

# Development and Validation of a Stability-Indicating HPLC Method for the Estimation of Artesunate and some known Degradants using Forced Degradation Studies

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## Abstract

*Malaria is an acute feverish illness caused by Plasmodium parasites and responsible for 1-3 million deaths per year. Artesunate, a part of the Artemisinin group of drugs, has been found to be among the most effective drug against this parasite. A stability indicating method was developed using a reversed phase high performance liquid chromatography (RP-HPLC) to quantify the Active Pharmaceutical Ingredients (API) and its possible degradants. The chromatographic separation was done on a Gemini C18 (100x 4.6mm, 3µm) column. The mobile phase used was mixture of Phosphate buffer (pH 3.0) and acetonitrile in the ratio of 48:60 (v/v) at isocratic mode and detection of the eluents was done using the UV detector at 216nm. The method shows a good linearity in the concentration range of 50-150% for Artesunate and the impurities (Artemisinin, α-arteminol and β- arteminol) with regression co-efficient ranging from 0.9979 to 0.9999. LOD and LOQ for all the analytes had respective ranges of 0.00531-0.00125 and 0.00377 – 0.134747. Artesunate had LOD and LOQ of 0.04582 and 0.13747 respectively. The Artesunate tablets and raw material were subjected to various stress tests and the tablets were found to be susceptible to acidic, alkaline and oxidation hydrolysis with the formation of degradants. The proposed stability indicating method was accurate, precise, robust, stable, and specific. The developed method was validated in accordance with ICH guidelines and hence can be successfully applied to the determination of Artesunate and its degradation impurities in tablet formulation.*

**Keywords:** Artesunate; forced degradation; high performance liquid chromatography; malaria; stability indicating method

## 1. Introduction

Malaria is an acute feverish illness which can spread to people when bitten by infected female Anopheles mosquitoes as a result of transfer of the Plasmodium parasite (WHO, 2021). This illness causes over 400,000 deaths per year (WHO 2019). There are 5 parasite species that responsible for this disease in humans namely; *Plasmodium falciparum*, *Plasmodium knowlesi*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium vivax* (Kwenti *et al.*, 2017). Artesunate, which is one of the Artemisinin group of drugs, is among the most effective drug against this parasite (Li and Weina, 2010). It is a water-soluble derivative of Artemisinin and can be administered by injection (NCBI, 2022). It is sometimes abbreviated as AS (Artesunate). It is on the list of essential medicines as the most important medications needed in a basic system (WHO, 2019). Nigeria, being a malaria-endemic country, is located in the tropical regions of Africa. Thus, subjecting drug products to unsuitable environments during storage and transportation could result in the breakdown of the products. This situation necessitates stability studies on Artesunate to ensure that the drug can maintain its quality when exposed to harsh conditions in the process of storage, transportation, and usage (Ahmad *et al.*, 2016). Stability indicating method is a key element in stability studies to guarantee the wholesomeness and durability of drug products. As stated in the



International Conference on Harmonization (ICH) guidelines (ICH Q2 R1), a Stability Indicating Assay Method (SIAM) must be able to estimate active constituents accurately and precisely in the presence of impurities, additives, and degradants (Burgess and Hammondb 2015). Forced degradation studies are usually carried out to develop and validate stability indicating methods for the Active Pharmaceutical Ingredient (API). Forced degradation of the drug product may be through heat, light, acidic and alkaline hydrolysis, oxidation etc. (Ali *et al.*, 2012). Other usefulness of force degradation studies includes the prediction of the possible degradants of a drug product, to detect impurities associated with drug substances or excipients, and to resolve stability-related issues (Brummer, 2011).

Some HPLC methods have been developed and validated for the determination of artesunate either singly or in combination with other pharmacons (Nogueira *et al.*, 2013; Mondal *et al.*, 2019; Desai *et al.*, 2021; Saeed *et al.*, 2020; Mathew *et al.*, 2018; Jyothi *et al.*, 2014). Of all these, only those developed by Mathew *et al.* (2018), Jyothi *et al.* (2014) and Mondal *et al.* (2019) were stability indicating i.e. involved force degradation studies. Studies have, however, rarely been done to develop and validate methods to estimate the impurities associated with the drug. Also, the force degradation of the drug raw material has scarcely been done in comparison with the tablet forms to assess the influence of the excipients on the stability of the active ingredients in the formulations.

The aim of this study was therefore to develop and validate a stability indicating High Performance Liquid Chromatography (HPLC) method for the estimation of artesunate and some of its degradants. Force degradation studies were also carried out on the tablet formulation and the raw materials according to the ICH guideline, ICHQ2B (Jagirapu *et al.*, 2019) and the specificity of the developed method was affirmed.

## 2. Materials and method

### Chemicals and reagents; The following reagents were used during the analysis

HPLC grade Acetonitrile (Merck) 99.9%, Orthophosphoric acid (BDH) 98.5%, Potassium dihydrogen Orthophosphoric (BDH) 99.6%, Demineralised water (conductivity < 0.05 $\mu$ s/cm), Sodium hydroxide (BDH), Conc. Hydrochloric acid (37%), System Suitability Test (SST) solution

**Table 1. Instrumentation and analytical conditions were stated as below**

Parameters	Settings
Water Alliance 2695 HPLC	Reversed phase
Water Alliance Detector module 2489	216nm
HPLC soft ware	Integrator
Potassium Buffer (KH <sub>2</sub> PO <sub>4</sub> )	pH 3.0
Mobile Phase	Acetonitrile + Buffer ( 52:48)v/v
Column temperature	25 <sup>0</sup> C
Column used	Gemini, C18,
Column dimension	100 x 4.6 mm, 3 $\mu$ m
Grant ultrasonicator	25 <sup>0</sup> C
Injection volume	20 $\mu$ L
Mettler Toledo Analytical balance	NA
Toledo pH meter	NA

### Preparation of the Buffer solution

1.36g of potassium dihydrogen phosphate was dissolved in 900ml of demineralized water and the pH was adjusted to 3.0 using orthophosphoric acid. The solution was made up to 1000ml with demineralized water (Al-Hakkani *et al.*, 2021).



### Preparation of standard solutions

A standard mixture of Artesunate, Artemisinin,  $\alpha$  and  $\beta$  arteminol were prepared according to International pharmacopoeia 10th Edition (WHO, 2021). The chromatogram of the standard mixture of Artesunate and its impurities are shown in Figure 1.

### Sample preparation for unstressed tablet

A portion of the finely milled tablet powder equivalent to about 40 mg Artesunate was transferred into 10ml volumetric flask and diluted to the mark with acetonitrile, shake and sonicated for 15 minutes. The resultant solution was centrifuged to obtain a clear supernatant solution and later injected under the stated chromatographic conditions (Brummer, 2011).

### Method Validation

Validation of the stability-indicating method was done according to the stipulations in International Conference on Harmonization (ICH) Guidance for Industry (Q2 R1) Validation of Analytical Procedures: Text and Methodology (ICH, 2011, Gupta et al., 2015). Parameters evaluated include: accuracy, linearity, precision, Limit of Detection (LOD) and Limit of quantitation (LOQ).

### Linearity

A stock solution at ten times normal working concentration solution containing a mixture of 40mg/ml Artesunate RS & 10mg/ml impurities standard solution was prepared (Solution S) in acetonitrile. Aliquots of the stock solution (Solution S) were diluted into separate 50ml volumetric flasks, to obtain 50% to 150% matrix concentrations.

### Precision

Method precision was carried out by injecting six replicates of the standard mix solution into the HPLC. The retention times and peak areas of the replicates for the four analytes were noted. The precision is expressed as the %Relative Standard Deviation of peak areas and it should not exceed 2%.

### Accuracy

For this validation parameter, three different concentrations of Artesunate and impurities standard solution (80-120%) were prepared and spiked into a placebo and the mean % recoveries for each of the standard were calculated.

### LOD and LOQ

LOD and LOQ was calculated from each calibration curve using formulae:

$$\text{LOD} = 3.3 \cdot \sigma / \text{Slope} \dots\dots\dots \text{Equation 1}$$

$$\text{LOQ} = 10 \cdot \sigma / \text{Slope} \dots\dots\dots \text{Equation 2}$$

Where  $\sigma$  = the standard deviation calibration curve response.

### Forced degradation and preparation of sample solutions

The Artesunate tablets were subjected to various stress tests and the solutions of the degradation samples were prepared as described by Blessy et al. (2014)

#### Hydrolysis Stress

For each of acid, alkaline, aqueous and oxidative hydrolyses, 100 mg of Artesunate was weighed into 10 mL volumetric flask and labelled accordingly. To each of acid and alkaline hydrolysis flasks, 1 mL of 1 M HCl and 1 mL of 1 M NaOH was added respectively, heated to 50 °C in water bath for 4 hours, neutralized and made to the mark with acetonitrile. For aqueous and oxidative hydrolysis, 5 mL of demineralized water and 1 mL of 0.1% peroxide solution was added respectively to the samples, flasks heated to 50 °C in the water bath for 4 hours, cooled and made up to the mark with acetonitrile. Afterwards, 4 mL clear solutions from each of the hydrolyses were diluted to 10 mL with acetonitrile and 20  $\mu$ L of the resulting solutions were injected into the HPLC for analyses.

### Thermal Stress

200 mg of Artesunate powder model tablets was spread on a clean and dry petri dish and heated in an oven at 80 °C for 24 hours and cooled. Tablet powder sample containing an equivalent of 100 mg of Artesunate was weighed from the heated and cooled sample into a 10 mL volumetric flask and made up to mark with acetonitrile. 4 mL of the clear solution was diluted to 10 mL with acetonitrile and 20 µL of the resulting solution was tested according to the HPLC method.

### Photolytic Stress

200 mg of Artesunate powder model tablets was exposed to sunlight for 24 hours. Tablet powder sample containing an equivalent of 100 mg of Artesunate was weighed into a 10 mL volumetric flask and made up to mark with acetonitrile. 4 mL of the clear solution was diluted to 10 mL with acetonitrile and 20 µL of the resulting solution was tested.

## 3. Results and Discussion

### Method development and optimization

The isocratic optimized stability indicating HPLC method was used to resolve the mixture of Artesunate standard and its known impurities. The results of the chromatogram obtained with this condition are shown in figure 1 that represents the system suitability (SST) chromatogram.

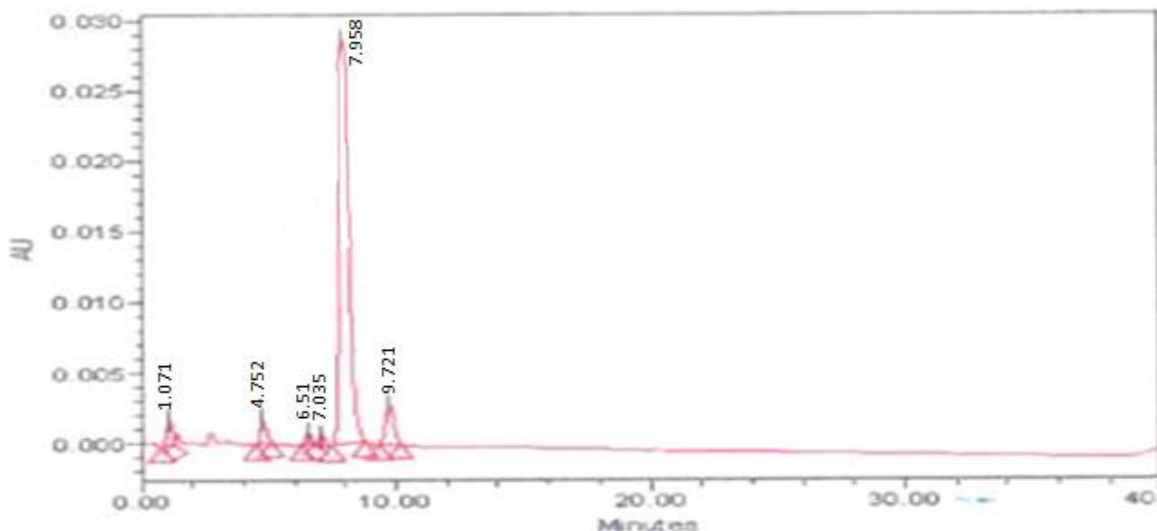


Figure 1.0: Chromatogram for SST Solution (Contains Artesunate and known Impurity substances;  $\alpha$ -Artemimol,  $\beta$ -Artemimol and Artemisinin in acetonitrile)

### Verification Parameters

The parameters verified were Linearity, Precision, LOD, LOQ and Accuracy tests.

The linearity studies were determined at different concentration (50%-150%) for Artesunate,  $\alpha$ -Artemimol,  $\beta$ -Artemimol and Artemisinin standards. Figures 2, 3, 4 and 5 below show the calibration curves for all the standards.

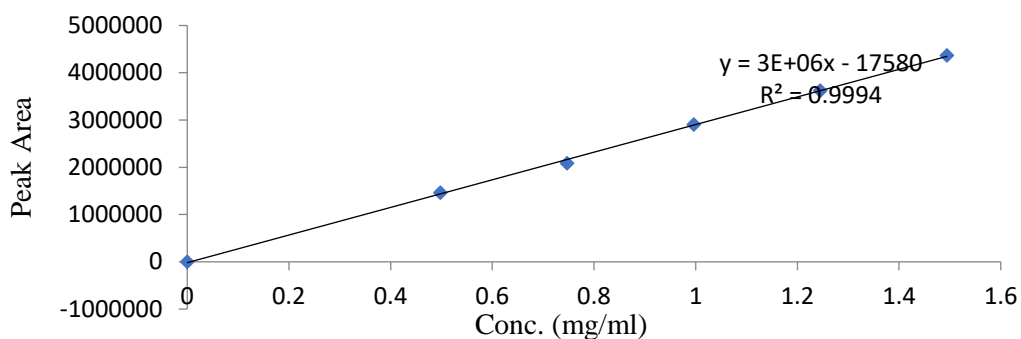


Figure 2.0: Linearity plot for Artesunate  
The R-squared value of this calibration curve is 0.9994 which is within the acceptable limit.

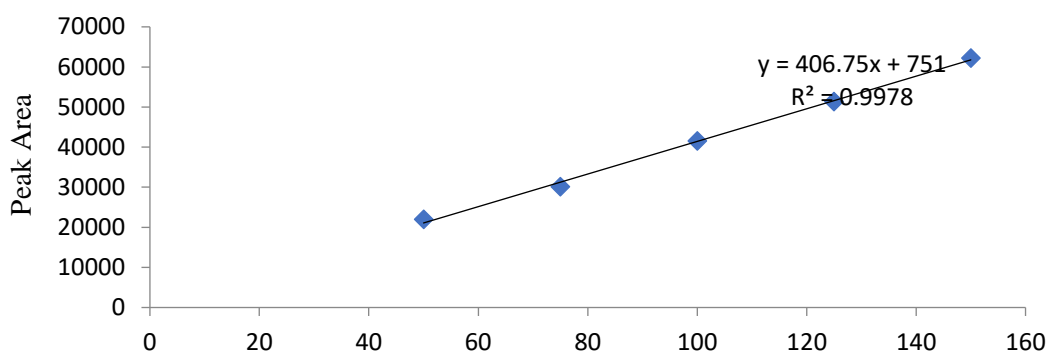


Figure 3.0: Linearity plot for  $\alpha$ -Arteminimol  
The coefficient of determination for the  $\alpha$ -Arteminimol calibration curve is 0.9978 which is a good R-squared value.

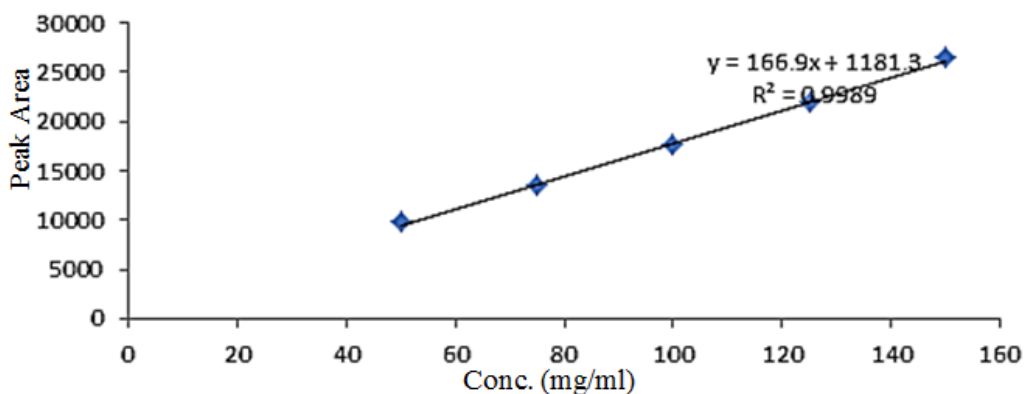


Figure 4.0: Linearity plot for  $\beta$ -arteminol  
The  $\beta$ -arteminol calibration curve also had a good fit with the R-squared equal to 0.9989.

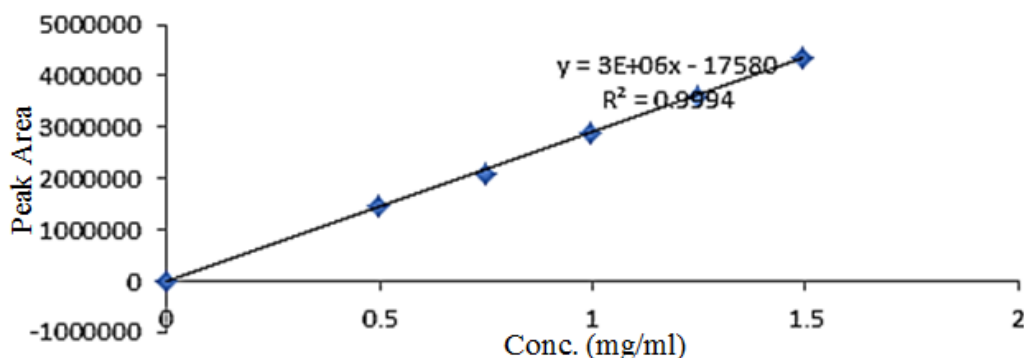


Figure 5.0: Linearity plot for Artemisinin

The R-squared value for the Artemisinin calibration curve is also 0.9994 which falls within the accepted range.

**Accuracy:** The result for the accuracy testing is displayed in table 2 below.

Table 2.0: %recoveries for the analytes

Matrix prepared (%)	Analytes Conc. (mg/mL)							
	$\alpha$ -Artemimol spiked conc.n=3	$\alpha$ -Artemimol recovered conc. (%recovery)	$\beta$ -Artemimol spiked conc. n=3	$\beta$ -Artemimol recovered conc. (%recovery)	Artesunate Spiked conc. n=3	Artesunate recovered conc. (%recovery)	Artemisinin Spiked conc. n=3	Recovered Conc. (%recovery)
80	0.062	0.060 (96.77%)	0.021	0.02 (95.24%)	0.800	0.791(98.88 %)	0.079	0.077 (97.47%)
100	0.077	0.073 (94.81%)	0.026	0.025 (96.15%)	0.996	0.986 (98.99%)	0.099	0.097 (97.97%)
120	0.092	0.093(101.09 %)	0.031	0.030 (99.77%)	1.200	1.206 (100.5%)	0.119	0.117 (98.31%)

The %recoveries for each of the analyte ranged from 94.81% to 101.09%. as contained in Table 2.. The ranges of the results obtained are within the acceptable limit and in accordance with the recommendation of compendia and other literatures (WHO, 2021; Rao *et al.*, 2011).

### Precision

Method precision was determined by injecting six replicates of the Artesunate and impurities standard solution into the HPLC. The retention times and peak areas of six replicates for the four analytes are as shown in Table 3.

Table 3.0: Results for the precision of the method

Analyte	$\alpha$ -Artemimol	$\beta$ -Artemimol	Artemisinin	Artesunate
1	41515	17622	322835	2905594
2	40789	17558	321970	2905528
3	40875	17086	321765	2939600



Analyte	$\alpha$ -Artemimol	$\beta$ -Artemimol	Artemisinin	Artesunate
4	41549	17609	319870	2906727
5	40975	17549	318999	3003565
6	41064	17026	320765	2913679
Mean	41127.8	17408.3	321034	2929115.5
SD	326.6	275.02	1429.9	38734.5
%RSD	0.8	1.6	0.45	1.3

The results of the precision obtained showed that the high degree of reproducibility of the replicate injections because the the percent relative standard deviation (%RSD) was below 2.0% which is an indication that the method developed is précised (Gupta et al., 2015)

**Limit of detection and Limit of quantitation:**

The LOD for this method for artesunate, artemisinin,  $\alpha$ -arteminol and  $\beta$ -arteminol were 0.04582, 0.00138, 0.00531 and 0.00125 respectively while the LOQ for these analytes were 0.13747, 0.00415, 0.01591 and 0.00377 respectively. The LOD and LOQ reported in this study for Artesunate was lower than those reported for the same drug product by Jyothi et al. (2014).

**Force Degradation Studies (FDS)**

FDS for Artesunate tablet

The results obtained from Artesunate tablet powder after subjected to various stress conditions are as shown in Tables 4.0 and 5.0.

Table 4.0: Results of Stress Testing Parameter for Artesunate tablet

Stress type	Conditions	Total impurities/Degradants
Acid hydrolysis	1M HCl at 60°C for 4hrs	35.52%
Alkaline hydrolysis	1M NaOH at 60°C for 4hrs	30.58%
Aqueous hydrolysis	60°C for 4hrs	No known impurities detected
Oxidative stress	1ml of 0.1% v/v H <sub>2</sub> O <sub>2</sub> at 60°C for 4hrs	8.97%
Thermal stress	80°C for 24hrs	0.57%
Photolytic stress	24hrs exposure	No known impurities detected

From Table 4.0 above, it was observed that acid and alkaline stress conditions brought significant degradants from the forced degradation study. This is because the impurities (degradants) are much greater than 10% that is usually used to assess the effectiveness of degradation (Steven et al., 2013). There was little or no significant degradation with thermal, aqueous and photolytic stress conditions and these might be attributed to the behaviour of Artesunate tablets under these conditions (Chandrashekhar et al., 2017). The results of oxidation stress showed relatively low degradation process as the values for the impurities were less than 10% (Manish et al., 2017).

**Mass balance**

This is usually used to access the effectiveness of stability indicating method and the degradation pathway of pharmaceutical products (Steven et al., 2013; Alsante et al., 2011). The Table showed the results for the mass balance obtained during the stress tests.



Table 5.0: Mass balance of Degradative Stress test Parameters

Parameter	Acid Stress			Alkaline Stress			Oxidation stress		
	Retention Time (mins)	Peak Area	(% of Unstressed Analyte Peak Area)	Retention Time (mins)	Peak Area	(% of Unstressed Analyte Peak Area)	Retention Time (mins)	Peak Area	(% of Unstressed Analyte Peak Area)
Unstressed Tablet (Artesunate)	7.97	2905594	100	7.97	2905594	100	7.97	2905594	100
Stressed Tablet (Artesunate Peak)	8.06	1182632	40.70	8.08	1353706	46.59	8.06	2600641	89.50
Degradants	-	1031961	35.52	-	905827	30.58	-	260497	8.97

It can be deduced from Table 5 that total amount of the Artesunate and the impurities were not up to 90%. This implied that acid stress condition might have resulted in loss of the active ingredient through some other mechanisms during acid stress process. After the stress testing, no degradations were observed in thermal and photolytic stress conditions. However, degradations were observed in acidic stress, alkaline stress and oxidation stress degradations and agrees with limit described by Hamrapurkar et al., 2011. Figure 6 represents the chromatogram from tablet that undergone oxidation stress.

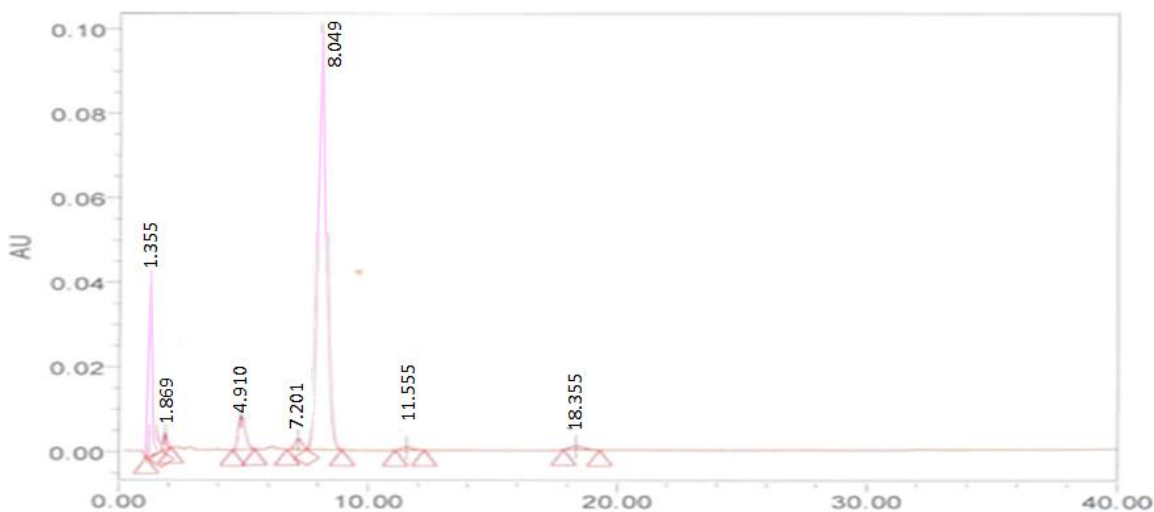


Figure 6.0: Chromatogram for Oxidative degradation of the Artesunate tablet.

**FDS for Artesunate raw material was carried out in a similar way as in Artesunate table.**

The results obtained from Artesunate raw material powder after subjected to various stress conditions are as shown in Tables 6.0 and 7.0.



Table 6.0: Results of Stress Testing Parameter for Artesunate raw material powder

Stress type	Conditions	Total impurities/Degradants
Acid hydrolysis	1M HCl at 60°C for 4hrs	45.55 %
Alkaline hydrolysis	1M NaOH at 60°C for 4hrs	37.25%
Aqueous hydrolysis	60°C for 4hrs	No known impurities detected
Oxidative stress	1ml of 0.1% v/v H <sub>2</sub> O <sub>2</sub> at 60°C for 4hrs	19.30%
Thermal stress	80°C for 24hrs	5.95%
Photolytic stress	24hrs exposure	25.02%

Table 7.0: Mass balance of Degradative Stress test Parameters for Artesunate raw material

Parameter	Acid Stress			Alkaline Stress			Oxidation stress		
	Retention Time (mins)	Peak Area	(% of Unstressed Analyte Peak Area)	Retention Time (mins)	Peak Area	(% of Unstressed Analyte Peak Area)	Retention Time (mins)	Peak Area	(% of Unstressed Analyte Peak Area)
Unstressed (Artesunate raw material)	7.54	3025394	100	7.54	3025394	100	7.54	3025394	100
Stressed (Artesunate raw material)	7.60	1089142	35.08	7.59	1216208	40.43	7.55	2373421	78.45
Degradants	-	1361427	45.55	-	1128472	37.25	-	583901	19.30

### Acidic and alkaline hydrolysis of AS raw materials

Studies have shown that Artesunate raw material can undergo hydrolysis under acidic and alkaline conditions which depend mostly on the PKa of the drug material (Rajab & Saeed 2017). The results of acid and alkaline stress testing showed that there was an appreciable level of degradation as seen in Tables 6.0 and 7.0 above. The degradation profile for the crushed tablet and Artesunate raw material follow similar path way in terms of the amount of degradants obtained in both studies. The solubility of artesunate is more enhanced under acidic condition (Agnihotri et al., 2013; Blessy et al., 2014). The chromatograms in figures 7, 8 & 9 showed that the retention times for the unstress Artesunate raw material (7.54mins) is closer to that of the acidic and alkaline stress. (see the attachment)

### Oxidation stress of AS raw materials

There was a significant degradation under oxidation stress of Artesunate raw material. The degradants formed was around 20% which is an indication of appropriateness of degradation under stress condition. In comparison of this result with that obtained in milled tablet, showed that there was an appreciable level of degradation in Artesunate raw material as reflected in Table 7.0. The chromatogram in Figure 10.0 showed that the retention time for Artesunate under oxidation condition was 7.55mins which is very close to that of unstressed Artesunate raw material. (Camargo et al., 2022).



### **Photolytic stress of AS raw materials**

The electronic transition of Artesunate material is prone to be affected upon exposure to light which can reflect on the chromophoric compound present. The result of photolytic stress for Artesunate raw material showed that there was measurable level of degradants formed (25%) during this process that is greater than the minimum requirement for degradation as indicated in Table 6.0 above. The chromatogram in Figure 11.0 showed that the retention time of Artesunate raw material is 7.57mins which also very closed to that of unstressed raw material. (see the attachment)

### **FDS and Specificity of the HPLC Method**

The chromatograms presented in Figures 6.0 – 12.0 shows the separation of the degradants arising from the various stress testing and the API (Artesunate) can easily be identified and estimated in the presence of the degradants and other impurities. This implies that the method developed is specific.

### **Comparison of the FDS on the AS raw material and on the AS tablet**

From the result of the respective forced degradation studies of both the AS raw material and tablet, greater degradation was observed for the raw samples than those of the tablets. This could be likely due to the stability of excipients-active interaction and therefore led to slight resistance to degradation that the excipients provide to the drug (Jordan et al., 2021).

### **4. Conclusion**

A simple, precise, accurate and sensitive Reversed Phase High Performance Liquid Chromatography (RP-HPLC) method was developed for the determination of Artesunate and its degradants. A comprehensive stability study was carried out by subjecting samples of Artesunate powdered tablets and raw material to degradation by acid, alkaline, oxidation, heat, and light. The degradation of Artesunate under acidic, alkaline and oxidation conditions were observed in both the raw material and milled tablet. Also, the result of photolytic stress has however showed that Artesunate raw material could be much more susceptible to degradation than the milled tablet. The chromatographic method showed good specificity for the artesunate in the presence of its degradants produced from the stressing tests on both the raw material and the tablet. The SIAM has been proven to have met the acceptance criteria for Linearity, Precision, and Accuracy test parameters with Artesunate peaks clearly resolved without interference from other components in the tablet formulation as shown in the results obtained. The results obtained from this study showed that the SIAM could be routinely used in assessing the stability of Artesunate tablet powder.

### **Recommendation**

It is recommended that stability indicating analytical method should be employed in pharmaceutical analysis in order to ensure the quality and safety of the pharmaceutical products.

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