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Liquid chromatography–mass spectrometry assay of a thiadiazole derivative in mice: application to pharmacokinetic studies

Hong Wong[a], Lee Jia[a,*], Jim B. Camden[b], Steve D. Weitman[a]

[a]*Institute for Drug Development/CTRC, 14960 Omicron Drive, San Antonio, TX 78245, USA*
[b]*The Procter & Gamble Company, Cincinnati, OH 45217, USA*

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Abstract

Modern atmospheric pressure ionization (API) ion-trap mass spectrometry in connection with fast chromatographic separations using a short narrow-bore C_8 column was developed to determine 5-phenyl-3-thioureido-1,2,4-thiadiazole (301029), a novel virus inhibitor in serum. Both 301029 and an internal standard (I.S.) were separated from serum samples by acetonitrile deproteinization and extraction without time-consuming reconstitution. The chromatographic separation was achieved on a C_8 reversed-phase narrow-bore column using acetonitrile–water–acetic acid (90:10:0.01, v/v/v) as a mobile phase. The mass spectrometric analysis was performed by atmospheric pressure chemical ionization (APCI) mode with positive ion detection. Single ion monitoring (SIM) scan mode of *m/z* 237 and 158 was used to quantitatively determine 301029 and I.S., respectively. The low limit of quantitation was 25 ng/ml. The assay exhibited a linear range of 25–2500 ng/ml. Recovery from serum proved to be 100–113%. The precision (C.V.) and accuracy (RE) of the method were 2–12% and 94–112%, respectively. The present method was applied to determine the pharmacokinetic parameters of 301029 following oral administration of the agent to mice at 5 g/kg. The results revealed that the elimination half-life of 301029 was 413 min and the area under serum concentration–time curve was 354 μg/ml/min.

Keywords: Thiadiazoles; 5-Phenyl-3-thioureido-1,2,4-thiadiazole

1. Introduction

Thiadiazole derivatives have been known to be a carbonic anhydrase inhibitor with corneal permeability [1,2] and a matrix metalloproteinase [3,4]. More recently, it is reported that they are potent anti-viral agents [5,6]. 301029 (Fig. 1), a representative thiadiazole derivative, is thus found to be a highly effective inhibitor of bovine viral diarrhea virus (BVDV). This candidate drug exhibits a therapeutic index 100-fold greater than that of the existing approved hepatitis C virus (HCV) inhibitor ribavirin based on its high efficacy and low toxicity. Mechanistic studies revealed that 301029 does not inhibit virus entry into target cells, instead, 301029 inhibits intermediate steps in the virus replication cycle, which results in significant reduction in RNA synthesis. Our recent studies demonstrated that 301029 has antiviral properties against HCV and BVDV replication (unpublished observation) that would

*Corresponding author. Tel.: +1-210-677-3830; fax: +1-210-677-0058.
E-mail address: ljia@saci.org (L. Jia).

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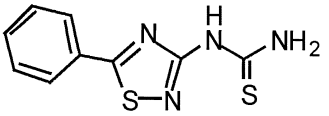
301029
MW 236



Internal Standard
MW 157

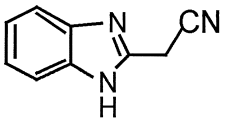


Fig. 1. Chemical structures of 301029 and I.S.

provide advantages over existing therapies with ribavirin. In order to proceed the development of this novel drug, an analytical method was needed to assess in vivo blood concentrations of 301029.

Both gas chromatography and microbiological analytical procedures exist for detecting derivatives of 301029 in biological fluids. However, both are time-consuming and labor-intensive. Although a more useful analytical high-performance liquid chromatography (HPLC) method was developed to detect thiadiazole derivatives [7–9], the method involves several steps including freezing, evaporating the sample which may lead to degradation of the compound and consequently loss of the drug. In addition, UV detection was not feasible due to interference of plasma components in the same range of wavelengths as 301029. The validation procedure for measurement of 301029 in plasma was limited to the detection, precision and accuracy. A simpler sample preparation coupled with faster LC–mass spectrometry analytical method was thus developed in our laboratory [10].

The method was based on a standard reversed-phase HPLC method and MS detection using an ion trap mass analyzer. With the ion trap technology, the collection of ions is distinct from the ejection of ions; thus the wide isolation window does not result in decreased spectral resolution. Because of its ability to select one particular ion, the LC–MS method required less sample cleaning procedures than a UV detection method [11]. To facilitate the pre-clinical development of this drug, we initiated the development LC–MS method that allowed us to establish pharmacokinetic profile of 301029 after oral administration of the drug to mice. The results of these studies are reported here.

2. Experimental

2.1. Materials and reagents

301029 was synthesized by our chemical team, and the internal standard (I.S.) 2-Benzimidazolyl-acetonitrile (Fig. 1) was purchased from Aldrich (Milwaukee, WI, USA). Acetonitrile, methanol, chloroform, and acetic acid of HPLC grade were purchased from EM Science (Gibbstown, NJ, USA). *N*,*N*-Dimethylformamide solution was bought from Allied Signal (Muskegon, MI, USA)

2.2. Instrument conditions

Liquid chromatography (LC) was performed using a Surveyor reversed-phase LC system coupled with a LCQ Duo ion trap mass spectrometer (ThermoQuest, San Jose, CA, USA), equipped with a APCI interface. The Surveyor MS pump is a quaternary pumping system with a low dead volume of 80 μl, which is ideal for the use of 2 mm (I.D.) columns. With less volume, this column shortens chromatography analysis time and increase system sensitivity. The separation was achieved on a 3.5 μ Kromasil C_8 column (150×2 mm I.D., Phenomenex, Torrence, CA, USA) eluted with a mobile phase consisted of acetonitrile, water and 0.01% acetic acid (90:10:0.01, v/v/v). The mobile phase was isocratically pumped at 100 μl/min for 6 min with the solvent front diverted to waste to prevent contamination of the mass spectrometer from the aqueous soluble salts. The atmospheric pressure chemical ionization interface was used to generate positive ion ($[M+H]^+$). The vaporizer temperature was set at 200°C, the ion source corona needle voltage was set at 4.5 kV and the sheath gas (N_2) was set at 0.5 l/min at 100 p.s.i. Ions were guided through the heated capillary by

setting the temperature and the voltage at 170°C and 3 V, respectively. The signal was amplified by adjusting the ion electron multiplier at −695.37 V and the dynode at −14.87 kV. Lastly, vacuum was maintained at 0.92×10^{-5} Torr. The mass spectrometer was programmed to scan singly the 301029 ion at 237 m/z and the I.S. at 158 m/z. Ion chromatograms were created by using a filter which monitors the ion of interest. The above conditions optimized the analysis of 301029. The peak area ratios for curves and quantitation were processed by using a PC compatible using Xcalibur software (ThermoQuest, San Jose, CA, USA).

2.3. Preparation for standard and quality control samples

301029 solubility in water is approximately 50 μg/ml. Stock solutions of 301029 and internal standard were prepared in *N*,*N*-dimethylformamide at 1.32 mg/ml and 10 mg/ml, respectively. Stock solutions were further diluted with mobile phase to obtain desired concentrations. Serum was spiked with 301029 stock solution and I.S. in order to extract 301029 and to precipitate plasma components.

The nominal plasma concentrations (7 points) of calibration standards were 25, 50, 100, 200, 500, 1000 and 2500 ng/ml. Quality control (QC) levels were chosen at 50, 200 and 1000 ng/ml.

2.4. Sample preparation

Each plasma sample (0.1 ml) was placed into a 0.5 ml test tube and vortex-mixed for 30 s. The mixture was vortexed for 30 s, spiked with 0.1 ml of mobile phase containing I.S. at 510 ng/ml. The sample was vortexed for 30 s and centrifuged for 10 min at 10 000 *g*. The supernatant was transferred to an auto-sampler vial and 20 μl was injected into the LC–MS system.

2.5. Method validation

Plasma calibration curves and three replicates of QCs were analyzed. The peak area generated by 301029 and I.S. were gathered and ratios were calculated. The calibration curves were constructed by weighted $(1/Y)$ least-squares linear regression analysis of the peak area ratios of 301029 and I.S. versus the concentrations of 301029. Calibration curve equations were used to calculate the concentrations of 301029 in the samples and QCs from their peak area ratios.

The intra-batch precision and accuracy were determined by analyzing, on the same day, three replicates of spiked samples at three concentrations of 50, 200 and 1000 ng/ml against a calibration curve. The inter-batch precision and accuracy were also carried out by analyzing three replicates of spiked samples at the same concentrations, on different days, as the intra-batch samples, against a calibration curve.

2.6. Recovery

The deproteinization recoveries of 301029 from mouse serum were calculated by comparing the peak area ratios of the mouse serum with solvent samples. The recovery study was carried out by spiking mouse blank samples with 301029 at 50, 200 and 1000 ng/ml. Solvent sample data were obtained in the same way as for plasma samples, except that the drug was added after deproteinization.

2.7. Dosing and sampling

301029 was orally administered to BDF-1 mice (5 g/kg). Blood samples were withdrawn at 0.25, 0.5, 1, 2, 4 and 8 h post dosing. The serum was obtained by centrifugation at 10 000 *g* for 10 min, and then mixed with one-fold excess of acetonitrile for deproteinization as described in Section 2.4.

2.8. Data analysis

The ratio of the peak area of 301029 to that of I.S. was used as the assay parameter. Peak-area ratios were plotted against theoretical concentrations. Standard calibration curves were obtained from unweighted least-squares linear regression analysis of the data. Mouse serum concentrations of 301029 were back-calculated from the standard calibration curves. Pharmacokinetic parameters were calculated using the computer program WinNonlin (Pharsight, Mountain View, CA, USA). The serum concentration–time

data of 301029 were analyzed by using non-compartmental model based on the goodness-of-fit criteria set by the lowest standard errors of the fitted parameters. The WinNonlin used the trapezoidal rule to calculate the area under the serum concentration–time curves (AUC) up to the last measured serum concentration.

3. Results and discussion

LC–MS for the determination of 301029 in mouse serum was investigated. The mass spectrum of 301029 (Fig. 2) showed a protonated molecular ion (MH+) at m/z 237.0. The mass spectrum of the I.S. showed a protonated molecular ion (MH+) at m/z 158.0.

3.1. Separation and specificity

To demonstrate specificity of the method, four typical chromatograms from the study of 301029 in mouse plasma are shown in Fig. 3, in which chromatograms of blank extracted plasma were compared with spiked plasma. Retention times of less than 4 min were achieved for both 301029 and I.S. 301029 eluted at 3.1 min and I.S. at 3.3 min. For both drug and the I.S., the chromatograms were free of interfering peaks at their respective retention times.

3.2. Optimization of MS conditions

301029 was first directly infused at 5 μl/min into the mass spectrometer using APCI. Parameters such as corona discharge needle, heated capillary temperature and voltage, nitrogen sheath gas and auxiliary gas were optimized in order to obtain the best signal-to-noise ratio of 301029. The compound was then infused into the mobile phase flow (100 μl/min) from the LC by using a tee union and the same parameters were further fine-tuned: the sheath gas setting had to be changed from 0.1 l/min to 0.5 l/min. The heated capillary temperature and voltage was adjusted from 170°C to 200°C and 2.8 V to 3 V, respectively. SIM scan mode chromatograms were used to determine 301029 and the I.S. in serum. SIM spectra selectively filter out ions not related to the target compounds and a very clean ion chromatogram can thus be obtained due to the great selectivity and sensitivity of this scan mode.

3.3. Linearity, precision and accuracy

Table 1 presents the accuracy, precision and linearity of three standard curves. Calibration curves were plotted as the peak area ratio (drug/I.S.) vs. drug concentration. The assay was linear in the concentration range of 25–2500 μg/ml. The C.Vs were less than 24%. The correlation coefficients (R^2) were greater than 0.998 for all curves.

The precision and accuracy of this method were verified by calculating intra-batch variation at three concentrations (50, 200 and 1000 ng/ml) in three replicates. As shown in Table 2, the C.Vs were less than 12%. Intra-assay accuracy was excellent with values ranging between 94 and 112%, which is well within the acceptance criteria of 80–120% set by US Food and Drug Administration. Inter-day accuracy and precision, compared at three concentrations on three separate occasions is also shown in Table 2, and exhibited good reproducibility on all three occasions with accuracy ranging from 96 to108% and precision ranging between 2 and 6%. These results indicate that the method was reliable within the analytical range, and the use of the I.S. was very effective for reproducibility by LC–MS.

3.4. Deproteination recovery

Deproteination recovery was calculated by comparing the peak area ratios of 301029 in plasma samples with the peak area ratio of solvent samples. As shown in Table 3, the recovery of 301029 was determined at three different concentrations (50, 200 and 1000 ng/ml). The recovery of 301029 were 100–113%. The overall average was 105%, demonstrating the efficiency of the sample preparation with little variation.

3.5. Pharmacokinetics evaluation

The method described was rugged for the analysis of all samples collected during pharmacokinetic investigations [12]. A typical serum concentration vs. time profile for BDF-1 mice after oral administration (5 g/kg) is depicted in Fig. 4. The serum con-

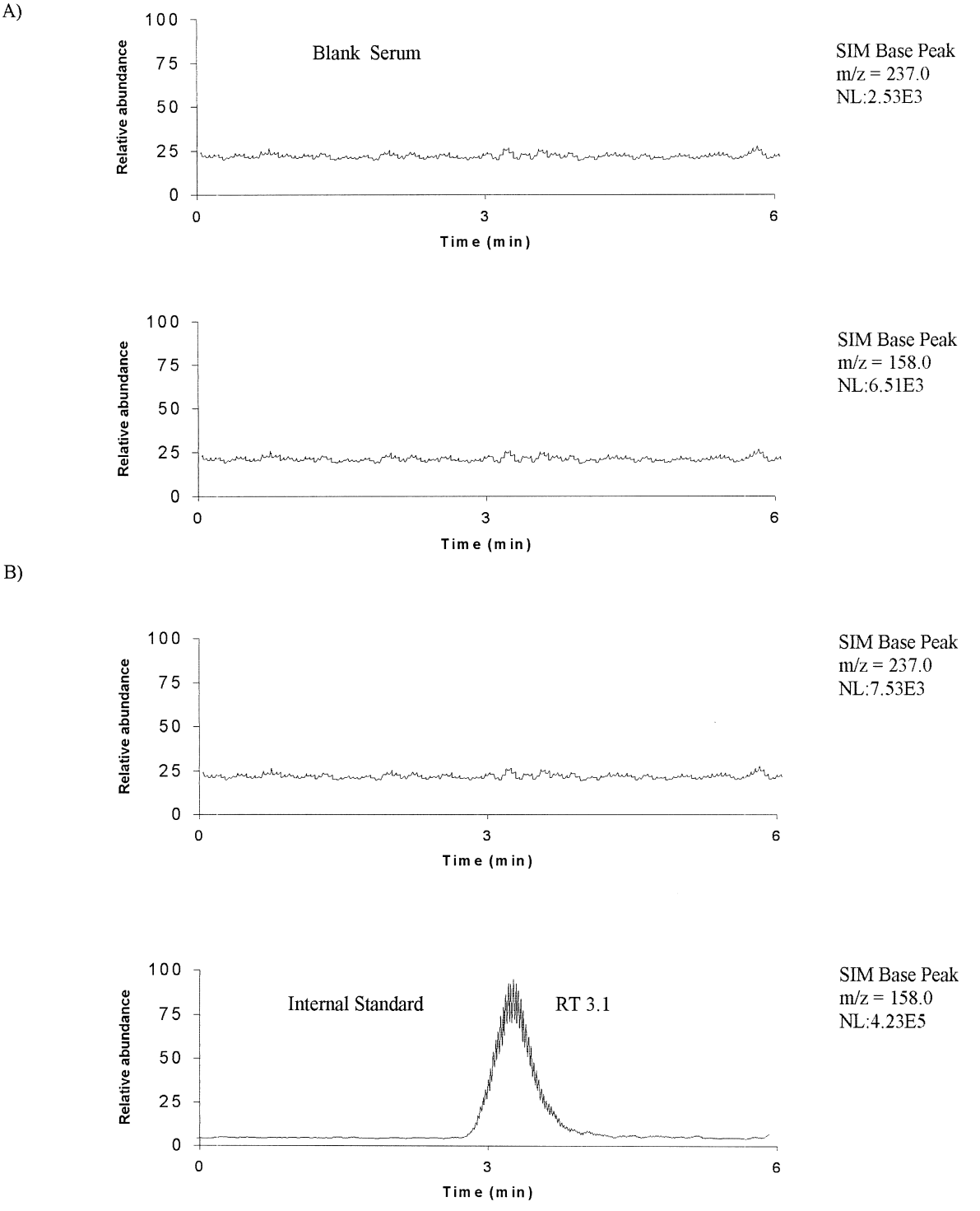
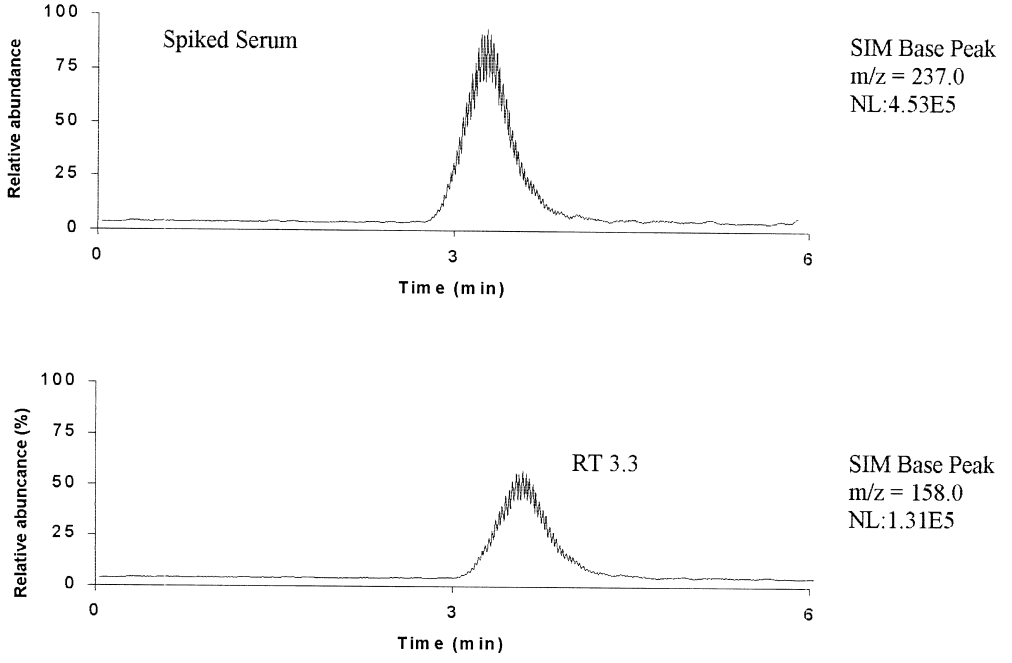


Fig. 2. Representative mass chromatograms of 301029 in mouse serum: (A) blank serum, (B) blank serum spike with the I.S., (C) mouse serum spiked with 301029 and I.S., and (D) a sample from BDF-1 mouse serum 1 h after dosing.

C)



D)

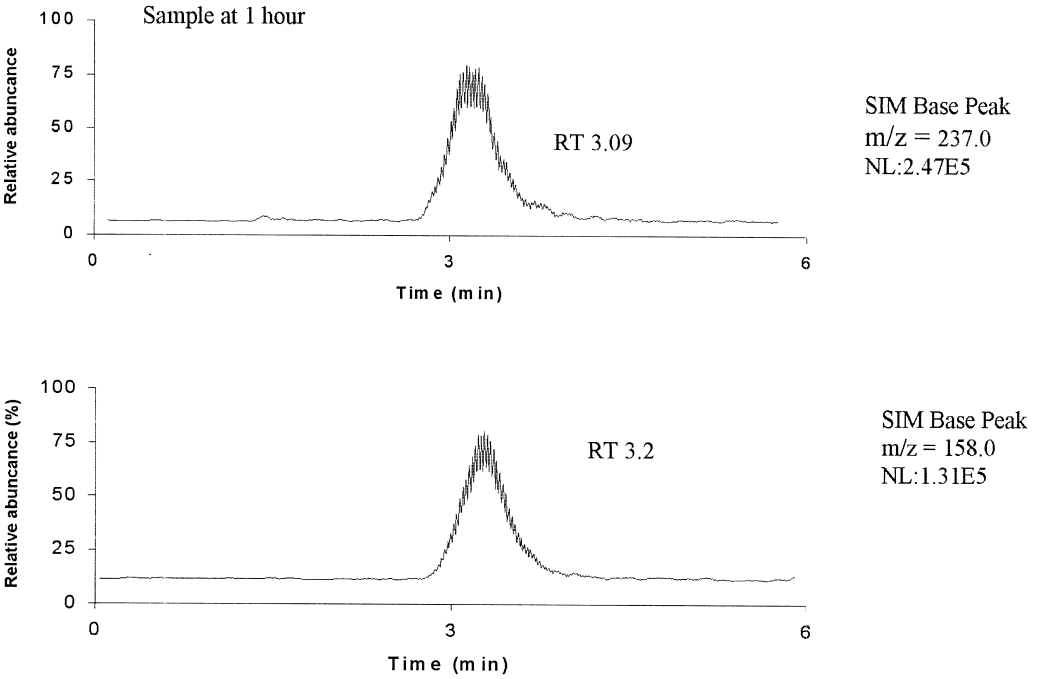


Fig. 2. (*continued*)

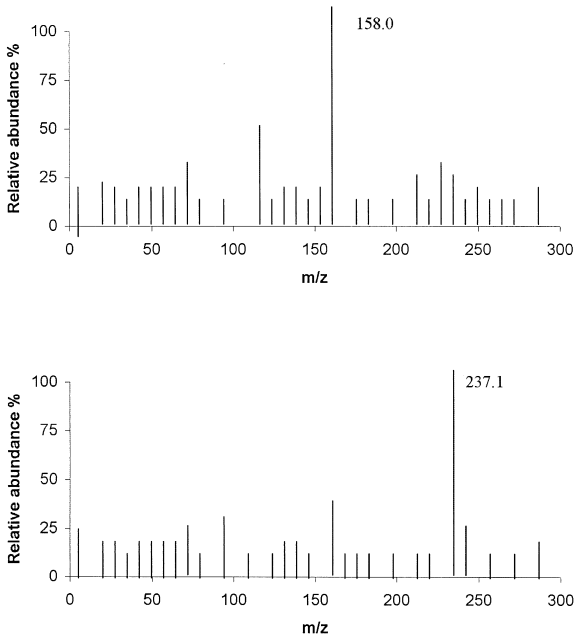


Fig. 3. Mass spectrums of 301029 and I.S.

Table 1
Calibration curve statistics for 301029 in mouse serum

Conc. (ng/ml)	Mean peak area ratio	STD	C.V. (%)
25	0.1991	0.0487	24
50	0.2899	0.0423	15
100	0.4465	0.0289	6
500	2.5443	0.4255	17
1000	5.2392	0.4403	8
2500	14.4352	1.5083	10

Table 2
Batch precision and accuracy for 301029

Theoretical conc. (ng/ml)	Calculated conc. (ng/ml)	RE (%)	C.V. (%)
Intra-batch precision (n=3)			
50	56.3±1.5	112	2
200	210.0±25	105	12
1000	932.0±32	94	3
Inter-batch precision (n=3)			
50	53.6±0.01	106	2
200	216.0±13	108	6
1000	964.0±21	96	2

Table 3
Recovery of 301029 from mouse serum

Conc. (ng/ml)	Mean peak area ratio		Recovery (B/A, %)
	Solvent samples (A)	Plasma samples (B)	
50	0.2724	0.2773	100
200	0.9286	0.9063	102
1000	4.6421	5.2870	113

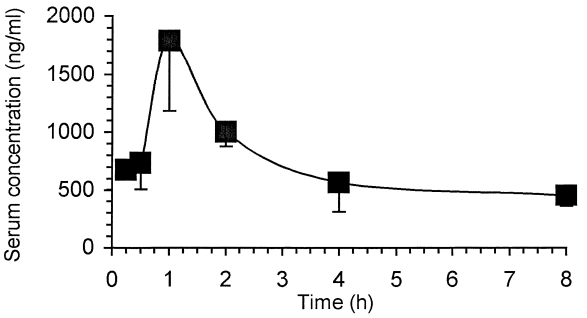


Fig. 4. Serum concentration–time profiles of 301029 after oral administration to BDF-1 mice (5 g/kg). Each point represents mean±SD of three mice.

centration–time course of 301029 system was fairly slow with half-life averaged 413±25 min. The total clearance was about 160±20 nl/min, which is close to the physiological clearance rate of mice. The maximum blood concentration was 1790±148 ng/ml. The area under the serum concentration–time curve (AUC) was estimated at 354±35 μg/ml/min for up to 8 h (Table 4).

The present pharmacokinetic studies revealed that 301029 declined at a moderate rate from the sys-

Table 4
Serum pharmacokinetic parameters (mean±SD) of 301029 after oral dosing to BDF1 mice

Dose (g/kg)	5
Half-life (min)	413±25
T_{max}(min)	60±3
C_{max} (μg/ml)	1.79±0.14
$AUC_{0\rightarrow 8\ h}$ (μg/ml/min)	354±35
Total body clearance (nl/min)	160±20
Mean residence time $_{0\rightarrow 7}$ (min)	189±35
V_z (ml)[a]	96±11

A non-compartmental input model was used to compute the PK parameters of 301029.

[a] V_z denotes volume of distribution based on the terminal phase.

temic circulation after oral dosing, and the drug's bioavailability may need to be improved by formulation strategies.

4. Conclusions

The developed LC–MS method proved to be useful and reliable for the determination of serum concentrations of 301029. The sample pre-treatment procedure, involving a direct deproteinization with acetonitrile, is simple and rapid, avoiding degradation of the drug. Assay performance was assessed both on the basis of the statistical characteristics of individual calibration lines and from the results of quality control samples. This method, validated for concentrations ranging from 25 to 2500 ng/ml, has a good reproducibility and accuracy and low limits of quantitation and detection. The method is useful for clinical therapeutic drug monitoring.

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