Journal of Pharmaceutical and Biomedical Analysis 55 (2011) 1024–1030

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Determination of the cardioactive prototype LASSBio-294 and its metabolites in dog plasma by LC–MS/MS: Application for a pharmacokinetic study

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ARTICLE INFO

Article history:
Received 20 October 2010
Received in revised form 21 February 2011
Accepted 25 February 2011
Available online 3 March 2011

Keywords:
LASSBio-294
LC–MS/MS
Pharmacokinetics
Canine plasma
Oral administration

ABSTRACT

In this work we describe the evaluation of the pharmacokinetics of a novel cardioactive compound of the *N*-acylhydrazone class, LASSBio-294, using high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) in dog plasma for the first time. Separation was achieved on a ZORBAX Rapid Resolution High Definition (RRHD) SB-C18 (50 mm × 2.1 mm, 1.8 μm) reversed-phase column at 20 °C with methanol-10 mM ammonium acetate solution (65:35, v/v) at a flow rate of 1.0 mL/min. Detection was performed using an electrospray ionization (ESI) operating in positive ion multiple reaction monitoring (MRM) mode by monitoring the ion transitions from m/z 275.2 → 149.1 (LASSBio-294) and m/z 152.0 → 110.0 (acetaminophen, internal standard). The calibration curve of LASSBio-294 in plasma showed good linearity over the concentration range of 1.25–800 ng/mL. The validated method was successfully applied to a pre-clinical pharmacokinetic study of the cardioactive prototype LASSBio-294 in beagles after oral administration. The main pharmacokinetic parameters $t_{1/2}$, C_{max} and AUC_{0-24} were (5.74 ± 0.55) h, (547.66 ± 35.12) ng/mL and (1621.77 ± 41.66) ng h/mL, respectively.

1. Introduction

Cardiovascular diseases are responsible for the largest number of natural death worldwide. Platelet adhesion and aggregation are key events in haemostasis and thrombosis, and they are also strictly related to pathological thrombotic processes, inflammation, immunological diseases and tumor metastasis [1]. Clinical evidence has clearly proven that antiplatelet therapy is useful for preventing thrombotic disorders and peripheral vascular diseases [2].

LASSBio-294 (2-thienylidene-3,4-methylenedioxybenzoylhydrazine; L-294) [3], was originally designed as phosphodiesterase (PDE) inhibitor candidate synthesized from natural safrole (Fig. 1), an abundant Brazilian natural product, obtained from Sassafras oil in high yield by classic distillation (*Ocotea pretiosa* and *Piper hispidinervum*) [4,5].

This *N*-acylhydrazone (NAH) derivative was characterized as a novel possible alternative for treatment of cardiac failure, once it was able to promote an effective positive inotropic and vasodilatory activity [6] through a mechanism different from that displayed by cardiac glycosides and β-adrenergic agonists. L-294 increased the spontaneous contraction of isolated hearts of Wistar rats in a dose-dependent manner (maximum effect at 25 μM) and the Ca^{2+} uptake into sarcoplasmic reticulum (SR) without changing the sensitivity of the contractile proteins to Ca^{2+} [7]. Moreover, this compound induced relaxation of isolated rat aorta with an IC_{50} of 74 μM [8] by increasing intracellular cyclic GMP levels. These pharmacological evidences suggest a novel mechanism of action, circumventing the toxic effects resulted from calcium homeostasis alteration [8]. More recently, L-294 was also described to prevent myocardial infarction induced cardiac dysfunction through improving intracellular Ca^{2+} regulation [9].

Taken together, the cardioinotropic and vasodilatory profiles of the *N*-acylhydrazone prototype strongly indicated that it is a novel drug candidate, effective and safer to treat pathological conditions in which cardiac muscle fatigue is a debilitating disorder [10]. In this context, the development of a new therapeutic candidate at the preclinical stage involves the investigation of its biotransformation profile in order to access important information about its pharmacokinetic behavior [11]. Despite the structure-based prediction and mammalian biomimetic biosynthesis of thiophene sulfoxide as the main metabolite of L-294 produced by fungus *Beauveria bassiana* ATCC 7159 strains [12] and the metabolites

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doi:10.1016/j.jpba.2011.02.031

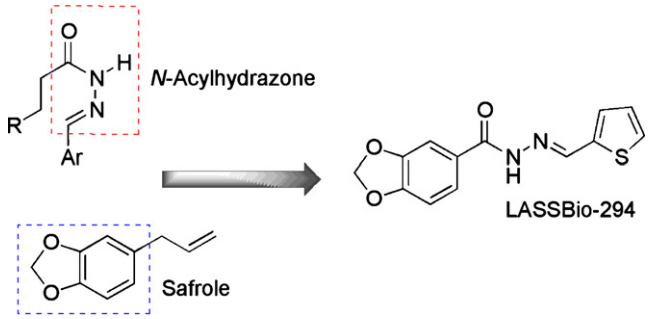

Fig. 1. Structural design concept of L-294.

evidenced from rat and human *in vitro* assays in our previous studies [13].

Studies of drug absorption, distribution, metabolism, excretion and toxicity (ADMET) as well as drug metabolism and pharmacokinetic (DMPK) [14,15] studies are widely used in drug discovery and development to help obtain the optimal balance of properties necessary to convert lead compounds into drugs that are safe and effective for human use [16]. Drug discovery efforts have been aimed at identifying and addressing metabolism issues at the earliest possible stage, by developing and applying innovative liquid chromatography mass spectrometry (LC–MS) based techniques and instrumentation [15,17], which are both faster and more accurate than prior techniques. Such new approaches are demonstrating considerable potential to improve the overall safety profile of drug candidates throughout the drug discovery and development process [17]. These emerging techniques streamline and accelerate the process by eliminating potentially harmful candidates earlier and improving the safety of new drugs.

In this study we report for the first time the development and validation of a simple, rapid and sensitive high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the determination of the cardioactive prototype L-294 in dog plasma. The method was evaluated with regard to its accuracy, precision, selectivity, sensitivity, reproducibility, and stability. Furthermore, it was successfully used in a preliminary pharmacokinetic study of orally administered L-294.

2. Experimental

2.1. Chemicals and reagents

LASSBio-294 (purity > 99.2%) was synthesized at LASSBio (Laboratório de Avaliação e Síntese de Substâncias Bioativas, Rio de Janeiro, Brazil). The internal standard (IS), acetaminophen (purity > 99%) was purchased from U.S. Pharmacopoeia (USA). Acetonitrile and methanol (HPLC grade) were purchased from Tedia (USA). Water was distilled and purified using a Milli-Q Water Purification System (Millipore, Bedford, MA, USA). All other chemicals were of analytical grade. Blank dog plasma (drug free, heparinized) was prepared in our laboratory and stored at −20 °C.

2.2. Instrumentation

The LC–MS/MS procedure was performed using an Agilent 1200 series HPLC and an Agilent 6410 Triple Quadrupole mass spectrometer equipped with an electrospray ionization source (Agilent Technologies, USA). The temperature of the sample cooler in the autosampler was 4 °C. The chromatographic separation was achieved on a ZORBAX Rapid Resolution High Definition (RRHD) SB-C18 (50 mm × 2.1 mm, 1.8 μm) column. All data were acquired employing Agilent 6410 Quantitative Analysis version Analyst data processing software.

2.3. Chromatographic conditions

The mobile phase was a mixture of methanol-10 mM ammonium acetate solution (65:35; v/v), which was pumped at a flow rate of 1 mL/min. The column oven temperature was set at 20 °C. The total run time was 1.5 min. The sample injection volume was 10.0 μL. Mass spectrometric detection was performed on a Series 6410 Triple Quad LC–MS/MS (Agilent Technologies, USA) in electrospray positive ionization (ESI^+) using multiple reaction monitoring (MRM). The mass transition was m/z 275.1 → 149.2 for L-294, and m/z 152.0 → 110.0 for acetaminophen (IS), respectively. The other working parameters of the mass spectrometer were as follows: dwell time 200 ms; gas flow 10 L/min; gas temperature 350 °C; nebulizer pressure 50 psi; fragmentor voltage 60 V (LASSBio-294) and 90 V (IS), collision energy 36 eV (L-294) and 14 eV (IS).

2.4. MS

In order to develop ESI conditions for L-294 and IS, quadrupole full scans were carried out in positive ion detection mode. The solutions containing L-294 and IS were injected directly into the mass spectrometer respectively. The first step was determining the MRM transition and an optimization flow injection analysis (FIA) were carried out. The FIA gives us a better evaluation of the instrument signal-to-noise ratio (S/N). The results showed that the most sensitive mass transition were m/z 275.2 → 149.1 for L-294 (analyte) and m/z 152.0 → 110.0 for acetaminophen (IS) (Fig. 1). A resolution of 1 mass unit is sufficient to distinguish the ions (e.g., A typical definition of unit resolution is when the peak width at half-height is about 0.6–0.8 mass unit). This kind of low-resolution mass filter covers almost 90% of the instruments commonly used for qualitative and/or quantitative analysis of small molecules.

All metabolites structures propositions were achieved in an Agilent 6210 TOF MS, orthogonal acceleration time-of-flight mass spectrometer with dual sprayer interface for mass calibration for acquisition of molecular mass data with highest accuracy. ESI in positive mode with dual spray was applied during the experiments and the equipment was set as the following conditions: dry gas, dry temp 300 °C, nebulizer 25 psi, scan 50–950 Da, fragmentor 150 and capillary 5000 V.

2.5. Sample preparation

An aliquot of 200 μL of the samples plasma was transferred to an eppendorf tube, 50 μL of IS and 720 μL of methanol. The mixture was vortex-mixed for 1 min, and then centrifuged at 16,000 rpm for 10 min at 4 °C. 10 μL aliquot of the supernatant was injected into the LC–MS/MS system for analysis.

2.6. Preparation of calibration curves and quality control samples

Stock solutions of L-294 (10 mg/mL) and IS (10 mg/mL) were prepared in methanol and stored at 4 °C. The stock solution of L-294 was diluted quantitatively with methanol to give working standards at concentrations of 12.5, 25.0, 50.0, 100, 400, 1600, 6400 and 8000 ng/mL. The working IS solution was prepared by diluting the IS stock solution with methanol giving a concentration of 2000 ng/mL. Calibration standards of L-294 were prepared by dissolving the evaporated working standards with blank dog plasma yielding final concentrations of 1.25, 2.50, 5.00, 10.0, 40.0, 160 and 800 ng/mL. The quality control (QC) samples used in the validation and in the plasma level determinations were prepared in the

same manner, using a separately weighed stock solution. The nominal plasma concentrations of QC samples were at low (2.50 ng/mL), medium (10.0 ng/mL) and high (640 ng/mL) concentrations.

2.7. Assay validation

A thorough and complete method validation for determination of L-294 in dog plasma was done following the FDA guidelines [18]. The method was validated for specificity, sensitivity, linearity, accuracy and precision, recovery, matrix effect and stability.

The calibration curves were prepared in the range of 1.25–800 ng/ml for L-294 as described above. The L-294 calibration curve was constructed by plotting peak area ratio (y) of L-294 to the internal standard, versus L-294 concentration (x). Linearity was assessed by weighted ($1/x$) linear regression of calibration curves generated in triplicate on three consecutive days using analyte–internal standard peak area ratios. The acceptance criterion for a calibration curve was a correlation coefficient (r) of 0.99 or better, and that each back-calculated standard concentration must be within 15% deviation from the nominal value except at the lower limit of quantification (LLOQ), for which the maximum acceptable deviation was set at 20%. The LLOQ was defined as the lowest concentration in the standard curve at which the relative standard deviation (RSD) ($n = 6$) was within 20% and relative error (RE) was within ±20%. Calibration curves were prepared with each batch of animal samples. The LLOQ for L-294 in plasma was 1.25 ng/ml.

The accuracy and precision of the method was evaluated by replicate analysis of spiked quality control samples. QC samples were prepared as described above. The QC samples were analyzed five times a day to evaluate intra-day precision. The same procedure was performed once a day for three consecutive days to determine inter-day precision. The precision was calculated by using the RSD and a one-way analysis of variance (ANOVA). Accuracy was determined by comparing the calculated concentration using calibration curves with known concentration. The accuracy was expressed as relative error (RE%). It was calculated using the formula: $RE\% = (E - T)/T \times 100$ (by dividing a deviation by the nominal value and multiplying by 100).

Recovery presents the extraction efficiency of a method, which was performed at three QC levels (five samples each). The recoveries were evaluated by comparing peak areas of analytes in spiked plasma samples with those of samples to which the analytes had been added after extraction.

The matrix effect was defined as the direct or indirect alteration or interference in response due to the presence of unintended or other interfering substances in the samples. It was evaluated by comparing the peak area of the analytes dissolved in the blank plasma sample's precipitated solution with that of the analytes dissolved in the mobile phase. Three different concentration levels of L-294 (2.50, 10.0 and 640 ng/mL) were evaluated by analyzing three samples at each level. The blank plasma used in this study was from six different batches of the blank plasma. If the peak area ratio is less than 85% or more than 115%, a matrix effect is implied.

Stability experiments were performed to evaluate the stability of the analyte and IS in plasma under different conditions. QC samples were subjected to short-term room temperature condition for 24 h, to long-term storage conditions (−20 °C) for 2 months, to post-preparative stability of the processed samples after 24 h and to three freeze–thaw stability studies. All stability studies were conducted at three QC levels (2.50, 10.0 and 640 ng/mL as low, middle and high values) with five determinations for each.

The selectivity of the assay was checked by analyzing blank plasma samples of six dogs. Each blank plasma sample was tested using the proposed extraction procedure and LC–MS/MS conditions to ensure no interference of L-294 and IS from plasma.

Prior to running each batch of pre-clinical plasma samples, the instrument performance (e.g., sensitivity, reproducibility of chromatographic retention and separation, plate number and tailing factor) was determined by analysis of the L-294, IS, blank plasma and plasma spiked with L-294 and IS.

2.8. Pharmacokinetic study

The animal handling protocol of this pharmacokinetic study had been reviewed and approved by the Institutional Animal Care and Use Committee of the Federal University of Goias (UFG). Six healthy beagles dogs (six females) weighing 9 ± 1 kg were used in a pharmacokinetic study. They were kept in a environmentally controlled breeding room for 30 days before starting the experiments and fed with standard laboratory food and water. All standard laboratory food was gently sponsored by Guabi Alimentos, Brazil. Prior to the experiments, the dogs were fasted and maintained with physiological saline for 12 h. Dogs were randomized according to body weight, and capsule of L-294 were orally administered at a dosage of 10 mg (the capsules were prepared by Farmácia Escola Laboratoy at University of Goias following the L-294's patent [10]). Dog blood samples were collected after administration of L-294 at 0, 0.25, 0.50, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 12, and 24 h. Blood samples (1 mL) were transferred to heparinized tube and centrifuged at 5000 rpm at 4 °C for 10 min. Plasma was separated and stored at −80 °C until analysis. Concentrations of L-294 were determined by LC–MS/MS method developed in this study.

The profiles of plasma concentrations versus time were acquired for each subject. The peak plasma concentration (C_{max}) and the time to C_{max} (t_{max}) were obtained directly from the data. The other major pharmacokinetic parameters were calculated by non-compartmental method. The elimination rate constant (λ) was estimated from the least-squares regression slope of the terminal portion of the plasma concentration–time curve. The apparent elimination half-life ($t_{1/2}$) was calculated as $\ln 2/\lambda$. The area under the plasma concentration–time curve from time zero (pre-dose) to the last measurable concentration (AUC_{0-24}) was calculated using the linear trapezoidal rule. The AUC from 0 to infinity ($AUC_{0-\infty}$) was calculated as the $AUC_{0-\infty} = AUC_{0-t} + C_t/\lambda$. C_t is the last plasma concentration evaluated in plasma greater than the LLOQ. The mean residence time (MRT) was obtained by dividing the area under the first moment–time curve ($AUMC_{0-\infty}$) by the area under the curve ($AUC_{0-\infty}$). $V_{z/F}$ was the apparent volume of distribution. $C_{l/F}$ was the clearance rate. C_{last} was the last measured plasma concentration. The data analysis of pharmacokinetic parameters was performed by using The R Project for Statistical Computing (Version R-2.11.1) [19] with the package "PK" [20] and the PKSolver, an add-in program for pharmacokinetic and pharmacodynamic data analysis in Microsoft Excel (Version 2.0) [21].

3. Results and discussion

3.1. Method development

3.1.1. Selection of IS

To find a compound that could ideally mirror the analyte and serve as a suitable IS we screened several compounds. Acetaminophen was found to be the most appropriate IS for the present study as its retention time was similar to that of L-294 (Fig. 2).

No significant interference in the MRM channels at the relevant retention times was observed during the study.

3.1.2. Sample pre-treatment

In this method, the plasma sample was precipitated with methanol and then the extracts were injected into the mobile

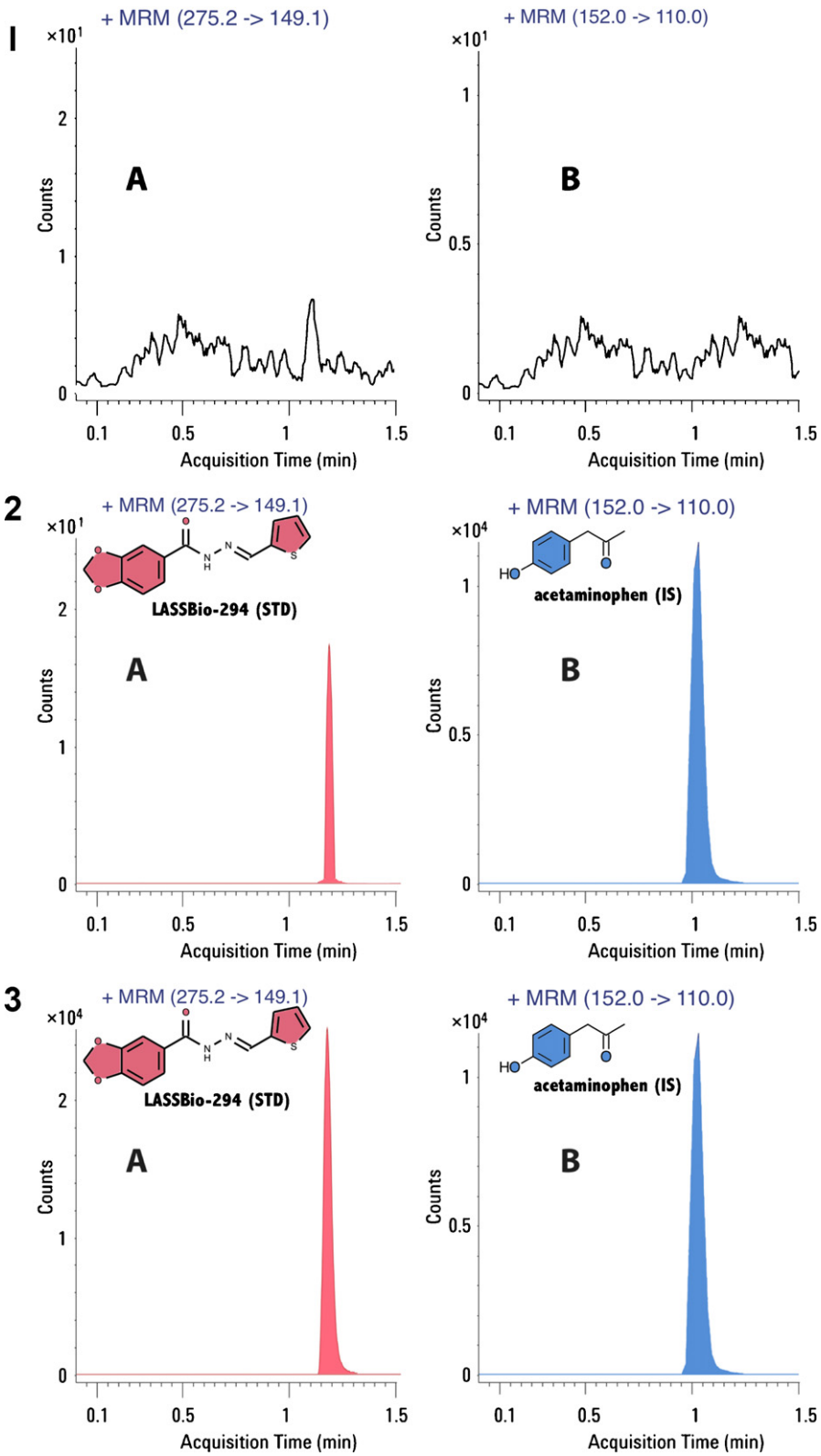

Fig. 2. Typical chromatograms of L-294 and IS in beagle plasma: (1A–B) a blank plasma sample extracted; (2A–B) a plasma sample of 1.25 ng/mL (LLOQ) + internal standard (IS); (3) (A) a plasma sample obtained from a beagle at 2 h after oral administration of 10 mg of L-294 (B) a plasma sample + internal standard (IS).

phase stream without evaporation and reconstitution. It could simplify the sample preparation procedure significantly and also meet the requirements of the assay. No interference was observed from any endogenous or exogenous plasma matrix.

3.1.3. *Liquid chromatography*

Chromatographic analysis of the analyte and IS was initiated under isocratic conditions aiming at develop a simple separation process with a short run time. Feasibility of various mixture(s) of solvents such as acetonitrile and methanol using different buffers

Table 1
Precision and accuracy for the determination of L-294 in dog plasma by LC–MS/MS method.

Spiked (ng/mL)	Intra-batch precision and accuracy ($n = 5$)			Inter-batch precision and accuracy ($n = 3$)		
	Measured (mean ± S.D.) (ng/mL)	RSD (%)	RE (%)	Measured (mean ± S.D.) (ng/mL)	RSD (%)	RE (%)
2.50	2.58 ± 0.08	3.3	1.2	2.65 ± 0.12	4.6	2.4
10.0	10.22 ± 1.28	4.5	−0.4	10.45 ± 0.23	5.2	1.9
640	641 ± 0.51	6.1	3.4	641 ± 0.48	7.3	4.1

such as ammonium acetate, ammonium formate and formic acid, along with altered flow rates (in the range of 0.2–1.5 mL/min) were tested to identify the optimal mobile phase that produced the best sensitivity, efficiency and peak shape. It was found that a mixture of methanol–10 mM ammonium acetate solution (65:35, v/v) could achieve this purpose and was finally used as the mobile phase. A flow rate of 1.0 mL/min permitted a run time of 1.5 min.

3.1.4. Specificity and sensitivity (LLOQ)

The specificity of the method towards endogenous plasma matrix was evaluated in six different batches of dog plasma. Fig. 1 shows the typical chromatograms of blank plasma, spiked plasma with L-294 and the IS, and dog plasma after oral administration. The retention times of L-294 and IS were 1.21 and 1.07 min, respectively. No significant direct interference in the blank plasma traces was observed from endogenous substances in drug-free dog plasma at the retention time of L-294 or the IS.

In our pilot study, the lowest plasma concentrations of the 10 mg oral dose do not exceeded 1.25 ng/ml. In order to obtain the good linearity and avoid instrument carry-over resulting from high concentration, we defined the linear range as 1.25–800 ng/mL. Samples exceeding 800 ng/mL were diluted by the same blank plasma before pre-treatment. The LLOQ of L-294 was established at 1.25 ng/ml (Fig. 2), which was sensitive enough for pharmacokinetic study of L-294 for oral administration in dogs. The precision and accuracy at this concentration was acceptable, with 9.1% of the RSD and 0.7% of the RE ($n = 6$).

The limit of detection (LOD), defined as a signal to noise ratio of 3:1, was 0.1 ng/mL (Fig. 3).

3.2. Linearity, accuracy and precision

The L-294 calibration curve was constructed by plotting peak area ratio (y) of L-294 to the internal standard, versus L-294 concentration (x). Linearity was assessed by weighted ($1/x$) linear regression of calibration curves generated in triplicate on three consecutive days using analyte-internal standard peak area ratios, a typical equation of the calibration curve was $y = 0.0061x - 4.5929$. Good linearity was obtained over the concentration range of 1.25–800 ng/mL with correlation coefficients (r) = 0.9988.

The intra- and inter-batch precision and accuracy of the assay were assessed by analyzing QC samples. The precision was calculated using one-way ANOVA. The results, summarized in Table 1, demonstrate that the precision and accuracy values are within the acceptable range and the method is accurate and precise.

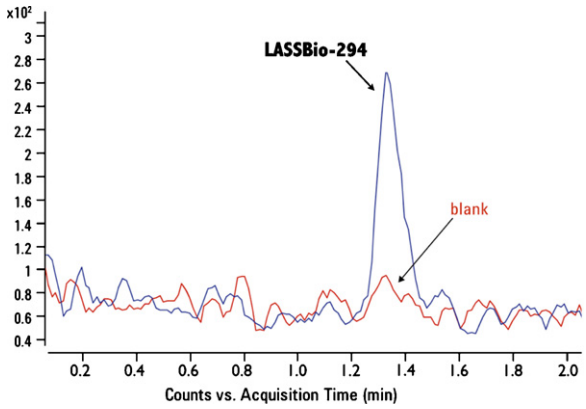

Fig. 3. Overlay of 0.1 ng/mL of L-294 (LOD) chromatogram recovered from dog plasma (blue trace) overlaid with a blank (red trace) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

3.3. Recovery and matrix effect

The extraction recovery of L-294 was calculated by analyzing five replicates at 2.50, 10.0 and 640 g/mL. The extraction recoveries of the assay were 83.6 ± 4.9%, 89.8 ± 4.3% and 92.3 ± 3.6% for the low, middle and high concentrations, respectively.

To study the effect of matrix on analyte quantification with respect to consistency in signal suppression, matrix effect was checked with six different batches of heparinized plasma. Three replicates for each of three concentrations (2.50, 10.0 and 640 g/mL) were prepared from different batches of plasma. The results were 95.3 ± 3.9, 93.8 ± 2.4 and 97.0 ± 2.0 for the low, middle and high concentrations, respectively. In this study, there was no matrix effect of the analytes.

3.4. Stability

The stability of L-294 was studied under a variety of storage and handling conditions. The results are summarized in Table 2. The data indicated that L-294 was stable under the conditions evaluated, reflecting actual sample handling and analysis. Stability of the QC samples after 24 h at room temperature and after the three

Table 2
Short-term, post-preparative, and freeze–thaw stability of L-294 in beagle plasma.

Spiked concentration of LASSBio-294 (ng/mL)	Mean of percentage remaining (%)		
	2.50	10.0	640
Short-term stability (24 h, room temperature, $n = 5$)	108.1	106.9	101.2
Post-preparative stability (24 h, room temperature, $n = 5$)	96.4	97.9	97.9
Freeze and thaw stability (three cycles, −20 °C/room temperature, $n = 5$)	101.7	106.7	96.3
Long-term stability (60 d, −20 °C, $n = 5$)	104.6	102.5	107.1

freeze and thaw cycles, and post-preparative stability of the processed samples after 24 h were acceptable. L-294 in plasma stored at −20 °C was stable for 2 months. The stock solutions of L-294 and IS at −20 °C were stable for 2 months at least.

3.5. Assay selectivity

The selectivity of the assay was checked by analyzing blank plasma samples, with and without the acetaminophen internal standard. Plasma from six health dogs donors were tested to be blank for L-294 (see Fig. 1 for a double blank). The internal standard did not contribute a measurable response to the L-294 signal. In addition, plasma samples spiked with caffeine (1 μg/mL), dexchlorpheniramine (76 ng/mL) and metamizole (5 μg/mL) were also evaluated against the standard sample (STD) at the lower limit of quantitation (LLOQ). Each blank plasma sample was tested using the proposed extraction procedure and LC–MS/MS conditions to ensure no interference of L-294 and IS from plasma.

3.6. Pharmacokinetic studies

No pharmacokinetic study of L-294 has been reported so far as we know. The developed method in this paper was successfully used for a pharmacokinetic study in which plasma concentration of L-294 up to 24 h after oral administration a dosage of 10 mg in 6 healthy beagles dogs. Mean plasma concentration–time profiles are presented in Fig. 4.

The data analysis of pharmacokinetic parameters was performed by using The R Project for Statistical Computing (version R-2.11.1) package "PK" and the PKSolver (version 2.0). The non-compartmental pharmacokinetic parameters were calculated and are listed in Table 3. These results demonstrate that L-294 is rapidly absorbed and eliminated.

3.7. Metabolites elucidation

The major circulating components in plasma after an oral dose were the parent compound, a sulfoxide (M1) and a hydroxylated (M2) and metabolites. The metabolites were characterized by ESI-MS/MS and their structures confirmed by infusion of the M1 and M2 samples in electrospray–high-resolution mass spectrometry ESI-HRMS. L-294 and its metabolite M1 structures were elucidated by ^{13}C, ^{1}H [22] NMR and ESI-MS/MS analysis as reported in our previous work [12].

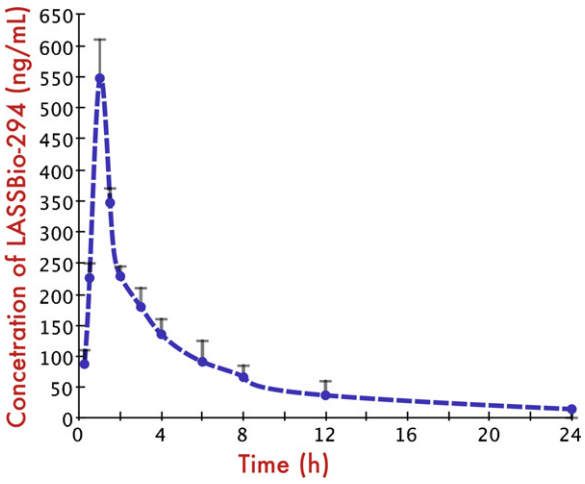

Fig. 4. Plasma concentration profile of L-294 in dogs after oral administration of a dose of 10 mg (mean ± S.D., $n = 6$).

Table 3
The pharmacokinetic parameters of L-294 in beagles following an oral administration of 10 mg L-294 ($n = 6$).

Parameters	Mean	S.D.
$AUC_{(0-24)}$ (ng h/mL)	1621.77	41.66
$AUC_{(0-\infty)}$ (ng h/mL)	2010.13	55.12
MRT (h)	7.06	0.47
$T_{1/2}$ (h)	5.74	0.55
T_{max} (h)	1.00	0.11
C_{max} (ng/mL)	547.66	35.12
$C_{l/F}$ (mg)/(ng/mL)/h	4.97	0.01
$V_{z/F}$ (ng)/(ng/mL)	41.25	0.04

According to the MS analysis, the thiophene ring moiety from L-294 was hydroxylated giving the metabolite M2. The main peak for metabolite M2 was found at a retention time of 0.94 min. From the MS total ion chromatogram (TIC), the extracted ion chromatograms (EIC) for all compounds. The EIC for metabolite M2 shows the measured $[M+H]^+$ at m/z 291.0437 with a relative mass error of 0.6 ppm (Fig. 5B). For the first time we could propose this new product of metabolism of this prototype, measured with accurate mass ESI TOF data.

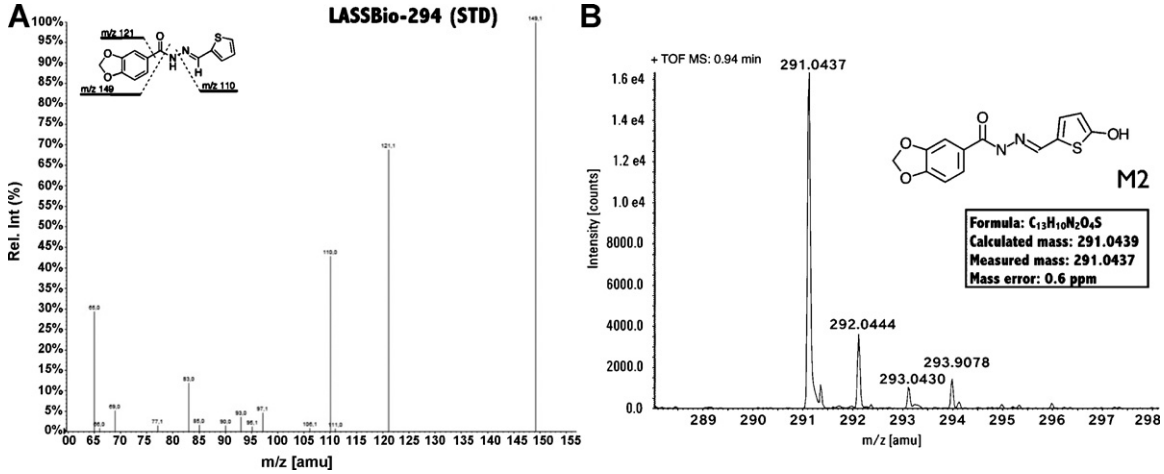

Fig. 5. (A) The product ion mass spectra of L-294, (B) accurate mass and elemental composition of metabolite M2, a hydroxylated thiophene ring portion of L-294 found in beagle plasma after 1 h of oral administration.

4. Conclusion

A sensitive, specific and accurate method is first described for the quantification of the cardioactive prototype L-294 in dog plasma by LC–MS/MS in positive electrospray ionization mode using MRM and fully validated according to commonly accepted criteria. The method exhibited excellent performance in terms of high selectivity, low LLOQ (1.25 ng/mL), wide linear range (1.25–800 ng/mL), small organic solvent consumption (720 μL) and small plasma volume (200 μL). Moreover, the method has been successfully used for a pre-clinical pharmacokinetic study in dogs after oral administration of L-294. The pharmacokinetic parameters obtained from this study can give some useful information for further research of L-294.

Acknowledgement

The authors thank CAPES, CNPq and INCT-INOFAR (#573.564/2008-6) for financial support and fellowships.

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