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Development and validation of a liquid chromatography–mass spectrometry (LC–MS) assay for the determination of the anti-cancer agent *N*-[2-(dimethylamino)ethyl]-2,6-dimethyl-1-oxo-1,2-dihydrobenzo[*b*]-1,6-naphthyridine-4-carboxamide (SN 28049)

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ABSTRACT

N-[2-(Dimethylamino)ethyl]-2,6-dimethyl-1-oxo-1,2-dihydrobenzo[*b*]-1,6-naphthyridine-4-carboxamide (SN 28049) is a potent topoisomerase II poison being developed to treat solid tumours. A reliable and sensitive LC–MS method has been developed and validated for the determination of SN 28049 in plasma using a structurally similar internal standard. This method had acceptable intra- and inter-assay accuracy (95–105%) and precision (R.S.D. < 6.5%) over the range 0.062–2.5 μM (using a 100 μl sample), and had a lower limit of quantitation of 0.062 μM. Both aqueous and plasma solutions of SN 28049 were stable during short-term (24 h at room temperature or 4 °C) and long-term storage (8 months at −80 °C), and following freezing and thawing (three cycles). The method was applied to study the pharmacokinetics of SN 28049 in mice after iv administration (8.9 mg/kg; $n = 3$ mice per time point). The maximum plasma concentration achieved was 1.22 ± 0.05 μM, and concentrations were measurable up to 12 h post-administration. A bi-exponential concentration-time curve was observed with an elimination half-life of 2.3 ± 0.2 h (mean ± S.E.), a volume of distribution of 34.5 ± 2.2 l/kg, and a plasma clearance of 12 ± 0.5 l/h/kg.

1. Introduction

Due to its inherent selectivity and sensitivity, liquid chromatography (LC) coupled with mass spectrometry (MS) has become the premier analytical tool for *in vitro* and *in vivo* absorption, distribution, metabolism and excretion (ADME) studies [1–3]. Most commonly liquid chromatography–mass spectrometry (LC–MS) has been applied to preclinical and clinical pharmacokinetic samples, in addition to the identification of drug metabolites [4–6].

N-[2-(Dimethylamino)ethyl]-2,6-dimethyl-1-oxo-1,2-dihydrobenzo[*b*]-1,6-naphthyridine-4-carboxamide (SN 28049; Fig. 1a) is a member of a series of benzonaphthyridine derivatives that was synthesized as part of a programme to develop new highly active DNA binding drugs [7]. SN 28049 targets the enzyme topoisomerase II [8] and induces complete regression of subcutaneous colon-38 murine tumours following administration of doses of 8.9 mg/kg and lower [7]. SN 28049 is both more dose potent (~50-fold *in vitro* and ~20-fold *in vivo*) and more active against this tumour when compared to previously described topoisomerase II poisons such as *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide(DACA) [9]. This high dose potency leads to challenges in the detection of sub-micromolar drug concentrations *in vivo*. Chromatographic methods utilising UV absorption, fluorescence and electrochemical detection were all investigated (data not shown) but found to have insufficient sensitivity for pharmacokinetic studies in mice. We report here the development of a sensitive LC–MS method for measurement of SN 28049 in plasma and its application to the pharmacokinetics in mice following iv administration (8.9 mg/kg).

2. Experimental

2.1. Materials

SN 28049 (Fig. 1a) (free base, 99% pure by LC; MW, 338) and the internal standard (IS) SN 28507 (Fig. 1b) (free base, 98% pure

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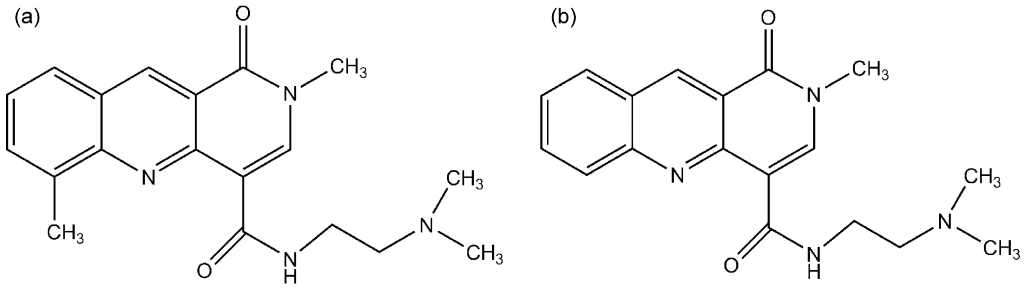

Fig. 1. (a) Chemical structure of SN 28049 and (b) SN 28507.

by LC; MW, 324) were synthesized in the Auckland Cancer Society Research Centre by Graham Atwell [7]. Unless otherwise stated, all other chemicals were commercially available and of analytical grade. Acetonitrile (MeCN) and formic acid were purchased from Merck, KGaA, Darmstadt, Germany. Ethylene disodium tetra acetic acid (EDTA) (anticoagulant) from Bio Chemed (Winchester, VA) was used for preparation of plasma. Water used in all experiments was purified by filtering through ion exchange columns and a 0.22-μ filter (Milli-Q purification system, Millipore Corporation, Bedford, MA, USA). Drug-free human plasma was obtained from the Regional Blood Transfusion Centre, Auckland Healthcare, NZ, and was centrifuged ($3000 \times g$ for 10 min) prior to use to remove any fibrin clumps.

2.1.1. Plasma collection

All animal procedures were approved by The University of Auckland Animal Ethics Committee. Mouse plasma was prepared from the blood of anaesthetised healthy (C57 Bl/6 strain) mice obtained from the Vernon Jansen Unit, The University of Auckland, NZ, and stored at −80 °C until analysis. To prevent coagulation, mouse blood was collected from the ocular sinus under isofluorane anaesthesia in 1.5 ml microfuge tubes containing 20 μl of 7.5% EDTA, and the plasma separated from red blood cells by centrifugation at $5000 \times g$ for 10 min.

2.2. Instrumentation

2.2.1. Liquid chromatograph–mass spectrometer

An Agilent® 1100 series capillary LC system was used with a capillary pump, an auto sampler, and an ion trap mass spectrometer (Agilent®, Avondale, PA). System control and data acquisition were carried out with the Agilent LC–MSD trap software ver. 5.3, incorporating the MSD Trap Control software from Bruker Daltonics (Fahrenheitstr, Bremen, Germany). An Agilent Zorbax C18 5 μm (150 mm × 0.5 mm) column pre-equilibrated for 30 min at 20 μl/min was used to achieve chromatographic separation of the analytes. Isocratic elution with 20% MeCN, 79.9% Milli-Q water, 0.1% formic acid and 5 mM ammonium formate was employed with the first 2 min of the run diverted to waste. The elution time was 9 min for SN 28049 and 4.5 min for the IS. A post-run equilibration of 3 min was used to remove any interference for the subsequent run. The MS2 fragments of SN 28049 $[M+H]^+$ (m/z 339$\hat{2}$94) and IS $[M+H]^+$ (m/z 325$\hat{2}$80) were selectively monitored using the ion trap mass spectrometer with the following conditions:

Fragmentation amplitude: 1.0 V; interface: electrospray; mode of ionization: positive; drying gas (nitrogen): 5 l/min; Nebulizer pressure: 10 psi; drying temperature: 250 °C; capillary voltage: 40.5 V; skimmer voltage: 45.66 V; capillary exit: 172.95 V; ion charge control target: 20,000; maximum accumulation time: 20 ms; scan range: 200–400 m/z; scan mode: standard + enhanced; scan speed: 13,000 (m/z)/s.

2.3. Preparation of standards and quality controls

Calibrants were prepared by adding known concentrations of working solutions prepared from 1 mM stock solution of SN 28049 (3.38 mg dissolved in 2 ml of MeCN and 8 ml of Milli-Q water) to freshly thawed particle-free human plasma, and mouse plasma. The IS was prepared as a 1 mM stock solution by weighing 3.24 mg of SN 28507 and dissolving in 2 ml of MeCN and 8 ml of Milli-Q water. The working IS solution (0.2 μM) was prepared by adding 20 μl of the 1 mM stock solution to 100 ml. Working solutions of SN 28049 were diluted in mobile phase (20% MeCN, 0.1% formic acid and 5 mM ammonium formate) and stored at 4 °C. The latter were stable over a period of 8 months. The plasma with known concentrations of SN 28049 (0.062–2.5 μM) were used to construct the calibration curve as follows: Duplicate 100 μl samples of the plasma calibrants were added to 12 mm × 75 mm glass tubes (Biolab Scientific, Auckland, NZ) followed by 100 μl of IS (0.2 μM). The plasma sample was then deproteinated by addition of 10 volumes of 3:1 ice cold MeCN:MeOH, and thoroughly mixed for 30 s using a vortex mixer (Heidolph Reaxtop, Schwabach, Germany). After centrifugation ($3000 \times g$ at 4 °C) for 15 min, the clear supernatants were transferred to 12 mm × 75 mm glass tubes and concentrated in a centrifugal vacuum concentrator. The concentrated extract was then reconstituted in 100 μl of mobile phase and injected (5 μl) in to the LC–MS for analysis. To construct the calibration graph, the peak area ratios of SN 28049 to IS were plotted against SN 28049 concentrations. The best-fit straight line was then obtained using linear regression analysis by SigmaPlot® (Systat Software Inc., San Jose, CA).

Freshly prepared working solutions of SN 28049 from an independently weighed 1 mM stock were used to prepare quality control (QC) samples. Appropriate volumes were then added to freshly thawed particle-free human plasma to give concentrations of 0.062, 0.5 and 1.0 μM (10 ml of each concentration). Aliquots (400 μl) of each QC samples were stored at −80 °C immediately after the preparation. During each subsequent analytical run, one set (triplicate) of each QC concentration was included (scattered in between the calibrants and the unknown samples) and processed with the calibrants and unknown samples.

2.4. Validation procedures

Analytical specificity was tested by inspection of chromatograms of extracted drug-free human and mouse plasma for interfering peaks. Absolute recoveries were assessed by comparing peak areas of SN 28049 and IS from extracted plasma QC samples, to standards prepared in the mobile phase. All recovery studies were performed at three different concentrations and in triplicate. To determine intra-day reproducibility, 5–6 replicates of the QC samples were analysed, including the lower limit of quantitation (LOQ). Inter-day precision was calculated from QC samples analysed on

three or more different days. At each concentration, precision was calculated as the relative standard deviation (R.S.D.) and accuracy as the percentage of the true value. Acceptable precision was defined by a R.S.D. within 15.0%, and accuracy within ±15.0% of the true value. The LOQ was defined to be the lowest concentration that could be measured with adequate accuracy (i.e., ±20.0% of the true value), and precision (R.S.D. within 20.0%). Matrix effects on each analyte were assessed in triplicate by spiking different batches of extracted plasma (mouse and human). Each analytical run consisted of a single calibration curve, triplicate QC samples at three concentrations, one reagent blank, one plasma blank and one zero-level standard. The stability of SN 28049 was measured in duplicate at room temperature over 24 h in plasma. At each timepoint, plasma samples containing SN 28049 were removed, and then extracted as described above. In addition, the stability of SN 28049 in plasma was assessed at three different concentrations in triplicate when left on ice or on the bench top for 0–24 h. Similarly, stability during storage in the autosampler was determined at three different concentrations in triplicate over 24 h at 4 °C. Three freeze–thaw cycles at −80 °C were used to test the stability SN 28049 in plasma. Long-term plasma stability was assessed at −80 °C over 8 months. For short-term, long-term and freeze–thaw stability, mean concentrations of triplicate samples were compared to the initial values.

2.5. Application to pharmacokinetic evaluation

An intravenous pharmacokinetic study using SN 28049 was undertaken in female C57 Bl/6 mice (20–25 g). SN 28049 (8.9 mg dissolved in 10 ml of phosphate-buffered saline) was administered at 8.9 mg/kg (26.3 μmol/kg) intravenously via the tail vein as a fast bolus injection (10 μl/g of mouse body weight). This dose was curative in mice with subcutaneously implanted colon-38 tumours. Blood samples were collected into EDTA tubes at 0.08, 0.5, 1, 2, 4, 8 and 12 h (three mice per timepoint) after drug administration. Plasma was separated and SN 28049 concentrations were measured in 100 μl samples as described above (see Section 2.3). Non-compartmental pharmacokinetic parameters were calculated using WINNONLIN®, Version 5.0.1. Values for area under the plasma concentration–time profile curve (AUC) were calculated using the log trapezoidal rule with extrapolation of the terminal slope to infinity by log-linear regression. The elimination half-life (T 1/2) was calculated by the equation $\ln 2/\lambda$ (λ is the terminal slope determined by log-linear regression). The terms Cmax and Tmax represent the maximum concentration achieved and the time to maximum concentration respectively. The model-independent pharmacokinetic parameters, clearance (CL), volume of distribution at steady state (Vss) were calculated by the following equations: CL = dose/AUC; Vss = (dose × AUMC)/(AUC)^2; where AUMC represents the total area under the first moment of the concentration–time curve, computed in a similar fashion to that used for AUC.

3. Results and discussion

3.1. Validation of the assay

3.1.1. Linearity

SN 28049 calibration curves over a range of 0.062–2.5 μM were constructed in human and mouse plasma. The calibration curves were fitted using a logarithmic plot to enhance performance over the maximal assay range. The calibration curves constructed from the human ($y = -1.06x - 2.52$) and mouse plasma ($y = -1.03x - 2.51$) were superimposable, indicating that these matrices are interchangeable, and thus justifying the use of human plasma for assay validation and production of quality controls (due to the limited availability of mouse plasma). Acceptable linear regressions were obtained for SN 28049 ($r^2 = 0.998$). The reproducibility of the calibration curves in human plasma was acceptable as shown in Table 1. Accuracy and precision data obtained from the calibration curves prepared on six different occasions over the concentration range (0.062–2.5 μM) were also acceptable (Table 2).

Table 1
Slope, intercept and correlation coefficient for calibration curves prepared in human plasma over 6 days.

Day	Slope	Intercept	r^2
1	1.02	−2.48	0.9965
2	1.12	−2.67	0.9947
3	1.06	−2.52	0.9971
4	1.00	−2.33	0.9975
5	1.03	−2.41	0.9980
6	1.05	−2.47	0.9977
Mean ± S.D.	1.05 ± 0.04	−2.48 ± 0.01	0.997 ± 0.001

Table 2
Precision (R.S.D., %) and accuracy data over the calibration range (0.062–2.5 μM) back-calculated from calibration curves prepared in human plasma.

Concentration (μM)	Mean back calculated concentration (μM)	R.S.D. (%)	Accuracy (%)
0.062	0.06 ± 0.003	3.4	95.7 ± 3.2
0.125	0.12 ± 0.005	5.0	99.9 ± 5.0
0.25	0.24 ± 0.03	2.9	101.0 ± 2.9
0.375	0.37 ± 0.04	4.8	100.1 ± 4.8
0.5	0.5 ± 0.004	4.4	104.1 ± 4.6
0.75	0.75 ± 0.03	5.7	103.2 ± 5.9
1.0	1.0 ± 0.03	4.5	101.0 ± 4.6
2.5	2.3 ± 0.05	5.2	92.0 ± 4.8

Results are expressed as mean ± S.D. ($n = 6$).

3.1.2. Specificity

The MS2 fragments of SN 28049 and IS were selectively identified through the LC–MS multiple reaction monitoring when spiked and extracted from plasma. The retention time of IS was 4.5 and 9 min for SN 28049. A representative total ion chromatogram showing the separation of SN 28049 and IS is shown in Fig. 2a. No interference was observed from the matrix (human or mouse plasma). A representative chromatogram (Fig. 2b) acquired from

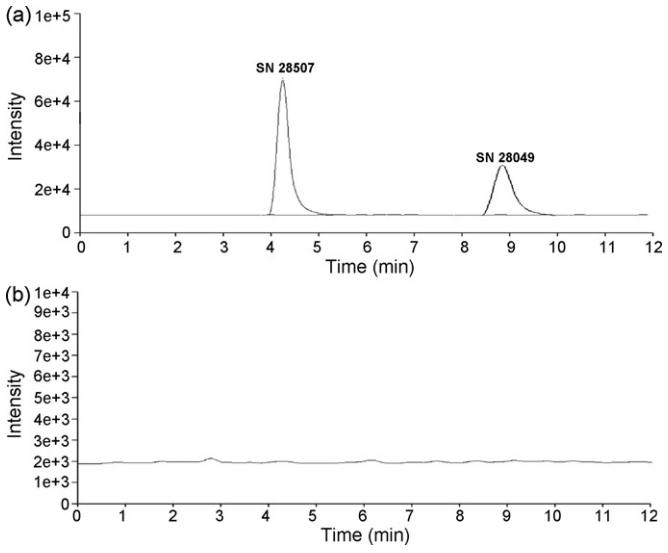

Fig. 2. (a) Total ion chromatogram of SN 28049 (0.062 μM) and IS (0.2 μM SN 28507) in human plasma and (b) chromatogram demonstrating no interference at the retention time for IS and SN 28049 from blank human plasma extract.

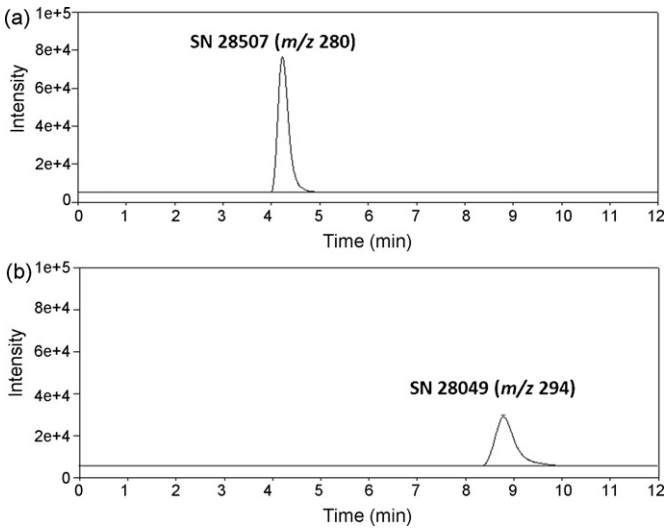

Fig. 3. (a) Extracted ion chromatogram of IS (0.2 μM SN 28507) and (b) SN 28049 (0.062 μM) in human plasma.

the blank human plasma extract demonstrates the absence of possible interfering peaks. An extracted ion chromatogram with the traces of IS and SN 28049 is shown in Fig. 3.

3.1.3. Fragmentation of SN 28049 and IS

SN 28049 ionizes in the positive ionization mode, gaining a proton and entering the metastable state of $[M+H]^+$ and can be seen in the spectrum as *m*/*z* 339. The latter can then be fragmented and characterized in MS2 mode to give an ion at *m*/*z* 294. Similarly the IS ionizes positively to *m*/*z* 325 which upon fragmentation (MS2 mode) yields *m*/*z* 280. Structure of these fragments were simulated and established with the help of ACD/MS fragmenter (ver. 9, ACD Labs, Toronto, Ontario, Canada). The fragmentation pattern and the mass spectra are shown in Fig. 4.

3.1.4. Recovery

The recovery was determined by comparing peak areas of authentic standards prepared in mobile phase to those extracted from spiked human plasma. Both authentic standards and spiked human plasma were prepared in triplicate in the same manner over three concentrations 0.062, 0.5 and 1.0 μM. The absolute recoveries of SN 24089 (0.062–1.0 μM) and the IS (0.2 μM) were found to be >85.0 ± 2.1% and >86.5 ± 3.5%, respectively.

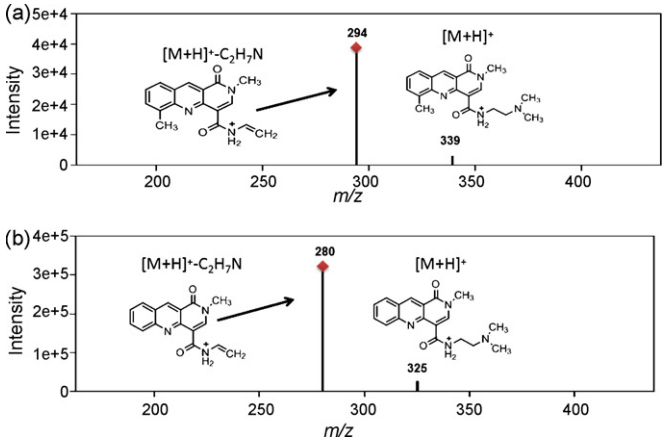

Fig. 4. (a) Mass spectra of the precursor, product ion and simulated structures of the fragments of SN 28049 (*m*/*z* 339→294) and (b) SN 28507 (IS) (*m*/*z* 325→280).

Table 3
Intra-assay precision (R.S.D., %) and accuracy at low, medium and high concentration in human plasma.

Actual concentration (μM)	Measured concentration (μM)	R.S.D. (%)	Accuracy (%)
0.062	0.05 ± 0.02	3.5	90.8 ± 5.3
0.5	0.49 ± 0.2	4.4	98.1 ± 3.2
1.0	1.02 ± 0.6	6.4	102.2 ± 5.0

Results are expressed as mean ± S.D. (*n* = 8).

3.1.5. LOQ

The LOQ in human and mouse plasma samples analysed by the LC–MS was 0.062 μM.

3.1.6. Precision and accuracy

The intra-assay precision was determined by calculating the R.S.D. of 8 repeat measurements for SN 28049 in human plasma on 1 day at three different concentrations (0.062, 0.5 and 1.0 μM), and was found to be less than 6.4% for all concentrations. The intra-assay accuracy was determined by comparing the means of the measured concentrations to their true concentrations on the same day. The intra-assay accuracy was also acceptable over the three concentrations, varying between 90.8 ± 5.3% and 102.2 ± 5.0% of the true values (Table 3).

The inter-assay precision was determined by replicate measurements performed on ten different occasions over an 8-month period for three concentrations in human plasma. The inter-assay precision was less than 5.2%. The inter-assay accuracy for SN 28049 was calculated over 10 different occasions and was between 94.1 ± 5.1% and 105.0 ± 5.0% as shown in Table 4.

3.1.7. Stability

3.1.7.1. Freeze–thaw stability. SN 28049 in human plasma was tested for stability by subjecting it to multiple freeze–thaw cycles over 48 h. Three concentrations of SN 28049 (0.062, 0.5 and 1.0 μM) were prepared in human plasma and frozen at −80 °C. These samples were then thawed and frozen three times at 24, 36 and 48 h after preparation. Samples were analysed in triplicate after the third freeze–thaw cycle and concentrations were determined from the calibration curve. The mean accuracies were 97.9 ± 2.8% (0.062 μM), 106.1 ± 0.3% (0.5 μM) and 99.5 ± 4.4% (1.0 μM) indicating acceptable stability in plasma over 3 freeze–thaw cycles.

3.1.7.2. Long term storage stability. SN 28049 was stable in human plasma over the 8-month validation period when stored at −80 °C. Plasma QC samples prepared during the intra-assay analysis were stored for 8 months and subsequently analysed. The results showed a precision <6.5% R.S.D. and an accuracy of 98.0 ± 5.1%.

3.1.7.3. Stock solution stability. Three concentrations of SN 28049 (0.062, 0.5 and 1.0 μM) were prepared in 20% MeCN from the stock solution (1 mM). The stability of these three concentrations was tested at room temperature and 4 °C. The solutions were found to be stable at room temperature (95.7 ± 0.5% to 101.5 ± 0.1% of the initial values) as well as at 4 °C (91.9 ± 0.5% to 94.9 ± 0.2% of the initial values) over a period of 24 h.

Table 4
Inter-assay precision (R.S.D., %) and accuracy at low, medium and high concentration in human plasma.

Actual concentration (μM)	Measured concentration (μM)	R.S.D. (%)	Accuracy (%)
0.062	0.058 ± 0.003	5.2	94.1 ± 5.1
0.5	0.5 ± 0.01	2.3	100.8 ± 2.8
1.0	1.05 ± 0.05	4.2	105.0 ± 5.0

Results are expressed as mean ± S.D. (*n* = 10).

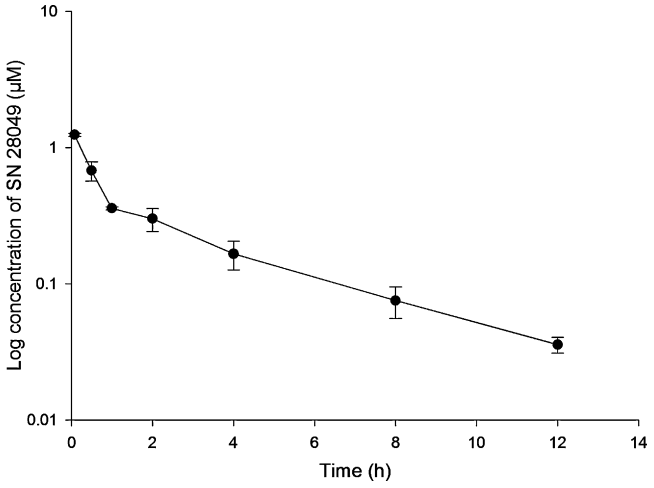

Fig. 5. Plasma concentration–time profile in C57 Bl/6 female mice after iv administration of SN 28049 (8.9 mg/kg). Each point represents the mean ± S.E. ($n = 3$).

3.1.7.4. Bench top stability. SN 28049 was tested for stability in human plasma over 24 h at room temperature. Three concentrations of SN 28049 (0.062, 0.5 and 1.0 μM) were prepared in human plasma and left at room temperature for 24 h. Triplicate aliquots of each concentration were taken at 0, 9 and 24 h and the concentration measured was found to be 93.4 ± 3.6% to 101.2 ± 2.8% of the initial values, indicating that SN 28049 was stable in plasma over 24 h at room temperature.

3.1.7.5. Post preparative stability. With an LC–MS analysis time of 12 min, processed plasma samples from pharmacokinetic experiments may be held in the refrigerated autosampler (4 °C) for at least 24 h for large batch analysis. Hence, the post-preparative stability of SN 28049 was determined by processing the three plasma quality control samples (0.062, 0.5 and 1.0 μM). Triplicate samples at each concentration were held in the autosampler and injected at 0, 16 and 24 h. The concentrations were determined from a standard curve in human plasma run at the same time and varied from 96.0 ± 0.5% to 101.0 ± 1.4% of the concentration at 0 h. Stability of IS over 24 h was calculated by comparing it with a plasma-extracted sample at 0 h and was 99.1 ± 1.4%.

3.1.8. Matrix effects

The matrix samples (human and mouse plasma) used to evaluate recovery were compared to SN 28049 at the same nominal concentrations in extraction solvent. This demonstrated a recovery of 86% (0.062 μM), indicating a lack of ion suppression by matrix components.

3.2. Application to pharmacokinetic evaluation

The plasma concentration–time profile following an iv bolus administration of 8.9 mg/kg SN 28049 to mice is shown in Fig. 5. The AUC was 2.2 ± 0.1 μM.h. The maximum concentration (Cmax) observed at the 5 min (Tmax) time point was 1.22 ± 0.05 μM, and concentrations were measureable up to 12 h. Non-compartmental analysis indicated a relatively high volume of distribution (Vss) (34.6 ± 2.2 l/kg), a moderate plasma clearance (CL) (12.0 ± 0.5 l/h/kg) with an elimination half-life (T 1/2) of 2.3 ± 0.2 h (mean ± S.E.).

4. Conclusion

A relatively rapid and sensitive LC–MS method has been developed which allows the measurement of SN 28049 concentrations in plasma as low as 0.062 μM with acceptable precision and accuracy. In addition it has been demonstrated that SN 28049 is stable in plasma at room temperature over 24 h, during three freeze-thaw cycles and over 8 months when stored at −80 °C. Using 100 μl plasma sample this method was able to measure SN 28049 for up to 12 h after the iv administration of a therapeutic dose, and is eminently suitable for further investigation of the pharmacokinetics of SN 28049 by other routes of administration, or in other small laboratory animals, or in clinical studies.

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