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Rapid titrimetric and spectrophotometric methods for the determination of artesunate in bulk and tablet formulations

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ABSTRACT

A titrimetric and a spectrophotometric methods are developed and validated for the assay of artesunate in pure and in tablets. The titrimetric method is based on the reaction of artesunate with acid resulting in the cleavage of the endoperoxide bond of the molecule, hence hydrogen peroxide is generated in situ; this in the presence of potassium iodide liberates iodine which is then titrated against standardized sodium thiosulphate. The spectrophotometric method is based on the opening of the lactone ring by sodium hydroxide resulting in an alkali decomposed product which absorbs light at 300nm. In the titrimetric method the percentage content of active ingredient per tablet was ≥ 99.5% ≤ 105% ±1. In the spectrophotometric method Beer's law was obeyed in the range of 0.5µg/ml - 50µg/ml with linear regression equation of A = 0.02e and correlation coefficient of (r) 0.9998 (n = 10). The apparent molar absorptivity and Sandell sensitivity were 1.36x10^3.L/mol/cm and 0.282µg/cm^2. The limits of detection (LOD) and quatification (LOQ) determined as per the current International Conference on Harmonization (ICH) guidelines were 0.375 and 1.25µg/ml respectively. The precision (RSD%) and accuracy (RE%) determined by evaluating the inter and intra day variations at three artesunate concentration levels were < 2.5% and < 3.0 respectively. These validated methods were successfully applied to assay tablets procured locally and the results found to be in good agreement with the reference titrimetric method in the international pharmacopoeia. The accuracy and validity of these methods were further confirmed by performing recover studies via standard addition procedure with the results showing that excepients have no effects on the methods.

Keywords: Artesunate, Assay, Endoperoxide Bond Spectrophotometric, and Titrimetric

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INTRODUCTION

Malaria remains a major source of concern within the tropics as it is the major causes of morbidity and mortality within the region. Over 300 million new malaria infections and 3 million deaths are recorded each year predominantly afflicting vulnerable people such as children under five, pregnant women and people living with HIV [1,2]. Healthy adults are not spared as they are incapacitated during malarial attack. The emergence and spread of resistance to current quinoline antimalaria [3] and antifolate has been a serious obstacle toward global malaria control. The emergence of multidrug resistant *Plasmodium falciparum* and the potent threat to Artesunate is also a major source of worry. Artesunate is the hemi succinate derivative of Artemisinin derived from the plant *Atemisia annua*. It is the most widely distributed of the three major derivative of artemisinin. Just like the other two (artemether and arteether). It is readily metabolised to dihydroarteminin [4]. Artemisinin derivatives are the fastest acting antimalarial drugs [5,6] these compound have improve parasiticidal properties *in vitro*, rapidly arresting

parasite metabolism in concentrations within the lower nano-molar range and killing parasite more quickly than any other antimalarial drug [7]. The emergence of multidrug resistant *Plasmodium* and the potential threat to the artemisinins lead to the strong recommendation of Artemisinin combination therapy by WHO and authorities of endemic countries for the treatment of severe malaria. Artesunate being the most widely distributed and used makes it a major candidate for adulteration and counterfeiting just like any other very successful drug. There has been reports of widespread distribution of fake and counterfeit artesunate in South East Asia [8,9,10). East Africa [11]. The widespread distribution of counterfeit Artesunate is not likely to be localized in South East Asia and East Africa alone and since fake drug syndicates are sophisticated chances are that fake and adulterate artesunate may soon follow the genuine ones into the West Africa sub region, if not already here [12,13,14]. The fight against widespread distribution of counterfeit/fake artesunate tablets demand the use of sensitive, accurate and reliable methods and equipment. In the international pharmacopoeia Artesunate is assayed by HPLC and acid base titration [15]. Some workers have assayed artemisinin and its derivatives using HPLC [16,17,18]. Unfortunately most pharmaceutical outlet involved in the sales and distribution of Artesunate in this region can ill afford HPLC equipment. The second method acid-base titration is simple, accurate, affordable and reproducible. The application of this proposed iodometric titrimetric method can serves as complimentary while the application of the proposed spectrophotometric method could serve as a confirmatory method. In pharmaceutical analysis titrimetric and spectrophotometric methods are usually methods of choice for some drugs owing to their simplicity, accuracy, affordability and reproducibility. In the proposed titrimetric method Artesunate is reacted with potassium iodides in acid medium, iodine is liberated *in situ* which is then titrated against a standardized sodium thiosulphate using boiled starch as the indicator. In the proposed spectrophotometric method Artesunate is reacted with sodium hydroxide resulting in the opening of the lactone ring of the molecule and production of alkali decomposition product which is then measured spectrophotometrically. Both proposed method are versatile cost effective and environmentally friendly, devoid of the use of toxic solvents which could be hazardous to the analyst and the environment.

EXPERIMENTAL SECTION

All spectrophotometric measurements were carried out using Heylos β model of UV-Vis. Spectrophotometer from Thermo electron cooperation U.S.A. equipped with 1cm quartz cell to match.

Chemical and Regents
All reagents and chemical used for this work were analytical grade and their stock solutions were prepared and diluted in double distilled water.

Potassium iodide: (10%) BDH, England was prepared by dissolving 10g in sufficient distilled water to make up to 100mls.

Sodium thiosulphate: 0.05M. The solution was prepared by dissolving 7.910g of the chemical (BDH. England) in sufficient distilled water and diluted to 1 litre of distilled water and standardized using potassium iodate.

Absolute ethanol (Dterck Germany):
Starch indicator (1%), 1g of soluble starch (BDH. England) was made into slurry with 20ml of cold water and mixed with 80ml of boiled water.

Sodium hydroxide
Sulphuric Acid (IM) concentrated sulphuric acid (BDH, SP.gr 1.84) was diluted appropriately with distilled water to obtain IM.

Artesunate powder (pharmaceutical grade) 99.8% was obtained from the Directorate of Pharmaceutical service of the University of Uyo Teaching Hospital; as a kind gift and used as received. Commercial brands of artesunate used were procured locally from pharmacy shops within Uyo.These include;

(1) Lever artesunate - (Geneith Pharmaceutical)
(2) Askasunate (Diamond Remedies Nig. – LTD.
(3) Gsunate (Greenlife Pharmaceuticals).
(4) Malmeter (Evans Health Care)

Method A – Titrimetry
A 150ml aliquot of the drug solution in absolute ethanol containing 5 to 70mg of artesunate was accurately measured into a 100ml iodine flask and 3ml of 10% potassium iodide was added. The content of the flask was then acidified using 2mls of IM sulphuric acid – the resulting mixture was shaken and corked properly and placed in a dark cupboard for 30 minutes. At 10minutes interval the content of the flask was swirled gently. At the expiration of 30 minutes the released iodine was titrated with 0.05M of freshly prepared and standardized sodium thiosuphate solution. In the course of titration 4 drops of 1% starch solution was added near the end point. The titration procedure was repeated two more times. A blank titration was carried out without the drug from where the amount of artesunate per aliquot was calculated.

Amount (mg)= VMrS/n

V=Volume of sodium thiosulphate
Mr=Relative molecular mass of drug
S=Concentration of sodium thiosulphate
n=the moles of thiosulphate reacting with 1 mole of the drug.

Method B – Spectrophotometry
Different aliquot 0.5 – 5.0ml of standard 100mg/ml were transferred into a series of 10ml standard volumetric flasks. The total volumes in the different flasks were adjusted to 5ml using absolute ethanol. Then 2ml of IM sodium hydroxide was added and shaken vigorously for 1 minute and made up to the mark with absolute ethanol. The resulting mixture was place in a water bath maintained at 60^0C for 30minutes. The absorbance was measured at 300nm against reagent blank and a calibration curve was generated by plotting the increasing absorbance versus the concentration, from where the concentration of the unknown was determined or calculated from the regression equation obtained from the Beer's law data.

PROCEDURE FOR ASSAY OF ARTESUNATE IN TABLET
Twenty tablets of artesunate (commercial brand) were pulverized into very fine powder. A quantity equivalent to 100mg was transferred into a 100ml beaker and 20ml of absolute ethanol was added and sonicated for 5minutes. The content of the beaker was then transferred into a 100ml standard volumetric flask. Another 20ml of absolute ethanol was used to rinse the beaker and transferred to volumetric flask; another 20ml of absolute ethanol was added and shaken vigorously. The resulting mixture was made up to the mark using absolute ethanol with gently swirling. The resulting mixture was filtered using Watman filter paper No 42. The first 10ml portion of the filtrate was discarded and a convenient aliquot was taken and analyzed titrimetrically as described above. The tablet extract containing 1mg/ml of artesunate was further diluted stepwise to obtain 10µg/ml using absolute ethanol from where a convenient aliquot was analyzed spectrophotometrically as described above.

PLACEBO BLANK ANALYSIS
Placebo blank based on commonly used exipients were prepared. The composition made up of 10mg starch, 10mg talc, 10mg ethylene cellulose, 10mg sodium alginate, and 10mg magnesium stearate were mixed together and a solution of this as described in the procedure for tablets was prepared and analyzed titrimetrically and spectrophotometrically.

PROCEDURE FOR THE DETERMINATION OF ARTESUNATE IN SYNTHETIC MIXTURE
A quantity of pure artesunate powder equivalent to 100mg was weighed accurately and transferred to a 100ml beaker containing the composition of excipient as described above. The resulting mixture was homogenized and transferred into a 100ml standard volumetric flask and a solution prepared as described under the procedure for tablets above. The solution was mixed well and filtered using Whatman filter paper No.42. The resulting solution was assayed by titrimetric procedure described above. The synthetic mixture solution containing 1mg/ml was then diluted stepwise with absolute ethanol to obtain a working concentration of 10µg/ml for the spectrophotometric procedure described above. A convenient aliquot was analyzed spectrophometrically, to study the effect of pharmaceutical excipients such as talc, sodium cellulose, starch, magnesium stearate, etc on these assay procedures.

RESULTS AND DISCUSSION

Titrimetry

The determination of ART as described in the international pharmacopoeia is acid – base titration using sodium hydroxide. From a careful search of the literature, there is no report for the assay of ART by iodometric titrimetry. Attih *et al* described a similar procedure but this was for dhydroartemisinin [19].

In acid medium the oxygen centre of the endoperoxide moiety is protonated leading to the generation of hydrogen peroxide *in situ*.

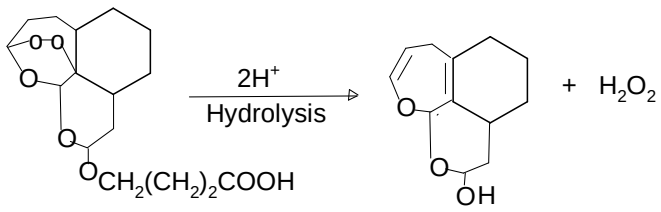


Figure 1. Scheme for the generation of hydrogen peroxide *in situ*

The hydrogen peroxide generated then reacts with potassium iodide liberating iodine. The liberated iodine is then titrated against standardized sodium thiosulphur

$$KI + 2H^+ + H_2O_2 \longrightarrow 1_2 + 2H_2O\ (l)$$

$$2I^-_{(aq)} + 2H^+_{(aq)} + H_2O_2 \longrightarrow I_2 + 2H_2O$$

Iodide H_2O_2 = I_2 = 2e

$$1_2 + 2S_20^{2-} \longrightarrow 21^- + S_20^{2-}$$

Iodide dithionate

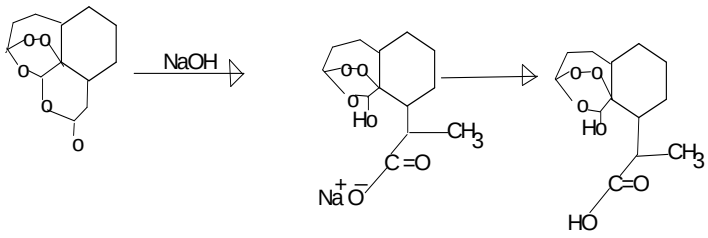
$$2S_20_3^{2-} \equiv 1_2 \equiv 2e \equiv$$

$$2S_20_3^{2-} \equiv 1_2 \equiv 2e \equiv H_2O_2$$

The amount of ART in the aliquot is calculated from this equation or from the reaction stoichiometry which is 1:1:2 for [Drug (H_2O_2): [iodine] : [$2S_2O_3^{2-}$]

SPECTROPHOTOMETRY

Artesunate and dihydroartemisinin lack isolated convalently unsaturated group that show a characteristic absorption in the UV-Vis region. That is (chromophore) conjugated double bonds or basic centre as nitrogen and lone pair election but when incubated in sodium hydroxide they give alkali decomposed product which can be determined spectrophotometrically. As typical alkali, sodium hydroxide opens up the lactone ring of artesunate and dihydroartemisinin to form bifunctional compound (hydroxy acid) which can be measured spectrophotometrically at 300nm.



Alkali- decomposed product absorbing at 304nm

Scheme for alkali hydrolysis of dihydroartemisinin

OPTIMISATION OF REACTION CONDITIONS

The reaction conditions were studied and carefully optimized. A particular variable (e.g. Temperature, Time, Concentration) was varied while others were kept constant to ascertain the effect of that particular variable on the performance of the proposed method.

Effect of Temperature

In the spectrophotometric method, it was observed that there was a steady increase in the absorbance as the temperature was increased, when a fixed concentration of ART (30µg/ml) was made to react with 1ml of IM NaOH. (25^0 30^0, 40^0c, 50^0c, 60^0.)

Effect of Time

To study the effect of time a fixed concentration of artesunate (30mg/ml) was reacted with 1ml of 1 molar sodium hydroxide solution at reaction temperature of 60^0C. The absorbance readings were taken at 5, 10, 15, 20, 30 and 40 minutes. The absorbance was observed to increase with time up till 30minutes, beyond this there was no further increase in absorbance. The complete alkali hydrolysis of artesunate was completed within 30 minutes given an alkaline decomposed product measured spectrophotometric at 300nm.

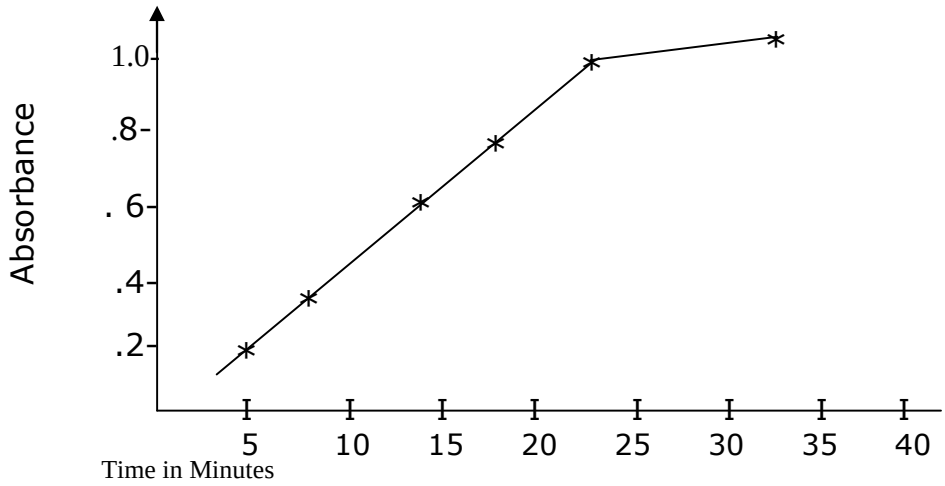


Fig. 2: Effect of time on the absorbance of the alkaline decomposed product of 30µg/ml Artesunate by 1ml of IM sodium hydroxide

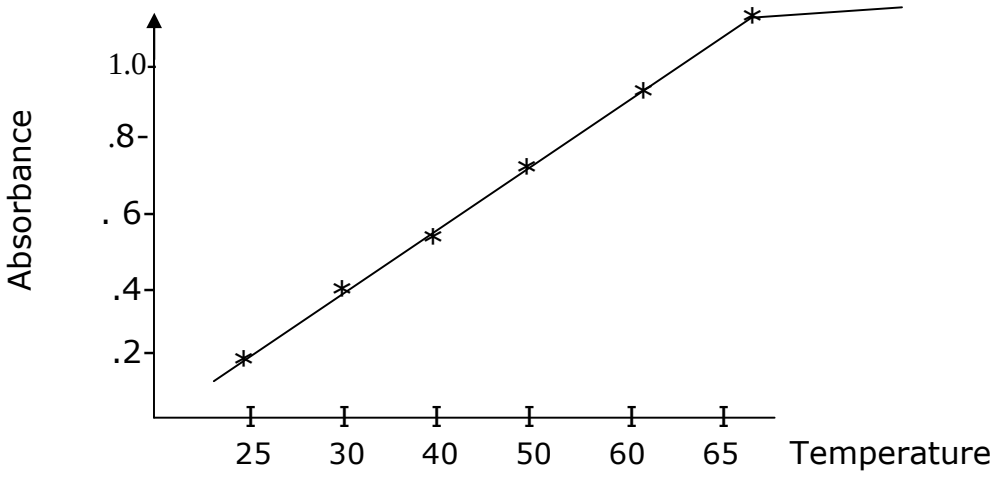


Fig.3 effect of temperature on the absorbance of the alkaline decomposed product of 30mg/ml Aresunate by 1ml IM NaOH

Effect of sodium hydroxide concentration

To study the effect of NaOH concentration, a fixed concentration of Artesunate (30μg/ml) was made to react with 1ml of varying concentration of NaOH 0.25M, 0.5M, 1M and 2M) kept in the water bath maintained at 60^0C for 30mins. The absorbance was observed to increase with increasing concentration of sodium hydroxide up to 1M. When the maximum concentration absorbance was recorded; beyond 1ml of IM NaOH., erratic results were obtained. The volume of sodium hydroxide was also considered it was also found that Iml of IM NaOH was most suitable for the reaction.

Potassium hydroxide was also used for the alkaline hydrolysis of Artesunate. A measurable alkaline decomposition product was obtained at lower concentration of KOH. Iml of 0.25M potassium hydroxide was also found to be suitable for the spectrophotometric analysis of artesunate.

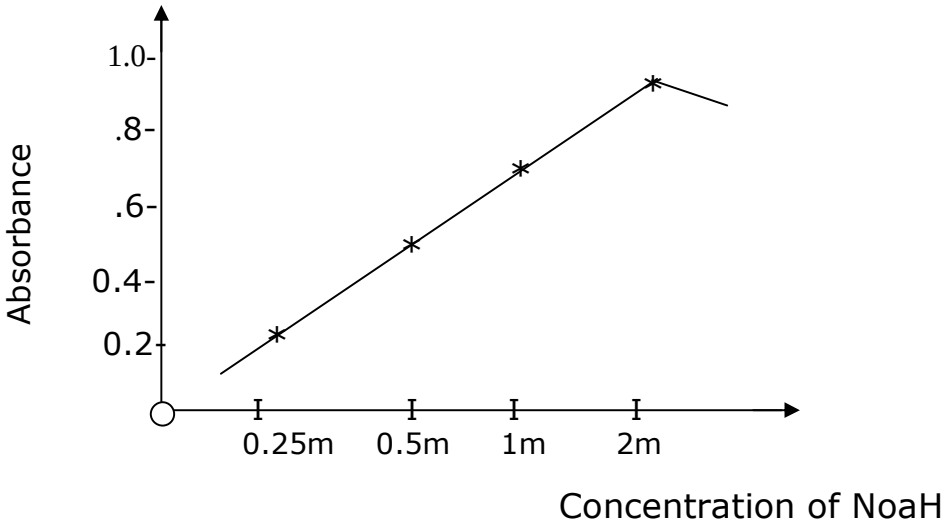


Fig. 4 effect of lml of varying concentration of NaOH on the absorbance of the alkaline decomposed product of Arestunate

Analytical Data

The prepared methods were validated for linearity, sensitivity, precision, accuracy, selectivity and recovery.

Linearity and Sensitivity:

A linear relationship was obtained between the absorbance and concentration of Artesunate within the range of 5 and 60mg/ml of Artesunate, under optimum conditions. The calibration graph generated by plotting absorbance versus concentration is discussed by the equation.

$$A = BX + C$$

where A=absorbance, B=slope, X=concentration and C the intercept obtained by the least square method. The correlation coefficient, slope and the intercept are recorded in table 1.

To evaluate the sensitivity of the spectrophotometric method. The following parameters – molar absorptivity and sandell sensitivity were determined. The limits of detection and quantification were determined as per the current ICH guidelines (ICH 2005) and recorded also in table 1. The limit of detection (LOD) and limit of quantification were calculated from the formula LOD =3.3σ/s and 10σ/s LOQ =

where σ is the standard deviation of five reagent blank determination and S is the slope of the calibration curve generated.

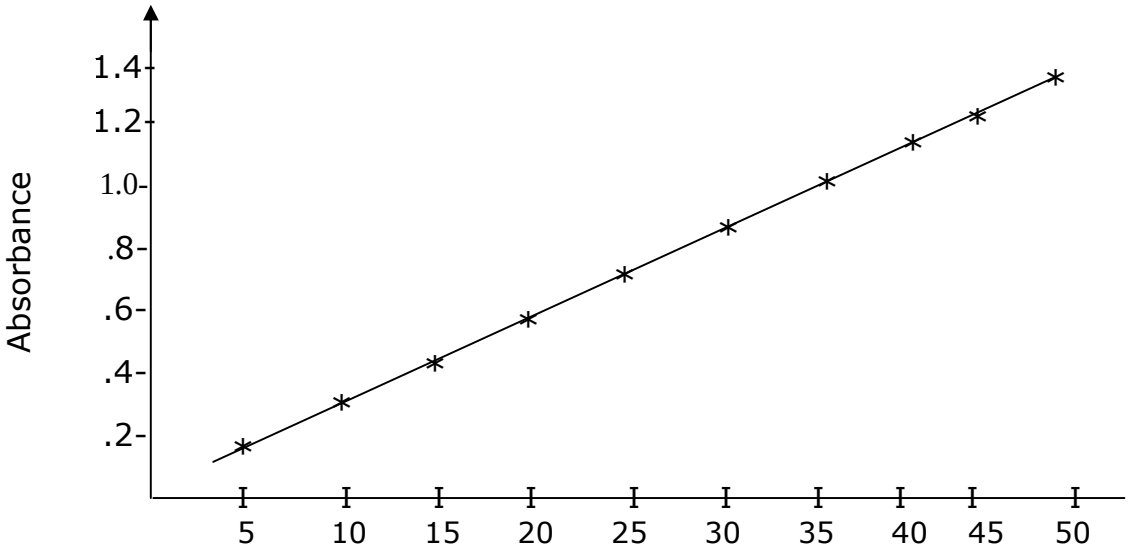


fig.5 Absorbance Vs Concentration of Artesunate (µg/ml) (calibration curve.)

PARAMETER

λmax	300nm
Linear range µg/ml	2.0-60
Molar absorptivity (E) /mol/cm	1.365×10^3
Sandell sensitivity mg/cm^2	0.282
Limit of detection (LOD)	0.375
Limit of qualification (LOQ)	1.25
Regression equation	y = 0.024x
Intercept	0.0001
Slope	0.024
Coefficient of correlation (r)	0.9998

PRECISION AND ACCURACY

To evaluate precision and accuracy of the two methods proposed, solutions containing three different concentrations of pure Artesunate were prepared and analyzed in five replicate (n=5) within the same day, (intra-day). The same determination was carried out for five consecutive days (inter-day); precision and accuracy. The % relative standard deviation (%RSD) i.e., precision and the percentage standard error % R. E. i.e accuracy were very low indicating the good precision and accuracy of the proposed methods. The percentage relative error was determined using the formular.

$$\% \text{ R. E.} = \left(\frac{\text{Amount found} - \text{Amount taken}}{\text{Amount Taken}} \times 100 \right) \quad 1$$

The results are recorded in Table II.

TABLE II: Results of the Recovery Studies by Standard Addition Method

Method A (Titrimetry)					Method B(Spectrophotometry)			
Tablets studied	**Amount of drug (mg/ml)**	**Amount of Drug Added**	**Total amount found/mg**	**% recovery of pure drug + SD.**	**Amount of Drug µg/ml**	**Amount of Drug Added µg/ml**	**Total amount found**	**% recovery of pure Drug +SD.**
1) Lever Artesunate (50mg)	2.10 2.10 2.10	4.0 8.0 12.0	6.21 10.30 14.30	102.7+1.15 103+ 1.50 101 + 1.11	20.24 20.24 20.24	10.0 30.0 50.0	30.19 50.32 70.50	99.5+1.12 100.3+1.0 100.5+1.1
2) Askasunate (Diamond Remedies) (50mg)	3.10 3.10 3.10	4.0 8.0 12.0	7.25 11.30 15.50	103.7+2.65 102.5+1.76 103.3 +2.36	30.15 30.15 30.15	10.0 30.0 50.0	40.25 61.0 80.37	101.0+1.1 103.0 +1.2 100.4+1.1
3) Gsunate (Greenlife) (50mg)	4.20 4.20 4.20	4.0 8.0 12.0	8.25 12.45 16.50	101.3+0.86 103. 0+2.20 102.5+1.76	40.10 40.10 40.10	10.0 30.0 50.0	50.0 70.31 91.00	99.0+1.15 101.0+1.0 101.8+1.12
4) Malmeler (Evans) (50mg)	4.50 4.50 4.50	4.0 8.0 12.0	8.60 12.65 16.60	102.5+1.76 102.0+1.32 102.1+1.	40.50 40.50 40.50	10.0 30.00 50.00	50.60 71.20 91.10	101.0+0.68 102.3+0.54 101.2+1.13
5) Articin (Embasy) (50mg)	4.60 4.60 4.60	4.0 8.0 12.0	8.62 12.70 16.65	100.5+1.1 101.3+0.86 100.4+1.12	50.10 50.10 50.10	10.00 30.00 50.00	60.08 81.10 101.20	99.8+1.11 103.3+0.84 102.2+0.76

Table 1: Evaluation of intra day and inter day Accuracy and precision

S/N	Method	Amount of ART taken	Intra Day Accuracy And Precision			Inter Day Accuracy And Precision		
			Amt of ART found	RE%	RSD%	Amt of ART Found	RE%	RSD%
1. 2 3	Titrimetry	4.0 8.0 12.0	4.15 8.20 12.3	3.75 2.50 2.80	1.88 1.25 1.41	4.16 8.22 12.4	4.0 2.75 3.33	2.0 1.37 1.67
1 2 3	Spectrophotometry	2.0 4.0 6.0	2.06 4.08 6.10	3.0 2.0 1.66	1.5 1.0 0.83	2.05 4.06 6.12	2.5 1.5 2.0	1.25 0.75 1.00

Table 2: Result of Analysis of tablet by Titrimetry method

S/N	**Tablets Analysed**	**Label Cla----(mg)**	**Reference Method**	**Titrimetric Method**	**Spectrophotometric Method**
1.	Lever Artesunate (Geneith)	50	110.5+1.0	111.5+1.12 F = 1.25 t = 1.48	111.0+1.15 F = 1.15 t = 0.73
2.	Askasunate (Diamond Remedies)	50	110.0+1.11	111.0+1.21 F = 1.19 t = 1.17	110.9 +1.25 F = 1.27 t = 1.20
3.	G Sunate (Greenlife)	50	110 +1.11	111.2+0.84 F = 1.75 t = 1.93	111.0+0.86 F = 1.67 t = 1.60
4.	Malmeter Evans	50	110.0+0.86	111.4+1.12 F = 1.70 t = 1.58	110.5 +1.10 F = 1.64 t = 0.80
5.	Articin (Embassy)	50	110.0+1.11	111.0+1.21 F = 1.19 t = 1.36	111.0 + 1.12 F = 1.02 t = 1.42

SELECTIVITY

The two methods were evaluated for selectivity as discussed by placebo blank and synthetic mixture analyses. It was discovered that the pharmaceutical excipients tested had no noticeable interference with the developed analytical methods. The synthetic mixture analyses gave excellent recovery ranging from 99.00+ 0.85 to 103.6+ 1.25; indicating high accuracy and non interference of the pharmaceutical excipients on the developed methods.

ROBUSTNESS AND RUGGEDNESS

The robustness of the methods were evaluated. In the titrimetric method, the reaction time and the volume of the acid (H_2SO_4) were slightly altered (30+1min and 2.5ml). In the spectrophotometric method the reaction time was slightly altered (30+1min). The ruggedness of these methods were evaluated by two different analysts using

different burettes in the titrimetric method and different spectrophotometer. The robustness and ruggedness analysed at three different drug concentration levels (2.0, 4.0, 6.0mg/ml) for the titrimetric method and 30, 60, 90µg/ml in the spectrophotometric method. The intermediate precision (RSD) being a measure of the robustness and ruggedness, showed no statistical differences between different analysts and instruments suggesting that both proposed methods were robust and rugged.

APPLICATION TO TABLETS

The proposed methods were used to analyze five commercially available artesunate tablets procured in local Pharmacies in Uyo, Nigeria. The results obtained were statistically compared to a reference titrimetric method (International Pharmacopoeia) for the assay of artesunate. Via student's t-test and the variance – ratio f-test. The calculated t and f values at 95% confidence level and at 4 degrees of freedom were below the tabulated values of 2.77 and 6.39 respectively as shown in table 2.

RECOVERY STUDIES

The accuracy and validity of the proposed methods were further tested by performing recovery studies via standard addition method. A calculated amount of pure artesunate was used to spike a pre analyzed artesunate powder at three concentration levels and the total was found using the proposed methods repeating each determination three times. The percentage recoveries of the added artesunate ranged between 99.0%s to 103.3% with standard deviation of 0.54 to 1.76 as shown in Table 4. This confirms the excellent recoveries and the non interference from other pharmaceutical excipients co-formulated with the drug.

CONCLUSION

The proposed methods are reproducible sensitive and affordable in the phase of counterfeit and fake artesunate being distributed in Africa and Asia. The titrimetric method can be used to complement the official titrimetric method in the international pharmacopoeia. The method uses very simple Laboratory Bench Chemicals/reagents, which are ecofriendly and which are not hazardous to the analyst and the environment. The method is devoid of excessive extraction steps using hazardous organic solvent.

The spectrophotometric method is very simple and affordable also using simple and inexpensive laboratory bench reagents. The two methods can be used to complement each other. The proposed methods were not affected by pharmaceutical excipients used to formulate artesunate. Since they are affordable they are recommended for use in routine quality control laboratories.

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