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Validated HPLC method for determination of LASSBio-581, a new heterocyclic *N*-phenylpiperazine derivative, in rat plasma☆

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Abstract

A rapid, simple and accurate high performance liquid chromatography (HPLC) method was developed and validated for the determination of LASSBio-581 (1-[1-(4-chloro-phenyl)-1H-[1,2,3]triazol-4-ylmethyl]-4-phenyl-piperazine) in rat plasma using ketoconazole as internal standard. Plasma samples were deproteinized with methanol. A good chromatographic separation was achieved using a reversed phase C_{18} column. Mobile phase consisting of sodium dihydrogen phosphate monohydrate (pH 4.5, 0.02 M) and methanol mixture (35:65, v/v) was used at a flow rate of 1.0 ml/min. The eluate was monitored using a UV detector at 248 nm. The retention times of LASSBio-581 and the internal standard were approximately 3.8 and 5.6 min, respectively. The calibration curves were linear over the concentration range of 0.25–8.0 μg/ml with correlation coefficients >0.99. The limit of quantitation was 0.25 μg/ml. The accuracy of the method was $>90\%$. The intra-day relative standard deviation (R.S.D.) ranged from 6.15 to 10.52% at 0.4 μg/ml, 7.44 to 13.81% at 1.5 μg/ml and 6.10 to 13.94% at 6.0 μg/ml. The inter-day R.S.D. were 9.54, 8.42 and 8.25% at 0.4, 1.5 and 6.0 μg/ml, respectively. No interference from endogenous substances or metabolites were observed. The method has been used to measure plasma concentrations of LASSBio-581 in pharmacokinetic studies in rats.

Keywords: LASSBio-581; Validation; HPLC-UV detector; Pharmacokinetics

1. Introduction

Clozapine is an atypical antipsychotic agent, which shows therapeutic profile diverse of the classic antipsychotic and binds to different receptors, including dopaminergic ones [1]. The main side effects of this drug are the hematological

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disorders, such as agranulocytosis, which restrict the use of clozapine to some categories of psychotic patients. The search for new more efficient dopaminergic agents with lower adverse effects is a promising research field. Aiming to obtain new compounds with similar therapeutic profile to clozapine without the hematological side effects, three different families of hetorocyclic compounds were designed and synthesized as possible ligands to dopamine receptors [2]. One of these compounds is LASSBio-581 (Fig. 1).

In vitro studies with binding of QNB, H-EB, 3H-SCH-23390 and 3H-YM-09151-2 as regards mAchR, nChR, D_1 and D_2 receptors in rat brain were carried out in Baltimore, USA. The compound LASSBio-581 showed affinity according to dopaminergic receptors [3], with LD_{50} of 707 mg/kg [2].

The compound LASSBio-581 was assayed (dose range of 15–40 mg/kg) in three experimental models: (1) blockade of amphetamine (30 mg/kg, ip) induced stereotypy in rats; (2) the catalepsy test in mice and (3) apomorphine (1 mg/kg, ip) induced hypothermia in mice. On the basis of the results of catalepsy and amphetamine-induced stereotypy, the compound LASSBio-581 demonstrated an inhibitory effect on dopaminergic behavior, which hypothermic response that was not prevented by haloperidol (0.5 mg/kg, ip) [4].

Besides the evaluation of pharmacodynamics it is important to determine the pharmacokinetic properties of the compound LASSBio-581. The support of pre-clinical pharmacokinetic investigations requires reliable bio-analytical methodology for the measurement of the drug involved. In order to investigate LASSBio-581 concentrations in rat plasma, a high performance liquid chromatography (HPLC) method was developed using chromatographic conditions based on the assay for quantitation of the ketoconazole in biological fluids [5]. The analytical methodology was validated regarding linearity, accuracy, precision (intra-day and inter-day) and limit of quantitation. The method has been used for the analysis of the compound in rat plasma. Representative plasma concentration versus time profile resulting from oral administration of LASSBio-581 to male Wistar rats are presented in this communication.

2. Experimental

2.1. Chemical and reagents

LASSBio-581 (93.5%) was synthesized at the Laboratório de Avaliação e Síntese de Substâncias Bioativas (LASSBio, Rio de Janeiro, Brazil). Ketoconazole (99.5%) was purchased from Delaware (Porto Alegre, Brazil). NaH_2PO_4 (Merck®, São Paulo, Brazil), LC-grade methanol (Omnisolv®, Porto Alegre, Brazil) and LC-grade water (Milli-Q system, Millipore®) were used for the mobile phase preparation.

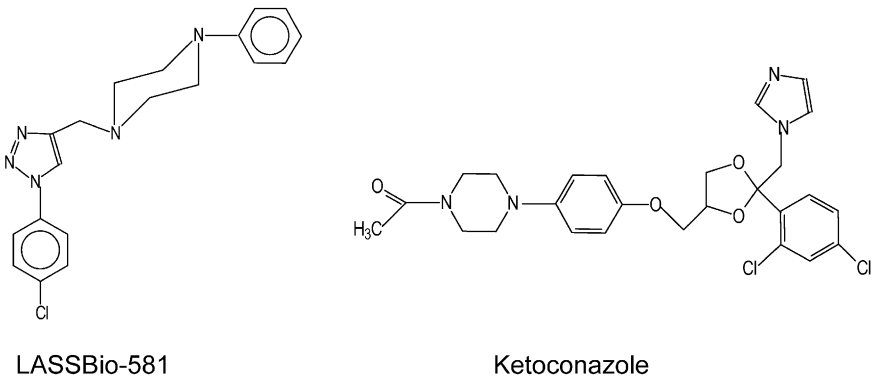

Fig. 1. Molecular structure of LASSBio-581 and ketoconazole.

2.2. Chromatographic equipment and conditions

A Shimadzu HPLC system equipped with LC-10ADVP pump with a low-pressure gradient flow control valve FCV-10ALVP, SCL-10AVP system controller, SIL-10ADVP auto-sampler, SPD-M10AVP photodiode array detector and a DGU-14A degasser was used. SHIMADZU CLASS-VP software (Version 6.12) was used for data acquisition and mathematical calculations. The chromatography was performed on a Waters Bondapack® C_{18} HPLC column (3.9 × 150 mm) preceded by a guard column (3.9 × 20 mm, 4 μm particle size) packed with the same material. All samples and standard solutions were chromatographed at room temperature using a mixture of sodium monohydrogen phosphate (pH 4.5, 0.02 M) and methanol (35:65, v/v) as mobile phase, with detection at 248 nm where the maximum absorbance of the substance investigated was observed (Fig. 2). A flow rate of 1.0 ml/min was used and 150 μl samples were injected. The auto-sampler was set up to inject sample aliquots every 15 min. The ratio of peak area of analyte to internal standard was used for the quantitation of plasma samples.

2.3. Standard solutions

A standard stock solution of LASSBio-581 was prepared in methanol with final concentration of 500 μg/ml. Appropriate dilutions of the stock solution were made with methanol. The calibration curve samples were prepared by spiking 100 μl of Wistar rat plasma with 10 μl of the appropriate standard working solution to result the final concentrations of LASSBio-581 of 0.25, 0.3, 0.5, 0.75, 2.0, 4.0, 8.0 μg/ml. The standard stock solutions were prepared in duplicate from separate weightings: one set was used to prepare the calibration curve samples and one set was used to prepare the quality control (QC) samples. A standard stock solution of ketoconazole (I.S.), which molecular structure is shown in Fig. 1, was prepared in methanol to produce concentration of 500 μg/ml. This solution was successively diluted to give 7.5 μg/ml. The standard stock solutions, calibration curve and QC samples were freshly prepared on each analysis day.

2.4. Sample preparation

Samples were deproteinized by the addition of 200 μl of methanol (containing the 7.5 μg/ml of

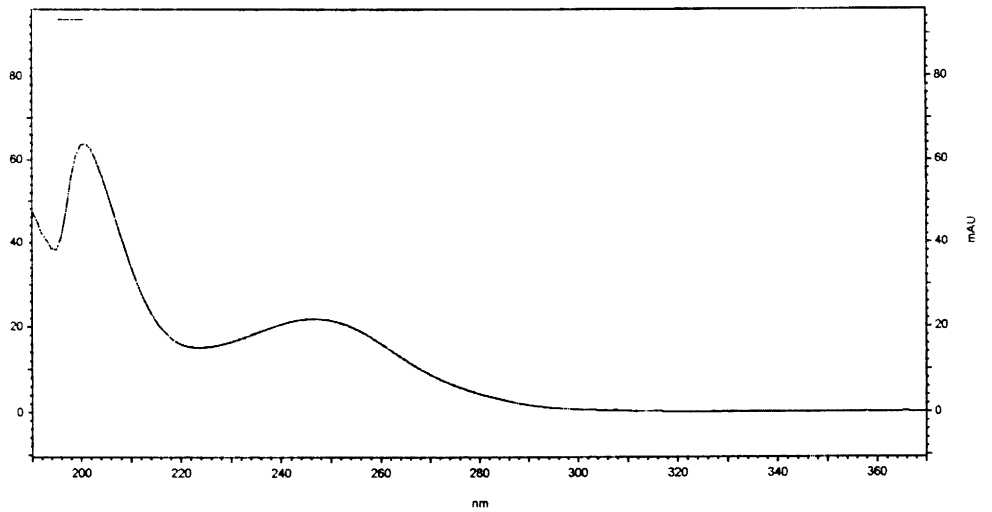

Fig. 2. Spectrum of the compound LASSBio-581 in methanol.

internal standard) and vortexed for more 25 s followed by centrifugation at 8000 rpm for 10 min at 21 °C. The supernatant was transferred into another Eppendorf tube and frozen at −20 °C until analysis by HPLC. The final concentrations of ketoconazole in the samples was 5 μg/ml. All samples, including QC samples, were processed in the same manner. Preliminary studies showed that LASSBio-581 is stable in plasma under the conditions investigated (data not showed).

2.5. Validation procedures

Validation samples were prepared and analyzed to evaluate the intra-day and inter-day precision and accuracy of the analytical method in rat plasma. Precision was reported as percentage of relative standard deviation (% R.S.D.) of the estimated concentrations and the accuracy was determined by comparing the measured concentrations of the plasma samples to the true concentration spiked into the sample.

To determine the accuracy of the method, QC samples of LASSBio-581 (QC) were prepared in triplicate at three concentration levels (0.4, 1.5 and 6.0 μg/ml) and analyzed using the procedure outlined above.

In order to determine the linearity, three determinations of each concentration level of the LASSBio-581 calibration curve were performed each day for three consecutive days. Calibration standards were freshly prepared in triplicate every day during ongoing analysis. Standard curves were obtained from the linear least square regression analysis of drug/internal standard peak area ratio as a function of the theoretical concentration. Slopes, intercepts and correlation coefficients were determined.

The selectivity of the assay was investigated by processing and analyzing blanks prepared from six independent lots of control plasma as well as by analyzing plasma samples obtained after oral administration of the compound to Wistar rats. The blanks and the animal samples were surveyed for interfering peaks.

2.6. Pharmacokinetic study

The pharmacokinetic study was carried out in three male Wistar rats. The animals were fasted overnight (∼12 h) and had access to water throughout the experimental period. Animals were given feed 4 h after drug administration. LASSBio-581 was administered through a gavage at a dose of 30 mg/kg oral (p.o.). Blood samples were collected into heparinized tubes at different time points (0, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10 and 12 h) from the tail vein and centrifuged at 8000 rpm at 21 °C for 10 min to separate the plasma. Plasma (100 μl) samples were prepared as described under Section 2.4.

3. Results and discussion

3.1. Separation

The molecular structures of LASSBio-581 and ketoconazole are shown in Fig. 1. The specificity/selectivity of the method can be illustrated in Fig. 3 by comparing the chromatogram of the independent blank plasma (A) with that obtained after analysis of LLOQ standard of LASSBio-581 and internal standard in spiked plasma (0.25 and 5 μg/ml, respectively) (B). Fig. 3C shows the highest standard concentration of the calibration curve. As it can be seen LASSBio-581 and the internal standard were well separated from each other. A representative chromatogram from 1 h plasma sample (3.9 μg/ml) after oral administration of 30 mg/kg to rats is shown in Fig. 3D. It can be observed that the metabolites produced by the oral administration of the compound did not interfere with the analyte peak, showing the specificity of the method. The mean retention times for LASSBio-581 and the internal standard were 3.8 and 5.6 min, respectively.

3.2. Quantitation limit

The lower limit of quantitation (LLOQ) was 0.25 μg/ml, which was the lowest concentration of analyte in a sample that could be determined with

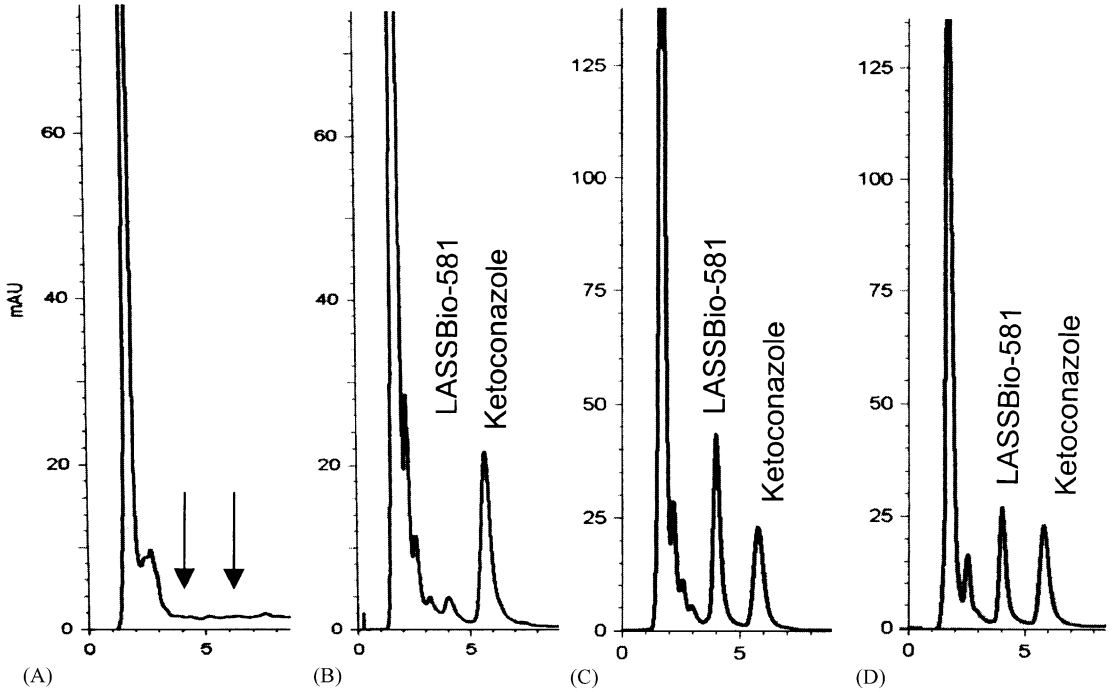

Fig. 3. Representative HPLC chromatograms of the method: (A) blank rat plasma, (B) rat plasma spiked with LASSBio-581 and I.S. (0.25 and 5 μg/ml, respectively), (C) rat plasma spiked with LASSBio-581 and I.S. (8.0 and 5 μg/ml, respectively) and (D) 1 h plasma sample (3.9 μg/ml) from rat dosed with LASSBio-581 at 30 mg/kg p.o. and I.S. (5 μg/ml).

acceptable precision and accuracy [6] under the stated experimental conditions for this method.

3.3. Linearity

The linearity of the method was observed in the investigated concentration range demonstrating its suitability. Regression results and statistics from calibration standard curves on three successive days are shown in Table 1. The correlation coefficients (r) were found to be >0.99 indicating functional linear relationship between the concentration of analyte and area under the peak. Using the average linear calibration equations determined for each validation day, the standard concentrations of the calibration curve were back-calculated. All back-calculated values of the individual calibration standards were within 15% of the spiked value, except the LLOQ, which was within 20%.

Table 1
Calibration curve parameters and statistics for LASSBio-581 in rat plasma[a]

Curve	Slope	y-Intercept	Correlation coefficient
Day 1			
1	0.1421	0.00140	0.9999
2	0.1575	0.00007	0.9999
3	0.1322	0.01660	0.9987
Day 2			
1	0.1425	0.00650	0.9978
2	0.1310	0.00270	0.9999
3	0.1384	0.00990	0.9991
Day 3			
1	0.1685	0.00250	0.9980
2	0.1688	0.01600	0.9972
3	0.1543	0.00480	0.9997
Mean ($n=9$)	0.1484	0.00156	
S.D.	0.0144	0.00928	
R.S.D. (%)	9.76		

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

Table 2
Intra and inter-day variation of LASSBio-581 in rat plasma

Spiked concentration	Day	Measured concentrations[a]		
		Mean (μg/ml)	S.D.	R.S.D.
Intra-day variation				
0.4 μg/ml	1	0.37	0.02	6.15
	2	0.44	0.03	7.34
	3	0.38	0.04	10.52
1.5 μg/ml	1	1.37	0.19	13.81
	2	1.41	0.16	11.16
	3	1.60	0.12	7.44
6.0 μg/ml	1	6.06	0.75	12.49
	2	5.45	0.76	13.94
	3	6.43	0.39	6.10
Inter-day variation				
0.4 μg/ml		0.40	0.04	9.54
1.5 μg/ml		1.46	0.12	8.42
6.0 μg/ml		5.97	0.49	8.25

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

3.4. Precision and accuracy

A summary of the results on precision and accuracy as derived from the measured concentration for the validation samples is given in Tables 2 and 3. The within-run R.S.D. and between-run R.S.D. were below 13.94 and 9.54%, respectively. Considering analyte measurement in biological matrix the criteria of acceptance for accuracy and precision are $\pm 15\%$ at all concentration levels, except at the LLOQ where it can be $\pm 20\%$ [6].

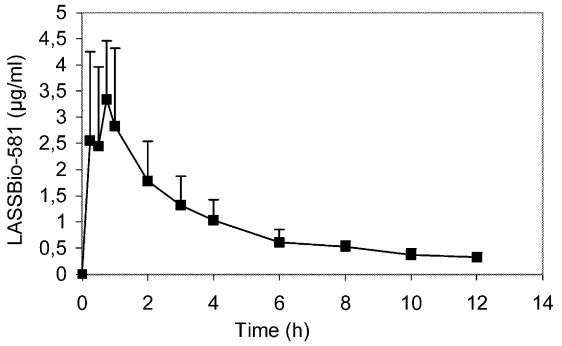

Fig. 4. Mean plasma concentration–time profile of LASSBio-581 after single oral dose of 30 mg/kg to male Wistar rats. The data points are means and positive error bars are S.D. of three animals.

Table 3
Accuracy for the analysis of LASSBio-581 in rat plasma

Concentration (μg/ml)	Accuracy (%)	Range (μg/ml)
0.4	97.50	0.37–0.44
1.5	97.33	1.37–1.60
6.0	99.50	5.45–6.43

3.5. Applicability in rat plasma sample

In order to demonstrate the applicability of the method for pharmacokinetic studies, male Wistar rats received LASSBio-581 as a single dose (30 mg/kg) by oral route. The mean concentration–time profile for LASSBio-581 in plasma obtained from three rats is shown in Fig. 4. The peak plasma concentration was 3.34 ± 1.48 μg/ml, obtained at 0.75 ± 0.0 h. The estimated elimination half-life was 4.6 ± 2.7 h. Detailed pharmacokinetic data for all animals enrolled in the pre-clinical study will be reported in a separated article.

4. Conclusions

An HPLC method with UV detection has been developed for quantitation of LASSBio-581 in rat plasma. All results were within the acceptable ranges for bio-analytical purposes. The chromatography system used provided clear separation of the compound and internal standard with no interfering peaks. The assay was validated and the results demonstrated that the standard curve is linear over the concentration range investigated. This simple assay is reproducible and accurate, has a short analysis time and has been successfully applied to the analysis of plasma samples from rats in support of pre-clinical pharmacokinetic studies.

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