Original Research Article

A Novel Derivatization Ultraviolet Spectrophotometric Method for the Determination of Dihydroartemisinin using *p*-Nitroaniline

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Abstract

Purpose: To develop a novel ultraviolet (UV)–spectrophotometric method for the determination of dihydroartemisinin (DHA) in tablets using p-nitroaniline (PNA) as a derivatizing agent.

Methods: Derivatization was based on the reaction between methanol solutions of dihydroartemisinin (DHA) and p-nitroaniline (PNA) in acid medium (1M HCl) at elevated temperature and for a short reaction time. Optimal detector response was obtained within 15 min when the reaction was carried out at 90 ^{0}C in a molar ratio of 2:1 (DHA:PNA). The method used for analysis was validated and a linear calibration curve constructed in the range of 30 – 100 µg/mL for the reaction mixture at an absorbance of 290 nm.

Results: Separation of adduct from PNA was better achieved on reversed phase thin layer chromatography (TLC) using acetonitrile : water (60:50) or on high performance liquid chromatography (HPLC) with retention times of 2.8 min for PNA and 5.8 min for the adduct. The limit of detection was 6 µg/mL. The method was precise and accurate in the range 100.70 - 100.96 %, with intraday and interday precisions of less than 2 % at concentrations of 40 and 80 µg/mL, respectively. The new method was applied to the assay of two brands of dihydroartemisinin tablets with accuracy similar to that of the International Pharmacopoeia (IP) UV-spectrophotometric method (p > 0.05).

Conclusion: The derivatization method is simple, direct, devoid of dilutions and inexpensive in terms of reagent requirements and analyte volume, and has a shorter reaction time, cpmpared with IP method. Based on the foregoing, the method can be adopted as an alternative to the official assay method for routine quality control of dihydroartemisinin tablets.

Keywords: Derivatization, Ultraviolet spectrophotometry, Dihydroartemisinin, p-Nitroaniline, Analysis, Assay, Quality control

INTRODUCTION

Dihydroartemisinin is a semi-synthetic derivative and the major metabolite of the parent drug artemisinin (Qinghaosu) obtained from the plant *Artemisia annua* [1]. Owing to the increase in drug resistance, the World Health Organization (WHO) in 2005 recommended that all uncomplicated *P. falciparum* infections be treated with artemisinin-based combination therapies (ACTs) but DHA and the other artemisinin

derivatives lack chromophores, and therefore are UV-transparent and require derivatization procedures before UV-spectrophotometry can be applied [1].

Analytical procedures for the detection and assay of DHA and the other artemisinin-based drugs have been developed during the last 25 years but these either involve multiple dilution stages, long reaction hours or costly gadgets. In this paper, we report a novel UV-spectrophotometric method for the assay of Dihydroartemisinin Tablets using *p*-nitroaniline as the derivatizing agent. The new method is simple, direct, devoid of dilutions and inexpensive in reagents and analyte volumes and has a shorter reaction time with minimal instrumentation. The method compares well with the current International Pharmacopoeia IP (2003) method ($p > 0.05$). Consequently, we deem the method suitable for routine quality control analysis, especially routine field assay of DHA-based drugs.

EXPERIMENTAL

Reagents and chemicals

Methanol, ethyl acetate, chloroform, acetone, hydrochloric acid, *p*-nitroaniline, (BDH, UK), acetonitrile (Philips and Hams England), hexane (Hopkins and Williams, Birmingham, England), liquid paraffin, distilled water, Alaxin® 60 mg (dihydroartemisinin tablets, GVS Labs, India) and Cotecxin® 60 mg (dihydroartemisinin tablets, Jiaxing Nanhu Pharm, China).

Equipment

UV-visible spectrophotometer (Unicam Aurora, Helios Scan Software v 1.1, Pye Unicam, England), Buck Scientific Fourier transform infrared spectrophotometer model M500, HPLC (HP series 1100), Mettler analytical balance H80 (U.K), water bath, UV lamp light sources, vortex mixer.

Derivatization

The derivatization reaction was based on the reaction between methanol solutions of dihydroartemisinin (DHA) and *p*-nitroaniline (PNA) in acid medium (1M HCl) at the elevated temperature of 90 °C.

Stock solutions of DHA (100 μg/mL) and PNA (500 μg/mL) were prepared for the derivatization reaction. In order to determine the optimum conditions for the derivatization reaction, the following critical response parameters were optimized; (i) the strength of HCl required for the reaction (ii) stoichiometric ratio of drug – reagent (DHA-PNA) (iii) temperature of the reaction and (iv) reaction time

Optimization of acid reaction condition

Aliquot volumes (1 ml) each of 0.5, 1, 2, 3, 4 and 5.0 M HCl were put into identical test-tubes with 1 mL of DHA stock solution (100 μg/mL) and 1 mL PNA stock solution (500 μg/mL) added to each test tube and vortexed for 10 s. 1 mL of methanol in a similar test tube served as blank. The test tubes were incubated in a water bath at 60 °C for 45 min. Again 1 mL each of DHA, PNA stock solutions and 0.2 mL 1M HCl were added in a test tube and vortexed for 10 s. The test tube was incubated in a water bath maintained at 60 °C for 45 min. After cooling to room temperature (25 °C), 4 mL of methanol was added and the UV spectrum recorded on a UV-Visible spectrophotometer (Unicam Aurora, Helios Scan Software v 1.1, Pye Unicam, England) between 190 - 800 nm.

Determination of the stoichiometric ratio of drug – reagent (DHA-PNA)

Equi-molar solutions (0.001M) of DHA (0.0284 g in 100 mL) and PNA (0.0138 g in 100 mL) were prepared in methanol. Into five different test tubes each containing 0.2 mL of 1M HCl, 0.3, 0.3, 0.3, 0.6 and 0.9 mL respectively of DHA were added and vortexed for 10 seconds. Thereafter 0.3, 0.6, 0.9, 0.3, and 0.3 mL respectively of PNA were added and again vortexed for 10 seconds. Five test tubes serving as controls and each containing same constituents except for DHA (which was replaced with methanol) were also prepared. All ten test tubes were incubated in a water bath at 60 °C for 45 min. The test tubes were left to cool at room temperature and 1 mL of methanol added. 0.5 mL was withdrawn into a tube, diluted with 3 mL of methanol and absorbance determined at 290 nm using a UV-visible spectrophotometer.

Optimization of reaction temperature and time

The temperature and time for optimum reaction were determined. Reaction mixtures containing 0.6 mL of DHA (10^{-3}M), 0.3 mL of PNA (10^{-4}M), and 0.2 mL of 1M HCl were vortexed for 10 s and incubated at varying temperatures ranging from 30 to 100 °C for 30 min. Methanol (1 mL) was added to each test tube to terminate the reaction and aliquots (0.5 mL) were withdrawn into sample tubes and again diluted to 3 mL with methanol and the absorbance of each sample tube was taken at 290 nm. Each reaction was

carried out in triplicate. The optimal reaction time followed the same procedure. Reaction tubes were incubated at 90 $^{\circ}$C and reaction was terminated by adding 1 mL methanol at 5, 10, 15, 30, 45 and 60 min, respectively. 0.5 mL of each test tube was withdrawn, diluted to 3 mL with methanol and absorbance reading taken at 290 nm. The test was also carried out in triplicate.

Determination of Rf values in different solvent systems

The aim was to compare the R_f values of the DHA–PNA adduct and the reagent in various solvent systems and to confirm the evidence of a new adduct formation. To 1 mL DHA (100 µg/mL), 0.2 mL 1M HCL and 0.1 mL PNA (500 µg/mL) solutions were added in a tube and vortexed for 10 s. In a second tube 1 mL of methanol, 0.2 mL 1M HCL and 0.1 mL PNA solution (500 µg/mL) were added and vortexed for 10 s. The two test tubes were then incubated at 90 ^{0}C for 15 min. The test tubes were cooled briefly and 3 mL of ethyl acetate and 3 mL of water were added and mixed again. From the ethyl acetate layer of each test tube about 5 µL was removed with capillary tube and spotted on silica gel plates separately. The following solvent systems were adopted during the investigation:

Normal phase – (i) ethyl acetate: methanol (6:4) (ii) ethyl acetate: hexane (5:5) (iii) chloroform: toluene: acetone (65:25:10) (iv) chloroform: methanol (95:5) (v) methanol: strong ammonia (100:50), (vi) liquid paraffin: ether (5:5).

Reverse phase – Acetonitrile: water (60:40, 40:60, 45:50, 60:50, 50:50 and 55:50)

IR Investigation

The aim of the investigation was to confirm the formation of a new chemical entity by the reaction between *p*-nitroaniline and dihydroartemisinin. *To* 5 mL of 1 mg/mL DHA solution, 0.2 mL 1M HCl and 2.5 mL of 1 mg/mL PNA solution were added in five test tubes and vortexed for 10 s. The test tubes were incubated at 90 ^{0}C for 15 min and thereafter allowed to cool to room temperature. To each test tube, 3 mL of dichloromethane was added and mixed, and same volume of water was added and mixed again. The dichloromethane layers were carefully withdrawn into petri dishes, using a pipette, and allowed to dry in air. The powdery adduct was further dried in an oven at 50 ^{0}C and this powder along with the PNA powder were scanned on an IR spectrophotometer.

Determination of Rt value in specified solvent system

The aim of the investigation was to find a suitable column and solvent system that will separate the DHA-PNA adduct and *p*-nitroaniline (PNA). The adduct was prepared using 1 mL DHA (100 µg/mL) solution, 0.2 mL 1M HCl and 0.1 mL PNA (500 µg/mL) solution vortexed for 10 s and the reagent was made using 1 mL of methanol, 0.2 mL 1M HCl and 0.1 mL PNA solution (500 µg/mL) vortexed for 10 s. Both tubes were incubated at 90 ^{0}C for 15 min, cooled to room temperature and 3 mL of ethyl acetate was added to each mixture, followed by addition of 3 mL of water. From the ethyl acetate layer, 100 µL were removed into separate sample bottles, allowed to dry in air and then covered and stored at –20 ^{0}C prior to HPLC analysis. For HPLC analysis, each sample tube had 0.5 mL methanol added into it and 20 µL was injected into the column.

Method validation

At the optimal analytical conditions described above, the calibration lines were obtained using standard solutions of 30, 40, 50, 60, 80 and 100 µg/mL. DHA. Linear regression analysis was used to calculate the slope, intercept and coefficient of determination (r^2) of each calibration line. The limit of detection was computed from the calibration equation as the analyte concentration giving a signal equal to the blank signal plus three standard deviations of the blank [18]. The accuracy and precision of the method were determined as documented by the United States Pharmacopoeia (USP) [17]: Multiple analysis (n = 5) of DHA at two different known concentrations (40 and 80 µg/mL) were analysed daily (intra-day) and repeated for three days (inter-day) using the optimized analytical method. The results were computed as mean ± standard deviation for both intra-day and inter-day analysis. Mean value within 15 % of the actual concentration value indicates the accuracy of the method and mean value within 15 % of the coefficient of variation ascertains the precision of the method.

Assay of dosage form

Two brands of DHA tablets, Cotecxin$^{\circledR}$ (60mg) and Alaxin$^{\circledR}$ (60mg) were used for the assay. An amount of powdered tablets (Alaxin$^{\circledR}$) equivalent to 10 mg of DHA was weighed and dissolved in 10 mL of methanol. After 15 min, the suspension was filtered to remove the excipients and the

filtrate was labeled as the stock solution (1 mg/mL). A portion of this stock solution (2 mL) was measured into a 20 mL volumetric flask, diluted to volume with methanol to give a 100 µg/mL working solution. Then 0.5 mL of the Alaxin® working solution was measured into four different test tubes and diluted to 1 mL with methanol to give a 50 µg/mL solution. To this, 0.2 mL of 1M HCl was added and vortexed, followed by 0.1 mL of 500 µg/mL PNA solution and vortexed again. The tubes were incubated at 90^{0}C for 15 minutes. The reaction was quenched with 3 mL of methanol and absorbance taken at 290 nm. The assay procedure was repeated for Cotecxin® tablets and these two tablets were also assayed using IP (2003) method. Both methods were compared and a two–tailed probability value ($p \leq 0.05$) was considered to be significant.

Statistical analysis

Statistical analysis was carried out by Student t-test (using Microsoft Excel 2007) when comparing the two assay methods with significance value set at $p \leq 0.05$.

RESULTS

Stoichiometric ratio of drug – reagent (DHA-PNA)

The coupling reaction of dihydroartemisinin and *p*-nitroaniline solution was optimum at ratio 2:1 of drug to reagent (Figure 1) and formed an intense yellow adduct under all coupling conditions tested.
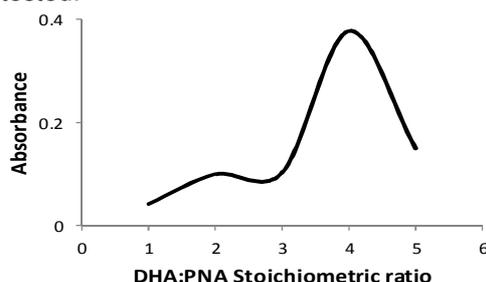
Figure 1: Plot of absorbance against DHA:PNA stoichiometric ratio (1 = 1:1; 2 = 1:2; 3 = 1:3; 4 = 2:1; 5 = 3:1 DHA:PNA respectively)

Optimum reaction conditions, temperature and time

The spectrum of the adduct indicated a hyperchromic shift with respect to that of the reagent with peaks at 205, 230 and 380 nm. The strength of HCl ideal for the reaction was 1M and the absorbance of the adduct was highest at 90^{0}C and 15 min (Figure 2).
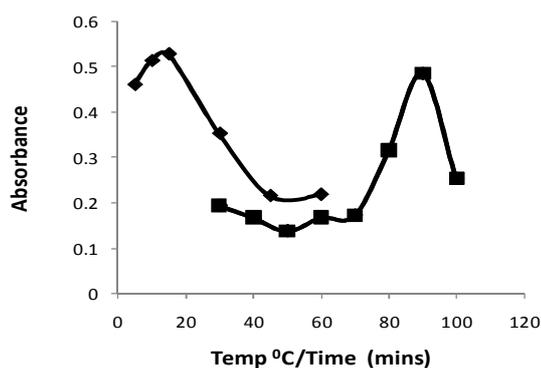
Figure 2: Plot of absorbance against temperature (^{0}C, ♦) and time (min, ■)

Chromatographic analysis showed that reversed phase systems gave a better separation of adduct from the reagent on both TLC and HPLC with acetonitrile:water ratios of 60:50, 40:50 and 55:50 giving good separation. The IR spectrum of the dried adduct was different from that of the reagent, thus confirming the success of the derivatization reaction (Figure 3).

Linear regression equation for the calibration line under optimal reaction conditions is $y = 0.0023x - 0.0018$ with r^2 of 0.9987. The limit of detection was 5.9 µg/mL. Validation studies show that the new method is precise and accurate. The intraday and interday precision was in the range 1.13 – 1.52 % and 0.84 – 1.13 % respectively. The mean percentage accuracy of the method was in the range 100.79 – 100.96 %.

Comparative analysis of the two brands of dihydroartemisinin by the official (IP 2003) and the new method is shown in Table 1. Using student t-test there is no significant difference between the official and the new method reported ($p = 0.08$).

Table 1: Content (mean ± SD) of dihydroartemisinin tablets obtained by the two assay methods

Brand	Proposed method (%w/w)	Official method (%w/w)	*P* value
Alaxin®	94.31± 2.12	95.47 ± 0.67	0.293779
Cotecxin®	97.35 ± 0.50	96.37 ± 0.58	0.082566

DISCUSSION

Figure 3 shows the IR spectrum of the reagent – PNA alone and when viewed simultaneously with the spectrum of the adduct, DHA-PNA. The adduct formed by the reaction of dihydro-artemisinin with *p*-nitroaniline has an IR spectrum with prominent peaks in the fingerprint region (1500- 600 cm^{-1}) that clearly differ from those of
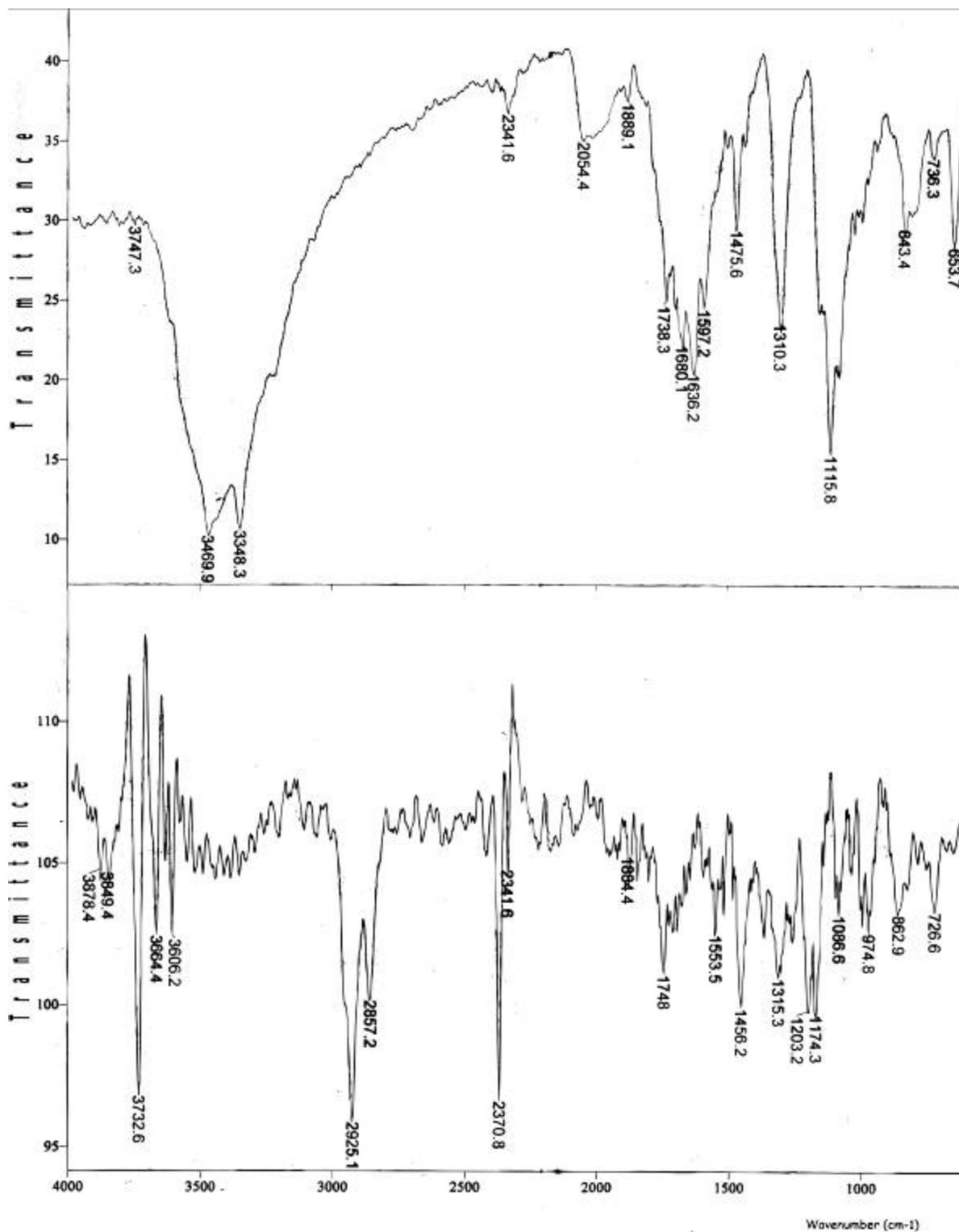
Figure 3: IR spectrum of PNA and DHA-PNA adduct viewed simultaneously
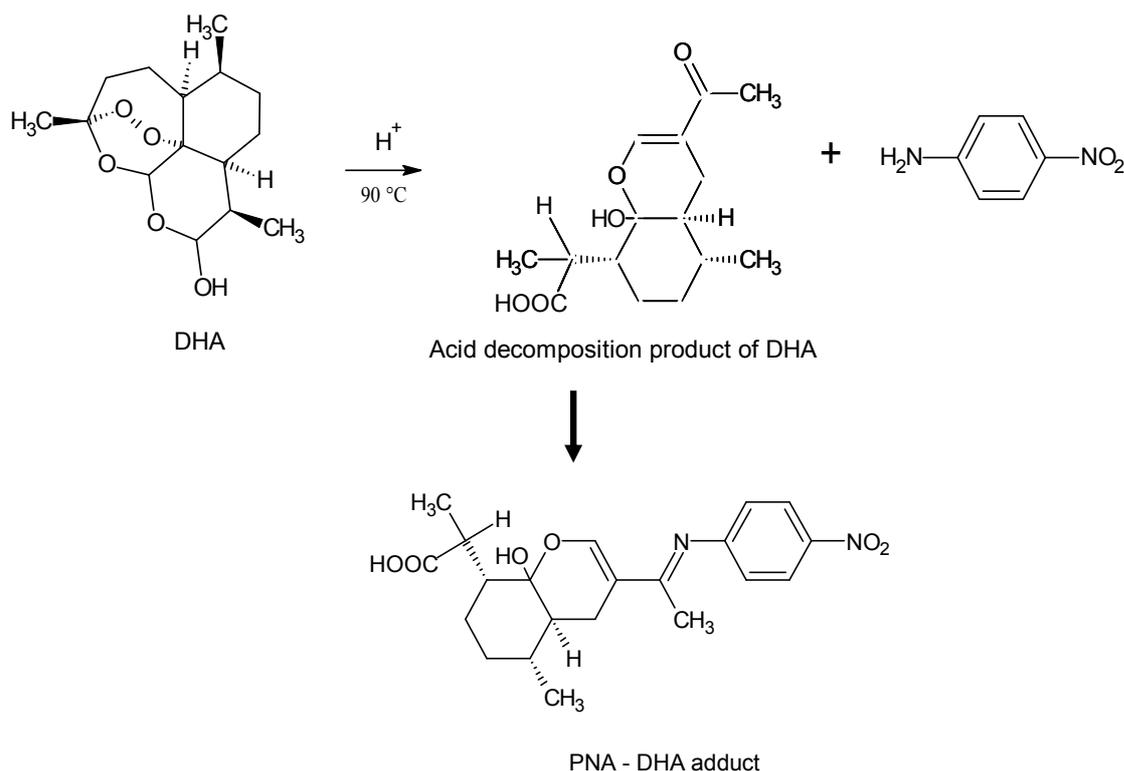
Figure 4: Proposed mechanism of reaction between DHA and PNA

the reagent spectrum. Notably, the UV absorption spectrum of adduct showed a hyperchromic shift relative to that of the reagent (not included). A possible mechanism of reaction for the adduct formation is, first, acid decomposition of DHA to form a product containing a carbonyl function which in turn reacts with the amine to generate an imine adduct. The presence of hyper-conjugation of bonds in the adduct thus formed accounts for the intense yellow color of the new compound (Figure 4). These considerations collectively suggest that the derivatization was successful and that a new chemical entity was formed. Indeed both the TLC and HPLC outcomes further confirm the formation of an adduct.

Using Job's method of continuous variation, a plot of absorbance against drug-reagent stoichiometric ratio showed maximum absorbance at a drug-reagent ratio of 2:1. While the possibility of multiple products can be suggested, TLC results did not indicate this. However, the HPLC separation on Hypersil column gave two separate peaks suggesting the formation of two main products. This corroborates the stoichiometric ratio determination. The optimum temperature and time for the reaction were 90 °C and 15 min, respwctively which compares with the reaction of artesunic acid with *p*-nitrobenzyl N, N diisopropylisourea at 80 °C for 2 h as reported by

Zou *et al* [15] and others [16]. However, their reaction time of 2 h is much longer than the 15 min recorded in this work. Also of importance is the 15 min reaction time in this experiment as against the IP method which requires at least 30 min reaction time [2].

A calibration curve constructed with our method at the absorbance at 290 nm, showed linearity in the range 30 - 100 µg/mL for DHA and 500 µg/mL for PNA, with an r^2 of 0.9987. The wavelength of 290 nm was selected because the two absorption maxima in the UV region (205 nm and 230 nm) were not suitable. Determination at 205 nm is subject to error since it is less than the cut-off point of methanol (210 nm), and at 230 nm, the peak is not visible at low concentrations of the drug and reagent. Our detection limit (6 µg/mL) can be accounted for by the reduced reactivity of artemisinin moiety since it lacks reactive functional groups.

Validation studies carried out on the new method showed that the proposed method is precise and accurate, thus making the method suitable for routine analysis of dihydroartemisinin tablets. The suitability of this new method for the analysis of dosage forms (tablets) was further quantified in the statistical comparison between it and the IP (2003) method for analysis of dihydroartemisinin tablets with a P-value = 0.08 , showing no significant difference, therefore implying their equivalence (*Table 1)*. On the

whole, the proposed method offers the advantages over the current official method of a shorter reaction time, and also is more direct, simpler, and consists of fewer dilution steps.

CONCLUSION

A validated novel UV-spectrophotometric method has been developed for the assay of dihydroartemisinin tablets. The method is low-cost, precise, accurate and simpler than the current official method. It is therefore recommended as an alternative method for routine quality control of dihydroartemisinin tablets

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