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Derivatization of Artesunate and Dihydroartemisinin for Colorimetric Analysis using *p*-dimethylaminobenzaldehyde

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Abstract

We set out to develop an alternative simple, accurate and precise method for the determination of artesunate and dihydroartemisinin in bulk samples and dosage forms. The method involves the reaction of the reactive methylene centres generated *in situ* from the acid decomposition of the artemisinin derivatives with *p*-dimethylaminobenzaldehyde (DMAB). DMAB was reduced to the purple-coloured alcohol and this was quantitatively used to estimate the concentrations of the artemisinin derivatives. The new procedure was carried out at 60^{o}C in less than 30 minutes with excellent calibration data. The method was accurate and precise and was found equivalent to the International Pharmacopoeia's method for the assay of these drugs. The method could find application as an alternative in-process and after manufacture method for the quality control of artesunate and dihydroartemisinin.

Keywords:

Artesunate, Dihydroartemisinin, *p*-dimethylaminobenzaldehyde, Colorimetric analysis

1. Introduction

Artemisinin is the antimalarial principle isolated by Chinese scientists from *Artemisia annua* L. Artemisinin is a drug used to treat multi- drug resistant strains of falciparum malaria [1]. Artemisinin (ginghaosu) is a sesquiterpene lactone with a peroxide bridge. It is poorly soluble in oils or water but the parent compound has yielded Dihydroartemisinin, the oil soluble derivatives artemether and artether and the more water soluble derivatives, sodium artesunate and artelinic acid. These derivatives have more potent blood schizonticidal activity than the parent compound and are the most rapidly effective antimalarial drugs known. They are used for the treatment of severe and uncomplicated malaria [2].

Several analytical methods have been reported for the determination of artemisinin and its derivatives in plant and biological samples. These methods include; TLC [3], HPLC with electrochemical detection [4-9], HPLC with UV detection [10-12], Enzyme-linked immunosorbent assay [13] as well as supercritical fluid chromatography [13, 14-15] among others. As relevant as these methods are many of them suffering from the disadvantages of extremely prolonged analysis time and utilization of sophisticated equipment which makes their application for routine analysis difficult. The artemisinin derivatives do not have any significant light absorption in the workable wavelength region of the UV-VIS spectroscopy and they do not have particular chemical groups that easily react with certain reagents to yield coloured products; however, they can be transformed by acid or base treatment to more

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reactive compounds such as enolate/carboxylates or α, β-unsaturated decalones [12, 16]. This transformation has been used as the basis for the determination of these drugs in dosage forms and biological fluids [17, 18, 19] using HPLC. The reactive methylene centres generated by acid or base treatment has also been used for the colorimetric detection of counterfeit artesunate, dihydroartemisinin [20] and artemether [21].

The ability of the reactive methylene centres, generated upon acid decomposition of the artemisinin derivatives, to reduce *p*-dimethylaminobenzaldehyde to the alcohol is investigated in this report as a means for the colorimetric determination of the artesunate and dihydroartemisinin.

2. Materials and Methods

Artesunate (ART) and dihydroartemisinin (DHA) chemical reference substances (Sigma), reagent grade *p*-dimethylaminobenzaldehyde (DMAB), sulphuric acid, ethylacetate, methanol, acetonitrile, 1, 4-dioxan and ethanol were purchased from BDH, Poole England.

Spectral measurements were recorded on a UV-VIS spectrophotometer (UNICAM uv1 v1.30, fitted with VISIONlite scan software version 2.2) while absorbance readings in the visible region were taken on a Jenway 6051 colorimeter.

2.1. Evidence of Reaction Between DMAB and the Artemisinin Derivatives

Sample solution of DMAB (0.3 % w/v) was prepared in 10 M Sulphuric acid while equimolar concentrations of the artemisinins (0.0201 mol L^{-1}) were made in methanol. 0.5 mL of each solution of ART and DHA were added into test tubes containing 0.5 mL DMAB. The immediate colour produced and after 20 minutes was noted.

Further evidence of reaction was obtained by thin layer chromatic analysis of the reaction mixture in both normal phase (ethylacetate: methanol, 9:1) and reversed phase (methanol: water, 4:6).

2.2. Selection of Analytical Wavelength

In order to select the appropriate wavelength for the coloured products, a spectrophotometric scan of the reaction mixture was done after diluting to 5 mL with acetonitrile. The absorption maxima of the reaction products relative to DMAB were noted.

2.3. Optimization studies

The temperature required for optimal colour formation was determined at 5 temperature levels of 30, 50, 60, 70 and 80 ^{o}C using two time levels of 5 and 20 minutes. The reaction mixtures were diluted to 5 mL with acetonitrile and the absorbances recorded at 540 nm and 470 nm for ART and DHA respectively on the colorimeter.

The time required for complete reaction was optimized at 60 ^{o}C using time levels of 0, 2, 5, 10, 15, 20, 25 and 30 minutes. Absorbance measurements were recorded as done above.

The amount of sulphuric acid required in preparing the DMAB solution for optimal colour development was optimized using acid concentrations of 0.625, 1.25, 2.5, 5, 6, 7, 8, 9, 10, 11 and 12 mol L^{-1}.

The reagent concentration required for optimal colour formation was determined using DMAB concentrations of 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 and 1 % in 10 mol $L^{-1}H_2SO_4$.

The diluting solvent required for the method development was determined using the optimized procedures as above and diluting the reaction mixtures each with water, methanol,

ethanol, *n*-propanol, acetonitrile or 1,4-dioxan. The absorbances were recorded in the visible region.

The optimal mole ratio required in the reactions was determined using Job's method of continuous variation. The optimal mole ratio was taken as one that gave the highest absorbance for the reaction products of DMAB with ART and DHA.

2.4. Validation studies

Calibration curves were prepared on each of three days using the optimized procedures described above. The slope, intercept and coefficient of determination (R^2) were determined by least squares method.

Recoveries of standard concentrations of ART and DHA from the reaction matrix were carried out on each of three successive days in order to determine the accuracy and reproducibility of the new method.

2.5. Dosage forms analysis

The new procedure was thereafter applied to the determination of ART and DHA in commercial brands of the tablets. The International Pharmacopoeia's [22] methods of alkalimetry (ART) and UV spectrophotometric method (DHA) were used as standard procedures. Data analysis was carried out using F-ratio and student's t-test at 95 % confidence level ($p = 0.05$).

2.6. Interference studies

The effect of commonly used tablet excipients was studied by carrying out sample determination from matrices containing each of lactose, starch, magnesium stearate, talc, gelatin and a mixture of the excipients.

3. Results and Discussion

Both ART and DHA formed an immediate purple colour with acidic solution of DMAB. The colours were stable for hours in the laboratory environment. TLC studies using both normal and reversed phases showed the formation a new compound more polar than the starting materials. Scanning on the UV spectrophotometer gave the spectra shown in Fig.1.

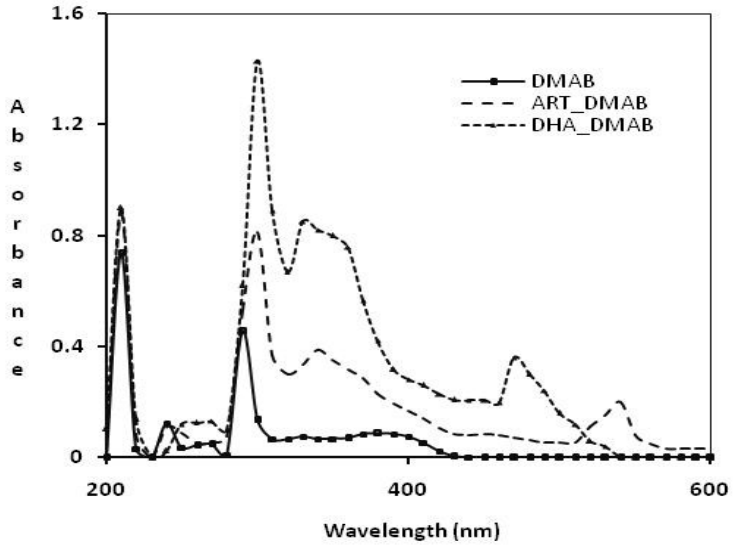


Fig. 1. Absorption spectra of DMAB compared to the reaction products with ART and DHA

As shown in Fig. 1, both ART and DHA produced new compounds with the reagent and pronounced bathochromic and hyperchromic shifts were observed. Optimal difference in absorptivity was found at 540 nm and 470 nm respectively for ART and DHA. The mechanism of the appearance of these new bands can be explained thus. All artemisinin derivatives lack chromophore that can absorb in the UV region and determinations have always focussed on HPLC with electrochemical detection or UV detection after pre-column [16, 19, 23] or post-column [24] reactions with acids or bases. The artemisinins were shown to be converted to reactive methylene centres upon contact with acid at elevated temperatures. The reactive methylene centres are known to readily release protons in chemical reactions. Thus the reactive centre generated by acid decomposition of ART and DHA reduces DMAB to form the alcohol as shown in Scheme 1. The formation of an alcohol from the reaction of DMAB with pyrrole derivatives having intact CH-group in the α or β position relative to the cyclic NH-group have been reported and DMAB reagent was utilised for the spectrophotometric determination of 2-phenylindole by Gillio-Tos *et al* [25]. The formation of [4-(dimethylamino)phenyl]methanol, reported in this paper, by the reaction of DMAB with acid decomposition products of ART and DHA is the first attempt at full colorimetric determination of these UV-inactive compounds with the aid of a common laboratory analytical reagent and presents the possibility of wide applicability since the artemisinins are the drugs of choice for treatment of malaria. The influx of several dosage forms of these drugs into the market will require a fast, simple and readily adaptable method especially in the third world countries where utilization of sophisticated equipment like HPLC are limited due to poor budget.

Optimization of temperature (Fig. 2a) and time (Fig. 2b) showed that optimal colour development occurs at 60^{o}C and 25 mins (ART) and 20 mins (DHA).

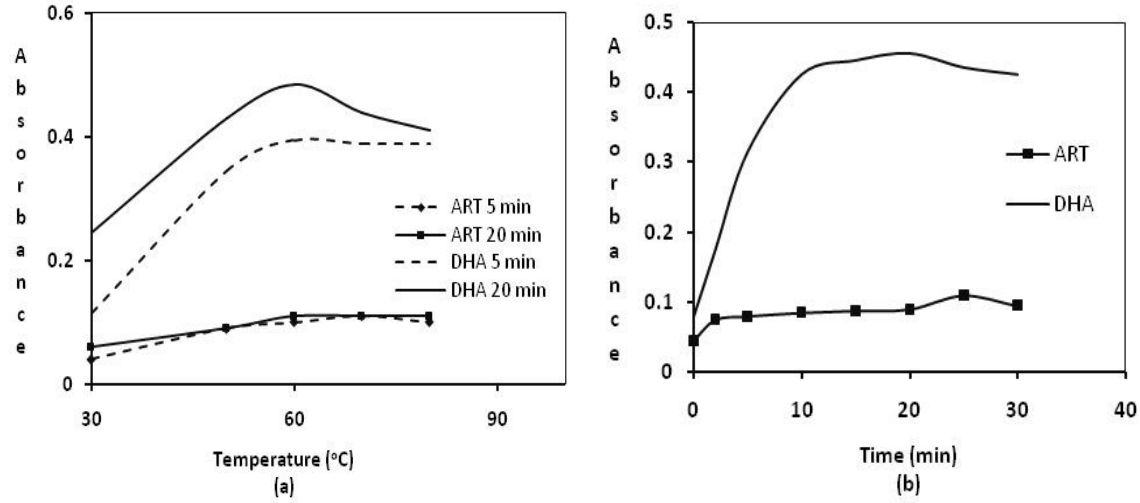
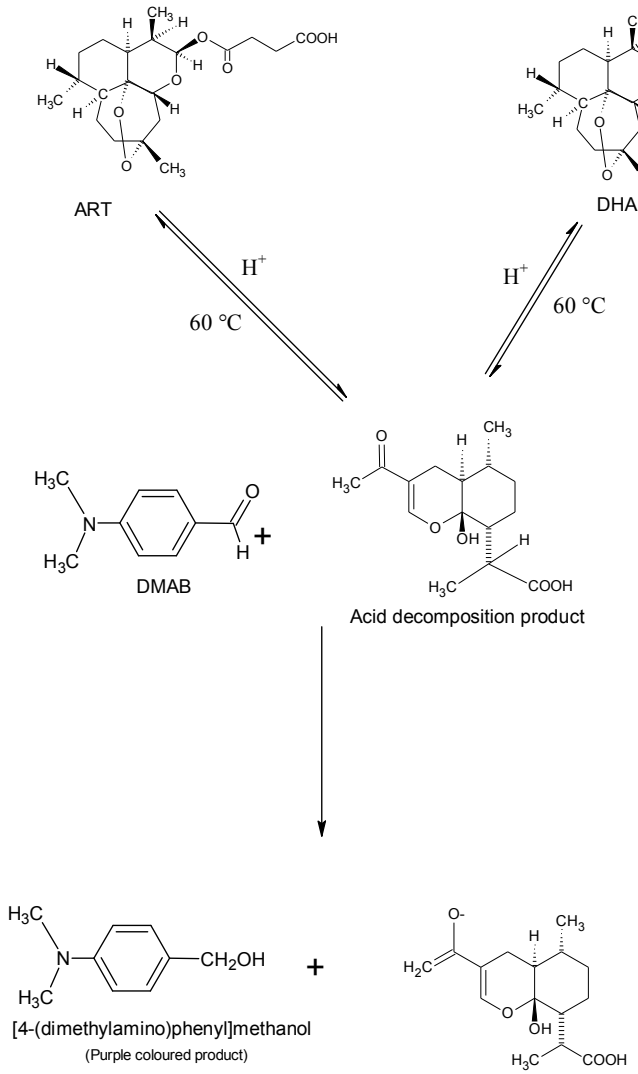


Fig. 2. Optimization of temperature (a) and time (b) for the reaction of DMAB with ART and DHA

Sulphuric acid concentration beyond 10 mol L^{-1} was found unnecessary at it imparted colour to the DMAB reagent. The optimization of acid concentration is presented in Fig. 3.

ART was found to produce optimal colour with DMAB when the latter was present at 1.0 % concentration and DHA required 0.05 % DMAB concentration (Fig. 4). Both reactants produced the maximum effects at mole ratios of 1:1.



Scheme 1. Reaction of ART and DHA with DMAB in acid

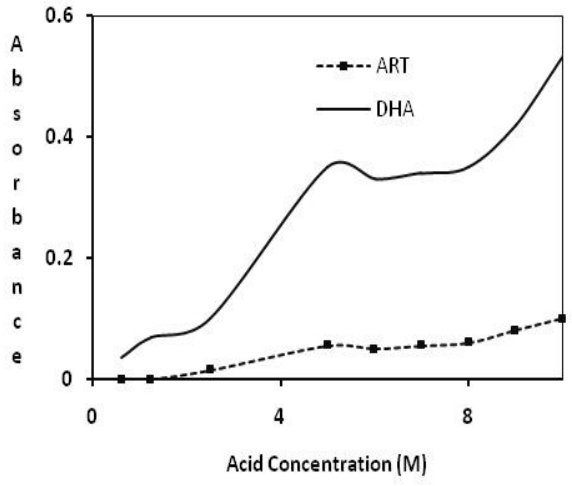


Fig. 3. Optimization of sulphuric acid for the reaction

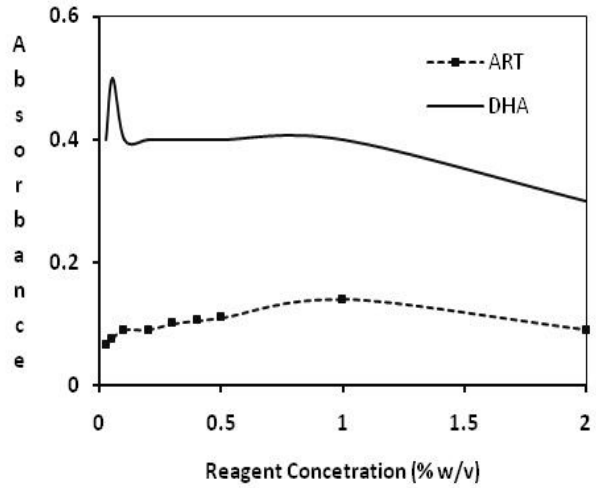


Fig. 4. Optimization of DMAB reagent concentration

Acetonitrile was found to be the best diluting solvent for the assay of ART while water proved the most superior solvent for the assay of DHA (Fig. 4). Validation studies were thereafter conducted on three successive days using the optimized conditions outlined above. Calibration lines were prepared over the three days period and the polled calibration data was used to generate the regression line equation for the assay of ART and DHA by the new colorimetric method.

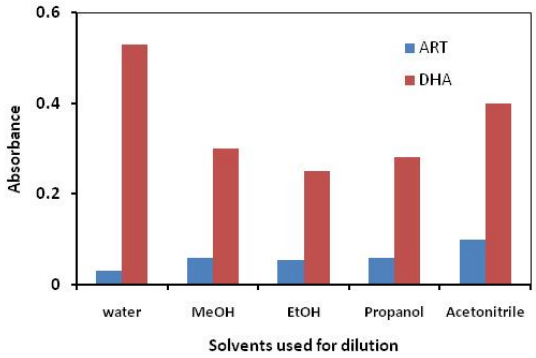


Fig. 4. Effect of diluting solvent on the absorbance of reaction mixture

Assays of ART were found linear over the concentration range of 15.4 – 77 µg mL^{-1} (R^2 = 0.9978) with limit of detection of 5.93 µg mL^{-1} and Sandell's sensitivity of 5133.5 ng mL^{-1} per 0.001A. The 95 % confidence intervals for the slope and intercepts are 0.001948 ± 0.0001687 and 0.019 ± 0.001485 respectively.

Molar absorptivity of (9.153 ± 0.59) x 10^2 $M^{-1}cm^2$ was obtained. The molar absorptivities of both DHA and ART are low as reported. But judging from the fact that both compounds do not have any light absorption in the workable region of the UV-VIS spectrum, a great advantage for full colorimetric analysis is envisaged. The molar absorptivity of ART was found to be higher than that of DHA. This can be explained based on the fact that higher molar concentrations of ART were adopted and coupled with the utilization of different wavelengths for the analysis. Similar validation parameters obtained for the assay of DHA are presented in Table 1 along with that of ART.

The accuracy and reproducibility of the new colorimetric procedure for the determination of artesunate and dihydroartemisinin from quality control samples are

presented in Table 2. Overall recoveries of ART and DHA are 101.80±1.53 and 101.25±1.60 % respectively.

Table 1: Analytical and Validation parameters for the reaction of ART and DHA with DMAB

Parameter	Artesunate (ART)	Dihydroartemisinin (DHA)
Beer's law limits, (μgmL^{-1})	15.4 – 77	11.4- 79.8
Limit of detection, (μgmL^{-1})	5.93	8.49
Molar absorptivity (mol^{-1} cm^2)	$(9.153 \pm 0.59) \times 10^2$	$(2.331 \pm 0.086) \times 10^2$
Sandell's sensitivity, (ng mL^{-1} per 0.001 absorbance unit)	5133.5	8000.0
Regression equation		
Intercept, a	0.019	-0.0127
Slope, b	0.001948	0.00125
Correlation coefficient, r	0.9989	0.9991
Coefficient of determination, R^2	0.9978	0.9982
Confidence interval of intercept, α	1.485×10^{-3}	1.39×10^{-3}
Confidence interval of slope, β	1.687×10^{-4}	3.15×10^{-5}

Table 2. Accuracy and Reproducibility of the new colorimetric method[a]

Concentration statistics (μgmL^{-1})	Day 1*		Day 2*		Day 3*		Inter-day	
	%Mean recovery	% RSD	%Mean recovery	% RSD	%Mean recovery	% RSD	%Mean recovery	% RSD
				Artesunate				
15.4	102.21±1.56	1.53	104.99±1.68	1.60	102.48±1.43	1.40	102.77±2.67	2.60
30.8	100.83±0.83	0.82	99.17±2.50	2.52	101.11±0.78	0.77	100.30±1.85	1.84
61.6	102.92±2.09	2.03	101.25±0.42	0.41	101.25±0.42	0.41	101.81±1.48	1.45
				Dihydroartemisinin				
11.4	100.61±1.74	1.73	100.93±2.50	2.48	100.93±2.50	2.48	100.87±2.30	2.28
34.2	101.61±1.26	1.24	99.21 ±0.37	0.37	100.15±1.26	1.26	100.32 ±1.44	1.44
57.0	102.25±0.50	0.49	105.14 ±0.21	0.20	100.44±0.76	0.76	102.61±2.01	1.96

*n = 12, [a] overall recoveries of ART and DHA are 101.80±1.53 (RSD= 1.50%) and 101.25±1.60 (RSD = 1.58%) respectively

The recoveries of pure samples of ART and DHA from the methods gave good results on the three-day assessment. Precision were better with higher concentration levels as it is easier to measure the larger volumes.

The new procedure was applied to the determination of artesunate and dihydroartemisinin in three different commercial tablet samples of each drug. The International Pharmacopoeia (Vol. 5) methods were used as standard procedures. The results are presented in Table 3. There was found to be no significant difference ($p>0.05$) between the assay results of the new colorimetric method described in this report and the official

methods. Thus the new method is of equivalent accuracy to the official procedures and could be used as an alternative analytical method.

There was no interference from the commonly used tablets excipients except for gelatin which gave abnormally high figures for the recovery studies. This is excepted as our previous work has proved that gelatin gets hydrolysed into amino acid units which readily condense with DMAB to give hydrazones [26].

Some clearly recognizable advantages of this new method for the assay of the artemisinin derivatives are simplicity, utilization of readily available laboratory reagents, good accuracy and precision as well as freedom from commonly utilized tablet excipients. This proposed method is the first attempt at a full colorimetric determination of artemisinin derivatives. It holds promise for ready utilization especially in third world economics where sophisticated facilities like HPLC with electrochemical detection are not available.

4. Conclusions

The new method for the colorimetric determination of artesunate and dihydroartemisinin using acidified *p*-dimethylaminobenzaldehyde proceeded readily and the method was suitable to determine the drugs in both bulk samples and dosage forms. The method is of equivalent accuracy to the official pharmacopoeia's methods and could be used as a fast and simple alternative method for the quality control of these important clinically useful agents.

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