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Simultaneous quantification of α-/β-diastereomers of arteether, sulphadoxine and pyrimethamine: A promising anti-relapse antimalarial therapeutic combination, by liquid chromatography tandem mass spectrometry☆

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Abstract

A rapid, sensitive, selective and specific HPLC/ESI-MS/MS assay method was developed and validated for the simultaneous quantitation of α-/β-diastereomers of arteether (AE), sulphadoxine (SDX) and pyrimethamine (PYR) in rat blood plasma using propyl ether analogue of β-arteether as internal standard. The method involved a single-step, liquid–liquid extraction with ethyl acetate and the analytes were chromatographed on a C_{18} chromatographic column by isocratic elution with methanol:ammonium acetate buffer (10 mM, pH 4) (90:10%, v/v) and analyzed by tandem mass spectrometry. The run time was 4.5 min and the weighted ($1/x^2$) calibration curves were linear over a range of 0.78–400 ng ml^{-1}. The method was validated fully and the lower limit of quantification (LLOQ) in plasma was 0.78 ng ml^{-1} for all the analytes. The intra- and inter-day precision and accuracy were found to be well within the acceptable limits (<15%) and the analytes were stable after three freeze–thaw (f–t) cycles. The absolute recoveries were consistent and reproducible. The assay method was applied to pre-clinical pharmacokinetic interaction studies of α-/β-AE, SDX and PYR in rats.

Keywords: Tandem mass spectrometry; Blood plasma; Sulphadoxine; Pyrimethamine; α-/β-Arteether

1. Introduction

Malaria control all over the world is threatened by widespread resistance of *Plasmodium falciparum* to chloroquine, the mainstay of antimalarial therapy for the past 50 years [1,2]. Most of the sub-Saharan and African countries, which bear the greatest burden of malaria morbidity and mortality, face an impending catastrophe because of rising cases of parasite resistance and associated increase in childhood mortality [3,4]. Thus, it is imperative to refurbish the existing therapeutic armamentarium against malaria and optimally utilize the existing chemotherapeutic agents to avoid or minimize the development of resistance.

It has been observed that indiscriminate use of antimalarial drugs, mainly as mono-therapies, for curative and more often as prophylactics lead to rapid development of parasite resistance. An alternative to this problem is to design and develop combination chemotherapies with drugs possessing differing mechanisms of action against the malarial parasite [5,6]. However, the therapeutic lifespan of the most widely used, cost effective fixed-dose combination of sulphadoxine (SDX)–pyrimethamine (PYR) (Fig. 1), which inhibits the two-folate pathway enzymes, is limited by the rapid emergence of resistant parasites [7]. This alarming situation of high level of parasite resistance to the common mono-therapies such as chloroquine and common combination therapies such as SDX–PYR have led to the evaluation and use of artemisinin-based combination therapies. These combinations have been found to be effective against drug resistance malaria in a variety of clinical settings [8].

In addition to preventing the emergence of parasite resistance and extending the therapeutic lifespan of the chemotherapeutic agents, artemisinin-based combinations of SDX–PYR is believed to minimize the chances of recrudescence to a significant extent. Thus, employing artemisinin-based therapeutic combinations can ensure complete clinical cure of

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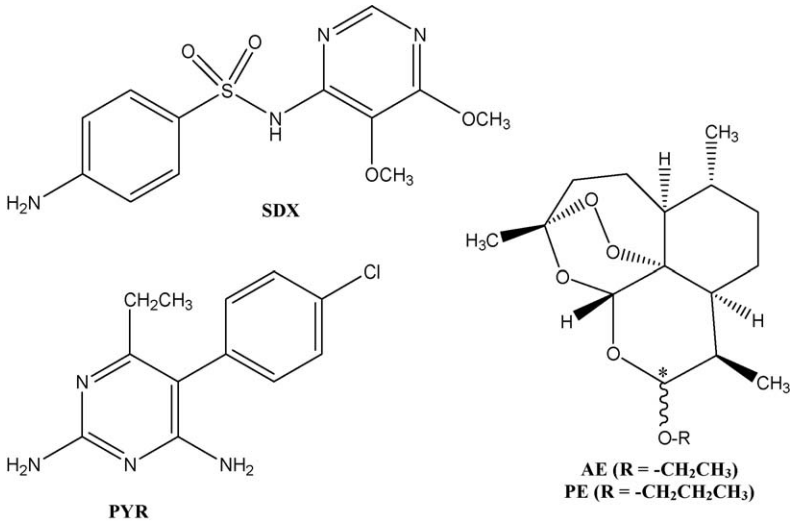

Fig. 1. Chemical structures of sulphadoxine (SDX), pyrimethamine (PYR), α-/β-arteether (AE) and propyl ether analogue of β-AE (PE used as internal standard). The asterisk denotes the position of the asymmetric center in AE.

plasmodium malaria. α/β-Arteether (AE) (Fig. 1), the oil soluble erythrocytic schizontocidal drug of artemisinin class is considered as the most rapidly acting antimalarials to date and is an obvious choice for developing a potent therapeutic combination. To date there are no reports of parasite resistance to α/β-arteether, nor to any of the artemisinin class of drugs, for that matter [9]. Its mechanism of action is entirely different from that of SDX and PYR, making it a perfect choice for combining with this established combination. However, before optimizing a therapeutic combination it is imperative to assess the interaction potentials, both pharmacodynamic and pharmacokinetic (PK/PD) interactions of the candidate drugs, when co-administered. The primary requirement to undertake a PK/PD interaction study is to have an analytical method which is reliable, reproducible, sensitive, selective, and if possible, compatible with high throughput pharmacokinetic (HTPK) approaches.

Earlier reports on bioanalytical methods for SDX and PYR are highly limited to HPLCs with conventional detection techniques and involve tedious sample clean-up procedures [10,11]. On the other hand, artemisinin derivatives pose challenging problems to bioanalysis due to the lack of UV or fluorescence sensitive chromophores for conventional detection techniques. The reported electrochemical detection methods for artemisinins, however, have many limitations and drawbacks [12,13]. But tandem mass spectrometry could overcome most of these shortcomings and many such methods are available in the literature for artemisinin class of compounds like dihydroartemisinin, artemether or arteether [14–16], including a highly sensitive assay reported earlier from our laboratory [17]. However, to date there are no reports on the simultaneous estimation of SDX and PYR with the diastereomers of arteether from rat blood plasma using tandem mass spectrometry. We intend to report, for the first time, a liquid chromatographic tandem mass spectrometric assay for the simultaneous estimation of α-/β-diastereomers of AE, SDX and PYR from blood plasma for their pre-clinical PK/PD interaction studies.

2. Materials and methods

2.1. Chemicals and reagents

Pure reference standards of α-/β-AE were obtained from Themis Medicare Limited (Mumbai, India). SDX and PYR pure reference standards were gifted by Cadila Pharma (India). Propyl ether analogue of β-AE (PE), used as internal standard (IS) was procured from the Medicinal and Process Chemistry Division of Central Drug Research Institute (Lucknow, India). Methanol, HPLC grade, was purchased from Thomas Baker (Chemicals) Limited (Mumbai, India). HPLC grade ethyl acetate was obtained from Mallinckrodt (KY, USA). 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 water purification system. Heparin sodium injection I.P. (1000 IU ml^{-1}, Biologicals E. Limited, Hyderabad, India) was obtained by local purchase. Sigmacote® CAT No. SL-2, used for silanising glassware was procured from Sigma Chemical Co. (St. Louis, USA). Blank drug-free rat plasma was collected from healthy, male *Sprague–Dawley* rats at Laboratory Animal Services Division of Central Drug Research Institute (Lucknow, India). Plasma was obtained by centrifuging the heparinised blood at 1000 × *g* for 15 min. Pooled or individual plasma samples were stored in glass vials at −60° C pending analysis or use. All ethical guidelines for maintenance and experimental studies with *Sprague–Dawley* rats were fully complied with during this procedure.

2.2. Calibration standards and quality control samples

Stock solutions (1 mg ml^{-1}) of α-/β-AE, SDX, PYR and the IS (PE) were prepared in 100% methanol and were used for preparing the respective working stock solutions. A mixed stock solution of the analytes in methanol containing 20 μg ml^{-1} each of α-/β-AE, SDX and PYR was used for constructing the analytical (AS) and calibration standard (CS) curves. To min-

Table 1
Optimized mass spectrometric conditions employed for the assay

Analyte	Q1 MS (*m/z*)	Q3 MS (*m/z*)	Dwell time (ms)	DP	CE	EP	CXP
Period I (0–2 min)							
SDX	311	156	200	95	24	10	10
PYR	249	198	200	95	50	10	10
Period II (2–4.5 min)							
α-AE	330[a]	267	200	35	13	10	15
β-AE	330[a]	267	200	35	13	10	15
PE (IS)	344[a]	267	200	40	13	10	15

DP: declustering potential, CE: collision energy, EP: entrance potential, CXP: collision cell exit potential.
[a] Ammonium adduct $[M + NH_4]^+$.

imize the non-specific adsorption of AE, the glassware used for the experiment was silanised prior to use. The volume of organic phase being spiked in to bio-matrix was less than 2.5% (v/v). The CSs were prepared in blank normal rat plasma over a concentration range of 0.78–400 ng ml^{-1} by serial dilution approach. Quality control (QC) samples at four different concentration levels (0.78, 1.56, 100 and 400 ng ml^{-1} as low$_1$, low$_2$, medium and high, respectively) were prepared separately in five sets independent of the calibration standards. During analysis these QC samples were spaced after every six to seven unknown samples to ensure the reliability of each analytical run.

2.3. Sample preparation

Sample preparation involved a simple, single-step, liquid–liquid extraction (LLE) with HPLC grade ethyl acetate (1 × 2 ml). The processing volume of plasma was fixed as 100 μl. The IS solution was spiked to such aliquots so as to get a final concentration of 30 ng ml^{-1} of PE and vortex mixed (Type 37600 mixer, Thermolyne, USA) prior to the addition of the extraction solvent. After mixing thoroughly with vortex mixer for 90 s and centrifugation (1000 × *g* for 5 min), the upper organic layer was transferred to another set of clean tubes after freezing the lower aqueous layer in liquid nitrogen. Then the organic phase was evaporated in Savant Speed Vac (USA) at 40 °C. The dry residue obtained was reconstituted in 100 μl of methanol and injected to HPLC–MS/MS.

2.4. Chromatographic conditions

A series 200 pump with flow control valves (Perkin-Elmer Instruments, Norwalk, CT, USA) was used to deliver an isocratic flow (90:10%, v/v) of methanol:10 mM ammonium acetate buffer of pH 4.0 at a flow rate of 0.4 ml min^{-1}. The chromatographic separation was achieved on a C-18 column (30 mm × 4.6 mm, 5 μm, Ultracarb, Phenomenex, USA). Samples were introduced into the chromatographic system using a Series 200 auto-sampler with peltier control tray. An automated column switching assembly (Vici Valco valve, USA) was used to control the mobile phase flow into the mass spectrometer. The analysis was carried out at ambient temperature and the pressure of the chromatographic system was ∼1500–1600 psig.

2.5. Mass spectrometric conditions

Mass spectrometric detection was performed on an API 4000 LC–MS/MS system (Applied Biosystems, MDS Sciex USA) equipped with an API electrospray ionization (ESI) source. Zero-air was used as source gas while ultra high pure (UHP) nitrogen was used as both curtain and collision gases. The mass spectrometer was operated at ESI positive ion mode and the analytes were quantified using multiple reactions monitoring (MRM). The transitions monitored were *m/z* 330 → 267, 311 → 156, 249 → 198 and 344 → 267 for α-/β-AE, SDX, PYR and IS, respectively. The declustering potential (DP), collision energy (CE) and other parameters for all the analytes as well as internal standard were optimized individually and are shown in Table 1. The ESI capillary was set at 5.5 kV and the source temperature was maintained at 200 °C. Data acquisition was performed using Analyst software platform version 1.4. Calibration and analytical standard curves were constructed using the peak area ratios of analyte to IS by weighted ($1/x^2$) least-square linear regression. Test samples and quality control samples are then interpolated from the calibration curve to obtain the concentrations of the respective analytes.

3. Results and discussion

3.1. Method development

Optimization of the declustering potentials (DP) of the ammonium adducts $[M + NH_4]^+$of α-/β-AE and PE at *m/z* 330 and 344, respectively, and protonated species $[M + H]^+$ of SDX and PYR at *m/z* 311 and 249, respectively, were carried out by continuous flow analysis. The influences of buffer molarity, pH and types of organic modifiers on the signal intensity were also studied at the optimized DPs for each of the analytes. Based on the peak intensities a 10 mM ammonium acetate buffer of pH 4 and methanol as the organic phase were selected for further studies. Since artemisinin type of compounds form multimers and cause a decline in the signal intensities of their respective monomers, the highest concentration of AE used in the present study was restricted to 400 ng ml^{-1}. It was observed that such multimer formation was insignificant at this concentration level under the mass spectrometric conditions employed. Also higher proportion of the organic modifier in the mobile phase was

found to improve the signal intensity. However, considering the need for chromatographic resolution between α- and β-AE the organic content in the mobile phase was optimized to be 90% (v/v). Since a reported method for the simultaneous estimation of both the isomers of AE is available [17], the major challenge was to incorporate SDX and PYR in the method without compromising on sensitivity and selectivity. Thus, a two-period assay was designed in multiple reaction monitoring mode (MRM), the most selective mode of detection, with the first period (0–2 min) dedicated for SDX and PYR and the latter (2–4.5 min) for AE isomers and IS, respectively.

Continuous flow MS/MS analyses were carried out to obtain the product ion spectra of the analytes. The collision energy and collision gas pressures were optimized such that the most abundant ion in the product ion spectra was of *m/z* 267 for α-/β-AE and PE and *m/z* 156 and *m/z* 198 for SDX and PYR, respectively. Monitoring these transitions of the parent molecules (*m/z* 330 and 344 → 267, *m/z* 311 → 156 and *m/z* 249 → 198) showed no interference in blank biological matrix (Fig. 2). So the mass spectrometer was set as follows: *m/z* 330, 311, 249 and 344 as precursor ions for α-/β-AE, SDX, PYR and IS, respectively, and *m/z* 267 as common product ion for α-/β-AE and IS and *m/z* 156 and *m/z* 198 for SDX and PYR, respectively, in the MRM mode. The propyl ether analogue of AE was selected as the IS as it is no longer used as an antimalarial drug in clinical situations, thus avoiding the chances of interference if the method is to be used in uncontrolled clinical trials.

The sample clean-up involved a single extraction solvent (ethyl acetate), but by using higher proportion of the extraction solvent (20 times the volume of bio-matrix) and by incorporating a thorough vortex mixing step (of 90 s duration), the extraction efficiency was enhanced. The recoveries obtained by this approach were similar to that reported earlier with the use of multiple extraction steps and/or with different extraction solvents [14,17]. As evident from Fig. 2, no interfering peaks were observed in the MRM chromatograms of blank plasma under the LC–MS/MS conditions described here.

The possibility of matrix effect on ionization was explored further by comparing the responses obtained from blank plasma extracts spiked with reference solutions with that of reference solutions of same concentrations in 100% methanol, which was used as the reconstitution solution. This study was carried out over the entire concentration range of 0.78–400 ng ml^{-1}. This proved that there was no appreciable matrix suppression on ionization with the ethyl acetate extract under the optimized mass spectrometric conditions.

The methanol content in the mobile phase was found to be critical for the signal intensity as well as for the elution of the analytes. So a balance between signal intensities and chromatographic resolution was achieved with 90% organic content in the mobile phase and the retention times of α-/β-AE, SDX, PYR and IS were found to be 2.40, 3.11, 1.44, 1.55, and 3.47 min, respectively, at the set flow rate of 0.4 ml min^{-1}. Maximum recoveries and signal intensities were achieved when 100% methanol was used as reconstitution solution.

The high organic content in the mobile phase (90% methanol) tempts us to think that the retention mechanism may be

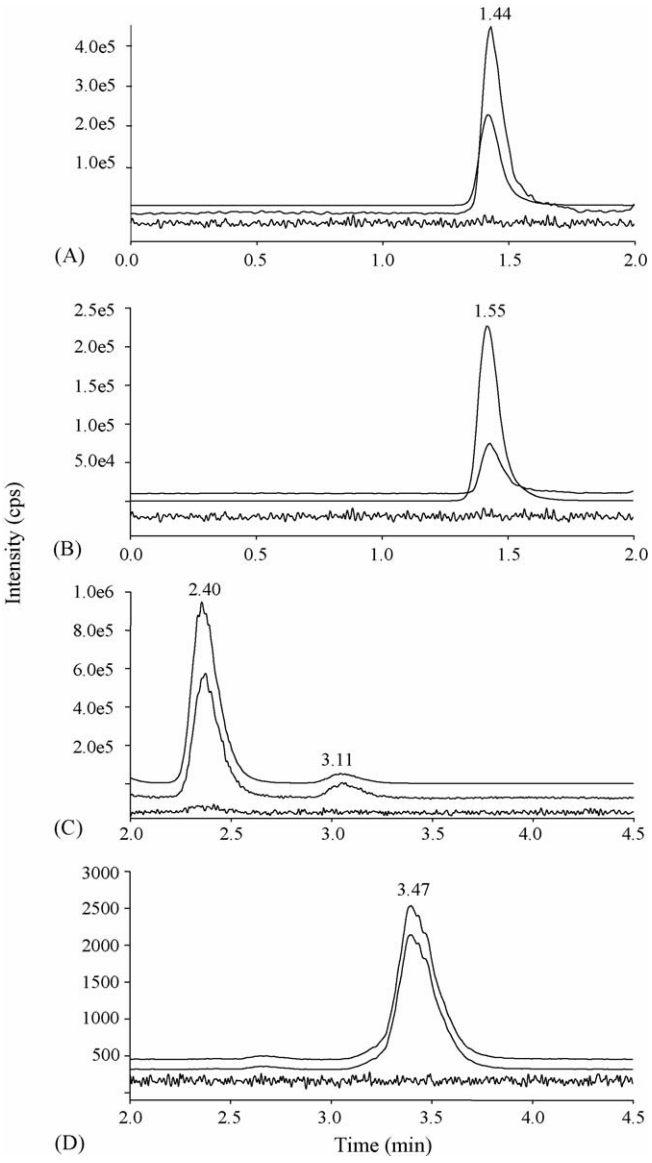

Fig. 2. Representative MRM chromatograms of (A) SDX, (B) PYR, (C) α-/β-AE and (D) PE, the internal standard, indicating selectivity of the assay. The chromatograms are shown in the following order: blank processed plasma, spiked plasma at LOQ (0.78 ng/ml) and 15 min rat test sample from pharmacokinetic studies.

hydrophilic interaction. In hydrophilic interaction chromatography (HILIC), compounds are separated by eluting a hydrophobic or mostly organic mobile phase across a neutral hydrophilic stationary phase, causing the solutes to elute in order of increasing hydrophilicity—the inverse of reversed phase chromatography. Moreover, an increase in the aqueous content in the mobile phase would result in a decrease in retention times [18]. A closer look would reveal that the order of elution in this case is same as in reversed phase chromatography, with more polar compounds eluting first followed by the less polar ones and any increase in water content in the mobile phase delayed the chromatographic elution of the analytes. Thus, at this point the present method may be considered as reversed phase chromatography.

Table 2
Assay linearity of the method (values are mean ± S.D., $N = 5$)

Analyte	Slope	Intercept	R^2
α-AE	0.0964 ± 0.0432	0.0778 ± 0.0139	0.999 ± 0.002
β-AE	0.0184 ± 0.0072	0.0085 ± 0.0036	0.995 ± 0.008
SDX	0.0088 ± 0.0036	0.0049 ± 0.0013	0.996 ± 0.002
PYR	0.0107 ± 0.0040	0.0070 ± 0.0007	0.995 ± 0.008

3.2. Assay performance and validation

Accuracy, precision, selectivity, sensitivity, linearity, recovery and stability were measured and used as the parameters to assess the assay performance [19]. The peak area ratios of α-/β-AE, SDX and PYR to IS in plasma were linear with the analyte concentration over a range of 0.78–400 ng ml^{-1}. The calibration model was selected based on the analysis of the data by linear regression with/without intercepts and weighting factors ($1/x$, $1/x^2$ and $1/\sqrt{x}$). The residuals improved by weighted ($1/x^2$) least square linear regression. Best fit for the calibration curve could be achieved by a linear equation of $Y = mX + c$ with $1/X^2$ weighting factor. The coefficient of determination (R^2) for α-/β-AE, SDX and PYR were above 0.995 over the concentration range used. Assay linearity of the method is presented in Table 2.

LC–MS/MS analysis of the blank plasma samples showed no interference with the quantification of α-/β-AE, SDX, PYR and IS. Specificity of the method was established with pooled as well as individual plasma samples from six different sources. Representative chromatograms of extracted blank plasma, blank plasma fortified with α-/β-AE, SDX, PYR and IS and test sample, indicating the selectivity of the method, are shown in Fig. 2. The retention times of all the analytes and the IS showed less variability with percent relative standard deviation (R.S.D.) well within the acceptable limit of 5% [19].

The limit of detection (LOD) demonstrated that all the analytes gave a signal-to-noise ratio (S/N) of 3 and above for 0.39 ng ml^{-1} extracted/injected. The lower limit of quantification (LLOQ), the lowest concentration in the standard curve, which can be measured with acceptable accuracy and precision, for α-/β-AE, SDX and PYR from normal rat plasma was established at 0.78 ng ml^{-1}. LLOQ was established with five samples independent of the standard curve.

Table 3
Accuracy and precision of the assay

Analyte	QC samples (ng ml^{-1})	Accuracy (% bias)		Precision (% R.S.D.)	
		Inter-day	Intra-day	Inter-day	Intra-day
α-AE	0.78	−5.7	5.2	5.8	7.1
	1.56	−4.2	3.4	6.3	4.8
	100	1.7	4.1	2.1	2.0
	400	2.5	−1.7	5.4	5.1
β-AE	0.78	2.5	3.0	1.7	3.9
	1.56	7.8	4.7	5.8	7.1
	100	−1.1	3.2	6.3	4.8
	400	−4.2	−3.7	2.3	7.3
SDX	0.78	5.7	7.2	2.0	4.8
	1.56	6.4	6.3	5.0	2.8
	100	3.5	−4.5	4.1	5.2
	400	4.7	7.0	5.4	3.5
PYR	0.78	−4.4	5.7	6.5	5.2
	1.56	3.8	5.0	4.5	1.0
	100	−5.3	3.9	5.2	1.7
	400	2.1	3.8	4.5	2.4

Accuracy and precision (intra- and inter-batch) were calculated with five (excluding blank) determinations per concentration level on five days (five each of duplicate low, medium and high QC samples) and are presented in Table 3. The precision was determined by within- and between-assay %R.S.D. [19] and accuracy was expressed as percentage bias. The results showed that the analytical 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 absolute recoveries of the analytes were calculated by comparing the mean peak areas from spiked plasma samples with that of reference solutions spiked in extracted drug-free plasma samples at similar concentration levels. The recovery of the IS (at 30 ng ml^{-1}) from the extracted calibration standards and QC samples during validation was 93.90 ± 6.9%. The average absolute recoveries for α-/β-AE, SDX and PYR over the concentration range of 0.78–400 ng ml^{-1} are shown in Table 4.

These results show that the analytical method is accurate and precise over the concentration range of 0.78–400 ng ml^{-1}.

Table 4
Table of average absolute recoveries (%) of α-/β-AE, SDX and PYR from spiked rat plasma samples (values are mean ± S.D., $N = 5$)

Concentration (ng ml^{-1})	α-AE	β-AE	SDX	PYR
0.78	79.91 ± 14.80	64.17 ± 10.35	76.90 ± 7.85	53.20 ± 12.75
1.56	83.25 ± 2.80	69.26 ± 4.81	73.00 ± 10.73	57.21 ± 8.50
3.13	90.00 ± 11.67	71.29 ± 15.76	76.4 ± 7.83	55.60 ± 9.61
6.25	86.73 ± 1.23	66.44 ± 11.67	67.80 ± 18.34	63.22 ± 14.53
12.5	87.15 ± 20.55	75.40 ± 23.41	69.40 ± 9.61	60.40 ± 10.73
25	90.21 ± 11.18	68.53 ± 18.78	69.70 ± 16.10	58.30 ± 7.16
50	85.54 ± 16.86	72.66 ± 18.11	70.10 ± 14.98	60.11 ± 10.51
100	86.28 ± 9.15	71.72 ± 19.74	68.5 ± 10.73	55.60 ± 17.44
200	83.58 ± 15.72	71.42 ± 20.41	70.2 ± 12.07	51.21 ± 9.61
400	84.21 ± 11.85	70.21 ± 10.51	68.10 ± 10.96	50.82 ± 16.99

3.3. Stability studies

QC samples were subjected to short-term room temperature, long-term storage condition (−60 °C) and freeze–thaw (f–t) stability studies. All the stability studies were carried out at three different concentrations levels (0.78, 100, 400 ng ml^{-1} as low, medium and high) in triplicate.

There was no significant difference between the responses of spiked standards at time zero and after 8 h for α-/β-AE, SDX and PYR (deviations <15%), indicating the stability of analytes at room temperature for over 8 h, which well encompasses the duration of typical sample handling and processing. Moreover, the analytes were found to be stable in the reconstitution solution for at least 12 h at 4 °C. The re-injection reproducibility was established to determine if an analytical run could be reanalyzed or repeated in case of any unexpected delays in analyses. The same set of QC samples were repeated after one analysis with 4 h gap in between during which the samples were stored at 4 °C and in all cases the deviations were less than 15%.

The deviations observed after first, second and third freeze–thaw cycles were within ±15% as is evident from the Table 5 at the concentration levels used for α-/β-AE, SDX and PYR, respectively, indicating adequate freeze–thaw stability. The spiked QC samples in triplicate, which were extracted and analyzed immediately (0 f–t) were used as the reference points to calculate the percent deviations after first, second and third f–t cycles. Also the QC samples stored at −60 °C were analyzed after 7, 15 and 30 days and there were no significant deviations with respect to the immediately analyzed samples. The long-term stability data at −60 °C are shown in Table 6. On similar lines, stability of the extracted dry residue was also established to be over 15 days (deviations observed <10% at all concentration levels studied).

Table 5
Freeze–thaw stability

Analyte	Concentration (ng ml^{-1})	Percent deviations after freeze–thaw cycles		
		1	2	3
α-AE	0.78	−2.3	6.4	4.8
	100	−3.7	2.3	7.3
	400	7.2	2.0	9.0
β-AE	0.78	6.3	5.0	2.8
	100	−4.5	4.1	5.2
	400	−1.3	3.8	1.2
SDX	0.78	5.7	−5.4	1.8
	100	0.8	−6.2	2.4
	400	1.5	0.25	6.0
PYR	0.78	4.3	−6.1	5.4
	100	−2.1	2.5	3.1
	400	−1.4	1.1	7.4

3.4. Application to pharmacokinetic interaction studies

The assay method was applied for the PK interaction studies of α-/β-AE, SDX and PYR in male *Sprague–Dawley* rats. Healthy male rats (225 ± 25 g) were divided into three treatment

Table 6
Stability data: bench top stability at room temperature and on long-term storage at −60 °C

Analyte	Storage condition	Nominal concentration (ng ml^{-1})	Concentration at $t = 0$ (ng ml^{-1})	Concentration recovered (ng ml^{-1})
α-AE	8 h at 25 °C	0.78	0.76	0.74
		100	100.5	97.5
		400	400.2	396.4
	30 days at −60 °C	0.78	0.77	0.74
		100	99.5	93.2
		400	401.2	390.2
β-AE	8 h at 25 °C	0.78	0.79	0.73
		100	99.5	93.2
		400	398.2	392.4
	30 days at −60 °C	0.78	0.78	0.75
		100	100.0	94.6
		400	402.5	395.8
SDX	8 h at 25 °C	0.78	0.76	0.77
		100	98.2	99.5
		400	396.5	398.4
	30 days at −60 °C	0.78	0.77	0.75
		100	100.4	96.4
		400	394.0	391.2
PYR	8 h at 25 °C	0.78	0.78	0.77
		100	100.3	99.8
		400	403.4	397.1
	30 days at −60 °C	0.78	0.79	0.77
		100	101.1	97.7
		400	410.1	401.6

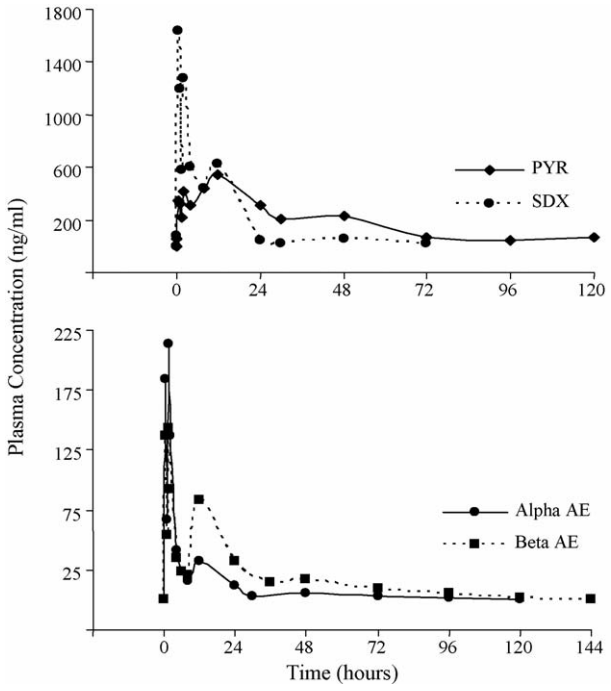

Fig. 3. Representative mean plasma concentration–time profile of SDX, PYR and α-/β-AE in rats during the PK interaction studies.

groups with the first group receiving α-/β-AE formulation, the second group receiving SDX–PYR combination and the third group receiving both α-β-AE and SDX–PYR concurrently. The α-/β-AE was administered intramuscularly into thigh muscles of rats at 30 mg kg^{-1} dose (EMAL injection containing both α- and β-AE in 30:70, w/w ratio), while SDX–PYR combination was administered orally (as extemporaneous aqueous suspension containing both SDX and PYR in 20:1 ratio without any suspending agent).

Blood samples were collected by sparse sampling approach ($N = 3$) and the plasma samples were analyzed by the validated LC–MS/MS assay. The plasma levels of all the analytes were monitored in blood plasma and the plasma concentration time data were subjected to model independent PK analyses. The mean plasma concentration time profile for SDX, PYR and α-/β-AE are shown in Fig. 3.

4. Conclusion

An LC–MS/MS method for the simultaneous quantification of α-/β-AE, SDX and PYR in rat plasma was developed and fully validated. This method offers significant advantages over those previously reported, in terms of selectivity, sensitivity, rapid sample clean-up procedures, faster run time and lower volumes of sample requirements. The established LOQ of 0.78 ng ml^{-1} is sufficiently low for pharmacokinetic interaction studies and could be further improved, if required, by sample concentration techniques. The internal standard, propyl ether analogue of AE, selected is acceptable in clinical trial situations also, as it is no longer used as a potential antimalarial drug. The results of the assay performance and the study conducted indicate that the method is precise and accurate enough for the PK/PD interaction studies of α-/β-AE, SDX and PYR as well as for their routine estimations as in therapeutic drug monitoring (TDM).

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