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Development and validation of a LC-MS/MS method with electrospray ionization for determination of LASSBio-579 in rat plasma

D.J. Conrado [a], E.C. Palma [b], C.A.M. Fraga [c], E.J. Barreiro [c], S.M.K. Rates [a], T. Dalla Costa [a,b,*]

[a] *Programa de Pós-Graduação em Ciências Farmacêuticas, Universidade Federal do Rio Grande do Sul, Av. Ipiranga, 2752 Porto Alegre, RS 90610-000, Brazil*
[b] *Centro Bioanalítico de Medicamentos, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga, 2752 Porto Alegre, RS 90610-000, Brazil.*
[c] *Laboratório de Avaliação e Síntese de Substâncias Bioativas, Faculdade de Farmácia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ 21944-971, Brazil*

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Abstract

A simple and sensitive LC-MS/MS analytical method was developed and validated for the determination of LASSBio-579 in plasma rat, using fluconazole as internal standard. Analyses were performed on a Shimadzu HPLC system using a Shimadzu C18 column and isocratic elution with acetonitrile–water (80:20, v/v), containing 0.4 mM ammonium hydroxide and 0.2 mM acetic acid at a flow rate of 1.0 ml/min (split ratio 1:5). A Micromass triple quadrupole mass spectrometer, equipped with an electrospray ionization interface, operated in the positive mode. Plasma samples were deproteinized with acetonitrile (1:2) and 50 μl of the supernatant were injected into the system. The retention times of LASSBio-579 and IS were approximately 4.7 and 2.4 min, respectively. Calibration curves in spiked plasma were linear over the concentration range of 30–2000 ng/ml with determination coefficient >0.98. The lower limit of quantification was 30 ng/ml. The accuracy of method was within 15%. Intra- and inter-day relative standard deviations were less or equal to 13.5% and 6.4%, respectively. The applicability of the LC-MS/MS method for pharmacokinetic studies was tested using plasma samples obtained after intraperitoneal administration of LASSBio-579 to male Wistar rats. No interference from endogenous substances was observed, showing the specificity of the method developed. The reported method can provide the necessary sensitivity, linearity, precision, accuracy, and specificity to allow the determination of LASSBio-579 in pre-clinical pharmacokinetic studies.

Keywords: LASSBio-579; Validation; LC-MS/MS; Pharmacokinetics

1. Introduction

The need of superior antipsychotic drugs with an improved therapeutic profile, i.e., improved efficacy on both positive and negative symptoms and decreased side effects, led to the development of the atypical antipsychotics of which clozapine is the prototype agent. Clozapine displays significant affinity for a large number of other neurotransmitter receptors such as D_4 and 5-HT. The drug clinical use has been restrict to some schizophrenic patients, because of the occurrence of clozapine-induced agranulocytosis in approximately 1–2% of patients [1,2].

The search for more efficient dopaminergic drugs lead to the design and synthesis of new *N*-phenylpiperazine derivatives by molecular hybridization of clozapine and L-741, aiming to obtain drugs with therapeutic efficacy similar to clozapine, devoid of its hematological side effects. Two of the compounds obtained were LASSBio-581 (1-[1-(4-chloro-phenyl)-1H-[1,2,3]triazol-4-ylmethyl]-4-phenyl-piperazine) and LASSBio-579 (1-[1-(4-chloro-phenyl)-1H-pyrazol-4-ylmethyl]-4-phenyl-piperazine) (Fig. 1) [3].

The selectivity of LASSBio-581 and LASSBio-579 to D_2 receptors was shown in binding and electrophysiological assays performed on brain homogenate and cultured hippocampal neurons [3]. The compounds were also assayed (dose range of 15–40 mg/kg) in three experimental models: (1) blockage of amphetamin (30 mg/kg, i.p.)-induced stereotypy in rats; (2) the catalepsy test in mice and (3) apomorphine (1 mg/kg, i.p.)-

* Corresponding author at: Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga, 2752 Porto Alegre, RS 90610-000, Brazil. Tel.: +55 51 3316 5418; fax: +55 51 3316 5437.
E-mail address: teresadc@farmacia.ufrgs.br (T. Dalla Costa).

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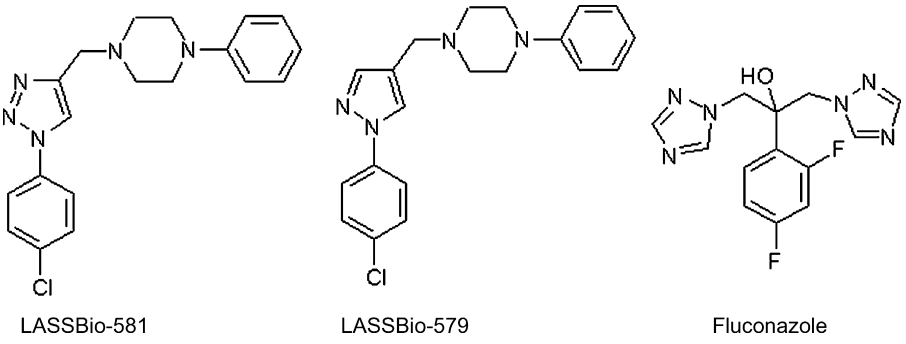

Fig. 1. Chemical structure of LASSBio-581, LASSBio-579 and the internal standard fluconazole.

induced hypothermia in mice. On the basis of the results of catalepsy and amphetamine-induced stereotypy, the compounds demonstrated an inhibitory effect on dopaminergic behavior [4].

The pharmacodynamic results obtained on dopaminergic system lead to the pre-clinical pharmacokinetic evaluation of LASSBio-581 and LASSBio-579 which can become prototype drugs to treat schizophrenia. The pharmacokinetic studies require a sensitive analytical method for the quantification of the drug in biological matrix. Tasso et al. validated a high performance liquid chromatography (HPLC) method for the determination of LASSBio-581 in rat plasma [5]. The method was successfully used to describe LASSBio-581 plasma pharmacokinetic profile and tissue distribuition in a pre-clinical study [6].

In this work, a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method was developed and validated for the determination of LASSBio-579 in rat plasma using fluconazole (Fig. 1) as internal standard (IS). The bioanalytical methodology was validated considering the specificity, linearity, precision, accuracy and lower limit of quantification. The applicability of the LC-MS/MS method for pharmacokinetic studies was tested using plasma samples obtained after intraperitoneal administration of LASSBio-579 to male Wistar rats.

2. Experimental

2.1. Solvents and chemicals

LASSBio-579 was synthesized by the Laboratório de Avaliação e Síntese de Substâncias Bioativas (LASSBio, Rio de Janeiro, Brazil). Fluconazole was purchased from Delaware (Porto Alegre, Brazil). Analytical grade ammonium hydroxide and glacial acetic acid were obtained from Merck (Darmstat, Germany). HPLC grade acetonitrile and methanol were purchased from Merck (Darmstat, Germany). HPLC water was obtained from a Millipore's Milli-Q System and used throughout the analysis.

2.2. Preparation of standard solutions and quality control samples

Two separate weighing of LASSBio-579 were used to prepare standard stock solutions at 500 μg/ml dissolved in methanol. One stock solution was used to prepare working solutions for the calibration curve standards. The other stock solution was used to prepare standard working solutions for the quality control (QC) samples. The calibration curve samples were prepared by spiking 100 μL of Wistar rat plasma with 10 μl of the appropriate standard working solution to obtain LASSBio-579 final concentrations of 30, 60, 125, 250, 500, 1000 and 2000 ng/ml. Quality control samples were prepared at 36, 180 and 1600 ng/ml. New standard stock solutions, calibration curve and QC samples were prepared on each analysis day.

Standard stock solution of fluconazole (IS) was prepared at 500 μg/ml in acetonitrile. This solution was successively diluted with acetonitrile to result in a final concentration of 3 μg/ml of fluconazole.

2.3. Instrumentations

Chromatographic analysis was carried out on a Shimadzu liquid chromatograph and Micromass Quattro LC mass spectrometer, using LC-10ADVP solvent delivery system with low-pressure gradient flow control valve FCV-10ALVP, SCL-10AVP system controller and DGU-14A degasser. The injections were performed by a Shimadzu SIL-10ADVP automatic injector and the analyses were performed using MassLynx software (Version 3.5).

2.4. Sample preparation

Prior to the chromatographic analysis, 100 μl of plasma samples were deproteinized by addition of 200 μl of acetonitrile (containing 3 μg/ml of IS), vortexed for 30 s and centrifuged at 6800 × *g*, 21 °C for 15 min. The supernatant was separated to inject into the LC-MS/MS system. Fluconazole final concentrations in the samples was 2 μg/ml. All plasma samples, including calibration curve, QC and samples from pharmacokinetic experiments were processed in the same manner.

2.5. Chromatographic and mass spectrometer conditions

The mobile phase used for the chromatographic separation was composed of acetonitrile–water (80:20, v/v) containing 0.4 mM ammonium hydroxide and 0.2 mM acetic acid (apparent pH 8.0). The mobile phase was filtered before using and it was delivered isocratically at a flow rate of 1.0 ml/min (split

ratio 1:5). The analysis was carried out at 40 °C using Shimadzu Shim-pack HPLC column (150 mm × 4.6 mm i.d.) packed with 5 μm ODS stationary phase, protected by Waters Novapak guard column packed with 4 μm RP18 material. The autosampler was set to inject 50 μl sample aliquots.

The analyte was monitored using a Micromass triple quadrupole mass spectrometer equiped with an electrospray ionization interface, operating in a positive mode (ESI^+). Samples were introduced into the interface through a heated nebulizer probe set at 280 °C. Nitrogen was the nebulizer and desolvation gas, and argon was used as collision gas. The other operating conditions were: nebulizer gas flow = 50 l/h; desolvation gas flow = 350 l/h; ion spray voltage = 3.2 kV; cone voltage = 30 V; ion source temperature = 130 °C; entrance potential = −3 V; collision energy = 18 V; collision cell exit potential = 6 V; collision gas pressure = 18 psi. The spectrometer was programmed in the multiple reaction monitoring (MRM) mode to allow the specific transition of precursor ion to fragment for each compound. The ions detection was performed by monitoring the decay of the mass-to-charge (*m/z*) ratio 353 precursor ion to the *m/z* 191 product ion for LASSBio-579 and the decay of the *m/z* 307 precursor ion to the *m/z* 220 product ion for IS. Fragment ions formatted from protonated LASSBio-579 [7] and fluconazole [8] molecules are showed in Fig. 2. The dwell time used for acquiring data for each MRM reaction was 0.5 s.

2.6. Validation procedures

Three determinations of each concentration level of the standard calibration curve and QC samples were analyzed in three consecutive days using different stock solution for each curve and quality control set. Linearity of calibration curves based on peak-area ratio (area of analyte/area of IS) as function of the nominal concentration was assessed by weighted (concentration $^{-1}$) least square regression. Slopes, intercepts and determination coefficients were calculated. A statistical analysis using ANOVA ($\alpha = 0.05$) was conducted to certify the linearity of the calibration curves. The selectivity of the method was investigated for potential interferences of endogenous substances by using eight independent batches of rat plasma samples. Moreover, the chromatograms of the experimental samples obtained after intraperitoneal administration of LASSBio-579 to Wistar rats were compared to the calibration curve standards chromatograms in order to detect interfering peaks. Intra- and inter-day precision and accuracy of the analytical method were shown by triplicate processing and following analysis of QC samples (36, 180 and 1600 ng/ml). Precision was calculated as relative standard deviation (R.S.D.) of the experimental concentrations and accuracy as the comparison between the experimental and nominal samples concentration. The criteria for acceptability of the data included accuracy within ±15% deviation from the nominal values and precision within ±15% R.S.D., except for the lower limit of quantification (LLOQ), where it should not exceed 20% of R.S.D. [9]. Recovery experiments (extraction efficiency) were performed by comparing the analytical results for extracted samples ($n = 3$) with unextracted standards that represent 100% recovery.

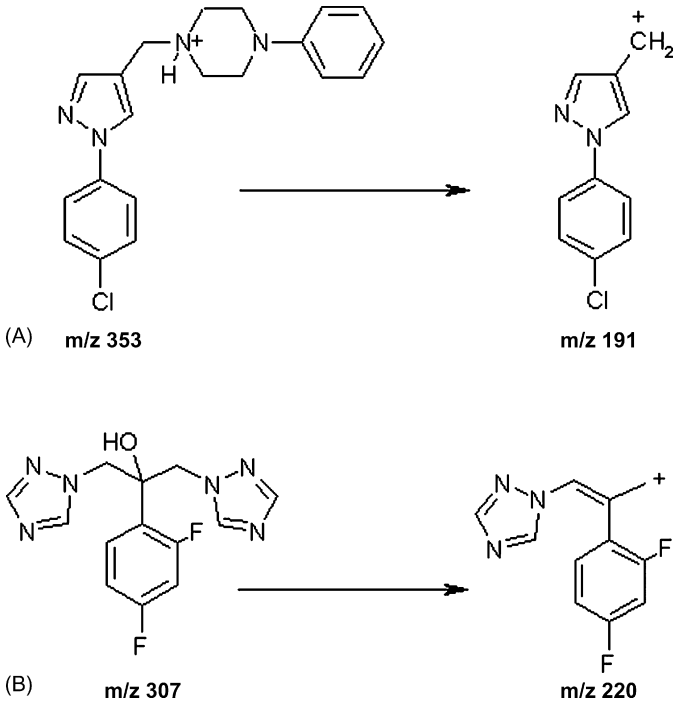

Fig. 2. Fragment ions formed from protonated LASSBio-579 (A) and fluconazole (B) molecules.

2.7. Preliminary stability studies

Preliminary studies were carried out to evaluate LASSBio-579 stability under the conditions used in this work. Analyte stability was determined during blood sample collection and handling at room temperature for 2 h and after freezing for 15 days at −20 °C. Furthermore, the stability of the processed samples was determined at 4 °C for 24 h in the autosampler.

2.8. Pharmacokinetic study

The applicability of the LC-MS/MS method developed for pharmacokinetic studies was tested using plasma samples obtained after intraperitoneal administration of LASSBio-579 to male Wistar rats ($n = 3$). The animals were kept under controlled 12 h light:12 h dark cycle during the acclimation period and had access to water and food ad libitum. For intraperitoneal administration, the suspension was prepared by dissolving LASSBio-579 in saline containing 3% (v/v) polysorbate 80. About 1.5 ml of the LASSBio-579 suspension was administered to rats corresponding at 60 mg/kg dose. Blood samples were withdrawn from lateral tail vein at 0; 0.25; 0.50; 0.75; 1; 1.5; 2; 4; 6; 12; 18; 24; 30 and 36 h after administration. Blood samples were collected into heparinized reaction tubes and centrifuged at 6800 g at 21 °C for 15 min to obtain plasma, which was stored at −20 °C until analysis. Plasma samples were processed as described in Section 2.4.

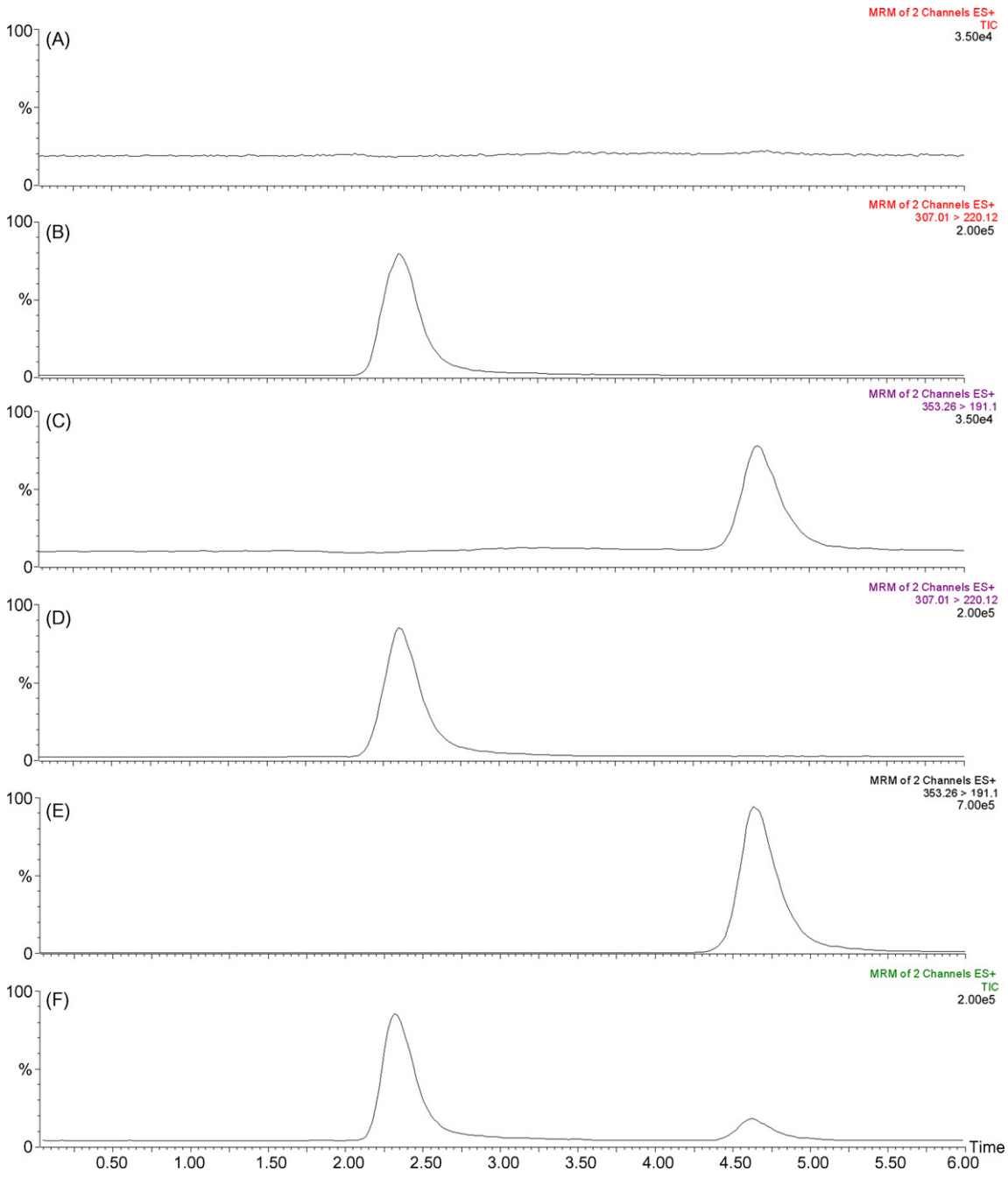

Fig. 3. Representative chromatograms in MRM-ESI$^+$ mode in rat plasma, using *m/z* ratios of 307 > 220 and 353 > 191 for IS and LASSBio-579, respectively. Total ion chromatogram (TIC) of blanck plasma (A). Plasma spiked with IS and LASSBio-579 (2.0 μg/ml and 30 ng/ml, respectively): *m/z* ratios of 307 > 220 (B) and 353 > 191 (C). Plasma spiked with IS and LASSBio-579 (2.0 μg/ml and 2.0 μg/ml, respectively): *m/z* ratios of 307 > 220 (D) and 353 > 191 (E). TIC of plasma sample 36 h post-administration of 60 mg/kg i.p. LASSBio-579 (plasma concentration 40 ng/ml) and IS (2.0 μg/ml) (F). The retention times observed were: IS, 2.4 min and LASSBio-579, 4.7 min.

3. Results and discussions

3.1. Selectivity and recovery

The selectivity of the analytical methodology can be seen in Fig. 3 by comparing representative chromatograms of independent blank plasma (A), blank plasma spiked with LASSBio-579 (30 ng/ml) and IS (2 μg/ml) (C and B) and blank plasma spiked with LASSBio-579 (2000 ng/ml) and IS (2 μg/ml) (E and D) and plasma sample 36 h post i.p. administration of 60 mg/kg of LASSBio-579 (plasma concentration of 40 ng/ml) and IS (2 μg/ml) (F). No additional peaks due to endogenous substances were observed that would interfere with the detection of the interesting compounds. The retention times of LASSBio-579 and IS were approximately 4.7 and 2.4 min, respectively. The extraction recovery of LASSBio-579 was 98.6%, independent of the analyte concentrations on the sample.

Table 1
Calibration curve parameters and statistics for LASSBio-579 in rat plasma[a]

Curve	Slope	*y*-Intercept	Determination coefficient
Day 1			
1	0.0024	0.0097	0.9998
2	0.0025	0.0156	0.9986
3	0.0021	0.0001	0.9820
Day 2			
1	0.0023	−0.0095	0.9992
2	0.0029	−0.0118	0.9998
3	0.0028	−0.0119	0.9997
Day 3			
1	0.0028	−0.0125	1.0000
2	0.0025	−0.0008	0.9996
3	0.0020	−0.0172	0.9980
Mean ($n=9$)	0.0025		
S.D.	0.0003		
R.S.D. (%)	12.2		

[a] S.D., standard deviation; R.S.D., relative standard deviation.

3.2. Linearity

The linearity of the method was observed in the investigated concentration range (30–2000 ng/ml). The calibration curve parameters determined in three consecutive validation days for LASSBio-579 are shown in Table 1. Calibration curves of peak area ratio (LASSBio-579/IS) as function of nominal concentration were linear using weighted (concentration $^{-1}$) linear regression, with a determination coefficient greater than or equal to 0.982 for all curves. The statistical analysis confirmed the linearity of the method developed ($p < 0.05$).

3.3. Lower limit of quantification

The lower limit of quantification (LLOQ) was 30 ng/ml, which was accepted as the lowest level on the calibration curve that could be determined with appropriate precision and accuracy under the experimental conditions of this analytical method (Tables 2 and 3) [9].

3.4. Precision and accuracy

The intra- and inter-day relative standard deviation values for LASSBio-579 are shown in Table 2. The intra-assay and inter-assay precision (R.S.D.) values for QC samples were less than or equal to 13.5 and 6.4%, respectively. The method showed an accuracy within 15%, which can be seen in Table 3. The results obtained for LC-MS/MS LASSBio-579 quantification method was within acceptable limits stated for bioanalytical methods validation [9].

3.5. Stability

Preliminary studies showed that LASSBio-579 was stable under the conditions used in this work. The analyte was stable in blood samples at room temperature for 2 h (99.4%) and in plasma samples after freezing at −20 °C for 15 days (98.2%). The stability of processed samples was determined at 4 °C in the autosampler for 24 h. LASSBio-579 and internal standard did not degrade at these conditions, showing 101.2 and 99.6% recoveries, respectively.

Table 2
Intra- and inter-day variation of LASSBio-579 in rat plasma

Nominal concentration	Day	Experimental concentrations[a]		
		Mean (ng/ml)	S.D.	R.S.D. (%)
Intra-day variation				
30 ng/ml (LLOQ)	1	28	2.1	7.7
	2	34	0.8	2.3
	3	31	3.05	9.8
36 ng/ml	1	36	4.9	13.5
	2	38	2.2	5.6
	3	39	3.0	7.8
180 ng/ml	1	175	16.2	9.2
	2	183	5.8	3.2
	3	180	10.3	5.7
1600 ng/ml	1	1483	74.5	5.0
	2	1681	211.1	12.6
	3	1623	42.7	2.6
Inter-day variation				
30 ng/ml (LLOQ)		31	3.2	10.4
36 ng/ml		38	1.3	3.3
180 ng/ml		179	3.6	2.0
1600 ng/ml		1596	101.3	6.4

[a] Values (mean and S.D.) represent $n = 3$ observations.

3.6. Applicability of the analytical method

To investigate the suitability of this analytical method for pharmacokinetic studies it was applied to determine LASSBio-579 plasma concentration after intraperitoneal administration of a single dose (60 mg/kg) to Wistar rats ($n = 3$). The mean plasma concentration-time profile of LASSBio-579 is shown in Fig. 4. The results indicated that the analytical method is suitable to measure plasma concentrations of the compound in pre-clinical studies. The peak plasma concentration (C_{max}) was 603 ± 384 ng/ml, attained at 45 min. The estimated half-life was found to be 9.6 ± 5.0 h. The area under the plasma concentration curve versus time from time zero to infinite ($AUC_{0-\infty}$) was 4.6 ± 2.2 μg h/ml. The results show that the lower limit of quantification of the method developed for LASSBio-579 was low enough to allow its use in pharmacokinetic studies because the extrapolated AUC was around 12 ± 8%, showing

Table 3
Accuracy for the analysis of LASSBio-579 in rat plasma

Concentration (ng/ml)	Range (ng/ml)	Accuracy (%)[a]
30 (LLOQ)	26–35	86.7–116.7
36	31–41	86.1–113.9
180	157–190	87.2–105.6
1600	1423–1829	88.9–114.3

[a] $n = 9$ Observations.

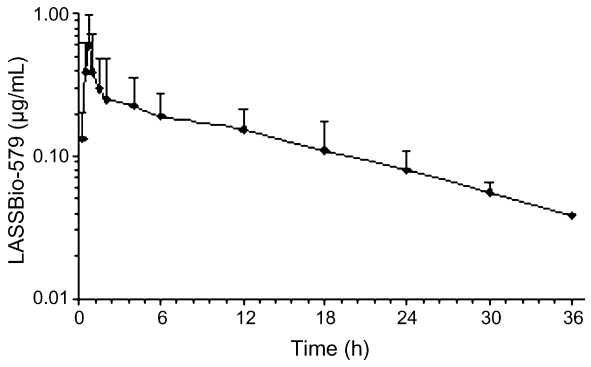

Fig. 4. Mean plasma concentration-time of LASSBio-579 after single intraperitoneal dose of 60 mg/kg to male Wistar rats. The data points are means and positive error bars are S.D. of three animals.

that the elimination phase of the compound was adequately characterized.

4. Conclusions

A LC-MS/MS bioanalytical method for the determination of LASSBio-579 in rat plasma using fluconazole as IS was established. This method showed adequate sensivity, linearity, precision and accuracy and it was succesfully applied to determine the concentration-time profiles of LASSBio-579 in pre-clinical pharmacokinetic pilot study. The full LASSBio-579 pre-clinical pharmacokinetic evaluation using the method described here is under way.

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