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A rapid and sensitive high performance liquid chromatographic assay of the new antimalarial compound 80/53 in serum with a novel sample clean-up method and its pharmacokinetics in rabbits[1]

Jyoti Kumar Paliwal, Ram Chandra Gupta *

Pharmacokinetics and Metabolism Division, Central Drug Research Institute, Lucknow-26001, India

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Abstract

The compound 80/53 (AM) is a new antimalarial agent synthesized by this institute as a safer and less toxic analogue of primaquine. It was found to exhibit fluorescence in acetonitrile solution and this finding was exploited to develop a selective and sensitive high performance liquid chromatographic (HPLC) assay of the AM in rabbit serum. The sample clean-up was done in a single step by simultaneous protein precipitation and extraction with acetonitrile in the presence of sodium sulfate. The lower limit of quantitation of the method was 50 ng ml^{-1} using 100 μl of serum sample. The method was fully validated from 50 to 1600 ng ml^{-1} concentration range with a recovery ranging from 70 to 75%. The within- and between-run variability was less than 10% and the drug in serum was stable over four freeze–thaw cycles and up to 24 h in injection solvent at 4°C. The method was applied to determine the pharmacokinetic parameters of AM in 5 rabbits receiving a single bolus intravenous and peroral dose in a crossover study. The concentration–time data after a 5 mg kg^{-1} i.v. dose in rabbits was best fitted to the two compartment body model with first order absorption and elimination rate constants. The terminal half-life and MRT of AM were 95.3 ± 43.5 and 104 ± 10.6 min respectively. After administering a single 20 mg kg^{-1} oral dose, the serum levels of AM in all the rabbits declined below the quantitation limit by 90 min and it was not possible to fit the data by the compartmental approach. The MRT of AM after oral dose was 31.1 ± 8.3 min. Application of the assay has also been extended to analyze the serum samples of rats, monkeys and humans.

Keywords: Antimalarial; Compound 80/53; Pharmacokinetics; Fluorescence; HPLC

1. Introduction

Chemotherapeutic agents of the 8-amino quinoline group are known to have tissue schizonticidal (causal prophylactic and radical curative) as well as gametocidal activity against malaria [1,2]. Of

* Corresponding author.
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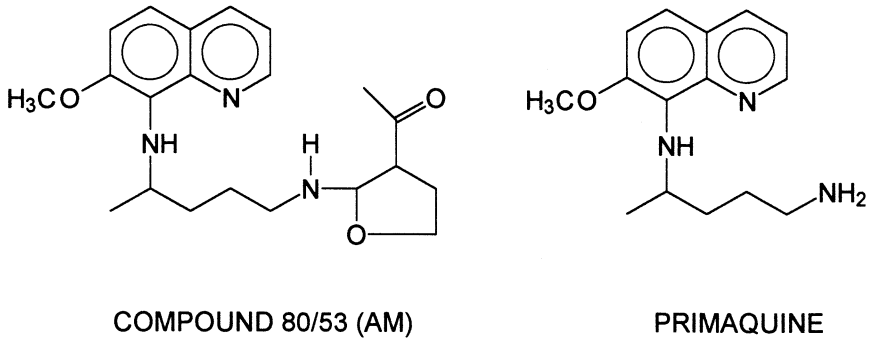

COMPOUND 80/53 (AM) PRIMAQUINE

Fig. 1. Chemical structure of AM (80/53) and primaquine.

the known analogues, only primaquine (Fig. 1) is in clinical use as an anti-relapse drug against *Plasmodium vivax* [3]. The therapeutic application of primaquine is limited because of adverse side effects, which include methemoglobinemia and cyanosis, hemolytic anemia in G-6-PD deficient individuals, hepatotoxicity and gastro-intestinal distress [4,5]. Attempts have been made to synthesize and evaluate analogues of primaquine, either by quinoline ring substitution or by side chain modification, in the search for compounds that would be more effective or less toxic [6,7]. Compound 80/53 (AM), *N*′-3′-(acetyl-4′,5′-dihydro-2′-furanyl)-*N*-(6-methoxy-8-quinolynyl)-1,4-pentanediamine, is a potent anti-malarial agent, synthesized as an analogue of primaquine by side chain modification (Fig. 1) [8,9].

AM has been evaluated for anti-relapse activity against sporozoite induced *Plasmodium cynomolgi* infection in rhesus monkeys. It is safer than primaquine and causes only one third as much methemoglobinemia [7]. It is also safe in subacute toxicity studies in rats and rhesus monkeys and has no teratogenic action [10]. Currently it is under phase II clinical trials. The compound AM is a weak base and undergoes hydrolysis which is catalyzed by hydrogen ion concentration under acidic conditions. UV spectra of AM in methanol, recorded in time mode scanning, shows that absorbance of the drug solution decreases at wavelength 302 nm but remains constant at 269 nm. [11]. In acidic medium, the compound is converted into primaquine [12]. Stability increases with increasing pH and optimum stability is found to be between pH 7–8. It is practically insoluble in water.

To date, only one HPLC assay method for simultaneous determination of AM and primaquine in 0.5 ml human serum has been reported [13]. The lower limit of quantitation of this method for AM is 50 ng ml^{-1}. In small animals like rats and rabbits, it is often difficult to obtain large samples. Hence, it was necessary to develop a new assay requiring 100 μl or less sample volume. The present work aims at developing and validating the new fluorescent HPLC assay for the determination of AM in rabbit serum for pharmacokinetic studies of the drug in rabbits.

2. Experimental

2.1. Chemicals and reagents

Sodium sulfate, propylene glycol, ethyl alcohol, potassium dihydrogen orthophosphate and orthophosphoric acid were of analytical grade and were procured from Glaxo (India). HPLC grade acetonitrile was obtained from S.D. Fine Chem (Boisar, India). Hexane was obtained from Merck (Worli, Bombay, India). Water was used after triple distillation through all-quartz distillation apparatus. *N*,*N*-dimethyl octylamine (DMOA) was synthesized, distilled and purified in our division. AM was purified in house to reference standard grade ($>99\%$).

A drug free rabbit serum pool was prepared from blood collected from the marginal ear vein of young healthy rabbits of the Laboratory Animal Division of the institute.

2.2. Instrumentation and chromatographic conditions

The HPLC system consisted of a Kontron HPLC System 600 (Kontron, Zurich, Switzerland), a 7125 injector with a 50 μl loop (Rheodyne, Berkeley, CA), an RF-530 fluorescence HPLC monitor (Shimadzu, Japan) operated at high sensitivity and excitation wavelength 269 nm, emission wavelength 480 nm and a C_{18} (5 μm) cartridge column (220 mm × 4.6 mm i.d.) preceded by a precolumn (30 mm × 4.6 mm i.d.) (Pierce, Rockford, IL). A model SVC-200H Savant Speed Vac concentrator (Savant, New York) was used to evaporate the organic solvent. AM was eluted with a mobile phase prepared by fortifying acetonitrile with 500 μl l^{-1} of DMOA. It was degassed before use and pumped at 0.8 ml min^{-1} flow rate at ambient temperature. Chromatograms were recorded and integrated by Nelson Analytical Chromatography software, Version 4.0 (Nelson Analytical, CA) on a PC/XT computer. Everyday the column was stabilized before use by allowing the mobile phase to run for 30 min, the baseline stability was checked by monitoring the baseline, and the system performance was checked by injecting a standard solution.

2.3. Stock and standard solution preparation

A stock solution containing 160 μg ml^{-1} AM w/v was prepared by dissolving 8 mg AM in 50 ml mobile phase. Working standards in the range 25–1600 ng ml^{-1} were also prepared in mobile phase by sequential dilution of the stock solution and stored at 4°C.

2.4. Optimization of sample clean up procedure and mobile phase composition

Preliminary studies indicated that fluorescence intensity of AM is quenched by the presence of water in the injection sample. Hence, the nature and the amount of salt, i.e. sodium chloride or sodium sulfate required during the protein precipitation step to minimize the water content in the supernatant, were determined. To prevent the conversion of AM into primaquine during sample clean up by the protein precipitation and extraction step, the amount of DMOA and/or potassium hydroxide solution required in acetonitrile was studied. The effect of the addition of water, methanol and/or hexane in the mobile phase on the resolution of AM from components of the serum matrix, peak response and retention time of AM were also studied.

2.5. Sample preparation

Serum samples were stored at −30°C until they were analysed. In a 1.5 ml disposable centrifuge tube, 40 mg of sodium sulfate was added to 100 μl serum and vortex mixed. It was vortex-mixed again for 1 min with 200 μl of the precipitation solvent (acetonitrile + 1 ml l^{-1} DMOA), stored in the dark at 4°C for 30 min and centrifuged at 3000 × *g* for 10 min. The 150 μl supernatant was injected into the HPLC fitted with 50 μl loop.

2.6. Method validation

The validation program for the new HPLC method for AM included within- and between-run precision and accuracy studies, freeze–thaw effects and stability of the drug in serum supernatant at 4°C. These studies were carried out in quadruplicate and at three concentration levels, namely low (100 ng ml^{-1}), medium (400 ng ml^{-1}) and high (1600 ng ml^{-1}).

2.6.1. Calibration graph

Calibration and quality control samples of AM from 50 to 1600 ng ml^{-1} were prepared by adding varying volumes of stock and working solutions in appropriate volumes of pooled, drug free rabbit serum so that the volume ratio of the organic phase added to the serum was less than 2%. Calibration and QC standards were stored at −30°C until they were analyzed. Peak height

Table 1
Precision, accuracy and recovery of the HPLC assay of compound AM in spiked rat serum

Concentration (ng ml^{-1})	Recovery (%) (mean ± SD, $n = 6$)	Between-run accuracy (bias %)	Within-run accuracy (bias %)	Between-run precision (RSD%)	Within-run precision (RSD%)
100	74.5 ± 3.0	+7.2	+7.7	3.0	6.4
400	73.8 ± 2.1	−1.9	−1.0	3.1	3.4
1600	70.8 ± 0.9	−2.5	−0.9	4.3	5.3

responses were used to prepare calibration curves for the drug in the serum and mobile phase each day before analyzing the unknown samples. The calibration curve was obtained by linear regression of the peak height of AM (y) versus its concentration (x) (Table 1). Recovery was calculated by comparing the peak heights obtained from the serum with corresponding mobile standards.

2.6.2. Accuracy and precision

The accuracy of this method was determined by the injection of calibration samples and the twelve QC samples (four each of the high, medium and low concentration) after sample clean up on 4 different days. The precision was determined by one way ANOVA as within- and between-run percent RSD. The accuracy was calculated as a percentage of the nominal concentration: accuracy = (concentration observed/nominal concentration) × 100%. All calibration samples were required to have a correlation value of at least 0.9990.

2.6.3. Stability of AM in the processed injection sample

The effect of sample clean-up and storage of supernatant (acetonitrile extract) at 4°C for up to 24 h prior to injection into the HPLC on the stability of AM was studied at low, medium and high concentration levels. One set comprising of quadruplicate QC samples of high, medium and low concentration was processed and the supernatant was analyzed after 2 h storage at 4°C. Similar sets were processed and injected after 4, 8 and 24 h storage at 4°C.

2.6.4. Freeze–thaw stability

QC samples of low, medium and high concentration, each containing 100 μl serum of the respective concentration were stored in disposable 1.5 ml centrifuge tubes at −30°C. One set comprised of quadruplicate samples of each concentration was analyzed on day 1 prior to storage (no freeze–thaw cycle) and other sets after 1, 2, 3 and 4 freeze–thaw cycles. Thawing was achieved by keeping the sample tubes at ambient temperature (20–30°C) for 1 h. The results of day 1 were taken as standard (100%) and the rest were compared as percent deviation.

2.7. Pharmacokinetic studies

Application of the new HPLC assay was demonstrated by determining the pharmacokinetic parameters of AM in young male rabbits. Young healthy male rabbits, weighing 2–2.5 kg were procured from the Laboratory Animal Division of the institute and acclimatized for three days prior to study with a 12 h alternate dark and light cycle. Each rabbit was given a single 5 mg kg^{-1} i.v. bolus dose and a 10 mg kg^{-1} peroral dose in a cross over study. A wash out period of seven days was allowed between the two doses to avoid any carryover effects.

2.7.1. Formulation

Solution formulation for i.v. and oral administration was prepared by dissolving 10 mg ml^{-1} of AM in a solvent containing 0.02 M phosphate buffer (pH 7.5), ethyl alcohol and propylene glycol in the ratio 1:4:5. The formulation was refridgerated at 4°C. The stability of the formulation was checked by injecting a diluted

aliquot into the HPLC. The formulation was stable without a decrease in peak response. After 15 days, up to 2% degradation was noticed. As a result, fresh formulations were prepared weekly.

2.7.2. Drug administration

For i.v. administration, rabbits were placed in a rabbit restrainer and 0.5 ml kg^{-1} of drug solution was administered via the marginal ear vein using a 21 G needle. The rabbits were fasted overnight prior to peroral administration. Using a syringe and flexible tubing (silastic) 1 ml kg^{-1} of AM formulation was administered orally. For complete delivery of the dose the canula was flushed with 2 ml water.

2.7.3. Sample collection and storage

Blood samples of nearly 500 μl were collected in glass tubes at 0, 15, 30, 45, 60, 90, 120, 180, 240 and 360 min post dose from the marginal ear vein. Serum was separated by centrifugation and stored at −30°C until analyzed.

2.7.4. Calculation of pharmacokinetic parameters

Model independent and dependent approaches were applied to estimate the pharmacokinetic parameters from the concentration–time profile of AM. The area under the concentration-time curve and the area under the first moment curve were calculated using a combination of linear and log trapezoidal methods. Ratios of AUMC and AUC gave the mean residence time (MRT).

2.8. Application of the assay in human, monkey and rat samples

The newly developed and validated HPLC assay with a fluorescence detection method was checked for the recovery of the drug and possible interference from the serum matrices of rats, monkeys and humans. Blank and spiked serum standards fortified with 400 ng ml^{-1} AM of each of the species were taken in triplicate, processed as described in Section 2.5 and analyzed on the HPLC. Patterns of the chromatograms of the blank serum of all the species were compared and checked for interference. Recovery from the spiked serum of each of the species was calculated and compared with the rabbit serum standard.

3. Results and discussions

3.1. Assay development

AM is a new antimalarial agent under phase II clinical trials. This compound exhibits native fluorescence in acetonitrile solution and the maximum fluorescence intensity is at excitation wavelength 269 nm and emission wavelength 480 nm. It prompted us to exploit the fluorescent properties of the drug to develop this new HPLC assay. The successful analysis of the drug in biological fluids by HPLC relies upon the optimization of the sample preparation, chromatographic separation and post column detection. Each of these three steps were carefully optimized for developing a sensitive, selective, reproducible and robust assay method of AM in 100 μl serum matrix. For optimizing the sample preparation, an organic precipitating agent, acetonitrile fortified with varying concentrations of DMOA and/or potassium hydroxide solution, was tried. All of these methods failed due to poor recovery, degradation of the drug and interference from the biological matrix. Addition of 0.1% DMOA in acetonitrile prevented degradation of AM into primaquine during the protein precipitation step but the serum water present in the supernatant quenched the fluorescence of AM leading to poor sensitivity. The addition of 40 mg sodium sulfate in serum saturates the serum water with salt and decreases its miscibility with acetonitrile. Three layers, namely protein and undissolved sodium sulfate in the bottom, serum water saturated with sodium sulfate in the middle and acetonitrile containing AM on the top, are separated during centrifuging the samples at $3000 \times g$ for 10 min. This upper acetonitrile layer was injected into the HPLC and the clean chromatograms are shown in Fig. 2. Addition of more than 40 mg (up to 80 mg) of sodium sulfate to the serum resulted in insignificant increases in peak height only, by decreasing the volume of supernatant (less than 100 μl) available for injection. The sample preparation steps of the method are simple and rapid to execute and yield chromatograms free from interference from the endogenous matrix constituents (Fig. 2). Sample clean-up is effected by simulta-

neous extraction and protein precipitation by acetonitrile in the presence of sodium sulfate.

Addition of solvents like methanol, hexane and water in the mobile phase (acetonitrile containing 0.05% DMOA), were found to influence the peak height and retention time of the drug. An increase in the proportion of water or methanol in the mobile phase decreased the peak response of the drug by fluorescence quenching. Up to 10% addition of water in acetonitrile has no effect on retention time, whereas greater than 10% (15% water) shows an increase from 4.2 to 5.3 min. The addition of methanol in the mobile phase (up to 15%) decreased the retention time of AM to 3.9 min. The presence of hexane (15%) in the mobile phase did not effect the fluorescence of AM but decreased the retention time of AM to 3.1 min.

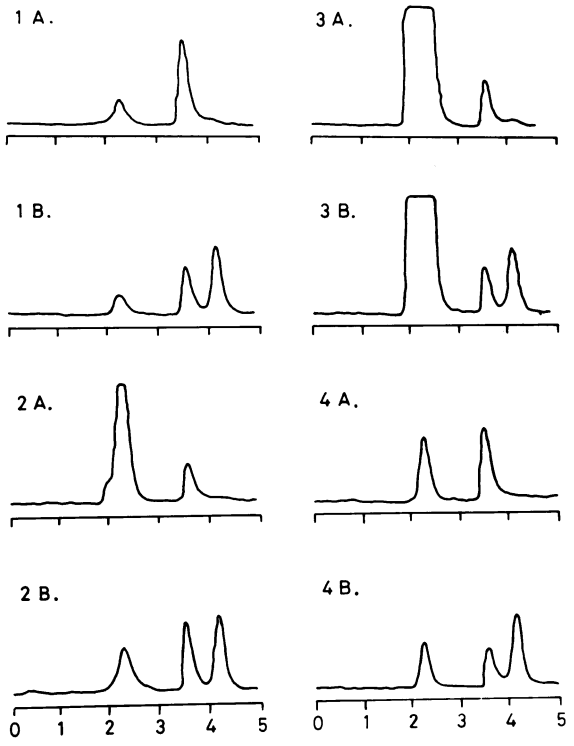

Fig. 2. Representative chromatograms of: (A) blank serum; (B) serum spiked with 400 ng ml^{-1} of AM from (1) monkey; (2) rabbit; (3) rat; and (4) human.

3.2. Method validation

The bioanalytical assay method of AM was validated to demonstrate the specificity, sensitivity, calibration linearity, recovery, precision and accuracy of the method. The lower limits of quantitation and detection of AM were 50 and 15 ng ml^{-1} in serum, respectively. Calibration curves of AM exhibited first order linear relationships in the entire range of analysis (50–1600 ng ml^{-1}). The correlation coefficient was always greater than 0.999 and the day to day variation in slope was negligible. The RSD of all 6 points on the calibration curve in 5 runs was less than 9%. Retention time of AM was 4.2 ± 0.16 min.

Table 1 summarizes the accuracy and the within- and between-run precision for the determination of AM in rabbit serum. The within-run RSD% (precision) and bias (accuracy) were less than 6.4 and 4.2%, respectively. Between-run RSD% was less than 4.3%. The recovery of AM was calculated by determining the peak height of the spiked control samples from the analytical curve and was calculated as a percentage of the nominal concentration, ranging between 70.8–74.5%.

Results of freeze–thaw studies are shown in Fig. 3A. The percent deviation was less than 8% at all three concentration levels studied over three freeze–thaw cycles. However, after the fourth freeze–thaw cycle, the percent deviation for the low (100 ng ml^{-1}) concentration was 15% whereas there was no effect on the medium and high concentrations. No extra peaks of any degradation were observed in the chromatograms. The variations observed in these results were of the same order as within- and between-run variation in accuracy and precision. Hence, it could be assumed that these variations are due to the inherent acceptable variability of the analysis. No trend was observed in the calculated concentrations after four freeze–thaw cycles, indicating stability of the compound under these conditions. At all concentrations, AM was stable in serum supernatant at 4°C, even after 24 h (Fig. 3B). The maximum percent deviation observed in this study was less than 8%. These observed variations were interpreted to be due to inter- and intra-batch variations which were within an acceptable limit.

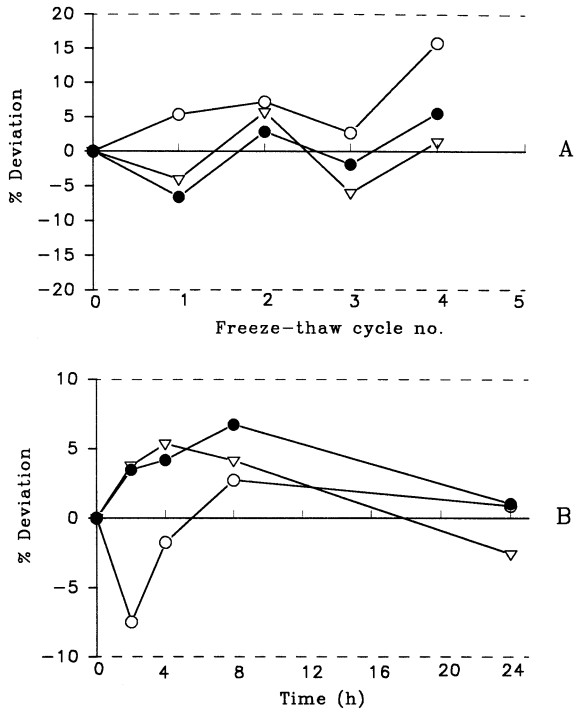

Fig. 3. Effect of: (A) freeze–thaw cycles of rabbit serum; and (B) storage of supernatant at 4°C on the stability of AM. (○) Low (100 ng ml^{-1}), (●) medium (400 ng ml^{-1}) and (▽) high (1600 ng ml^{-1}).

This assay method is capable of determining AM in human, rat and monkey serum. Although the pattern of the peaks appearing before the drug in the chromatograms of the serum of each species were different, there was no interference with the drug peak (Fig. 2). The relative recovery of AM was almost 100% from all the matrices as compared with the rabbit serum standard. Therefore, the same assay method can be used to analyze the serum samples in all these species without further full validation. Thus, the reported assay method for AM is simple, sensitive, reproducible and well suited for pharmacokinetic studies in the serum matrices from humans and laboratory animals.

3.3. Pharmacokinetics of AM in rabbits

Serum concentrations of the compound AM declined biexponentially following 5 mg kg^{-1} i.v. dose in rabbits. This trend was consistent in all five rabbits, as shown in Fig. 4. The serum concentrations fell below the quantitation limit after 6 h of i.v. dose and 90 min after oral dose. The concentration–time profile of AM in rabbits after i.v. dose was best described by a two compartment model and the best fit parameters were obtained by nonlinear least square regression of data on PCNONLIN Version 4 software [14]. The individual and mean pharmacokinetic parameters of AM following an i.v. and oral dose are given in Table 2. The terminal half-life of AM shows high variability amongst the rabbits and ranges between 59 and 168 min after i.v. dose. Compound AM is cleared rapidly from the blood at a rate of 30–40 ml min^{-1}. The value of the steady state volume of distribution, V_{ss} (2.29 l) is approximately half the volume of distribution V (4.21 l). The large difference between V_{ss} and V indicates that appreciable elimination occurs from the central compartment before distribution equilibrium has been achieved.

After oral administration of a 10 mg kg^{-1} dose, the AM concentrations in serum declined below the quantitation limit after 45 min in 3 rabbits and after 90 min in 2. This may be due to the variable degradation of AM under the acidic

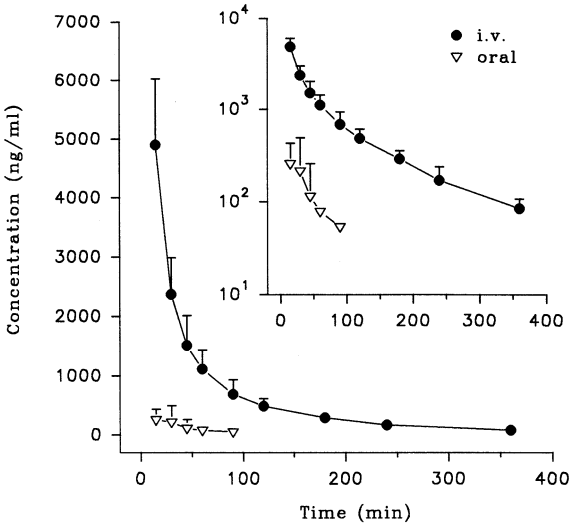

Fig. 4. Concentration–time plots of AM in rabbit receiving a single bolus (●) 5 mg kg^{-1} i.v. dose; (▽) 10 mg kg^{-1} peroral dose (each point represents mean ± SD of 5 rats).

Table 2
Pharmacokinetic parameters of compound AM in rabbits receiving a single bolus 5 mg kg^{-1} i.v. or 10 mg kg^{-1} peroral dose

Pharmacokitic parameters	Intravenous dose (5 mg kg^{-1})		Peroral dose (10 mg kg^{-1})	
	Mean	SD	Mean	SD
λ_1 (min^{-1})	0.074	0.017		
λ_2 (min^{-1})	0.0084	0.003		
λ_2 HL (min)	95.3	43.5		
k_{10}-HL (min)	19.1	4.7	16.6	7.0
V (l)	4.21	1.62		
V_{ss} (l)	2.29	0.37		
CL (l min^{-1})	0.032	0.004		
MRT (min)	104	10.6	31.1	8.3
$AUC_{(0-t)}$ (ng min^{-1} ml^{-1})	195711	33743	9490	9870

pH conditions of the stomach. The terminal half-life and MRT of AM were observed between 10 to 28 min and 23 to 43 min, respectively Table 2. Only 3–4 data points were available on the serum concentration–time curve of rabbits receiving oral dose, therefore it was not possible to estimate the pharmacokinetic parameters by the compartmental approach. For the same reason, the absolute bioavailability of AM in rabbits was not calculated. It is worth mentioning that AM is unstable in aqueous solutions and immediate flushing of the canula with water following oral administration might decompose significant amounts of the dose before being absorbed systemically. The overall variability in pharmacokinetic parameters as evident from the high value of SD in Table 2, might be due to variability in degradation/metabolism of the drug before and after absorption. It was primarily due to its degradation under gastric pH conditions. Hence, AM should not be given as an oral solution for its efficacy and safety studies. A similar variability in pharmacokinetic parameters of AM in healthy human subjects receiving 25, 50 or 75 mg single oral dose was observed. The terminal half-life was independent of dose and ranged between 1.85 and 7 h (unpublished data from this laboratory). In this study, only the parent drug was selectively measured and the major active metabolite of AM, primaquine, could not be determined due to the limitations of the assay method (primaquine is a non-fluorescent molecule).

To summarize, this study describes the simple, sensitive and robust fluorescent HPLC assay method for analyzing AM in rabbit, monkey, rat and human serum. The method was successfully validated from a 50 to 1600 ng ml^{-1} concentration range in rabbit serum. This method was capable of monitoring the serum levels of AM up to 6 h after a 5 mg kg^{-1} i.v. dose and was used to evaluate the pharmacokinetic parameters of AM in rabbits.

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