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Authors: Yinsong Zhu, Shu Yang, Juan Wang, Yujia Mao, Yan Xu, Jing An, Ziwei Huang



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LC–MS/MS assay for the determination of a novel D-peptide antagonist of CXCR4 in rat plasma and its application to a preclinical pharmacokinetic study

Yinsong Zhu^a, Shu Yang^a, Juan Wang^a, Yujia Mao^a, Yan Xu^{a *}, Jing An^{b *}, Ziwei Huang^{a,b *}

^a School of Life Sciences, Tsinghua University, Beijing, China

^b Department of Medicine, University of California at San Diego, La Jolla, CA, USA.

* Corresponding authors. Tel: (858) 822-0333; fax: (858) 822-3344 (Ziwei Huang). E-mail addresses: jan.ny.nsh@gmail.com (Jing An), zhuang@ucsd.edu (Ziwei Huang), xyupstate@gmail.com

Highlights

- A LC–MS/MS method was set up to assay DV1, a CXCR4 peptide antagonist, in rat plasma.
- Good recovery, minimal matrix effect was achieved by protein precipitation.
- Adsorption of peptide DV1 was tested at different conditions.
- The method was successfully applied to a pharmacokinetic study of DV1 in rats.

Abstract

DV1 is a potent and selective D-peptide antagonist of CXCR4 and being developed as a novel drug candidate molecule. For preclinical pharmacokinetic study of DV1, we

established an efficient and reliable liquid chromatography coupled to tandem mass spectrometric (LC–MS/MS) method for the assay of DV1 in rat plasma. Plasma samples were acidified by formic acid and then their protein content precipitated by acetonitrile. Sample separation was processed with a C18 column (4.6 mm × 100 mm, 5 µm) and washed by a water-acetonitrile gradient mobile phase containing 0.1% (v/v) formic acid at a flow rate of 0.4 mL/min. The mass spectrometer was operated in the multiple reaction monitoring mode and positive electrospray ionization. The assay had a good linearity over the range of 10-10000 ng/mL ($r > 0.998$) for DV1. The adsorption of the peptide was diminished by organic additives during the quantitative procedure. The intra- and inter-day precision was 1.9-9.8 % and the accuracy was 91.2-110.0%. No significant variation was observed under the optimized conditions. The recovery was above 52 % with low matrix effects. The method was successfully applied to a pharmacokinetic study of DV1 after subcutaneous injection at dose of 10 mg/kg in rats. The half-life and AUC_{inf} of DV1 were calculated as 8.7 h and 35553 ng/mL·h, respectively. It is the first report on the quantitative analysis and pharmacokinetic characterization of a D-peptide targeted CXCR4, which should be useful for further preclinical studies and development of this and other peptide therapeutics.

Keywords: DV1; CXCR4; LC-MS/MS; Pharmacokinetic; Peptide

1. Introduction

CXCR4 is a transmembrane protein belonging to the superfamily of G-protein-coupled receptors (GPCRs), which is expressed on multiple cells. Activation of CXCR4 by its only known chemokine ligand, stromal-cell derived factor-1 (SDF-1 α), governs important physiological processes, such as chemotaxis, organogenesis, as well as retention of hematopoietic stem cells (HSCs) in the bone marrow [1,2]. Deregulation of CXCR4/SDF-1 α signaling is associated with numerous pathological conditions, including various types of cancers, chronic inflammatory diseases, cardiovascular diseases, and immunodeficiencies [2]. Thus, CXCR4 is a promising

drug target. Effective antagonists of this receptor may be useful therapeutic agents [3]. To date, AMD3100 is the only CXCR4 antagonist approved by the Food and Drug Administration (FDA) for clinical application in mobilizing stem cells [4]. Our laboratory has a long standing interest in developing novel and effective CXCR4 antagonists. One of our focused natural chemokines as templates for CXCR4 inhibitor design is the viral macrophage inflammatory protein II (vMIP- II) encoded by human herpes virus 8 which is a natural antagonistic chemokine ligand of CXCR4 [5]. Our effort led to the discovery of DV1, a synthetic peptide composed entirely of D-amino acids derived from the first 21 residues of the N-terminus of vMIP-II [6,7]. The amino acid sequence of DV1 is *LGASWHRPDKCCLGYQKRPLP-NH₂*. Compared with the L-peptide with the same sequence (V1), DV1 displayed more than 10 times higher binding affinity of CXCR4. Importantly, DV1 was more stable than V1 in serum [8]. Previous studies also demonstrated that DV1 showed significant antiviral activity by blocking the entry of HIV-1 via CXCR4 co-receptor in the CXCR4-dependent HIV-1 strains [8,9]. In addition, DV1 was shown to be an effective mobilizer of hematopoietic progenitor cells from the bone marrow to the blood in C3H/HeJ mice (unpublished results). Thus, DV1 is a promising candidate molecule for further drug development.

The development of a bioanalytical method and characterization of the pharmacokinetic properties are necessary for a drug candidate before its preclinical pharmacological study. To date, there has been no report on the quantification methodology of DV1 or investigation of its pharmacokinetics. Recently, liquid chromatography coupled to tandem mass spectrometric (LC–MS/MS) has become an important analytical method for the quantification of peptides in biological samples. However, stability, adsorption and potential interference from endogenous molecules in complex biological matrices still present challenges in peptide quantitation

[10,11,12]. In the present study, an efficient and reliable LC–MS/MS method was developed for the assay of DV1 in rat plasma. Using the validated method, we characterized the pharmacokinetic property of DV1 in rats.

2. Materials and methods

2.1 Reagents and standards

DV1 was provided by GL Biochem Ltd. (Shanghai, China), and the purity was confirmed to be >98% by HPLC–UV analysis. Leuprolide (purity >98%) was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). HPLC-grade acetonitrile (ACN), acetone (ACT) and formic acid (FA) were all obtained from Fisher Scientific (Waltham, MA, USA). Ultra-pure water was generated by a Milli-Q water purification system (Billerica, MA, USA).

2.2 LC-MS/MS system and analytical conditions

The HPLC system consisted of a Shimadzu HPLC system (LC-30AD, Shimadzu, Kyoto, Japan) and an autosampler (SIL-30AC). Chromatographic separation was carried out on a Waters XBridge BEH C18 column (4.6 × 100 mm, 5 μm) maintained at 35 °C. The gradient mobile phase composed of 0.1% FA in water (v/v) as solvent A and 0.1% FA in ACN (v/v) as solvent B, with a gradient elution as follows: 0 - 1.5 min, 13 - 35 %; 1.5 - 5 min, 35 - 13 %; 5 - 6 min, 13% of solvent A at a flow rate of 0.4 mL/min. The autosampler temperature was kept at 8 °C, and 10 μL of a prepared sample was injected by the autosampler.

Mass spectrometric detection was operated on an AB Sciex API 4500 triple quadrupole mass spectrometer equipped with electrospray ionization (ESI) source (AB Sciex, Framingham, MA, USA). Positive electrospray ionization mode was utilized to detect both DV1 and leuprolide (used as internal standard, IS). Analyst™1.6 software was used to acquire and analyze the data. Optimized instrument parameters for DV1 and IS were as follows: capillary voltage, 4000 V; turbo heater temperature, 300 °C; curtain gas, 10 psi; ion source gas 1, 20 psi; ion source gas 2, 10 psi. The precursor ions of DV1 and IS were obtained using de-clustering potentials of 60 V and 105 V, respectively. Product ions of DV1 and IS

were formed at collision energies of 28.9 eV and 34 eV. The quadrupoles were set at unit resolution. The full-scan Q1 mass spectra of DV1 and IS showed a prominent ion $[M + 4 H]^{4+}$, m/z 606.7 for DV1 and m/z 605.4 for IS $[M + 2 H]^{2+}$, respectively. The most abundant fragmentation ion of the protonated precursors in the full-scan MS/MS spectrum of DV1 was at m/z 771.1, and for IS was at m/z 248.9. Quantization was performed by multiple reactions monitoring (MRM) of the transitions m/z 606.7-771.1 for DV1 and m/z 605.4-248.9 for IS, respectively.

2.3 Preparation of calibration curves and quality control (QC) samples

To prepare DV1 stock solution, approximately 10.0 mg of DV1 was dissolved in an appropriate volume of stock solution solvent (ACT: H₂O: FA, 20/79.9/0.1, v/v/v) to achieve the stock solution at concentration of 1.0 mg/mL. To obtain a series of sub-stock solutions, it was further diluted by the same solvent. The stock solution of IS was prepared by dissolving of leuprolide in water to obtain a final concentration of 1000 ng/mL. Stock solutions of DV1 and IS were stored at -20 °C for up to 30 days. To avoid potential degradation or adsorption problems of peptides, all the sub-stock solutions were prepared freshly and used immediately. For preparation calibration standards in plasma samples, sub-stock solutions (10 µL) were added into blank rat plasma (90 µL) to make the final DV1 concentrations of 10, 50, 200, 500, 1000, 5000 and 10000 ng/mL, respectively. The QC samples (10, 25, 500, 5000 and 8000 ng/mL) of DV1 were independently prepared in the same way of the calibration standards.

2.4 Sample preparation

After plasma samples were thawed at room temperature, aliquot (100 µL) of plasma was dispensed into a polypropylene tube and 10 µL of IS (1000 ng/mL), 20 µL of FA were added. And then, a volume of 300 µL of ACN was added and mixed for 2 min followed by centrifugation for 10 min (12000 rpm, 4 °C). The supernatant was transferred to a clean polypropylene tube and evaporated to dryness under a gentle nitrogen flow at 40 °C. The sample was then reconstituted in 100 µL reconstitution solution (ACT: H₂O: FA, 20/79.9/0.1, v/v/v), and mixed for 5 min. After centrifuged for 10 min (12000 rpm, 4 °C), the supernatant solution was transferred into a plastic (polypropylene) autosampler vial and aliquot of supernatant solution (10 µL) was injected for analysis.

2.5 Adsorption test

Adsorption of peptides to solid surfaces is a well known phenomenon [11,13]. In order to assess whether there is any adsorption of DV1 in aqueous solution, we tested the adsorption with series of DV1 solutions transferred in glass vials as described by Wang et al. [14]. Briefly, different concentrations of DV1 aqueous solutions (0.2 mL) were transferred from one glass vial to another corresponding vial and repeated the process for 1 to 5 times. The adsorption of DV1 was evaluated by comparing the peak areas before and after being transferred. After that, an additional experiment was conducted to investigate the effect of different types of vial materials on the binding capacity of DV1 (100 ng/mL). The adsorption of DV1 was assayed by transferred for five times in glass or plastic (polypropylene) vial. To solve the adsorption problem, different organic modifiers (ACN, Acetone and FA) were tested in the experiment.

2.6 Method validation

Method validation was performed according to the guidelines for bioanalytical methods of the FDA [15].

2.6.1 Specificity

The specificity of the method was evaluated by comparing chromatograms of blank plasma samples from five different rats, blank plasma samples spiked with DV1 (100 ng/mL) and IS, and plasma samples collected at 4 h after subcutaneous 10 mg/kg DV1 in rats to ensure no endogenous interferences at the peak region of the analyte and IS.

2.6.2 Linearity and lower limit of quantification (LLOQ)

Calibration curves were prepared by assaying rat plasma samples containing DV1 and IS at seven concentration levels ranging from 10 to 10000 ng/mL. The calibration curves ($y = ax + b$), were constructed by plotting the peak area ratio (y) of DV1 to IS

versus nominal concentrations (x) with the weighted ($1/x^2$) least square linear regression. The LLOQ of the method was determined as the lowest concentration on the calibration curves ($S/N > 10$) that could be calculated with an acceptable accuracy and precision within $\pm 20\%$.

2.6.3 Accuracy and precision

Intra- and inter-day accuracy and precision of the method were evaluated using five replicates of QC samples at the concentrations of 25, 500, 5000 and 8000 ng/mL on one day and three consecutive days. The acceptance criteria recommended for accuracy and precision were 85-115% and $\leq 15\%$ of the nominal concentrations, respectively.

2.6.4 Recovery and matrix effect

The recovery and absolute matrix effect were evaluated at 3 different levels for DV1 (25, 500, and 5000 ng/mL, $n = 5$) and 100 ng/mL of IS ($n = 5$). The recovery was estimated by comparing the peak areas before extraction with those of the analytes added to post-extracted blank plasma at corresponding concentration level. The matrix effects were measured by comparing peak areas of the same analytes in processed blank plasma extract with those of pure standards at equivalent concentrations.

2.6.5 Stability

The stability of DV1 in rat plasma was assessed by analysis of three replicates of QC samples (25, 500, and 5000 ng/mL) under the following conditions: storage at room temperature for 12 h prior to extraction, post-preparative samples leaving in the autosampler at 8 °C for 4 h, three freeze-thaw cycles (-80 to 25 °C), and storage at -80 °C for 30 days.

2.6.6 Dilution integrity

Analyte stock solution was spiked in blank plasma to get concentration equivalent to 1.6 times of the highest calibration standard and diluted with blank plasma to get 1/2 concentrations of the spiked sample. The diluted QC samples (DQCs, $n=5$) were then analyzed along with the calibration standards. The accuracy and precision of DQCs should be within $\pm 15\%$ of nominal values and $\leq 15\%$, respectively.

2.7 Application to a preclinical pharmacokinetic study

Male Sprague Dawley (SD) rats ($n = 5$, weight 200–220 g), provided by Charles River Laboratories (Beijing, China), were used in the pharmacokinetics studies. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Tsinghua University (Beijing, China). Rats were subcutaneously administered of DV1 (dissolved in physiological saline) at a single dose of 10 mg/kg. Blood samples were collected into heparin-coated polypropylene centrifuge tubes at 0, 5, 10, 15, 30 min and 1, 2, 4, 6, 8, 12, 24, 36 and 48 h after the dose. The plasma sample was obtained from blood collection by centrifugation at $3,500 \times g$ for 10 min at 4 °C, and then frozen at -80 °C until analysis. The samples and QCs were analyzed by the LC-MS/MS described above. The pharmacokinetic parameters of DV1 were determined by using WinNonlin v. 5.2 (Pharsight, Cary, NC).

3. Results and discussion

3.1 Optimization of LC-MS/MS conditions

DV1, a 21 D-amino acid residue peptide amidated at the C-terminus, was analyzed in the positive ion mode by an API 4500 triple quadrupole mass spectrometer. Fig. 1A illustrates the precursor ion full scan spectrum of DV1 in the positive ion mode. The predominant molecular ions are at m/z 1212.9, 809.1, 606.7 and 485.8 corresponding to $[M + 2H]^{2+}$, $[M + 3H]^{3+}$, $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$ of DV1, respectively. Leuprolide, a commercially available peptide, was used as the IS of this assay. Fig. 1B depicts a typical full scan spectrum of the IS with the major protonated molecular ion at m/z 1209.4 and 605.4, corresponding to $[M + H]^+$ and $[M + 2H]^{2+}$ of leuprolide. The most abundant ion at m/z 606.7 and 605.4 was chosen as a precursor ion of DV1 and IS, respectively. The full-scan product ion mass spectrum of DV1 is shown in Fig. 2A. The most abundant product ion observed in the MS^2 spectrum at m/z 771.1 corresponded to the ion y_{20}^{+3} of DV1 and was used for quantification. As shown in Fig. 2B, the most abundant product ion of the IS was at m/z 248.9, which was

consistent with previous report of leuprolide [16]. Therefore, MRM was performed using the transitions at m/z 606.7-771.1 for DV1 and m/z 605.4-248.9 for IS.

Several chromatographic parameters were optimized to achieve suitable chromatographic behavior. Mobile phases containing ACN-water resulted in a sharper peak than methanol-water. Trifluoroacetic acid (TFA) in the mobile phase is usually used to improve sharpness of the peptide chromatographic peak. In the study, we found that the addition of TFA suppressed the sensitivity of the method, and FA enhanced the intensity of peaks and improved peak shape. Finally, gradient elution with water-acetonitrile consisting of 0.1% (v/v) FA gave satisfactory peak shapes for DV1 and IS with retention times of 4.10 and 4.74 min, respectively. There was not obvious peak of DV1 observed in an extracted blank sample after the injection of an extracted sample of the highest calibration standard. The run time was 6 min with no carryover.

3.2 Adsorption test

Different from small molecular compounds, adsorption of peptides is a well known phenomenon [10]. Thus, it is necessary to evaluate adsorption when developing quantitative procedures of peptides. In the present study, we investigated the adsorption of DV1 in water by transferring it for different times in glass vials. From Fig. 3A, it can be concluded that the relative recovery of DV1 significantly reduced with the increase of transfer steps. Furthermore, we also observed more loss of peptide at low concentration (100 ng/mL) than that at high concentration (10000 ng/mL), possibly due to the saturability of binding capacity of the wetted solid surface area. Next, we investigated the adsorption of DV1 (100 ng/mL) being transferred for five times in glass or plastic vial. As shown in Fig. 3B, the result suggested that the adhesion problem was more severe in glass vials than that in plastic vials, consistent with previous report of other peptides [17]. Thus, the number of transfer steps of peptide solution needs to be restricted and plastic vials should be used during the assay. In order to minimize the adsorption, we found that the loss during transfers was reduced by the addition of ACN, TAC or FA as an organic modifier. When DV1 was resolved in 20% ACT (containing 0.1% FA), the adsorption of DV1 in the solution was significantly minimized after being transferred 5 steps in plastic vial but not in

glass vial. Therefore, 20% ACT (containing 0.1% FA) was used as reconstitution solution and plastic vials were used in the process of the study.

3.3 *Sample preparation*

Protein precipitation is a convenient and simple sample clean-up method and commonly used as a plasma sample preparation for peptide analytes [11,18,19]. Initially, plasma samples were deproteinized by mixing it with three-fold volumes of ACN, but the recovery of DV1 was less than 20%. The effects of adding different volumes of organic acids (FA, acetic acid and propionic acid) on the recovery of DV1 were evaluated. The results showed that FA significantly enhanced the recovery of the methods. During the sample preparation, the reconstitution solution was also optimized to achieve satisfactory chromatographic behavior. We found that when the percentage of ACN in a reconstitution solution was more than 30% (ACN: H₂O, v/v), two peaks of DV1 were observed in the chromatography. Surprisingly, this phenomenon could not be completely avoided by changing the composition of the mobile phase. This was probably due to the percentage of ACN in the reconstitution solution being too high relative to that in mobile phase at the point of DV1 eluted from the column. We also observed that the adsorption was more severe when the proportion of ACN was reduced in the reconstitution solution. In the optimized conditions, 20% ACT (containing 0.1% FA) as the reconstitution solution gave a sharper peak and low adsorption of the analytes.

3.4 *Method validation*

3.4.1 Specificity

Under the optimized conditions, no significant interference from rat plasma was observed at the elution times of DV1 or IS as shown in Fig. 4. DV1 and IS were totally separated by the conditions described above.

3.4.2 Linearity and LLOQ

To evaluate linearity of DV1, seven calibration standards (10, 50, 200, 500, 1000, 5000 and 10000 ng/mL) and weighted linear regression analysis were used. The calibration curve showed good linearity ($r > 0.998$) over the concentration range of 10–10000 ng/mL. Under these conditions, LLOQ was 10 ng/mL ($S/N > 10$) with an acceptable level of accuracy and precision (data shown in Table 1).

3.4.3 Accuracy and precision

Intra- and inter-day accuracy and precision values of this method are given in Table 1. The intra- and inter-day accuracies were in the ranges of 91.2–109.0% and 99.6–110.0%, respectively, with corresponding precisions in the ranges of 1.9– 9.0% and 1.2– 9.8%, respectively. The results were in the ranges of the guidelines specified by the FDA for bioanalytical methods.

3.4.4 Recovery and matrix effect

The data of recovery and matrix effect of the method are shown in Table 2. The recovery of DV1 was in the range of 52.0–66.9 % and matrix effect was within the range of 85.2–90.7 % at the three QC concentration levels, and the recovery of IS was (70.3 ± 5.8) % with a matrix effect of (87.6 ± 4.8) %. The results indicated reasonable recoveries with no obvious suppression or enhancement of ionization for DV1 or IS.

3.4.5 Stability

Results of stability studies of DV1 in rat plasma samples under various conditions are presented in Table 3. It can be seen that DV1 maintained reasonable stability in autosampler at 8 °C for 4 h, at room temperature (25 °C) for 12 h, at –80 °C for 30 days, and after three freeze–thaw cycles. No significant degradation of DV1 was observed under all the evaluated conditions.

3.4.6 Dilution integrity

Accuracy of the DQCs was found to be 101.6% of the nominal concentration, and the precision was below 7.9%. The results were acceptable.

3.5 *Application to the pharmacokinetic study*

The analytical method developed as described above was applied to the quantification of DV1 concentration in rat plasma for pharmacokinetic study. The mean plasma concentration-time profiles of DV1 after subcutaneous dose of 10 mg/kg in rats are shown in Fig. 5 and the pharmacokinetic parameters are summarized in the table inserted. The T_{\max} and C_{\max} were 0.83 ± 0.29 h and 8592 ± 1056 ng/mL, indicated that the absorption of DV1 was rapid. The high apparent distribution volume (V_z/F) suggested that DV1 was widely distributed in tissues. The long half time ($t_{1/2}$) and the slow reduction after 12 h were observed, possibly due to the release from the tissue compartment.

4. Conclusions

We developed a convenient HPLC-MS/MS method for the quantification of DV1, a novel D-peptide antagonist of CXCR4 in rat plasma. Good linearity of the method was achieved over the calibration range of 10-10000 ng/mL ($r > 0.998$). The adsorption of the peptide was diminished by organic additives during the quantitative procedure. The validation showed that the method was specific and sensitive, with a LLOQ of 10 ng/mL. The intra- and inter-day accuracy and precision were within the acceptable limit. No significant variation was observed under the evaluated conditions. The recovery was above 52 % with low matrix effects. The simplicity of the sample preparation made this method suitable for application to the pharmacokinetic study of DV1. The half-life and AUC_{inf} values of DV1 were as 8.7 h and 35553 ng/mL·h after a single dose (10 mg/kg) subcutaneous administration in rats. In conclusion, a convenient LC-MS/MS method for the quantification of DV1 was established and applied to evaluate the pharmacokinetic characterization of DV1 in rats. This method and the data obtained in this study may be useful for further preclinical development of DV1.

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Figure captions

Fig.1. MS spectrum of DV1 (A) and leuprolide (B, IS), acquired with an API 4500 mass spectrometer in positive ion mode.

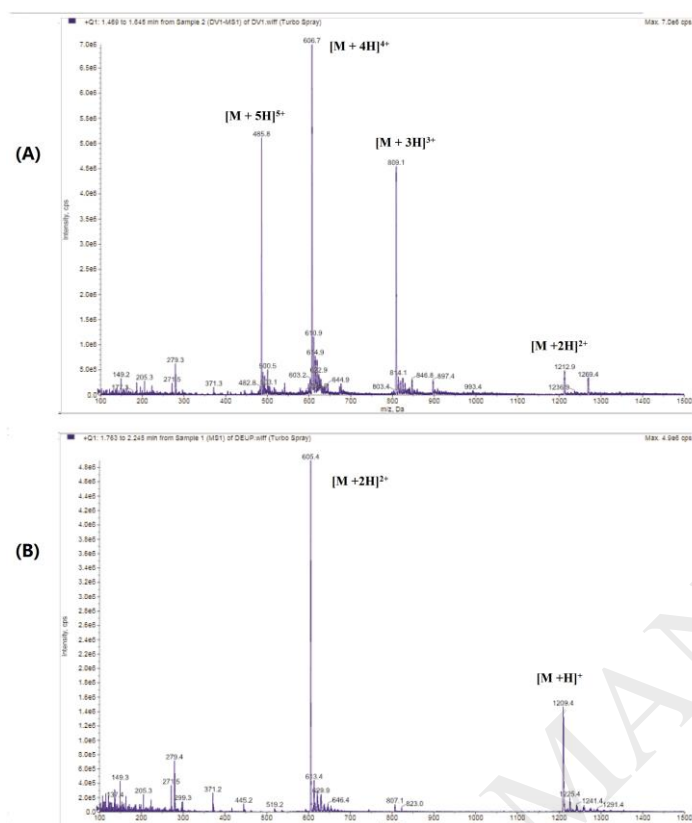


Fig.2. Full-scan product ion mass spectra of $[M + 4H]^{4+}$ ion for DV1 (A) and $[M + 2H]^{2+}$ ion for leuprolide (B, IS).

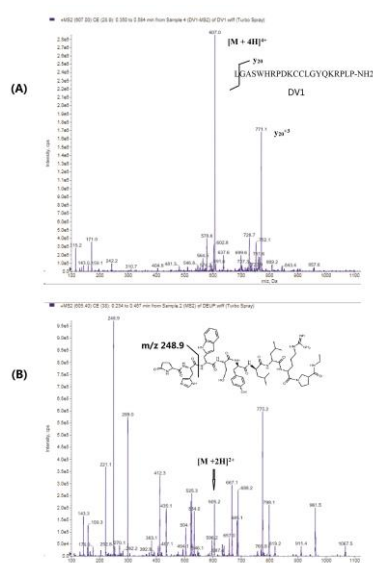


Fig.3. Adsorption test of DV1. (A) DV1 (100, 1000 and 10000 ng/mL) aqueous solutions were transferred from one glass to another corresponding vial and repeated the process for different times. The adsorption of DV1 was assayed by comparing the peak areas before and after being transferred. The results were expressed as relative recovery. (B) The adsorption of DV1 (100 ng/mL) in different solvents were evaluated after five transfer steps in glass or plastic vial. Each point represents the mean \pm SD ($n=3$). ACT: acetone.

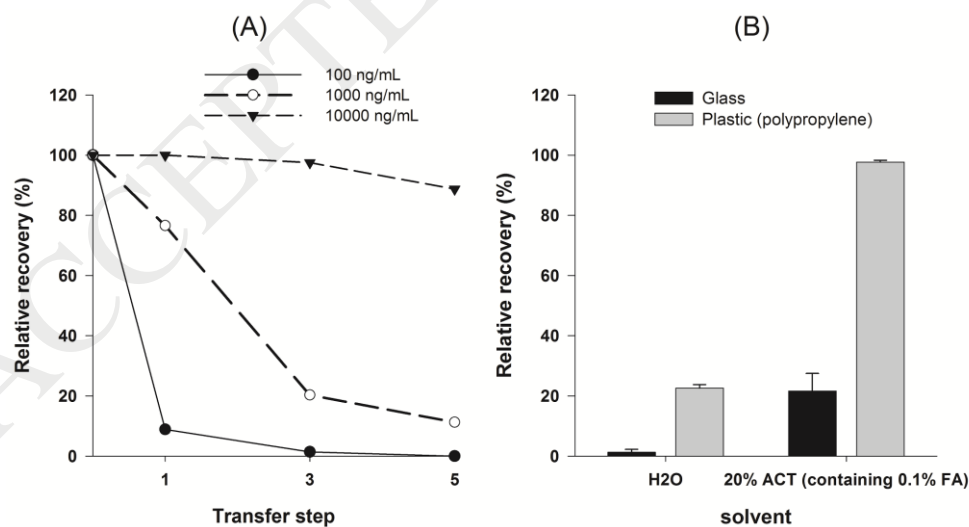


Fig.4. Typical MRM chromatograms of (A) blank rat plasma, (B) blank plasma spiked with DV1 (100 ng/mL) and IS (100 ng/mL) and (C) a plasma sample collected at 4 h after a single dose subcutaneous injection of 10 mg/kg DV1 in rat.

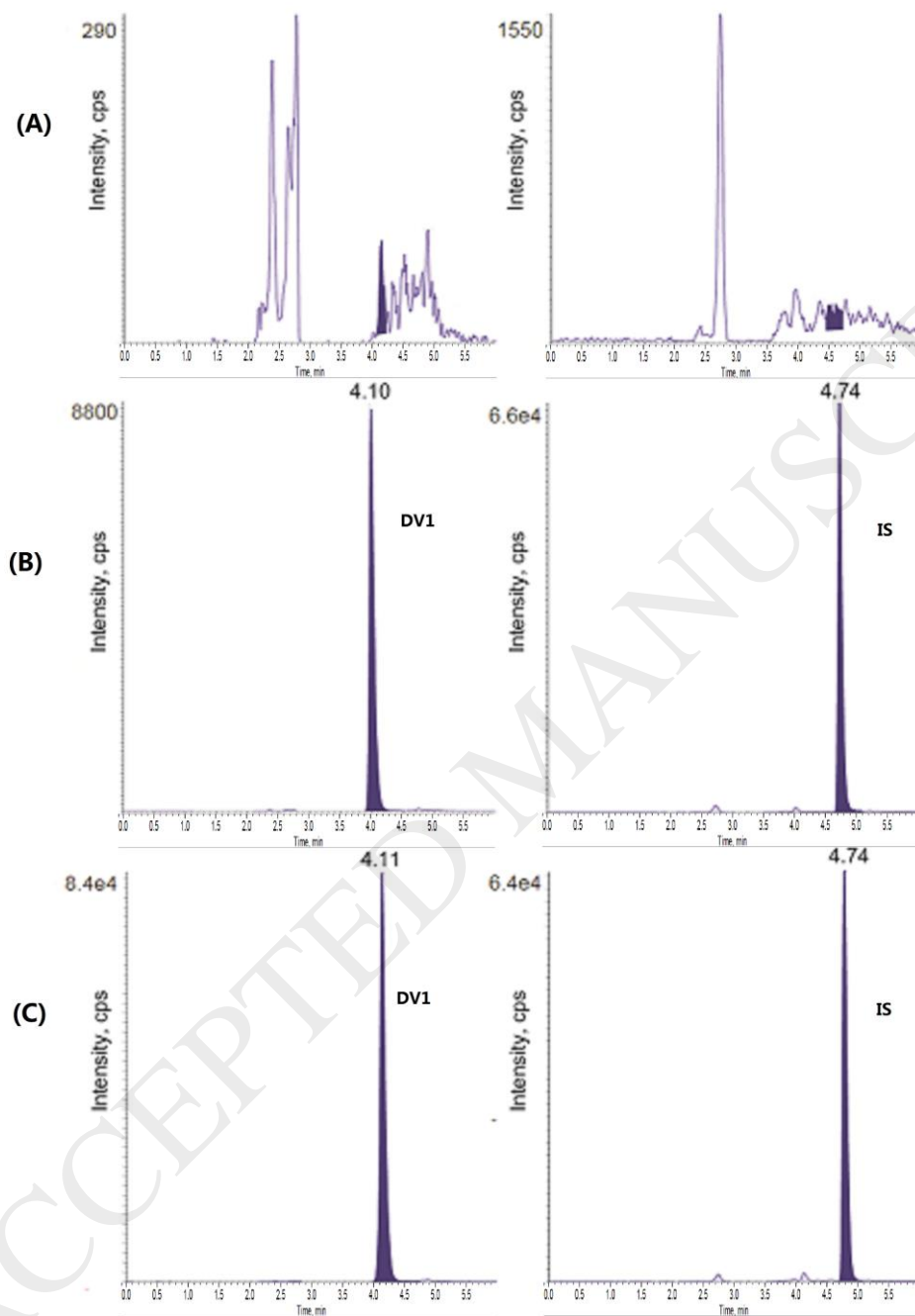
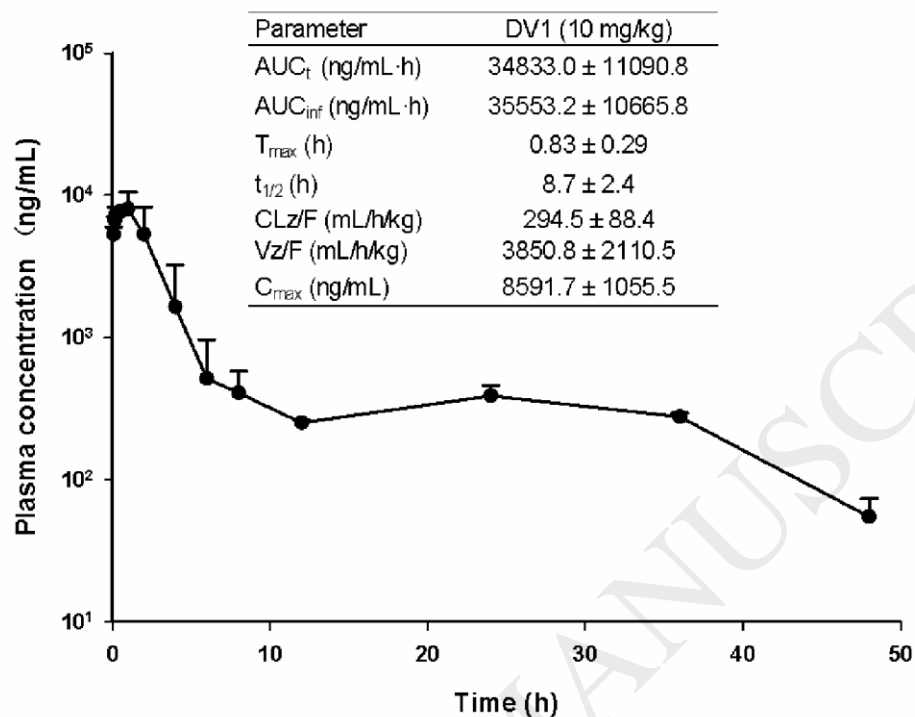


Fig.5. Mean (\pm SD) plasma concentration–time profile of DV1 after a single dose (10 mg/kg) subcutaneous administration in rats (n=5). Pharmacokinetic parameters are shown in the table inserted. Each point represents the mean \pm SD.



Tables

Table 1 Intra- and inter-run precision and accuracy for the quantification of DV1 in rat plasma by LC-MS/MS (n = 5)

Nominal concentration (ng/mL)	Test concentration (mean \pm SD, ng/mL)	Accuracy (%)	Precision (RSD %)
Intra-day			
10 (LLOQ)	10.4 \pm 0.8	103.8	7.6
25	25.7 \pm 1.2	102.7	4.8
500	456 \pm 8.8	91.2	1.9
5000	5448 \pm 191	109	3.5
8000	8522 \pm 722	106.5	9.0
Inter-day			
10 (LLOQ)	10.3 \pm 0.1	103.0	1.2
25	26.9 \pm 2.6	107.5	9.8
500	498 \pm 21	99.6	4.3
5000	5278 \pm 322	105.6	6.1
8000	8800 \pm 393	110	4.9

Table 2 Extraction recovery and matrix effect of DV1 and IS in rat plasma (n = 5)

	Concentration (ng/mL)	Recovery (mean \pm SD, %)	RSD (%)	Matrix effect (mean \pm SD, %)	RSD (%)
DV1	25	66.9 \pm 7.2	10.8	90.7 \pm 10.9	12.0
	500	59.5 \pm 4.1	6.9	86.3 \pm 8.1	9.4
	5000	52.0 \pm 6.6	12.8	85.2 \pm 11.7	13.7
IS	100	70.3 \pm 5.8	8.3	87.6 \pm 4.8	5.4

Table 3 Stability of DV1 in rat plasma under various storage conditions (n = 3)

Storage conditions	Nominal concentration (ng/mL)	Test concentration (mean \pm SD, ng/mL)	Accuracy (%)	Precision (RSD, %)
4 h at autosampler	25	21.7 \pm 1.1	86.9	5.0
	500	486 \pm 4.6	97.3	0.9
	5000	4990 \pm 174	99.8	3.5
12 h at room temperature	25	24.4 \pm 2.7	97.6	10.9
	500	507 \pm 17	101.5	3.4
	5000	5707 \pm 100	114.1	1.8
30 days storage at -80 °C	25	24.8 \pm 3.2	99.1	12.8
	500	563 \pm 21	112.5	3.8
	5000	5567 \pm 448	111.3	8.0
3 freeze-thaw cycles	25	22.9 \pm 2.3	91.5	9.9
	500	477 \pm 19	95.3	3.9
	5000	5530 \pm 433	110.6	7.8