Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 864 (2008) 52–60
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

A sensitive and selective liquid chromatographic tandem mass spectrometric assay for simultaneous quantification of novel trioxane antimalarials in different biomatrices using sample-pooling approach for high throughput pharmacokinetic studies☆

Rajendra Pratap Singh[a,b], S. Sabarinath[a,c], Shio Kumar Singh[a], Ram Chandra Gupta[a,*]

[a] *Pharmacokinetics & Metabolism Division, Central Drug Research Institute, Lucknow 226001, India*
[b] *Metabolism and Pharmacokinetics, Ranbaxy Research Laboratories, Gurgaon, India*
[c] *College of Pharmacy, University of Florida, Gainesville, USA*

Received 3 October 2007; accepted 24 January 2008
Available online 17 February 2008

Abstract

In the present studies, to give momentum to traditionally low throughput pharmacokinetic screening, a bioanalytical method based on the concept of sample pooling for simultaneous bioanalysis of multiple compounds is discussed. A sensitive, selective, specific and rapid HPLC/ESI-MS/MS assay method was developed and validated for the simultaneous quantitation of three novel trioxane antimalarials (99–357, 99–408 and 99–411) in rat plasma using trioxane analogue as internal standard. The suitably validated bioanalytical method was then further extrapolated to rabbit and monkey plasma by performing partial validation. Extraction from the plasma involves a simple two-step liquid–liquid extraction with n-hexane. The analytes were chromatographed on a cyano column by isocratic elution with acetonitrile:ammonium acetate buffer (pH 6) (85:15, v/v) and analyzed by mass spectrometry in multiple reaction-monitoring (MRM) mode. The chromatographic run time was 5.5 min and the weighted ($1/x^2$) calibration curves were linear over a range of 1.56–200 ng/ml. The limit of detection (LOD) and lower limit of quantification (LLOQ) in rat plasma, rabbit plasma and monkey plasma were 0.78 and 1.56 ng/ml, respectively, for all three analytes. The intra- and inter-batch accuracy and precision in terms of % bias and % relative standard deviation were found to be well within the acceptable limits (<15%). The average absolute recoveries of 99–357, 99–408 and 99–411 from spiked plasma samples were >90%, >70% and >60%, respectively. The assay method described here could be applied to study the pharmacokinetics of 99–357, 99–408 and 99–411 using sample-pooling technique.

Keywords: Electrospray ionization; Tandem mass spectrometry; Sample pooling; Biomatrices; Trioxane antimalarials

1. Introduction

The virulence of human malaria parasite *Plasmodium* species and its ability to develop drug resistance marks it out as one of the main threats to global public health. The increasing incidence of parasite resistance to the existing frontline chemotherapy for malaria has reached alarming heights over the past few years and has prompted World Health Organization (WHO) to initiate steps to supplement the existing therapeutic armamentarium [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. In recent years, artemisinin, isolated from Chinese medicinal herb *Artemisia annua* L., has emerged as a chemotherapeutic agent with potent antimalarial activity. Artemisinin and its derivatives or analogues are currently regarded as the most promising weapons against malaria. It is reported that their unique 1,2,4-trioxane sesquiterpene (endoperoxide) structure is indispensable for antimalarial activity [3–5].

Although natural artemisinin shows promising antimalarial activity, its poor bioavailability prompted the search for better analogues. Chemical insight in to artemisinin's biological

☆ CDRI Communication No.: 6630.

* Corresponding author. Tel.: +91 522 2612411–18x4277; fax: +91 522 223405/938/229504.
E-mail address: rcgupta@usa.net (R.C. Gupta).

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.01.054

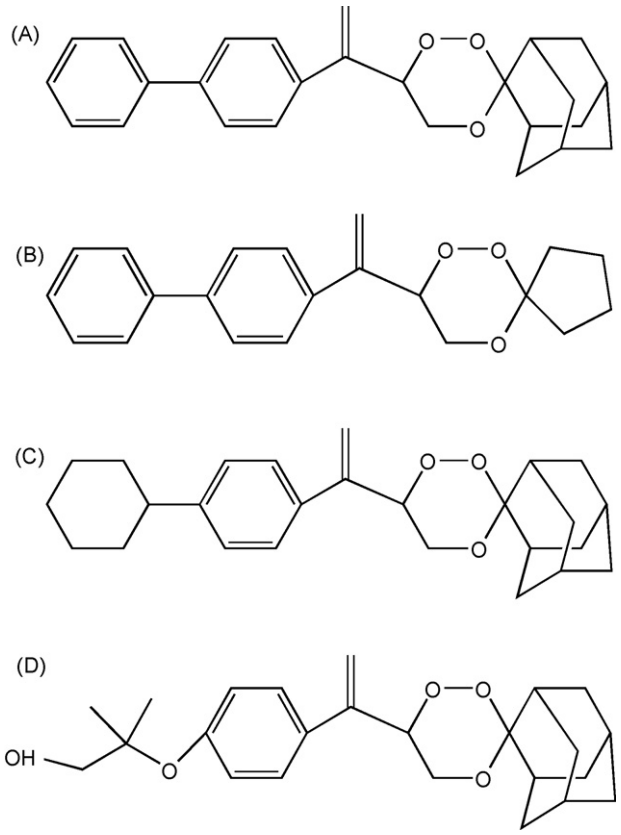

Fig. 1. Chemical structures of (A) 99–357, (B) 99–408, (C) 99–411 and (D) IS.

mechanism of action has allowed rational design of some new trioxanes and endoperoxide antimalarial drug candidates that are efficacious and safe. CDRI in its drug discovery program has developed promising antimalarial compounds 99–357, 99–408 and 99–411 of this class (Fig. 1) [6–11]. These candidates will be subjected to *in vitro* and *in vivo* pharmacokinetic (PK) screening for selecting promising candidates for further development.

Traditionally, PK screening is a low throughput process and produces bottleneck in drug discovery. Therefore, higher throughput approaches are required for rapid PK data generation. This led to development of novel concepts for performing high throughput PK (HTPK) studies, i.e. sample pooling and cassette dosing or N-in-one dosing.

Sample-pooling approach involves administration of one compound per animal followed by sample collection. The samples of same time point for all compounds are then pooled and subjected to simultaneous bioanalysis of all the compounds while cassette dosing involves simultaneous administration of a mixture of compounds, sampling and simultaneous bioanalysis. Sample-pooling technique avoids the possible complications resulting from *in vivo* drug–drug interactions as in the case of cassette dosing. Sample pooling has been shown to be a useful way to increase the speed with which compounds are studied and also reduces the overall cost to bring a new drug to the market [12–19].

In the present bioanalytical method, the usefulness of sample pooling has been demonstrated for applications to PK analysis by simultaneous estimation of mixture of compounds in different biomatrices. This paper presents the development and validation of a highly sensitive, selective and specific LC–MS/MS method for the simultaneous quantification of 99–357, 99–408 and 99–411 in rat plasma using a trioxane analogue as the internal standard (IS). This suitably validated method was further extrapolated to rabbit and monkey plasma with partial validation. The method can be applied to generate PK data as a part of preclinical screening in rats, rabbits and monkeys.

2. Experimental

2.1. Chemicals and reagents

Reference standards (>99% pure) of 99–357, 99–408, 99–411 and internal standard (Fig. 1) were synthesized by Dr. Chandan Singh, Medicinal Chemistry Division, Central Drug Research Institute Lucknow, India. Acetonitrile, HPLC grade, was purchased from Thomas Baker (Chemicals) Limited (Mumbai, India). HPLC grade n-hexane was obtained from E Merck (India) Limited and distilled freshly before use. 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, Biologicals E. Limited, Hyderabad, India) was obtained by local purchase. Blank, drug free plasma samples were collected from adult, healthy male *Sprague–Dawley* rats, *New Zealand* rabbits and *Rhesus* monkeys, respectively, at Laboratory Animal Division of Central Drug Research Institute (Lucknow, India). Plasma was obtained by centrifuging the heparinised blood at $1000 \times g$ for 15 min. Pooled plasma samples were stored at −60 °C till use. Prior approval from the Local Animal Ethics Committee was sought for maintenance and experimental studies with animals. All experiments, euthanasia and disposal of carcass were performed in accordance with the guidelines laid by local ethical committee for animal experimentation.

2.2. Calibration standards and quality control samples

Individual stock solutions (1 mg/ml) of 99–357, 99–408, 99–411 and the IS were prepared in acetonitrile and were used to prepare the respective working stocks. Calibration curves of 99–357, 99–408 and 99–411 were prepared in rat plasma, rabbit plasma and monkey plasma over a concentration range of 1.56–200 ng/ml. The individual plasma standards were prepared by spiking the plasma with working stocks of suitable dilution. Fifty microliters of individually spiked plasma with three compounds were then pooled to give the desired concentration range of 1.56–200 ng/ml. Quality control (QC) samples at five different concentration levels (1.56, 3.125, 6.25, 50 and 200 ng/ml as low_1, low_2, low_3, medium and high, respectively) were prepared separately in five sets, independent of the calibration standards and stored at −60 °C until assay. During analysis, these QC samples were spaced after every six to seven unknown samples. Test samples and quality control samples are then interpolated

from the calibration curve to obtain the concentrations of the respective analytes.

2.3. Sample preparation (sample pooling)

Calibration standards and test samples were prepared using a simple and efficient two-step liquid–liquid extraction process with n-hexane. Fifty microliters of the individually prepared calibration standards of the analytes of similar concentration were pooled (as described in previous section) to get pooled calibration standards over a range of 1.56–200 ng/ml. Internal standard solution (20 μl/ml of 1 μg/ml) was added to each of these pooled calibration standards and vortex mixed for 15 s. Similarly test samples of each time point of individual compound (collected from individually dosed animal) were also pooled to get one pooled sample per time point.

The processing volume of plasma was fixed as 150 μl. To each of these pooled samples 2 ml of n-hexane was added. The samples were vortex mixed for 2 min. 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 dry residue was reconstituted in mobile phase (100 μl) and injected on to HPLC.

2.4. Chromatographic 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 mobile phase at a flow rate of 0.85 ml/min. The chromatographic system consisted of a Spheri-10, Cyano column (100 mm × 4.6 mm, 5 μm), a cyano guard column (30 mm × 4.6 mm, 5 μm) and 85% acetonitrile-10 mM ammonium acetate buffer (pH 6) as mobile phase. The chromatographic run time was 5.5 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 ∼1500–1600 psi.

2.5. Mass spectrometric conditions

Mass spectrometric detection was performed on an API 4000 mass spectrometer (Applied Biosystems, MDS Sciex Toronto, Canada) equipped with an API electrospray ionization (ESI) source and Perkin-Elmer LC system. The mass spectrometer was operated at ESI positive ion mode and the analytes were quantified in multiple reaction-monitoring (MRM) mode. MS and MS/MS condition for pure standard 99–357, 99–408, 99–411 and IS was 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 of *m/z* 406.6 → 179.3, *m/z* 340.4 → 179.1, *m/z* 412.5 → 185.4 and *m/z* 418.4 → 119.2 were used for quantification of 99–357, 99–408, 99–411 and internal standard, respectively. The declustering potential (DP), collision energy (CE) and the collision gas were optimized for individual analytes. Zero air was used as source gas while nitrogen was used as both curtain and collision gas. Data acquisition and quantitation were performed using analyst software version 1.4 (Applied Biosystems, MDS Sciex Toronto, Canada).

2.6. Method validation

The present isocratic HPLC method was validated in terms of sensitivity, specificity, linearity, recovery, accuracy, precision, bench-top stability, freeze-thaw cycle stability and long-term stability for all three compounds in rat plasma through sample-pooling approach, instead of three separate validation regimens for the three compounds. The method validation was performed for 5 days at five different quality control (QC) concentrations (1.56, 3.13, 6.25, 50 and 200 ng/ml) in five replicates.

The lower limit of detection (LOD) was the quantity in plasma (of all species—rat, rabbit and monkey) after the sample cleanup corresponding to three times the baseline noise ratio (S/N > 3). The limit of quantitation (LOQ) is the concentration of the sample that can be quantified with less than 20% variation in precision. Linearity for calibration standards ($n = 8$) for 5 days was assessed by subjecting the spiked concentrations and the respective peak height to linear regression analysis ($Y = mX + c$) with different weighting schemes. The choice of proper calibration method depends on the residuals obtained and the coefficient of correlation. 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 ($1/x$, $1/x^2$, $1/y$, $1/y^2$ and $1/\sqrt{x}$) [20,21]. Calibration and analytical standard curves were constructed using the analyte to IS peak area ratios by weighted ($1/x^2$) least-square linear regression. The inter- and intra-batch accuracy was determined by calculating % bias of quality control samples 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.).

2.7. Stability studies

The freeze-thaw stability, bench-top stability and long-term stability for all analytes were determined at two concentration levels (QCs low_1 and high) in five replicates in rat plasma. To evaluate the impact of the freeze-thaw cycle, spiked controls 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 day's concentrations were calculated. Other sets were analyzed after 1, 2 and 3 freeze-thaw cycles. Bench-top stability of the samples was established for 8 h by keeping plasma samples at room temperature for 8 h, processing and analyzing. Deviation was calculated from freshly spiked and immediately processed samples. Long-term stability studies were performed over a period of 30 days. The results were expressed as % deviation with initial concentration.

2.8. Pharmacokinetic studies

Young, healthy adult male *Sprague–Dawley* rats weighing 225 ± 25 g (obtained from Laboratory Animal Division of the Institute) were housed in well ventilated cages and kept at room temperature on a regular 12-h light:12-h dark cycle. Animals were cared in accordance with the guidelines laid by local ethical committee for animal experimentation. The rats were acclimatized to laboratory environment for at least 2 days before conducting the experiment. In all the studies mentioned below, the dose was administered after overnight (~8–12 h) fasting. The oral dosing formulations of 99–357 (24 mg/kg), 99–408 (72 mg/kg) and 99–411 (24 mg/kg) were prepared by accurately weighing respective compounds and dissolving it in 4:6 mixture of dimethyl formamide and propylene glycol. The formulations were administered using a 2 ml glass syringe fitted with feeding cannula to overnight fasted rats. The strength of the formulations was adjusted such that the volume factor remained 1 ml/kg for all the three compounds. Blood samples were collected at pre dose, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12, 18, 24 and 48 h post dose The first sample was collected by cardiac puncture under light anesthesia. Terminal samples in rats were collected from the inferior venacava. Plasma was separated by centrifugation at 1000 × *g* for 10 min and stored at −60 °C prior to analysis. Analyses were performed within 30 days of sample collection by sample pooling.

3. Results and discussion

3.1. Method development

Full-scan spectra of analytes showed presence of signals of sodium adducts and protonated ions. The division of signal between sodium adducts and protonated ions resulted in compromised sensitivity. Sodium adducts are not normally employed as parent ions in MRM mode due to their high stability and erratic fragmentation pattern. Therefore, $[M+H]^+$ and/or $[M+NH_4]^+$ ions, which on fragmentation gave prominent and stable product ions were selected for further developmental work. Hence, possibility of formation of intense protonated or ammonium adduct was explored, using ammonium acetate buffer in combination with acetonitrile. Full-scan positive ion mass spectra of 99–357, 99–408, 99–411 and the IS showed ammonium adducts $[M+NH_4]^+$ of *m/z* 406.6, 340.4, 412.5 and 418.4, respectively. Declustering potential optimization of the ammonium adducts of 99–357, 99–408, 99–411 and the IS were carried out using full-scan acquisition over a mass range of 200–500 Da by continuous flow analysis.

Product ion spectra of the analytes were obtained by carrying out continuous flow MS/MS analyses. The most abundant ion in the product ion spectra of ammonium adduct of 99–357 (*m/z* 406.6), 99–408 (*m/z* 340.4), 99–411 (*m/z* 412.5) and the IS (*m/z* 418.4) were 179.3, 179.1, 185.4 and 119.2, respectively. The product ion spectra and the proposed fragmentation pathways of 99–357, 99–408, 99–411 and the IS are illustrated in Figs. 2 and 3, respectively.

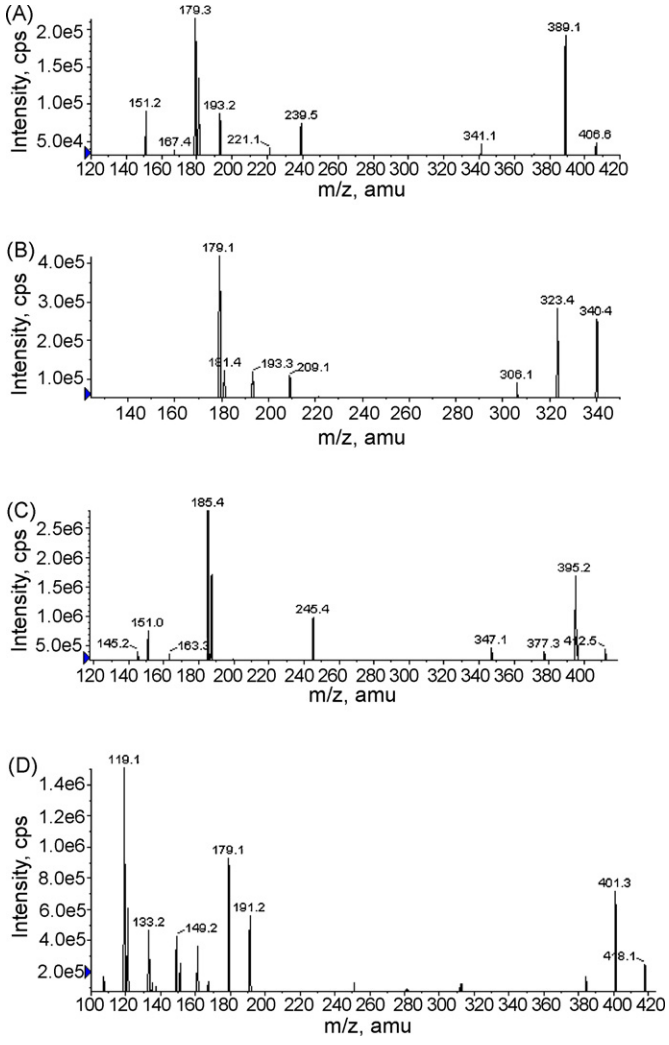

Fig. 2. Product ion spectra of (A) 99–357, (B) 99–408, (C) 99–411 and (D) IS.

The influence of buffer molarity, pH and types of organic modifier on the signal intensities was also studied at the optimized declustering potential. Based on the peak intensity of the respective ammonium adducts, 10 mM ammonium acetate buffer of pH 6 and acetonitrile, as the organic phase, at a flow rate of 0.85 ml/min were selected for further studies. Also higher proportion of the organic modifier in the mobile phase was found to improve the signal intensity. Initially, 95:5 (v/v) acetonitrile:ammonium acetate buffer at a flow rate of 0.85 ml/min was tried in isocratic elution. On the other hand, very high proportion of organic phase leads to improper elution leading to peak deformation. Therefore, the 85:15 (v/v) organic phase to buffer was selected as optimum. Moreover, reconstitution of the extracted dry residue in mobile phase improves peak symmetry. The dwell time was fixed at 200 ms and declustering potential was optimized to 30 for all the channels monitored (*m/z* 406.6, 340.4, 412.5 and 418.4). The collision energy was optimized for individual analyte for most intense fragment and was found to be 20 for 99–408 and 99–411, 22 for 99–357 and 30 for internal standard. The curtain gas was optimized to 13 while collision gas was optimized to 3. The Gas 1 (GS1), Gas 2 (GS2), entrance

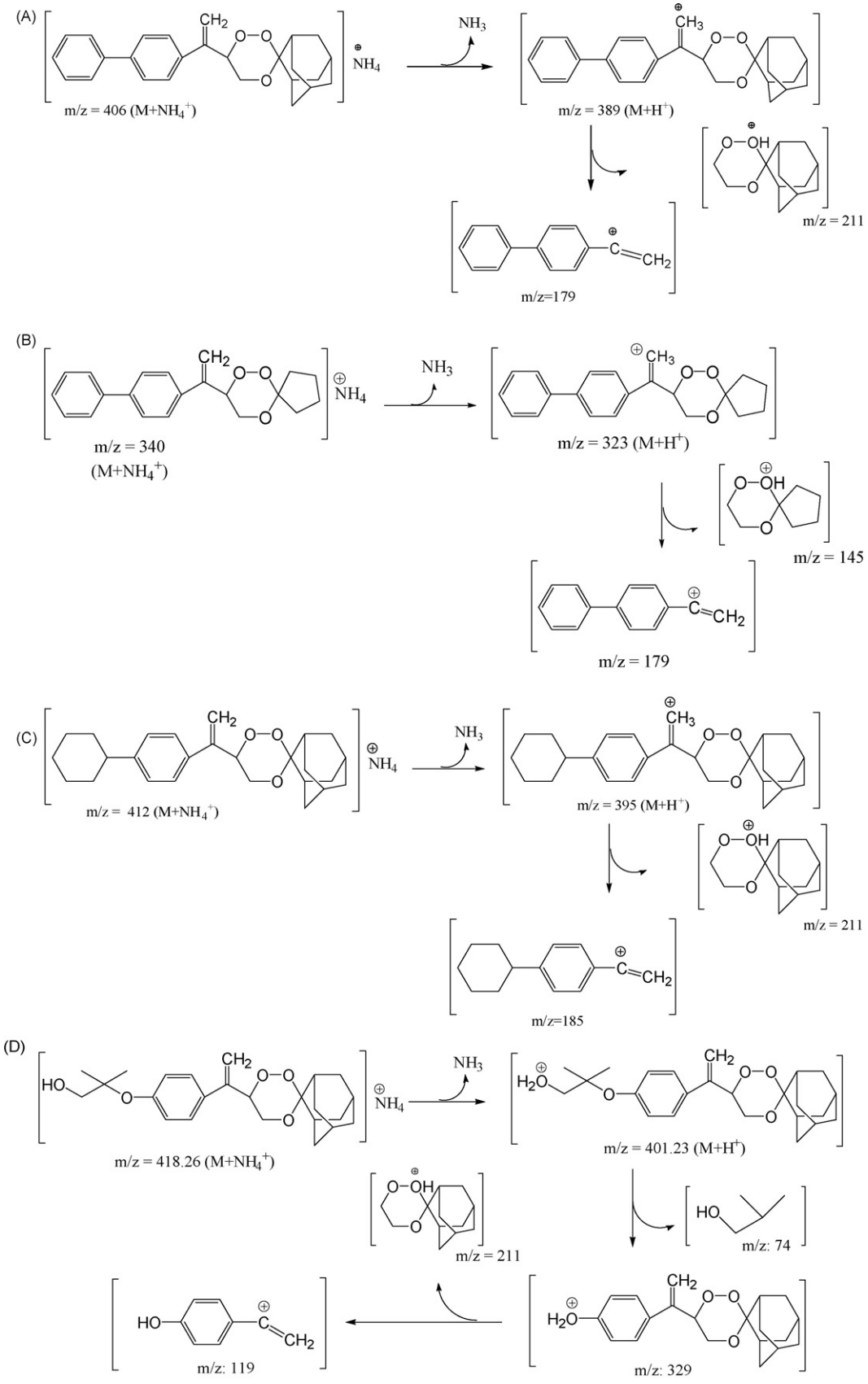

Fig. 3. Fragmentation pattern of (A) 99–357, (B) 99–408, (C) 99–411 and (D) IS.

potential (EP) and collision cell exit potential (CXP) were optimized to 30, 40, 4 and 10, respectively. The ion source potential was set at 5500 V and source gas temperature was optimized to 200 °C. The response functions of the analytes were found to be linear over a range of 1.56–200 ng/ml. To obtain highly specific and selective detection, multiple reaction-monitoring (MRM) mode was used for reliable quantification.

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 [22,23]. The peak area ratios of 99–357, 99–408, 99–411 with the IS in rat 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 (heteroscedasticity) and 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 99–357, 99–408 and 99–411 were above 0.998 over the concentration range used.

LC–MS/MS analysis of the blank rat plasma, rabbit plasma and monkey plasma samples showed no interference with the quantification of 99–357, 99–408, 99–411 and the IS. Specificity of the method was established in all matrices (from six different sources) with pooled as well as individual samples. Representative chromatograms of extracted blank plasma and blank plasma fortified with 99–357, 99–408, 99–411 and the IS indicating the specificity and selectivity of the method, are shown in Fig. 4. The chromatograms in Fig. 4 were obtained in final optimized condition (85% acetonitrile and 15% ammonium acetate buffer pH 6).

The limit of detection (LOD) demonstrated that all the analytes gave a signal-to-noise ratio (S/N) of 3 and above for 0.78 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 99–357, 99–408 and 99–411 from three matrices was established as 1.56 ng/ml. LLOQ was established with five samples independent of the standard curve.

The average absolute recoveries for 99–357, 99–408 and 99–411 over the concentration range of 1.56–200 ng/ml was found to be consistent and reproducible in rat, rabbit and mon-

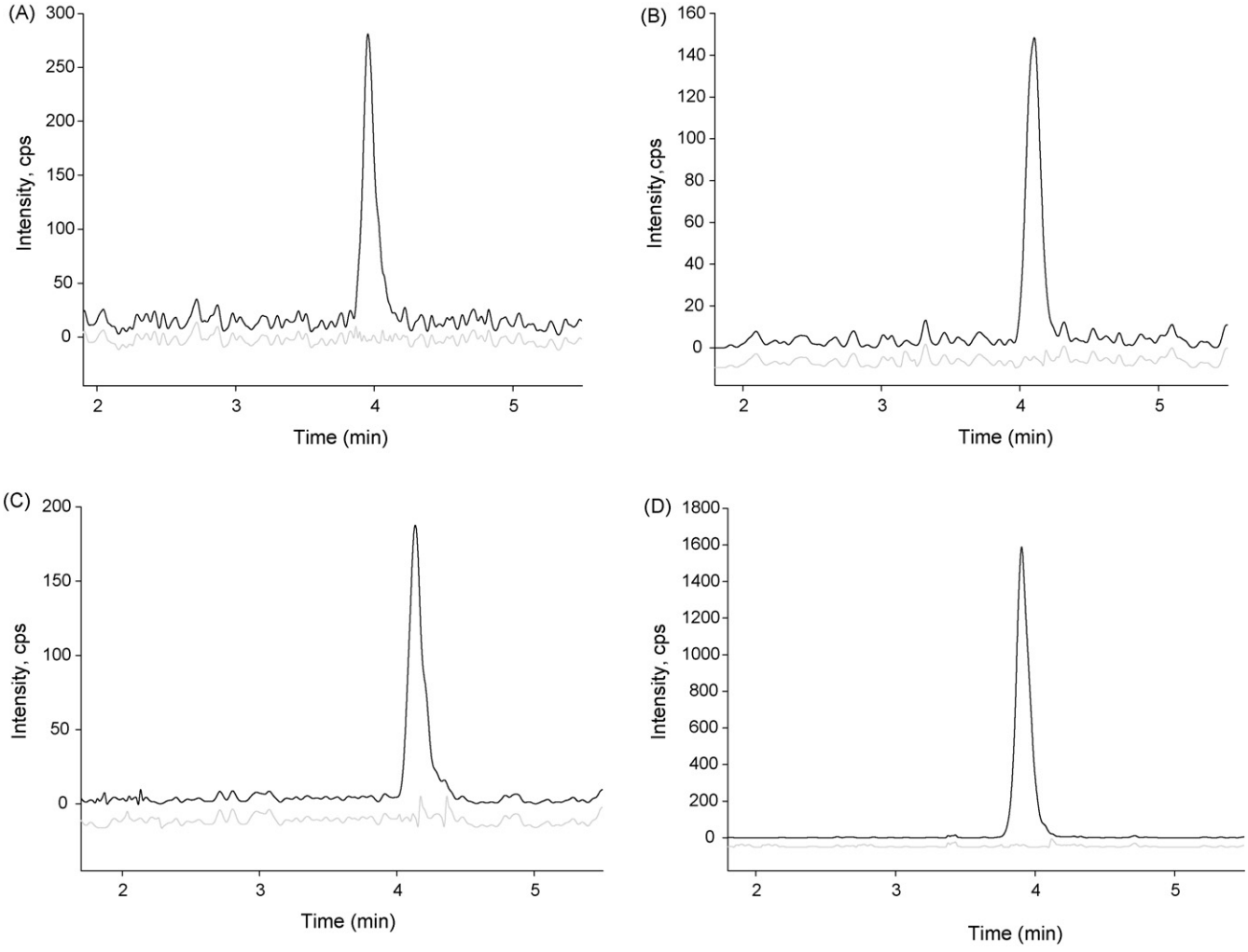

Fig. 4. Blank and fortified (1.56 ng/ml) MRM chromatograms of (A) 99–357, (B) 99–408, (C) 99–411 and (D) IS.

Table 1
Mean absolute recoveries of 99–357, 99–408 and 99–411 from rat, rabbit and monkey plasma

Species	Concentration (ng/ml)	% Absolute recovery (mean ± S.D., $n = 5$)		
		99–357	99–408	99–411
Rat	1.56	85.53 ± 6.30	72.45 ± 5.80	65.23 ± 8.65
	3.13	86.08 ± 8.06	74.53 ± 2.81	60.39 ± 5.65
	6.25	84.13 ± 5.31	73.53 ± 7.37	61.25 ± 3.36
	50	82.15 ± 5.36	73.57 ± 3.56	65.78 ± 6.72
	200	80.59 ± 3.87	72.17 ± 6.81	60.57 ± 7.01
Rabbit	1.56	82.56 ± 9.25	72.16 ± 8.23	62.25 ± 11.23
	3.13	81.23 ± 2.13	73.20 ± 8.81	61.24 ± 6.25
	6.25	80.25 ± 7.56	72.53 ± 5.56	62.31 ± 8.25
	50	81.78 ± 4.35	76.13 ± 6.02	60.12 ± 5.36
	200	84.56 ± 2.25	71.52 ± 1.97	60.42 ± 4.75
Monkey	1.56	85.17 ± 7.96	76.52 ± 7.72	64.46 ± 8.02
	3.13	83.24 ± 4.31	75.21 ± 6.87	63.17 ± 4.85
	6.25	81.82 ± 5.78	72.45 ± 4.28	66.25 ± 2.33
	50	84.56 ± 6.25	71.08 ± 4.52	61.62 ± 4.25
	200	80.02 ± 2.45	70.95 ± 3.21	64.25 ± 2.67

key plasma (Table 1). The recovery of the IS from the extracted calibration standards and QC samples during validation was 90.14 ± 5.7%. The recoveries were calculated from the peak area ratios of extracted fortified plasma samples read against the analytical standard curve. 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, which was used as reconstitution solution. This study was carried out over the entire concentration range. Peak area obtained from reference solution and analyte with blank matrices were subjected to statistical analysis (Student *t*-test). The differences were not found to be significant at 95% confidence interval at any concentration level. Thus, this study showed that there is no significant matrix suppression on ionization.

Accuracy in terms of % bias and precision in terms of % relative standard deviation for both intra- and inter-batch were calculated with five (excluding blank) determinations per concentration level on five days (five each of three lows, medium and high QC samples) in rat plasma and are presented in Tables 2 and 3. 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 method was further extrapolated to rabbit and monkey plasma by carrying out partial validation. The intra-day accuracy values for rabbit and monkey plasma are shown in Table 4. The results showed that the bioanalytical method for simultaneous estimation of 99–357, 99–408 and 99–411 is accurate and precise over the concentration range of 1.56–200 ng/ml.

3.3. Stability studies

All the stability studies were carried out at two concentrations levels (1.56 and 200 ng/ml as low and high QC) in five replicates. QC samples were subjected to freeze-thaw (f-t) stability studies in rat plasma. The deviation observed after first, second and third f-t cycles were within ±15% (Table 5) at the concentration levels used for 99–357, 99–408 and 99–411 indicating adequate freeze-thaw stability. Spiked QC samples in five replicates, which were extracted and analyzed immediately were used as the reference point to calculate the percent deviations after first, second and third f-t cycles. Also the QC samples stored at −60 °C were analyzed after 30 days and there were no significant deviations with respect to the immediately analyzed samples (Table 6).

There was no significant difference between the responses of spiked standards at time zero and after 8 h for 99–357, 99–408 and 99–411, indicating the stability of analytes at

Table 2
Intra- and inter-day accuracy for 99–357, 99–408 and 99–411 in rat plasma

Quality control	Concentration (ng/ml)	% Bias (accuracy)					
		Intra-day			Inter-day		
		99–357	99–408	99–411	99–357	99–408	99–411
QC low_1	1.56	0.52	4.30	5.11	−8.17	2.85	−6.45
QC low_2	3.13	−2.23	3.02	4.23	5.56	6.35	2.42
QC low_3	6.25	−1.75	−2.75	−0.89	−4.46	−4.53	3.46
QC medium	50	3.12	−4.08	−2.04	2.78	−5.51	2.01
QC high	200	2.12	0.73	−2.10	4.13	−6.23	0.69

Table 3
Intra- and inter-day precision for 99–357, 99–408 and 99–411 in rat plasma

Quality control	Concentration (ng/ml)	% R.S.D. (precision)					
		Intra-day			Inter-day		
		99–357	99–408	99–411	99–357	99–408	99–411
QC low_1	1.56	8.71	7.86	9.66	6.31	4.79	4.10
QC low_2	3.13	7.58	8.43	3.55	2.34	7.33	5.38
QC low_3	6.25	5.66	2.11	3.51	2.01	4.81	6.50
QC medium	50	6.21	1.21	4.84	5.02	2.78	4.51
QC high	200	1.72	2.89	6.23	6.27	4.81	4.10

Table 4
Intra-day accuracy for 99–357, 99–408 and 99–411 in rabbit and monkey plasma

Quality control	Concentration (ng/ml)	% Bias (accuracy)					
		Rabbit plasma			Monkey plasma		
		99–357	99–408	99–411	99–357	99–408	99–411
QC low_1	1.56	11.23	13.51	−4.80	−5.66	5.16	3.01
QC low_2	3.13	8.19	−7.32	2.77	−4.21	3.44	4.70
QC low_3	6.25	4.60	3.17	−1.49	1.66	4.11	3.17
QC medium	50	−5.13	−5.66	5.37	2.51	−1.70	−3.70
QC high	200	2.80	3.47	−7.20	−5.73	5.23	3.04

Table 5
Freeze-thaw stability data

Analyte	Concentration (ng/ml)	% Deviations after freeze-thaw cycles		
		1	2	3
99–357	1.56	9.24	3.77	−4.47
	200	−2.66	6.33	−0.44
99–408	1.56	−7.64	−6.92	−6.52
	200	−5.08	7.12	6.36
99–411	1.56	7.47	2.16	−1.86
	200	−2.11	5.40	−1.10

room temperature over 8 h, which well encompasses the duration of typical sample handling and processing (Table 6). Moreover, the analytes were found to be stable after reconstitution in acetonitrile for at least 12 h at 4 °C. 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 3 h gap in between wherein the samples were stored at 4 °C and in all cases the deviations were less than 15%.

3.4. Application to pharmacokinetic studies

The assay method was applied for the single dose PK studies of 99–357, 99–408 and 99–411 in male *Sprague–Dawley* rats. After single oral dose the 99/357, 99/408 and 99/411 in rats, blood samples were collected by sparse sampling approach ($N = 3$) over a period of 24 h and levels were detected up to 24 h in all cases. The plasma concentration–time profile for the three compounds is shown in Fig. 5.

Table 6
Bench-top and long-term stability data

Compound	Storage condition	Nominal concentration (ng/ml)	Concentration at $t = 0$ (ng/ml)	Concentration recovered (ng/ml)
99–357	8 h at 25 °C	1.56	1.53	1.62
		200	198.21	195.02
	30 days at −60 °C	1.56	1.48	1.40
		200	195.12	192.11
99–408	8 h at 25 °C	1.56	1.51	1.65
		200	205.58	208.97
	30 days at −60 °C	1.56	1.62	1.58
		200	208.29	207.47
99–411	8 h at 25 °C	1.56	1.59	1.43
		200	190.95	201.99
	30 days at −60 °C	1.56	1.67	1.59
		200	200.83	209.05

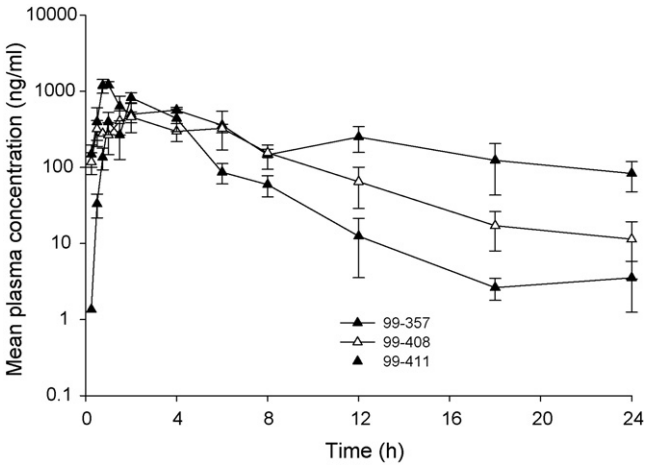

Fig. 5. Plasma concentration–time profiles of 99–357, 99–408 and 99–411.

4. Conclusion

An LC–MS/MS method for the quantification of 99–357, 99–408 and 99–411 in different biomatrices was developed and fully validated in rat plasma and partially validated in rabbit and monkey plasma using sample-pooling approach. This method offers significant advantages in terms of sensitivity and selectivity, faster run time (5.5 min) and lower volumes of sample requirements. Thus, the volume per samples to be collected per time point from an individual is reduced significantly enabling the inclusion of additional points. Moreover, this method significantly reduces the time and cost of analysis. The established LLOQ of 1.56 ng/ml is sufficient enough for PK studies and could be further improved, if required, by sample concentration. The results of the assay performance and the study conducted indicate that the method is precise and accurate enough for the routine assay of 99–357, 99–408 and 99–411. Thus, the method as well as the sample-pooling approach can be considered suitable for application to pre-clinical PK studies so as to get a plasma concentration–time profile useful for the realistic estimation of PK parameters of 99–357, 99–408 and 99–411.

Acknowledgements

The author wish to acknowledge Dr. Chandan Singh for providing authentic samples of 99–357, 99–408, 99–411 and IS. We are thankful to Council for Scientific and Industrial Research, India, for providing research fellowships and to the Director, CDRI, for providing facilities and infrastructure for the study.

References

[1] P. Olliaro, J. Cattani, D. Wirth, J. Am. Med. Assoc. 275 (1996) 230.
[2] WHO Fact sheets, World Health Organization, October 1998, http://www.who.int/inf-fs/en/fact094.html.
[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, N. Gupta, S.K. Puri, Tetrahedron Lett. 46 (2005) 205.
[7] A.G. Griesbeck, T.T. El-Idreesy, L.O. Hoinck, J. Lexa, R. Brun, Bioorg. Med. Chem. Lett. 15 (2005) 595.
[8] C. Singh, N.C. Srivastava, S.K. Puri, Bioorg. Med. Chem. 12 (2004) 5745.
[9] C. Singh, N. Gupta, S.K. Puri, Bioorg. Med. Chem. 12 (2004) 5753.
[10] C. Singh, H. Malik, S.K. Puri, Bioorg. Med. Chem. Lett. 14 (2004) 459.
[11] C. Singh, N. Gupta, S.K. Puri, Bioorg. Med. Chem. Lett. 13 (2003) 3447.
[12] R.P. Singh, S.K. Singh, R.C. Gupta, J. Pharm. Biomed. Anal. 37 (2005) 127.
[13] J. Berman, K. Halm, K. Adkinson, J. Shaffer, J. Med. Chem. 40 (1997) 827.
[14] M. Rajanikanth, R.C. Gupta, J. Pharm. Biomed. Anal. 26 (2001) 519.
[15] B.S. Kou, T.V. Noord, M.R. Feng, D.S. Wright, J. Pharm. Biomed. Anal. 16 (1998) 837.
[16] J.P. Atherton, T.J. Van Noord, B.S. Kuo, J. Pharm. Biomed. Anal. 2 (1999) 39.
[17] T.V. Olah, Mc Loughin, J.D. Gilbert, Mass Spectrom. 11 (1997) 17.
[18] Y. Hsieh, M.S. Bryant, J.M. Brisson, K. Ng, W.A. Korfmacher, J. Chromatogr. B 767 (2002) 353.
[19] Y. Zhao, G. Woo, S. Thomas, D. Semin, P. Sandra, J. Chromatogr. A 1003 (2003) 157.
[20] N.V. Nagaraja, J.K. Paliwal, R.C. Gupta, J. Pharm. Biomed. Anal. 20 (1999) 433.
[21] A.M. Almeida, M.M. Castel-Branco, A.C. Falcao, J. Chromatogr. B 774 (2002) 215.
[22] 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.
[23] R.D. McDowall, C. Hartman, W. Penninkx, Y.V. Hayden, V Pankeerberghen, D.L. Massart, in: H.H. Blume, K.K. Midha (Eds.), bio'94, Bio-International 2, Bioavailability, Bioequivalence and Pharmacokinetic Studies, Medpharm Scientific, Stuttguart, Germany, 1995, p. 331.