Novel spectrophotometric determination of some artemisinin derivatives using alcoholic ammonia solution generated insitu

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ABSTRACT

Background: Dihydroartemisinin (DHA) and Artesunate (ART) are frontline antimalarial drugs for treatment of uncomplicated malaria. A sensitive spectrophotometric method is here developed for the assay of these two antimalarials.

Methods: The reaction is based on the generation of alcohol Ammonia *in situ* by the action of sodium hydroxide on ammonium Nitrate. The lactone ring of the artemisinin derivatives opens producing a chromogen that can be measured spectrophotometrically; at λmax 350nm and 340nm for DHA and ART respectively.

Results: The absorbance is directly proportional to the drug concentration. Bears law is obey in the range of 5.0-10µg and 5.0 – 80µg/ml for DHA and ART respectively. The correlation coefficient of 0.9997 and 0.9998 respectively. The method was sensitive with molar absorptivity of 2.65 x 10^3 $LMOL^{-1}$ and 2.0 x 10^3 $lMOL^{-}CM^{-1}$ and Sandell sensitivity of 0.115 and 0.225 µg/cm^2 respectively for DHA and ART. The limit of detection and limit of quantification were determined as per the current ICH guidelines and found to be 0.485 and 0.430/ 1.27 and 1.32µg/ml for DHA and ART respectively. The precision and accuracy are excellent at $\leq$ 3.00 in all cases.

Conclusion: The method was compared statistically with official pharmacopoeal method via F-test and students T-test and was used to assay DHA and ART tablets procured local which showed good congruence. The aplicability of the method was ascertained by conducting recovery test via standard addition method with results showing no significant interference from excipients.

Keywords: Amminolysis, Antimalarials, Artesunate, Counterfeit medicine, Dihydroartemisinin

1. INTRODUCTION

The World Health Organization (WHO) defined counterfeit drug as medicines that are deliberately or fraudulently mislabeled with respect to identity, composition, and or source. This definition includes completely fake medicine and those that have been tampered with, adulterated, diluted, repackaged, or relabeled so as to misrepresent the dosage, origin, or expiration date. Substandard medicines that are cheaply produced in order to make unlawful profits also constitute counterfeit medicine [1]. Malaria remains the most important source of concern in the tropics strictly speaking in terms of morbidity and mortality. It attacks all ages and all manner of people including pregnant women and the greatest toll being on children below the ages of 5 years. Malaria kills about three thousand people daily with nine out of every ten cases in sub-Sahara Africa. About 85% of malaria deaths occur in children under the age of five years. Every 30 seconds a child dies of malaria as noted by the current WHO Report 2012. Malaria is the greatest cause of poverty in Africa [2]. The manufacture, export and distribution of fake and counterfeit antimalarials especially artemisinin derivatives from South East Asia is the real cause of complain and concern [3, 4, 5, 6, 7, 8, 9, 10]. The activity of these unscrupulous agents of darkness is putting the efforts geared towards the eradication of malaria in jeopardy. Patients are beginning to lose confidence on the health care system because of treatment failures and resistance to artesiminin antimalarials [11]. These acts will soon put the world public health system in trouble and because of this we may be setting
a stage for the worst crises in the world public healthcare system. The resistance of malaria parasites to the artesiminin derivatives has been reported by WHO in South East Asia in countries such as Myanmar, Cambodia, Vietnam, Thailand and Laos (WHO 2014). The counterfeiters are very sophisticated in their art. Their product could easily fool any government pharmacist or inspector in the field. They fake genuine blister packs from manufacturing dates to expiry date [7, 12]. The most painful is in the bid to make more profit they have very

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cheap and dangerous excipients some even are carcinogenic such as melamine and safrole. Some cause kidney failure [12]. These fake and counterfeit products are also found in Tropical or Sub-Sahara Africa. To check this influx simple methods must be developed, since the cost of acquiring highly technical analytical equipment is prohibitive, the cost of operating and servicing these equipment are equally out of the reach of the poor malaria endemic countries. A typical example is that hi-tech analytical equipment donated by WHO and other donors are moribund by epileptic power supply. Artesunate and Dihydroartemisinin are officially assayed by titrimetry, UV spectrophotometric and HPLC. Some workers have attempted to develop new simple methods for the assay of these antimalarials. Some of these methods are quite simple, sensitive and reproducible; while others though sensitive and reproducible have some obvious faults and suffer from one technical problem such as tight pH control, heating requirements and tedious organic solvent extraction which could be hazardous to the analyst and the environment [13, 14],. The artesiminin derivatives lack conjugated Double bonds and isolated basic centers such as nitrogen centre that can make them active in the UV-vis region but with derivatization reaction this can be achieved. In this case Alcohol Ammonia solution generated *in situ* by the reaction of Ammonium Nitrate and Sodium Hydroxide opens up the lactone ring of the Artemisinin derivation making them to yield products that can be assay spectrophotometrically.

2. MATERIALS AND METHOD

2.1 Materials

2.1.1 Equipment

All spectral measurements were carried out using spectro Uv-vis spectrophotometr Uv – 2500 made by Labomed Inc, USA. All weighing were done using Metlar electronic balance (0.001 – 200g).

2.1.2 Reagents

All reagents were of analytical grade with excellent shelf life. Sodium Hydroxide 0.1M was freshly prepared by desoiving 4.0g of the sodium hydroxide (BDH England in 100ml of distilled water and diluted appropriately to make 0.1M of sodium hydroxide. Ammonium Nitrate 0.1M was freshly prepared by drenching 200g of the Ammonium Nitrate BDH – England in distilled water and diluted appropriately to obtain 0.1M solution of Ammonium Nitrate. Absolute ethanol obtains from BDH England.

2.1.3 Drug Solutions – standard solution

Dihydroartemisinin powder (DHA) and Artesunate powder was kindly provided by the Director of Pharmaceutical services University of Uyo Teaching Hospital (UUTH), Uyo and was used as provided. The standard drug solution of DHA and ART were prepared by carefully transferring 100 µg of the drug (DHA or ART) into a 100 ml capacity volumetric flask containing 20 ml of ethanol (water in the case of ART). This was shaken vigorously to dissolve the drug completely and made up to the 100 ml mark using ethanol (or distilled water in the case of ART). The resulting drug solution was dilute appropriately to obtain a drug concentration of 1mg/ml. This standard drug solution was further diluted to obtain a working concentration of 200 µg/ml for DHA and 100 µg/ml for ART.

2.2 Methods

2.2.1 General Procedure

Different aliquot of the drug 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 with the drug concentration of 200 µg/ml of DHA was carefully transferred into a series of 10 ml capacity volumetric flask using a micro burette. The volume in the flask was brought up to the 6 ml mark. Then 3 ml of a mixture of freshly prepared 0.1M sodium hydroxide and 0.1M Ammonium Nitrate was carefully transferred to the flask containing the drug standard solution. The content of the flask was made up to the 10 ml mark with absolute ethanol and shaken gently, and placed in a thermostatically controlled water bath at a temperature of 60^0C for 30 minute with gentle swirling to mix well. The absorbance was measured at 350 nm against a reagent blank similarly prepared without the drug. A calibration graph generated by plotting the absorbance against the concentration from where the concentration of the unknown was evaluated or deduced from the regression equation from Beer's law.

2.2.2 Procedure for the Determination of Artesunate

Different aliquot of ART 0.5 – 4.0 with drug concentration of 100 µg/ml was transferred to a series of calibrated 10 ml capacity volumetric flask using micro burette. All other procedure as discussed above for DHA was followed carefully and a standard calibration was generated from where the concentration of the unknown was determined or evaluated from the regression equation derived from Beers Law.

2.2.3 Procedure for the Determination of the Drugs in tablets

Twenty tablets of the drugs were selected randomly and weighed singly determining the weight uniformity. The 20 tablets were pulverized using a ceramic mortar and pestle to fine powder. An amount of the powder equivalent to 100 mg was weighed and carefully transferred a 100 ml capacity volumetric flask containing 10 ml of ethanol (or distilled water in the case of ART) and shaken vigorously; another 60 ml of ethanol was further added and shaken vigorously for another 20 minutes to extract the drug; then the content in volumetric flask was made up to the 100 ml mark and finally shaken for 25 minutes to complete the drug extraction. The resulting drug mixture was filtered using No. 40 Watman filter paper. The first 10 ml of the filtrate was discarded. The concentration of the resulting drug solution was 1mg/ml which was diluted stepwise to obtain a working concentration of 100 µg/ml using ethanol (or distilled water in the case of ART). A suitable aliquot was then prepared and analyzed as discussed in the general procedure described above.

2.2.4 Procedure for the Determination of Placebo Blank

Some Pharmaceutical excipient usually used in the production of tablets were used to prepare the placebo blank; the composition of the placebo blank were 4 mg corn starch, talc 5 mg, microcrystalline cellulose 1mg, lactose 4 mg, magnesium stearate 0.5 mg, Acacia 10 mg. The mixture bulked up to 100 mg using pure cassava starch. The resulting mixture was shaken / agitated vigorously for 10 minutes and homogenized using an electromechanical mixer to form a homogenous powder mass. Then from the resulting mixture, 50 mg was weighed out and carefully transferred into a 50 ml capacity volumetric flask containing 10 ml of distilled water, this was sonicated for 10 minutes, then 10 ml of distilled water was further added to the mixture in the flask and shaken further, finally distilled water was used to make up to the 50ml mark of the volumetric flask to form the placebo blank solution which was then analyzed using the procedure for tablets.

2.2.5 Procedure for the Analysis of Synthetic Mixture

To determine the synthetic mixture, 100 mg of the pure drug powder was weighed carefully mixed with 100 mg powder of the placebo blank as composed above. This mixture was homogenized using an electromechanical mixer to form a homogenous mass. A quality of the homogenous mixture equivalent to 100 mg weighed out and carefully transferred to a 100 ml capacity volumetric flask containing about 20ml of ethanol (distilled water in the case of ART), this was sonicated and shaken vigorously and the content of this flask was made up to the 100 ml mark of the flask using ethanol (distilled water in the case of ART). The resulting synthetic mixture solution was further diluted appropriately from where a suitable aliquot was analyzed using the general procedure as discussed above.

3. RESULT AND DISCUSSION

This is a typical amminolysis reaction using alcoholic ammonia. In this case ammonia solution generated *in situ* open up the lactone ring of the Artemisinin derivative, dihydroartemisinin and Artesunate, given a dysfunctional group an alcohol and an acid a typical hydroxyacid. The initial reaction is that of Ammonium Nitrate and sodium hydroxide react generating alcoholic ammonia solution *in situ*. Then the

$$NH_4NO_3 + NaOH \longleftrightarrow NaNO_3 + NH_3 + H_2O$$

In the presence of ethanol the actual reagent ethanol ammonia is produced

$$NH_4^+ + OH^- \longrightarrow NH_3 + H_2O$$

Lactone ring react with the alcoholic ammonia, this basic property of ammonia first break the ester bond and then react with the COOH group to form a dysfunctional group i.e. an alcohol group and amide. Remember the artemisinin have no conjugated double bonds or any isolated basic centers that will make them active in the UV-VIS region but by this derivation reaction double bonds are created and basic centers are also created. This is the window created at this point for the determination of these artemisinin derivatives. As a side reaction the endoperoxide bond of the artemisinin is cleaved to produce hydrogen peroxide which could also react with ammonia solution. This is a very remote possibility.

$$NH_4 + OH^- \longrightarrow NH_3 + H_2O$$
$$H_2O_2 + NH_3 \longrightarrow NH_2OH + H_2O$$

3.1 Method Development

This reaction was studied and optimized to obtain reasonably trusted results. This was done by keeping some experimental variables constant while varying the particular variable studied to observe its effect on the overall outcome of the experiment.

3.2 Heating Time of Reaction

The reaction time of this experiment was studied. As the temperature was kept constant and the heating time varied; it was discovered that the maximum time for the optimum absorbance was 30 minutes. So 30 minutes heating time was adopted. Further increase in the time made the result obtained to be erratic.

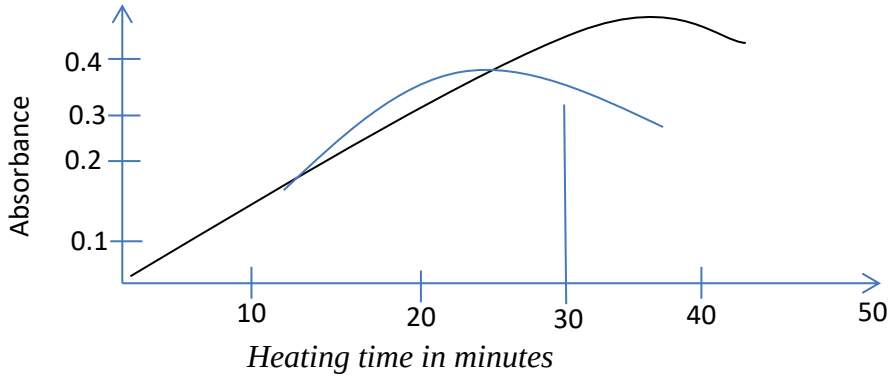

Fig. 1: Effect of heating time in the absorbance of the Amminolysis reaction

3.3 Temperature

The effect of temperature was studied while other variables were kept constant, it was discovered that as expected the absorbance increased as the temperature increased. It was further discovered that higher temperature gave results that were very erratic. A temperature of 60^0C with a heating time of 30 minutes was ideal for this reaction beyond that was erratic outcomes.

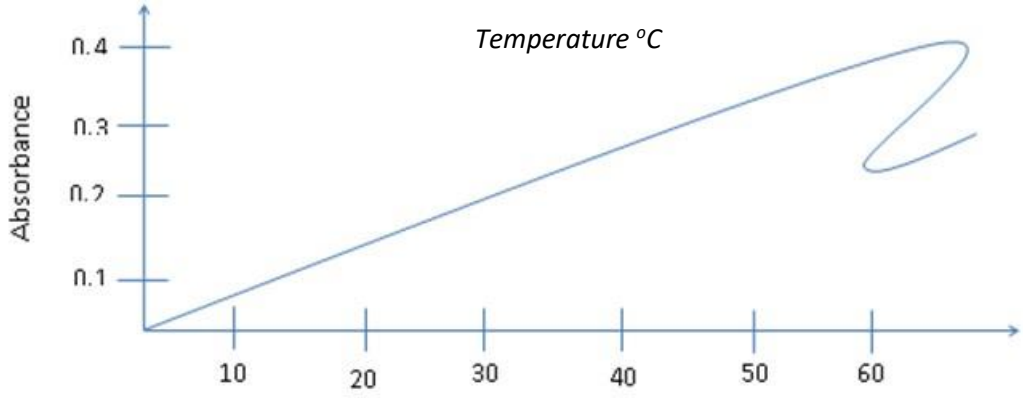

Figure. 2: Effect of temperature on the absorbance of the Amminolysis reaction based on aicoholic ammonia solution generated *in situ* with Artemisinin derivatives.

3.4 Effect of Concentration of Reactants

The concentration of the reactants were increased; other experimental variables were kept constant more ammonia solution was released *in situ* when the ammonium nitrate reacted with sodium hydroxide. The reaction became faster but so inconsistent absorbances were observed. The increases in the concentration of especially the sodium hydroxide lead to easier hydrolysis of the lactone ring. The absorbance became faster and higher but with very unreasonable absorbance values were noticed.

3.5 Method Validation

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

3.6 Linearity and Sensitivity

Under optimum experimental condition it was found that in fact as expected that the absorbance increased proportionally with the drugs concentration when measured at λmax 350 nm. Beer's Law was obeyed in the range of 5.0 – 100 µg/ml and 5.0 – 80 µg/ml respectively for DHA and ART. The calibration curved generated by

plotting absorbance versus drug concentration showed a typical linear graphs with straight line equations of A = MC to x where A is the absorbance, m is the slope, c is the concentration and x is the slope of the curve obtained via the least square method. The values of the slope, intercept, and correlation coefficient are recorded in table 1 above. The sensitivity of this proposed method were evaluated the molar absorptivity and the Sandell sensitivity were determined. The limits of detection (LOD) and quantification (LOQ) were determined based on the current ICH guidelines via the formula [15].

LOD = 3.3σ/5 and LOQ = 10σ/5

Where σ is standard derivation for the blank determination and S is the slope of the calibration curve. These values are also powdered in Table 1

Table 1: Analytical Parameters and Optical Characteristics of the Proposed Methods

S/N	Parameter	Value DHA	ARTS
1	λmax nm	350	352
2.	Beer's Law Limit (ug/ml)	5.0 – 100ug/ml	5.0 – 80ug/ml
3.	Molar absorptivity	2.65×10^3	2.0×10^3
4.	Sandel Sensitivity ug/cm^2	0.115	0.225
5.	Limit of Detection ug/ml	0.485	0.43
6.	Limit of Qualification ug/ml	1.27	1.32
7.	Regression Equation	A = mc + x	A = mc + x
8.	Slope	0.024	0.031
9.	Intercept	0.0007	0.0008
10.	Correlation coefficient	0.9997	0.9998

3.7 Accuracy and Precision

To evaluate the accuracy and precision of the proposed method six replicate determinations were determined at 3 concentration levels. This was performed 5 times within the same day (intraday) and for 5 consecutive days (interday). The accuracy was determined as percentage Relative Error (RE. %) using the equation.

$$\frac{\text{Amount found} - \text{Amount taken}}{\text{Amount taken}} \times \frac{100}{T}$$

While precision was evaluated as Relative Standard Derivation (RSD %) at 95% confidence level and at 4 degrees of freedom. The low values showed good accuracy and precision. The precision values evaluated as per RSD % were very low confirming the ruggedness of the method.

3.8 Application of the Method for the Assay of Commercial Tablets

The analytical applicability of this method was evaluated by its use in assaying the actual quality of DHA and ART in commercial tablets procured from local pharmacies in Uyo Metropolis in South-South Nigeria. The results obtained from the proposed method were statistically compared to official International Pharmacopoeal method via f-test (Variance Ratio test and student t test at 95% of confidence level and at 4 degrees of freedom. The results are recorded in table 6 showing that the calculated values were far lower than the official International Pharmacopoeal method.

3.9 Selectivity

The selectivity of this proposes method was determined using the results of the placebo blank and the synthetic mixtures which were described earlier. The placebo blank determination showed expressly that pharmaceutical excipients used in the tablets formulations had no effect on the results of the proposed method. Analysis of the synthetic mixture showed excellent recoveries within the range of 99.7$\pm$ 1.10% to 102.15 $\pm$ 0.89 showing high accuracy and non-interference of pharmaceutical excipients usually used when tablets are produced.

3.10 Robustness and Ruggedness

The robustness of this proposed method was determined by effecting small and deliberate changes or variation in some experimental parameters such as heating time and temperature on the absorbance value it was seen that minor changes in these parameters had no adverse effect on the absorbance. The Ruggedness of this proposed method was performed carrying out the experiment by two different analysts and using two different spectrophotometers.

Tables 2: Evaluation of Intraday and Inter day Accuracy and Precision for DHA

S/N	Amt of DHA taken	Intraday Precision and Accuracy			Interday Precision and Accuracy		
		Amt of DHA found	(Accuracy) RE%	(Precision) RSD %	Amt of DHA found	(Accuracy) RE%	(Precision) RSD %
1.	30	30.63	2.10	1.06	30.69	2.30	1.05
2.	60	61.59	2.65	1.30	61.78	2.96	1.20
3.	100	102.68	2.68	2.90	102.89	2.89	1.37

Tables 3: Evaluation of Intraday and Inter day Accuracy and Precision for ART

S/N	Amt of DHA taken	Intraday Precision and Accuracy			Interday Precision and Accuracy		
		Amt of DHA found	(Accuracy) RE%	(Precision) RSD %	Amt of DHA found	(Accuracy) RE%	(Precision) RSD %
1.	20	20.58	2.90	1.50	20.52	2.60	1.38
2.	40	41.18	2.95	1.40	41.17	2.92	1.62
3.	60	61.69	2.81	1.46	61.68	2.80	1.42

Table 4: Results of Analysis of tablets procured locally using developed method DHA

S/N	Tablets Brand Analyzed	Label Claim (mg)	Reference Method	Amount Found (Percentage of Label Claim) ±1.03
	DHA			
1.	Alaxin	60	110.0 ± 1.12	110.98 ± 1.20 F = 1.15 T = 1.48
2.	Santecxin	60	110.0 ± 1.16	111.0± 1.18 F = 1.03 T = 1.46
	ART			111.20± 1.30
3.	Lever Artesunate	50	110.0 ± 1.20	F = 1.17 T = 1.69
4.	Artesunate Nerus	50	110.0 ± 1.21	110.6± 1.18 F = 1.05 T = 0.87

Mean value of t_5 determinations. The value of tabulated at 95% confidence level and at 4 degree of freedom is 2.77, for F = 6.37

Table 5: Table of Recovery Studies via Standard addition Method

S/N	Tablets Studied	Amount of Drug (ug/ml)	Amount of Drug added (ug/ml)	Total Amount Found (ug/ml)	Recovery of Drug % ± SD
	DHA				
1.	Alaxin	40.20	20.0	61.00	104.0 ± 2.0
		40.20	40.0	81.10	102.0 ±1.0
		40.20	60.0	101.9	103.0 ± 1.09
2.	Santecxin	41.00	20.0	61.55	102±1.75
		41.00	40.0	81.40	101±0.50
		41.00	60.0	102.5	103± 1.25
	ART				
3.	Lever Artesunate	40.00	20.0	60.08	101.39±1.20
		40.00	40.0	80.01	100.05± 1.27
		40.00	60.0	99.98	99.97± 1.30
4.	Artesunate Nerus	50.10	20.0	70.65	102.70 ±1.63
		50.10	40.0	91.30	91.30 ± 1.57
		50.10	60.0	112.4	103.8± 1.92

3.11 Recovery Studies

The accuracy and the analytical performance of the proposed method were ascertained by performing recovery studies using standard addition method. This was performed by using a calculated amount of the pure drug powder to spike a pre-analyzed tablet powder at three different concentration levels and then analyzing the total three consecutive times via the proposed method. The percentage recovery value was found to be in the range of 99.97 $\pm$ 1.30% and 103 $\pm$ 1.09%; the values are recorded in table 5.

4. CONCLUSION

A simple, reproducible, precise, accurate and robust method has been developed for the determination of dihydroartemisinin and Artesunate. The method is adaptable in field stations and routine laboratories to effectively check the influx of fake and adulterated artemisinin derivatives being imported into Nigeria. The method is very simple as the chemicals and reagent used are not hazardous to the analyst and the environment.

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5. REFERENCES

[1] WHO Definition of Fake Drugs – WHO, 1998; Dr. Sabine Kopp (1992) Quality and Safety: Medicine Essential Medicines and Pharmaceutical Policies WHO.

[2] Coker H.A.B., Chukuani C. M., and Aina N.D. (2001). The Malaria Scourge Concept in Disease Management. Nigerian Journal of Pharmacy, 32:19-47.

[3] Rozendaal J, (2001) Fake Antimalarial Drugs in Cambodia Lancet 351 (923) 890

[4] Rozendaal (2006) Fake Antimalarial Drugs in Cambodia Lancet 351(923-980)

[5] Newton P.N., Dondorp A., Green M., White Maxay M., White NJ. (2003). Counterfeit Artesunate Antimalaria in Southeast Asia Lancot 362:169.

[6] Newton PN, Green M, Fernandez FM., Day NP. White NJ (2006). Counterfeit Drug Lancet Infectious Disease 6: 602 – 613.

[7] Karunamoothi, K. (2014). The Counterfeit Antimalaria is a crime against humanity: a systematic review of the scientific evidence. Malaria Journal 13(209); 1-13.

[8] WHO (2014) Global Malaria Programme status report on Artemisin resistance

[9] Dondorp et al (2009) Dondorp A.M., (2009) Artemisinin resistance in Plasmodium Falciparum Malaria, N Engl J. Med 2009 361(5); 455 – 67.

[10] Fairhust R.M. and Dondorp A.M. (2016) Artemisinin resistant Plasmodium Falciparum Malaria Microbiol Spectr. 2016; 4(3):10-1128/microbiolspec.1110-0013-2016.

[11] Adegoke O.A., Osoye A. (2011) Derivatization of Artesunate and Dihydroartemisinin for Colorimetric Analysis Using P- Methyl Aminobezaldehye. European Journal of Analytical Chemistry 2011: 6(2): 104 – 113.

[12] Ambroise – Thomas P. (2012). The Tragedy caused by Fake Antimalarial Drugs Mediterranean Journal of Haematology and Infectious Disease, 4:e2012027.

[13] Green M., Mount DL, Wartz R.A, (2001) Authentication of Artemether, Artesunate and Dihydroartemisinin Antimalarial tablets using simple colorimeter. Tropical Medicine International Health 6: (12) 980 – 1982.

[14] Attih, E. E. Usifoh E., and Umoh E. (2015) Novel Spectrophotometric Determination of Artesunate using Vanillin/sulphuric acid reagent. Journal of Chemical and Pharmaceutical Research 7(7) 1050 – 1058.

[15] International Committee for Harmonization (ICH) headlines Q_2 (R_1). Validation of Analytical Procedure: Text and Methodology, London.