Novel kinetic spectrophotometric determination of Artesunate using iodide/iodate mixture

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ABSTRACT

Background: Plant materials has been in use globally for medical reasons with little or no knowledge of phytochemicals therein present and often times no scientific basis.

Methods: A simple kinetic spectrophotometric determination of artesunate is develop. Four kinetic methods were evaluated, the initial rate method, the fixed time method, variable time method and the rate constant method. This kinetic spectrophotometric method was based on the redox reaction of artesunate with the iodate/iodide mixture; in acid condition hydrogen peroxide is generated in situ from the artesunate which then reacts with iodate/iodide mixture.

Results: The whole four kinetic methods tested all obeyed Beer's law and were quantitative. The initial rate and the fixed time methods were most sensitive. The calibration curve generated through the least square method were linear and the range was 20 – 70 µg/ml and 10 - 70µg/ml with correlation Coefficient of 0.9998 and 0.9997 respectively, the sensitivity of both kinetic methods were tested as per the ICH guidelines the limit of detection was 0.022µg/ml, 0.182µg/ml respectively. The accuracy and precision of both kinetic methods was determined as R.E% and RSD% which in all cases were $< 2.8\%$. The methods were statistically tested by comparing them with values from a pharmacopoeia standard via students T and variance ratio test (t and F) and was discovered that there were no significant difference in the results obtained.

Conclusion: The applicability of the methods was ascertained by standard addition method and was find that pharmaceutical excipients have no effect on the overall result of the developed methods.

Keywords: *Artesunate, Counterfeit, Kinetic spectrophotometry, Malaria, Redox reaction*

1. INTRODUCTION

The fight against malarial could be in jeopardy if the manufacture and distribution of fake/counterfeit artemisinin derivative is not stopped. The fight against this trade has to be frontal. The weak legislation against offenders by poor third world countries has worsen the already precarious situation. Daily sub-therapeutic, substandard and sometimes fake artemisinin are being imported to sub-Saharan African. Since there is treatment failure, the patient is beginning to lose confidence on our healthcare system. With sub-therapeutic doses malaria parasite will develop resistance to the use of these artemisinin derivatives for the treatment of malaria. If this situation is not controlled or stopped, we may be witnessing the emergence of untreatable malaria in these endemic countries which may likely precipitate serious public health crisis. Artesunate and Dihydroartemisinin are the two frontline drugs for the treatment of uncomplicated malaria at least in combination with other antimalarials. There have been reported cases of fake/counterfeit in Southeast Asia and Sub-Sahara Africa [1, 2, 3, 4, 5, 6]. To end these trade simple, reproducible and sensitive method for the determination of artesunate and dihydroartemisinin in pharmaceutical formulation must be developed. Many methods have been developed for the determination of these artemisinin derivatives have been developed these include: [7, 8, 9 10, 10, 11]. Most of these methods are very sensitive reproducible and quite effective but some of the methods have some obvious short comings which may include; tedious solvent extraction process, tight pH control etc. That apart very expensive highly technical analytical equipment which may not be affordable or readily available in these poor sub-Saharan countries has made the development of simple methods inevitable. This kinetic spectrophotometric method is sensitive, reproducible, selective, quantitative and easily affordable. The reagents are available, inexpensive and pose no hazardous effect on the environment and the analyst. The method is based on the redox reaction between the artemisinin derivative and the iodide/iodate mixture. Under acid condition, the endoperoxide bond in the arteminsinin derivative is cleaved, Hydrogen peroxide in generation in situ, the hydrogen peroxide then reacts with the iodide/iodate mixture leading to release of iodine which is then monitored and measured kinetically using Uv spectrophotometer.

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2. MATERIALS AND METHODS

2.1. Materials

2.1.1 Reagents

All reagents used were analytical grade with excellent shelf life. Potassium Iodide – A solution of 0.25M KI (Merck, Damstadt, Germany) was prepared freshly using distilled water and standardized iodometrically using standardized sodium thiosulphate. Potassium Iodate - A Solution of 0.10M KIO3 (Sigma, Germany) was freshly prepared using distilled water. Pure Artesunate powder was presented as a kind gift by the director of Pharmaceutical service University of Uyo Teaching Hospital and was used as provided. A standard artesunate solution of 1.0 mg/ml was prepared by disolving the 100 mg of artesunate powder in 100 ml of distilled water. This solution was further diluted stepwise to obtain a working concentration of 100 µg/ml for the preparation of the calibration curve.

2.1.2 Equipment

All spectral and absorbance measurements were carried out using Shimadzu UV – Visible Spectrophotometer 1601 Kyoto, Japan.

2.1.3 Biological materials

Female Wistar rats, *Ocimum gratissimum* leaf

2.2. Methods [8, 9, 10]

2.2.1 Initial Rate Method

Different aliquots of 0.2 – 1.6 ml of the standard artesunate (100 ug/mg) were carefully transferred into a series of 10 ml standard volumetric flasks using micro burette. Then 2.0 ml of 0.1M KI03 was added to the flask followed by the addition of 4.0 ml of 0.25M of KI. The system was acidified using 1ml of 1.0M of sulphuric acid and shaken to mix well and allowed to stand for 2 minutes at room temperature of 25+ 10c, thereafter the contents of the flask was shaken gently to mix well. The resulting solution was carefully transferred to the spectrophotometric cell and the rate of change of absorbance with time was measured at 350 nm against reaction blank similarly prepared without the artesunate. The initial rate (V) of this reaction was obtained from the tangent of the absorbance time slope. A calibration curve was generated by plotting the Logarithm of the initial rate against the Logarithm of the molar construction (Log C). The unknown drug concentration was determined from the calibration curve or from the regression equation .

2.2.2 Fixed Time Method

In this method the changes in absorbance caused by the effect of the Iodate/Iodide mixture on each of the drug (artesunate) solution at 350nm was recorded at the preselected fixed time of 2min intervals against reagent blank prepared similarly but without the drug. A calibration curve was generated by plotting the change in absorbance (DA) the initial concentration of the drug. The amount of the drug per sample was determined using the calibration curve or regression equation.

2.2.3 Procedure for the determination of Artesunate in Tablets

Twenty (20) tablets of artesunate tablets were randomly selected and weighed individually to establish weight uniformity. The tablets were pulverized using ceramic mortar and pestle. A quantity of the powder equivalent to 100 mg were weighed and transferred with a 100 ml capacity volumetric flask containing about 20 ml of water. This mixture was shaken vigorously and sonicated for 5 min; the volume in the volumetric flask was further increased to 80 ml and shaken vigorously to extract the drug. Finally the volume was made up to the 100 ml mark of the volumetric flask and filtered using Watman filter paper No.42. The first 10 ml portion of the mixture was discarded. The resulting mixture with the concentration of 1 mg/ml was further diluted to obtain a working concentration of 100 ug/ml from where a suitable aliquot was analyzed.

2.2.4 Procedure for Placebo Bank

A placebo blank was prepared using some pharmaceutical excipients normally used in formulation of tablet. The composition was talc 2 mg, magnesium stearate 4 mg, lactose 12.5 mg, acacia 20 mg, microcrystalline cellulose 15 mg. this was bulked up using corn starch, homoginized and mixed thoroughly to a homogenous powder mass. Then 20 mg of the mixture was carefully weighed out and dissolved in 20 ml of water and sonicate for a bout 20 mins and filtered. The resulting placebo blank solution was then analyzed using the recommended general procedure described above.

2.2.5 Procedure for the Analysis of Synthetic Mixture

In order to prepare a synthetic mixture 100 mg of pure artesunate powder and 100 mg of the placebo blank powder as prepared above were separately weighed and mixed together. The resulting two powder mixtures were homogenized to mix well. Then 100 mg of the mixture was carefully transferred into a 100 ml capacity volumetric flask containing 20 ml of distilled water and sonicate for 15 mins with intermittent agitation. The content of the flask was diluted and made up to 100 ml mark of the flask with distilled water and filtered discarding the first 10 ml of the filtrate. The resulting synthetic drug solution was further diluted. A suitable working concentration of this was obtained and analysed as described in the recommended procedure discussed above.

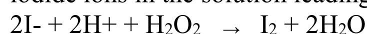
2.3 Data Analysis

The statistical analysis was done using SPSS software version 21. Descriptive statistics and graphical representations were used to describe and represent variables. Independent t-test was used to compare differences in mean between two groups while one way ANOVA was used to compare differences in mean between more than two groups. The level of statistical difference was set at $p < 0.05$

3.0 RESULTS AND DISCUSSION

Artesunate and dihydroartemisinin lack isolated covalently unsaturated group that show characteristic absorption in the UV-VIS region. The presence of the endoperoxide bond in the artesunate molecule is the source of hydrogen peroxide which reacts with the iodide/iodate mixture. In acid medium the oxygen centres of the endoperoxide bond of the artesunate are protonated leading to the cleavage of the endoperoxide bond leading to the generation of hydrogen peroxide in situ.

The hydrogen peroxide generated in situ then reacts with the iodide/iodate mixture practically oxidizing the iodide ions in the solution leading to the equation



$2I^- + 2H^+ + H_2O_2 \rightarrow I_2 + 2H_2O$

This reaction is dependent on the concentration of the hydrogen peroxide (in this case artesunate), the iodide ions (in this case the iodide/iodate mixture) the reaction is also time dependent.

The rate = $K \{I^-\}^q \{H_2O_2\}^2 \{H+\}^e$

Rate = $K^1 \{I^-\} \{H_2O_2\}$. Where $K^1 = K \{H^+\}^e$

Hence the basis for the kinetic spectrophotometry at 350 nm > max.

Optimization

The experimental factors affecting this redox reaction of hydrogen peroxide (in this case Artesunate) and the iodide (in this case the iodide/iodate) mixture were carefully studied and optimized. These parameters/factors include:

1*) Effect of Temperature*:- It is a fact that generally the rate of reaction increase with temperature ; increase in temperature in this case gave very erratic results because of instability of iodide with increased temperature degradation of artesunate is likely at increased temperatures. Since the reaction was spontaneous at room temperature. The experiment was performed at room temperature $25+5^0C$.

2) *Effect of Potassium Iodide Concentration*: - The effect of the volume of 0.25M KI was studied between 1 – 5 ml. It was discovered that the absorbance and the rate of reaction increased with increase in the volume of the KI, beyond 4.5mls the results obtained became inconsistent and erratic. Therefore 4ml of KI was suitable for 10ml reacting volume.

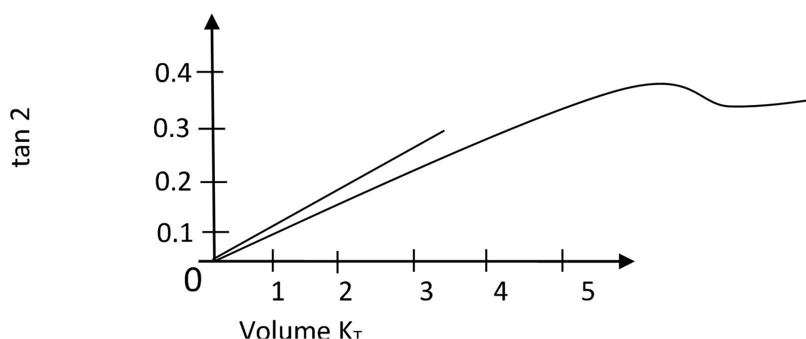


Fig. 1: Effect of Concentration of K_T on the rate of reaction

3). *Effect of Potassium Iodate*: - The effect of potassium iodate was studied using 100 ug of artesunate to react with 4.0ml of KI and then reacting this with varying volume of KIO_3 in the rang of 0.5 – 3.5ml. It was observed that the absorbance and the rate of reaction increased with increasing reaction of KIO_3 beyond 2.5 ml the result was no longer quantitative. The kinetic tangent of the absorbance give curve (tanØ =dA/dt) at different volumes of KIO_3 showed increase rate of reaction on the volumes increased but beyond 2.5ml no further increase was observed.

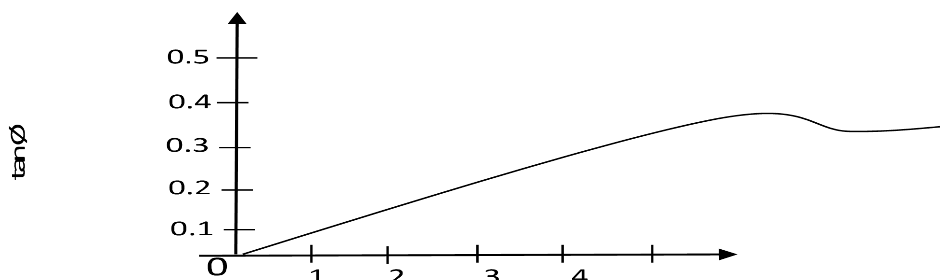


Fig. 2: Effect K_TO_2 on the rate of reaction

4). Effect of the acid used: - The different acids were used hydrochloric acid, sulphuric acid and ethanoic acid. Sulphuric acid and hydrochloric acid produced the best results. Ethanoic acid showed results that were not consistent. Nitric acid was not used because of its powerful oxidizing properties.

Quantitative Methods

Although four analytical methods were tested which include initial rate, fixed time, variable time and rate constant method. The sensitivity and correlation coefficient was used to chore the most appropriate of the analytic methods.

Initial Rate Method

Under optimum experimental condition as discussed earlier, the assay of artesunate was carried out in the presence of excess iodate/iodide concentration with respect to the artesunate concentration in aqueous medium at room temperature (25 ± 5^0C) at λmax 350nm. With respect to the KIO_3/KI concentration a Pseudo zero order of reaction was worked out. The order of reaction with respect to artesunate was determined by plotting the logarithm of the initial rate of the reaction vs. the logarithm of the molar concentration of artesunate. The initial rate followed a pseudo first order and obeyed the rate equation

Rate = [A = K^1C ….. (1)

V = DA/Dt = K^1C^n … (2)

Where V – Initial Rate of reaction. A is the absorbance, t is the time (secs) K^1 is the Pseudo- first order rate constant C is the molar concentration of the drug (artesunate) n is the order of reaction. Taking logarithm of equation (2)

Log V = Log DA/Dt =log K^1+ a log C

The initial kinetic plots of DA Vs Dt were all sigmoid in nature. The initial rate was obtained

The calibration curve was generated by plotting the logarithm of the initial rate log v vs. logarithm of the initial molar concentration of the drug (artesunate) which was observed to be linear with 20 µg – 70 µg/ml heat square method was used to analyze the linear precision of the calibration data (n = 5) the slopes, intercept and correlation coefficient were evaluated. The limit of detection was calculated based on the ICH guidelines using the equation. LOD = 3.3σ/5, where σ is the standard deviation of five replicate determination value under the same correlation 5 is the slope of calibration curves. The results of all these spectral and statistical data are recorded in Table I above.

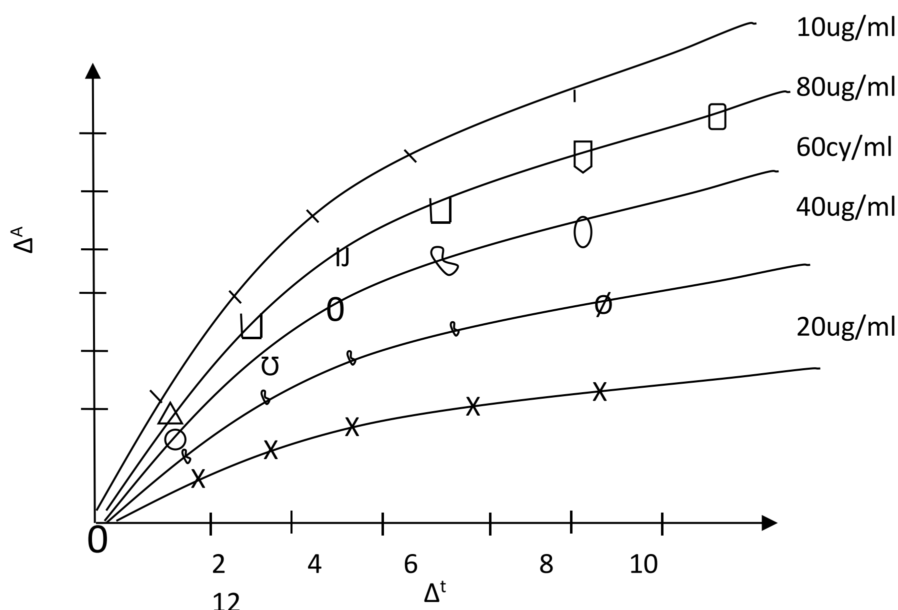


Fig. 3: Absorbance-time curves for the reaction of varying concentration artesunate with iodate/iodide mixture

Table 1: Spectral and Statistical Data for the Determination of Artesunate using the Initial Rate Method

Parameter	Initial Rate Method
> (max) mn	350
Bears Linear Range	20µg – 70µg
Precision Equation	A = ryx + c Log (rate) = Log DA/Dt = log K+n log C Log DA/Dt = log K + n log C Log Rate = 3.6510 + 1.0199 log C
Slope	1.0199
Intercept	3.6510
Correlation Coefficient	0.9997
Limit of detection (µg/ml)	0.022

Fixed Time Method

In this fixed time method, the absorbance of the reactions the iodate/iodide mixture with solutions of the drug containing varying concentrations were obtained at different preselected times of t_1 = 2 to t_5 = 10. Calibration curves were generated by plotting the absorbance against the molar concentrations of the drug solution in all cases the graphs were rectilinear. All curves obey Bears law, the molar absorptivity, the Bear's law limit, the linear precision equation correlation co-efficient detection limit the slope and intercept are recommended in table 2.

Table 2: Spectral Parameters and Statistical Data obtained by the Determination of Artesunate by Fixed Time Method

S/No	Parameter	2 Minutes	4 Minutes	6 Minutes	8 Minutes	10 Minutes
1.	λMax	350	350	350	350	350
2.	Beers Linear Range (ug/ml)	10 – 70	10 – 70	10 – 70	10 – 70	10 – 70
3.	Molar Absoptivity (L mol/cm	5.81×10^3	6.1×10^3	6.6×10^3	6.9×10^3	7.2×10^3
4.	Linear Precision Equation	A = 0.0057 + 1.120C	A = 0.0072 + 1.621C	A = 0.00086 + 1.833C	A = 0.0009 + 2.08C	A = 0.0001 + 2.140C
5.	Interception	0.057	0.0072	0.00086	0.0095	0.001
6.	Slope	1.420	1.621	1.833	2.08	2.14
7.	Correlation Coefficient	0.9998	0.9999	0.9999	0.9999	0.9999
8	Detection Limit (ug/ml)	0.182	0.237	0.270	0.290	0.288

From table 2 it can be observe that the correlation coefficient, the slope and the intercept which is not too significantly different from zero, the fixed time method is quite versatile for the analysis of artesunate in pharmaceuticals. Any of the fixed times will be suitable for the analysis of the artesunate in pharmaceutical formulation.

Variable Time Method or Fixed Absorbance Method

At different concentration levels of artesunate the general or recommended procedure was applied. The time in seconds required for the absorbance to reach 0.20 (preselected absorbance) was noted. A calibration curve was generated by plotting the reciprocal of time 1/Dt versus initial concentration of artesunate in each sample solution. Even though the curve obeyed Beers Law the correlation coefficient was about 0.9445 making this method less accurate as compared to the initial rate and the fixed time method have this method is not recommended.

Rate Constant Method

In this method the logarithm of absorbance change was plotted against time in seconds at room temperature of 25 + 50c. The pseudo first order rate constant (K1) was calculated from the slope by multiplying by the constant – 2.303. A calibration curve was generated by plotting pseudo first order constant (K1) versus the initial molar concentration of artesunate, the graph was rectilinear. The correlation coefficient (r) was found to be in the range of 0.9280 and 0.9290, hence this method was found less suitable for the analysis of artesunate in pharmaceutical formation.

Method Validation

Both the fixed time and the initial rate methods were suitable for the analysis of artesunate in pharmaceutical preparations. The fixed time method was chosen because of its high correlation coefficient (r) of 0.9999. The fixed time method was validated for linearity, accuracy, precision, selectivity.

Linearity and sensitivity

The calibration curves generated in the fixed time method by plotting the absorbance versus the molar concentrations were rectilinear with linear range of 10 – 70µg/ml in all the fixed time of 2, 4, 6, 8 and 10 minutes. The precision equation was determined as per least square method. The correlation coefficients were 0.9999 in almost all cases. The intercepts were very small values which were very close to zero in all

cases. The sensitivity parameters molar absorptivity was in the range of 5.81 x 103 and 7.2 x 103 other sensitivity parameters such as the limit of detection was determined as per the 1CH guidelines by evaluating the equation LOD = 3.3 σ/S, where σ is the standard deviation of five blank determination and S is the slope of the calibration curve. The range of the LOD was in the range of 0.182 to 0.288µg/ml. the values are recorded table II.

Accuracy and Precision

The accuracy and regression of this fixed time kinetic methods were evaluated by preparing the pure drug (artesunate) at three concentration levels and performing six replicate analyses within three days (intra day) and for three consecutive days (inter day). Percentage relative error or RE% was used to evaluate accuracy while percentage related standard deviation (RSD %) used to evaluate Precision. The value for accuracy and precision is as recorded in table III with accuracy being ≤ 2.10 % and the precision < 2.20 showing good accuracy and precision. Hence good repeatability and reproducibility. The value of accuracy was evaluated using the formula:

$$\text{RE\%} = \frac{(\text{Amount found} - \text{Amount taken})}{\text{Amount taken}} x \frac{100}{1}$$

Table 3: Evaluation of Accuracy and Precision

S/No	Amount of Artesunate	Intraday Accuracy and Precision Amount of Artesunate found	R.E.%	RSD%	Inter-day Accuracy and Precision Amount of Artesunate	R.E.%	RSD%
1	10µg/ml	10.16	1.6	1.70	10.20	2.0	1.78
2.	20µg/ml	20.25	1.25	1.16	2042	2.1	1.82
3.	40µg/ml	40.80	2.08	1.40	41.00	2.5	2.12

The accuracy and precision of the initial rate method was also evaluated by performing the intraday and inter-day analyses using the percentage Error (R.E. %) for accuracy and Relative Standard deviation (RSD %) for precision. The result is as shown in table IV

Table 4: Evaluation of Accuracy and Precision (Initial Rate Method).

S/No	Amount of Artesunate	Intraday Accuracy and Precision	R.E.%	RSD%	Inter-day Accuracy and Amount of Artesunate	R.E.%	RSD%
1	10µg/ml	10.20	2.00	2.10	10.26	2.6	1.18
2.	20µg/ml	20.43	2.15	2.02	20.48	2.40	2.00
3.	40µg/ml	40.88	2.2	1.80	40.87	2.18	2.16

In the initial method, the accuracy and precision values were ≤ 2.80 and ≤ 2.60 respectively, showing good accuracy and precision, hence also good repeatability and reproducibility.

Selectivity

The selectivity of both the initial rate and the fixed time methods were evaluated by using the placebo blank and the synthetic mixture methods as earlier described. Excipeints usually employed in pharmaceutical tablets formulations were used. The percentage recovery of artesunate for initial rate method and the fixed time method were 98±1.18 (n=7) and 97.5±1.30 (n=7). Showing that these pharmaceutical excipients have no interference with the developed method, indicating the reliability and the feasibility of the methods [8].

Robustness and Ruggedness

The robustness of both methods was tested by evaluating the influence of minor valuations on the sensitivity of the Methods. Minor changes were made in the concentration of potassium iodate, potassium iodide and the reaction time. It was discovered that these minor variations in these experimental variations had no major effect on the sensitivity and reproducibility of the developed methods. Ruggedness of the methods was tested by evaluating the RSD% on two different

spectrophotometers and by two different analysts for two consecutive days. There was no major or significant differences in the RSD% obtained compared with those obtained by the two analysts using two different instruments showing that both were rugged [10, 11, 13, 14,15].

Application of the Methods for the Analysis of Artesunate in Tablets

The developed methods were used successfully to assay some commercial brands of artesunate procured locally in Uyo South-South Nigeria. The results obtained via the developed methods were compared to official method (the UV method in the International Pharmacopoeia (IP 2003) [13] statistical via the variance ratio test for precision and the Students t test for accuracy, the values are recorded in table V. the value of the F-test and the students t-test at 95% confidence level and at 4 degrees of freedom showed that the values obtained were lower than the tabulated values (critical values) confirming no significant difference between the developed method and the pharmaceutical standard method. The results show no effects of excipient.

Recovery Studies

The feasibility and applicability of the developed methods was ascertained by evaluation using the standard addition method. In this method, a known quantity of the pure artesunate was used to spike a pre-analyzed tablet powder at three different concentration levels and the amount of the artesunate obtained analyzed by the proposed methods. The percentage recovery of the pure artesunate powder was determined with the standard deviation. The values are recorded in table VI

Table 5: Result of Analysis of Commercial brands of Artesunate tablets by the prepared methods

Tablets Analyzed	Label claim mg	Reference method	Results of develop method + SD	
			Initial Rate Method	Fixed Time Method
Lever Artesunate	50 -	110.0±1.21	111.2± 1.18 F = t = 1.78	111.0 ± 1.20 F = t = 1.62
Articin (Embassy)	50	110.0 ± 1.14	111.3 ± 1.17 F = 1.05, t = 1.63	111.0 ± 1.19 F = t = 1.70
Artesunate (Neros)	50	110.0 ± 0.98	111.0 ± 1.23 F = t = 1.48	111.0 ± 1.19 F = t = 1.70
Arsumax	50	110.0 ± 1.22	111.2 ± 1.16 F = t = 1.56	111.3 ± 1.18 F = t = 1.68

Mean of 5 determinations: The values of t (tabulated at 95% confidence level and at 4 degrees o freedom = 2.77. The value of f-test (tabulated at 95% confidence level and at 4 degrees of freedom = 6.37.

Table 6: Results of Recovery Studies via Standard Addition Method

Drug formulation studied	Initial Rate Method				Fixed Time Method			
	Amt of ART in tablet	Amt of pure ART added	Total found	Recovery or pure ARTs	Amt of ART in tablets ug/ml	Amt of pure ART Added	Total Amt found	% Recovery
Lever Artesunate	40.20	20.00	60.55	101.8 ± 0.88	40.20	20.00	61.00	104.0 ± 2.0
	40.20	40.00	81.26	102.5 ± 1.25	40.00	40.00	81.10	102.3± 1.0
	40.20	60.00	101.20	102.8 ± 1.43	40.20	60.00	101.90	103.0 ± 1.09
Articin	41.00	20.00	61.65	103.3 ± 0.88	41.00	20.00	61.55	102.3± 1.75
	41.00	40.00	82.20	100.3 ± 1.25	41.00	40.00	81.40	101 ± 0.50
	41.00	60.00	101.20	100.3 ± 0.20	41.00	60.00	107.20	103± 1.25
Artesunate (Neros)	40.20	20.00	61.00	104.0 ± 2.0	40.20	20.00	60.60	102 ± 1.41
	40.20	40.00	81.10	102.3 ± 1.0	40.20	40.00	81.25	103.0± 1.31
	40.20	60.00	109.90	103.0 ± 1.09	40.20	60.00	102.89	102.5 ± 1.76
	41.00	20.00	61.65	103.5 ± 1.44	41.00	20.00	61.55	103.3± 1.58
	41.00	40.00	82.50	103.0 ± 1.82	41.00	40.00	82.50	103.0 ± 1.88
	41.00	60.00	103	103.7 ± 2.45	41.00	60.00	103.10	103.10± 1.99

4.0. CONCLUSION

The proposed kinetic methods as developed (initial rate and fixed time) show great promise in the determination of artesunate. This can be used in routine laboratories and field stations for the assay of artesunate. The reagents are easily available and pose no serious hazard to the environment and the analyst. The methods are sensitive, reproducible and devoid of tedious extraction process and occur at room temperature and the method can easily be applicable in countries where no expensive analytical equipment is available.

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