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Development and validation of UV spectrophotometric method for the determination of artesunate and dihydroartemisinin by coupling

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Abstract
There is high rate of malaria treatment failure mostly due to the abundance of fake and substandard artesunate and dihydroartemisinin. Most of the instrument available for the determination of these products in pharmaceutical formulations are very sophisticated and expensive and occasionally nonexistence in most third world countries where malaria is endemic. The drug does not have a chromophore for easy UV analysis. We developed and validated a simple and reproducible ultraviolet spectrophotometric method that could be used to analyse artesunate and dihydroartemisinin in bulk and pharmaceutical formulations by coupling the drug with freshly prepared benzene diazonium chloride. The method was validated for linearity, accuracy, precision, reproducibility and specificity as stated in the International Conference on Harmonization (ICH) guidelines. It was also used to determine the content in two brands each of artesunate and dihydroartemisinin in Uyo metropolis, and the results agreed with that on the label. A calibration graph of coupled artesnate (ART) had a standard deviation of 0.0551 while that of dihydroartemisinin (DHA) was 0.0631. The regression data for the calibration graph showed a good linear relationship (r=0.9971) over a concentration range of 2.0 - 10.0$\mu g/ml$ using linear regression equation y = 0.075x + 0.018. The Limit of detection (LOD) were found to be 0.2451$\mu g/ml$ for ART and 0.3009$\mu g/ml$ for DHA, while limit of quantification (LOQ) were 0.7424$\mu g/ml$ for ART and 0.9118$\mu g/ml$ for DHA respectively. The Coefficient of Variation (CV) for both intra-day and inter-day accuracy expressed as percentage relative error was <2.8. Recovery studies using standard addition methods showed no interference with common excipients. The proposed method gave good validation results and statistical analysis proved that the method is precise, accurate and reproducible and could be used for routine analysis.

Keywords: Artesunate, Dihydroartemisinin, spectrophotometric method, validation, formulations

1. Introduction
The antimalaria drug artemisinin and its derivatives artesunate (ART) and dihydroartemisin (DHA) are frontline drugs recommended by the world health organisation [1] for use in the treatment of all forms of malaria affecting humans. The drugs has the fastest rate of *plasmodium* elimination and relieve of malaria symptoms [1, 2].

In recent time there has been reports of treatment failure while using these drugs in the treatment of malaria, [3, 4]. Most of these treatment failures are attributed to the use of fake and substandard drugs [5].

The instrument available for qualitative and quantitative analysis of these products are highly sophisticated and at times not available or out of reach for use in most developing countries including Nigeria where malaria disease is endemic, [6, 7, 8]. Naik *et al*., 2006 [9] developed an HPLC method for the determination of artesunate and dihydroartemisinin in human plasma using artemisinin as internal standard and atmosphere pressure and chemical ionization (APCI) as an interface**.** Bethy *et al.,* 1996 [10], Na-Bangchad *et al*. [11] (1998) Karburang *et al.,* 1997 [12], developed a simple rapid, sensitive, selective and reproducible HPLC method with reductive electrochemical detection for the simultaneous quantification of artesunate and dihydroartemisinin in plasma.

Diazonium salts are prepared by the treatment of primary amines such as aniline with nitrous acid (HNO_2) prepared from sodium nitrite ($NaN0_2$) and a mineral acid of HCl [13, 14].

Azo coupling is a reaction between a diazonium compound and other active (aromatic) compound which produces an azo-compound. In the reaction, the diazonium salt is an electrophile in an electrophilic aromatic substitution. In most cases, the products absorb longer wave length of light than the reactants because of increased conjugation [13, 14, 15].

All the existing methods of analysis use expensive equipment which may not be readily available in developing countries where malaria is endemic. The aim of this study is to develop and validate a simple, rapid, sensitive and reproducible UV spectrophotometric analysis method for the analysis of ART and DHA in pharmaceutical formulation by coupling the drug with freshly prepared benzene diazonium chloride.

2. Materials and Methods

2.1 Chemicals: All chemicals used were of analytical grade. Pure artesunate and dihydroartemisinin were gift from May and Baker Nigeria Plc, Lagos Nigeria. Concentrated HCl, HNO_2 (analytical grade), aniline and sodium hydroxide pellet were purchased from sigma Aldrich. Bi-distilled water was used for all dilution and analysis. A Cecil spectrophotometer model number CE7200 was used for the analysis.

2.2 Preparation of Benzene Diazonium Chloride (Diazotization): This is based on the method of Wheland [14], a mixture of 50ml of distilled water and 50ml of concentrated hydrochloric acid was incubated in ice bath for 30 minutes, and 20ml of aniline was added and stirred for five minutes. Twenty millilitres of 0.5M solution of sodium nitrite was gradually added to the solution and stirred while maintaining the temperature in ice at less than 10 ^{0}C.

2.3 Coupling of ART and DHA and search for λmax: Solution of pure ART and DHA each containing 5.0g/dm^3 were separately incubated in 50ml of 40.0% sodium hydroxide solution for thirty minutes with occasional swelling in 100ml beaker at ambient temperature. Freshly prepared benzene diazonium chloride solution (0.5ml) was added to each of the alkaline solutions and stirred for five minutes. ART-coupled complex gave an orange colour while DHA-coupled complex gave a yellow colour [13, 15]. 10ml of each solution was diluted to 100ml with NaOH(aq) and scanned for wave length of maximum (λmax).

2.4 Preparation of standard calibration curve of ART and DHA

Pure artesunate powder 5mg was dissolved in 5ml of ethanol, and 5mg of pure DHA was dissolved in 5ml of acetone. The solutions were diluted to 50ml with 0.1M NaOH (aq). Using a 5ml pipette, 1ml, 2ml, 3ml, 4ml and 5ml of the alkaline ART and DHA were measured into clean labelled test tubes. Appropriate volumes of 0.1M NaOH 9ml, 8ml, 7ml, 6ml and 5ml was added to make 10ml of solutions with concentrations of 2µg/ml, 4µg/ml, 6µg/ml, 8µg/ml and 10µg/ml respectively.
Using a micropipette, 0.1ml of freshly prepared benzene diazonium chloride was added to each tube and swelled for 5 minutes. The absorbance of the coupled ART and DHA solutions were measured at 220nm, against 0.1M NaOH (aq) as blank. A graph of concentration against absorbance was plotted for ART and DHA respectively.

2.5 Validation methods: The developed method was validated for linearity, sensitivity, precision, accuracy and recovery [16].

2.6 Linearity study

The 100µg/ml ART and DHA solutions used for wavelength of maximum absorption λmax determination was employed as a stock solution for linearity study. Aliquots in the range of 1.0 to 5.0ml of this solution were taken and diluted to 10ml with 0.1M NaOH (aq) to obtain different concentrations within the range of 10 to 50µg/ml. Using a micropipette, 0.1ml of freshly prepared benzene diazonium chloride was added to each tube and swilled for 10 minutes and the absorbance was read for linearity calibration plot.

2.7 Sensitivity study

The limit of detection (LOD) and limit of quantification (LOQ) were evaluated based on ICH guidelines, [16] using the equation
LOD = 3.3δ/s and LOQ = 10δ/s
Where δ is the standard deviation of five blank determinations and s is the slope of the calibration curve.

2.8 Intra-day Precision

Aliquots (1.0,2.0 and 3.0ml) of the 100µg/ml ART and DHA solutions were taken and respectively diluted to 50ml with the 0.1M NaOH to obtain three concentrations of 2.0, 4.0 and 6.0 µg/ml., respectively. Triplicate absorbance measurements of each were made five times within the same day and the mean, standard deviation and relative standard deviation (RSD) calculated.

2.9 Inter-day Precision

The selected concentrations for intra-day precision study were analysed again for five consecutive days and the mean, standard deviation and RSD calculated.

2.10 Recovery accuracy study

This was carried out using pre-formulated granules containing 80% w/w pure DHA and ART respectively, and maize starch and lactose. The granulation (60mg) was transferred into 100ml volumetric flaks and 50ml of 0.1M NaOH was added and shaken for 15min, using a vortex mixer and diluted to 100ml mark with the same solvent. The mixture was filtered to obtain sample stock solution and 0.1ml of freshly prepared benzene diazonium chloride was added. Aliquot of the stock solution 1.0ml was further diluted to three different concentrations with 0.1M NaOH(aq) and assayed for the content of ART and DHA. The content of DHA and ART was extrapolated from the standard curve.

2.11 Assay of content of DHA and ART in four selected marketed brands.

Accurately weighed tablet powder, equivalent to two different brands of 50mg of ART and DHA respectively were transferred into 100ml volumetric flask. Fifty millilitres of 0.1M NaOH(aq) was added to each flask and shaken for 15 minutes using a vortex mixer. The mixture was filtered and 50ml of the same solvent was used to rinse the residue and flask to make 100ml of ART and DHA solutions respectively, and 0.5ml of freshly prepared benzene diazonium chloride was added. One millilitre of the filtrate was further diluted to 100ml with the 0.1m NaOH(aq) and the absorbance of the coupled ART and DHA in each case was determined. The amounts of ART and DHA were extrapolated from the standard curve. Each assay was done in triplicate [16, 17].

2.12 Statistical analysis

Where applicable, the results were expressed as mean ± SD and analysed statistically using student t-test with the aid of excel 2007. Differences were considered significant at the 95% confidence limit and 4 degree of freedom.

3. Results and Discussion

The wavelength of maximum absorption (λmax) for the diazotized ART and DHA solutions was 220nm using 0.4µg/ml. 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 was plotted against concentration in the range of 0.05 to 3.5µg/ml. The calibration graph was a straight line with equation A = bc + x, and standard deviations of 0.0551 for ART solution and 0.0631 for DHA solutions respectively;

Where A = absorbance, b = slope, c = drug concentration in µg/ml and x = intercept obtained by least equation method.

The linearity parameters (Table 1) and the corresponding regression data, showed excellent linear relationship (r^2 = 0.997) over the working concentration range of 0.05 to 3.5µg/ml. The sensitivity parameters (Table 1) as calculated are in congruence with standards. Tables 2 and 3, presents the intra and inter-day precision of the new method, confirming adequate sample stability and method reliability. From the three selected concentrations for both ART and DHA, the calculated RSD, were all less than 7%, which show reliability of the new method.

Table 1: Linearity and sensitivity parameter data

Parameter	Result	
	ART	DHA
λmax (nm)	220	220
Beer's Law Linearity Rang (µg/ml)	0.05-3.00	0.05-3.00
Regression Equation	(A= 1.494c+0.03)	(A=0.692c-0.038)
Intercept (X)	0.03	-0.038
Slope (B)	0.742	0.692
Correlation Coefficient (R)	0.9971	0.9978
Limit of Detection (µg/ml)	0.2450	0.3009
Limit of Quantification (µg/ml)	0.7425	0.9118

Table 2: Intra-and inter-day precision data DHA (n=3, p=0.05)

Con. µg/ml	Mean Intra-day Absorbance ± SD	Relative Standard Deviate RSD %	Mean Inter-day Absorbance ± SD	Relative Standard Deviate RSD %
0.20	0.169 ± 0.002	0.97	0.171 ± 0.001	0.98
0.40	0.344 ± 0.004	1.72	0.342 ± 0.002	2.07
0.60	0.513 ± 0.003	1.32	0.520 ± 0.004	1.38

Table 3: Intra-and inter-day precision data ART (n=3, p=0.05)

Con. µg/ml	Mean Intra-day Absorbance ± SD	Relative Standard Deviate RSD %	Mean Inter-day Absorbance ± SD	Relative Standard Deviate RSD %
0.20	0.177 ± 0.002	0.96	0.171 ± 0.001	0.98
0.40	0.334 ± 0.014	1.77	0.340 ± 0.002	2.07
0.60	0.603 ± 0.003	1.32	0.590 ± 0.004	1.35

The recovery study was to assess the reliability of the method in the presence of common excipients used in tablet formulations. The mean recovery using the three excipients were all above 98.92% with RSD of 1.02%. This was not significantly different (P<0.05) from the expected recovery value. This shows that the method is reliable and the excipients do not interfere.

Application of the method to determine the percent absolute drug content of DHA and ART in some generics (Table 4) indicated a concentration of between 98.81 ± 2.02% to 100.90 ± 1.01% to that claimed on the various labels.

Table 4: Results of analysis of some commercial brands of ART and DHA by diazotization and comparison with BP standards

Tablet Analyzed	Label Claim(mg)	BP Standards (%)	Content by Diazotization Method (%)
Cotecxin (DHA)	60.00	110.00 ± 1.60	108 ± 2.11
Codisin (DHA)	60.00	110.00 ± 1.25	111 ± 1.31
Artesunate (ART)	50.00	110.00 ± 1.11	109 ± 2.30
Antesunart (ART)	50.00	110.00 ± 1.11	111 ± 1.50

4. Conclusion

Validation parameters of the developed method showed comparable accuracy and precision with standard pharmacopeal methods. The observed linearity rang agrees well with Beer-Lambert's law and the corresponding regression coefficient (r = 0.9971) is an indication of high degree of the method sensitivity. The method being simple, accurate, precise and highly selective could be used in the determination of artesunate and dihydroartemisinin in bulk and dosage formulations without interference from commonly used excipients and related substances whose λmax are not close to the test compound.

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