Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 7(7):1050-1058

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Novel spectrophotometric determination of artesunate using vanillin/ sulphuric acid reagent

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ABSTRACT

A sensitive, simple and reproducible spectrophotometric method is developed for the determination of Artesunate in pure and pharmaceutical formulation. The method is based on the conversion of artesunate to Dihydroartemisinin (a lactol) by acid hydrolysis and the subsequent reaction of the Dihydroatemisinin with Vanillin, a carbonyl compound, to form a hemiacetal product. This is an acid catalyzed nucleophilic, addition reaction. The chromogen generated absorbed the uv-vis light at 420nm. The absorbance of the chromogen varied proportionally with the Artesunate concentration. Beer's Law is obeyed between the range of 2-6 µg/ml with a Linear coefficient of 0.9998. The molar absorptivity and Sandell sensitivity were 1.19X10^4 L/mol/cm and 0.0323µg/cm^2 respectively. The limit of detection and quantification were 0.28µg/ml and 1.30µg/ml respectively. The intra and interday precision and accuracy expressed as percentage relative standard deviation and percentage relative error were ≤ 2. 73 and ≤ 1.93. The method was successfully used to assay Artesunate in tablets procured locally and the results when compared statistically with a pharmacopoeia method showed good congruence. The accuracy and applicability of the method was confirmed by performing recovery studies via standard addition method with the result showing no interference from pharmaceutical excipients.

INTRODUCTION

Malaria remains the deadliest of all parasite diseases in sub-Saharan and other tropical regions of the world. The twin factors of international travel and global warming has led to the spread of malaria to over 40% of the world's population [1, 2, 3]. Current projections suggest that if global warming remains unchecked, it could re-establish itself in Europe and North America [4].

However, the most critical problem facing the treatment of malaria is the development of resistance to Antimalaria compounds such as chloroquine and the antifolates. [5,6]. Consequently, to overcome the resistance problem, the world Health Organization (WHO) and health authorities in malaria endemic countries recommend the Artemisinin Combination therapy (ACT).The aim was to provide better efficacy and to avoid resistance [6,7,8]. The artemisinin antimalarial drugs have been very useful because of its strong parasiticidal action on the malaria parasites. The success recorded by the novel antimalarial activity of the artemisinins is almost short lived because of reports of the reduced susceptibility of some strains of plasmodium in southeast Asia due to the distribution of fake and counterfeit Artemisinin derivatives especially Artesunate [9,10,11,12,]. The presence of fake Artemisinin Antimalarials has also been reported in Africa [13, 14,15].

Poor quality antimalarial drugs leads to drug resistance and inadequate treatment which pose an urgent threat to vulnerable populations and jeopardize progress and investment in combating malaria [16]. WHO defines counterfeit medicine as one which is deliberately and fraudulently mislabeled with respect to identity and/or source. [17]. Both branded and Generic products are counterfeited or faked. The counterfeiters are sophisticated; they produce identical holograms, blister packs with batch numbers and expiry date looking absolutely genuine. Their packaging is seen as a perfect copy of the original, hence their quality can fool anybody even experts in the pharmaceutical industries [12]. These fake artemisinin antimalarials can only be detected in the laboratories with high technical facilities which are hardly available in third world countries where malaria is prevalent.

The aim of this research work is to attempt to solve this malaise by developing a sensitive, reproducible and affordable method for the determination of Artesunate in bulk and tablet formulations. Artesunate of all available artemisinin derivatives is the most faked reported [18]. Artesunate is a succinate derivative of Artemisinin and it is assayed in the international pharmacopoeia by HPLC and titrimetry. In literature, some methods have been developed for the assay of Artesunate [18,19,20,21,22]. To the best of our knowledge, no spectrophotometric method using vanillin/sulpheric Acid reagent has been developed. This method being very simple can complement the titrimetric method in field stations or conventional laboratories for the quantification of Artesunate as sophisticated equipment are hardly found in this part of the world.

EXPERIMENTAL SECTION

All spectral determinations were made using Helios β (Thermo electron inc. USA) spectrophometer with 1cm matched quartz cell.

REAGENTS: All reagents and chemicals used in this work were analytical grade.
1.Vanillin (Merck Darmstadt Germany), 4% solution of vanillin was prepared by dissolving 4mg of the substance with 10mls of absolute ethanol and made up to 100ml with ethanol. The reagent was prepared fresh daily.
2. Absolute ethanol (Merck Darmstadt Germany) was prepared and used without dilution.
3. Concentrated sulphuric Acid (Sp.gr 1.83) B.D.H was prepared appropriately by dilution of the acid to obtain 5M solution.

Standard Artesunate solution pharmaceutical grade of Artesunate was donated by the Director of pharmaceutical services, university of Uyo Teaching Hospital, Uyo as a kind gift and used as received.

A standard stock solution of Artesunate was prepared by carefully weighing out 100mg of the substance and transferred into a 100ml volumetric flask and dissolved in enough distilled water to make up 100ml mark of the volumetric flask to obtain a concentration of 1mg/ml. the resulting stock solution was diluted further to obtain a working concentration of 100µg/ml.

GENERAL PROCEDURE FOR THE DETERMINATION OF ARTESUNATE

Different Aliquots (0.5-5ml) containing 10µg/ml of standard Artesunate solution were carefully transferred into series of 10ml capacity (Calibrated) Volumetric flask, using a micro burette. The volume in the volumetric flask was brought up to 6ml by adding absolute ethanol then 1ml of 4% vanillin solution was added and shaken properly to mix , 1ml of concentration sulphuric acid was also added to each flask and mixed well and allowed to stand for 5minutes. Finally the volume in each flask was made up to the mark and swirled gently to mix well and the absorbance of the resulting Chromogen was measured at 420nm against reagent blank prepared similarly but carefully omitting the drug. A calibration curve was generated by plotting the absorbance against the Artesunate concentration. The unknown concentration of Artesunate in a given sample was determined from the calibration curve generated or evaluated from the regression equation derived from Beer's Law.

PROCEDURE FOR ASSAY OF ARTESUNATE IN TABLETS

Twenty tablets of lever® Artesunate were weighed individually to obtain weight uniformity then all the tablets were pulverized using ceramic mortar and pestle. A quanlity of the power equivalent to 100mg of the Artesunate was carefully weighed out and transferred into 100ml capacity volumetric flask containing 20ml of distilled water and sonicated for 10minutes.

A further 60ml of distilled water was added and shaken vigorously for another 10minutes and finally the volume in the flask was made up to the 100ml mark with distilled water and filtered using whatman filter paper No 42. The first 100ml portion of the filtrate was discarded. The resulting concentration of Artesunate was 1mg/ml. This was diluted further to obtain a working concentration100µm of Artesunate from where a convenient aliquot was analyzed using the general procedure discussed above.

METHOD FOR PLACEBO BLANK AND MIXTURE

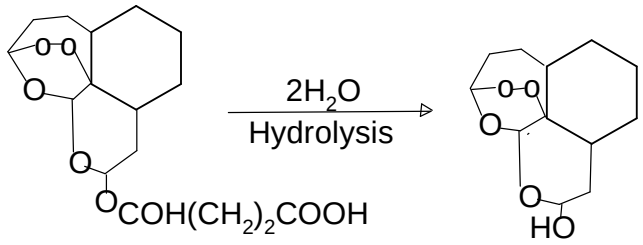
A placebo blank powder containing some pharmaceutical excipients such as Lactose 15mg, Magnesium stearate 0.5mg, talc 1mg, micro crystalline cellulose 12.8mg, acacia 2mg and maize starch added to bulk up the mixture to 100mg. this mixture was mixed appropriately and homogenized to form a homogenous mixture and transferred into a 100ml capacity volumetric flask containing 40ml of distilled water. The resulting mixture was shaken vigorously and sonicated for 20minutes. A further 40ml was added and shaken vigorously for another 10minutes. Finally, the mixture was made up to the 100ml mark and filtered using whatman filter paper number 42.The resulting placebo blank solution was analyzed by exactly following the procedure for tablet dosage form as described above.

PROCEDURE FOR ANALYSIS OF SYNTHETIC MIXTURE

The synthetic mixture was prepared by carefully measuring and adding of 100mg of pure Artesunate powder and transferred into a beaker containing 100mg of the placebo powder as prepared above. The resulting mixture of the two was homogenized and 100mg of the resulting mixture was carefully transferred from there to a 100ml calibrated volumetric flask containing 50ml of distilled for a further 10minutes.Thereafter two equal volumes of 25ml of distilled water was added and shaken at each time. The resulting synthetic mixture containing the drug was filtered using whatman filter paper No 42. The first 10ml of the filtrate was discarded. The resulting synthetic drug solution was diluted appropriately to obtain a working concentration of 100µg/ml from where a suitable aliquot was analyzed as described in the procedure for tablet above.

RESULTS AND DISCUSSION

The reaction between Artesunate and Vanillin/Sulphuric reagent is based primarily on the conversion of Artesunate to Dihydroartemisinin (Lactol) by hydrolysis using concentrated sulphuric Acid. Dihydroartemisinin, being an alcohol, then reacts with the Vanillin which is a carbonyl compound forming a condensation product Hemiacetal compound. The reaction being catalyzed by concentrated sulphuric Acid. The Mechanism of this reaction is most likely an Acid catalyzed Nucleophilic addition reaction. A proton first attaches to the carbonyl Oxygen leading to the development of positive charge in carbonyl compound. Next a Molecule of DHA (DihydroArtemisinin), an alcohol, joins the positively charged carbon centre to form a hemiacetal group.



Artesunate Dihydroartemisinin (lactol)

Figure 1 Artesunate is converted Dihydroartemisinin (a lactol)

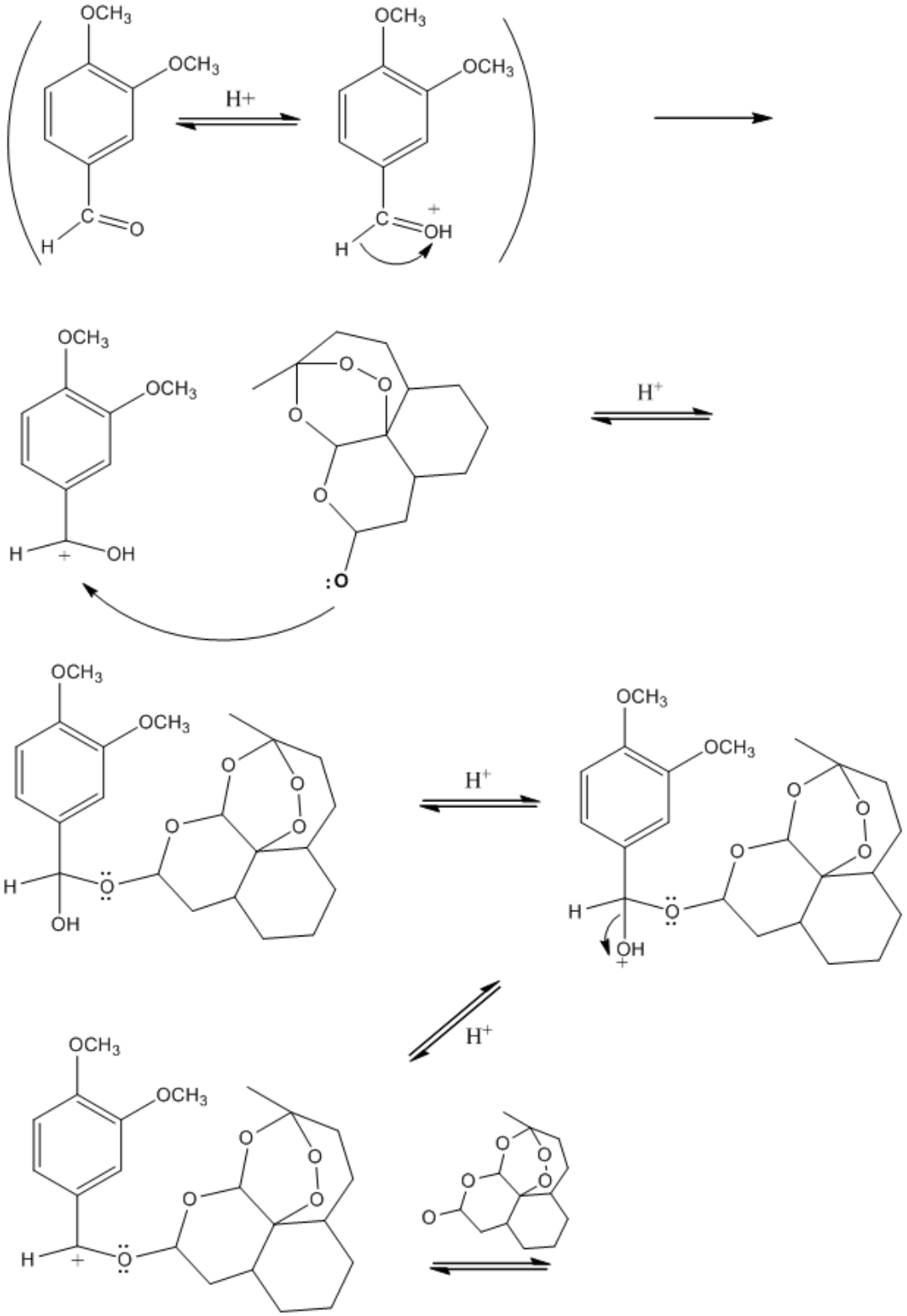


Figure 2: Mechanism of Reaction between DHA and Vanillin / Sulphuric Acid

Reagent
The colour produced pink to reddish brown coloured chromogen which absorbed maximally at 420nm at room temperature. Under these conditions the linearity between the absorbance and the drug concentration was established leading to the use of this developed method to determine the concentration of the drugs per sample of pure Artesunate and in tablet formulations.

OPTIMIZATION OF EXPERIMENTAL CONDITIONS
Various experimental conditions leading to the formation of this chromogen were carefully studied and optimized. This was done by varying a particular parameter while keeping others constant and its effect on absorbance observed and measured.

EFFECT OF TEMPERATURE ON THE REACTION
The effect of temperature on the formation of colored chromogen was measured by increasing the temperature from room temperature (25 ± 1^0 C) to 50^0C.it was discovered that the colored was formed instantly when the reagents were added when the temperature was increased beyond 30^0C, there was a marked colour change which was no longer stable and the absorbance was no longer linear with respect to the concentration of the drug.

EFFECT OF CONCENTRATION OF REAGENTS
Vanillin Reagent: all other reagents concentrations were kept constant while the concentration of vanillin was increased gradually from 1% through to 10% and the volume of 4% vanillin reagent was varied from 0.5-3.0mls. It was observed that there was a gradual increase in the absorbance as the concentration of vanillin was increased. Maximum absorbance was recorded at 4% reagent used. Beyond 5%, the results were erratic as deeper colored chromogen was formed. The absorbance was no longer linear.

VOLUME OF SULPHURIC ACID
This reaction is an acid catalyzed reaction both for the hydrolysis of the Artesunate and for the formation of the hemiacetal complex. The volume of the concentrated sulphuric Acid was varied between 0.5ml to 30ml. it was discovered that 1ml of concentrated sulphuric Acid was adequate for the formation of good quality colored chromogen that was very stable. Addition of more H_2SO_4 from 2ml gave deep dark color suggesting that the solution was getting changed. At 420nm, the absorbance was erratic due to an unstable colored chromogen

SOLVENTS: Water, ethanol, methanol were used to perform the reaction. Aliquots containing 10, 20µg/ml, 50µg/ml of the drug was prepared and analyzed three times each using water, ethanol and methanol and the absorbance 4hours at 1hour intervals. All three gave very useful results but the chromogen formed when water was used was not as stable as the color of chromogen formed when absolute ethanol and methanol were used. Absolute ethanol was used because it gave the best absorbance and the colored chromogen was more stable.

COLOUR STABILITY: To test the stability of the coloured complex formed. The drug was assayed at 3 concentration levels of 20µg, 40µg and 60µg/ml. The absorbance of the chromogen was then observed for 80minutes at 20minutes interval and at 420nm. See results in table 1

Table 1: Test for stability of the colured complex formed per time

S/N	Concentration of Artesunate (µg/ml)	Absorbance at 420nm AT 20minutes interval				
		1	20	40	60	80
1	20	0.215	0.214	0.212	0.210	0.210
2	40	0.321	0.320	0.320	0.319	0.319
3	60	0.400	0.395	0.394	0.393	0.393

Within 80minutes the absorbance was very stable *Table 1*

METHOD VALIDATION PROCEDURES
The developed method was validated for linearity and sensitivity, precision and accuracy, selectivity and robustness, ruggedness and recovery.

LINEARITY AN SELECTIVITY: At optimal experimental conditions, the change in the drug concentration was directly proportioned to the absorbance. The calibration curve so generated was linear and it obeyed Beer's law

within the range of 2-60µg/ml. The regression equation was obtained by the least square method in the form of y=mc + b where y= Absorbance, c= Concentration, m= Slope and b= intercept.

These values including the correlation coefficient are recorded in table 2. Parameters defining the sensitivity of the method including molar absorptive, Sandell sensitivity, Limit of Detection (LOD) and limit of qualification (LOQ) were also determined. The limit of detection (LOD) and limit of Qualification (LOQ) were determined as per the current ICH guidelines with the formulae;

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

Where σ is the standard deviation of five replicate reagent blank determinations and S = Slope of the calibration curves.

Table 2: Sensitivity and regression parameters

	PARAMETER	VALUE
1.	λmax (nm)	420
2.	Beers Law Range (µg/ml)	2-60µg
3.	Molar Absorptivity (L/mol/cm)	$1.19X10^4$
4.	Sandell Sensitivity (µg/cm^2)	0.0323
5.	Limit of Detection (µg/ml)	0.47
6.	Limit of Qualification (µg/ml)	1.45
7.	Regression Equation (µg/ml)	A= mc + b
8.	Slope	0.018
9.	Intercept	0.001
10.	Correction Coefficient	0.9997

ACCURACY AND PRECISION

To determine accuracy and precision of this method, solution continuing three different concentration of Artesunate were prepared and Analyzed in five replicate determinations three times within a day (intra-day) and three times for three consecutive days (inter-day). The accuracy was evaluated as percentage Relative error (RE %) calculate using the formular;

$$RE\% = \left[\frac{\text{Amount found} - \text{Amount Added}}{\text{Amount Added}}\right] \times 100$$

The Precision was calculated as the percentage relative standard deviation %RSD and the results are recorded in Table 3

Table 3: Determination of intraday and interday Precision and Accuracy

S/N	Amount 0f Atesunate Acid (µg/Ml)	Intra Day Accuracy And Precision			Inter Day Acuracy And Precision		
		Amount of Artesunate Found (µg/ml)	RE %	RSD %	Amount of Artesunate Found (µg/ml)	RE %	RSD %
1	40	40.95	2.23	1.23	41.09	2.73	1.93
2	80	81.65	2.06	1.46	81.58	1.98	1.40
3	120	121.98	1.66	1.17	122.09	1.74	1.71

SELECTIVITY: The selectivity of the proposed method was tested as described earlier. Analyses of a convenient aliquot prepared as described earlier showed no significant change in absorbance meaning that these pharmaceutical ingredients tested did not interfere with the proposed method.

When the synthetic mixture spiked with pure Artesunate as prepared was analyzed, the results showed excellent recoveries of between 99.50 to 101.00 with standard deviation of 0.92 and 1.14. Meaning that the pharmaceutical excipient used in the synthetic mixture had no effect on the developed method.

ROBUSTNESS AND RUGGEDNESS

The developed method was evaluated for robustness by deliberate but minor variations in the reagent and reaction parameters. The volume of the concentrated sulphuric acid, reaction time and the volume of absolute ethanol varied slightly and the effects on the absorbance were evaluated. The ruggedness was evaluated by performing the analysis

using three instruments by three analysts. The robustness and ruggedness were evaluated at three different concentration levels of the pure drug and the precision determined, as percentage relative Standard Deviation (% RSD) and the results recorded in Table 4

Table 4: Determination of Robustness and Ruggedness

ROBUSTNESS (RSD %)					RUGGEDNESS (RSD %)	
Amount of Artesunate Studied	H_2So_4 volume (n=3)	Reaction Time (n=3)	Volume of Absolute Ethanol used (n=3)	Amount of Atesunate Studied	Inter instrument n=3	Inter Analysts n=3
40	1.21	1.07	1.11	40	1.47	185
80	1.13	1.15	1.67	80	1.15	1.31
100	1.72	1.25	1.54	100	1.08	1.44

- H_2SO_4 volume used = 1.0ml, 1.5ml, 1.8ml
- Reaction Time = 2min, 5min, 10minutes
- Volume of Absolute Ethanol used = 1.0ml, 1.5ml, 2.0ml

APPLICATION TO TABLETS

The developed and validation method was successfully used to evaluate 6 commercial brands of Artesunate tablets procured from Local Pharmacies in Uyo, South-South Nigeria. The results obtained were statistically compared with the titrimetric method stated in the international pharmacopeia (2005) for the determination of Artesunate using the student's t-test and the variance ratio F-test. The calculated t and F values were observe to be below the tabulated values of t=2.77 and f=6.39 at 95% confidence level and at 4 degrees of freedom. The results showed no significant difference with that obtained by applying the international pharmacopoeia Titrimetric method.

Table 5: Results of Artesunate Tablets Using the Proposed Method

COMMERCIAL ATESUNATE TABLET ANALYZED		Label Claim (mg)	Found (percent of Label/claim ± S.D)	
			REFERENCE METHOD	PROPOSED METHOD
1.	Arsumate®(Saniofi)	50	99.60± 1.43	101.0±1.00 F=204 t=1.64
2.	Artesunate® (Neros)	50	99.89±1.44	101.31±1.06 F=2.64 t=1.38
3.	Gsunate® (Greenlife)	50	99.70±1.87	101.28±1.15 F=2.64 t=1.25
4.	Lever Artesunate (Gemeith)	50	99.72±1.30	101.30±0.89 F=2.13 t=1.70
5.	Articin® (evans)	50	99.80±1.52	101.63±1.14 F=1.78 t=1.24
6.	Malmeter (Evans)	50	99.87±1.40	100.92±1.01 F=1.92 t=1.05

Mean of five determinations. The values of tabulated at 95% confidence level and at 4 degree of freedom is 2.77. The value of F (tabulated) at 95% confidence level at 4 degree of freedom = 6.37.

RECOVERY STUDIES: recovery studies was performed using the standard addition method to confirm the accuracy and applicability or otherwise of the method. Pure Artesunate powder at three concentration levels was used to spike an already analyzed tablet powder and the total amount of the Artesunate in the mixture determined via the proposed method. The percentage recovery of the drug and the standard deviation are recorded in table 6. The results ranged from 99.8 to 102.5 with standard deviation of 0.92 – 1.14. This shows a good recovery confirming that pharmaceutical excipients had little or no effect on the developed method.

Table 6: Results of the recovery study by the standard addition method

TABLETS STUDEIED	AMOUN OF DRUGS	AMOUNT OF PURE DRUG ADDED (μg/ml)	TOTAL AMOUNT OF DRUG FOUND	RECOVERY OF THE PURE DRUG (Artesunate) % ISD
1. Arsumax (Sanofi)	30.00 30.00 30.00	20.00 40.00 60.00	50.20 69.80 89.89	101.01 ± 1.10 99.50 ± 1.13
2. Arteunate (Neros)	40.00 40.00 40.00	20.00 40.00 60.00	59.90 81.00 99.88	99.50 ± 1.07 102.50 ± 1.10 99.80 ± 1.11
3. Gsunate (Greenlife)	40.00 40.00 40.00	20.00 40.00 60.00	60.18 9.89 100.82	100.90 ± 0.92 99.73 ± 1.12 101.36 ± 1.14
4. Lever Artesunate (Gemeith)	50.00 50.00 50.00	20.00 40.00 60.00	70.20 90.90 111.10	101.00 ± 1.10 102.30 ± 1.02 101.80 ± 1.03
5. Articin (Embassy)	50.00 50.00 50.00	20.00 40.00 60.00	69.95 89.93 111.00	99.80 ± 1.11 99.80 ± 1.01 101.70 ± 1.14
6. Malmeter (Evans)	60.00 60.00 60.00	20.00 40.00 60.00	80.18 101.00 121.00	100.90 ± 1.02 102.50 ± 1.04 101.70 ± 1.12

- *Mean of three determination*

CONCLUSION

A new sensitive, reproducible and affordable method is developed. The method is devoid of tedious extraction procedures using hazardous organic solvents. The reagents used were eco-friendly and posed no danger to the environment or the analyst. The simplicity of this method makes it to be very useful in routine quality assurance laboratories or even in field stations to check the influx of counterfeit fake Artesunate.

Acknowledgement
The authors will like to thank the Director of Pharmaceutical service of the University of Uyo Teaching Hospital for the provision of the pure Artesunate used in this work.

We are also grateful to Prof. J.N. Eloff of the phytomedicine laboratory of the University of Pretoria who provided the laboratory space and equipment for the research.

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