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HPLC-UV method for assaying 99/357, a synthetic trioxane antimalarial derivative in rat and rabbit serum

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Abstract

In the present study an accurate and precise HPLC-UV assay method in rat and rabbit serum has been developed and validated for determination of 99/357—a new synthetic analogue of artemisinin developed by Central Drug Research Institute (CDRI), of the class of trioxane derivative. Separation was achieved using a C-18 reversed phase column with a mobile phase comprising of acetonitrile and deionized water (80:20%, v/v) using a UV detector, set at a wavelength of 266 nm. The method, applicable to 200 μl serum, involved double extraction of the samples with 20% isopropyl alcohol (IPA) in *n*-hexane. The recovery of 99/357 in the two matrices was >90%. The method was sensitive with a limit of quantitation of 25 ng/ml in both rat and rabbit matrices. Precision and accuracy were within the acceptable limits, as indicated by relative standard deviation (accuracy) varying from −12.7 to 5.7% and bias (precision) values ranging from 0.6 to 11.8%. Moreover, 99/357 was stable in serum up to 30 days of storage at −60 °C and after being subjected to three freeze/thaw cycles. The method will be applied to perform pharmacokinetic studies of 99/357.

Keywords: HPLC-UV; Synthetic trioxane derivative; Antimalarial; Serum; Pharmacokinetics

1. Introduction

Malaria remains world's most dreaded infectious disease in terms of human sufferings and death. The world health organization (WHO) estimates 300–500 million cases of malaria around the world yearly. Parasite resistance to existing chemotherapy is becoming an increasing problem in Southeast Asia and in African continents. There is a dire need for design and identification of new drugs with enhanced activity to combat and control of malaria. Several semi-synthetic derivatives of artemisinin – the active ingredient of the Chinese herb 'qinghao' (*Artemisia annua*) used traditionally for treating fevers – have been used increasingly over the past two decades. Artemisinin and its derivatives are considered as the most rapidly acting antimalarials to date and are being used clinically world over [1–3]. The endoperoxide sesquiterpene lactone moiety of this class of compounds is found to be indispensable for the erythrocytic schizontocidal activity and reacts with the intraparasitic heme and form free radicals. These free radicals appear to damage intracellular targets and perform their antimalarial activity [4,5]. However, the existing artemisinin class of the drug had to be improved regarding efficacy, neurotoxicity, stability and pharmacokinetic behaviors. Many synthetic antimalarial peroxides have been prepared but most suffers from low oral activity and toxicity, a defect shared in part by semi-synthetic artemisinins. Therefore, a need exists to identify novel peroxide antimalarial agents with high oral activity, devoid of neurotoxicity and moreover affordable.

Central Drug Research Institute (CDRI), Lucknow, in the quest for more potent antimalarials, developed an artemisinin class compound, code named 99/357 (Fig. 1). It possesses a 1,2,4-trioxane nucleus similar to the endoperoxide lactone of artemisinins, essential for antimalarial activity. To carry out the preclinical pharmacokinetic studies for this new chemical entity (NCE) an HPLC assay method has been developed and

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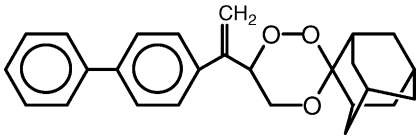

Fig. 1. Chemical structure of 99/357.

validated fully in rat serum. This method was extrapolated to rabbit serum and cross validated. These methods will be applied for further pharmacokinetic studies of 99/357 in rats and rabbits.

2. Materials and methods

2.1. Chemicals

Chemical structure of 99/357 (a synthetic trioxane derivative) is shown in Fig. 1. 99/357 (purity >99%) was synthesized at the Medicinal Chemistry Division of Central Drug Research Institute, Lucknow. HPLC grade acetonitrile (ACN) and *n*-hexane were procured from J.T. Baker (Philipsburg, USA). HPLC grade isopropyl alcohol (IPA) was obtained from Spectrochem Pvt Ltd. (Mumbai, India). Deionized water (DW) was obtained from Milli Q PLUS system (18.2 MΩ cm). All other chemicals were of analytical grade and procured from local sources unless specified. Serum was obtained from drug free male rats (Sprague Dawley) and rabbit (New Zealand), which were procured from the Laboratory Animal Services Division, CDRI.

2.2. Bioanalytical method

The HPLC system consisted of a pump (LC-10 ATvp with SCL 10Avp system controller, Shimadzu, Japan) with quaternary flow control valve system (FCV-10ALvp) and a degasser (DGU-14A) to pump the mobile phase. The detection was performed using SPD-10Avp UV–vis detector set at 266 nm. A Model 7725i syringe loading injector (Rheodyne, USA) with a fixed 100 μl loop was used to inject the samples. Chromatographic separations were performed on Spheri-5, RP-18 column, Applied Biosystems Inc. (100/4.6 μm i.d. 5 μm), coupled with a guard column packed with the same material (30/4.6 mm i.d., 5 μm). The mobile phase was composed of acetonitrile:TDW :: 80:20% v/v at a flow rate of 1.2 ml/min. Mobile phase was filtered and degassed (20 min) in a sonicator (Bransonic cleaning Co., USA) before use. Data was analyzed using CLASS-VP software (Shimadzu, Japan) running on a Compaq PC. The HPLC system was equilibrated for approximately 30 min at a flow rate of 1.2 ml/min before the commencement of the analysis. A vortex-mixer (Thermolyne, USA), Model SVC-220H speed vac concentrator (Savant, NY, USA) and Model K130 centrifuge (BHG Hermle) were used for sample preparation. Serum samples were stored at −60 °C in Ultra Freeze U41085, Ultra Low Freezer (New Brunswick Scientific, USA).

2.3. Stock and standard solution

Stock solution was prepared by dissolving 5 mg of 99/357 in 5 ml acetonitrile to give final concentration of 1 mg/ml. Working stock solutions were prepared from the mother stock (1 mg/ml) using mobile phase (ACN:TDW :: 80:20). Mobile Phase standards (25–1000 ng/ml) were prepared by appropriate dilution of the working stock solutions using mobile phase (ACN:TDW :: 80:20). These analytical standards were used to determine HPLC system reproducibility and absolute recovery for 99/357 from biomatrices used. All solutions were stored at 4 °C for a maximum period of 1 month. Stock solutions and analytical standards were prepared in HPLC grade acetonitrile.

2.4. Calibration curve

Calibration and quality control (QC) samples of all the analytes from 25 to 1000 ng/ml in both biomatrices (rat and rabbit serum) were prepared by adding various volumes of mixed stock solutions in appropriate volume of pooled rat and rabbit serum so that the volume ratio of the organic phase added was less than 2.5%. Calibration and QC standards were stored at −60 °C until analysis. Quality control samples at low (25 ng/ml), medium (250 ng/ml) and high (1000 ng/ml) concentrations were used for the method validation program. Prior to HPLC analysis these calibration standards and QC were processed according to method outlined in the following paragraph.

2.5. Sample preparation

A simple and efficient two-step liquid–liquid extraction process is employed to isolate 99/357 from the rat as well as rabbit serum. To 200 μl of blank or the spiked serum 3 ml of 20% IPA in *n*-hexane was added. The tubes were vortex mixed for 2 min and then centrifuged at 2000 rpm at 4 °C for 10 min. The organic layer was transferred into another tube by snap freezing the aqueous layer in liquid nitrogen and evaporated to dryness under reduced pressure in speed vac concentrator (Savant Instrument, Farmingdale NY, USA). The aqueous phase was re-extracted with 3 ml of 20% IPA in *n*-hexane and organic phase was transferred to same tube and finally evaporated to dryness. The residue was reconstituted in 0.2 ml of mobile phase and injected into HPLC. The calibration curve was obtained by linear regression ($y = mx + c$) of the peak heights of 99/357 versus concentration.

3. Method validation

The HPLC-UV method was validated for 5 days at low (25 ng/ml), medium (250 ng/ml) and high (1000 ng/ml) in five replicate. The method developed was validated in terms of HPLC system reproducibility, sensitivity, specificity, linearity, absolute recovery, accuracy, precision, freeze-thaw

cycle stability and long-term stability in spiked serum samples stored at −60 °C. The assay method was extrapolated to rabbit serum by performing cross validation in terms of specificity and intra and inter-assay accuracy and precision. The lower limit of detection (LOD) for 99/357 was the quantity in the serum in both species after the sample cleanup corresponding to three times the baseline noise (S/N > 3). The limit of quantitation is the concentration of the sample that can be quantified with less than 20% variation in precision [6].

The specificity was defined as non-interference in the region of compounds of interest with the endogenous substances, drug metabolite or other compounds in the determination of concentration [7].

Linearity for calibration standards ($n = 6$) in five replicates for 5 days was assessed by subjecting the spiked concentrations and the respective peak height to linear regression analysis ($y = mx + c$) with and without intercepts. The choice of proper calibration method depends on the residuals obtained and the coefficient of correlation [8,9].

The recovery of an analyte from sample prior to analysis was determined by preparing QC samples of low, medium and high concentrations. The recovery of 99/357 from serum was calculated by comparing the peak height response obtained by the QC standards in serum with those from calibration curve in mobile phase.

The accuracy was determined by injection of calibration standards and QC standards in five replicates on five different days ($n = 75$, five each of low, medium and high concentration). The inter- and intra-batch accuracy was determined by calculating %bias from the theoretical concentration using the following equation.

$$\%\ \text{Bias} = \frac{(\text{Observed Concentration} - \text{Spiked Concentration})}{\text{Spiked Concentration}} \times 100$$

The inter and intra day precision was determined by subjecting the data to one-way analysis of variance (ANOVA) in terms of relative standard deviation (%R.S.D.).

Freeze thaw stability of serum samples was determined by preparing calibration standards of strengths low, medium and high in five replicate for four different days. One set of three concentrations was analyzed on day of preparation (no freeze-thaw cycle) before storing the remaining sets at −60 °C. Other sets were analyzed after one, two and three freeze-thaw cycles. Thawing was achieved by keeping the sealed tubes at room temperatures for 30 min. The results were expressed as percent deviation with initial concentration.

The effect of storage on dry residue stability is determined at 25 ng/ml (low), 250 ng/ml (medium) and 1000 ng/ml (high) concentration levels. All the samples (five replicates samples of three concentrations × four sets) were prepared and dry residue was stored in glass tubes sealed with aluminum foil and then with parafilm. One set in triplicate was reconstituted on day 1 with mobile phase and analyzed on the same day (without storage). The remaining sets were similarly analyzed on days 2, 4, and 6 and results are expressed as percent deviation.

For the long-term stability of 99/357 in the spiked samples stored at −60 °C, the QC samples at low, medium and high concentrations in five replicate for four different days were prepared and stored at −60 °C. These sets of samples were analyzed after 0, 7, 15 and 30 days of storage and their concentrations read from the respective calibrations standard curve on that day and results expressed as percent deviation from 0 days concentration.

The method developed was cross validated in rabbit serum (change in species within matrix, i.e. from rat serum to rabbit serum), in term of intra- and inter-assay accuracy and precision [6].

4. Results and discussion

An accurate specific and validated assay method is prerequisite for estimation of any analyte in a biomatrices. The successful analysis of the analytes in biological fluids using HPLC relies on the optimization of sample preparation, chromatographic separation and post-column detection. Each of these steps was carefully optimized for developing sensitive, selective, and reproducible assay methods in different species.

The chromatographic conditions were modified to get better selectivity and sensitivity. Thus molarity and pH of buffer and the type of column material were optimized. The effect of molar strength and the nature of buffer in the mobile phase response on peak shape of 99/357 were studied [10]. Buffers like potassium dihydrogen orthophosphate and ammonium acetate were used for the preparation of the mobile phase. However, there was no significant effect on peak shape and retention time by using buffer of either type. The use of buffers with different pH ranging from 4 to 6 could also not affect peak shape. During optimization of the mobile phase the best results in term of peak shape and run time were obtained using as mobile phase a mixture (80:20) of acetonitrile:water. Different columns (Cyano and RP-18) of varying length were used to check the peak shape and retention time. The isocratic elution with 80:20 acetonitrile:water at 1.2 ml/min on Spheri-5, RP-18 column shows no interference with any endogenous impurity. The new assay method developed for 99/357 was found to be specific. The HPLC system reproducibility was checked with five replicate injections of each analytical standard. The variations in the peak heights of 99/357 was maximal at 25 ng/ml (3.9%) and was less than 2.0% at all other concentration levels indicating that the system yields reproducible data. The result showed that the variation were within acceptable limits of $P > 0.05$ in one way ANOVA.

Sample clean up techniques were also optimized to get rid of interfering endogenous substances without sacrificing the recoveries of 99/357. A proper selection of solvent was

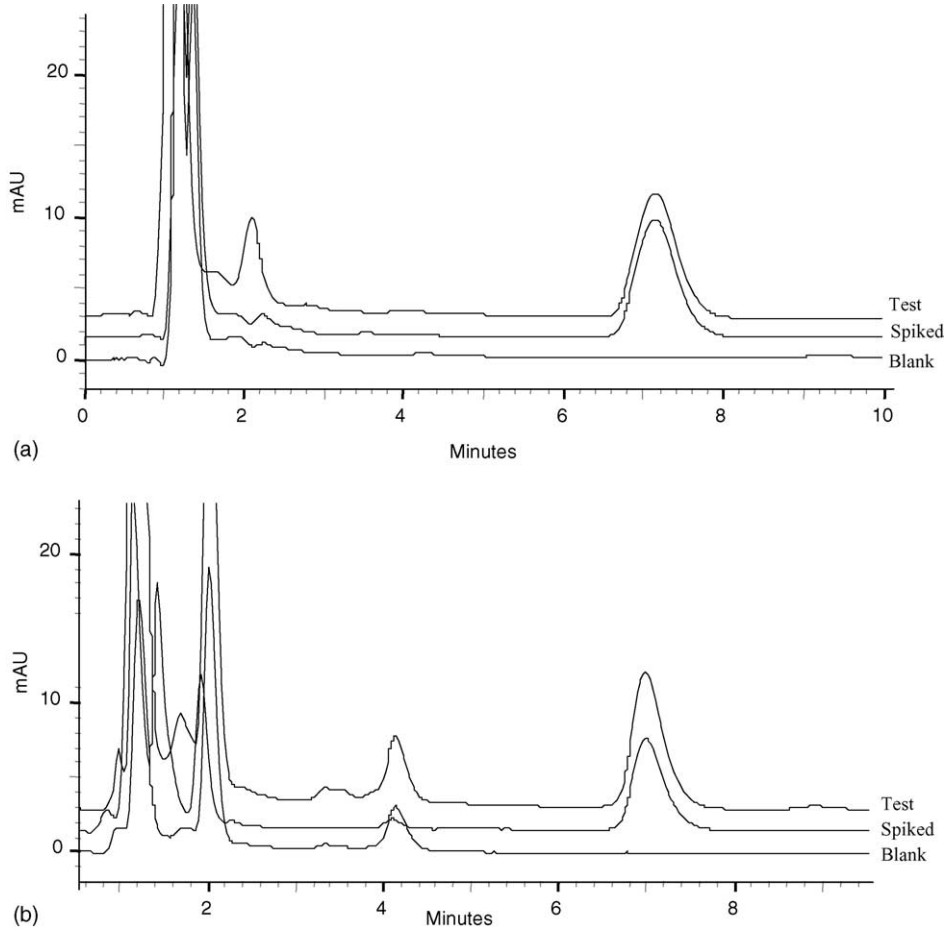

Fig. 2. Representative chromatogram of blank, test (30 min) and spiked (400 ng/ml) in (a) rat serum and (b) rabbit serum.

essential for yielding maximum recoveries. Extraction solvents like *n*-hexane, ethyl acetate and different composition of isopropyl alcohol in *n*-hexane were used. The best result were obtained with 20% IPA in *n*-hexane without compromising recoveries. The HPLC chromatogram (Fig. 2(a) in rat and Fig. 2(b) in rabbit serum) shows no serum endogenous peak interferes with peak of interest. The chromatographic conditions and extraction procedure yielded a clean chromatogram for analyte. The recovery of 99/357 from spiked serum sample was calculated by comparing the peak height with those obtained from mobile phase standards and found to be >90% at all the concentration in both the matrices. The mean recoveries from serum are summarized in Table 1.

Table 1
Mean recoveries of 99/357 in rat and rabbit serum

Concentration (ng/ml)	%Absolute recovery (mean ± S.D., n = 3)	
	Rats	Rabbits
25	102.4 ± 10.10	91.6 ± 6.12
50	97.2 ± 4.76	95.3 ± 6.38
100	94.3 ± 5.37	95.2 ± 5.68
250	93.1 ± 5.34	105.8 ± 2.90
500	99.6 ± 3.58	102.4 ± 5.39
1000	93.5 ± 3.82	97.4 ± 3.67

The peak height of 99/357 in rat and rabbit serum varied linearly with concentration over the range tested (25–1000 ng/ml). The calibration model was selected based on individual calibration data by linear regression with or without intercept and weighing factor. A linear equation $y = mx + c$ without weighing schemes was used to perform standard calibration. Limit of detection and limit of quantitation for 99/357 was 12.5 and 25 ng/ml, respectively. The percent relative standard deviation (precision) and percent bias values (accuracy) at three concentrations in five replicate for 99/357 are presented in Table 2 for complete validation in rat serum and cross validation in rabbit serum. The result show the analytical method is accurate in both the cases as the intra and inter batch variation in two species is within the acceptable limit of ±20% for low and ±15% for all other concentrations. Similarly the %R.S.D. for different QC samples was within ±15% at all concentration level analyzed.

The 99/357 is found to be stable over a period of 30 days in normal serum when stored at −60 °C (Fig. 3(a)). The analyte was found to be stable after three freeze-thaw cycles (Fig. 3(b)) and the dry residue after extraction was also found to be stable over a period of 6 days when stored at −60 °C (Fig. 3(c)). The percent deviation calculated was

Table 2
Accuracy and Precision of 99/357 in rat and rabbit serum

Species	Concentration (ng/ml)	%Bias (accuracy)		%R.S.D. (precision)	
		Intrabatch	Interbatch	Intrabatch	Interbatch
Rat	25	−7.8	−12.7	4.9	11.8
	250	4.4	7.0	4.5	7.8
	1000	2.4	3.3	0.6	4.5
Rabbit	25	4.3	5.7	3.6	1.8
	250	−2.1	−3.5	2.1	6.6
	1000	−7.1	−6.6	2.6	2.6

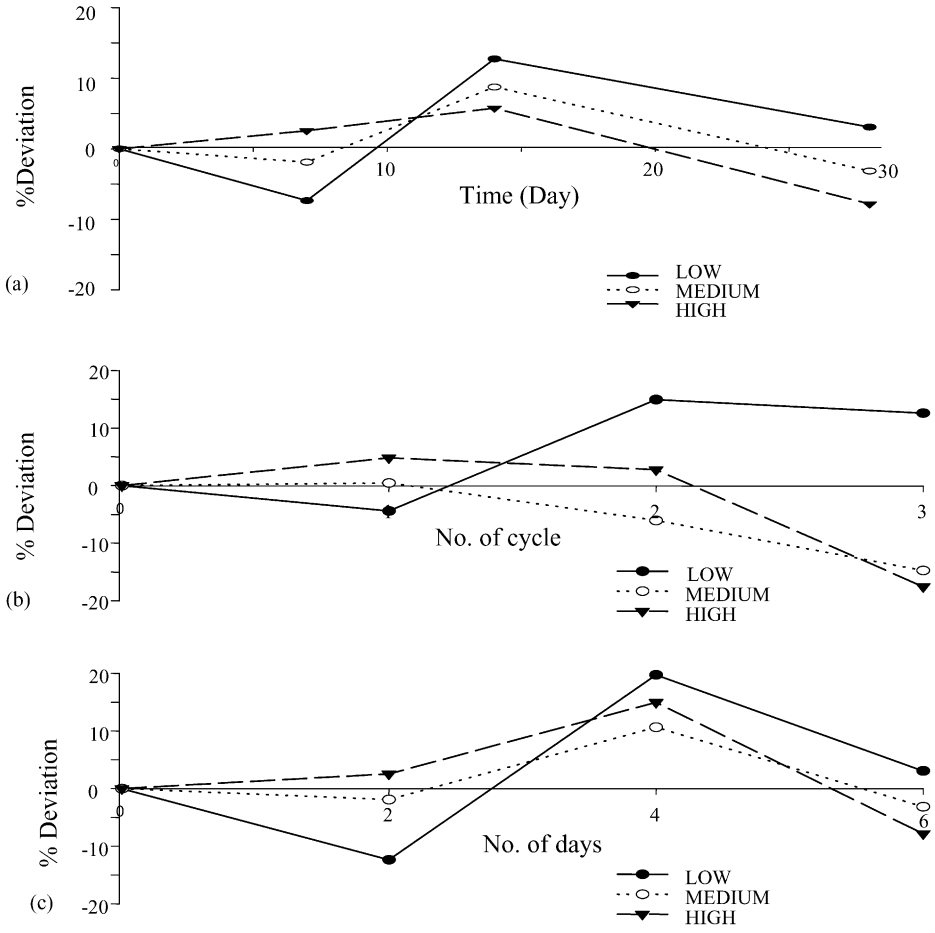

Fig. 3. (a) Long-term stability; (b) freeze-thaw stability; and (c) dry residue stability.

within ±20% at low concentration while it was found to be within ±15% for all other concentrations.

5. Conclusion

The relevance of pharmacokinetics in drug development has been well recognized and systematic interpretation of disposition behavior is of considerable use in reducing the expenditure and time involved in drug development besides optimization of drug therapy. Thus a suitably validated method in different species can lead to rapid pharmacokinetic studies. The present assay method was found to be specific, accurate and precise over the linearity range of 25–1000 ng/ml. There were no stability problems for 99/357 during storage and sample processing hence fulfilling the criteria for bioanalytical methods. This suitably validated method will be applied further to carry out pharmacokinetic studies in rats and rabbits.

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