measured as a function of time. It was discovered that the formed complex was stable for over 60 minutes.

Effect of temperature

While keeping other experimental parameters constant and gradually increasing the temperature of the reaction, the study showed that there was no significant change in the rate of oxidation of iron II to iron III and its subsequent complexation as shown in the absorbance studied.

Method of validation

The proposed method was validated for linearity, sensitivity, selectivity, accuracy, precision, robustness and ruggedness.

Linearity

There is a linear relationship between absorbance and drug concentration. The increased absorbance is directly proportional to the drug concentration. Beer's law is obeyed when absorbance is plotted against concentration in the range of 0.5 to 50 µg/ml. the calibration curve was typical of a straight line graph with the equation:

$$A = bc + x$$

where A = absorbance; b = slope; c = drug concentration in µg/ml and

x = the intercept obtained by least square method. (Figure 5).

The correlation coefficient, slope and intercept are recorded in Table 1.

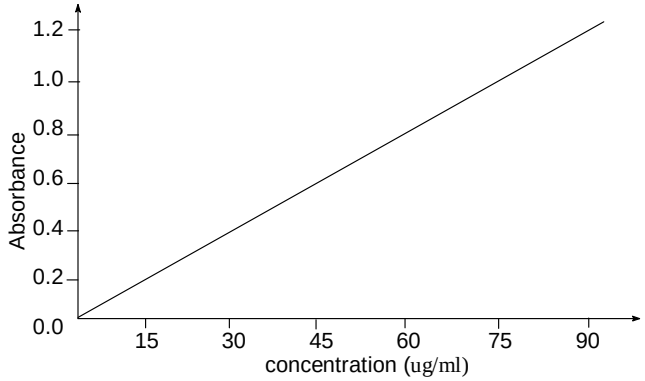


Figure 5: Calibration curve for spectrophotometric method

Table 1: Analytical parameters and optical characteristics of the proposed method

S/n	Parameter	Value
1	λ_{max} nm	472
2	Molar absorptivity($Lmol^{-1}cm^{-1}$)	2.18×10^4
3	Sandel sensitivity($Mgcm^{-2}$)	0.013
4	Limit of detection(Mg/ml)	0.37
5	Limit of quantification(µg/ml)	1.22
6	Regression equation	$A = bc + \chi$, ($A = 0.0188c + 0.006$)
7	Slope	0.0188
8	Intercept	0.006
9	Correlation coefficient	0.9979

Sensitivity

Parameters for sensitivity of the developed method e.g. apparent molar absorptivity and Sandell sensitivity were determined and the values recorded in Table 2. The Limits of Detection (LOD) and Limits of Quantification (LOQ) were calculated based on the current ICH guidelines using the formulae:

$$LOD = \frac{3.3\sigma}{S} \text{ and } LOQ = \frac{10\sigma}{S}$$

Where σ is the standard deviation of 5 blank determinations and S, the slope of the calibration curve. The values are also recorded in Table 2.

Accuracy and precision

The accuracy and precision of the developed method was determined by preparing solutions containing five different concentrations of DHA and analyzed in five replicate determinations within the same day (intra-day) and five consecutive days (inter day). The accuracy was calculated as percentage relative error (R.E. %), using the formula:

$$\text{R.E. \%} = \frac{\text{Amount Found} - \text{Amount Taken}}{\text{Amount Taken}} \times \frac{100}{1}$$

The precision was calculated as percentage relative standard deviation (RSD %). The inter- and intra-day accuracy values are recorded in Table 2.

Selectivity/Interferences

The reliability of the results in the presence of interferences which is a measure of selectivity was studied using common excipients in tablet formulation such as lactose, talc, magnesium stearate, gum acacia, sucrose, glucose, sodium citrate and starch. Two methods were used: (1) preparation of placebo blank and (2) preparation of synthetic mixture spiked up with a specific concentration of the DHA. The placebo blank was prepared by using a specific amount of the excipient and made into a solution and assayed as described in the "preparation of tablets". In the second method, 90 mg of the excipients (mixed) in different composition was spiked up with 10 mg of DHA, mixed and homogenized and dissolved in enough ethanol to make up 100 ml. the resultant mixture was assayed via the procedure for tablets. On both occasions, there was clearly no interference from the excipients mentioned. The selectivity of the proposed method was further ascertained by studying the interference from some ionic species. Some anions and cations were individually measured and transferred into specific concentration of the DHA and the absorbance measured at 472 nm based on the fact that only a specific ionic species is the only extra ionic species in the system. The specificity was measured using percentage error as the only criterion in comparison with the absorbance obtained for that concentration. The result showed that there was insignificant error (Er % $\leq$ 4 %). Few cations such as Cu^{2+} and HPO_4^{2+} showed error $\geq$ 5%.

Table 2: Statistical evaluation of accuracy (R.E. %) and precision (RSD %)

S/N	Amount of DHA taken µg/ml	Intra-day accuracy and precision			Inter-day accuracy and precision		
		Amount of DHA found	RE %	RSD %	Amount of DHA found	RE %	RSD %
1	40.00	40.00	2.5	1.25	40.80	2.0	1.96
2	80.00	81.85	2.31	2.17	81.78	2.19	1.57
3	120.00	122.00	1.66	1.17	122.2	1.83	0.87

Table 3: Result of analysis of commercial brands of the tablets by the proposed method

S/N	Tablets analyzed	Label claim (mg)	Reference method	Results of the developed methods ± standard deviation
1	Alaxin	60.00	110.00 ± 1.100	110.90 ± 1.20 F = 1.19, t = 1.24
2	Cotecxin	60.00	110.00 ± 1.16	111.00 ± 1.15 F = 1.02, t = 1.37
3	Santecxin	60.00	110.00 ± 1.00	111.00 ± 0.96 F = 1.09, t = 1.6
4	Codisin	60.00	110.00 ± 1.25	111.00 ± 1.10 F = 1.29, t = 1.34

Mean of five determinations. The value of t (tabulated at 95% confidence level and at four degrees of freedom) = 2.77. The value of F (tabulated at 95% confidence level and four degrees of freedom) = 6.37.

Robustness and ruggedness

The robustness was evaluated by small and deliberate changes in the parameter that could affect the overall results generated from the developed method. These parameters include slight changes in acid concentrations and volumes, pH of the medium and slight changes in the temperature of reaction (from 25^o to 30^o). The capacity of the developed method was not affected by these small but deliberate variations. The ruggedness of the method was evaluated via RSD % on two instruments by two different analysts on two different days. There was no significant statistical difference from the RSD % results obtained by the two analysts using two equipment, confirming the ruggedness and the robustness of the developed method.

Table 4: Results of the recovery study by the standard addition method.

S/N	Tablets studied	Amount of drug (μg/ml)	Amount of pure drug added (μg/ml)	Total amount found (μg/ml)	Recovery of pure drug in % ± SD
1	Alaxin	40.20	20.00	60.45	101.00 ± 0.88
		40.20	40.00	80.75	101.40 ± 0.69
		40.20	60.00	101.00	101.30 ± 0.67
2	Cotecxin	41.00	20.00	62.00	105.00 ± 2.50
		41.00	40.00	82.50	103.00 ± 1.88
		41.00	60.00	103.50	104.00 ± 2.08
3	Santecxin	45.10	20.00	65.60	102.50 ± 1.25
		45.10	40.00	86.00	102.30 ± 1.13
		45.10	60.00	106.20	101.80 ± 0.92
4	Codisin	50.10	20.00	70.50	102.95 ± 1.25
		50.10	40.00	91.30	103.00 ± 1.50
		50.10	60.00	112.10	101.80 ± 1.67

The value of determinations

Method application

The developed method was successfully applied to assay four commercially available brands of DHA in tablets (i.e. Cotecxin, Codisin, Alaxin and Santecxin) procured locally in Uyo, South-South Nigeria. The results obtained by the developed method were statistically compared with the standard method in (IP 2nd) via student t-test for accuracy and F-test as a measure of precision. The results are shown 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) values, showing no significant difference between the developed and reference method showing a good congruence between the two.

Recovery studies

Recovery studies were performed via standard addition made to ascertain further the validity and accuracy of this method. A pre-analyzed tablet powder was spiked with pure DHA at three different concentration levels and the total amount of drug determined by the proposed method. The recovery percentage of the added pure DHA was determined with the standard deviation and recorded in Table 5. The result ranged between 105.60 and 112.50% with a standard deviation of 1.04 and 1.48% respectively, showing a good recovery indicating little or no interference from the pharmaceutical excipients.

CONCLUSION

In this work a simple, sensitive, precise, accurate and robust spectrophotometric method was developed. This method used ecofriendly chemicals which are not hazardous to the analyst and the environment. The advantage of this method is that it is devoid of exhaustive and tedious chemical extractive stages. The method was simple and required no heating. The proposed method is of immense value in routine quality control laboratories and in field stations especially in developing countries where sophisticated equipment is not available.

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