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LC–MS/MS assay and dog pharmacokinetics of the dimeric pyrrolobenzodiazepine SJG-136 (NSC 694501)

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Abstract

The dimeric pyrrolobenzodiazepine SJG-136 (NSC 694501) has potent in vitro cytotoxicity and in vivo antitumor activity. SJG-136 binds in the minor groove of DNA and produces G–G interstrand cross-links via reactive N^{10}–C^{11}/$N^{10'}$–$C^{11'}$ imine/carbinolamine moieties. We have developed a sensitive, specific liquid chromatography tandem mass spectrometry (LC/MS/MS) method for the quantitative determination of SJG-136 in plasma. SJG-136 was isolated by solid phase extraction through a C8 column, reverse-phase HPLC separation was accomplished on a C18 column with isocratic elution and MS/MS detection, monitoring the *m/z* 557–*m/z* 476 transition after electrospray ionization. The linear range and lower limit of quantitation from plasma standard curves were 2.8–1800 nM, and 5 nM, respectively. SJG-136 plasma protein binding was species-dependent. Values of the unbound fraction in human, rat and mouse were 25%, 16.2% and <1%, respectively. Protein binding was saturable in dog plasma where the unbound fraction increased from 10.8% to 22.3% over a 22–720 nM concentration range. SJG-136 pharmacokinetics after a single intravenous dose were best fit to a two-compartment open model with elimination half-life and plasma clearance values of 97 min and 6.1 mL/min/kg, respectively. SJG-136 did not accumulate in plasma following intravenous administration of 1.0 μg/kg doses for five consecutive days.

Keywords: LC/MS/MS; Pharmacokinetics; Anticancer agents; Benzodiazepine

1. Introduction

The dimeric pyrrolobenzodiazepine SJG-136 (8,8′-[[(propane-1,3-diyl)dioxy]-bis[(11aS)-7-methoxy-2-methylidene-1,2,3,11a-tetrahydro-5H-pyrrolo[2,1-c][1,4]benzodiazepin-5-one], NSC 694501, Fig. 1) has potent in vitro cytotoxicity (mean GI_{50} = 7.4 nM) and in vivo antitumor activity [1,2]. This activity is associated with binding in the minor groove of DNA and formation of covalent G–G interstrand cross-links via reaction with its N^{10}–C^{11} and $N^{10'}$–$C^{11'}$ imine moieties (Fig. 1) [3]. Consequently, preclinical investigations of activity and toxicity have been performed with extremely low doses of drug, as will subsequent Phase I clinical trials for cancer patients.

Characterization of SJG-136 pharmacokinetics and metabolism requires a sensitive, specific assay to determine plasma drug concentrations likely to be in the low nanomolar range. Previous investigations of drugs with high potency, such as the cyclopropylisoindole analogs adozelesin [4] and bizelesin [5,6] and marine natural products ecteinascidin 729 [7] and dolostatin 10 [8], utilized non-specific bioassays to achieve the necessary sensitivity. Availability of LC/MS/MS instrumentation offers the capability to quantitate potent drugs with high specificity and sensitivity. We herein describe an LC/MS/MS assay for SJG-136 that achieves high sensitivity, and illustrate the utility of that assay with plasma protein binding and with plasma drug concentration data in dogs administered intravenous injections of SJG-136.

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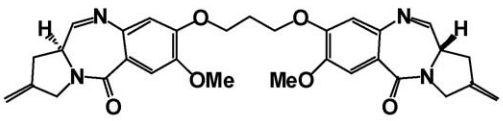

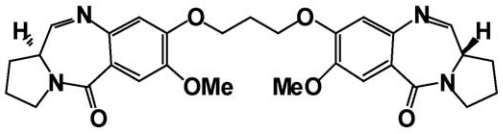

Fig. 1. Structures of SJG-136 and the internal standard, DSB-120.

2. Experimental

2.1. Reagents and materials

SJG-136 and DSB-120 were provided by the National Cancer Institute, Pharmaceutical Resources Branch, Division of Cancer Treatment (Bethesda, MD, USA). HPLC-grade acetonitrile, methanol, and water were purchased from EM Science (Gibbstown, NJ, USA). Formic acid (minimum 95%), citrate-phosphate dextrose solution, human serum albumin fatty acid free and gamma-globulin free from fraction V (96–99%), human α_1-acid glycoprotein purified from Cohn fraction VI (99%), and potassium phosphate monobasic anhydrous, minimum 99.0%, *N*,*N*-dimethyl-acetamide 99+%, were purchased from Sigma (St. Louis, MO, USA). Heparin sodium 1000 units/mL was purchased from American Pharmaceutical Partners Inc. (Los Angeles, CA, USA). Potassium hydroxide ACS was purchased from Curtin Matheson Scientific Inc. (Houston, Texas, USA). 0.9% sodium chloride was purchased from Baxter Healthcare Corporation (Deerfield, IL, USA). Deionized and distilled water was used to prepare buffer solution.

Drug-free human plasma was obtained from healthy volunteers and frozen at −20 °C. Rat and mouse whole blood was collected using 10% heparin in citrate–phosphate dextrose solution as an anticoagulant. The plasma was separated by centrifugation (8000 × *g*, 3 min, 4 °C) and stored at −20 °C for later analysis.

2.2. LC/MS/MS instrumentation

The LC/MS/MS system consisted of a Shimadzu liquid chromatograph (Wood Dale, IL, USA) with two LC-10ADvp pumps (flow rate 0.200 mL/min), and a SIL-10ADvp autoinjector (injection volume 20 μL) coupled to a triple quadrupole Quattro Micro mass spectrometer (Waters Corporation, Milford, MA) fitted with a switching valve and an electrospray ionization probe operating in the positive mode. SJG-136 detection was accomplished by MS/MS using the parent ion *m*/*z* of 557.0 and the daughter ion *m*/*z* of 476.0. The dwell time, cone voltage, and collision energy values were 0.2 s, 40 V, 30 eV, respectively. The internal standard (DSB-120) was detected by MS/MS using parent ion signal of *m*/*z* 533.20 and daughter ion signal of *m*/*z* 247.0. The dwell time, cone voltage and collision energy values were 0.2 s, 40 V, and 45 eV, respectively. The source temperature, desolvation temperature, cone gas flow and desolvation gas flow were 100 °C, 120 °C, 100 L/h and 250 L/h, respectively. LC/MS/MS data were collected for 5 min after sample injection.

2.3. Chromatographic conditions

Separation of SJG-136 and DSB-120 was achieved using a Haipeek Clipeus C18 precolumn (20 mm × 2.1 mm i.d., 5 μm) (Chrom Tech, Apple Valley, MN, USA) and a Genesis Lightning C18 analytical column (10 cm × 2.1 mm, 120 Å, 4 μm) (Jones Chromatography, Lakewood, CO, USA). The elution program is shown in Table 1.

2.4. Sample preparation

Stock solutions (1.0 mg/mL) of SJG-136 and DSB-120 were prepared in acetonitrile (SJG-136) and absolute ethanol (DSB-120) and stored at −20 °C in silanized amber glass vials. Working standard solutions were prepared daily by adding aliquots of the 1.0 mg/mL stock solutions to ice-cold acetonitrile. Plasma standards containing SJG-136 (2.8–1800 nM) and DSB-120 (89.9 nM) were prepared by adding aliquots of the working standard solutions to plasma (200 μL) measured into 12 mm × 75 mm glass tubes. SJG-136 and DSB-120 were isolated from plasma using solid phase extraction (SPE) through BondElute C8 (100 mg, 1 mL) cartridges (Varian, Harbor City, CA, USA). The SPE columns were pre-rinsed with MeOH (2 × 1 mL) and 50 mM KH_2PO_4, pH 6.0 (2 × 1 mL); loaded with plasma samples diluted with an equivalent volume of 50 mM KH_2PO_4, pH 6.0 (200 μL); rinsed with 50 mM KH_2PO_4, pH 6.0 (1 × 1 mL) and water (2 × 1 mL); dried for 5 min, and eluted with MeOH (2 × 1 mL). Extracts were concentrated to dryness using a slow stream of nitrogen and reconstituted with 50:50 MeOH–H_2O (100 μL).

2.5. Data analysis and standard curves

Mass spectra and chromatograms of SJG-136 and DSB-120 were processed using the MassLynx v4.0 software with Quan-

Table 1
Column elution program

Time (min)	Conditions
0–4	40:60 (v/v) acetonitrile–water containing 0.1% formic acid. Flow switched to waste 0–1 min after injection
4–7	100% acetonitrile containing 0.1% formic acid
7–10	40:60 (v/v) acetonitrile–water containing 0.1% formic acid

Lynx. Peak areas were determined using a multiple reaction monitoring (MRM) scan for the parent ion to a specific product ion transition for SGJ-136 and DSB-120. Standard curves were prepared by plotting peak area ratios of SJG-136/DSB-120 against SJG-136 concentration over the range of 2.8–1800 nM. Standard curves were analyzed by linear least-squares regression analysis weighted by the inverse of the SJG-136 concentration.

2.6. Assay validation

The lower limit of detection (LOD) was defined as the lowest concentration of SJG-136 that yielded a signal-to-noise ratio of three. The limit of quantitation (LOQ) was defined as the lowest concentration that could be measured with percent relative standard deviation (%RSD) and percent relative error (%RE) of <20% as determined from spiked plasma samples.

Recoveries of SJG-136 and DSB-120 from plasma were evaluated by comparing the SJG-136 and DSB-120 peak areas from spiked human plasma standards (2.8–180 nM) in duplicate to peak areas from standards (2.8–180 nM) prepared in 50:50 MeOH–H_2O. Contributions of ionization suppression to recovery of SJG-136 and DSB-120 were evaluated by monitoring ion intensity of the *m/z* 557.1 → 476.2 transition and the 533.1 → 247.3 transition, respectively, following injection of mobile phase or blank plasma extract onto the chromatographic system set up for post-column continuous infusion of 1 μg/mL SJG-136 or DSB-120 at a flow rate of 5 μL/min.

Precision and accuracy were determined by repeated analysis of 5, 75 and 600 nM QC samples ($n = 3$ per concentration) on three separate days. Precision was measured as the relative standard deviation for the three measurements. Accuracy was calculated as the mean difference between the measured and labeled concentration where the difference was calculated using the following equation, difference = |(measured concentration − labeled concentration)/labeled concentration × 100%|.

2.7. Stability

Short-term stability of SJG-136 in plasma was determined over 48 h at 4 °C and 37 °C. SJG-136 concentration was 89.93 nM in plasma and 250 μL sample aliquots were transferred to 1.7 mL microcentrifuge tubes at selected time points and stored at −20 °C for later analysis.

Long-term stability of SJG-136 in plasma was determined at −20 °C over 4 weeks. Plasma samples were prepared at two concentrations (5.62 nM and 89.93 nM) of SJG-136. The samples were then aliquoted to 1.7 mL silanized microcentrifuge tubes and transferred to a −20 °C freezer. Samples were thawed and analyzed in duplicates weekly for 4 weeks.

2.8. Protein binding

SJG-136 binding to proteins was measured in thawed dog, human, mouse and rat plasma, human serum albumin (40 mg/mL) dissolved in phosphate buffered saline, pH 7.4 and α_1-acid glycoprotein (1 mg/mL). After 30-min incubation at 37 °C, samples were added to the reservoir of a YM-30 Amicon Centrifree micropartition device (Millipore, Bedford, MA). Ultrafiltration was accomplished by centrifugation (1500 × *g*) in a fixed angle rotor for 30 min at 4 °C after a 30-min incubation period at room temperature. Drug concentrations were measured in samples before (sample reservoir) and after (filtrate cup) centrifugation and the percentage of drug recovered and protein binding were calculated by the equations:

$$\text{Protein recovered} = \left[1 - \frac{\text{filtrate cup concentration}}{\text{sample reservoir concentration}}\right] \times 100\%$$

$$\text{Protein binding} = \left[1 - \frac{\text{percentage recovered(plasma)}}{\text{percentage recovered(ultrafiltrate)}}\right] \times 100\%$$

2.9. Pharmacokinetics

Beagle dogs were given SJG-136 dissolved in 5% *N*,*N*-dimethyl-acetamide in 0.9% saline via intravenous injection. Blood (2.0 mL) was collected from the jugular vein, transferred to tubes containing heparin and placed on wet ice until centrifugation. Plasma was isolated by centrifugation and stored at −70 °C in cryogenic vials for later analysis.

3. Results and discussion

3.1. Chromatographic conditions

Positive electrospray ionization of SJG-136 and DSB-120 produced abundant protonated molecular ions $[M + H]^+$ at *m/z* 557 and 533, respectively. Product ion spectra for SJG-136 (Fig. 2A) and DSB-120 (Fig. 2B) included prominent product ion peaks at *m/z* 476 and 247, respectively. Based on these data, quantification of SJG-136 and DSB-120 was performed in the multiple reaction monitoring (MRM) mode by monitoring the *m/z* 557 → 476 transition for SJG-136 and the *m/z* 533 → 247 transition for DSB-120. The highest signal intensities for SJG-136 were achieved with cone voltage and collision energy set at 40 V and 30 keV, respectively. The highest signal intensity for DSB-120 was achieved with cone voltage and collision energy set at 40 V and 45 keV, respectively.

Reverse phase chromatography using a Genesis Lightning C18 analytical column and a mobile phase composed of 40/60 water/acetonitrile containing 0.1% formic acid permitted rapid separation of SJG-136 and DSB-120 (Fig. 2C). The retention times of DSB-120 and SJG-136 were 2.3 min and 2.7 min, respectively. The lower limit of detection (S/N > 3) was 2.8 nM.

3.2. Sample preparation

When compared with liquid extraction and protein precipitation, solid phase extraction yielded the highest recovery and

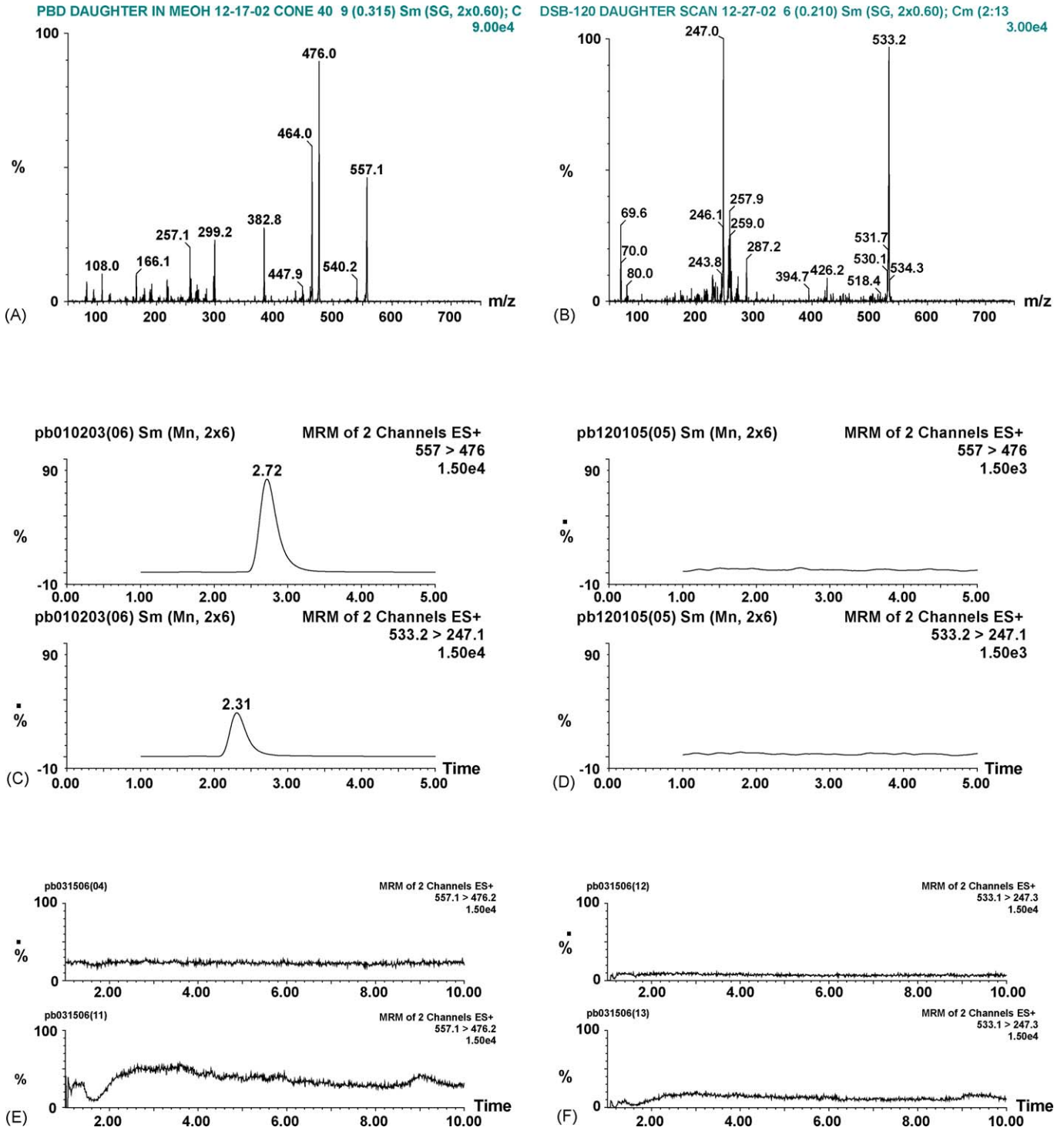

Fig. 2. LC/MS/MS analysis of SJG-136: (A) fragmentation pattern produced by positive ion electrospray ionization of SJG-136; (B) fragmentation pattern produced by positive ion electrospray ionization of DSB-120; (C) LC/MS analysis of SDJ-136 (180 nM) and DSB-120 in plasma extracts. Upper panel: ion chromatogram for the *m/z* 557 → *m/z* 476 transition for SJG-136. Lower Panel: ion chromatogram for the 533 → *m/z* 247 transition for DSB-120; (D) LC/MS analysis of a plasma extract without added SJG-136 or DSB-120. Upper panel: ion chromatogram for the *m/z* 557 → *m/z* 476 transition for SJG-136. Lower Panel: ion chromatogram for the 533 → *m/z* 247 transition for DSB-120; (E) ion intensity for the *m/z* 557 → *m/z* 476 transition for SJG-136 following post-column continuous infusion of SJG-136. Upper panel: injection of mobile phase. Lower panel: injection of human plasma extract; and (F) ion intensity for the 533 → *m/z* 247 transition for DSB-120 following post-column continuous infusion of DSB-120. Upper panel: injection of mobile phase. Lower panel: injection of human plasma extract.

best reproducibility of the three isolation methods for SJG-136. Mean recoveries of SJG-136 and DSB-120 from plasma standard curve samples following solid phase extraction were 83% and 87%, respectively. Injection of plasma extract caused 20% reduction in the SJG-136 ion intensity 1.6 min after injection that returned to baseline 2.2 min after the injection (Fig. 2E). Similarly, the ion intensity produced by continuous flow of DSB-120 was reduced by 5% following injection of plasma extract.

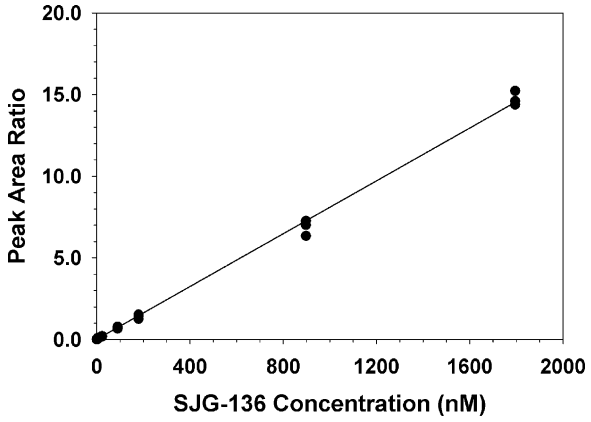

Fig. 3. Standard curve prepared with 1.4–1800 nM SJG-136 with DSB-120 as the internal standard.

The maximum effect was detected 1.6 min after injection that returned to baseline 2.2 min after the injection (Fig. 2F). These data are consistent with a minimal effect of ionization on the net recovery of SJG-136 and DSB-120 from plasma following solid phase extraction.

3.3. Assay linearity and limits of quantitation

Standard curves were linear range in the range 2.8–1800 nM with r^2 values of >0.9900 for standard curves (Fig. 3). The lower limit of quantitation was 5 nM. Under the chromatographic and MRM conditions selected for separation and detection of SJG-136, analysis of blank mouse rat dog and human plasma samples did not show any interferences at the retention times of DSB-120 and SJG-136 as illustrated for human plasma in Fig. 2D.

3.4. Precision and accuracy

Within-day and between-day reproducibility of the SJG-136 LC/MS/MS assay was excellent. Precision and accuracy measurements for the 5 nM QC samples were less than 15%, while those same measurements for the 75 nM and 600 nM QC samples were less than 10% (Table 2).

3.5. Stability

Stability of SJG-136 in rat, mouse, human, and dog plasma (Fig. 4) was evaluated at 4 °C and 37 °C for a 48-h period. SJG-136 was stable in rat and mouse plasma during the 48-h incubation at 4 °C and 37 °C. The SJG-136 concentration in human plasma fell to 75% and 50% of the initial concentration after 48 h incubation at 4 °C and 37 °C, respectively. SJG-136 was stable in dog plasma at during incubation at 4 °C, but concentration values fell to 70% of the initial value during incubation at 37 °C. The SJG-136 half-life estimates of 43 h and 109 h, respectively, during incubation in human and dog plasma at 37 °C indicate that less than 5% degradation should occur during sample work-up. SJG-136 was stable in human plasma stored at −20 °C for 4 weeks (Fig. 5).

3.6. Protein binding

SJG-136 plasma protein binding was evaluated in human, dog, rat and mouse plasma, as well as in aqueous solutions containing human serum albumin and α_1-acid glycoprotein (Table 3). Protein binding in human plasma was high (75%) and independent of drug concentration in the 22.5–719 nM range. The percent bound value of 78% in human serum albumin and absence of binding to α_1-acid

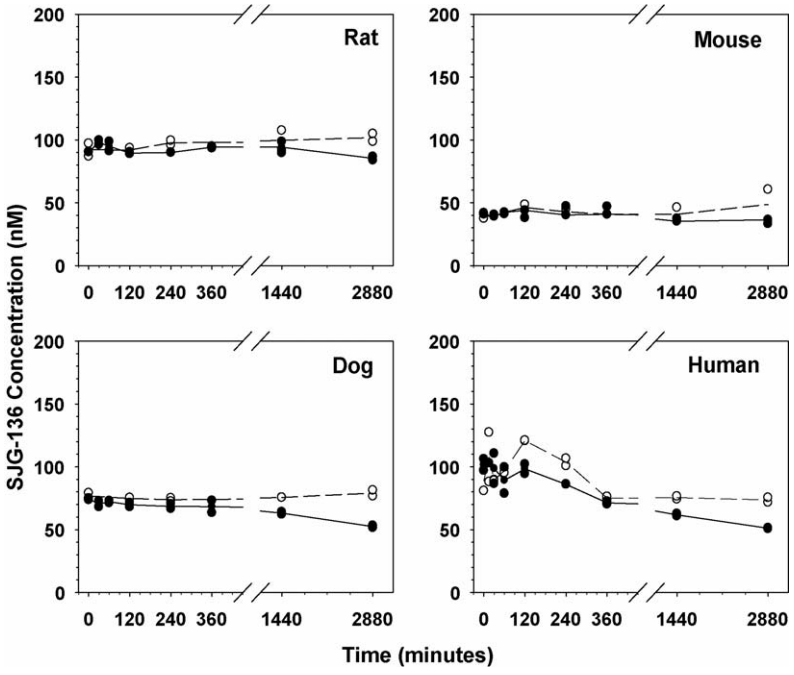

Fig. 4. SJG-136 stability in rat, mouse, human, and dog plasma during incubation at 4 °C (closed circles) and at 37 °C (open circles) for a 48-h period.

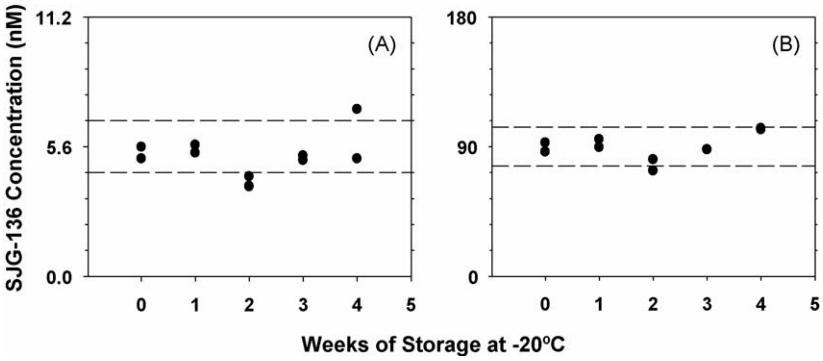

Fig. 5. Long-term storage of SJG-136 in Plasma. Specimens were stored at −20 °C. Aliquots were analyzed weekly. Dashed lines represent quality control limits of ±20% for the 5.6 nM concentration (A) and ±15% for the 89.9 nM concentration (B).

glycoprotein suggests that SJG-136 principally bound to albumin in plasma. Protein binding in dog plasma was very high. Saturation was observed over the concentration range, with a small decrease in binding when the drug concentration was increased from 22.5 nM to 719 nM. Protein binding values in rat and mouse plasma were 94% and 100%, respectively. Saturation of protein binding was not observed for rat and mouse plasma.

3.7. Single-dose pharmacokinetics in Beagle dogs

SJG-136 plasma profiles for Beagle dogs administered a single 10 or 20 μg/kg dose are illustrated in Fig. 6. SJG-136 was detected in dog plasma samples for at least 150 min after administration of 10 μg/kg SJG-136 and for at least 210 min after administration of 20 μg/kg SJG-136. After achieving peak (2-min) plasma concentrations of 220 nM and 580 nM, respectively, SJG-136 plasma concentrations declined in a biphasic manner (Fig. 6) with a terminal elimination half-life of 97 min (range, 41–156 min) and a mean plasma clearance value of 6.1 mL/min/kg (Table 4).

Table 2
SJG-136 precision and accuracy data for LC/MS/MS assay

	Nominal concentration (nM)		
	5	75	600
Within-day ($n = 3$)			
Measured concentration (mean ± SD, nM)	5.46 ± 0.48	76.2 ± 4.3	553 ± 7
Precision (%)	8.8	5.6	1.3
Accuracy (%)	10.3	3.7	7.9
Between-day ($n = 3$)			
Measured concentration (mean ± SD, nM)	5.01 ± 0.67	77.7 ± 6.6	592 ± 42
Precision (%)	13.3	8.5	7.0
Accuracy (%)	10.2	6.1	6.6

Table 3
SJG-136 protein binding (percent bound)

Treatment	Concentration (nM)		
	22.48	89.93	719
Human plasma	71.8	76.5	77.6
Dog plasma	89.2	82.2	77.7
Mouse plasma	100.0	100.0	98.1
Rat plasma	94.4	93.4	93.5
Human serum albumin	88.3	70.2	75.2
Human α_1-acid glycoprotein	0	0	0

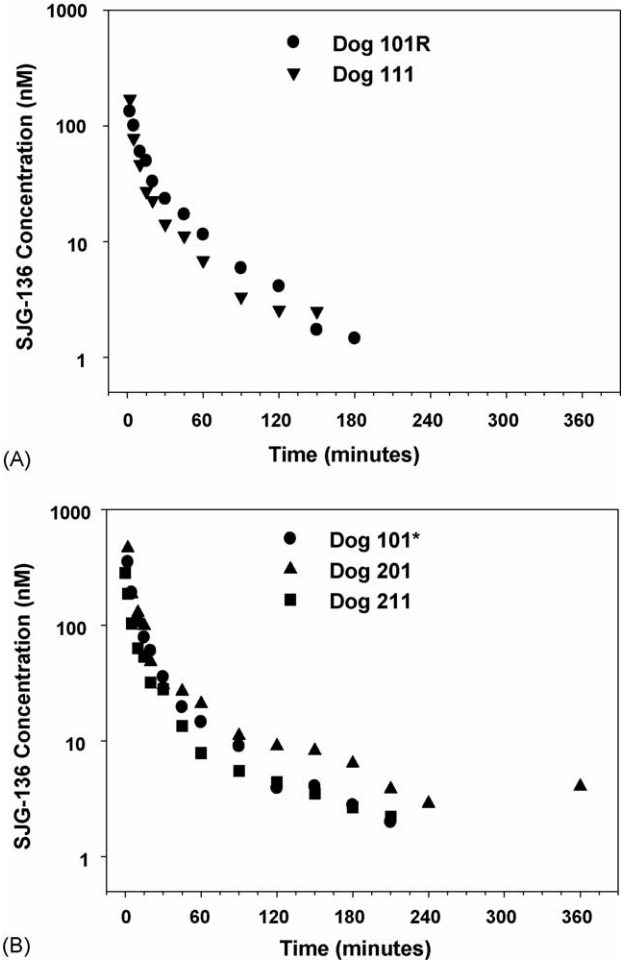

Fig. 6. Graphs of SJG-136 plasma concentration vs. time following i.v. bolus administration of 10 μg/kg (A) or 20 μg/kg (B) drug to dogs.

Table 4
Pharmacokinetics of SJG-136 (PBD) in Beagle dogs

Dose (μg/kg)	Dog	C_{max} (nM)	Half-life (min)	AUC (nM min)	Cl (mL/min/kg)	V_{ss} (mL/kg)
10	111	287	142.8	2790	6.43	551
10	101R	160	41.1	2960	6.08	233
20	201	849	155.6	8020	4.47	509
20	211	370	83.3	5200	6.91	362
20	101*	526	61.9	5420	6.62	249

3.8. Multiple-dose pharmacokinetics in Beagle dogs

During a 5-day schedule of intravenous injection of SJG-136, blood samples were drawn 10 min after drug administration on days 1 and 5. SJG-136 was detected in plasma after administration of the 0.2 μg/kg dose, but concentrations were below the assay detection limit. Mean ± SD SJG-136 plasma concentrations after administration of the 1.0 μg/kg dose were 5.11 ± 0.68 nM and 5.35 ± 1.14 nM on days 1 and 5, respectively.

4. Conclusions

A sensitive, specific LC–MS/MS assay was developed for determination of the potent dimeric pyrrolobenzodiazepine SJG-136 in plasma. The assay was linear in the range of drug concentrations expected during preclinical pharmacology studies in dogs, mice and rats. SJG-136 was stable in plasma stored frozen and when kept on ice during the work-up procedure. We found species-dependent protein binding in rodents, and saturable protein binding in dogs. SJG-136 was quantified in plasma from dogs administered as little as 1.0 μg/kg intravenously. SJG-136 pharmacokinetics (Beagle dogs) were best described by a two-compartment open model, and the parent drug did not accumulate when administered for five consecutive days. These data may be important if species differences in SJG-136 pharmacokinetics are observed between animals and humans.

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