Journal of Chromatography B, 877 (2009) 2074–2080

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Liquid chromatographic tandem mass spectrometric assay for quantification of 97/78 and its metabolite 97/63: A promising trioxane antimalarial in monkey plasma☆

R.P. Singh, S. Sabarinath, N. Gautam, R.C. Gupta, S.K. Singh*

Pharmacokinetics and Metabolism Division, Central Drug Research Institute, Lucknow, India

ARTICLE INFO

Article history:
Received 31 March 2009
Accepted 21 May 2009
Available online 29 May 2009

Keywords:
LC–MS/MS
Validation
Monkey plasma
Antimalarial
Trioxane

ABSTRACT

The present manuscript describes development and validation of LC–MS/MS assay for the simultaneous quantitation of 97/78 and its active *in-vivo* metabolite 97/63 in monkey plasma using α-arteether as internal standard (IS). The method involves a single step protein precipitation using acetonitrile as extraction method. The analytes were separated on a Columbus C_{18} (50 mm × 2 mm i.d., 5 μm particle size) column by isocratic elution with acetonitrile:ammonium acetate buffer (pH 4, 10 mM) (80:20 v/v) at a flow rate of 0.45 mL/min, and analyzed by mass spectrometry in multiple reaction-monitoring (MRM) positive ion mode. The chromatographic run time was 4.0 min and the weighted ($1/x^2$) calibration curves were linear over a range of 1.56–200 ng/mL. The method was linear for both the analytes with correlation coefficients >0.995. The intra-day and inter-day accuracy (% bias) and precisions (% RSD) of the assay were less than 6.27%. Both analytes were stable after three freeze-thaw cycles (% deviation <8.2) and also for 30 days in plasma (% deviation <6.7). The absolute recoveries of 97/78, 97/63 and internal standard (IS), from spiked plasma samples were >90%. The validated assay method, described here, was successfully applied to the pharmacokinetic study of 97/78 and its active *in-vivo* metabolite 97/63 in *Rhesus* monkeys.

1. Introduction

Malaria remains world's one of the most infectious disease in terms of human sufferings and death. An estimated 250 million people are infected annually with approximately one million deaths each year. The majority of infections and deaths occur in sub-Saharan Africa [1,2]. The increasing incidence of resistance in malaria prevalent areas against classical antimalarials has prompted worldwide research to design and develop new drugs, of ideally different molecular mechanism(s) of action from those against which the malaria parasite has developed resistance. 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. The endoperoxide sesquiterpene lactone moiety of this class of compounds is found to be indispensable for the erythorocytic 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 [3–5]. 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 high 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.

Artemisinin and its derivatives or analogues are currently regarded as the most promising weapons against malaria. It is reported that their unique sesquiterpene (endoperoxide) structure is indispensable for antimalarial activity [3–5]. Several synthetic trioxanes, simplified analogs of artemisinin retaining the crucial endoperoxide bridge, have been reported. In the quest for new antimalarials, CDRI, Lucknow, developed an artemisinin class compound, code named 97/78 (Fig. 1) having potent antimalarial activity [6–7]. It possesses a 1,2,4-trioxane nucleus similar to the endoperoxide lactone of artemisinins, essential for antimalarial activity [8–10]. To carry out the pre-clinical studies of 97/78 and its metabolite 97/63 using LC–MS/MS assay; a sensitive, selective and specific method has been developed and validated in monkey plasma. This method was successfully applied for generation of pharmacokinetic parameters of 97/78 and its *in-vivo*

☆ CDRI Communication No.: 7711.

* Corresponding author. Tel.: +91 522 2612411–18x4389; fax: +91 522 223405/223938/229504.
E-mail address: shiokumar@yahoo.com (S.K. Singh).

1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2009.05.044

metabolite 97/63 in *Rhesus* monkeys after oral administration of 97/78.

2. Experimental

2.1. Chemicals and reagents

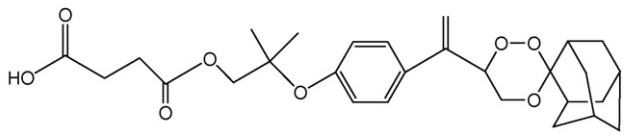
Pure reference standards of 97/78, and metabolite 97/63 (Fig. 1) were procured from medicinal and process chemistry division, Central Drug Research Institute (CDRI) India. α-Arteether used as internal standard was obtained from Themis Chemicals Limited (Mumbai, India) (Fig. 1). Acetonitrile, HPLC grade, was purchased from Thomas Baker (Chemicals) Limited (Mumbai, India). Ammonium acetate and glacial acetic acid AR were purchased from E Merck (India) Limited. Ultra pure water of 18.2 MΩ cm was obtained from a Milli-Q PLUS PF system. Heparin sodium injection I.P. (1000 IU/mL) was purchased from Biologicals E. Limited (Hyderabad, India). Blank monkey plasma was collected from adult healthy, drug free male *Rhesus* monkeys (age 5–6 years) at Laboratory Animal Division of CDRI (Lucknow, India). Plasma was obtained by centrifuging the heparinised blood at 1000 × *g* for 10 min. Pooled plasma samples were stored at −60 °C till use.

2.2. LC–MS/MS instrumentation and analytical conditions

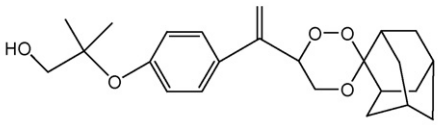
The HPLC system consists of Series 200 pumps and auto sampler with temperature controlled Peltier-tray (Perkin–Elmer instruments, Norwalk, CT, USA). Isocratic mode was used to deliver the mobile phase at a flow rate of 0.45 mL/min. Chromatographic separation was achieved on Columbus C_{18} (50 mm × 2 mm i.d., 5 μm particle size) column with guard, (Phenomenax USA). Elution was carried out using acetonitrile:ammonium acetate buffer (pH 4, 10 mM) (80:20 v/v) as mobile phase. The chromatographic run time was 4.0 min and injection volume was optimized to 20 μL. The analysis was carried out at ambient temperature and the pressure of the chromatographic system was ∼600–800 psi.

Mass spectrometric detection was performed on an API 4000 LC–MS/MS mass spectrometer (Applied Biosystems, MDS Sciex USA) with Analyst 1.4 software. The mass spectrometer was operated at electrospray ion source (ESI) positive ion mode and the analytes were quantified using multiple reactions monitoring (MRM) mode. Zero air (air minus moisture) was used as source gas while nitrogen was used as both curtain and collision gas. MS and MS/MS conditions for pure standard 97/78, metabolite 97/63 and IS (α-Arteether) were optimized by continuous infusion at 5 μL/min using syringe pump (Model '11', Harvard apparatus). Optimized precursor (ammonium adducts of analytes, $M+NH_4^+$) to product ion transitions monitored *m/z* 518.4 → 173.1; 418 → 119.1 and 330 → 267 were used for quantification of 97/78, metabolite 97/63 and IS (α-Arteether) respectively. The Declustering potential (DP), collision energy (CE) were optimized for individual analytes. Data acquisition and quantitation were performed using analyst software version 1.4 (Applied Biosystems, MDS Sciex Toronto, Canada).

97/78 Parent Compound



97/63 Metabolite



IS (α-Arteether).

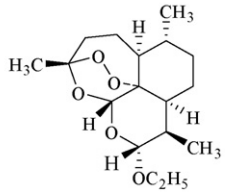


Fig. 1. Chemical structure of parent compound 97/78, metabolite 97/63 and IS (α-Arteether).

2.3. Preparation of standard and quality control (QC) samples

Individual stock solutions of 1 mg/mL concentration for 97/78, metabolite 97/63 and IS (α-Arteether) were prepared in acetonitrile. The internal standard α-Arteether was of choice because of non-availability of the deuteriated compound. Being of same class of drug with same mechanism of action IS can not be used as a therapeutic drug with 97/78. Working solutions of all analytes were obtained by step-wise dilution of the stock solution. Calibration curves of 97/78 and metabolite 97/63 were prepared in blank normal monkey plasma over a concentration range of 1.56–200 ng/mL. The individual plasma standards were prepared by spiking the working stocks of suitable dilution into the plasma to give the desired concentration range of 1.56–200 ng/mL. Quality control (QC) samples at three different concentrations (1.56, 25 and 200 ng/mL as low, medium and high respectively) were prepared separately in five replicates, independent of the calibration standards and stored at −60 °C until analysis. Test samples and quality control samples were then interpolated from the calibration curve to obtain the concentrations of the respective analytes.

2.4. Sample preparation

Calibration standards, quality control samples and test samples were prepared by using a simple one step protein precipitation extraction process with acetonitrile. The processing volume of plasma was fixed as 100 μL and to it 10 μL IS solution was added to each sample to get final concentration of 4 ng/mL followed by vortex mixing for 15 s. Protein precipitation of plasma samples was carried out by addition of 390 μL acetonitrile in order to make the final volume 0.5 mL. The extraction tubes were then vortex mixed for 60 s and centrifuged at 12,000 × *g* for 5 min. 100 μL supernatant was transferred to auto-injector vials and injected for analysis.

2.5. Assay validation

The present LC–MS/MS assay was validated in terms of sensitivity, specificity, linearity, recovery, accuracy, precision, freeze-thaw cycle stability, long-term stability and auto-injector stability for 97/78 and metabolite 97/63 in monkey plasma. The method validation was performed for 3 days at three different quality control (QC) concentrations (1.56, 25 and 200 ng/mL) in five replicates.

The lower limit of detection (LOD) was the quantity in plasma, after the sample cleanup, corresponding to three times the baseline noise ratio (S/N >3). LLOQ is the lowest concentration of the standard curve that can be measured with acceptable accuracy and precision (±20% variation). The lower limit of quantitation (LLOQ) was the concentration where analytes signal is atleast 10 times of noise i.e. signal to noise (S/N) ratio >10.

Linearity for calibration standards ($n = 8$) for 3 days was assessed by subjecting the spiked concentrations and the respective peak area ratios. Calibration and analytical standard curves were constructed using the analyte to IS peak area ratios by weighted ($1/x^2$) least-square linear regression [11].

The inter- and intra-batch accuracy was determined by calculating % bias (% bias = (Observed concentration − Nominal concentration)/Nominal concentration × 100) of quality control samples. 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 (% RSD).

The extraction recoveries of the analytes at three QC levels were determined by comparing peak areas obtained from plasma samples with those found by direct injection of standard solutions prepared in mobile phase of the same concentration.

2.6. Stability studies

The freeze-thaw stability (−60 °C), auto-injector (4 °C) and long-term (−60 °C) stability for analytes were determined at three concentration levels (1.56, 25, and 200 ng/mL) in five replicates. To evaluate the impact of the freeze-thaw cycles, spiked control in the plasma were prepared at low, medium and high concentrations. One set was analyzed without being subjected to freeze-thaw cycle and considered as reference value from which percent deviation for other set's concentration was calculated. Other sets were analyzed after 1, 2 and 3 freeze-thaw cycles. After sample preparation, stability of the analytes in the auto-injector was evaluated for upto 4 h, the maximum duration for which a sample may have to wait in the peltier tray for pending analysis and results were expressed in % deviation with initial injection ($t = 0$ h). Long-term stability study was also performed over a period of 30 days. The results were expressed as % deviation with freshly prepared QC samples of same concentration.

2.7. Pharmacokinetic study

Young, adult and healthy male *Rhesus* monkeys (age 5–6 years) weighing 6.0 ± 1.5 kg, (obtained from Laboratory Animal Division of Institute) were housed in well ventilated cages and kept at room

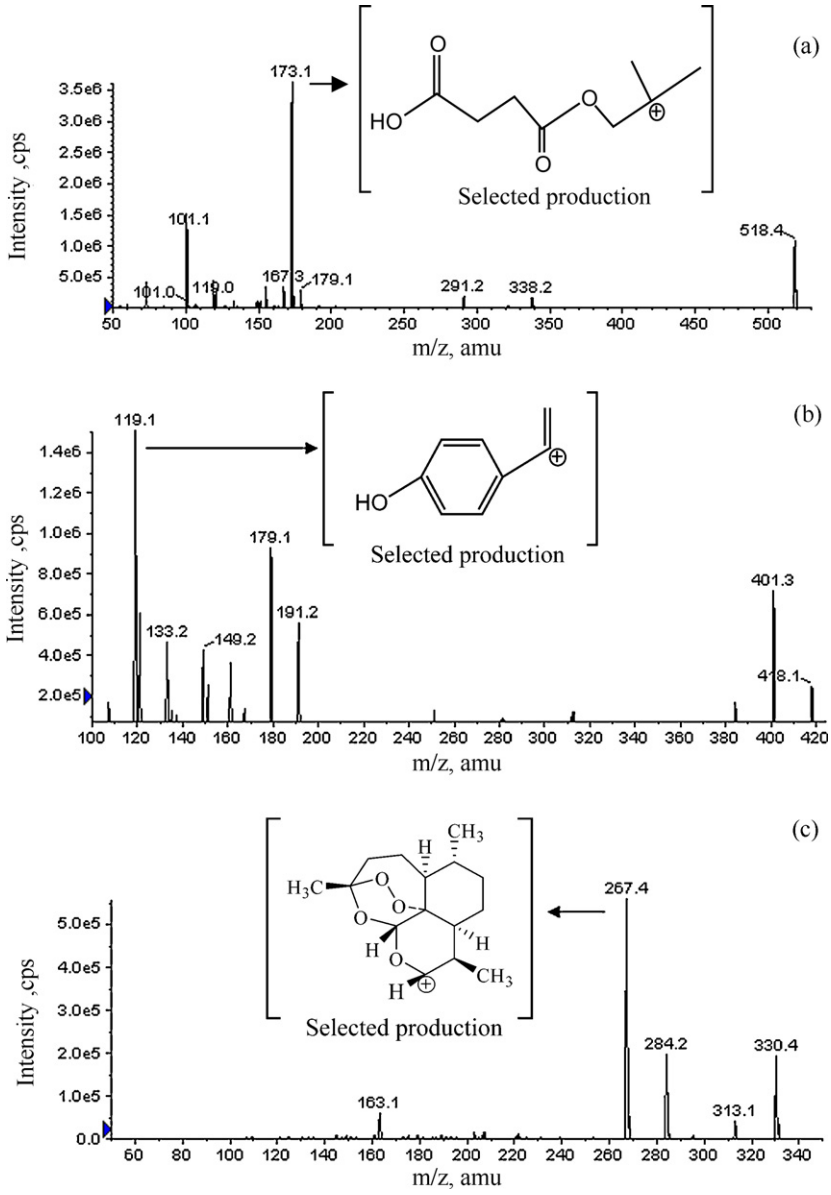


Fig. 2. Product ion mass spectra of (a) 97/78, (b) metabolite 97/63 and (c) IS (α-Arteether).

temperature on a regular 12 h light dark cycle. Prior approval from the Local Animal Ethics Committee was sought for maintenance and experimental studies with animals. Animals were cared in accordance with the guidelines laid by local ethical committee for animal experimentation and follow principles of the *guide for care and use of laboratory animals* (Department of health education and welfare, number [NIH] 85-32). The oral dosing formulation of 97/78 was prepared in 5% $NaHCO_3$ to dose administration at 20 mg/kg. The strength of the formulation for oral administration was 25 mg/mL. Oral dose was administered using a 10 mL syringe and an orogastric catheter to monkeys fasted for approximately 12 h prior to administration. The catheter was flushed with approximately 10 mL water after dose administration. Serial sample approach was used to collect blood samples through femoral vein. Blood samples (~1.5 mL) were collected in heparinzed glass tubes at different time point viz. 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 8, 12, 18, 24, 30, 48, 55, 72 and 96 h after oral dose administration. Plasma was separated by centrifuging blood samples at 1000 × *g* for 10 min at 4 °C and stored at −60 °C until analysis. Pharmacokinetic parameters were determined by data fitting approach applying non-compartmental analysis using WinNonlin (5.1) software program (Pharsight Corp, USA).

3. Results and discussion

3.1. LC–MS/MS optimization

Continuous flow analysis was carried out to obtain parent ion and product ion mass spectra of analytes. Full-scan mass spectra of 97/78, metabolite 97/63 and IS (α-Arteether), with ammonium adducts ($M+NH_4^+$) shows most significant intensity of *m*/*z* 518.4, 418.1, and 330.4 respectively for parent ion. The ammonium adduct is found to be robust enough for monitoring the transitions. The robustness of the ammonium adduct was checked at pH range 3–5. Also the robustness was checked at different percent of the buffer ranging from 15 to 25% in final mobile phase. The collision energy and collision gas were optimized such that the most abundant ion in the product ion spectra of ammonium adduct of 97/78 (*m*/*z* = 518.4), metabolite 97/63 (*m*/*z* = 418.1) and IS (α-Arteether) (*m*/*z* = 330.4) were 173.1, 119.1 and 267 respectively. Declustering potential opti-

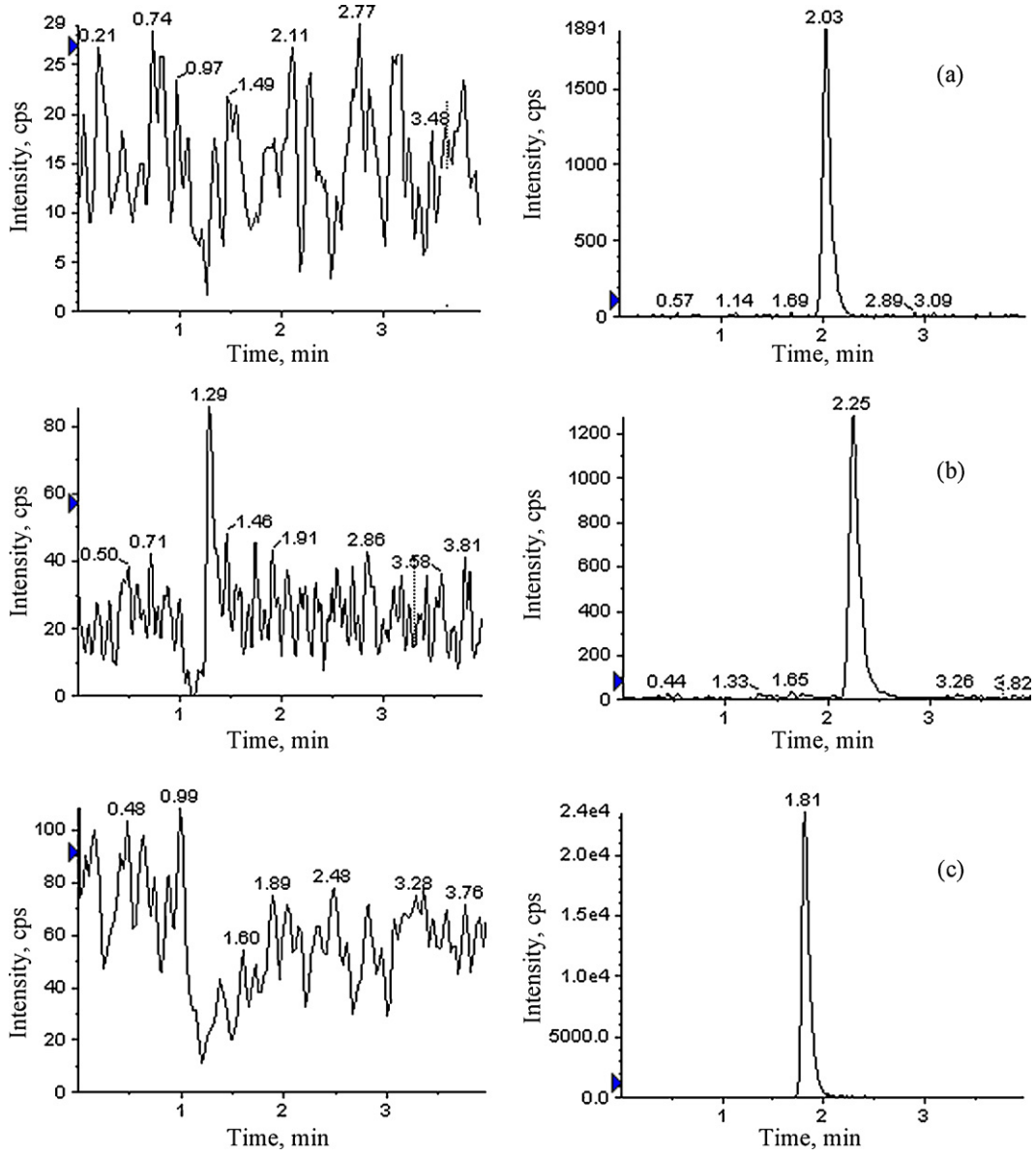


Fig. 3. Representative blank and spiked (at 1.56 ng/mL i.e. LLOQ level) chromatogram of (a) 97/78 (b) metabolite 97/63 and (c) IS (α-Arteether) at conc. 4 ng/mL in monkey plasma.

Table 1
Recoveries, accuracy and precision of 97/78 and its metabolite 97/63 in monkey plasma ($n = 5$).

Analytes	Concentration (ng/mL)	Recoveries	Accuracy (%bias)		Precision (% RSD)	
			Inter-day	Intra-day	Inter-day	Intra-day
97/78	1.56	96.33 ± 2.25	94.37 ± 3.24	93.86 ± 2.34	3.43	2.49
	25	92.50 ± 1.80	97.58 ± 6.12	97.55 ± 4.43	6.27	4.54
	200	93.17 ± 5.06	97.11 ± 0.44	96.5 ± 4.03	0.45	4.18
97/63	1.56	97.17 ± 1.76	96.65 ± 2.04	94.72 ± 3.85	2.11	4.06
	25	95.33 ± 2.36	97.09 ± 0.35	95.85 ± 2.86	0.36	2.98
	200	96.00 ± 2.00	96.28 ± 1.44	95.69 ± 2.65	1.50	2.77

mization of the ammonium adducts of analytes were carried out using full-scan acquisition over a mass range of 300–600 Da by continuous flow analysis.

The product ion spectra with proposed chemical structure of selected product ion were shows in Fig. 2. The dwell time was fixed at 200 ms and Declustering potential was optimized to 50 V for m/z 518.4, 418.1 and 35 V for 330.4. The collision energy was optimized for individual analyte for most intense fragment and was found to be 24 V for 97/78, 30 V for metabolite 97/63 and 13 for IS. Based on the peak intensity of the respective ammonium adducts, 10 mM ammonium acetate buffer of pH 4 and acetonitrile as the organic phase, at a flow rate of 0.45 mL/min were suitable for all analytes.

The curtain gas (CUR) and collision gas (CAD) were optimized to 15 and 3 psi respectively. The Ion source gas 1 (GS1), Ion source gas 2 (GS2), entrance potential (EP) and collision cell exit potential (CXP) were optimized to 30 psi, 45 psi, 4 V and 10 V respectively. The ion source potential was set at 5500 V and source gas temperature was optimized to 200 °C. To obtain highly specific and selective detection, multiple reaction-monitoring (MRM) in positive ion mode was used for reliable quantification. Monitoring these transitions of the parent molecules showed no interference in six blank biological matrix. The response of the individual ions (parent and metabolite) was checked individually and is further determined in combination (with or without internal standard). Qualifier ion was not included in the analysis as no interference was observed as responses were similar in each case. The interference check was conducted with both plasma and analytical standard.

3.2. Assay performance and validation

Selectivity, sensitivity, linearity, accuracy, precision and stability were measured and used as the parameters to validate and assess the bioanalytical assay performance [12–16]. The peak area ratios of analytes with the IS in monkey plasma were linear with the analyte concentration over a range of 1.56–200 ng/mL. It was found that the variance was not constant across the calibration standard (heteroscedasticityand hence weighting was used to improve residuals and to counter variance. Best fit for the calibration curve was achieved by a linear equation of $Y = mX + c$ with $1/x^2$ weighing factor. The coefficient of correlation (r^2) for 97/78 and metabolite 97/63 were 0.9983 ± 0.0009 and 0.9983 ± 0.0010 over the concentration range used.

LC–MS/MS analysis of the blank monkey plasma samples showed no interference with the quantification of analytes and the IS. Specificity of the method was established in monkey plasma (from six different sources) with pooled as well as individual samples. The interference with the common antimalarials like sulfadoxine (m/z 311 → 156) and pyrimethamine (m/z 249 → 198) was also determined however no interference was observed. The capacity factor obtained for 97/78 and 97/63 were 0.81 and 1.01 respectively. Representative chromatograms of extracted blank plasma and blank plasma spiked with 97/78, metabolite 97/63 and the IS indicating the specificity and selectivity of the method, are shown in Fig. 3.

The limit of detection (LOD) demonstrated that all the analytes gave a signal-to-noise ratio (S/N) of 3 and above at a concentration level of 0.5 ng/mL. The lower limit of quantification (LLOQ), the lowest concentration in the standard curve which can be measured with acceptable accuracy and precision, for 97/78 and metabolite 97/63 from matrices was established as 1.56 ng/mL. LLOQ was established with five samples independent of the standard curve.

The recoveries were calculated from the peak area of extracted spiked plasma samples read against the analytical standard curve spiked into plasma blank. The absolute recoveries were calculated by taking the matrix effect into the account though no significant matrix effect was observed. The recovery of 97/78 and metabolite 97/63 were 96.33 ± 2.25/97.17 ± 1.76, 92.5 ± 1.8/95.33 ± 2.36 and 93.17 ± 5.06/96.0 ± 2.0% at concentration of 1.56, 25 and 200 ng/mL respectively. The recovery of the IS from the extracted calibration standards and QC samples during validation was 92.14 ± 6.7%. The possibility of the matrix effect on ionization was explored further by comparing the responses obtained from blank plasma extract spiked with reference solutions with that of same reference solutions in mobile phase. This study was carried out over the entire concentration range. The matrix suppression obtained was less than 3%. The effect on ionization of phospholipids and possible interference from late eluting endogenous compound was also evaluated by repeated injection. Further the product ion transition of the 184 from most of lipids (496, 704, 758, 806) was also monitored. However no significant effect was observed on ionization. Thus, these studies showed that there is no significant matrix suppression and/or enhancement on ionization.

Accuracy in terms of % bias and precision in terms of % relative standard deviation (%RSD) for both intra- and inter-batch were calculated with five determinations per concentration level on 3 days (five replicates of low, medium and high QC samples) in monkey plasma and are presented in Table 1. The results show that the bioanalytical method is accurate, as the bias is within the acceptance limits of ±20% of the theoretical value at LLOQ and ±15% at all other concentration levels. The precision around the mean value never exceeded 15% at any of the concentrations studied. The results showed that the bioanalytical method for simultaneous estimation of 97/78 and metabolite 97/63 is accurate and precise over the concentration range of 1.56–200 ng/mL.

Table 2
Freeze-thaw stability of 97/78 and metabolite 97/63 in monkey plasma.

Analytes	Concentration (ng/mL)	% Deviations after freeze-thaw cycles		
		1	2	3
97/78	1.56	−2.41	−1.32	−3.07
	25	−1.69	−2.10	−3.31
	200	−4.05	−5.71	−5.81
97/63	1.56	−5.22	−4.58	−5.64
	25	−5.39	−5.66	−6.99
	200	−5.25	−5.25	−8.21

Table 3
Long-term stability at −60 °C of 97/78 and metabolite 97/63 in monkey plasma.

Analyte	Nominal concentration (ng/mL)	19 days		30 days	
		Concentration recovered (ng/mL)	% Deviation	Concentration recovered (ng/mL)	% Deviation
97/78	1.56	1.51	−0.66	1.45	−4.61
	25	24.45	−0.81	24.2	−1.83
	200	193	−4.22	193	−4.22
97/63	1.56	1.5	−4.15	1.49	−4.37
	25	24.78	−1.20	23.83	−4.86
	200	191.5	−5.67	189.33	−6.73

Table 4
Stability of 97/78 and metabolite 97/63 in the auto-injector at 4 °C.

Analyte	Concentration (ng/mL)	0 h	4 h	
		Average observed concentration (ng/mL)	Average observed concentration (ng/mL)	% Deviation
97/78	1.56	1.52	1.49	−1.97
	25	24.65	24.47	−0.73
	200	201.5	192.0	−4.71
97/63	1.56	1.57	1.52	−3.18
	25	25.05	24.13	−3.67
	200	203.0	192.3	−5.27

3.3. Stability studies

97/78 and metabolite 97/63 shows freeze-thaw (f-t) stability in monkey plasma which were carried out at three concentrations (1.56, 25 and 200 ng/mL) in five replicates. The deviation observed after first, second and third f-t cycles were within ±8.3% (Table 2) with freshly prepared (no f-t) QC samples of same concentrations in five replicates. Also the QC samples stored at −60 °C were analyzed after 19 and 30 days and there were no significant deviations (<±7%) with respect to the immediately analyzed samples (Table 3). The re-injection reproducibility was established to determine if an analytical run could be re-analyzed in case of unexpected delay in analyses. The same set of QC samples were repeated after one analysis with 4 h gap in between within the samples were stored at 4 °C and in all cases the deviations were less than ±6% (Table 4). The 97/78 and metabolite 97/63 were also shown to be stable in monkey plasma at room temperature for at least 8 h (at bench-top) where deviations were less than 10% freshly spiked sample.

3.4. Application of the analytical method to pharmacokinetic studies

The LC–MS/MS method developed was used to investigate the pharmacokinetics of 97/78 and metabolite 97/63 after oral administration of 97/78 (Dose 20 mg/kg) in monkey ($N = 3$). Pharmacokinetic parameters were determined using a non compartmental analysis of plasma concentration time data using WinNonlin 5.1 software (Pharsight Corp, USA). Table 5 shows the main pharmacokinetic parameters of 97/78 and metabolite 97/63. Fig. 4 shows the mean plasma concentration–time profiles of 97/78 and its metabolite 97/63 (inset of Fig. 4). Percentage extrapolation in all the cases from the last measured time point to infinity (i.e. % extrapolation between AUC_{0-all} and $AUC_{0-\infty}$) was less than 2% which suggested that the parameters derived were well estimated. The parent compound 97/78 could be detected up to 8 h in plasma, while metabolite 97/63 was measurable up to 96 h. The plasma concentration time profile of the compound showed that 97/78 remains above minimum effective concentration (MEC) for more than 2 h and its active metabolite remain above MEC (unpublished data) for more than 12 h making it a promising candidate. Following oral administration of 97/78, a high and rapid conversion to metabolite 97/63 was observed, which evident by lesser T_{max}, higher C_{max} and higher systemic exposure ($AUC_{0-\infty}$) of metabolite.

Table 5
Pharmacokinetic parameters for 97/78 and its metabolite 97/63 following oral dose administration of 97/78 at 20 mg/kg (Mean ± SEM, $N = 3$).

Pharmacokinetic parameters	97/78	Metabolite 97/63
C_{max1} (ng/mL)	242.67 ± 64.26	1165.67 ± 348.18
T_{max} (h)	1.33 ± 0.70	1.83 ± 0.17
AUC_{0-t} (ng h/mL)	426.48 ± 9.0	4390.97 ± 895.87
$AUC_{0-\infty}$ (ng h/mL)	460.35 ± 27.04	4430.39 ± 905.17
$T_{1/2}$ (h)	0.96 ± 0.14	7.48 ± 0.37
MRT (h)	2.18 ± 0.27	8.03 ± 0.18
Cl/F(L/h/kg)	43.23 ± 2.63	*

* Not applicable.

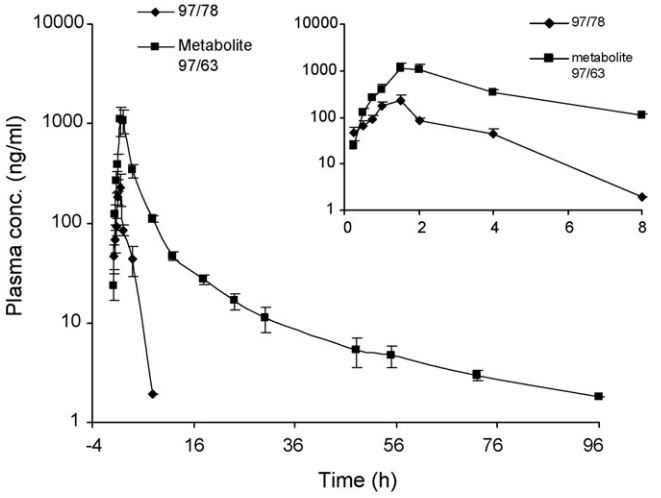


Fig. 4. Plasma concentration versus time profile of 97/78 and metabolite 97/63 in male *Rhesus* monkeys after oral administration of 97/78 at 20 mg/kg dose (Mean ± SEM, $N = 3$).

3.5. Conclusion

A sensitive, accurate and precise procedure based on LC–MS/MS has been developed and validated for the simultaneous determination of 97/78 and its *in-vivo* active metabolite 97/63 in monkey plasma with the lower limit of quantification of 1.56 ng/mL. A good

linearity was obtained over a concentration range 1.56–200 ng/mL. There were no stability and matrix problems for 97/78 as well as its metabolite 97/63 during storage and sample processing. This method was successfully applied for the single dose pharmacokinetic study of 97/78 in *Rhesus* monkeys following oral administration. Shorter run time of 4 min. is helpful in carrying out analyses rapidly. Further application will be carried out in various pre-clinical evaluations of these candidate drug molecule(s) viz. protein-binding estimation, drug–drug and drug–food interactions, toxicokinetics and multiple-dose pharmacokinetic studies.

Acknowledgement

The authors wish to express their sincere gratitude to Dr. Chandan Singh, Medicinal Chemistry division, Central Drug Research Institute, Lucknow, for the authentic samples of 97/78 and metabolite 97/63. The authors are thankful to Director, CDRI for his constant encouragement and support. We also acknowledge Council of Scientific and Industrial Research (CSIR) for providing research fellowships.

References

[1] World Health Organisation Expert Committee on Malaria 20th Report, WHO,Geneva, 2000.
[2] J.G. Breman, M.S. Alilio, A. Mills, Am. J. Trop. Med. Hyg. 71 (2004) 1–15.
[3] W. Yikang, Acc. Chem. Res. 35 (1999) 255.
[4] A. Robert, D.C. Odile, J. Cazelles, B. Meunier, Acc. Chem. Res. 35 (2002) 167.
[5] F. Cheng, J. Shen, X. Luo, W. Zhu, J. Gu, R. Ji, H. Jiang, K. Chen, Bioorg. Med. Chem. 10 (2002) 2883.
[6] C. Singh, S.K. Puri. United State Patent 6316493 B1.(2001).
[7] P. Olliaro, T.N.C. Wells. Clin. Pharmacol. Ther., in press.
[8] C. Singh, N. Gupta, S.K. Puri, Tetrahedron Lett. 46 (2005) 205.
[9] A.G. Griesbeck, T.T. El-Idreesy, L.O. Hoinck, J. Lexa, R. Brun, Bioorg. Med. Chem. Lett. 15 (2005) 595.
[10] C. Singh, N.C. Srivastava, S.K. Puri, Bioorg. Med. Chem. 12 (2004) 5745.
[11] A.M. Almeida, M.M. Castel-Branco, A.C. Falcao, J. Chromatogr. B 774 (2002) 215.
[12] M. Rajanikanth, R.C. Gupta, J. Pharm. Biomed. Anal. 26 (2001) 519–530.
[13] R.P. Singh, S. Sabarinath, S.K. Singh, R.C. Gupta, J. Chromatogr. B 864 (2002) 52.
[14] B.S. Kou, T.V. Noord, M.R. Feng, D.S. Wright, J. Pharm. Biomed. Res. 16 (1998) 837–846.
[15] W. Xu, Y. Zhang, M. Yang, Z. Shen, X. Zhang, H. Li, J. Pharm. Biomed. Anal. 45 (2007) 667–672.
[16] V.P. Shah, K.K. Midha, J.W.A. Findlay, H.M. Hill, J.D. Hulse, I.J. McGilveray, G. McKay, K.J. Miller, R.N. Patnaik, M.L. Powel, A. Tonelli, C.T. Viswanathan, A. Yacobi, Pharm. Res. 17 (2000) 1551–1557.