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A high throughput approach for simultaneous estimation of multiple synthetic trioxane derivatives using sample pooling for pharmacokinetic studies

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Abstract

The present study describes the application of concept of sample pooling to increase the throughput of pharmacokinetic screening at drug discovery and development stage. An HPLC-UV method for the simultaneous estimation of three synthetic antimalarial compounds 99/357, 99/408 and 99/411 has been developed and validated in rat serum with internal standard for pharmacokinetic profiling. Drug compounds in serum were extracted by two-step liquid–liquid extraction with 2% isopropyl alcohol in *n*-hexane and quantitated using a validated gradient HPLC-UV method, which was made feasible for all compounds using gradient elution scheme. The method was validated in terms of HPLC reproducibility, linearity, specificity, recovery, accuracy and precision, freeze thaw stability and long-term storage stability. Excellent linear relationships ($r > 0.99$) were obtained for calibration as well as analytical standards over a concentration range of 25–1000 ng/ml for three analytes. Recoveries were fond to be >85% for 99/408 and 99/357 and >70% for 99/411. The method developed for three analytes was found to be accurate and precise as bias and percent relative standard deviation (% R.S.D.) values were within limits (<20%). By employing sample pooling approach, plasma level – time profile following single intravenous dose of all three compounds were obtained in a fraction of the time required by conventional single compound dosing and analysis.
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Keywords: Sample pooling; Reversed phase liquid chromatography; Pharmacokinetics; Serum; Antimalarial

1. Introduction

The synthesis of large number of compound with the advent of robotics and combinatorial chemistry and HTS results in rapid identification of leads, which requires further preclinical studies before entering to developmental stages. Good pharmacokinetic and metabolism properties are fundamental to the success of the drug candidate, and it is important to have supporting preclinical data before entering to the clinical trials. The success rate of the candidate molecules relies on desired attributes of bioavailability, chemical tractability selectivity and potency. Effective analysis method for generation of the rapid pharmacokinetic data prevents unsuitable candidates reaching later stages and thus maximizing the cost effective utilization of resources. High throughput pharmacokinetic (HTPK) screening approaches provides the rapid and effective ways to generate pharmacokinetic (PK) information and thus economize the time and resources required for the drug discovery. It is therefore necessary to bring the 'traditional' low throughput activities of drug metabolism and pharmacokinetics into the higher throughput arena [1,2]. This leads to birth of novel concept for performing PK studies, i.e. sample pooling and cassette dosing. Sample pooling and Cassette (N-in-one) dosing are the techniques for higher throughput screening in drug development to rapidly assess pharmacokinetic and metabolic profiles of large number of compounds [3].

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Cassette dosing involves administration of multiple compounds to a single animal – sampling – analyzing the samples simultaneously for all the compounds. While cassette dosing seems to be an efficient way to simultaneously screen many compounds, the potential for drug–drug interactions is a problem even at lower doses. Alternatively the sample pooling approach involves administration of one compound per animal, followed by sampling. The samples of same time point for all compounds are pooled and subjected to simultaneous bioanalysis. This one-in-one in vivo approach to accelerate the acquisition of the concentration time data required for pharmacokinetic screening is devoid of potential drug–drug interaction as in N-in-one in vivo pharmacokinetics [4–6].

Both sample pooling and cassette dosing capitalize heavily on tandem liquid chromatography/mass spectrometry due to its high selectivity and sensitivity. In the present study traditional HPLC-UV method is used for simultaneous estimation of multiple synthetic compounds owing to limited availability of LC MS/MS. The size of the sample pool that could be employed under the sample pooling concept depends entirely on the selectivity, specificity and sensitivity of the bioassay developed. In the present method the applicability of conventional bioanalytical techniques like HPLC-UV to sample pooling concept is described using three novel 1,3,4-trioxane antimalarials of artemisinin class [7–12], 99/408, 99/411 and 99/357, developed at Central Drug Research Institute (CDRI), Lucknow.

The present method differs from previously reported HPLC-UV method for 99/357 [13] as it includes two more pharmacologically active trioxane congener and will be applied in estimation of all three compound by novel sample pooling concept, thus giving higher throughput to preliminary pharmacokinetic screening. The assay method reported here was fully validated as per ICH guidelines so as to facilitate reliable and accurate pharmacokinetic profiling. The method was applied to generate intravenous pharmacokinetic profiles of these candidate molecules in male *Sprague-Dawley* rats to illustrate the applicability of sample pooling concept.

2. Materials and methods

2.1. Chemical, reagents and apparatus

The structure of three compounds 99/357, 99/411 and 99/408 (synthetic trioxane derivative) and internal standard is shown in Fig. 1. These compounds (purity >99%) along with internal standard were synthesized by Dr. Chandan Singh in house at the Medicinal Chemistry Division of Central Drug Research Institute (CDRI), Lucknow, India. HPLC grade acetonitrile was obtained from J.T. Baker (USA). *n*-Hexane was procured from J.T. Baker (Philipsburg, USA). HPLC grade isopropyl alcohol (IPA) and dimethyl formamide (DMF) were obtained from Spectrochem Pvt. Ltd. (Mumbai, India). Deionized water (DW) was obtained from Milli Q

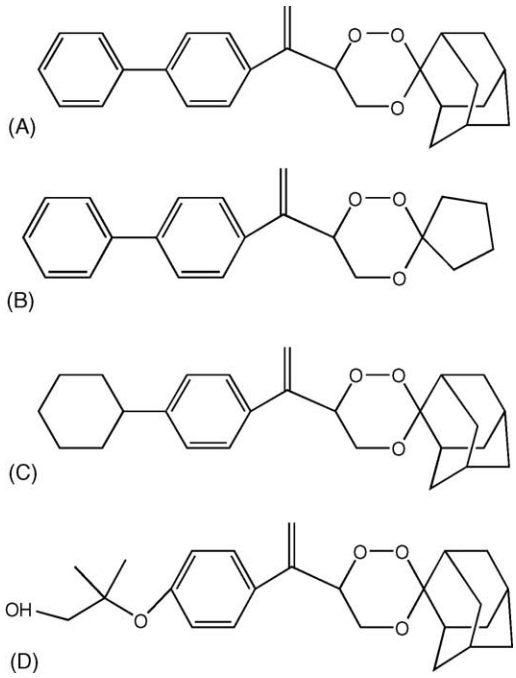

Fig. 1. Chemical structures of (A) 99/357, (B) 99/408, (C) 99/411 and (D) internal standard.

PLUS system (18.2 MΩ cm). All other chemicals were of analytical grade and procured from local sources unless specified.

The HPLC (Shimadzu, Japan) system consisted of a system controller (SCL 10Avp), pump (LC-10Atvp) along with quaternary flow control valve system (FCV-10ALvp) and a degasser (DGU-14A) to pump the mobile phase. The detection was performed using a two-channel UV/vis detector (SPD-10Avp) set at wavelengths 247 and 266 nm. The samples were injected through a syringe loading injector (Model 7725i, Rheodyne, USA) with a fixed 100 μl loop. Chromatographic separations were performed on Spheri-5, RP-18 column, Applied Biosystems, Inc. (100/4.6 mm i.d., 5 mm), coupled with a guard column packed with the same material (30/4.6 mm i.d., 5 mm). The mobile phase was composed of acetonitrile and TDW at a flow rate of 1.5 ml/min. with initial condition of 70% acetonitrile. Before use mobile phase was filtered through 0.22 μm filter and degassed for 20 min in sonicator (Bransonic cleaning Co., USA). Data was analyzed using CLASS-VP software (Shimadzu, Japan) running on a Compaq presario PC. The HPLC system was equilibrated for approximately 30 min at a flow rate of 1.5 ml/min on initial gradient condition 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). Blank serum was obtained from drug free male rats (Sprague-Dawley), which were procured from

the Laboratory Animal Services Division, CDRI Lucknow, India.

2.2. Stock and standard solution preparation

Individual stock solutions of three compounds (1 mg/ml) were prepared by dissolving 10 mg of individual compound in 10 ml of HPLC grade acetonitrile. Mixed stock solution of all the three compounds (100 μg/ml) was prepare by transferring 1 ml of each and finally making up the volume to 10 ml with acetonitrile. The mobile phase standards were prepared by appropriately diluting the stocks in the range of 25–1000 ng/ml using 80:20 acetonitrile: TDW. The stock of internal standard was prepared in acetonitrile by dissolving 1mg in 10 ml. The working stock was prepared by taking 20 μl of stock and making the volume 10 ml to give final concentration of 2 μg/ml.

2.3. Calibration and quality control standards

The calibration and quality control (QC) standards for three components were prepared by pooling the individually spiked rat serum sample of same concentrations. The individual serum standards were prepared by spiking the serum with working stocks of suitable dilution. An amount of 100 μl of individually spiked serum with three compounds was pooled to give final concentration range of 25–1000 ng/ml. Quality control samples of 25 ng/ml (lower limit of quantitation, LLOQ), 50 ng/ml (QC low), 200 ng/mL (QC medium) and 1000 ng/ml (QC high) were also prepared and stored −60 °C until assay. The calibrations standards carrying all three preleads were freshly made before each assay. The quality of bioanalytical data is highly dependent on the quality of standard curve and calibration model used to generate it. The calibration curve was prepared by linear regression with different weights ($1/x$, $1/x^2$ and $1/\sqrt{x}$) using Microsoft Excel software [14].

2.4. Sample preparation and extraction procedure

Calibration standards and test samples were prepared using a simple and efficient two-step liquid–liquid extraction process. The test samples of each time point and the calibration standards of similar concentration were first pooled and internal standard was added to these samples. To each of these pooled samples 2 ml of 2.5% isopropyl alcohol in n-hexane was added. The samples were vortex mixed for three minute. The organic layer was transferred to another tube by snap freezing the aqueous layer in liquid nitrogen and evaporated to dryness under reduced pressure in speedvac concentrator (Savant Instrument, Farmingdale, NY, USA). The method was repeated and re-extraction was carried out in similar manner. The volume of the tubes were made up to 200 μl using 80:20 ACN:TDW mixture.

3. Method validation

The present gradient HPLC method was validated in terms of HPLC system reproducibility, sensitivity, specificity, linearity, absolute recovery, accuracy, precision, freeze thaw cycle stability, dry residue stability and long-term stability for all three compounds with internal standard in serum through the sample pooling approach, instead of three validations for three compounds. The method validation was performed for 5 days at four different concentrations (25, 50, 400 and 1000 ng/ml) in five replicate. Pre-study validation was acceptable for all three preleads as evidenced by inter- and intra-assay precision and accuracy obtained for five replicates of the three quality control samples. The lower limit of detection (LOD) for all three preleads was the quantity in the serum in both species after the sample cleanup corresponding to three times the baseline noise ratio (S/N > 3). The limit of quantitation is the concentration of the sample that can be quantified with less than 20% variation in precision. Linearity for calibration standards ($n = 7$) 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. The accuracy was determined by injection of calibration standards and QC standards in five replicates on five different days ($n = 100$, five each of LLOQ, QC low, QC medium and QC high). The inter- and intra-batch accuracy was determined by calculating % bias from the theoretical concentration. 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.) [15].

4. Stability studies

The freeze thaw stability and long-term stability for all three analytes was determined at three concentration levels (QC low, QC medium and QC high) in five replicates. To evaluate the impact of the freeze thaw cycle, spiked control ($n = 5 \times 4$ assays = 20) in the serum 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 day's concentration was calculated. Other sets were analyzed after 1, 2 and 3 freeze thaw cycles. Long-term stability studies were also performed over a period of 30 days. The results were expressed as % deviation with initial concentration.

5. Pharmacokinetic studies

Young and healthy male *Sprague-Dawley* rats weighing 250 ± 25 g, (obtained from Laboratory Animal Division of institute) were housed in well ventilated cages and kept at

room temperature on a regular 12 h light dark cycle. Animals were cared in accordance with principles of the guide for care and use of laboratory animals (Department of health education and welfare, number [NIH] 85-32). The intravenous dosing formulation (6 mg/ml) of 99/357, 99/408 and 99/411 were prepared by accurately weighing 24 mg dissolving it in 1.6 ml of DMF and the solution was made up with 2.4 ml of propylene glycol. A uniform 12 mg/kg doses was administered with these formulation using a tuberculin glass syringe fitted with 26G needle via caudal vein in rats. Blood samples collected at different time ranging from 0.08 to 12 h post-dose the sample were collected by cardiac puncture under light anesthesia. Terminal samples in rats were collected from the inferior venacava. All blood samples were allowed to clot at room temperature for 30 min and centrifuged at 2000 × *g* for 5 min at 4 °C. Serum was separated and stored at −60 °C.

6. Result and discussion

6.1. Optimization of the chromatographic conditions and sample cleanup

Spectroscopic analysis of compounds showed that 99/357 and 99/408 have maximum UV absorbance at 266 nm (λ_{max} 266 nm) while 99/411 has maximum absorbance at 247 nm (λ_{max} 247 nm). Therefore the chromatographic detection is performed at two different wavelengths using a two channel UV–vis detector. The successful analysis of the analytes in biological fluids using HPLC relies on the optimization of chromatographic conditions like sample preparation, chromatographic separation and post-column detection, etc. Thus for better selectivity and sensitivity different types of column made and the mobile phase were used. The length of the column was varied for appropriate run time and proper resolution of preleads and internal standards. In the beginning 20 cm C-5 column (cyano) was used with isocratic 80:20 ACN:TDW mobile phase at a rate of 1.2 ml/min. The compounds were eluted in first 10 min with suitable resolution but the peaks showed interference with the endogenous serum impurities. The alteration of mobile phase composition and the rate of flow did not have appreciable effect on the interference of endogenous impurities. The change of column from C-5 to C-18 has a marked effect on the interference of the serum impurities but the retention time of three preleads markedly increases (25 min for 99/411) and the peak height was also reduced markedly. Use of mobile phase with different buffers having different molar strength and pH did not have any effect on peak shape and the peak height or the specificity. However shifting from isocratic elution to gradient elution technique significantly reduces run time (fifteen minutes), improves peak height and provides optimum resolution. With gradient elution there was no interference with any endogenous serum impurity. Gradient elution at a rate of 1.5 ml/min with mobile phase comprising of acetonitrile and TDW was found to be optimum in terms of peak height, resolution and run time. The gradient varies form 70% of acetonitrile to 95% over a period of 6 min and come back to 70% in 3 min after running for 5 min at 95% acetonitrile level. The use of acetonitrile for reconstitution as well as standard preparation caused peak fronting. The peak symmetry was improved by preparing the standards and reconstituting residue in 80:20 ACN:TDW.

The sample cleanup technique was also optimized in order to get minimum interference of endogenous impurities. Different techniques like protein precipitation, liquid–liquid extraction and solid phase extraction were used for sample cleanup. A two-step liquid–liquid extraction was found to be best suitable for sample cleanup. Extraction was carried out with diethyl ether, hexane, ethyl acetate and different percentage of isopropyl alcohol in *n*-hexane. It was observed that with *n*-hexane the recoveries of the 99/411 was very less while with ether and ethyl acetate the serum impurities interfere with the compounds peaks and hence selectivity is compromised. Back washing of the dry residue after extraction to reduce endogenous impurities with acid and base was performed. This back washing did not showed any significant decrease in the serum impurities. Thus for optimum recoveries of all three preleads 2% of isopropyl alcohol in *n*-hexane was selected as extraction solvent. Moreover the two-step liquid–liquid extraction technique and the chromatographic condition yielded a clean chromatogram. The endogenous impurities do not interfere with the elution region of the compound indicating that method is selective as shown in Fig. 2 for blank and spiked (400 ng/ml) while in Fig. 3 for test samples (60 min).

All criteria commonly applied during validation of a bioanlytical method were assessed, which includes linearity, recovery, accuracy, and precision and stability studies. Inclusion of all above mentioned parameters are in accordance to the GLP guidelines, which are routinely followed for the assay validation. To verify the linearity of the chromatographic responses (peak height) against concentration of the compounds in spiked serum, a calibration curve was prepared in the concentration range 25–1000 ng/ml. In some cases, manipulation of the calibration data by applying weights or by transformations becomes necessary. In the present study, linear least square regression analysis with intercept showed highly reproducible relationship with coefficient of correlation > 0.99 indicating the response is linear over the concentration range (25–1000 ng/ml). The residuals did not improve much without intercept and with weighted linear regression ($1/x$, $1/x^2$ and $1/\sqrt{x}$). Thus a linear regression model with intercept was chosen for routine analysis of data.

6.2. Recoveries, accuracy and precision

Recoveries (calculated as percent recovery) of the compounds from the spiked serum samples were calculated at low, medium and high concentrations with the standard curve prepared from analytical standard prepared in mobile phase.

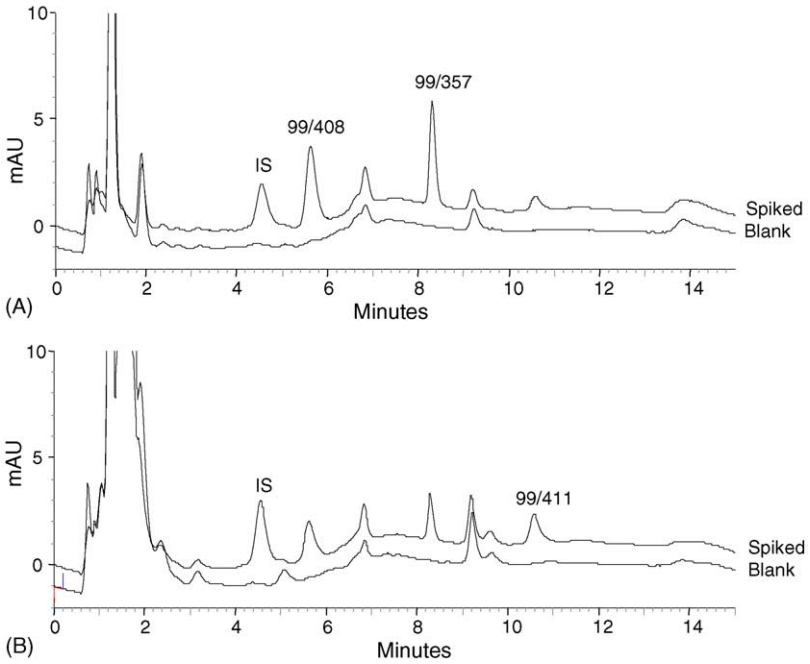

Fig. 2. Representative chromatogram of blank and spiked in rat serum at two wavelengths (A) λ_{max} 266 nm, (B) λ_{max} 247 nm.

The recoveries of 99/357 and 99/408 was found to be >85%, while of 99/411 was >70% with CV less than 15% for all the concentrations (Table 1).

The accuracy and precision was determined by injection of QC standards (LLOQ, low, medium and high) in five replicates on five different days (n = 100, five each of LLOQ, low, medium and high concentration). Accuracy and the precision values of the method are summarized in Tables 2 and 3, respectively. The inter- and intra-batch accuracy in terms of % bias was determined by comparing the concentration of spiked controls estimated from the calibration curve to their true value. Five samples were analyzed at each concentration on the same day. To determine the variance in inter- and intra-day precision, data were subjected to one-way analy-

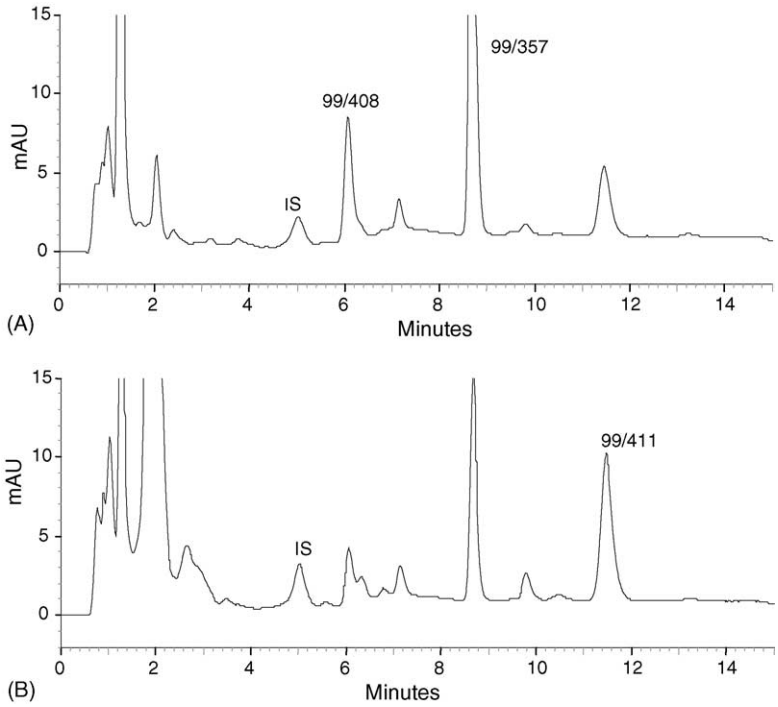

Fig. 3. Representative chromatogram of test (60 min, after single intravenous dose) in rat serum at two wavelengths (A) λ_{max} 266 nm, (B) λ_{max} 247 nm.

Table 1
Mean absolute recoveries of 99/357, 99/408 and 99/411 in rat serum

Concentration (ng/ml)	%Absolute recovery (mean ± S.D., $n = 5$)		
	99/357	99/408	99/411
25	97.66 ± 8.99	106.23 ± 2.80	82.93 ± 10.69
50	89.23 ± 4.89	99.08 ± 8.20	73.39 ± 9.69
100	92.19 ± 5.45	98.94 ± 7.37	75.67 ± 7.63
200	98.42 ± 9.99	99.35 ± 8.12	83.61 ± 3.36
400	91.02 ± 7.59	93.63 ± 7.90	76.67 ± 1.61
800	85.93 ± 8.35	91.08 ± 5.19	77.38 ± 6.71
1000	89.50 ± 8.65	90.83 ± 7.23	73.76 ± 9.15

Table 2
Intra- and inter-day accuracy for 99/357, 99/408 and 99/411

Concentration (ng/ml)	% Bias (accuracy)					
	Intra-day			Inter-day		
	99/357	99/408	99/411	99/357	99/408	99/411
25	0.62	−2.02	−2.92	−0.06	−2.47	−1.6
50	−2.58	2.95	−10.02	−2.16	−1.73	−12.54
400	−2.02	−0.98	−4.87	−3.72	0.69	−8.28
1000	−2.20	−1.41	−2.29	−2.35	−1.58	−2.29

sis of variance (ANOVA), and relative standard deviation (% R.S.D.) was calculated using mean square value. The results showed satisfactory intra- and inter-day accuracy and precision for three preleads as indicated by % bias and % R.S.D. of ≤20% for QC low and ≤15% for all other concentrations, which is well within specified limit [16].

6.3. Stability studies

Freeze thaw stability, in process dry residue and long-term stability studies for 99/357, 99/408 and 99/411 were performed. The results of first day analysis were considered as reference and subsequent results were compared with the first day result and finally expressed as percent deviation. The three compounds were found to be stable after three freeze thaw cycles and changes in concentration were found to be within acceptable limits (≤20%) (Fig. 4). Long-term stability studies were also performed over a period of 1 month. The relative percent deviation was found to be within specified limits of ≤20% (Fig. 5). Thus the stability studies showed that the three synthetic trioxane CDRI preleads are stable under the condition evaluated.

Table 3
Intra- and inter-day precision for 99/357, 99/408 and 99/411

Concentration (ng/ml)	% R.S.D. (precision)					
	Intra-day			Inter-day		
	99/357	99/408	99/411	99/357	99/408	99/411
25	3.3	3.7	5.4	7.3	10.6	13.0
50	2.3	5.2	4.4	6.7	3.7	6.4
400	3.0	3.4	5.0	7.1	5.7	14.1
1000	2.1	2.8	4.1	7.3	7.3	10.8

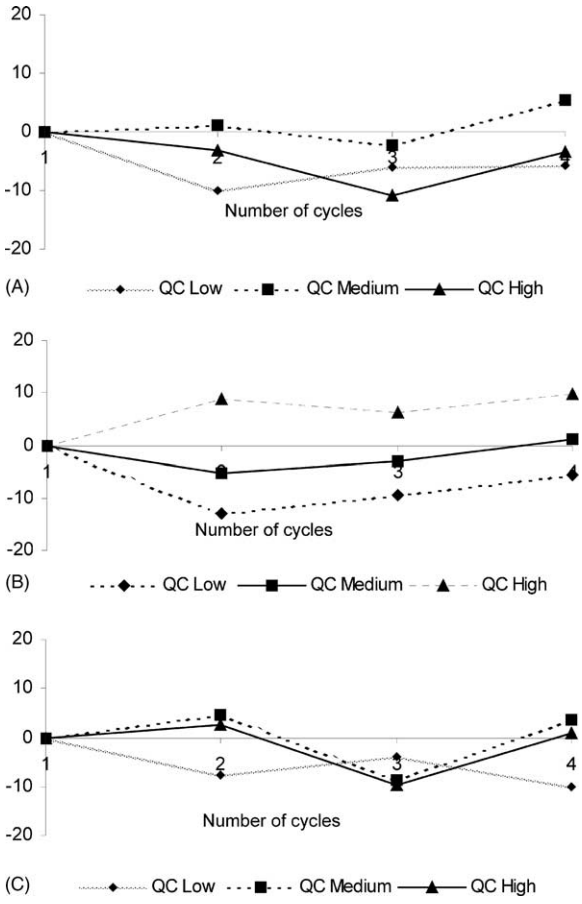

Fig. 4. Freeze thaw stability of (A) 99/357, (B) 99/408 and (C) 99/411 in rat serum.

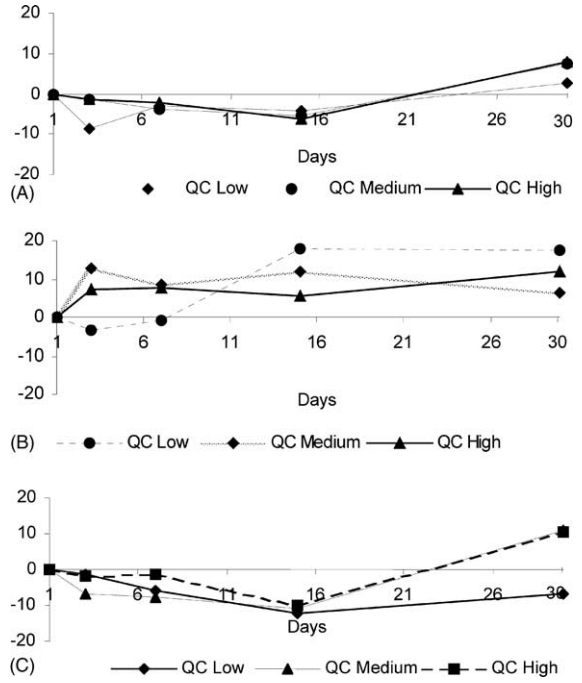

Fig. 5. Long-term stability of (A) 99/357, (B) 99/408 and (C) 99/411 in rat serum.

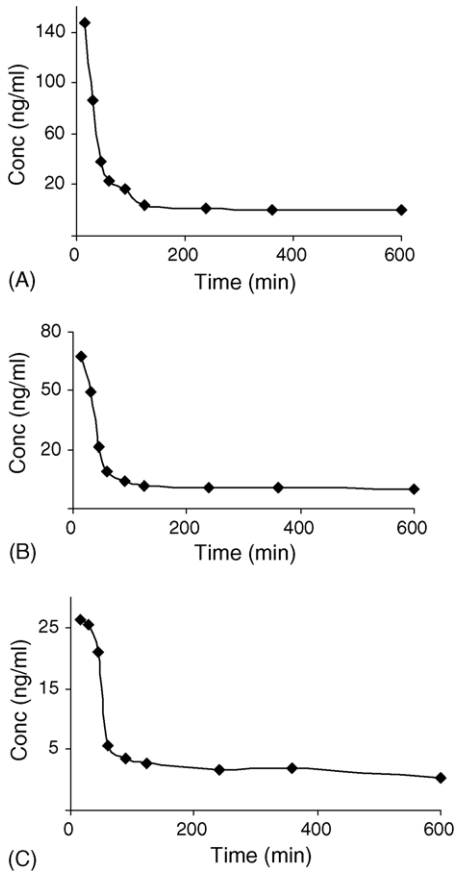

Fig. 6. Concentration versus time profile of (A) 99/357, (B) 99/408 and (C) 99/411 after single intravenous dose in rat.

6.4. *In vivo application*

The present validated bioanalytical method-using sample pooling approach has been applied for rapid pre-clinical pharmacokinetic studies of the compounds 99/357, 99/408 and 99/411 after single intravenous dose for pilot screening. After single intravenous dose the 99/357, 99/408 and 99/411 in rats, the samples were collected over a period of 12 h and levels were detected up to 10 h in all cases. The purpose of the pilot studies was two fold firstly to timely modify the existing HPLC method, if required, and secondly to generate early information about pharmacokinetic parameters. There was no apparent formation of any metabolites, which interfered chromatographically with any of the parent compounds. The plasma concentration time profile for the three compounds is shown in Fig. 6.

7. Conclusion

The relevance of pharmacokinetics in drug development has been well recognized thus there is a need for rapid generation of pharmacokinetic parameters, which can reduce the cost and time required for drug development. In the present studies the concept of sample pooling was successfully applied to single dose intravenous study of three CDRI developed synthetic trioxane preleads. 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, 99/408 and 99/411 during storage and sample processing hence fulfilling the criteria for successful bioanalytical method. The use of concept of one-in-one dosing followed by sample pooling markedly reduces the sample processing and the analysis time as all three compounds were processed and analyzed simultaneously instead of processing and analyzing one at a time.

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