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QUANTITATIVE SPECTROPHOTOMETRIC DETERMINATION OF ARTESUNATE IN PHARMACEUTICALS USING THE IRON-THIOCYANATE COMPLEX FORMATION REACTION

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ABSTRACT

A sensitive, accurate and reproducible spectrophotometric method is developed for the elimination of Artesunate in pharmaceutical formulations. This novel method is based on the oxidation of FeII to FeIII by artesunate in acid medium and the subsequent formation of Ferric-thiocyanate complex (blood red) chromogen which absorbs, Uv-vis light maximally at 480nm. Under optimized experimental conditions, the absorbance was found to increase linearly with the artesunate concentration. A calibration curve was generated which obeyed beer's law within the range of 0.4 – 50 µg/ml with a linear co-efficient of 9997, a slope of 0.0113 and a negligible intercept. The molar absorptivity and Sandell sensitivity were 1.08×10^4 $LMol^{-1}$ CM^{-1} and 0.0356 µg/cm respectively. The limit of detection (LOD) and Limit of Qualification (LOQ) were 0.15 and 1.29 µg/ml respectively. The accuracy expressed as percentage relate error (ER%) and the corresponding precision expressed as relative standard deviation (RSD %) were <3.0% and <2.5% respectively. The proposed method was validated and used to assay artesunate in tablet formulations procured locally, the result obtained was statistically compared with official international pharmacopoeia method for artesunate; the result showing no difference in their means. The t and f value were <1.50 and > 2.76 respectively. The accuracy and practicability of the method was ascertained by

recovery studies performed via standard addition method with the results showing high accuracy and virtually no interference from pharmaceutical excipients.

KEYWORDS: Artesunate, Counterfeit, Malaria, Pharmaceutical, Spectrophotometry.

INTRODUCTION

Malaria continues to be a major global public health problem. It is still the number one killer among all parasitic diseases in sub-Saharan Africa. It has been estimated that nearly half of the world's population (34.5 billion) are at risk of malaria infection .[1] WHO as at December 2014 update declared that in 2013 alone there was 198 million malaria cases and estimated deaths of 584,000 from the disease.[2] People living in the poorest countries are the most vulnerable. In 2013 90% of all malaria death occurred in WHO African Region, mostly among children below the age of 5 years, pregnant women and people living with HIV. The fight against malaria has been a long and bitter one because of the ease with which the parasitic *plasmodium sp* developed resistance to anti-malarial drugs. First line drugs for the treatment of malaria especially chloroquine, sulphadoxime /pyrinthamine (Fansidar) are no longer effective. The World Health Organization and authorities in malaria endemic countries base on the effectiveness of Artemsimin and its derivative recommended treatment based on the Artemisimin combination therapy.(ACT).[3],[4],[5] Unfortunately Artemsimin resistance is now emerging in the greater Mekong, the same sub region where resistance emerged for all previous, frontline treatments, including chloroquine and sulphadoxime – pyumethamine (Fansidar). There are currently no available replacement drugs for a Artemsimin.[6],[7],[8] The emerging resistance is due partly to the administration of sub therapeutic doses of Artemsimin based drugs to unsuspecting patients. Recently poor quality anti-malaria drug has been reported in Southeast Asia and sub-Saharan Africa.[9],[10],[11][12],[13] Poor quality anti-malaria drugs lead to resistance and inadequate treatment pose a serious threat to vulnerable populations putting the progress made so far and investments in combating malaria in jeopardy. About 100,000 deaths a year in Africa are linked to the counterfeit drug fade.[14] WHO defined counterfeit medicine as one which is deliberately and fraudulently mislabeled with respect to identity and source; both branded and generic products are faked. In counterfeit products, there non or subtherpentic quantities of Artemsimin derivatives and sometimes curtain potentially dangerous substances such as metamizole, melamine and sofrole.[15] Counterfeiters are very sophisticated they produce identical holograms, batch numbers, expiry dates, blisters and tablets looking absolutely genuine, making detection of

fake drugs particularly difficult. Their packaging is always a perfect copy and their quality cannot be assessed readily by lay persons or even experts of pharmaceutical industry without the aid of quality testing laboratory. These obviously require laboratories with high level of technical facilities which are rarely available in developing countries[15], hence the development of this simple low cost spectrphrtemetric method. Of all available artemisinin derivatives, artesunate has the most favourable pharmalogical profile for use an ACT, partner for the treatment of uncomplicated malaria.[16] This makes it a perfect candidate for outright faking or counterfeiting.

The international pharmacopoeia prescribes titration or HPLC for the assay of artesunate.[17] Some workers have developed some methods for assay of artesunrate[18],[19],[20] This proposed method is based on the generation of hydrogen peroxide from artesunate, by the cleaving of the endpoxide bond of artesunate. The hydrogen peroxide generated insitu now oxidizes Iron II to Iron III. The ferric III generated then form a blood red complex with thiocynanate which is spectrophotometrically determined at λmax 480.

EXPERIMENTAL

Apparatus

All spectral measurement were carried out using a Hey los β Uv-visible, spectrophotometer, Thermo electron corp. U.S.A. with 1cm quartz cell.
All pH measurement was done by (Pw 9 x 20 pH meter Philips – England.

CHEMICAL AND REAGENTS

All chemical and regents were Analar grade

1. Ferrous ammonium sulphate (Merck, Darmstadt Germany). A 0.08M solution of FAS was prepared by dissolving 31.36g of the substance in 5ml of dilute of hydrochloric acid (0.1M) and made up for 1 litre of distilled water.
2. Ammonium thiocyanate (3M). This solution was prepared by dissolving 3.45grams of the chemical (B. D. H England) in distilled water and made up to 100ml with the same distilled water.
3. Hydrochloric Acid (Sigma). A 2M solution of this acid was prepared by diluting the concentrated acid (Sp. Gr. 1.18) appropriately with distilled water.

PURE ARTESUNATE SAMPLE

Pharmaceutical grade artesunate was donated by the directorate of pharmaceutical services University of Uyo, Teaching Hospital, Uyo; as a kind gift. The drug was used as given.

A standard stock solution of artesunate containing 200µg/ml was prepared by weighing accurately and dissolving 20mg of artesunate in 100ml of ethanol. This stock solution was further diluted to 50µg/ml for the analytical delimitations.

EXPERIMENTAL PROCEDURE

Different aliquots (0.25, 0.50, to 5 ml) containing 50µg/ml of the standard artesunate solution were accurately transferred into a series of 10ml calibrated volumetric flasks using a microburette. The total volume in the flasks were adjusted to 5ml using absolute ethanol. The content of the flask was acidified with 1ml of 2M hydrochloric acid and shaken gently; followed by the addition of 1ml of 0.08M solution of (FAS) ferrous Ammonium sulphate. The resulting mixture was swirled gently and allowed to stand for 10mins.

Then 1ml of 3M Ammonium thiocyanate solution was added to each of the flasks and shaken to mix well. The resulting mixture was made up to the 10ml mark with with absolute ethanol and the absorbance was measured at 480nm; against absolute ethanol as blank. A calibration curve was generated by plotting the absorbance as a function of the artesunate concentration. The concentration of the unknown was deduced from the calibration curve or computed from the regression equation obtained from Beer's law data.

PROCEDURE FOR TABLETS

Twenty tablets of lever artesunate were weighed and powdered using a ceramic motar and pestle. A quantity of the powder equivalent to 100mg of the drug artesunate was weighed and transferred into a 100ml volumetric flank containing 30ml of absolute ethanol and sonicated for 10minutes a further 50ml of absolute ethanol was added and shaken vigorously to extract the drug. Then the content was made up to the 100ml mark and filtered using Whatman filter paper No. 42 the first 10ml of the filterate was discarded. The concentration of the artesunate solution was 1mg/ml. This was diluted stepwise to a working concentration of 100µg/m1 from where a convenient aliquot was analyzed using the experimental procedure discussed above.

EXPERIMENTAL PROCEDURE FOR PLACEBO BLANK

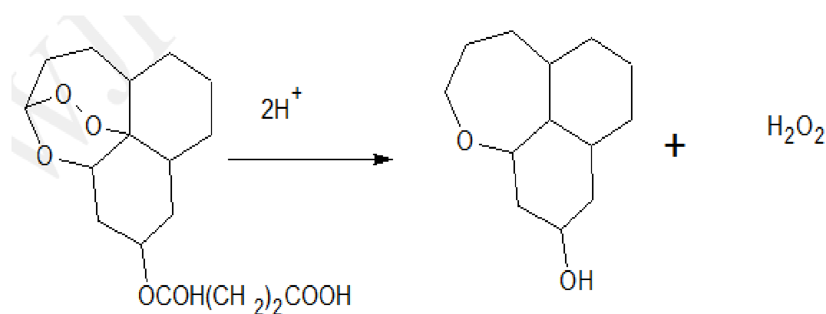
A placebo powder containing (Pharmaceutical excipients) talc, 4mg, magnesium stearate 0.2mg, lactose 12.8mg microcrystalline cellulose 15mg acacia 20mg and bulk up with maize starch to make up 100mg. This was mixed to form a homogeneous mixture and 20mg was carefully weighed and, dissolved in 20ml of water and sonicated for 15miniute and filtered. The resulting placebo blank was analyzed exactly following the procedure for tablets as discussed above.

PROCEDURE FOR THE ANALYSIS OF SYNTHETIC MIXTURE

A synthetic mixture was prepared by measuring 100mg of pure artesunate and 100mg of the placebo powder as prepared above. The two where homogenized and 100mg of the mixture was carefully transfered into 100ml volumetric flask containing 50ml of absolute ethanol and sonicated for 20minutes with intermittent shaking. The content of the flask was made up to 100 mark of the flask with the same etanol and filtered. Ten (10) ml of the filtrate was discarded. The resulting synthetic drug solution was further diluted to obtain a suitable working concentration and then analyzed as described in the experimental procedure above.

RESULTS AND DISCUSSION

The proposed method is based on the generation of hydrogen peroxide from the cleavage of the Endoperoxide Bridge of artesunate in acid medium (Sheme1). The hydrogen peroxide subsequently oxidize Iron II to Iron III which complexes with thiocyanate ion forming a red coloured complex which absorbs the uv-vis light maximally at 480nm. The reaction is a typical diagnostic reaction for the detetion of Iron III. This reaction has been used for the determination of some pharmaceuticals.



Stucture of Artesunate

Scheme 1: for the generation of Hydrogen Peroxide in situ from Artesunate in Acid medium

OPTIMIZATION OF REACTION CONDITIONS

The experimental conditions for the complete oxidation and the eventual complex formation of ferric thiocyurnate complex were carefully studied and optimized. This was done by keeping all other factor or variables constant while varying the particular variable under study and observing its effect on the absorbance.

Type of acid and thiocyanate reagent

The reaction was performed under acid medium to prevent iron (III) hydrolysis and to cause the protanation of the two oxygen atoms in the endoperoxide bond of artesunate. Based on this demand the effect of the following acid was studied perchloric acid ($HCLO_4$), sulphric acid, H_2SO_4, hydrochloric acid (HCI), Nitric acid (HNO_3) and acetic acid. HAc the results were very close for all the acids used but HCI was used because it gave a better result, 1ml of 2M HCI was adequate for a total of 10ml of the whole reagent. Futher increase gave high absorbance but with erratic results. To achieve rapid oxidation and eventual complexation three common thiocyanate salts were also investigated alongside the acid type used. The three thiocyanate salts studied include NaSCN, KSCN and NH_4SCN. This study was conducted by reacting 5ml to 5µg/ml artesunate with 1ml of 2M HCI, and 1m of 0.08M FAS and each of the thiocyanate salt in turn and the absorbance measured. It was discovered that at this condition Ammonium thiocyanate gave the most favourable result. Hence 1ml of 3M NlH_4SCN was used for the complexation reaction. At higher pH the colour intensity decreased because of the hydrolyses iron that was taken place.

Effect of Ferrous Ammonium Sulphate

The effect of the variation on the concentration of FAS on the absorbance of the complex was determined by varying the concentration of FAS added while keeping the concentration of the ammonium thiocyanate, HCI and the pH constant, 1ml 0.08M, 1ml of 2M and 2.5) respectively it was found that 1ml of 0.08m of FAS was adequate for the formation of stable complex.

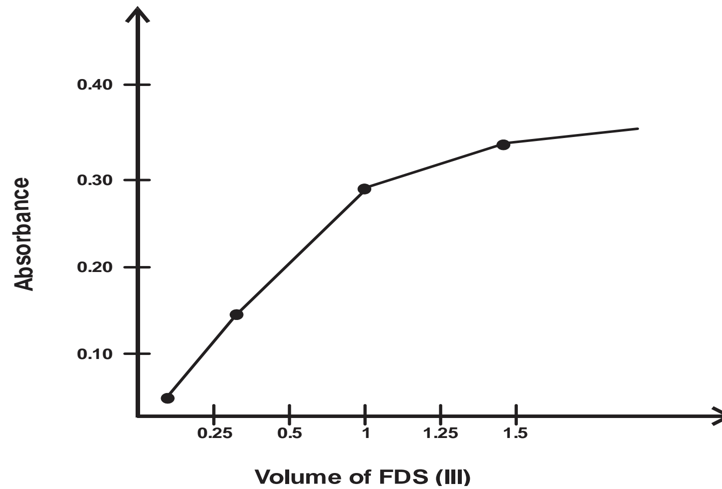


Figure 1: Effect of concentration of FAS on the absorbance of the thiocyanate Ferric thiocyanate complex formed (stock solution of 0.08 M FAS).

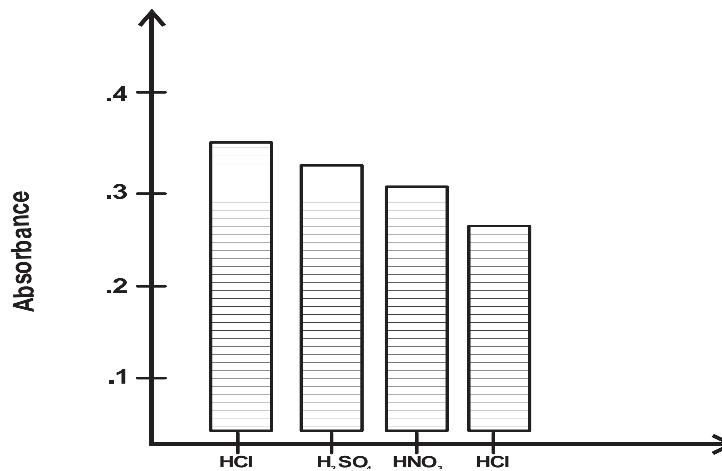


Figure 2: Variation of absorbance of 5µg/m1 of drug with,

(a) 1ml of 2M HCI (b) 1ml of 2M H_2SO_4 (c) 1ml of 2M HNO_3 and (d) 1ml of 2M HAc and 1ml of 0.08 FAS and 1ml of 3M Ammonium sulphate.

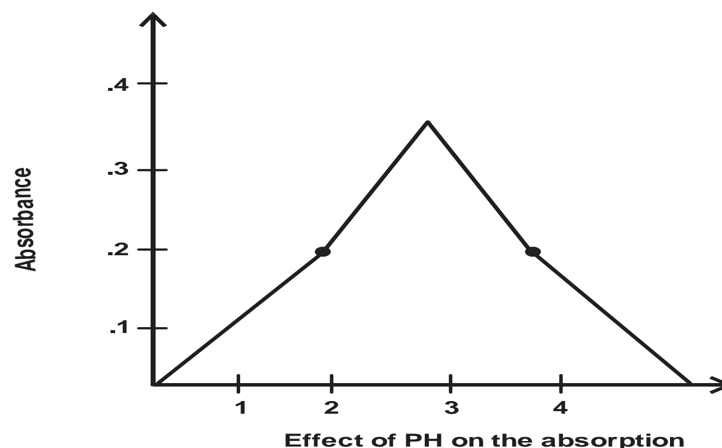


Figure 3: Effect of pH of the medium on the Absorbance of the formed complex.

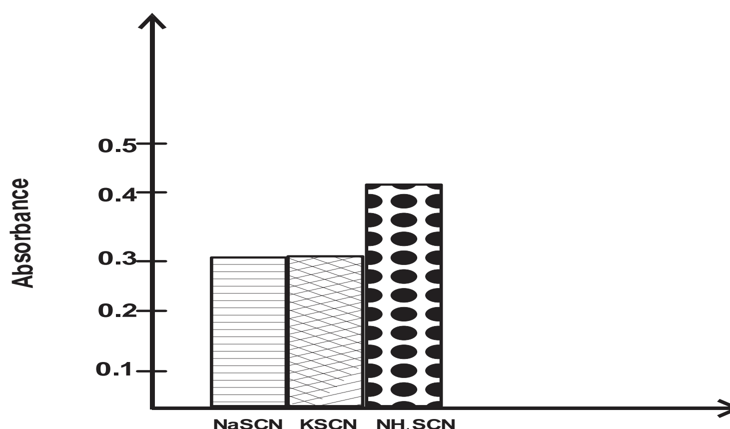


Figure 4: Variation of absorbance after reaction of 5ug/ml of Artesunate with 1ml of 2ml HCI, 1m of 0.08m Ferric Ammonium sulphate and (a) 1mlg of 3m NasCN, (b) 1ml of 3m KSCN and (c) 1ml of 3m NH4SCN.

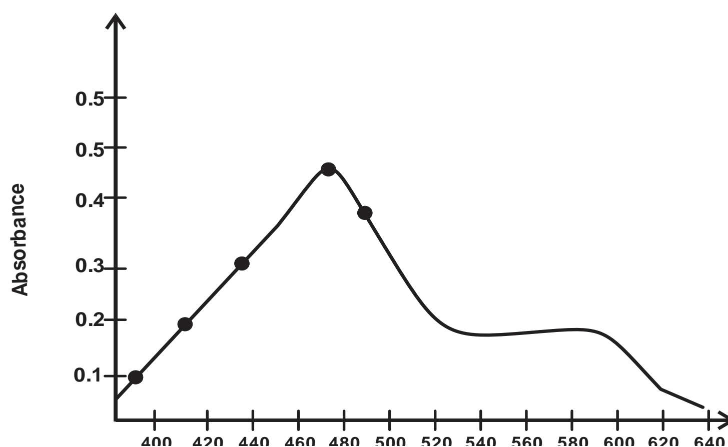
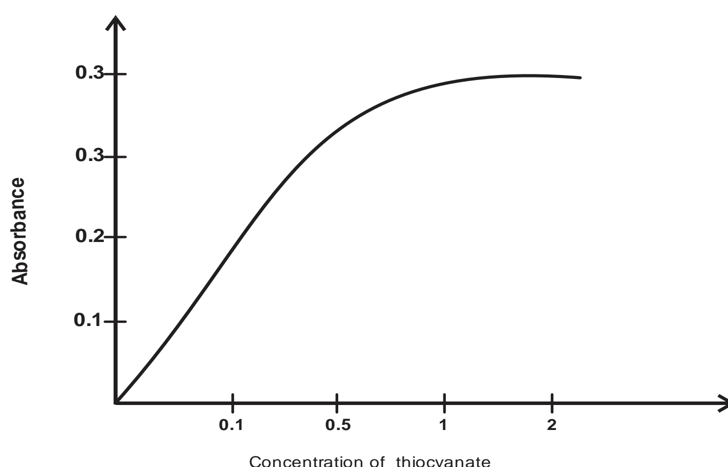


Figure 5: Absorption spectra of 5mg/ml of artesunate using the proposed method



Effect of thiocyanate on the absorbance

Figure 6: Variation of absorbance after reaction of 5µg/ml of Artesunate with 1ml of 2M HCI, 1ml of 0.08M Ferric Ammonium sulphate and (a) 1ml of 3M NaSCN, (b) 1ml of 3M KSCN and (c) 1ml of 3M NH_4SCN.

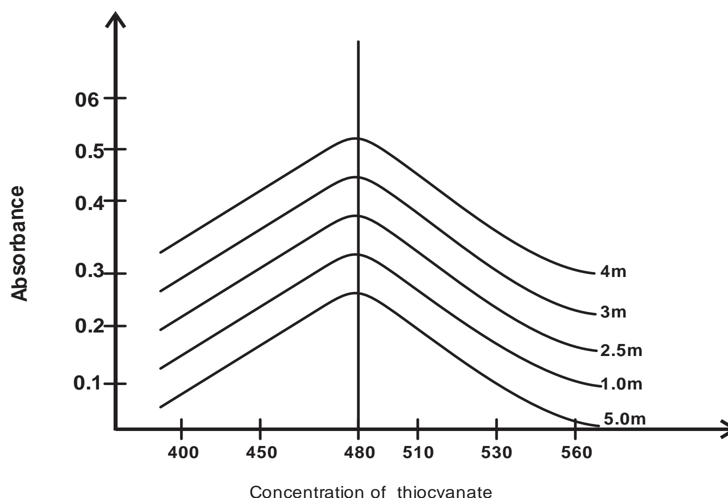


Figure 7: Effect of thiocynate volume added on the absorbance of the Ferric thiocynate complex formed.

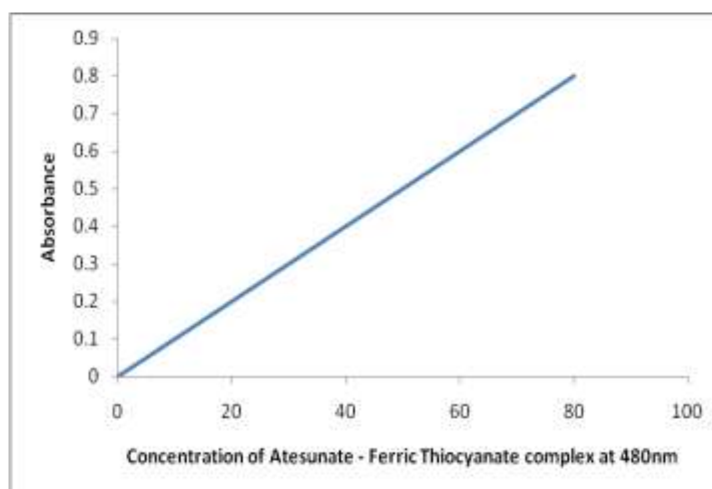


Figure: 8: Calibration curve of Absorbance Vs Artesunate – Ferric-Thiocyanate complex at 480nm.

EFFECT OF TEMPERATURE

Though rate of reaction are increased by increase in temperature. There was no significant increase in oxidation of Iron II to III at higher temperatures and the consequent complexation. Therefore the reaction was performed at room temperature.

STABILITY OF THE COMPLEX FORMED

Under optimum experimental condition at 25°C (room temperature) 1) and normal daylight the stability of the formed. Artesunate - Ferric - thiocynate complex was studied using distilled water, methanol and ethanol. Samples for the analysis were prepared by taking 5µg/ml of the drug + 1ml, 2M HCI + 1ml 0.08M FAS and 1ml 3M Ammonium thiocynate in

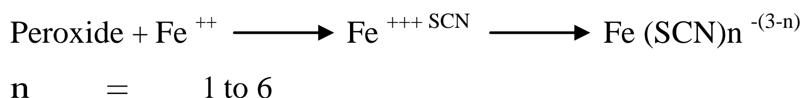
distilled water, ethanol, methanol, respectively, and observing the absorbance change per time at specific time of 10 min, 20 mins, 30 mins, 50 mins, 60 mins - 360 mins using distilled water, methanol and absolute ethanol respectively. The stability of the formed complex was more stable in both methanol and absolute ethanol almost in equal measure than distilled water. Hence absolute ethanol used made. The Drug-Ferric-Ammonium complex formed was very stable for over 6 hours. Other organic solvent such as chloroform and acetone were not used because of cost.

Order of Addition of Reagents

This was studied by changing the order of addition of reactants and then observing the absorbance. It was observed that the order of addition. Artesunate + HCl + F AS + SCN was more suitable with the best absorbance.

STOICHIOMETRY

In the presence of excess ammonium thiocynate the stoichiometry of Fe 3+ and Artesunate are determined by Jobs method of continuous variation. Various proportions of the Artesunate and F AS solutions were mixed maintaining the total volume (l0m1) of the reactant, with other species remaining constant. The absorbance was measured at optimal conditions. The result showed (1 : 1) Artesunate: Fe ratio complex formed.



n = 1 to 6

The mole ratio of the Artesunate and the thiocyanate ion was also determined using the Job's method; by varying the concentration of Artesunate and Ammonium thiocyanate within a constant volume (10ml) of reactants. The result showed 4: 1 ratio for SCN- and Artesunate.

Table 2: Stability of the formed complex as a function of time in distilled water, absolute ethanol and menthol respectively.

	5Min	10	20	30	50	60Min	100Min	180Min	360Min
Distilled water	0.372	0.372	0.372	0.392	0.392	0.392	0.396	0.398	0.399
Absolute ethanol	0.390					0.380	0.790	0.793	0.796
Methanol	0.391				.			0796	0.41

Order of Addition

Order	Reagent	Absorbance
1	Drug + HCl + FAS + SCN	0.3960
2	HCl + FAS + Drug + SCN	0.3050
3	HC1 + FAS + SCN + Drug	0.2110
4	FAS + HCl + Serv + Drug	0.218
5	F AS + SCN + HCl + Drug	0.211

METHOD VALIDATION

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

LINEARITY AND SENSITIVITY

The standard calibration curve generated by plotting absorbance vs artesunate concentration showed a linear correlation. Beer's law was obeyed in the range of 0.4- 70 µg/ml. Regression analysis of Bear's Law data was carried out using the least square method to evaluate slope, intercept and the correlation co-efficient; the value obtain are recorded in table 5. The regression equation was in the form of y = mx + C. where y represent the absorbance, m the gradient (slope) and C the intercept which was negligible. The sensitivity parameters such as molar absorptivity

Sandell sensitivity, limit of detection (LOD) and limit of qualification (LOQ) where determined as per the current ICH guidelines using the formular.

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

Where σ is the standard deviation of five blank determination and S representing the slope of the calibration graph. These parameters are recorded in table 6 as shown below:

Table 6: Analytical and regression Parameters of proposed method

PARAMETER	
max	480nm
Bears law limit graph µg/ml	0.4-70
Molar absorptivity L ml-l cm-l	1.08 x 104
Sandell sensitivity µg *Icm*	0.03559
Limit of detection (LOD) µg *Iml*	0.15
Limit of Quantification (LOD) µg *Iml*	1.20
Regression equation	Y = m x + c (Y = 0.0113 r + 0.0009)
Slope	0.0113
Intercept	0.0009
Coefficient of correlation (r)	0.9998

SELECTIVITY AND INTERFERENCES

The selectivity of this method was evaluated by preparing and analyzing the placebo blank and synthetic mixture containing pure artesunate as described earlier. There was no difference between the absorbance of the placebo blank and the reagent blank showing no interference from the excipients used. The result for the synthetic mixture as assayed by the proposed method gave a mean percentage recovery of 99.945 (n=5) with relative standard deviation (% RSD) 2.02%. Showing that the method was highly selective.

In the process of the interference study some anions and cations were individual used to test their effect on the absorbance of specific aliquots prepared and analysed at 480nm. Under the same optimum conditions. The anions and cations studied were SO_4^{2-}, NO_3^- OH, ClO^- and K^+, NH_4^+ , Ca^{2+}, Zn^2+. When added individually to specific aliquot up to 1: 1 ratio showed no significant difference in absorbance. When the ratio was increased to 1 :2. Analyte aliquot: Cation or anion; the error was less than 3.0% showing that the anions and cations analysed had no significant effect on the proposed method.

ACCURACY AND PRECISION

The accuracy and precision of this proposed method was evaluated by preparing solutions containing three concentrations of pure artesunate and analysed in five replicate determinations. The accuracy was determined as percentage relative error calculated using the formular.

$$Er\% = \left\{ \frac{\text{Amount found} - \text{Amount added}}{\text{Amount Added}} \times \frac{100}{1} \right\}$$

The precision was determined as percentage relative standard deviation RSD%. The accuracy and precision where performed three time within a day (intra day) and for three consecutive days (inter day) within 7 days the results are shown in table 7.

SIN	AMOUNT	ACT	INTRADAY	ACCURACY		INTER DAY ACCURACY				
	ADDED (UG/ML)		PRECISION							
			AMOUNT	RE	RSD	AMOUNT	OF	PU	RE%	RSD%
			FOUND			ART FOUND				
1	30		30.88	2.93	2.90	30.86			2.78	1.90
2	60		60.98	1.63	1.15	61.02			1.70	1.20
3	100		102.00	2.00	1.40	102.98			2.98	2.09

ROBUSTNEES AND RUGGEDNESS

The proponed method was evaluated for robustness by deliberate minor variations in the concentration of HCI used, concentration of FAS and the reaction time and effects on the

absorbance evaluated. The ruggedness was evaluated by carrying out the analysis in three different machines by three analysts. Both the robustness and ruggedness were evaluated at three different artesunate concentration levels and the precision determined (RSD %)

Table 7: Results for Robustness and Ruggedness.

ROBUSTNESS (RSD)				RUGGEDNESS (RSD)		
Amount of Art Studied	HCL VOLUME (n=3)	Reaction Time (n=3)	FAS Concentration (n=3)	Amount of ART Studied	Inter Instruments (n=3)	Inter Analyst (n=3)
30	1.99	2.01	1.53	30	2.13	2.60
60	1.50	1.84	1.62	60	1.65	1.54
100	1.73	1.62	.176	1.00	2.87	1.98

HCI acid concentration of lM, 1.5M, 2.5M were used. Standing reaction time of 5min, 8mins, and 10mins.

FAS concentration of O.06M, 0.07M, 0.09M was used

Application of the method to analysis of artesunate in tablets.

The proposed method was successfully used to analyze artesunate in commercial tablet brands procured in Uyo, South South Nigeria the results from this was statistically compared with the international pharmacopeial titrimetic method (2005) for artesunate using student's t-test and the variance ratio F-test the calculated t and F values were below the theoretical tabulated values of t = 2.77 and F = 6.39 at 95 confidence level and at 4 degrees of freedom. The proposed method showed some congruence with the pharmacopoeial method as there was no significant difference between the results of both methods (table 8).

Table 9: Result of artesunate table using the proponed method

COMMERCIAL TABLET ANALYSED	LABEL CLAIM mg	FOUND (PERCENT LABEL CLAIM ± SD) REFERENCE METHOD	PROPOSED METHOD
Artsunate (Jubilee) Analysed	50	99.80± 1.18	100.0 ± 0.84
			F = 1.97, t = 1.01
Arinate (Reals)	50	99.92 + 1.15	100.0 ± 0.58
			F = 2.86, t = 1.22
Arsumax (Sanafi)	50	99.95 + 1.25	100.09 ± 0.89
			F = 1.97, t = 1.23
Artesunat (News)	50	99.85 ± 1.16	100.22 ± 0.83
			F=1.95t=361.41
Askasunate (Dcam remedies)	50	99.85 ± 1.28	100.32 ± 1.01
			F = 1.61, t = 1.41
Articin (Embrany)	50	99.90 ± 1.26	100.10 ± 0.86
			F = 2.15, t = 1.50

Arthlon (Goldmoaro)	50	99.90 ± 1.34				100.21 ± 0.82
						F = 2.67, t = 1.15
Gsunate	50	99.89 ± 1.38				100.20 ± 1.01
						F = 1.87, t = 0.81
Leverartesunate (Geneith)	50	99.92 ± 1.36				100.28 ± 0.87
						F = 2.44, t = 141
Malmeter (Evans)	50	99.92 ± 1.38				100.25 ± 0.83
						F = 2.76, t = 1.22

Table Results of the Recovery studies by standard addition method

S/N	TABLET STUDIED	AMOUNT OF DUNG (UG/ML)	AMOUNT OF PURE ARTESUNATE POWDER ADDED (CG/ML)	TOTAL AMOUNT FOUND (MG/ML)	RECNERY PF PURE DUNG ±SD.
1	Artesunate (Jubilee)	30.00 30.00 30.00	20.00 40.00 60.00	50.65 71.00 91.80	103.0 ± 1.00 103.0 ± 0.76 101.6 ± 1.15
2	Arinate (Reals)	40.00 40.00 40.00	20.00 40.00 60.00	60.20 80.50 101.00	101.0 ± 1.41 103.0 ± 0.76 10 1.6± 1.46
3	Arsumax (Sanafi)	40.20 40.20 40.20	20.00 40.00 60.00	60.00 40.00 60.00	100.5 ± 1.16 102.0 ± 1.20 101.3 ± 1.08
4	Artesunat (Neros)	41.00 41.00 41.00	20.00 40.00 60.00	61.30 81.00 101.90	101.5 ± 1.90 100.2± 1.12 101.5 ± 1.08
5	Articin (Embrany)	42.00 42.00 42.00	20.00 40.00 60.00	62.1 83.00 102.50	100.5 ± 0.81 102.5 ± 0.92 100.8 ± 1.15
6	Lever Artesunate (Henieth)	50.10 50.10 50.10	20.00 40.00 60.00	70.08 91.00 111.00	99.9 ± .21 102.2 ± 1.46 101.5 ± 1.12
7	Gsunate (Greenlife)	52 52 52	20.00 40.00 60.00	72.28 91.91 113.00	101.4±1.27 99.9 ± 1.17 10 1.6± 1.15

Mean value of three determinations

RECOVERY STUDIES

Recovery studies was carried out via standard addition procedure to confirm the applicability and accuracy of the proposed method. Pure artesunate powder at three different concentration level with added to per-analyzed tablet powder and the total amount was determined using the purposed method. The **** showed excellent **** of (> 99.9:1.13) meaning that usual pharmaceutical ingredient had no effect on the results generated by this method. Seven local bunds of artesunate tablets were used for the recovery study for table.

CONCLUSION

A new spectphotometric method is developed here for the assay of artesunate tablets. The method is sensitive and economical requiring no extra cost as regards heating no extensive chemical extraction. The reagents are eco-friently the analyst faces no danger as the reagents are non carcinogenic and has no hazardous effect on the environment. The method is also very simple it has a reasonable linearity range it is reproducible. Common pharmaceutical excipients have no effect on the capacity of the method for the use in analyses of artesunate in routine laboratories and in the field, especially in developing counties where more sophisticated analytical equipment are hardly available.

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