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Electrospray LC–MS/MS quantitation, stability, and preliminary pharmacokinetics of bradykinin antagonist polypeptide B201 (NSC 710295) in the mouse

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Abstract

B201 (NSC 710295), [SUIM-(Darg-Arg-Pro-Hyp-Gly-Igl-Ser-Digl-Oic-Arg)$_2$], a third generation of bradykinin (BK) antagonist, has been found to possess high potency. We report the development of a highly sensitive electrospray LC–MS/MS assay method for the analysis of B201 in plasma for the first time, using an ion-trap mass spectrometer. Human or mouse plasma (0.2 ml) was spiked with B201 and the internal standard, substance P. The compounds were extracted with a preconditioned C-18 reversed-phase column and analyzed by LC–MS/MS. The analytes were separated on a 50 × 2 mm (i.d.) BetaBasic C8 column, using a gradient elution. The positive ion selected reaction monitor mode was used monitoring the transitions of ions at m/z $938.9^{3+} \rightarrow 816.0^{2+}$ for B201 and $674.3^{2+} \rightarrow 665.7^{2+}$ for substance P. Assay validation was performed, and the limit of quantitation (LOQ) for B201 was found to be 1 ng/ml for human plasma and 2.5 ng/ml for mouse plasma. The recovery was 78% for B201 and 88% for substance P. The assay was linear from 2.5 to 1500 ng/ml for mouse plasma monitored. Using a 0.2 ml plasma, the within-day CVs were 9.3% at 2.5 ng/ml, 6.5% at 100 ng/ml, and 3.8% at 1000 ng/ml for human plasma ($n = 6$). For mouse plasma, the respective within-day CVs were 17.6, 9.6, and 6.2% ($n = 6$). The between-day CVs for human plasma were 8.2, 10.9, and 2.4%, respectively, ($n = 3$) and the respective values for mouse plasma were 11.9, 8.6 and 6.5% ($n = 6$). Pharmacokinetics of B201 in the mouse was studied following i.v. administration at 5 mg/kg and found to conform to a two-compartment model with an initial half-life of 14 min and a terminal half-life of 44 h. Plasma B201 peak level was detected at μg/ml range and the levels were detectable for a least 24 h. Preliminary oral bioavailability was found to be about 1%. This method demonstrates that an ion trap mass spectrometer can be a powerful tool to quantify large peptides at low nanogram per mililiter with a non-isotopically labeled internal standard.

Keywords: LC–MS/MS assay; Ion-trap mass spectrometry; B201 polypeptide; Bradykinin antagonist

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1. Introduction

B201 (NSC 710295), a synthetic polypeptide dimer [SUIM-(Darg-Arg-Pro-Hyp-Gly-Igl-Ser-Digl-Oic-Arg)$_2$], is a third generation of bradykinin (BK) antagonist that acts as a biased agonist of BK receptor. There are considerable ongoing efforts to develop agents which inhibit growth by interfering with ligand-receptor interactions. A number of first generation of BK antagonists were developed in 1980s [1,2]. Potency and stability issues prompted the development of the second [3,4] and third [5,6] generations. Among the third generation compounds, B201 analogs have been found to possess high potency and oral activity. Preclinical pharmacology and pharmacokinetics of B201 have not been investigated, because of a lack of analytical methodology. We report the development of a highly sensitive electrospray LC–MS/MS assay method for the analysis of B201 in plasma and its pharmacokinetics in the mouse.

Recently, there has been an increasing interest to quantify large peptides having a molecular weight higher than 1500 amu using ESI/LC–MS [7–14] and ESI/LC–MS/MS [8]. Triple quadrupole mass [7–9,14] spectrometers are usually used for the analysis. More recently, ion trap mass spectrometers [11,13] have also been used for this purpose. Comparisons between the ion trap mass spectrometer and triple quadrupole mass spectrometer have been made [15–18], and an isotope-labeled [8] or structurally similar analog [7,8,10–14] as the internal standard is generally needed for the method with an ion trap instrument. The suitability of an ion trap mass spectrometer for quantitation of organic compounds is still under debate [19–23]. In this study, we report the use of an ion trap mass spectrometer for the quantitation of a large peptide, B201, using a structurally-different polypeptide, Substance P, as the internal standard.

2. Experimental

2.1. Materials

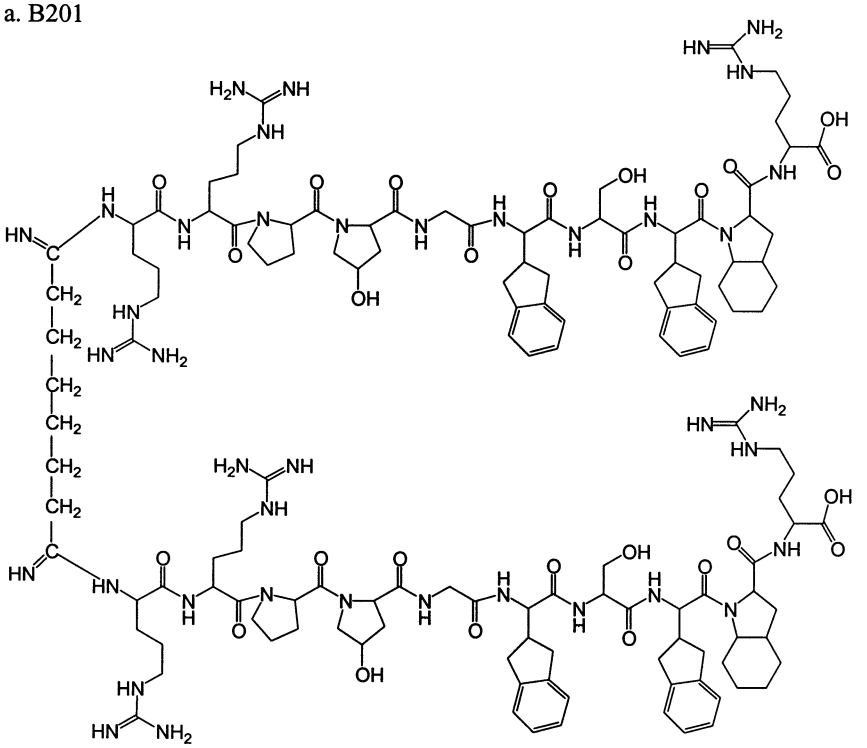
BK antagonist B201, SUIM-(Darg-Arg-Pro-Hyp-Gly-Igl-Ser-Digl-Oic-Arg)$_2$ (purity > 92% by HPLC), was provided by the National Cancer Institute (Bethesda, MD) and used without further purification. The internal standard, substance P, Arg-Pro-Lys-Pro-Glu-Glu-Phe-Phe-Gly-Leu-Met-NH_2 (purity > 98%) was obtained from Sigma (St. Louis, MO) and used without further purification. Structures of B201 and substance P are shown in Fig. 1. All organic solvents, except for trifluoroacetic acid (TFA), which was from J.T. Baker, were obtained from Fisher Scientific (Pittsburgh, PA) and were of HPLC grade. The HPLC-grade water (> 18 mΩ) was obtained from an E-pure water purification system (Barnstead, Dubuque, IA).

2.2. LC–MS/MS assay

The LC–MS system used consisted of a Finnigan LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA), coupled to a Shimadzu HPLC system (Shimadzu, Columbia, MD). The HPLC system was equipped with two LC-10ADvp Pumps (Shimadzu, Columbia, MD) and a SIL-10ADvp autoinjector (Shimadzu, Columbia, MD). The temperature of the autoinjector was set at 5 °C during operation. B201 and substance P were separated on a BetaBasic 5-μm C8 (octyl silane) 50×2 mm (i.d.) stainless steel column (Keystone Scientific, Bellefonte, PA), which was coupled to a pre-column filter (0.5-μm frit fits a classic column filter) and a 5-μm C8 10×2 mm (i.d.) guard column (drop-in guard cartridge fits a uniguard holder) (Keystone Scientific). The components were eluted with a gradient mode at a flow rate of 0.20 ml/min. Mobile phase, $A = 0.05\%$ TFA in H_2O (v/v); $B = 0.05\%$ TFA in CH_3CN (ACN) (v/v). Each gradient elution run took 33 min (0–15 min 10% B, 15–20 min 60% B, 20–23 min 10% B, which was being maintained to 33 min). The LC elute was introduced into electrospray ion source without splitting.

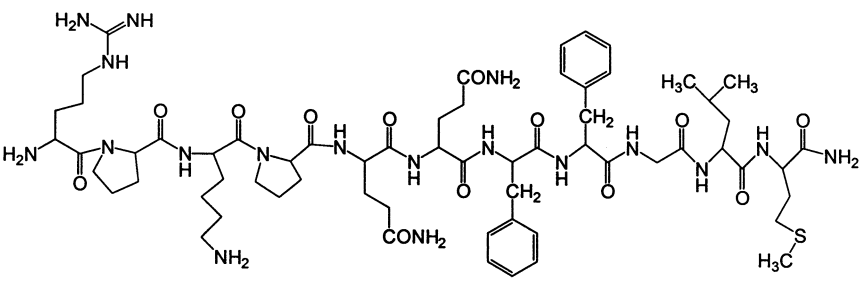
The mass spectrometer was operating with a background helium pressure of 1.75×10^{-3} torr, a typical electrospray needle voltage of 6 kV, a sheath nitrogen gas flow of 80 (arbitrary unit), and an auxiliary nitrogen gas flow of 20 (arbitrary unit) (ratio 4:1), and a heated capillary tempera-

a. B201



HN—D-Arg—Arg—Pro—Hyp—Gly—Igl—Ser—D-Igl—Oic—Arg
(CH2)6
HN—D-Arg—Arg—Pro—Hyp—Gly—Igl—Ser—D-Igl—Oic—Arg

b. Substance P



Arg—Pro—Lys—Pro—Glu—Glu—Phe—Phe—Gly—Leu—Met—NH2

Fig. 1. Structures of B201 (a) and substance P (b).

ture of 230 °C. Selected reaction monitoring (SRM) mode was chosen for monitoring daughter ions. The precursor ions were isolated and activated for 30 ms to produce daughter ions with relative collisional energies of 21.5% for B201 and 21.0% for substance P. The transitions of ions were at m/z $938.9^{3+} \rightarrow 816.0^{2+}$ for B201 and $674.3^{2+} \rightarrow 665.7^{2+}$ for substance P. An automatic gain control was set to ensure high sensitivity, but avoid the space charge effects. The mass spectrometer was tuned to its optimum sensitivity by infusion of B201. All operations were controlled by Finnigan Navigator 1.2 software on a Windows NT 4.0 system.

2.3. Sample extraction

Solid phase extraction (SPE) method was chosen, since B201 is soluble in water. A 13×100 mm polystyrene plastic tubes (Fisher Scientific) for the eluate from the SPE column and 12×32 mm Unison polypropylene vials (Sun International, Wilmington, NC) for the autoinjector were used. A stock solution of B201 at1 mg/ml in 50% acetonitrile was prepared and stored at -80 °C. SPE columns, custom-packed with 300 mg Bakerbond octadecyl C18 40-μm PrepLC Packing (J.T. Baker, Phillipsburg, NJ) in Bio-Spin chromatographic columns (BioRad, Hercules, CA) were used. Human or mouse plasma, 0.2 ml each in a 0.5 ml microcentrifuge polypropylene tube (Perfector Scientific, Atascadero, CA), was spiked with B201 and substance P. The resultant plasma samples were then loaded onto the SPE columns, which were washed sequentially with 1 ml of water and 1 ml of 0.1% TFA containing 10% (v/v) ACN. The analytes were eluted from the columns with 1.8 ml of 0.1% TFA containing 80% ACN. After evaporation of the solvent, the residue was dissolved in 100 μl of 50% ACN in 0.05% (v/v) TFA. The mixture was centrifuged at $3500 \times g$ for 5 min with a AccuSpin centrifuge (Beckman, Palo Alto, CA). A 50 μl aliquot was introduced into LC–MS/MS system for analysis.

2.4. Assay validation

Plasma samples for the standard curves were prepared by spiking 0.2 ml human plasma or mouse plasma with various amounts of B201 and a constant amount of substance P. The standard curves were obtained in the concentration range of 1–1500 ng/ml in human plasma and 2.5–1500 ng/ml for mouse plasma. The within-run precision values were determined in six replicates for each of the concentrations of 2.5, 100, and 1000 ng/ml of B201 separately in human and mouse plasma. The between-run precision was determined across the same three concentrations on different days and the mean concentrations and the coefficients of variation (CVs) were calculated. The accuracy of the assay was determined by comparing the nominal concentrations with the corresponding calculated concentrations. The specificity of the assay was established by simultaneously monitoring more than one major product ions derived from the molecular ion of B201 in blank plasma. No trace of interference substance was found at the same retention time as the substance P. The recovery was evaluated at the concentration level of 200 ng/ml for B201 and 100 ng/ml for substance P in human plasma. Plasma containing the analyte in question was extracted as before without the addition of the internal standard, which was added before the LC–MS analysis. The peak area ratio of the extracted analyte to the internal standard was compared with those of the unextracted pairs. B201 and substance P were used interchangeably as the analyte and the internal standard in this case.

2.5. Pharmacokinetic study of B201 in mouse

$CD2F_1$ mice (Harlan, Indianapolis, IN) weighing 21.0–27.4 g were used in the pharmacokinetic studies. Since the plasma volume obtained was smaller than 0.2 ml and variable, it was necessary to use four animals per time point with the plasma from each time point pooled. An appropriate amount of B201 (13.0 mg) was dissolved in normal saline (18.6 ml), and approximately 0.15 ml (adjusted to weight) was injected through the tail vein to result in an i.v. bolus dose of 5 mg/kg. The animals were anesthetized by CO_2 and the blood was removed from the heart through a

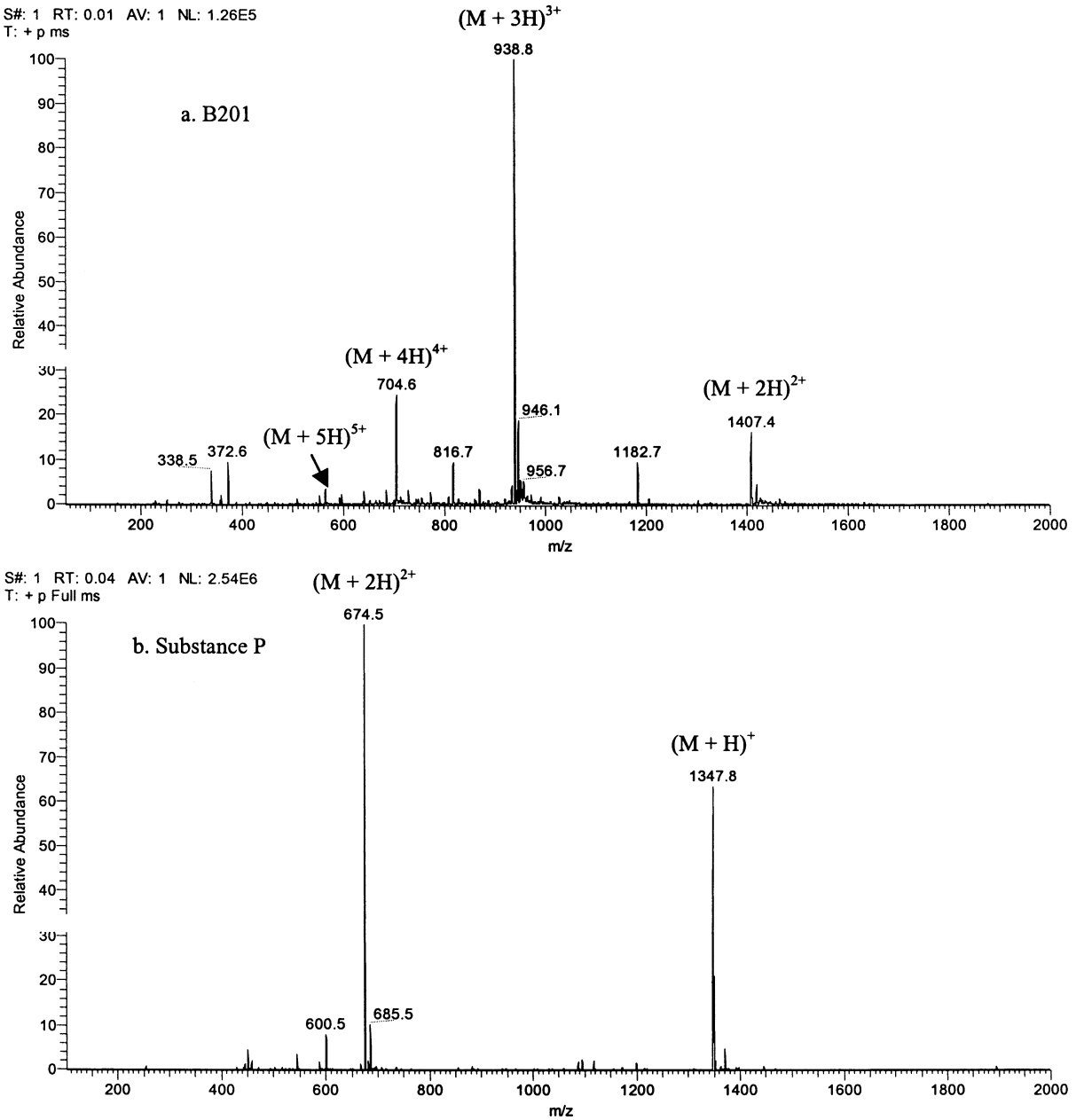


Fig. 2. Electrospray mass spectra of B201 (a) and substance P (b).

thoracic opening according to a typical schedule of 0 (pre-dose), 5, 10, 15, 30, 60, 120, 180, 210, 240, 480, 720, 1260, 1440 min after dosing. The blood samples were centrifuged at $1000 \times g$ for 5 min and the supernatant of each was collected and kept at -20 °C until analysis. A 100 μl aliquot from each animal from the same time point was pooled. A 200 μl aliquot of the pooled

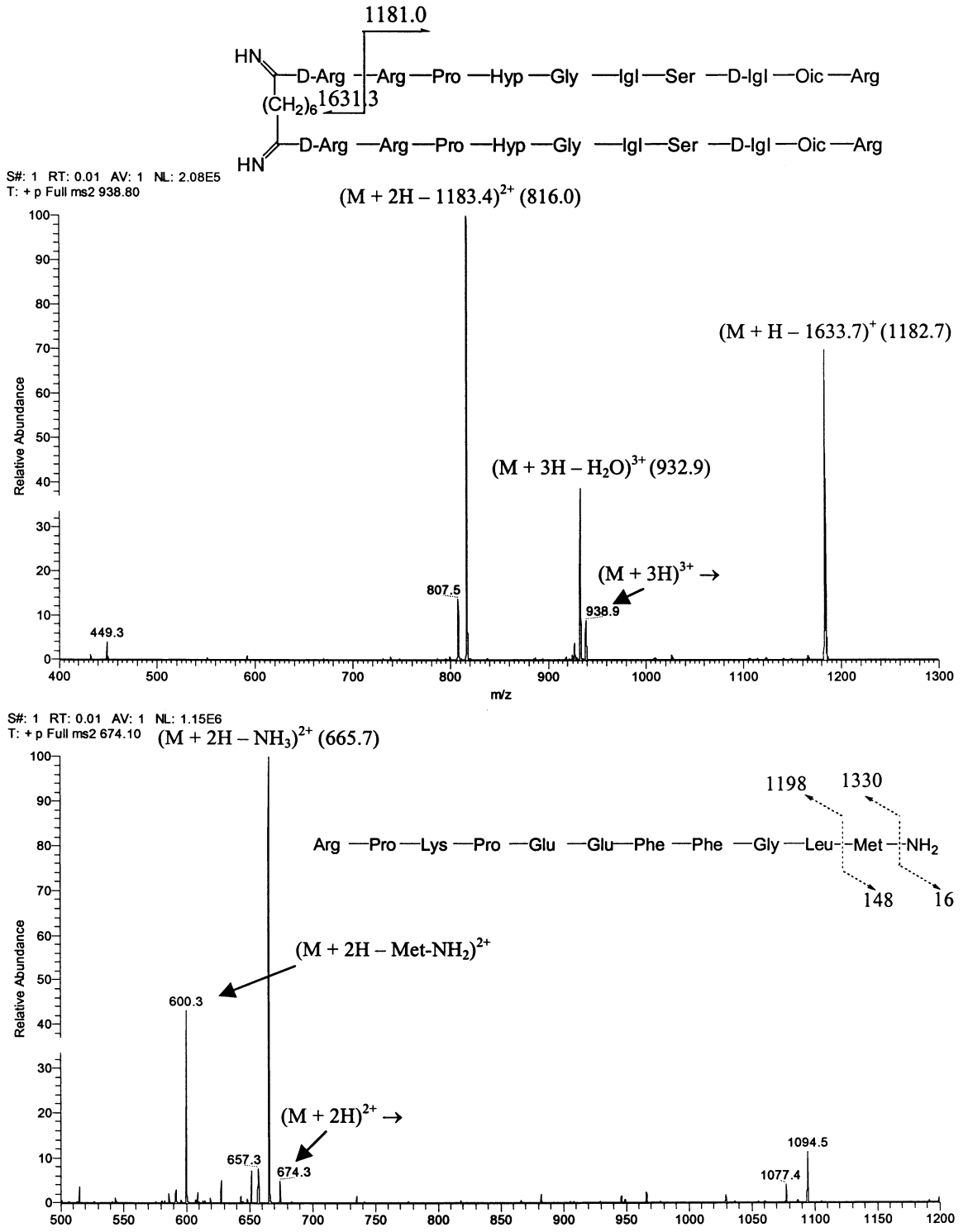


Fig. 3. Collision-induced dissociation mass spectra of the triply charged B201 (top) and doubly charged substance P (bottom).

plasma samples was analyzed for B201 by the LC–MS/MS. The oral pharmacokinetics were studied at 10 mg/kg. B201, 3.36 mg, was dissolved in 1.8 ml normal saline and only one animal was used per time point. Approximately 0.14 ml of the dosing solution was administrated to each animal by gavage to result in a dose of 10 mg/kg. The blood samples were withdrawn at the time schedule of 0 (pre-dose), 15, 30, 45, 60, 90, 120, 240, 480 min after dosing. The animals were anesthetized by CO_2 and the blood was removed as in the case of the i.v. study. The pharmacokinetic parameters were obtained by fitting the plasma concentration-time data to an appropriate pharmacokinetic model with the WinNonlin Computer software (Pharsight, Mountain View, CA).

2.6. Stability of B201 in plasma and buffer solution

Stability studies of B201 were carried out in mouse plasma at 4 and 37 °C. B201 in mouse plasma at 50 and 1000 ng/ml was incubated separately at 37 °C in a water bath (Precision Scientific, Chicago, IL). At the time schedule of 0, 0.5, 1, 3.5, 7.5, 20 and 24 h following incubation, a 0.2 ml aliquot of each was removed and processed for the B201 assay. A similar study was carried out in a 4 °C refrigerator. A stability study of B201 in 0.067 M NaH_2PO_4 buffer, pH 7.4, was also carried out at 37 and 4 °C. B201 solutions at 10 and 100 μg/ml in 0.067 M buffer solution were prepared by diluting 1 mg/ml B201 stock solution in

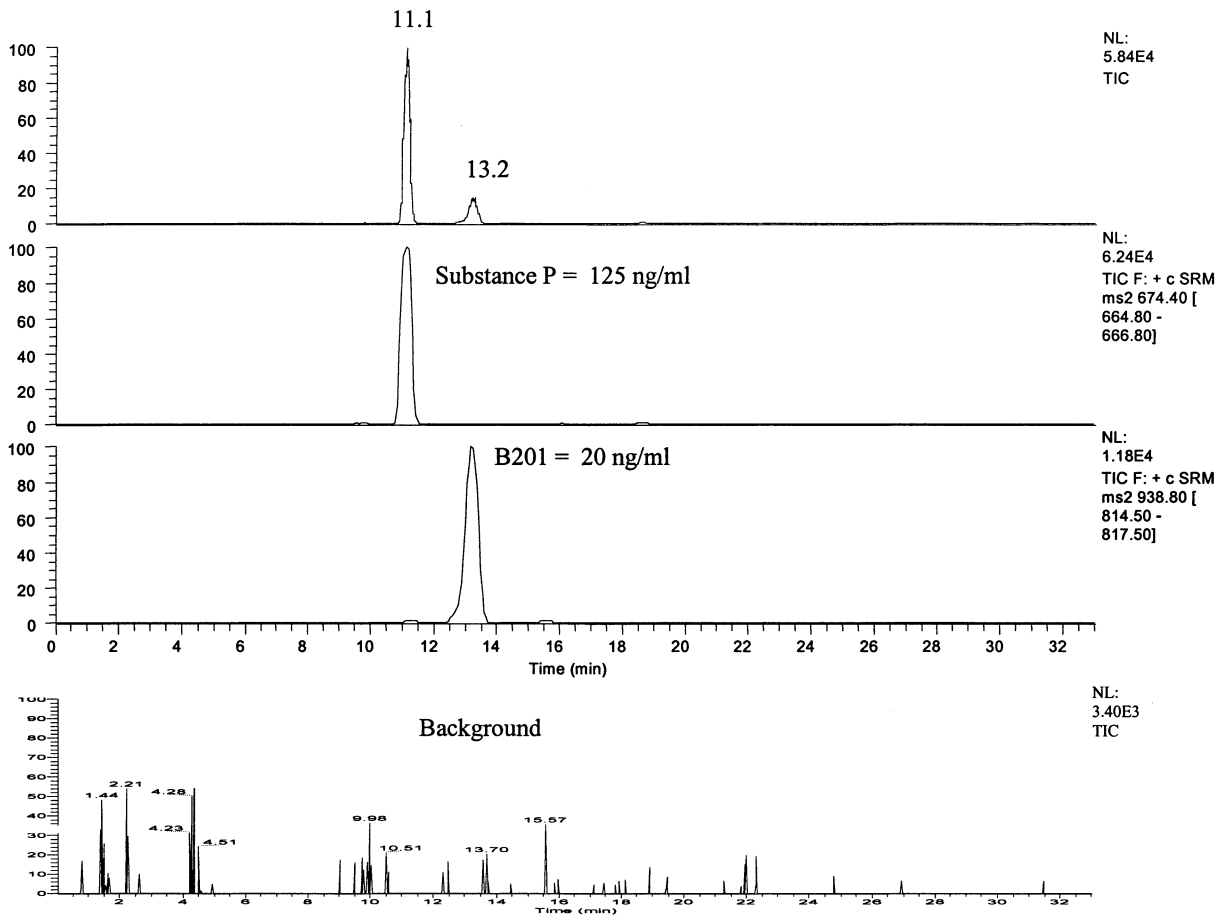


Fig. 4. SRM mass spectra of B201 and substance P in 0.20 ml mouse plasma.

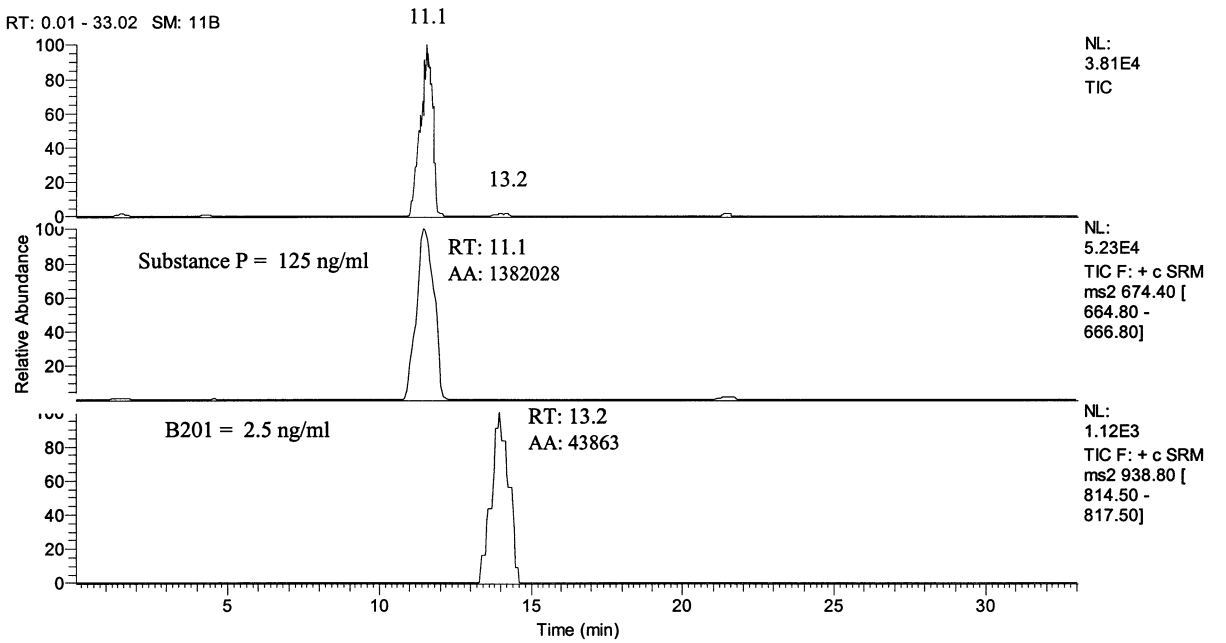


Fig. 5. SRM mass spectra of B201 at 2.5 ng/ml in 0.20 ml mouse plasma.

50% acetonitrile solution with appropriate volumes of 0.067 M sodium dihydrogen phosphate solution. The resulting B201 solutions were incubated separately in a 37 °C water bath and 4 °C refrigerator as before. At time schedule of 0, 0.083, 0.25, 0.5, 1, 2, 4, 6, 6, 12, 22.83 and 24 h following incubation, a 10 μl aliquot each was removed, diluted by 100 times with 50% acetonitrile containing 0.05% TFA, and processed for B201 assay. A calibration curve in 0.06 M NaH_2PO_4 buffer was also performed at the same time.

3. Results and discussion

3.1. LC–MS/MS assay

Under the ESI condition, B201 in 1:1 acetonitrile:TFA gave a number of ions having different charge states, $[M+4H]^{4+}$ at $m/z = 704.6$, $[M+3H]^{3+}$ at $m/z = 938.8$, and $[M+2H]^{2+}$ at $m/z = 1407.4$. As shown in Fig. 2, the most abundant peak is the triply charged ion, $[M+3H]^{3+}$. This ion was selected for the collisional-induced disso-

Table 1
Method validation of B201 in 0.20 ml mouse plasma

	2.5 ng/ml	100 ng/ml	1000 ng/ml
Within-run number/concentration			
1	2.35	100.0	1061
2	2.18	113.0	951.8
3	2.07	89.0	928.6
4	2.89	90.6	1048
5	2.13	90.3	1022
6	3.09	101.3	1088
Average ± S.D.	2.45 ± 0.43	97.3 ± 9.30	1017 ± 63.3
%C.V.	17.6	9.6	6.2
Accuracy	98.1	97.4	101.7
Between-run number/concentration			
1	2.98	101.3	984.5
2	2.56	96.3	942.5
3	2.10	85.5	1057
4	2.59	106.4	1075
5	2.81	100.3	912.4
6	2.65	87.3	1029
Average ± S.D.	2.65 ± 0.31	96.2 ± 8.25	1000 ± 64.7
%C.V.	11.9	8.6	6.5

Table 2
Method validation of B201 in 0.20 ml human plasma

	2.5 ng/ml	100 ng/ml	1000 ng/ml
Within-run number/concentration			
1	3.13	94.8	965.0
2	3.10	96.0	1019
3	2.96	108.0	1027
4	2.72	108.2	968.7
5	3.22	110.7	934.5
6	2.46	105.1	948.0
Average ± S.D.	2.93 ± 0.29	103.8 ± 6.75	977.1 ± 37.8
%C.V.	9.87	6.51	3.87
Accuracy	117.3	103.8	97.7
Between-run number/concentration			
1	2.87	91.0	926.1
2	2.89	112.6	971.0
3	2.46	107.4	958.2
Average ± S.D.	2.74 ± 0.24	103.7 ± 11.3	951.8 ± 23.1
%C.V.	8.86	10.9	2.4

Table 3
Relevant pharmacokinetic parameters of B201 in mouse plasma

PK parameter	Intravenous administration i.v. (5 mg/kg)	Oral administration p.o. (10 mg/kg)
C_{5min}/C_{max} (ng/ml)	5773	53.6
T_{max} (min)	–	30
α (1/h)	2.85	4.2
$T_{1/2\alpha}$ (min)	14.4	9.0
β (1/h)	0.0156	0.4336
$T_{1/2\beta}$ (h)	44.3	1.6
A (ng/ml)	4030	395
B (ng/ml)	3.3	19.8
AUC_t (ng/ml × min)	164 405	3422
AUC_∞ (ng/ml × min)	174 366	3540
CL_t (ml/min/kg)	30	–
V_{dss} (l/kg)	26.3	–
F ($AUC_{p.o.\infty} \times Dose_{iv}/AUC_{i.v.\infty}$ $Dose_{po}$)		1.02%

ciation (CID) experiment, that generated four major daughter ions at $m/z = 807.5^{2+}$, 816.0^{2+}, 932.9^{3+}, 1182.7^{+} (Fig. 3). The cleavage between two arginine residues is responsible for the formation of two strongest peaks at m/z 816.0^{2+}, 1182.7^{+} (Fig. 3 inset). The precursor/product ion pair at $m/z = 938.9$ and 816.0 was selected in SRM mode for quantitation of B201. Similarly, substance P generated the doubly charged ion as the most abundant peak, $[M + 2H]^{2+}$ at $m/z = 674.5$ in the full scan (Fig. 2b). CID of $[M + 2H]^{2+}$ produced two major peaks at $m/z = 665.7^{2+}$ and 600.3^{2+}. The losses of ammo-

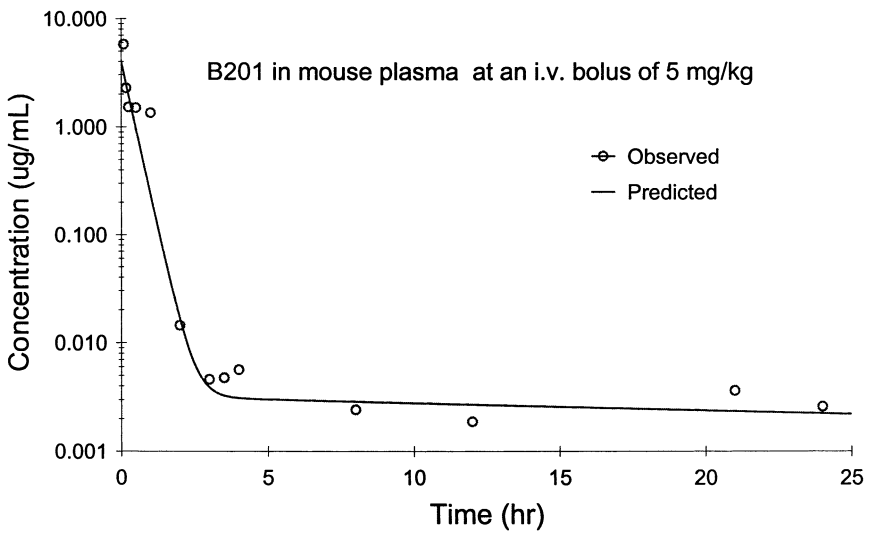


Fig. 6. Concentration-time profile of B201 in mouse following i.v. administration at 5 mg/kg.

nia and methionine amide at the C-terminus resulted in the formation of these two major peaks, respectively. The precursor/product ion pair at $m/z = 674.5$ and 665.7 was selected in SRM mode for quantitation of substance P.

3.2. Assay validation

The assay was validated in both human plasma and mouse plasma. The SRM chromatograms of plasma spiked with substance P and blank mouse plasma are shown in Fig. 4. Under the LC condition, B201 and substance P were baseline separated with retention times of 13.2 and 11.1 min, respectively, with no interference from the blank plasma (Fig. 4). This lack of interference established the specificity of the assay. Similar results were found in human plasma (data not shown). The limit of detection (LOD) of the assay was found to be about 0.25 ng/ml, as defined by a signal to noise ratio 550:1. The signal was based on the integration of the peak area performed with the software of the instrument. The LOQ for B201 was assigned to be 1 ng/ml for human plasma and 2.5 ng/ml for mouse plasma based on a signal to noise ratio 4800:1, using a 0.2 ml of plasma (Fig. 5). Due to the jaggedness of the peak at low concentration, we elected to be conservative to assign the LOQ. The recovery was found to be 78% for B201 at 200 ng/ml and 88% for substance P at 100 ng/ml. The assay was found to be linear from 1 to 12500 ng/ml for human plasma, and 2.5–1500 ng/ml for mouse plasma, with regression coefficients all > 0.995. The method was validated and the results are summarized in Table 1 for mouse plasma and Table 2 for human plasma. The within-day CVs were 9.9, 6.5, and 3.8% ($n = 6$) for 2.5, 100, and 1000 ng/ml, respectively, for human plasma. The respective between-day CVs were 8.9, 10.9, and 2.4% ($n = 3$). For mouse plasma, the within-day CVs were 17.6, 9.6, and 6.2% ($n = 6$), while the between-day CVs were 11.9, 8.6, and 6.5% ($n = 6$), for 2.5, 100, and 1000 ng/ml, respectively. In the same medium, the accuracy values were 98.1, 97.4, and 101.7%, respectively.

Stability of B201 was studied in mouse plasma and 0.06M sodium dihydrogen phosphate solution at 4 and 37 °C. B201 concentrations in mouse plasma both at 50 ng/ml and at 1000 ng/ml were found to decline monoexponentially with half-lives ($t_{1/2s}$) of 12.1 and 14.5 h (mean 13.3 ± 1.2 h), respectively. At 4 °C, B201 concentrations in mouse plasma at 50 and 1000 ng/ml also declined monoexponentially with $t_{1/2s}$ of 40.6 and 36.9 h (mean 38.8 h), respectively. Thus, B201 was found to be unstable in mouse plasma. However, B201 at 1000 ng/ml in 0.067 M, pH 7.4, NaH_2PO_4

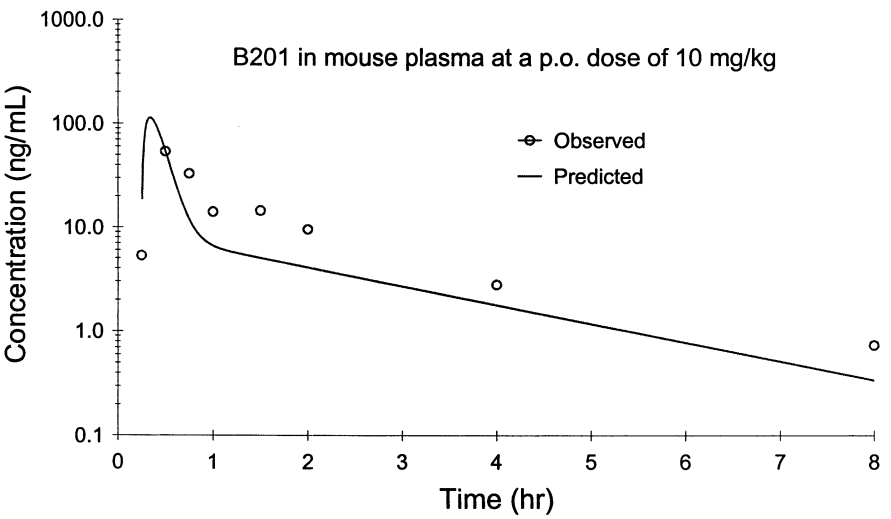


Fig. 7. Concentration-time profile of B201 in mouse following p.o. administration at 10 mg/kg.

buffer was found to be more stable than in plasma with a $t_{1/2}$ of 38.2 h at 37 °C and no decrease in concentration at 4 °C for at least 24 h.

3.3. Preliminary pharmacokinetics of B201 in the mouse

Using this LC–MS/MS method, a preliminary pharmacokinetic study of B201 in $CD2F_1$ mice was carried out following intravenous administrations at 5 mg/kg and p.o. administration at 10 mg/kg. For i.v. bolus dosing at 5 mg/kg, plasma concentration-time profiles of B201 showed a biexponental decay and the concentrations were detectable up to 24 h (Fig. 6). The concentration-time profiles were fitted to a two-compartment model (WinNonlin) and the relevant pharmacokinetic parameters were computed (Table 3). As shown, the initial half-life time was about 14.4 min and the terminal half-life time was about 44.3 h. The total clearance was found to be 30 ml/min/kg. The volume of distribution at steady-state (V_{dss}) was 26.3 l/kg. For p.o. dosing at 10 mg/kg, plasma concentration-time profiles of B201 showed a biexponental decay after reaching the peak, and the concentrations were detectable up to 8 h (Fig. 7). The concentration-time profiles were fitted to a two-compartment oral absorption model and the relevant pharmacokinetic parameters were computed (Table 3). As shown, the initial half-life was about 9 min and the terminal half-life was about 1.6 h. The preliminary oral bioavailability was found to be 1.0%.

4. Conclusion

A highly sensitive ESI/LC–MS/MS method for the quantification of B201 in plasma has been developed. This method has been validated and can be applied for the pharmacokinetic study of B201 in plasma. Preliminary pharmacokinetics of B201 in the mouse indicated that the peptide conforms to a two-compartment behavior with a rather long terminal half-life and is detected in plasma after oral dosing, although quantitative bioavailability was only 1%. An ion trap mass spectrometer can be used to quantify large peptides at low nanogram per ml concentration using a non-isotopically labeled internal standard.

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