Validated Spectrophotometric Determination of Artesunate and Dihydroartemisinin Using Anisaldehyde/Sulphuric acid Reagent

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ABSTRACT

Background: To check the widespread manufacture and distribution of counterfeit Artemisinin derivatives in Nigeria, a simple sensitive spectrophotometric method has been developed.

Method: The method was based on the nucleophilic reaction of Artesunate (ART) and Dihydroartemisinin (DHA) with Anisaldehyde/Sulphuric acid reagent, monitored spectrometrically at λ_{max}500nm and 490nm for ART and DHA respectively.

Results: Beers Law was obeyed within the range of 5.0 - 50µg/ml and 5.5 - 60µg/ml for ART and DHA respectively. The linear calibration curve determined through the least square method had regession equation of A= mx + c with the correlation coefficient of 0.996 and 0.9980 for ART and DHA respectively. Sensitivity parameters – Molar Absorptivity and Sandell Sensitivity were determined with the value 2.28×10^4 and 2.25×10^4, and 0.83 and 0.59 µg/cm^2 for ART and DHA respectively. Limits of Detection (LVD) and Qualification (LOQ) determined as per the current international committee on Harmonization (ICH) were found to be 0.37 and 0.30 for ART and DHA respectively. The inter-day and intraday precision and accuracy were ≤ 2.9. The method was used to assay commercial tablets produced for local Pharmacies in Uyo, Nigeria, and was statistically compared with an official method via student T test and various F tests. The results showed good congruence, the performance of the method on further test using recovery studies via standard addition method showed no interference by pharmaceutical excipients.

Conclusion: A simple, sensitive and reproducible method based on the nucleophilic reaction of the drugs with Anisaldehyde/Sulphuric acid has been developed for the determination of ART and DHA in tablets distributed in local pharmacies in Uyo metropolis.

Keywords; Artesunate, Anisaldehyde, Counterfeit, Dihydroartemisinin, Malaria, Resistance, Sulphuric acid

INTRODUCTION

Artesunate and Dihydroartemisinin remain the two major frontline drugs for the treatment of uncomplicated falciparum malaria. The two drugs are derivatives of artemisinin obtained from the Chinese plant *Artemisia annua*. These artemisinin derivatives have very high parasiticidal effect on plasmodium, killing the parasite rapidly. This factor coupled with the near absence clinical adverse effect has made these drugs major candidates for adulteration and counterfeiting. Quite recently, there have been several reports of substandard, counterfeit and fake Artesunate in

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Southeast Asia especially in the greater Mekong Sub region. These countries include Cambodia, Vietnam, Thailand, Laos etc. [1-4]. This problem has eventually spread to Sub-Saharan Africa [5-6]. Based on the activities of these sophisticated counterfeiters, fake and substandard Artemisinin derivatives are consistently being given to unsuspected patients leading to the development of resistance to artemisinin derivatives in Southeast Asia. ([7, 8]). This has also been reported by WHO in Southeast in countries such as Cambodia, Myamman, Vietnam, Thailand, Laos [9]. The same report showed there is a delayed parasite clearance during routine ACT therapeutic efficacy studies [9]. These artemisinin derivatives have become the target of extremely sophisticated and professional counterfeit drug trade. [5, 10]. If these trends are not checked, there are may be facing serious public health crises be when the resistance and intending resist enters Africa being and endemic area for malaria. Then the whole achievement by World Health Organization (WHO) in Malaria control will be in complete jeopardy, hence, the attempt to develop very sensitive, simple and dependable method that is affordable by endemic countries to check the influx of artemisinin derivatives for endemic African countries. ART and DHA are officially assayed by HPLC and titrimetry (international pharmacopoeia), some works have also developed some method for the assay of ART and DHA in tablets ([11,12, 13]. In some of the method proposed, sophisticated analytic machines are used. These machines are too expensive, using machines such as HPLC, GCMS, and LCMS are prohibitive in terms of cost as most of the endemic countries may not have the capacity to procure. That apart, some of these machines were donated by donor agencies but most machines are moribund because of epileptic nature of power supply in these poor endemic countries. The simpler methods developed are reasonably sensitive and reproducible but suffer from one technical problems or the other. Some of these problems being tight pH control, heating requirements, and tedious extraction steps using some organic solvent that could be hazardous to the analyst and the environment. In this work, a simple, sensitive and reproducible method has been developed for the determination of ART and DHA in tablets distributed in local pharmacies in Uyo metropolis. The method is based on the nucleophilic reaction of the drugs with Anisaldehyde/Sulphuric acid reagent.

MATERIALS AND METHOD

2.1 Materials

2.1.1 *Apparatus*
All absorbance measurements were made using Labomed spectrophotometer, INC. USA, with 1cm matched quartz cell.

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

2.2 Methods

2.2.1 Preparation of Anisaldehyde/Sulphuric acid reagent.

This reagent was freshly prepared by measuring 0.5ml of p-anisaldehyde (BDH) in 50ml of glacial acetic acid; and 1ml of concentrated sulphuric acid.

2.2.2 Preparation of Standard drug solution;

The standard solutions of the drugs (Artesunate and Dihydroartemisinin) were prepared by carefully measuring 100ml of the drug and transferring same to a 100ml volumetric flask, containing 20ml of distilled water (Ethanol in the case of DHA) and shaken well to dissolve completely. Then the resulting solution was made up to the 100ml mark using distilled water (Ethanol in the case of DHA). The concentration of the drug was 1mg/ml. The pure drugs were provided by the Director of Pharmaceutical service, University of Uyo Teaching Hospital, and used as provided. The drug solutions were finally diluted to give a working concentration of 200µg/ml.

2.2.3 General Procedure

Different aliquot of the drug (0.5, 1.0, 1.5, 2.0, 4.5 and 5.0), with concentration of 200µg/ml were carefully transferred to a series of 10ml capacity calibrated volumetric flask, with micro burette. The volume in the flask was brought up to 5.0ml distilled water (or ethanol in the case of DHA). Then anisaldehyde reagent was added and made up to the

10ml mark of the volumetric flask and placed in a water bath maintained at 40℃, and allowed to stand for 10 minutes with gentle swirling to mix well. The absorbance was measured at … against reagent blank, which was prepared exactly as the sample but without the drugs.

2.2.4 Assay of ART and DHA Tablets

Twenty tablets of Artesunate (Dihydroartemisinin) commercial brands were weighed singly to determine average weight of one tablet (weight uniformity). The twenty tablets were pulverized into a fine powder and an amount of the powder equivalent to 100mg was accurately weighed and transferred into a 100ml capacity volumetric flask containing 30ml of distilled water (ethanol in the case of DHA). The mixture was sonicated for 10 minutes and further shaken vigorously for another 20 minutes, then the mixture in the flask was made up to the 100ml mark using distilled water (Ethanol in the case of DHA). The resulting mixture was filtered using Whatman filter paper Nº 42. The first 10ml portion of the filtrate was discarded. The drug extract solution containing 1mg/ml was diluted appropriately to obtain a working concentration of 200µg/ml from where an appropriate aliquot was analysed using the general procedure.

2.2.5 Procedure Determination of Placebo blank

Pharmaceutical excipients mostly used for the formulation of tablets were used for the preparation of placebo blank. The compositions included Acacia 15mg, talc 10mg, microcrystalline cellulose 1mg, magnesium stearate 0.4mg and lactose 4mg. The composition mixture was bulked up to 100 mg with the use of maize starch. The resulting mixture was agitated for 10 minutes and homogenized using electromechanical mixer to form a homogenous mass of powder. Then, 50 mg of the mixture was weighed carefully and transferred into a 50 ml volumetric flask containing 10 ml of distilled water, this was sonicated for about 10 minutes. Twenty (20 ml) of distilled water was added to the mixture in the flask and shaken vigorously to dissolve to form a solution. Finally, distilled water was added to make up to the 50 ml mark and shaken to form a placebo blank solution. This was then analysed using the procedure for tablets as explained earlier.

2.2.6 Procedure for the analysis of the Synthetic mixture

One hundred (100mg) of the pure drug powder was carefully weighed out and mixed with 100mg of the placebo blank powder as composed above. This mixture was homogenized to form a homogenous powder mass. A quantity of the resulting mixture equivalent to 100mg was carefully weighed and transferred to a 100ml capacity volumetric flask containing about 250ml of distilled water (ethanol in the case of DHA). This was then shaken vigorously and made up to 100ml with distilled water (ethanol in the case of DHA). The resulting synthetic solution was further diluted appropriately from where a suitable aliquot was analysed using the general procedure.

3. RESULTS

Table 1: Analytical Parameters and optical characteristic of the proposed method.

Parameters	ART	DHA
Wavelength λ_{max}(nm)	500	490
Beers Law Range µg/ml	5.0 – 50	5.5 – 60
Molar Absorptivity	2.28×10^4	2.275×10^4
Sandell Sensitivity (µg/cm^2)	0.83	0.59
Limit of detection (µg/ml)	0.37	0.30
Limit of qualification (µg/ml)	1.22	1.16
Regression equation		
A = mx + c	A = 0.0093x + 0.0023	A =0.0190x + 0.006
Slope	0.0093	0.0190
Intercept	0.0023	0.0059
Correlation coefficient	0.9996	0.9980

The colour change from yellow to red when anisaldehyde was reacted with DHA and ART was the basis for the development of this spectrophotometric method.

Table 2: Evaluation of intraday and inter-day Accuracy and Precision for ART

S/N	Amount of ART taken µg/ml	Intraday Precision and Accuracy			Inter-day Precision and Accuracy		
		Amount of ART found	RE%	RSD%	Amount of ART found	RE%	RSD%
1	30	30.65	2.16	1.07	30.69	2.30	1.02
2	60	61.65	2.75	1.36	61.70	2.83	1.14
3	100	102.91	2.91	1.43	102.82	2.82	1.39

Table 2: Evaluation of intraday and inter-day Accuracy and Precision for DHA

S/N	Amount of DHA taken µg/ml	Intraday Precision and Accuracy			Inter-day Precision and Accuracy		
		Amount of DHA found µg/ml	RE%	RSD%	Amount of DHA found	RE%	RSD%
1	20	20.60	3.00	1.49	20.58	2.90	1.43
2	40	41.10	2.75	1.36	41.2	3.00	1.02
3	60	61.73	2.87	1.42	61.75	2.92	1.43

Table 4: Results of Analysis of tablets procured using the developed method

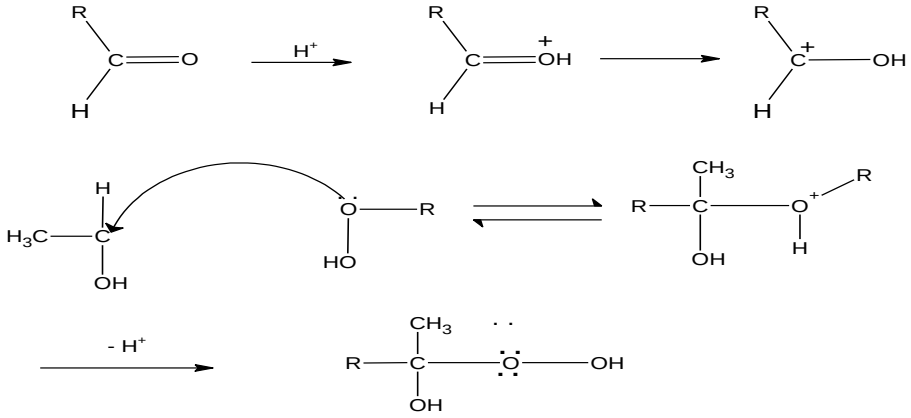
S/N	Tablet brand Analysed ART	Label claim (mg)	Reference method	Amount found (percentage of label claim) ± SD by developed method
1	Artesunate Neros	50	110.00 ± 1.22	111.90 ± 1.03 F = 1.40 t = 1.46
	Lever Artesunate	50	110.60 ± 0. 97	111.40 ± 1.14 F = 1.38 t = 1.07
2	DHA Cotecxin	60	110.00 ± 1.12	11.48 ± 1.40 F = 1.56 t = 1.04
	Santecxin	60	110.00 ± 1.20	111.58 ± 1.31 F = 1.19 t = 1.12

Table 5: Result of Recovery studies via standard addition method

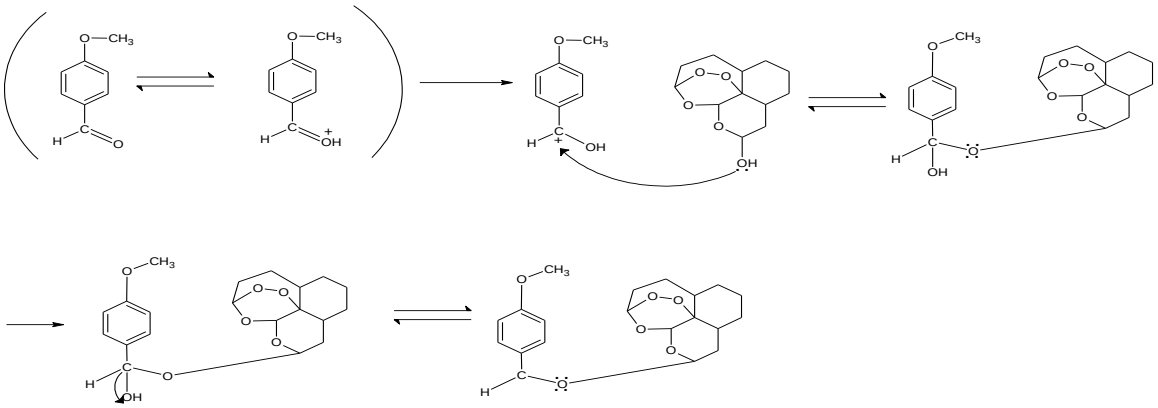
S/N	Tablet studies	Amount of drug (µg/ml)	Amount of pure drug added	Total amount found (µg/ml)	Recovery of pure drug ± SD
1	Artesunate (Neros)	40.00	20	60.15	101.00 ± 1.20
		40.00	40	80.70	101.75 ± 1.16
		40.00	60	101.15	101.92 ± 1.02
	Lever Artesunate				
		40.20	20	60.36	100.80 ± 1.10
		40.20	40	80.01	99.80 ± 1.67
		40.20	60	101.09	101.50 ± 1.18
2	Cotecxin	40.00	20	60.08	100.40 ± 1.10
		40.00	40	80.01	100.03 ± 1.21
		40.00	60	99.98	99.97 ± 1.23
	Santecxin	42.00	20	61.98	99.90 ± 1.48
		42.00	40	82.10	100.25 ± 1.12
		42.00	60	101.98	99.97 ± 1.60

4. DISCUSSIONS

Anisaldehyde is an aldehyde and a carbon compound. DHA is an alcohol and ART is a succinate but in acidic solution there is hydrolysis of succinate group converting ART to acid and alcohol. The mechanism of this reaction is most likely to be an acid catalyzed nucleophilic addition reaction. A proton first attaches to the carbonyl oxygen leading to a development of positive charge on the carbonyl compound, next a molecule of DHA being carbon centre to form a hemiacetal group.



The hemi-actal group formed finally condenses with a molecule of alcohol (DHA) to form the acetal group



4.1 Method Development

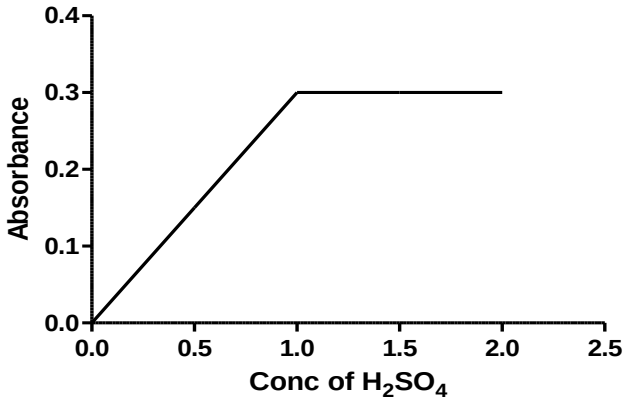
Some experimental variables were studied and optimized to obtain reasonable results. The experimental variables were kept constant while varying the particular variable under study and observing its effect on the process. Since this particular reaction was feasible in acid medium especially sulphuric acid. The reaction was performed in sulphuric acid within the concentration range of 0.5 –1.5M. A concentration of 1.0M Sulphuric acid gave a maximum and stable absorbance values.

4.2 Validation of the Method

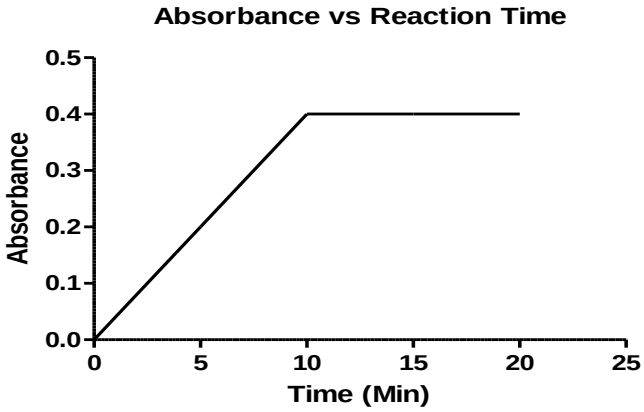
The method was validated for linearity, sensitivity, accuracy and precision.

4.3 Linearity

The absorbance varied proportionally with drug concentration under optimum conditions. A calibration curve was plotted from where the concentration of the unknown was calculated. Beers Law was obeyed within the range of 5.0 - 50µg/ml for ART and 5.5 -60µg/ml for DHA.The calibration curve was straight line with equation A= mx + c, where A is the absorbance, m is the slope and c the intercept, obtained via the least square method. The values are in table 2



The reaction time seemed instantaneous but a maximum absorbance which was stable was 10 minutes. Hence, the experiment was performed at 10 minutes standing time.



Temperature; The temperature for the … was studied; within the range of 25℃ - 40℃. At room temperature, the reaction seemed instantaneous, but at 40℃, maximum and stable absorbance values were obtained.

4.4 Sensitivity

The sensitivity of the proposed method was determined by using the following parameters; Molar absorptivity, Sandell sensitivity. Limit of detection (LOD) and Limit of qualification was evaluated as per the current ICH guidelines [14], using the formula.

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

Where σ is the standard deviation for 5 blank determination and S is the slope calibration graph. The values for all these parameters are in table 1

4.5 Accuracy and Precision

To determine the accuracy and precision of the proposed method, six replicate determinations at 3 concentration levels were evaluated. This was done five times within the same day (intraday) for five consecutive days (inter). The accuracy was evaluated as relative error percent (R. E. %), Using the formula.

$$\frac{Amount\ found - Amount\ taken}{Amount\ taken} \times \frac{100}{1}$$

The accuracy was evaluated as the relative standard deviation RSD% determined at 95% confidence level and at four degrees of freedom. The method was found to be accurate and precise done to the Law values obtained.

4.6 Selectivity

This was determined using placebo blank determination and the synthetic mixture determination which was earlier described. The placebo blank determination showed that the pharmaceutical excipients had no effect on the proposed method. Analysis of the synthetic mixture showed very high recovery rate, this was within the range of 99.6 ± 1.10% to 102.15± 0.89%, including high accuracy and noninterference of excipients usually used when producing tablets.

4.7 Robustness and Ruggedness

To test the Robustness, small and deliberate changes or variation of some experimental parameters such as Reaction time, Temperature and Volume of sulphuric acid and evaluated the effect of this change in absorbance. It was observed that this minimum change had no serious effect on the absorbance. To test the ruggedness, the experiment was performed by two different analysts using two different spectrophotometers. The precision values determined as per RSD% were very low, showing the ruggedness of the method.

4.8 Application of the method for commercial tablets

The applicability of the proposed method was evaluated by using it to analyse and evaluate some commercial brands of ART and DHA tablets bought from local pharmacies in Uyo metropolis in South-South Nigeria. The result obtained were statistically compared with an official standard pharmacopoeial method via students T test and variance ratio test F at 95% confidence level and at 4 degrees of freedom. The calculated values were found to be lower than the tabulated value. The value shows that there is good congruence between the developed method and the official method.

4.9 Recovery Studies

The accuracy and analytical performance of the proposed method was further confirmed by performing recovery studies via standard deviation method. A calculated amount of the pure powder was used to spike a pre-analyzed tablet powder at the concentration levels and the total analysed by the proposed method. The percentage recoveries was excellent within the range of 99.89 ± 1.10% − 102 ± 1.02%. The values are in the table 6

CONCLUSION

A simple, precise, accurate and robust method has been developed for the determination of DHA and ART. This method can be adaptable for use in routine Laboratories and field stations to check the influx of fake and adulterated DHA and ART tablets currently being imported to Nigeria. The method is simple and the chemicals and reagents used are not hazardous to the analyst and the environment.

Acknowledgement

The authors are grateful to the Director of Pharmaceutical service University of Uyo Teaching Hospital for providing the pure DHA and ART powder for use in this work.

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