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NOVEL SPECTROPHOTOMETRIC DETERMINATION OF SOME AR TEMISININ DERIVATIVES IN TABLET USING 2,4-DINITROPHENYL HYDRAZINE REAGENT

[1]*Attih Emmanuel, [1]Paschal Chidera Anthony, [1]Etim Emmanuel, [1]Igboasonyi Alnold, [2]Bassey Augustine and [1]Fortune Chinaemezu Bartholomew

[1]Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmacy University of Uyo.

[2]Department of Pharmacology, Faculty of Clinical Sciencies University of Uyo Teaching Hospital.

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***Corresponding Author**
Attih Emmanuel
Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmacy University of Uyo.

ABSTRACT

Background: A novel spectrophotometric method is developed for the determination of Artesunate (ART) and Dihydroartemisinin (DHA). These two frontline drugs have been used for the treatment of malaria. Their success in the treatment of malaria makes ART and DHA excellent candidates for adulteration and counterfeiting. **Objective:** To develop a method to add to the arsenal of methods already developed to check the influx of counterfeit ART and DHA. **Materials and Methods:** Different aliquots (0.5-5.0 ml) of the pure artesunate solution with concentration of 100 µg/ml were carefully transferred to a series of 10ml capacity calibrated volumetric flask. Absolute methanol was added to each flask to bring up the content to 5 ml and 1 ml of 0.1M of sulphuric acid was added to the mixture and shaken appropriately; then 1ml of sodium acetate buffer was added to each of the volumetric flask. Finally, 2 ml of 0.02 M, 2,4-diphenylhydrazine was added and methanol added to make up to the 10 ml mark of the flask and swirled gently to mix well. The Absorbance was measured at 310 nm, against reagent blank, prepared similarly but without the drug. **Results:** This system obeyed Beers law. The calibration curve obtained via least square method was linear with correlation coefficient of 0.9979 and 0.9997 respectively for DHA and ART. Molar absorptivity and standard sensitivity were $2.8x10^4$LMol ^{1}/ 1.0142µ g/cm^2 and 2.930z10^{4}LMol-1/1.312ug/cm^2 for DHA and ART respectively. The limit of

detection and qualification were 0.41/1.15 and 0.32/0.58 for DHA and ART respectively. The accuracy and precision of the method were determined and were found to be ≤ 3.0%. The developed methods were statistically compared with established method using students T-test and Variance ratio F-test showing good congruence. **Conclusion**: The method was used to analyze tablets procured locally from Pharmacies within Uyo Metropolis in Nigeria with excellent recoveries obtained.

KEYWORDS: Artesunate, Counterfeit, Dihidroartemisinin, Nucleophilic, Spectrophotometric.

INTRODUCTION

Malaria remains the most deadly parasitic disease in the world. This condition may remain for a long time as over 300 million new malaria infections and several millions of malaria deaths are recorded yearly.[1]

Due to global warming, massive intercontinental travels, manufacture and massive distribution of substandard and fake artemisinin antimalarials.

Global warming is the major cause of this problem. Current projections suggest that if global warming remains unchecked, malaria could reestablish itself in Europe and North America.[2]

Traditionally, malaria has always been a tropical problem. As international travel become more and more common, malaria is no longer confined to the tropical zone of the world alone as "imported malaria" is an increasing world health problem.[3] The manufacture and effective distribution of substandard, fake or counterfeit Artemisinin derivative from South East Asia is a major cause of the development and spread of multi drug resistance.[4] The fake, counterfeit and substandard Artemisinin derivatives are imported into Africa. Sub-Sahara African countries have weak legislation which could deter the sophisticated counterfeiters. These counterfeiters are very technically sound and can produce counterfeits that can easily pass as genuine drugs. They produce blister packs with holograms showing manufacturing and expiring dates that easily fool government agents and even pharmacist to the field.[5] Officially these artemisinin derivatives are analyzed using HPLC, Spectrophotometry and titrimetry (International pharmacopeia).

Many workers have developed some methods for the assay of artemisinin derivatives ranging from UV-VIS methods.[6] Chromatographic methods has also been developed.[7] Some of the

methods developed are novel being simple and reproducible, but with some technical problems such as tight pH control and tedious extraction process requiring some serious skills. The proposed method is very simple, reproducible and only requires basic technical expertise. The method uses simple reagent with excellent shelf life.[8]

The method is based on the typical reaction of aldehydes and ketones with 2,4-Dinitrophenyl Hydrazine which gives colored species which is measured spectrophotometrically.[9]

MATERIALS AND METHODS

Equipments

All Spectral measurements were carried out using; Spectrophotometer UV-VIS, RS spectrophotometer UV-2500, Labomed Inc, USA. All weighing were done using, Metler Electronic balance (0.001-200g).

Reagents

All reagents used were analytical grade with excellent shelf life. 2,4-Dinitrophenyl Hydrazine reagent (0.002 M). the solution was prepared by dissolving 0.1981 g of 2,4-Dinitrophenyl Hydrazine (BDH) in 5 ml of concentrated sulphuric acid (1M) and the volume completed to 100 ml in a volumetric flask with distilled water, then 20 ml of the solution diluted to 100 ml with distilled water to obtain a solution of concentration of 0.002 M. Sodium Acetate buffer, this was prepared by adding 1.36 g of anhydrous sodium acetate (BDH England) powder, 0.6 ml of glacial acetic acid in sufficient water to produce 100 ml. Sulphuric acid 0.1M, concentrated sulphuric acid (BDH England) is diluted approximately to obtain 0.1M solution. Absolute methanol made by James Borr Limited, London.

Standard drug solution

Standard drug solution, Pure artesunate (Sigma Aldrich USA), and Dihydroartemisinin (DHA) a gift from the Director of Pharmaceutical services of the University of Uyo Teaching Hospital, was used as received. An equivalent of 100 mg was accurately weighed and transferred into 100 ml capacity volumetric flask and dissolved in enough dissolved water to obtain a concentration of 1mg/ml. this was further diluted approximately to obtain a working concentration of 100 µg/ml, of ARTS and DHA.

General procedure

Different aliquots (0.5-5.0 ml) of the pure artesunate solution with concentration of 100 µg/ml were carefully transferred to a series of 10ml capacity calibrated volumetric flask. Absolute methanol was added to each flask to bring up the content to 5ml and 1ml of 0.1M of sulphuric acid was added to the mixture and shaken appropriately; then 1ml of sodium acetate buffer was added to each of the volumetric flask. Finally, 2 ml of 0.02 M, 2,4-Dinitrophenyl Hydrazine was added and methanol added to make up to the 10 ml mark of the flask and swirled gently to mix well. The Absorbance was measured at 310 nm, against reagent blank, prepared similarly but without the drug.

Procedure determination of artesunate (or dihydroartemisinin) in Tablets

Twenty (20) tablets of artesunate (or Dihydroartemisin) were weighed singly (to determine weight uniformly) and crushed in a ceramic mortar using a ceramic pestle. A quantity of the powder equivalent to 100 mg was transferred to a 100 ml capacity volumetric flask. About 20 ml of distilled water was added and shaken vigorously to extract the drug. Then a further 60 ml was also added and shaken vigorously. Finally more distilled water was added to make up to 100 ml mark of the volumetric flask, after mixing well, the Artesunate mixture was filtered using Whatmans filter paper No. 42. The first 10 ml of the filtrate was discarded. The resulting artesunate solution with concentration of 1 mg/ml was then diluted stepwise to obtain 100 µg/ml from where the general procedure was used to analyzes.

Procedure for placebo blank

A pacebo blank was composed using pharmaceutical excipients. The blank as constituted, include; Talc (5 mg), Lactose (10 mg), Sodium Alginate (5 mg), magnesium stearrate (5 mg) and Methyl Cellulose. Maize starch was then used to bulk up the mixture to 100 mg. These constituents were thoroughly mixed and homogenized together to form a composite powder mixture. This mixture was made into a solution and processed as described above in the procedure for tablets from where a suitable aliquot was prepared and analyzed using general procedure as described above.

Procedure for the determination of the drug in synthetic mixture

A synthetic drug mixture was prepared carefully mixing 100mg of the drug with 100 mg of the pacebo blank as constitute above. The mixture was shaken vigorously and homogenized. The 100 mg of the resulting synthetic mixture was carefully transferred to 100 ml capacity volumetric flask containing about 40ml of distilled water. This was sonicated for 10 minutes

and shaken vigorously to extract. Then more distilled water was added to make up to the 100 ml mark of the flask and filtered using Whatman filter paper No.42. The first 10 ml of the filtrate was discarded. The resulting synthetic solution with a concentration of 1mg/ml was further diluted appropriately to obtain 100 µg/ml for the spectrophotometric analysis as described in the general procedure.

RESULTS AND DISCUSSION

The reaction of 2,4-Dinitrophenyl Hydrazine and artesunate (or Dihydroartemisinin) is a typical reaction of aldehyde and ketone. Artesunate is a Semi-Succinate derivative of artemisinin. On hydrolysis the succinate group is cleaved and the carbonyl group is created in position 12 of the artesunate molecule where the reaction is likely to occur.

The reaction is a typical Nucleophilic addition-elimination reaction. The -NH_2 in the hydrazine is characteristically Nucleophilic. The nitrogen with its unshared pair of electrons attacks the carbon atom of the carbonyl group leading to the removal of proton and the elimination of water. “Fig 1” This reaction under room temperature provides a bright yellow coloured species measured at 310nm.

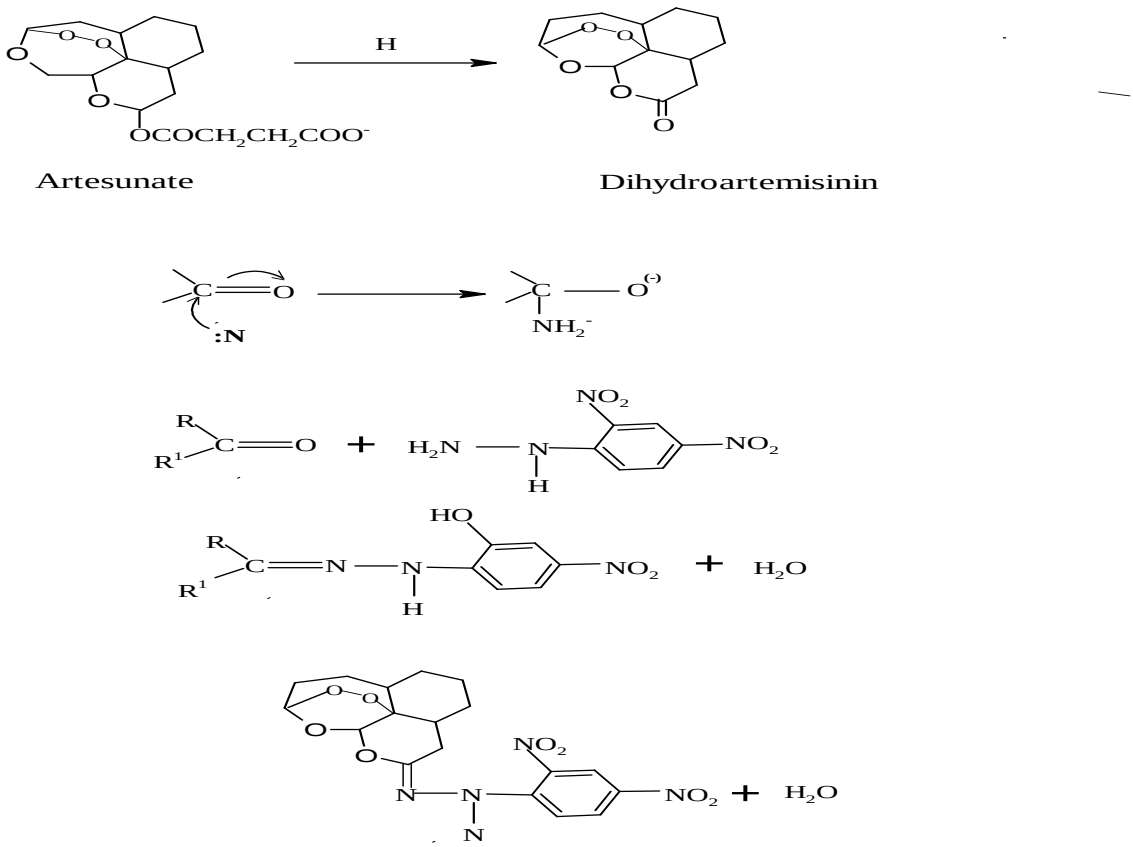

Figure 1: Nucloephilic addition- elimination reaction of aretesunate.

Optimization of reaction and experimental condition

Conditions for this experiment were studied and carefully optimized. To obtain optimum condition for the best result some experimental variables crucial to the experiment were kept constant while varying the particular variable under study.

Effect of temperature

The reaction was studied in the temperature range 25^{o}C to 40^{o}C keeping other experimental variables constant and observing its effects on the absorbance. In this particular case it was observed that the reaction was spontaneous at room temperature. Higher temperature gave results that were erratic and non quantitative absorbance value.

Effect of time

To study the effect of reaction time (t) on absorbance "Fig 2", a fixed concentration of the drug (ART) 100 µg/ml with a volume of 5.0 ml was reacted with 0.02 M of 2,4-Dinitrophenyl Hydrazine described in the general procedure at different time intervals of 5 minutes, 10 minutes, 20 minutes, 25 minutes and 30 minutes. The value of the absorbance increased giving by 10 minutes. The absorbance was optimum. No further increase in absorbance was observed after 10 minnutes.

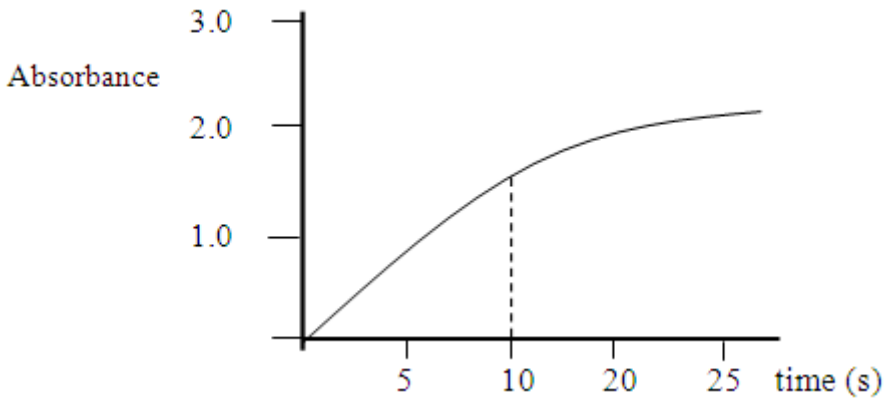

Figure 2: Graphical representation of the effect of reaction time (t) on absorbance ART.

Effect of acid type and pH

This reaction is formed by the presence of acid and it's quite sensitive to the pH medium. Sulphuric acid concentrations were varied between the ranges of 0.05M-0.15M. It was discovered that sulphuric acid concentration of 0.1M was found to produce yellow coloured specie that was stable giving a best absorbance value.

The system is pH sensitive requiring a constant pH of about 4.0. The pH of the system was varied between 1 and 7 acid pH it was found that the pH of 4 gave the best absorbance value. Hence the use of Sodium acetate-acetic acid buffer. Other acids such as hydrochloric acid and phosphoric acid were used. It was found that sulphiric acid was to be most appropriate.

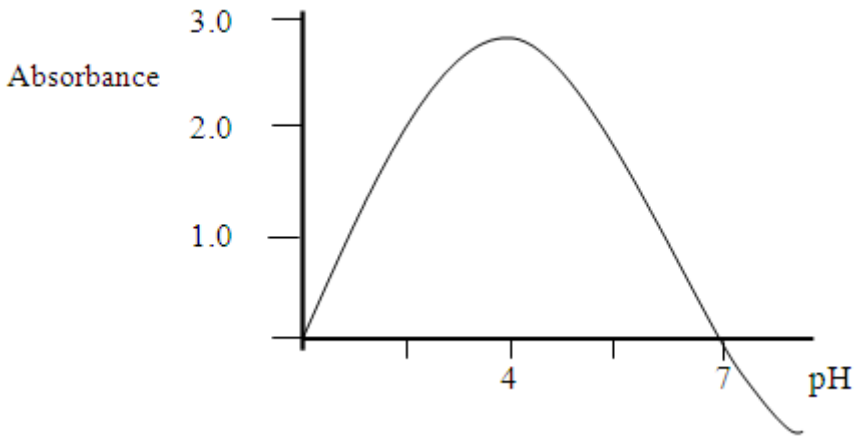

Figure 3: Graphical representation of the Effect of pH on the Absorbance.

Effect of concentration on 2,4-Dinirophenyl Hydrazine

Since it is a Nucleophilic reaction, the lone pair electrons in the nitrogen makes the reaction favourable. The concentration of 2, 4- dinitrophenyl hydrazine was varied in the range while other experimental variables were kept constant. It was found that in the reaction volume of 10ml a concentration of 0.002M 2, 4-dinitophenyl hydrazine was found suitable for maximum absorbance.

Validation of the proposed method

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

Linearity and sensitivity

It was observed that a linear relationship existed when absorbance was plotted against drug concentration. (i.e DHA and ARTs). Beers law was observed in the range of 10 µg - 50µg/ml and 0.5µg/ml - 60µg/ml respectively for DHA and ARTs. A calibration of curve obtained by Least square method had a linear equation of the form A=Mx +C, where A = absorbance, x=slope, c= drug concentration (DHA or ARTs) and m is intercept. The correlation coefficient was 0.9979 and 0.9997 for dihydroartesinin and artesunate respectively. The molar absorptivity, Sandell sensitivity and other sensitivity parameters such as; Limit of

Quantification (LOQ) and limit of detection were determined based on the current ICH (international committee on hamonization) using the formula.

LOD= 3.3 σ/5

LOQ = 10σ/5

Where σ is the standard deviation of 5 reactions blank while S is the slope of the calibration curve. These values of all parameters are recorded in the Table 1.

Table 1: Sensitivity and regression parameters.

Parameters	DHA	ART
λmax	300nm	310nm
Beer Law Range	0.5-50µg/ml	0.5-60µg/ml
Molar Absorptivity $Lmol^{-1}$ cm^{-1}	$2.8x10^4$	$2.930x10^4$
Sandel Sensitivity $\mu g/cm^2$	1.0142	1.312
Limit of detection	0.41	0.32
Limit of Quantification	1.15	0.58
Regression Equation	A =mc + c A=0.001x + 0.0007	A= 0.002x +0.0006
Slope	0.001	0.002
Intercept	0.0007	0.0006
Correlation Coefficient	0.9979	0.9997

Accuracy and precision

Intraday and interday accuracy of the developed method were evaluated by analyzing the pure standard drug solution by performing 6 (n = 6) replicate determination at three concentration levels. The relative error (R.E%) which is accuracy was calculated using the formula.

$$\text{R.E \%} \quad \frac{\text{Amount of drug found} - \text{Amount of drug taken x 100}}{\text{amount of drug taken}}$$

The relative standard deviation (RSD %) was determined as the precision of the developed method. The results of both accuracy and precision were low in fact ≤ 3.0% in all cases showing high precision and accuracy of this developed method. These values are recorded in table 2 below.

Table 2: Evaluation of intraday and interlay accuracy and precision.

	Amount of drug taken	Amount found	R.E%	RSD%	Amount found	R.E%	RSD%
	30	30.68	2.27	1.01	30.80	2.27	2.59
DHA	60	61.56	2.60	2.55	61.80	3.00	1.00
	90	92.70	3.00	2.96	91.83	2.03	2.14
	30	30.00	2.67	2.62	30.90	3.00	1.87
ART	60	61.72	2.87	2.12	61.68	2.80	2.65
	90	92.68	2.98	2.86	92.70	3.00	2.83

Selectivity

Two methods were used to determine the selectivity of this proposed method namely; the placebo blank and the synthetic mixture. The two methods gave good values in terms of R.E% and R.S.D% and excellent recoveries between ≥ 98.8 ≤105% with a standard deviation of 1.20 – 1.62. Showing that there was no interferences from pharmaceutical excipient used in tablets formulation process.

Robustness and ruggedness

Acid concentration and the reaction time were deliberately altered in small quantities or increment and to observe their effects on the overall results and recoveries just to test the robustness of the proposed method as observed. These minor deliberate alterations had no major effect on the overall result.

To test the ruggedness of this method, the whole experimental processes were replicated by two different analysts using two different spectrophotometers. The method results showed no major difference in the value obtained as per the recoveries showing that the developed method is quite rugged.

Application of the proposed method to the assay of tablets

The applicability and suitability of the proposed analytical method was tested by analyzing DHA and ART in tablet preparation procured locally from community pharmacist within Uyo metropolis (South – South Nigeria). The result obtain were compared statistically with international pharmacopeia via students t- test and variance ratio test F- test at 95% confidence and at 4 degrees of freedom. The results are recorded in table 3. The results show clearly that the calculated value were lower that the tabulated values, meaning that the values had no significant difference with official method.

Table 3: Results of analysis of DHA and ART tablets procured locally.

S/N	Tablet brand Analysed	Label claim (mg)	Reference method	Amount formed (% of tablet Claim) ± SD. By proposed method.
	DHA			
1	Alaxin	60	110.00±1.40	110.90±1.04 F =1.81, t = 1.15
	Santecxin	60	110.00±1.35	110.82± 1.20 F =1.27, t = 1.02
	ART			
2	Leverartesunate	50	110.00±1.28	110.99±1.62 F = 1.60, t = 1.06
	Arsumax	50	110.00±0.98	109.90±1.18 F = 1.45, t = 1.31

Mean of five determinations

The value of tabulation at 95% confidence level and at four degree of freedom is 2.77. The value of F-test (Tabulated of 95% confidence level and at 4degrees of freedom is 6.39).

RECOVERY STUDIES

To confirm the accuracy and applicability of the developed method recovery studies was performed via standard addition technique. A calculated amount of pure artesunate and dihydroartemisinin were used to spike a pre-analyzed tablet powder at three different concentrations levels. The resultant drug mixture was then analyzed three consecutive times using the developed method. The percentage recovery values were found to be in the range of 99.50±1.09 to 101.75± 1.20% as shown in Table 4.

Table 4: The percentage recovery values.

S/N	Tablets studied	Amount of Drug(µg/ml)	Amount of pure Drug added	Total amount Found (µg/ml)	Recover pure drugs ± SD
	DHA				
		40.00	20	60.25	101.25±1.19
	Alaxin	40.00	40	80.36	100.90±2.10
		40.00	40	99.60	99.50±1.46
	Santecxin	40.20	20	60.50	101.50±0.76
		40.20	40	80.78	101.45±1.24
		40.20	60	100.92	101.79±1.26
	ART				
	Leverartesunate	60.00	20	79.96	99.80±1.76
		60.00	40	100.78	101.90±2.14
		60.00	60	111.00	101.60±1.14
	Arsumax	50.00	20	69.98	99.90±1.27
		50.00	40	91.12	102.80±2.11
		50.00	60	111.05	101.75±1.20

CONCLUSION

A sensitive, reproducible and selective method is developed for the assay of dihydroartimisinin and Artesunate in tablet formulations. This method can be adopted for routine laboratory analysis of these artemisinin derivatives to check the influx of substandard and counterfeit artesunate into Nigeria since the method is very direct with no hazard posed to the analyst and environment.

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