



ORIGINAL ARTICLE

Assay method for quality control and stability studies of a new antimalarial agent (CDRI 99/411) [☆]

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Abstract CDRI compound no. 99/411 is a potent 1,2,4-trioxane antimalarial candidate drug under development at our Institute. An HPLC method for determination of CDRI 99/411 with its starting material and intermediates has been developed and validated for in process quality control and stability studies. The analytical performance parameters such as linearity, precision, accuracy, specificity, limit of detection (LOD) and lower limit of quantification (LLOQ) were determined according to International Conference on Harmonization ICH Q2(R1) guidelines. HPLC separation was achieved on a RP-select B Lichrosphere[®] column (250 mm × 4 mm, 5 μm, Merck) using water containing 0.1% glacial acetic acid and acetonitrile as the mobile phase in a gradient elution. The eluents were monitored by a photo diode array detector at 245 and 275 nm. Based on signal to noise ratio of 3 and 10 the LOD of CDRI 99/411 was 0.55 μg/mL, while the LLOQ was 1.05 μg/mL. The calibration curves were linear in the range of 1.05–68 μg/mL. Precision of the method was determined by inter- and intra-assay variations within the acceptable range.

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1. Introduction

Malaria is still one of the major infectious diseases caused by a protozoa *Plasmodium falciparum*, with billions of people at risk for this deadly disease especially in underdeveloped countries [1–4]. The developing world urgently needs new, safe, and effective antimalarial drugs as the failure of the contemporary drugs due to the increasing resistance of the malaria parasite has further complicated the problem of treating malaria [5]. Semisynthetic derivatives of artemisinin are effective against both the chloroquine-sensitive and chloroquine-resistant malaria, hence used for the treatment of malaria caused by multidrug resistant *P. falciparum* [6]. CDRI 99/411 (Fig. 1) is a potent orally active

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1,2,4-trioxane antimalarial candidate compound for the treatment of malaria [7–8]. 1,2,4-Trioxane ring system in artemisinin is essential for the antimalarial activity due to the presence of peroxide group [9]. A synthetic analog of this is a better replacement for management of *falciparum* malaria. Patent for CDRI 99/411 is granted as it has shown a good profile for development as a drug and hence has been taken up for further development at our Institute [10]. Synthesis of CDRI 99/411 is shown in Fig. 2. In this reaction 1-(4-cyclohexylphenyl) ethanone (**1**) is converted to ethyl 3-(4-cyclohexylphenyl)-but-2-enoate (**2**) and the resulting ester is converted to 3-(4-cyclohexylphenyl)-but-2-en-1-ol (**3**), which finally results into CDRI 99/411 after peroxidation with 2-admantanone. In a multistep synthesis, the starting material and the intermediates may form the process impurities; thus there is a need for an analytical tool for identification and quantification of CDRI 99/411 [11]. Liquid chromatography is usually an analytical method of choice for pharmaceutical quality control [12], so the present study was undertaken to develop and validate an HPLC method for CDRI 99/411 having good separation of starting material, intermediates for process quality control and degradation products in stress stability studies.

2. Experimental

2.1. Reagents and standards

Standard CDRI 99/411, starting material (**1**) and intermediates (**2**, **3**) were supplied by the Medicinal and Process Chemistry Division of our Institute. HPLC grade acetonitrile was obtained from Spectrochem Pvt. Ltd. (Mumbai, India) and glacial acetic acid (analytical grade) from E. Merck (India) Ltd. (Mumbai, India). Milli-Q pure water was obtained from a Millipore Elix water purification system

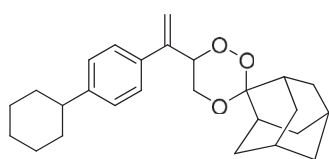


Fig. 1 Chemical structures of CDRI 99/411.

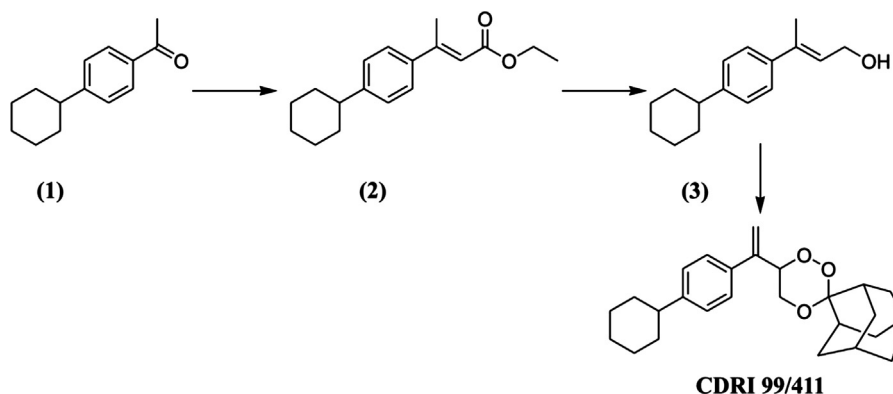


Fig. 2 Schematic representation of synthesis of CDRI 99/411.

purchased from Millipore India Pvt. Ltd. (New Delhi, India). Other reagents used were of analytical grade.

2.2. Apparatus and chromatographic conditions

Waters HPLC (Milford, MA, USA) system was equipped with a binary gradient pump (model 515, Waters), an auto sampler injector (Model 2707, Waters) and a diode array detector (Model 2998, Waters). The sample and standard solution were analyzed by HPLC after injecting 20 μ L. The following gradient was used with (A) water containing 0.1% glacial acetic acid and (B) acetonitrile: 0 min, 30:70 (A:B); 5 min, 10:90; 15 min, 10:90; 17 min, 30:70; and 20 min, 30:70. HPLC separation was achieved on a RP-select B Lichrospher[®] column (250 \times 4 mm, 5 μ m, Merck, Germany). Column effluent was monitored at 245 and 275 nm. The solutions were filtered and degassed before use. Chromatography was performed at 30 $^{\circ}$ C at a flow rate of 1.0 mL/min. Data were acquired and processed using Waters HPLC interface and software, Empower 2.

2.3. Preparation of stock and working standard solutions

Primary stock solutions of compounds **1**, **2**, **3** and CDRI 99/411 were prepared by dissolving the respective compounds in acetonitrile to achieve concentrations of 2.06, 1.92, 1.42 and 1.36 mg/mL respectively. These solutions were further diluted by transferring 1 mL each of the above stocks into 10 mL volumetric flask and making up to the volume with acetonitrile. Finally, solutions were prepared by diluting the diluted solution with acetonitrile to achieve different dilutions required for calibration of these compounds.

2.4. Preparation of sample solution

The stock solutions for samples of bulk candidate drug prepared for preclinical studies and samples from the stability chambers were prepared by dissolving these samples in acetonitrile. These samples were analyzed after dilution of stock with acetonitrile.

2.5. Method validation

2.5.1. Specificity and selectivity

The chromatographic interferences were assessed by comparing chromatograms of blank acetonitrile with those of samples spiked with compounds **1**, **2**, **3** and CDRI 99/411 in acetonitrile.

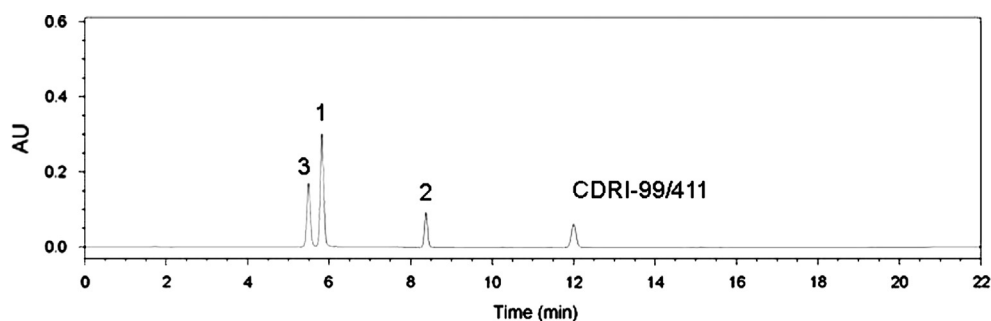


Fig. 3 HPLC chromatogram of mixture of compounds 1, 2, 3 and CDRI 99/411.

Table 1 Regression data, limit of detection (LOD) and lower limit of quantification (LLOQ) for compounds CDRI 99/411, 1, 2 and 3.

Compounds	LOD ($\mu\text{g/mL}$)	LLOQ ($\mu\text{g/mL}$)	Linear range ($\mu\text{g/mL}$)	Regression equation	r^2
1-(4-Cyclohexylphenyl) ethanone (1)	0.4	0.80	0.8–25.7	$y = 74,344x + 2464$	0.999
Ethyl 3-(4-cyclohexylphenyl)-but-2-enoate (2)	0.38	0.75	0.75–24.0	$y = 22,148x - 2266$	0.999
3-(4-Cyclohexyl- phenyl)-but-2-en-1-ol (3)	0.28	1.10	1.1–35.5	$y = 72,196x - 1041$	0.999
CDRI 99/411	0.52	1.05	1.05–68.0	$y = 37,764x - 1694$	0.999

Table 2 Intra- and inter-day assay variation of compounds 1, 2, 3 and CDRI 99/411.

Compounds	Concentration taken	Intra-day assay variation ^a			Inter-day assay variation ^b		
		Conc. found (mean \pm SD; $\mu\text{g/mL}$)	RSD	DFA (%)	Conc. found (mean \pm SD; $\mu\text{g/mL}$)	RSD	DFA (%)
Compound-1	25.70	25.72 \pm 0.07	0.26	0.07	25.76 \pm 0.02	0.06	0.22
	6.40	6.39 \pm 0.03	0.41	-0.68	6.37 \pm 0.03	0.54	-0.91
	0.80	0.77 \pm 0.02	2.98	-3.21	0.74 \pm 0.03	4.20	-6.18
Compound-2	24.00	24.08 \pm 0.23	0.95	0.34	24.08 \pm 0.23	0.95	0.34
	6.00	6.02 \pm 0.08	1.31	0.30	6.09 \pm 0.04	0.68	1.43
	0.75	0.79 \pm 0.01	0.78	4.91	0.79 \pm 0.01	0.66	5.44
Compound-3	35.50	35.72 \pm 0.30	0.83	0.63	35.80 \pm 0.24	0.67	0.84
	8.80	8.93 \pm 0.05	0.55	1.43	8.94 \pm 0.06	0.71	1.63
	1.10	1.13 \pm 0.13	0.94	2.47	1.15 \pm 0.01	1.01	4.82
Final product (CDRI 99/411)	34.00	34.07 \pm 0.07	0.21	0.20	34.08 \pm 0.06	0.18	0.22
	8.50	8.58 \pm 0.07	0.85	0.98	8.58 \pm 0.13	1.55	0.95
	1.05	1.09 \pm 0.02	1.90	3.60	1.12 \pm 0.03	2.93	6.60

^aDetermined with three replicates at each concentration.

^bDetermined with nine replicates (day 1, $n=3$; day 2, $n=3$; day 3, $n=3$) at each concentration.

2.5.2. Linearity of calibration curves

The linearity of the method was established by constructing calibration curves over a concentration range of 0.8–25.7, 0.75–24.0, 1.1–35.5 and 1.05–68.0 $\mu\text{g/mL}$ for compounds 1, 2, 3 and CDRI 99/411, respectively. Plots of peak area (response) against analyte concentration were used. The slope, intercept and the correlation coefficient of each calibration curve were determined using linear regression analysis.

2.5.3. Accuracy and precision

In order to assess the intra- and inter-day precision and accuracy of the assay, quality control (QC) samples at low, medium and high

concentrations were prepared as described above. The intra-day precision of the assay was assessed by calculating the relative standard deviation (RSD) for the analysis of QC samples in three replicates and inter-day precision was determined by the analysis of three replicates QC samples on three consecutive days. The accuracy was calculated on the basis of the difference in the mean calculated concentration and concentration taken (% deviation from actual concentration, % DFA).

2.6. Stability studies

Forced degradation studies were performed to evaluate the stability indicating properties and specificity of the method [13]. All solutions

used for stress studies were prepared with acetonitrile at an initial concentration of 1 mg/mL of CDRI 99/411 and heated at 80 °C and then diluted with acetonitrile to give a final concentration of 50 µg/mL.

The acidic degradation of CDRI 99/411 was studied in hydrochloric acid (0.1 M) at 80 °C for 8 h and alkaline degradation was studied in sodium hydroxide (0.1 M) at 80 °C for 1 h and the stressed samples were instantly cooled and diluted with acetonitrile. Oxidation was conducted by refluxing with 3% H₂O₂ for 1 h at 80 °C and then diluted with acetonitrile.

3. Results and discussion

3.1. Chromatography

The purpose of this study was to develop an HPLC method for in process quality control and for stability studies. During the method development, priority was given for the complete separation of the compound of interest CDRI 99/411 from other starting material and intermediates involved in the process of synthesis of the final

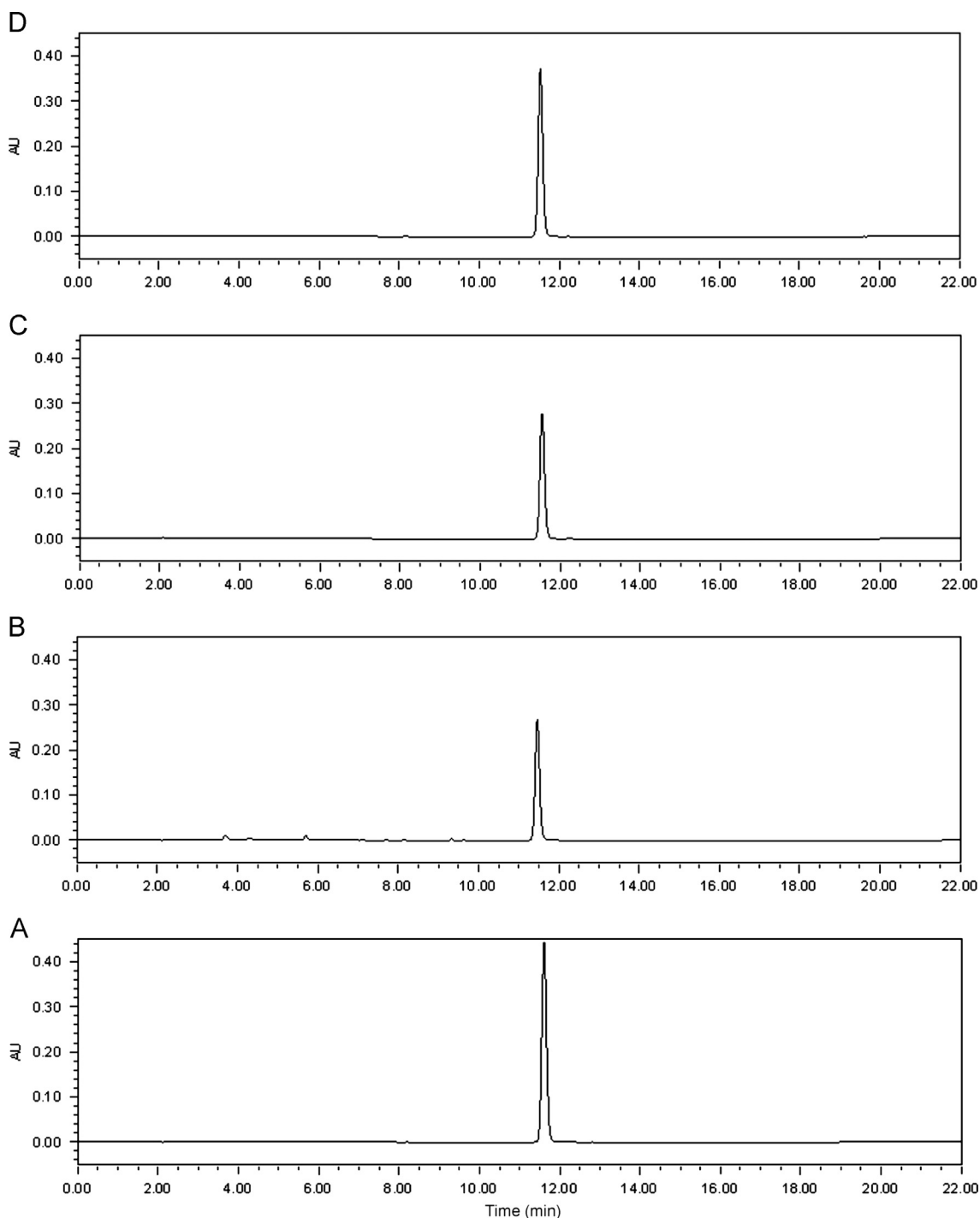


Fig. 4 Chromatogram of standard CDRI 99/411(A) and representative chromatograms of CDRI 99/411 on acidic (B), alkaline (C) and oxidative (D) degradations.

Table 3 Forced degradation studies of CDRI 99/411.

Stress conditions	Drug recovered ^a (%)	Drug decomposed ^a (%)
Standard drug	100.00	0.00
Acidic hydrolysis	60.17	39.83
Alkaline hydrolysis	62.95	37.05
Oxidative degradation	83.50	16.50

^aDetermined with three replicates.

product. Optimum chromatographic conditions were obtained after running various combinations of acetonitrile and water with glacial acetic acid as the mobile phase with C18 (Lichrocart[®] column; 250 × 4 mm, 5 μm, Merck), Cyno (Lichrocart[®] column; 250 × 4 mm, 5 μm, Merck) and RP-select B (Lichrospher[®] column; 250 × 4 mm, 5 μm, Merck) columns. C18 and Cyno columns resulted in peak with tailing and higher flow rate was required. Finally RP-select B Lichrospher[®] column was used for the study as it gave sharp peak for compounds CDRI 99/411, **1**, **2** and **3**. Gradient elution was preferred over the isocratic method as separation of compounds **1** and **2** was not proper.

3.2. Selectivity and specificity

The retention times of compounds **1**, **2**, **3** and CDRI 99/411 were 5.4, 5.8, 8.35 and 11.93 min, respectively. On a RP-select B Lichrospher[®] column, compounds were separated on the basis of their polarity. Compound-**3** contains hydroxyl group which makes it more polar thus eluting first i.e., with less retention time, next comes compound-**1** which has a ketonic group followed by compound-**2** which is a conjugated ester, less polar than ketone, and hence elutes after compound-**1**. CDRI 99/411 has a substituted peroxide bond and an ether linkage; consequently it is least polar and therefore it has the longest retention time. It should be recalled that compound **1** is the starting material, whereas compounds **2** and **3** are intermediates in the synthesis of final compound CDRI 99/411. None of the above compounds interfered with the peak corresponding to CDRI 99/411 or with each other (Fig. 3).

3.3. Linearity, limits of detection and quantification

The calibration curves ($n=3$) showed a linear relationship between peak area and concentration over the ranges of 0.8–25.7, 0.75–24.0, 1.1–35.5 and 1.05–68.0 μg/mL for compounds **1**, **2**, **3** and CDRI 99/411, respectively. The correlation coefficient (r^2) of the calibration curves for all the analytes was found to be more than 0.999. The limits of detection (LOD) and quantification (LOQ) were measured according to the ICH guidelines. The LOD and LLOQ of the analytes were determined based on a signal to noise ratio of 3 and 10 respectively (Table 1).

3.4. Accuracy and precision

Accuracy and precision data for intra- and inter-day assay are given in Table 2. The assay values on both occasions (intra- and inter-day) were found to be within the acceptable limits [14–16].

3.5. Stability studies

Chromatograms obtained from the assay of stressed samples are shown in Fig. 4(A)–(D). CDRI 99/411 shows significant degradation in alkaline and acidic hydrolysis and oxidative stress conditions (Table 3).

4. Conclusions

The developed and validated HPLC method is reproducible and can be routinely used for quality control of CDRI 99/411 in bulk manufacture, with ongoing stability studies for drug development process. It provides base line separation of the compound with all the starting materials, intermediates and product of interest. Several bulk samples of compound 99-411 from the Medicinal and Process Chemistry Division and bulk preparation, required for pharmacological and toxicological studies, were analyzed by the reported method.

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