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Highly Sensitive Liquid Chromatographic Analysis of Artemisinin and Its Derivatives after Pre-Column Derivatization with 4-Carboxyl-2,6-Dinitrobenzene Diazonium Ion

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Summary. A highly sensitive and reproducible isocratic liquid chromatographic method has been developed for the analysis of artemisinin and its three commonly used derivatives (artesunate, dihydroartemisinin, and artemether). The method involves a precolumn derivatization reaction with 4-carboxyl-2,6-dinitrobenzene diazonium ion to produce azo adducts that are UV-active. The critical parameters for the derivatization such as temperature, reaction time, and reagent concentrations were studied and optimized. The chromatographic separations were carried out on a C-18 column with mobile phase consisting of acetonitrile–0.1% acetic acid (60:40) at a flow rate of 1 mL min^{-1}. UV detection was set at 254 nm. Dynamic linear calibration range was obtained at concentrations of artemisinins ranging from 0.26 to 1.44 μg mL^{-1}. The low limits of detections of artemisinin, artesunate, dihydroartemisinin, and artemether were found to be 0.091, 0.0125, 0.0489, and 0.0128 ng μL^{-1}, respectively. The developed methods were precise (RSD <3%) and accurate (% error <5%). The developed methods may find application in dosage form analysis and pharmacokinetic studies.

Key Words: artemisinin derivatives, 4-carboxyl-2,6-dinitrobenzene diazonium ion (CDNBD), precolumn derivatization, HPLC

Introduction

Malaria still remains one of the most debilitating diseases of the tropics with high morbidity and mortality [1]. Many of the strains of *Plasmodium* are now resistant to conventional therapies (chloroquine, pyrimethamine/sulfadoxine). The artemisinins are increasingly being used against multi-drug resistant malaria because of their effectiveness. This family of drugs is derived from the natural product artemisinin (ATS) obtained from the plant *Artemisia annua* L. and semi-synthetic derivatives artemether (ATM), artesunate (ART), and dihydroartemisinin (DHA).

The artemisinins do not have particular functional groups that easily react with certain reagents to yield UV active products, in a derivatization procedure. However, they can be transformed by acid or base treatment to more reactive compounds such as enolate/carboxylates or α,β-unsaturated decalones [2]. This transformation has been used as the basis for the determination of these drugs in dosage forms and biological fluids [3]. The reactive methylene centers generated by acid or base treatment have also been used for the colorimetric detection of counterfeit artesunate, dihydroartemisinin [4], and artemether [5] as well as HPLC techniques with precolumn derivatization [6, 7, 8] or post-column derivatization [9, 10].

Several analytical methods, such as thin layer chromatography [11, 12], HPLC with electrochemical detection [13–17], ultraviolet detection [9, 18], polarographic detection [19], thermospray [20], and electrospray [21] mass spectrometric detection, evaporative light scattering detection (ELSD) [22], and atmospheric pressure chemical ionization [23] have been reported for the qualitative and quantitative determination of artemisinin and its derivatives in dosage forms, biological fluids, and crude plant extracts. Some other recent methods for the assay of these drugs alone or in combination with other antimalarials have also been reported [24–28]. However, these methods still had some limitations related to the chromatography and detection in terms of resolution, time-consuming, and requiring prolonged derivatization.

Recently, we have demonstrated the ability of the reactive methylene centers to couple with 4-carboxyl-2,6-dintrobenzene diazonium ion (CDNBD) [29]. Varying shades of reddish brown color were produced and the formation of new compounds different from artemisinin derivatives and CDNBD was revealed on TLC. The developed spectrophotometric methods for the three artemisinin derivatives were found to be simple and accurate with reproducible results.

The purpose of the present study was to develop and validate a simple, isocratic, accurate, and highly sensitive HPLC–UV method for the analysis of artemisinins, in order to take advantage of the sensitive determination obtained in the UV spectrophotometric method. The high reactivity of CDNBD to form azo adducts with artemisinins was explored as a preliminary step to dosage forms analysis and related pharmacokinetic studies.

Experimental

Materials and Reagents

Artemisinin, artesunate, dihydroartemisinin, and artemether CRS (kindly donated by Professor Ying Li of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences, China, HPLC purity ≥98.5%), HPLC grade methanol, and acetonitrile were obtained from Merck (Darmstadt, Germany). Sodium nitrite, urea, sulphuric acid, orthophosphoric acid, ethylacetate, and sodium hydroxide were all of analytical reagent grade from Sinopharm Chemical reagent company, China. Water was purified from a Milli-Q water purification system (Millipore AS, Bedford, MA, USA).

Instrumentation

Chromatography was carried out using Varian Prostar equipped with a variable UV wavelength detector, autosampler, and a column thermostat. Operation and data acquisition were carried out through the Varian MS workstation Ver. 6.8 (Varian Associates, Palo Alto, CA, USA).

Preparation of Stock Solutions

The diazonium reagent (CDNBD) was prepared in nitrosyl sulphuric acid as previously reported [30] at a molar concentration of 9.14×10^{-4} M. Equimolar solutions of ATS (0.26 mg mL^{-1}), ART (0.36 mg mL^{-1}), DHA (0.26 mg mL^{-1}), and ATM (0.27 mg mL^{-1}) were prepared by dissolving the compounds, respectively, in 10 mL of glacial acetic acid at ambient temperature.

Chromatographic Conditions

A C-18 column (150 × 4.6 mm, 5 μm) (LUNA Phenomenex, USA) was used for separation. The temperature of the column was kept at 30 °C. All injections were performed using a 20-μL loop.

Mobile Phase

Optimized mobile phase consisting of acetonitrile–0.1% acetic acid was pumped through the column at a flow rate of 1.0 mL min^{-1}. The column effluent was monitored with the UV detector at 254 nm.

Precolumn Reaction between Artemisinins and CDNBD

500 μL aliquot of CDNBD reagent in a test tube were added into 50 μL of each of the artemisinin stock solutions. The immediate formation of reddish-brown color was observed. The sample tubes were allowed to stay at room temperature for 20 min to complete the reaction. The reaction solution was transferred into ice-bath, with 5 mL of ice-cold water and 500 μL 8.67 M NaOH added. The reaction mixture was shaken and then partitioned using 2 mL of ethylacetate. A 200 μL aliquot of the ethylacetate layer was transferred into glass tubes and evaporated to dryness using a gentle stream of nitrogen gas at room temperature. The residue was dissolved using 1 mL of HPLC grade methanol for further chromatographic analysis.

Validation Procedure

Calibration Curves and Standards

Calibration curves were prepared for each of the four artemisinin derivatives. Linear regression analysis was carried out using Origin Ver. 6.1. The lowest limit of detection (LLOD) and lowest limit of quantitation (LLOQ) were calculated from the pooled calibration data using the expressions $3.3\sigma/s$ and $10\sigma/s$, respectively, where σ is the standard deviation of the blank signal ($n = 4$) and s is the slope of the calibration curve.

Accuracy and Precision

Standard solutions of the four artemisinin derivatives were spiked into CDNBD reagent solution and each mixture was carried out under the optimized conditions. The recovery of each drug from the matrix was monitored. Assay precisions were assessed using the percent relative standard deviations (RSD). Intra- and inter-day precision of each concentration was carried out on two successive days with three replicates each.

Method Selectivity for Derivative Formation

The selectivity of the diazonium ion to azo-dye derivatization of the artemisinin derivatives was assessed by carrying out the reaction of the derivatives in blank medium. The blank medium was prepared by adding sodium nitrite in mixture solution of sulphuric acid and orthophosphoric acid with the same time period as the diazotization reaction described and stopping the reaction with the addition of urea to prevent the excess nitrous acid. The

precursor amine for CDNBD (4-amino-3, 5-dinitrobenzoic acid) was omitted in the preparation of the blank medium. Sample solutions of ATS, ART, DHA, and ATM were made at the optimized conditions and then chromatographic separations were performed as done before.

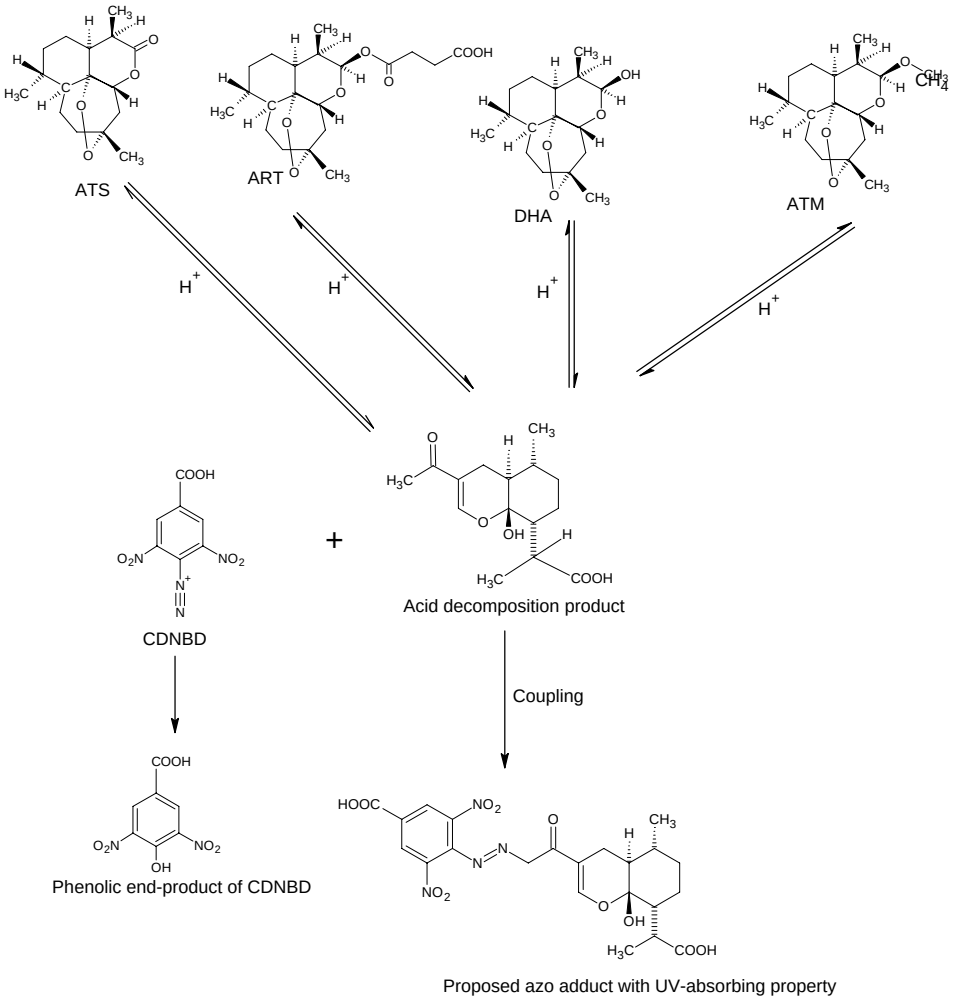
Derivative Stability

Sample solutions of derivatized artemisinins were prepared and the chromatographic runs were carried out for 4 h to study the stability of the azo adducts at room temperature.

Results and Discussion

Chemistry of the Precolumn Derivatization Reaction

The artemisinin derivatives produced immediate reddish-brown azo adducts with CDNBD and the intensity of the color increased at higher temperatures. The conversion of artemisinins to α,β-unsaturated decalones has been previously reported by Thomas et al. [18]. The decalones possess reactive methylene centers and have been adopted as means of detection of artesunate using fast red [4]. In the present study the ability of artemisinin and its three regularly used derivatives (ART, DHA, and ATM) to react with CDNBD upon acid decomposition *in-situ* was investigated. The CDNBD as a diazonium ion has been previously utilized as a highly reactive diazonium ion. Presence of two nitro groups and a carboxylic acid moiety at *ortho* and *para* positions to the diazonium ion makes it highly reactive. Judging from the chromatographic separation where azo adducts with similar retention times were produced at different rates, the reaction of CDNBD with the artemisinin derivatives is therefore proposed to take place through the acid-decomposition intermediate to form the azo adducts. This is presented in *Scheme 1.* Further justification for the new azo adduct was obtained from ^{1}H-NMR (not shown) where the only set of aromatic protons was that from the CDNBD molecule. When compared with the near zero absorptivity of the artemisinin derivatives, CDNBD will be a useful derivatizing reagent for these drugs. The preparation of CDNBD in acidic medium offers the advantages that the acid decomposition of the artemisinin derivatives will take place *in situ*. This will eliminate the need for prior decomposition by non-specific acid or base hydrolysis, before the azo adduct formation takes place unlike previously reported precolumn derivatization procedures.



Scheme 1. Proposed coupling reaction between CDNBD and artemisinin derivatives

Chromatographic Analysis and Optimization Studies

The goals of the chromatographic separation procedures were to achieve the resolution between the reagent residual (excess CDNBD) and the phenolic compound likely to be formed from the diazonium ion upon the reaction processing. Since the compounds to be separated are weakly acidic compounds, the first mobile phase selection consisted of acetonitrile–0.01 M phosphate buffer pH 3.0 (50: 50) at a flow rate of 0.6 mL min^{-1} and detection wavelength of 254 nm. The reagent peaks were not well resolved from the azo adducts produced by artemisinins with CDNBD. Also the retention times were close to the dead volume. Further optimization of the mobile

phase was then carried out by replacing the phosphate buffer with water maintaining the other conditions (C-18 column, 254-nm detection, mobile phase ratio 50:50 and flow rate of 0.6 mL min^{-1}). This new adjustment gave retention times that were far removed from the dead volume (CDNBD at 6.055 min and the azo adducts at times ranging from 5.013 to 5.849 min). Adjustment of the ratio of the acetonitrile to water at 65:35 improved the resolution between the peaks. While further adjustments to 60:40 and a flow rate of 1 mL min^{-1} produced the perfect separation between the residual CDNBD, the azo adducts, and the phenolic produced from the diazonium ion. However, the chromatographic bands had broad peaks. As shown in *Scheme 1,* all the compounds to be separated contain carboxylic acid functional groups. Thus the line broadening may be due to the ionization of this functional group. The water component of the optimized mobile phase composition was thereafter replaced with 0.1% acetic acid in water. This produced the best resolution and all the compounds were separated from each other and far away from the dead volume. The resulting chromatograms using these optimized conditions are presented in *Fig. 1.* As evident from the chromatograms, CDNBD residual has a peak at retention times varying around 5.2–5.4 min between the individual chromatographic runs. The artemisinin-CDNBD adduct has retention time of 7.45 ± 0.15, ART 7.454 ± 0.19, DHA 7.51 ± 0.25, and ATM 7.45 ± 0.15 min. The phenolic compound produced from CDNBD upon processing of the reaction mixture had reproducible retention times around 11.29–12.11 min between runs. The changes in the retention times for the phenolic end-product of CDNBD following successive chromatographic runs may be due to its potential for ionization on the column. The various chromatographic parameters are summarized in *Table I.* The retention factors, resolutions, and plate efficiencies all fall within acceptable ranges for good chromatographic runs. The reaction of CDNBD with the acid hydrolysis products of the artemisinins produced the same compound as evidenced from the chromatographic retention times that are very close to each other.

Table I. Chromatographic parameters for the azo adducts of artemisinin derivatives using the optimized separation conditions

Drug	Retention time (min)[a]	Efficiency[b]	Resolution[a]	Retention factor (k')[a]
Artemisinin	7.45 ± 0.15	881	3.1 ± 0.1	6.39 ± 0.22
Artesunate	7.454 ± 0.19	22,107	4.07 ± 0.25	5.74 ± 0.12
Dihydroartemisinin	7.51 ± 0.25	23,113	4.03 ± 0.41	5.97 ± 0.12
Artemether	7.45 ± 0.15	18,785	3.73 ± 0.12	6.09 ± 0.06

[a]Mean ± SD, n = 4; [b]normalized to 1 m of length of column.

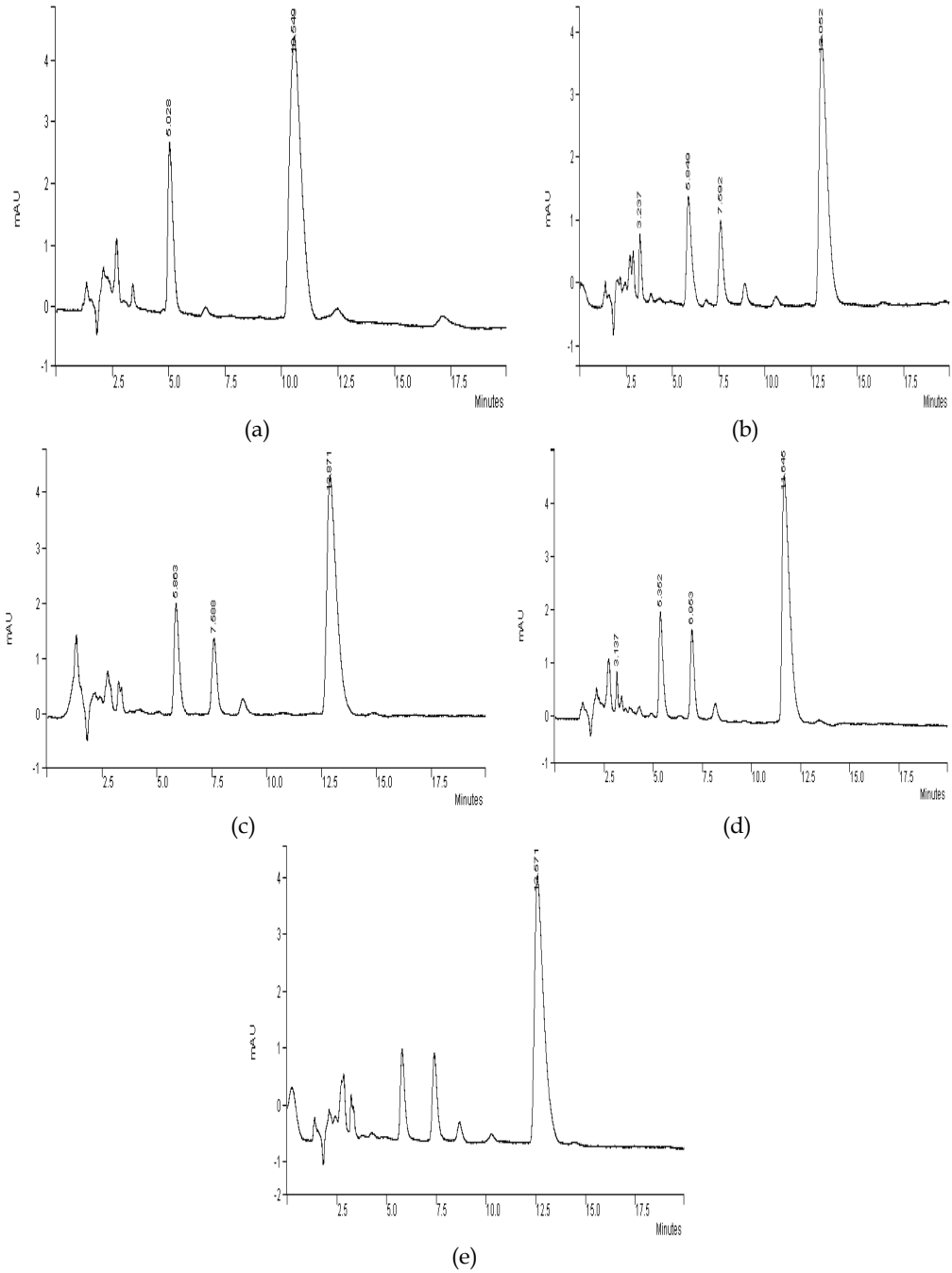


Fig. 1. Representative chromatograms using optimized chromatographic separation conditions; (a) CDNBD, (b) ATS (CDNBD–ATS peak 7.45 min), (c) ART (CDNBD–ART peak 7.45 min), (d) DHA (CDNBD–DHA peak 7.51 min) and (e) ATM (CDNBD–ATS peak 7.45 min)

Further attempt at varying the acetic acid concentration in the mobile phase using concentrations 0.05%, 0.2%, 0.5%, and 1.0% gave similar results. The influence of varying the concentration of acetic acid was more evident on the baseline than on the resolution and peak characteristics. Best baseline was still achieved with 0.1% acetic acid while there was no statistical significant difference among the mean peak areas when the results were analyzed using ANOVA ($p > 0.05$). The optimized mobile phase was therefore adopted as acetonitrile–0.1% acetic acid at a flow rate of 1 mL min^{-1}.

The efficiency of the extraction procedure into ethylacetate from the reaction mixture was judged by the clear solution of the aqueous layer which turns colorless. In our previous UV method development [29], TLC analysis of the reaction mixture revealed absence of any residual product following the optimal temperature and time of reaction.

Three response parameters that can influence the precolumn reaction were studied and optimized. These parameters are coupling temperature, time required for coupling at optimum temperature and the reagent concentration. Using the method of steepest ascent [31] and based on our previous study on the derivatization of artemisinin derivatives with CDNBD [29], four temperatures (25, 50, 60, and 70 °C) were investigated at time levels of 5 and 20 min. The result of the temperature required for coupling reaction is presented in *Fig. 2a*. The peak area for each of the four azo adducts increased gradually from 25 to 70 °C. Indeed some products were already present at room temperature and investigating the possibility of room-temperature precolumn derivatization reaction was attempted. However, the results were not consistent. Thus 70 °C which we had earlier in the spectrophotometric method achieved as optimum temperature was again adopted. Temperature (80 °C) higher than 70 °C led to instability of both the azo adducts and the CDNBD peaks. The conversion of artemisinin derivatives to the reactive methylene centers that can react with such groups as diazonium ions have always been carried out at elevated temperatures. The advantage of the present procedure is that the conversion of the artemisinins to compounds possessing the reactive methylene centers takes place in a one-step reaction. Thus the acid medium aided by the high temperature ensures the conversion in-situ. This holds the promise of improved accuracy since the method does not involve much sample work-up.

The optimum time required for coupling reaction to proceed to completion was carried out at 2, 5, 10, 15, 20, 25, and 30 min at 70 °C. The peak response is presented against the time of coupling in *Fig. 2b*. Artemisinin required 20 min for optimum reaction while 15 min was required by both DHA and ATM. Artesunate was optimally converted after 10-min reaction time. Both DHA and ATM attained a plateau in their responses at 15 min. While for artesunate a second peak produced at 25 min was less than the re-

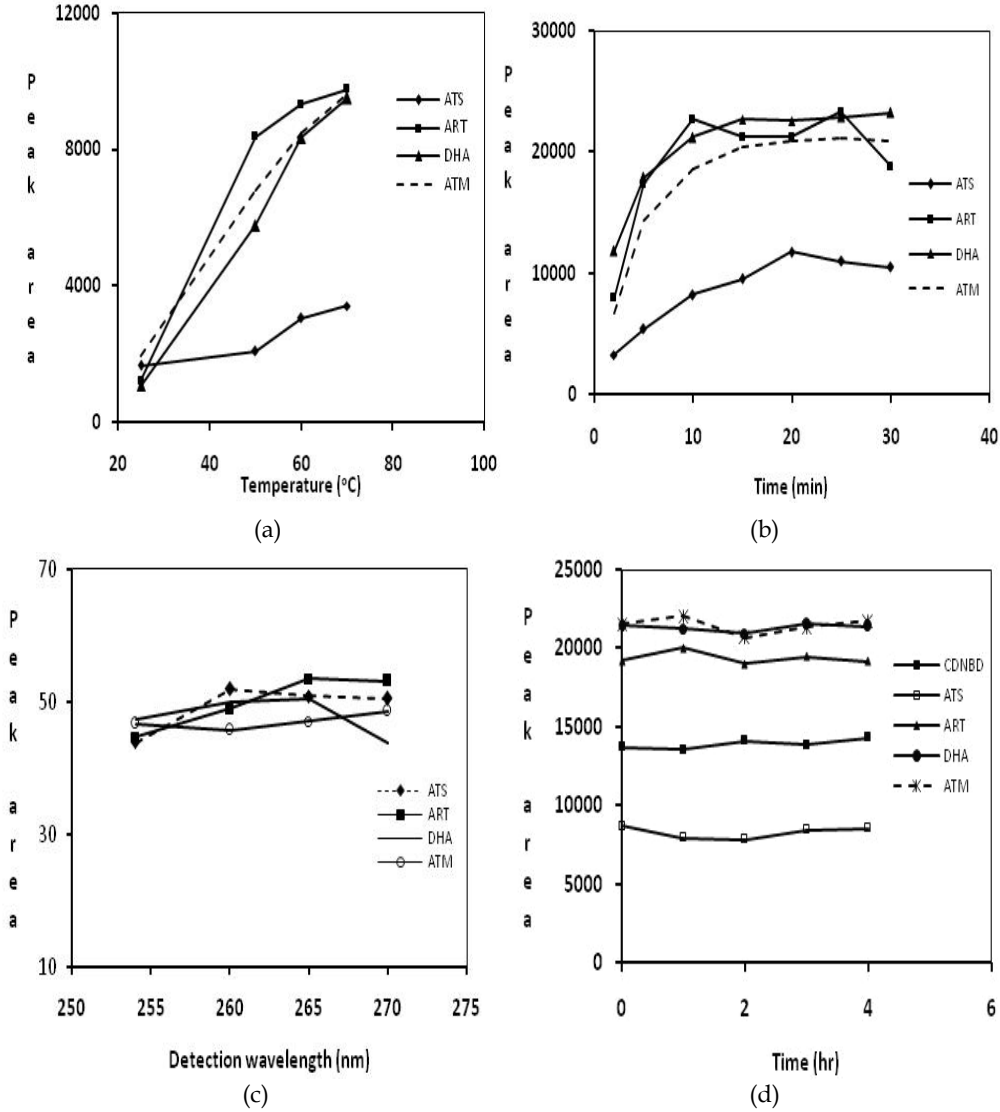


Fig. 2. Optimization studies; (a) temperature at 5 min, (b) coupling reaction time at 70 °C, (c) detection wavelength and (d) analytical signal stability

sulting one at 10 min. 10-min reaction time was therefore selected. Despite the fact that the same azo adducts are produced by the reaction of CDNBD with artemisinin and its three derivatives, the rate of formation of the azo adducts differs and the other functional groups present in the drugs appear to have influenced the conversion. The succinic acid moiety in ART did not drastically affect its hydrolysis into the reactive methylene derivative required for coupling since the reaction medium contains a stronger acid.

The absence of additional functional groups in the artemisinin molecule when compared to its three other derivatives makes it the least reactive. Indeed, the peak areas for equimolar concentrations with CDNBD gave the least values compared to the other three compounds.

The effect of detection wavelength on the chromatographic characteristics of the azo adducts was studied by varying the wavelength using 254, 260, 265, and 270 nm. The result is presented in *Fig. 2c*. Average peak values were monitored and then ANOVA was adopted to test the differences in the values of each of the four adducts. There was found to be no significant difference among the peak areas at the four wavelength values. Detection wavelength of 254 nm was therefore subsequently adopted. This will make the procedure readily adaptable to HPLC conditions in which a single wavelength detector is available.

The effect of reagent concentration on the peak characteristics was investigated using reagent concentrations of 5.74, 11.48, 22.45, 34.43, 45.9, and 57.38 μM. The reaction was carried through as done previously. In all cases the reaction medium was neutralized with equimolar amounts of 8.67 M NaOH. This neutralization step was adopted for all the reactions in order to protect the column from excess acid present in the reaction medium. The peak area varied with the reagent concentration and a plateau was obtained at a concentration of 34.43 μM (300 μL), which was thereafter used as the optimized reagent concentration. Further increment was observed at higher reagent concentrations but consideration of the peak widths showed that the peak base was broader hence contributing significantly to the higher peak areas.

Validation Studies

Dynamic Range and Limit of Detection

Calibration curves were prepared on each of three days for six data points. Linear regression analysis was carried out to determine the slope, intercept, and the correlation coefficient. The dynamic linear range was arrived at as the range that gave the highest slope, least intercept, and excellent correlation coefficient. The results generated for the four artemisinin derivatives are presented in *Table II*. All the four artemisinin derivatives yielded excellent calibration with the diazonium ion. Noteworthy is the low limits of the calibration range. These low concentration ranges have only been reported for such methods as HPLC coupled to mass spectrometer. The high sensitivity of this new method of determining artemisinins via azo dye formation with CDNBD is further corroborated by the extremely low limits of detection and limits of quantitation. For a 20-μL injection volume sub-nanogram quantities

of the artemisinins are detected. This holds promise for a ready application to pharmacokinetic studies of these artemisinin derivatives. In our described UV spectrophotometric method previously reported [29] low limits of detection were also recorded. This indicates the suitability of CDNBD to determine these derivatives. Despite the possibility of formation of the same or similar intermediate reactive methylene compound by the four artemisinin derivatives, the different stoichiometric concentrations relative to CDNBD may have resulted in the different dynamic calibration ranges obtained for the four derivatives.

Table II. Validation parameters for the chromatographic analysis of the artemisinin derivatives

Parameter	Artemisinin	Artesunate	Dihydroartemisinin	Artemether
Range (μg mL^{-1})	0.26–1.04	0.54–1.44	0.26–0.65	0.27–0.675
Regression equation[a]				
Intercept, *a* Slope, *b*	6179.62 1445.85	2518.95 9852.91	2107.92 15,947.88	937.90 19,043.97
Correlation coefficient, *r*	0.99998	0.99903	0.99915	0.9985
LLOD (μg mL^{-1}) LLOD (ng per 20-μL injection)	0.091 1.81	0.0124 0.249	0.049 0.978	0.0128 0.256
LLOQ (μg mL^{-1})	0.274	0.038	0.148	0.0387
LLOQ (ng per 20-μL injection)	5.48	0.75	2.96	0.774

[a] $Y = bX + a$, where Y is the peak area counts for concentration X μg mL^{-1}.

Intra-Day Accuracy and Precision

The stability of the calibration curve for each azo adduct of the four compounds within a given day was evaluated by carrying out repeated measurements of spiked samples spanning the entire dynamic working range of the calibration curve. Relative standard deviation was used as a measure of the precision of three replicate samples at each concentration levels while the error between concentration added and that found constitutes the accuracy. The results are presented in *Table III*. For the four azo adducts the RSD was

all less than 3%, indicating good reproducibility while low errors less than 5% were also recorded in the accuracy assessment.

Table III. Determination of intra-day accuracy and precision

Drug	Concentration added (μg mL^{-1})	Concentration found ± SD (μg mL^{-1})[a]	% Found	% RSD[b]	Error[c]
Artemisinin	0.39	0.416 ± 0.003	106.67	0.673	0.026
	0.65	0.627 ± 0.006	96.46	0.877	−0.023
	0.91	0.888 ± 0.020	97.58	2.25	−0.022
Artesunate	0.648	0.664 ± 0.017	102.47	2.53	0.016
	1.008	1.011 ± 0.016	100.30	1.58	0.003
	1.368	1.381 ± 0.011	100.95	0.796	0.013
Dihydroartemisinin	0.338	0.334 ± 0.007	98.82	2.10	−0.004
	0.468	0.469 ± 0.015	100.21	3.20	0.003
	0.598	0.627 ± 0.0097	104.49	1.55	0.029
Artemether	0.351	0.3403 ± 0.007	96.95	1.998	−0.0107
	0.486	0.4833 ± 0.002	99.44	0.352	−0.0027
	0.621	0.611 ± 0.010	98.39	1.65	−0.01

[a]n = 4; [b]precision; [c]accuracy.

Inter-Day Accuracy and Precision

Variation within replicate injections on two days was assessed and pooled together to give the inter-day accuracy and precision. The results are presented in *Table IV*. The repeatability as measured by % RSD was less than 5% for all the four artemisinin derivatives. Also, quantitation of the controls (accuracy) yielded results with low errors.

For both the intra- and inter-day accuracy and precision assessments, higher accuracies, and precisions were obtained for the mid and high concentration levels of the calibration curves.

Table IV. Assessment of inter-day accuracy and precision

Drug	Concentration added (μg mL^{-1})	Concentration found ± SD (μg mL^{-1})[a]	% Found	% RSD[b]	Error[c]
Artemisinin	0.39	0.4195 ± 0.0052	107.56	1.23	0.0295
	0.65	0.659 ± 0.029	101.38	4.40	0.009
	0.91	0.913 ± 0.030	100.33	3.28	0.003
Artesunate	0.648	0.637 ± 0.0302	98.30	4.74	−0.011
	1.008	0.988 ± 0.0261	98.02	2.64	−0.02
	1.368	1.414 ± 0.041	103.36	2.90	0.046
Dihydroartemisinin	0.338	0.334 ± 0.006	98.82	1.78	−0.004
	0.468	0.481 ± 0.016	102.78	3.41	0.013
	0.598	0.598 ± 0.025	101.51	4.17	0.009
Artemether	0.351	0.335 ± 0.008	95.36	2.38	−0.016
	0.486	0.477 ± 0.008	98.07	1.75	−0.009
	0.621	0.609 ± 0.011	98.07	1.93	−0.012

[a]n = 8; [b]precision; [c]accuracy.

Analytical Signal Stability

Due to the azo linkage, many azo derivatives are extremely light-sensitive and in dye industry, an assessment of light fastness is commonly done as a quality control procedure for the suitability of a given dye on fabric or as adjuvant. However, in analytical applications, protection from undue exposure to broad day light is often sufficient as a quality assessment. Since this is the first comprehensive application of azo dye derivatization of artemisinin using a diazonium ion, the analytical signal stability of the azo adducts was monitored at hourly intervals for samples kept at room temperature for a four-hour period. The results are presented in *Fig. 2d*. All the four azo adducts were stable for the period. Analysis of variance (ANOVA) yielded no significant difference in the peak areas for the analysis period. This therefore confirms that the azo adducts can be determined over a four-hour period without any significant loss of accuracy. The reagent signal was also discovered to be stable for the period of analysis. Further comprehensive stability studies at other temperature levels will be assessed in our future work.

Assessment of Method Selectivity

The selectivity of the azo coupling reaction between the artemisinin derivatives and CDNBD was investigated by carrying out the coupling reaction using the blank medium prepared without 4-amino-3,5-dinitrobenzoic acid (the precursor amine of CDNBD). This aspect was conceived from the standpoint of assessing the effects of the acidic medium on the artemisinin and since previous derivatization reactions have focused mostly on acid decompositions. The chromatograms generated are presented in *Fig. 3* for the

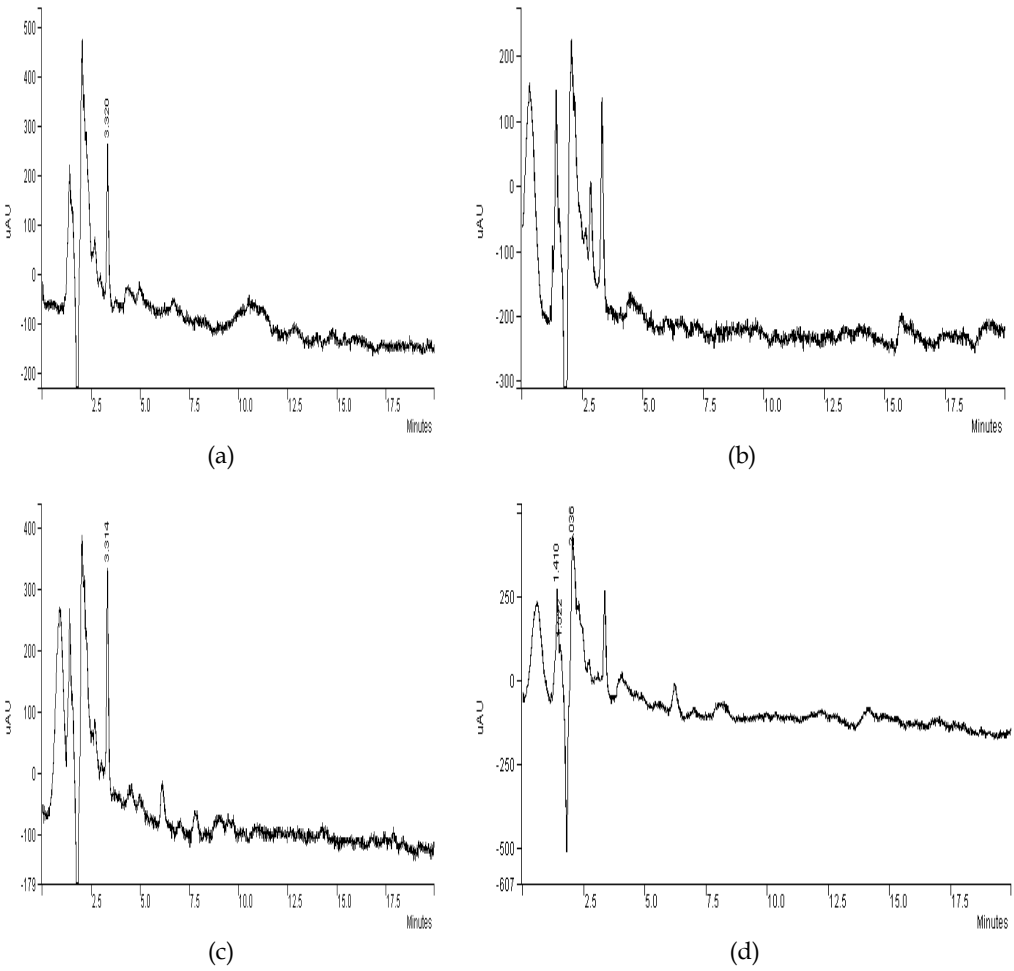
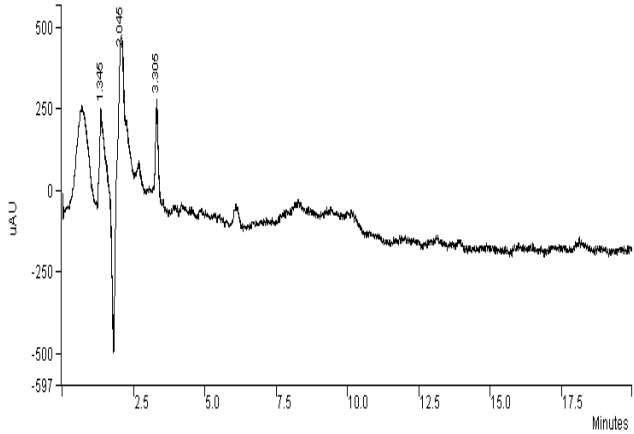


Fig. 3. Chromatograms of the blank medium and those with the artemisinin derivatives; (a) blank medium, (b) ATS, (c) ART, (d) DHA and (e) ATM



(e)

Fig. 3. (Continued)

blank medium and the four artemisinins. It can be observed that the non-specific peaks close to the dead volume of the separation were actually due to the interaction of the blank medium with the artemisinin derivatives. There were no detectable peaks at time intervals of 5–12 min where the azo adducts and CDNBD residues are eluted. Thus, CDNBD has proven to be a highly reactive and selective reagent for the assessment of the artemisinin derivatives.

This new isocratic HPLC with precolumn derivatization compares favorably with previously reported methods and the low LOD values are consistent with previous reports involving analysis in biological fluids as reported by Thomas et al. [18], Naik et al. [23], and Batty et al. [32]. This report is the first precolumn derivatization that will be applied to artemisinin and its derivatives. Previous procedures have focused on one or two of the artemisinins. It provides a quick and sensitive means of quantitation for the artemisinins as single sample injection run takes just 20 min to complete on HPLC.

Further studies on the utilization of this diazonium ion will focus on the applications of the coupling reaction to real sample analysis in dosage forms and biological fluids. However, one major drawback envisaged in the application of this new precolumn derivatization procedure to real sample analysis will be analysis of samples containing a mixture of artemisinin derivatives. One way of circumventing this may require prior separation before coupling with CDNBD is attempted.

Conclusions

The HPLC method developed for the analysis of artemisinins was highly sensitive, accurate and precise. The precolumn reaction is fast, quick, and readily adaptable. The method was found adoptable for the analysis of artemisinin and three of its derivatives in pure samples.

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