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Quantitative analysis of a novel HIV fusion inhibitor (sifuvirtide) in HIV infected human plasma using high-performance liquid chromatography–electrospray ionization tandem mass spectrometry

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ABSTRACT

A sensitive method for measuring sifuvirtide, a novel HIV fusion inhibitor peptide drug in HIV-1$^+$ human plasma by liquid chromatography–tandem mass spectrometry (LC–MS/MS) was developed. The plasma samples were treated by solvent/detergent (S/D) method to inactivate viral activity before analysis. After protein precipitation sifuvirtide was determined by LC–MS/MS. A structure analog was used as internal standard (IS). The mass spectrometer was operated in positive ion and multiple reaction monitoring mode with transitions m/z 946.3 → 159.0 for sifuvirtide and 951.7 → 159.2 for IS. The intra-day precision ranged from 2.74% to 7.57% with accuracy from 91.63% to 102.53%. The inter-day precision ranged from 2.65% to 3.58% and the accuracy from 95.53% to 105.28%. Stability studies showed that sifuvirtide was stable both during the assay procedure and long-term storage. The lower limit of quantitation (LLOQ) was 9.75 ng ml^{-1}. The method was used for analyzing samples from phase IIa clinical study of sifuvirtide in China.

1. Introduction

According to recent data, roughly 40 million individuals worldwide are living with the rapidly spreading HIV (human immunodeficiency virus) [1]. Due to lack of effective vaccines, development of novel anti-HIV drugs is important to save lives of people with HIV infection. So far, more than 31 anti-HIV drugs and combinations have been approved for clinical use, and most of them belong to reverse transcriptase inhibitors (RTIs) and protease inhibitors (PIs). Application of these drugs in various combinations, known as highly active antiretroviral therapy (HAART), has significantly reduced the morbidity and mortality of HIV/AIDS [2]. However, more and more HIV/AIDS patients have failed to respond to HAART regimens because of the emergence of variants resistant to the current treatment regimens [3]. Therefore, the development of new classes of anti-HIV drugs is urgently needed.

Enfuvirtide (MW 4492 Da) is the first HIV-1 fusion inhibitor approved for clinical use [4]. However, it has several weaknesses. Its short half-life (3.8 h) requires more frequent subcutaneous injections. High dosage (90 mg) and twice daily usage of enfuvirtide may result in the injection site reactions and financial burden for HIV/AIDS patients. Based on the 3 dimensional structural information of HIV-1 gp41, a novel anti-HIV fusion inhibitor sifuvirtide was designed (Ac-SWETWEREIENY TRQIYRI LEESQ EQQDRNERDL LE-NH2, MW 4727 Da). This inhibitor has been engineered with different sequence from enfuvirtide. The anti-HIV-1 potency *in vitro*, mechanism of action and results from phase Ia clinical study of sifuvirtide have been reported [5]. Due to much better inhibition activity, lower dosage will very likely be used (only 10–20 mg/day). Such low dose administration of large peptide brings a big challenge to biopharmaceutical analysis in clinical studies.

Nowadays, liquid chromatography–tandem mass spectrometry (LC–MS/MS) has become the preferred technique for sensitive and selective quantification in biological matrices [6]. However, multiple charges on a large peptide, sample extraction and poor chromatography present challenges to the development of methods to quantitate large peptides in plasma by LC–MS/MS. D'Avolio et al. [7] reported a HPLC method with fluorimetric detector for the quantification of enfuvirtide in plasma of HIV infected subjects. Chang et al. [8] and van den Broek et al. [9] reported quantification of enfuvirtide in HIV-1$^+$ human plasma by LC/MS/MS. The clinical plasma samples were heat-treated to inactivate viral activity before analysis of enfuvirtide. The LLOQ of the method was 10 ng/ml.

He et al. [5] reported results from phase Ia clinical study of sifuvirtide, but no details of the quantitation method was presented. Dai et al. [10] reported quantification of sifuvirtide in monkey plasma by an on-line solid-phase extraction procedure combined with liquid chromatography coupled with electrospray ionization ion-trap mass spectrometry. In particular, tandem triple quadrupole mass spectrometry used in the multiple reaction

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monitoring mode provides unrivaled sensitivity and selectivity. Meanwhile, installation of on-line SPE system and preparation of SPE columns are difficult. If an extraction cartridge is used for repetitive sample extraction, contamination of the micro-column with endogenous material is detrimental for its chromatographic performance and capacity. The phase IIa clinical samples need be treated to inactivate viral activity before analysis for safety, which has not been taken in consideration for preclinical studies.

We report here a quantitative LC–triple quadrupole MS/MS method for analysis of sifuvirtide in HIV-1^+ human plasma.

2. Experimental

2.1. Chemicals and reagents

All chemicals were HPLC grade or reagent grade unless otherwise stated. Methanol, ethanol and acetonitrile (ACN) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Formic acid (FA) was from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA). Sifuvirtide and internal standard (Ac-SWETWEREIENYTR QIYRILEENQEQQDRNERDLLE-NH_2, MW 4754 Da) were provided by FUSOGEN, Inc. (Purity ≥99.5%, Tianjin, P.R. China). Blank healthy human plasma was obtained from the affiliated hospital of the Academy of Military Medical Sciences, Beijing, China. Protease inhibitor cocktail tablets without ethylenediaminetetraacetic acid (EDTA) were purchased from Roche Diagnostics, Mannheim, Germany. Nondenaturing lysis buffer was purchased from Biolife Company (Beijing, P.R. China). Distilled water, prepared from demineralized water, was used throughout the study. The whole blood was collected to vacuum heparin sodium anticoagulant tubes (Vacuette, 4 ml, Lot: 020802, Gelena Company, Austria).

2.2. Preparation of stock solutions

Stock solutions were prepared by dissolving 2.0 mg sifuvirtide or internal standard in 1 ml reconstitution solution (1% FA (v/v) in water/ACN (90:10, v/v). The stock solution of internal standard was diluted to a final concentration of 2 μg ml^{-1} as the working solution. The stock solutions were stored at 4 °C.

2.3. Preparation of calibration curve and quality control (QC) standards

One protease inhibitor cocktail tablet was dissolved in 150 μl distilled water. 15 μl protease inhibitor solution was added into each 500 μl plasma according to the introduction of the protease inhibitor cocktail tablets. Calibration standards were prepared by spiking stock solution into blank healthy human plasma for generation of the calibration curve samples (9.75, 19.5, 78.1, 312.5, 1250 and 5000 ng ml^{-1}). QC samples were also prepared in blank healthy human plasma. Concentrations of the lower, middle, higher QC samples were 9.75, 312.5 and 5000 ng ml^{-1}.

2.4. Sample processing

2.4.1. Virus inactivation

20 μl nondenaturing lysis buffer was added to 500 μl plasma and they were mixed for 10 s. The mixture was kept at 4 °C for 1 h. Then, the plasma was centrifuged at 12,000 × *g* at 4 °C for 15 min and the supernatant was transferred to a fresh tube.

2.4.2. Sample pretreatment

As plasma sample pretreatment protein precipitation was carried out. 200 μl of study samples, calibration curve standards, or QC samples were pipetted into 2 ml EP tubes. The internal standard, 40 μl, was introduced and mixed gently. 0.4 ml of ACN was added to all samples in the tube. The tube was capped, and mixed for 5 min, and centrifuged at 19,600 × *g* for 10 min at 4 °C. The supernatant was transferred to another tube and evaporated to dryness under nitrogen. All samples were reconstituted in 200 μl reconstitution solution, capped, and mixed for 1 min. The reconstituted samples were centrifuged at 19,600 × *g* for 5 min at 4 °C, the supernatant was transferred to an autosampler vial and 50 μl was injected onto the LC–MS/MS system.

2.5. LC–MS/MS

LC–MS/MS experiments were conducted on an Agilent 1100 LC system (Agilent Technologies) coupled to a MDS Sciex API 4000 triple quadrupole MS/MS system (Applied Biosystems) operating in electrospray ion source (ESI) mode. The LC system was outfitted with a binary pump, an autosampler, and an in-line mobile phase vacuum degasser. A reverse-phase Ultra Aqueous C18 analytical column (2.1 mm × 100 mm, 5 μm particle size) and guard column (2.1 mm × 12.5 mm, 5 μm particle size) were purchased from RESTEK Corporation. The LC elution conditions were as follows (all solvent percentages were volume fractions): mobile phase A, 0.1% FA in water; mobile phase B, 0.1% FA in ACN; time program, 0 min, 95% A/5% B; 1.0 min, 95% A/5% B; 5 min, 5% A/95% B; 5.5 min, 5% A/95% B; 10 min, 95% A/5% B and 12 min, 95% A/5% B. The flow rate was 200 μl/min at ambient temperature. The retention time was 9.21 min for sifuvirtide and 9.16 min for IS. The MS/MS operating parameters for analyte and IS were obtained and optimized via positive ion mode. The transitions for multiple reaction monitoring were m/z 946.3 → 159.0 for sifuvirtide and m/z 951.7 → 159.2 for IS. The operational parameters of the mass spectrometer were as follows: Collision activated dissociation (CAD) = 5 psi, Curtain gas (CUR) = 30 psi, Gas1 = 20 psi, Gas2 = 20 psi, TurboIonspray voltage (IS) = 5000 V, TEM = 200 °C, Collision energy (CE) = 65 eV, CXP = 30 eV, Declustering potential (DP) = 40 V for analyte, 20 V for IS, Entrance potential (EP) = 15 V for analyte, 10 V for IS, Resolution Q1: Unit and Resolution Q3: Unit.

2.6. Data acquisition and analysis

Data acquisition was performed using Analyst 1.4 software (Applied Biosystems-SCIEX). Ratios of analyte peak area versus IS peak area were calculated for each point and standard curves were constructed by least square linear regression analysis using a weighting factor of $1/x^2$, in which x is the concentration in ng ml^{-1}. Test samples and quality control samples were then interpolated from the calibration curve to obtain the concentrations of the respective analytes.

Pharmacokinetic data analysis was performed by the non-compartmental method. The maximum serum concentration (C_{max}) and the time to C_{max} (T_{max}) were determined from the observed serum concentrations. The terminal elimination half-life ($t_{1/2}$) was calculated as $0.693/k_{el}$, where the k_{el} was apparent elimination rate constant of sifuvirtide from serum. The area under the serum concentration–time curve ($AUC_{0-\infty}$) from zero to infinity was calculated as the sum of $AUC_{0-t} + AUC_{t-\infty}$. AUC_{0-t} from zero to the last measurable time was calculated by trapezoidal rule and $AUC_{t-\infty}$ was calculated as C_t/k_{el}, where C_t was the last observed serum concentration after administration. The apparent total clearance (CL) was calculated as dose/$AUC_{0-\infty}$. The volume of distribution at steady state (V_{ss}) and the mean residence time (MRT) were calculated by the non-compartmental method.

2.7. Validation procedures

The specificity of assay was investigated by processing and analyzing six independent blank (drug-free) samples. The intra-day

precision, evaluated as relative standard deviation (R.S.D.), and the accuracy of each method were determined from results for replicate analyses ($n = 6$) of QC samples. Similarly, the inter-day precision (R.S.D.) and accuracy were determined replicate analyses ($n = 6$) of the QC samples performed on six separate runs. The LLOQ was chosen as the concentration of the lower calibration standard with an acceptable limit of variance (within 20% for both precision and accuracy). Matrix effects for analytes were evaluated by comparing the peak areas of the analyte at three QC concentrations spiked post-extraction into plasma extract, to the corresponding peak areas of the same analyte present in the mobile phase. In order to observe the extent of change in the analyte response in the presence of the interference under HPLC–MS/MS assay conditions, a post-column infusion scheme is developed. This experiment uses an infusion pump to deliver a constant amount of analyte into the HPLC stream entering the ion source of the mass spectrometer. The mass spectrometer is run in MRM mode to follow the infused analyte. Blank sample extracts are injected onto the HPLC column under conditions chosen for the assay. Any endogenous component that elutes from the column and causes a variation in ESI response of the infused analyte(s) is seen as a suppression/improvement in the response of the infused analyte. In this way, the analyst is able to detect specific endogenous sample components that affect the target analyte. In addition, this experiment provides detailed information about the time profile of the interference as it elutes from the column under the chromatographic conditions of the assay.

The extraction recoveries of sifuvirtide at three QC levels were determined by comparing peak area of the analytes obtained from plasma samples spiked before extraction to those spiked after the extraction. Stability after freezing and thawing was assessed by analyzing QC samples ($n = 5$) over three freeze/thaw cycles. Stability at room temperature was assessed using QC samples ($n = 5$) that had been stored at room temperature for more than 12 h. Stability during storage at −4 and −70 °C was assessed by using QC samples ($n = 5$) that had been placed in a storage freezer for 48 h (−4 °C) and 30 days (−70 °C). Stability of HIV virus inactivation was assessed by analyzing QC samples ($n = 5$) by solvent/detergent (S/D) inactivation.

3. Results and discussion

3.1. Sample progressing

3.1.1. Virus inactivation

According to Chang et al. [8], the clinical samples were to be heat-treated to inactivate viral activity before analysis of enfuvirtide. In our experiment, however, the plasma samples were slightly faded by this method. In addition, there was obvious decrease on LC–MS/MS response of sifuvirtide. The sample may be denatured by this method. All of significant transfusion-transmissible pathogens, such as HIV, HBV, and HCV, are lipid-enveloped viruses which are extremely susceptible to membrane disruption by S/D [11]. Nondenaturing lysis buffer containing nonionic detergent, salt and organic solvent was chosen as S/D reagent. The volume ratio of 20 μl nondenaturing lysis buffer to 500 μl plasma sample was used to inactivate HIV viral activity. The LC–MS/MS response of samples decrease when treated by high nondenaturing lysis buffer to plasma sample volume ratio, whereas it cannot effectively inactive HIV viral activity with the low volume ratio according to viral activity assay (data not shown).

3.1.2. Sample extraction

van den Broek et al. [9] extracted enfuvirtide from plasma by SPE for LC/MS/MS analysis. Initially, sifuvirtide was extracted with C18 and HLB OASIS (Waters) SPE cartridges. The recoveries of both analytes and IS were about 80% by HLB SPE cartridges. However, protein precipitation was chosen as a convenient and simple sample cleanup method for the peptide-type analytes. In addition, protein precipitation in a close tube is safer than SPE to handlers, though the HIV-1^+ plasma was inactivated. D'Avolio et al. [7] extracted enfuvirtide from 50 μl plasma by addition of 450 μl of extraction solution (1% TFA in acetonitrile) in a PTFE microfuge tube. Chang et al. [8] isolated enfuvirtide and IS from plasma by protein precipitation with two volumes of acetonitrile to plasma for LC–MS analysis.

In our experiment, the optimal volume ratio of organic solvent to plasma was determined by a series of samples with increasing amounts of three different organic solvents (methanol, ethanol and ACN). Because of the relatively large molecular size, the peptide was found to precipitate with the plasma proteins with high organic solvent ratios. When the ratio was 4:1 and 8:1 of methanol or ethanol or ACN:plasma, the recoveries of sifuvirtide were between 45% and 60%. When the ratio was 2:1 of ACN:plasma, the results were satisfactory with consistently good recovery and relatively clean supernatant after centrifugation at high speed. With methanol or ethanol at the same or lower ratios interference was observed in the blank plasma around the retention time of sifuvirtide.

3.2. MS/MS

The mass spectrometer was operated in the positive ion multiple reaction monitoring mode in the LC/MS/MS analysis. The most abundant ion was the $[M+5H]^{5+}$ ion at m/z 946.3 for sifuvirtide. The transition 946.3 → 159.0 for sifuvirtide was selected, since it showed the highest selectivity and signal-to-noise ratio (Fig. 1). Chang et al. [8] reported that while the fragment with the transition of m/z 1124 → 159 for enfuvirtide (the immonium ion of tryptophan) was more abundant with high collision energy 200 eV, resulting in high signal intensity, there was interference from the endogenous substances of the control plasma. In our experiment, CE value yielding 946.3 → 159.0 for sifuvirtide is not high (65 eV) and no interference from the endogenous substances of the control plasma was observed. Dai et al. [10] selected the transition 946.3 → 871.87 for sifuvirtide with ion-trap mass spectrometry. The detection ways between ion-trap and triple quadrupole MS are different. Therefore, the transitions of the same molecule from two different mass spectrometers may be different.

3.3. Liquid chromatography

For chromatographic resolution from other potentially coeluting substances, C18, phenyl, and amino columns were evaluated. In general, the capacity factor of sifuvirtide was higher on C18 column compared with the phenyl and amino columns. C18 column was used because of better resolution. The retention time was 9.21 min for sifuvirtide and 9.16 min for IS.

3.4. Internal standard

The structures of the sifuvirtide and IS are similar. The difference of amino acid sequence between analyte and IS is the 23th residue from N terminus, which is Ser for sifuvirtide and Asn for IS. The isoelectric points of sifuvirtide and IS are both 4.42. The transition 951.7$[M+5H]^5$ → 159.2 was selected (Fig. 2), since it showed the highest selectivity and signal-to-noise ratio. The IS shows similar behavior in HPLC, MS and physicochemical characteristics as sifuvirtide. IS interferences were examined by introducing plasma samples containing only IS. No IS interference and no additional ion suppression were observed (Fig. 3). Thus it is suitable for quantitative analysis.

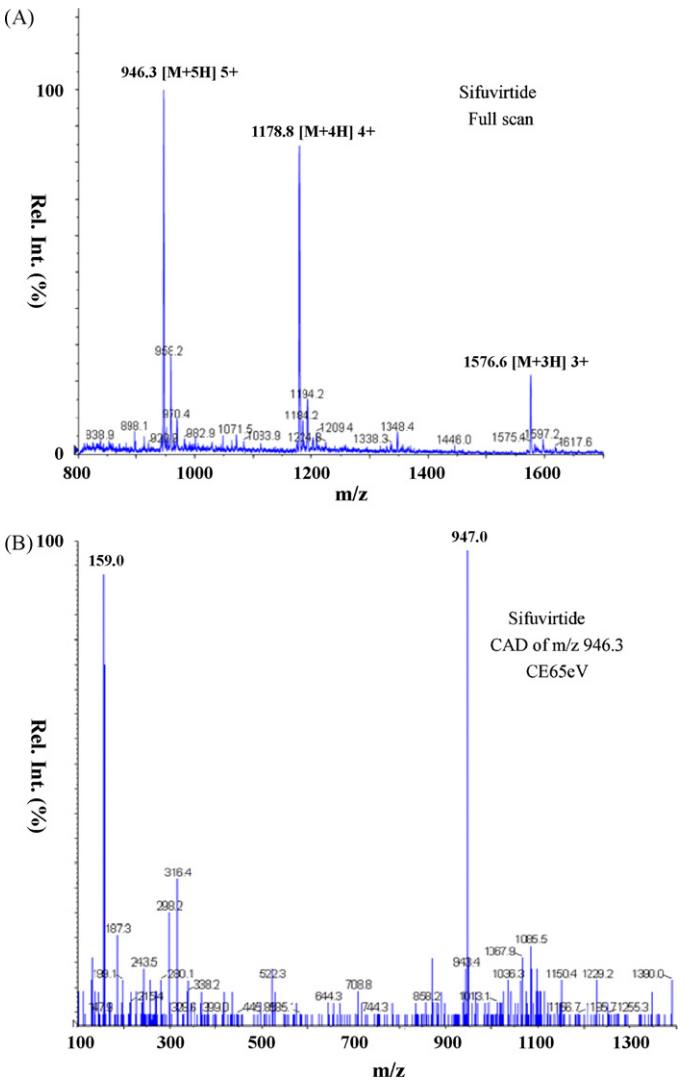

Fig. 1. ESI full scan mass spectra of sifuvirtide (A) and product ion spectra of $[M+5H]^{5+}$ at *m/z* 946.3 for sifuvirtide (B).

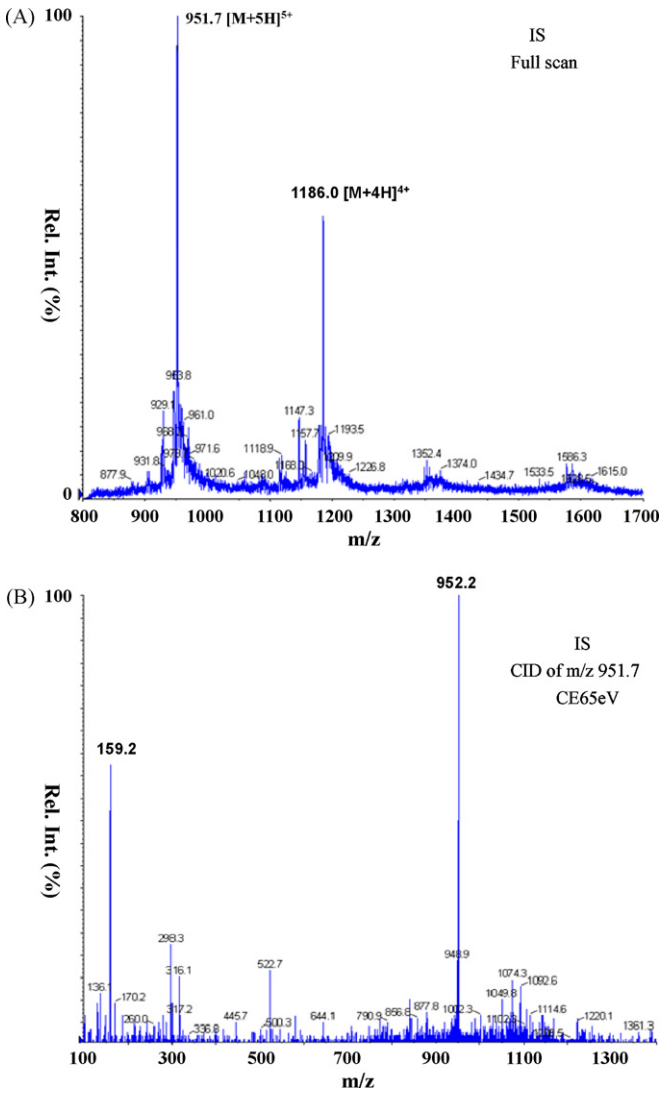

Fig. 2. ESI full scan mass spectra of IS (A) and product ion spectra of $[M+5H]^{5+}$ at *m/z* 951.7 for the IS (B).

3.5. Method validation

Validation was based on the FDA guidelines for Bioanalytical Method Validation [12].

3.5.1. Specificity

No significant interfering peaks from six different lots of plasma were found on the retention time of either the analyte or the IS which is shown in Fig. 3.

3.5.2. Linearity and sensitivity

Regression was linear over the tested concentration range (9.75–5000 ng ml^{-1}). The average correlation coefficient, R^2 was 0.998 (0.001 S.D.), calculated from five calibration curves. The standard curve was $Y = (0.12 \pm 0.03) + (4.84 \pm 0.08)x$. The LLOQ of analyte was only 9.75 ng ml^{-1} for the peptide (MW, 4727 Da) with S/N above 12. MRM chromatograms of LLOQ samples are shown in Fig. 3.

3.5.3. Assay precision and accuracy

The accuracy and precision of the method were determined by analyzing QC samples at low, middle, high concentrations. Six replicate samples at each concentration were analyzed in six separate runs. Intra-day and inter-day precision and accuracy results are presented in Table 1. Intra-day and inter-day precision values were <7.57% and 3.58%, respectively. Intra-day and inter-day accuracy ranged from 91.6% to 102.53% and from 95.53% to 105.28%, respectively.

3.5.4. Matrix effect and recovery

Matrix effects for analytes were evaluated by comparing the peak areas of the analyte at three QC concentrations spiked pos-

Table 1
Intra-day and inter-day accuracy and precision of sifuvirtide.

	QC1	QC2	QC3
Added (ng/ml)	9.75	312.5	5000
Intra-day ($n = 6$)			
Mean found (ng/ml)	9.63	286.33	5126.67
CV (%)	6.88	2.74	7.57
Accuracy (%)	98.75	91.63	102.53
Inter-day ($n = 6$)			
Mean found (ng/ml)	9.63	329.0	4776.67
CV (%)	2.71	2.65	3.58
Accuracy (%)	98.80	105.28	95.53

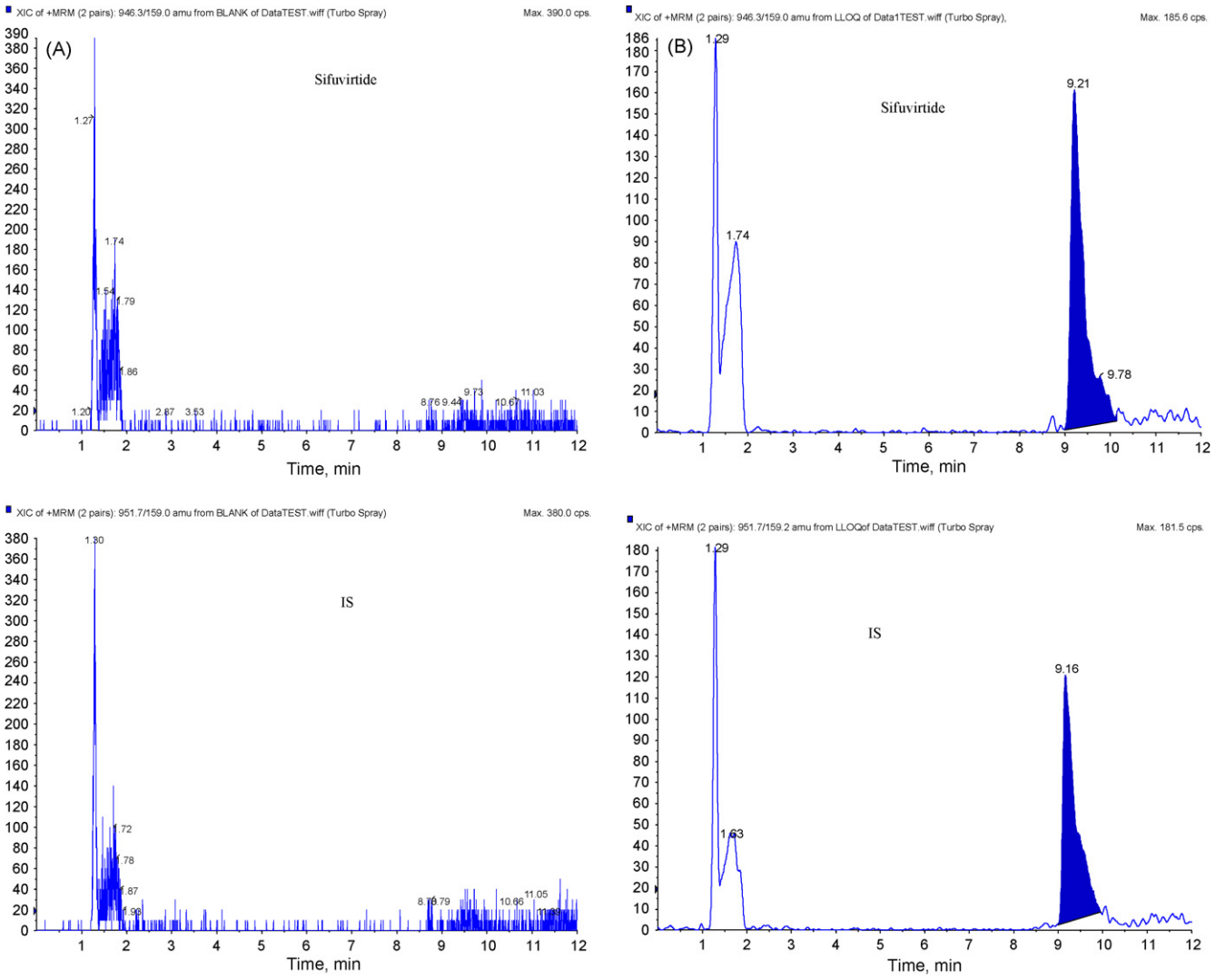

Fig. 3. Representative chromatograms: (A) blank plasma without IS, (B) LLOQ sample with IS and (C) subject sample with IS.

textraction into plasma extract, to the corresponding peak areas of the same analyte present in the mobile phase [12]. The mean matrix effect of sifuvirtide was between 110% and 124%. The CV of matrix effect of sifuvirtide was between 5.8% and 10.1%. The mean value should be within 15% of the actual value except at LLOQ and HLOQ (the higher limit of quantitation), where they should not deviate by more than 20%. However, the mean value of HLOQ was slightly higher than acceptable value. Therefore, post-column infusion experiments were used to determine whether there was matrix effect for sifuvirtide. 50 μl of mobile phase and blank plasma extracted was injected in HPLC. Sifuvirtide solution in the mobile phase at 5 μg ml^{-1} was infused post-column with the syringe pump at a flow rate of 5 μl min^{-1}. In Fig. 4, the signal was slightly suppressed between 1 and 2 min for the extracted plasma (the retention time of sifuvirtide is 9.21). Therefore, ion enhancement was not influenced by matrix components.

The extraction recoveries of sifuvirtide at three QC levels were determined by comparing peak area of the analytes obtained from

Table 2
Matrix effect and extraction recoveries of sifuvirtide from human plasma.[a].

Nominal concentration (ng/ml)	Mean peak area[b] (A)	Mean peak area[c] (B)	Mean peak area[d] (C)	Matrix effect[e] (%)	Recovery[f] (%)
9.75	984	846	650.6	116.3(CV10.12)	76.91
312.5	4,400	4,020	3,230	109.6(CV5.78)	80.35
5000	46,490	37,460	28,320	124.1(CV6.91)	75.60
2000 (IS)	8,200	6,740	5,218	121.6(CV7.18)	77.42

[a] $n = 6$.
[b] Standard in mobile phase.
[c] Standard spiked after extraction into extracts from five different plasma lots.
[d] Standard spiked before extraction into extracts from five different plasma lots.
[e] Matrix effect (%) expressed as the ratio of the mean peak area of an analyte spiked postextraction (B) to the mean peak area of the same analyte standards (A) multiplied by 100.
[f] Extraction recovery (%) expressed as the ratio of the mean peak area of the analytes spiked into plasma before extraction (C) to the mean peak area of the analytes spiked into plasma after extraction multiplied by 100 (B).

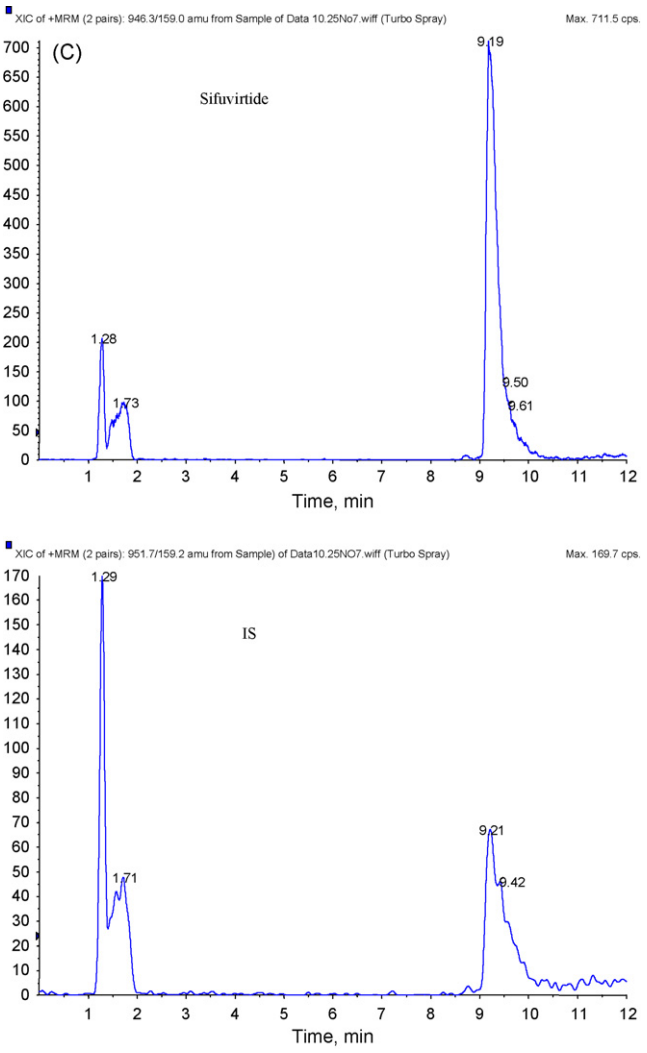

Fig. 3. (*Continued.*)

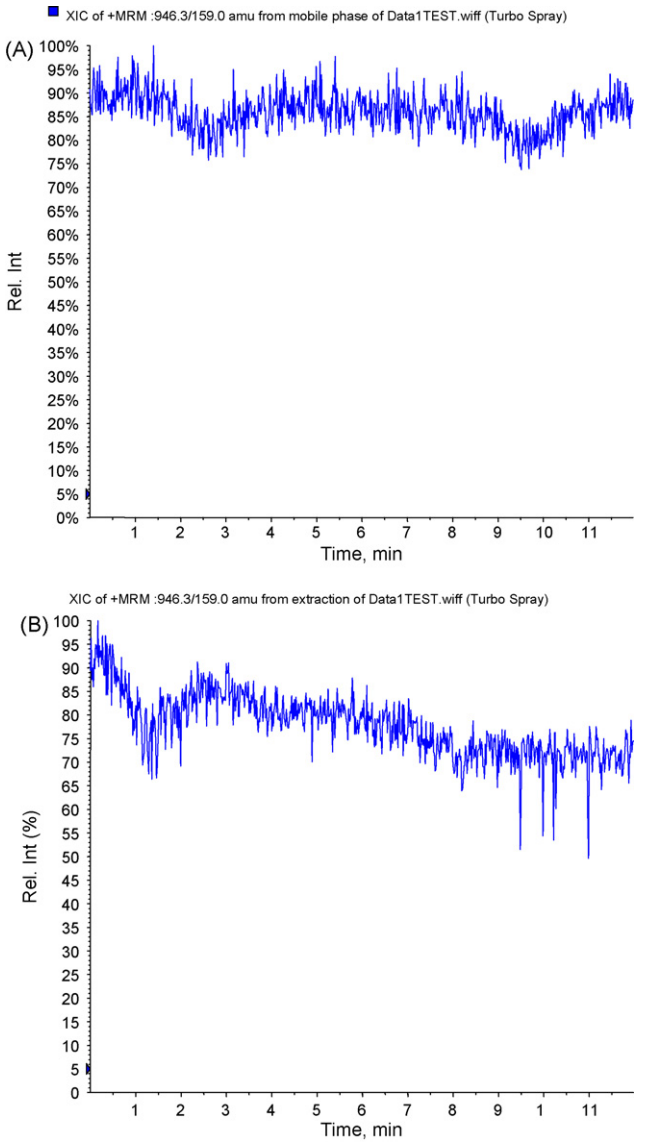

Fig. 4. LC–ESI-MS post-column infusion chromatograms: (A) mobile phase and (B) blank plasma extraction.

plasma samples spiked before extraction to those spiked after the extraction [12]. The mean extraction recovery of sifuvirtide was more than 75.6%. The results are shown in Table 2.

3.5.5. Stability

The stability of benchtop, freezer storage, freeze/thaw and inactivation of the QC samples were evaluated. The analyte was stable for at least 12 h at room temperature, at least 48 h at 4 °C, at least one month at −70 °C, and after three freeze–thaw cycles. HIV viral inactivation did not affect the stability of the samples. The results are summarized in Table 3.

3.6. Clinical study

The method was successfully used for analyzing samples from clinical study of a 28-day continuous subcutaneous injection (10 or 20 mg once daily) to provide pharmacokinetic profiles of the drug in 20 HIV-1 infected adults. Fig. 5 shows the concentration versus time curve of sifuvirtide of continuous sc 10 and 20 mg/day for 28 days of two patients. The main pharmacokinetic parameters of the two patients are listed in Table 4. After the 28th injection, sifuvirtide in plasma was detectable until 72 h in 10 mg/day group, and 120 h for 20 mg/day group. The plasma drug concentration reached steady state in the subjects after five consecutive doses of sifuvirtide (10 or 20 mg/day).There were no significant differences between T_{max}

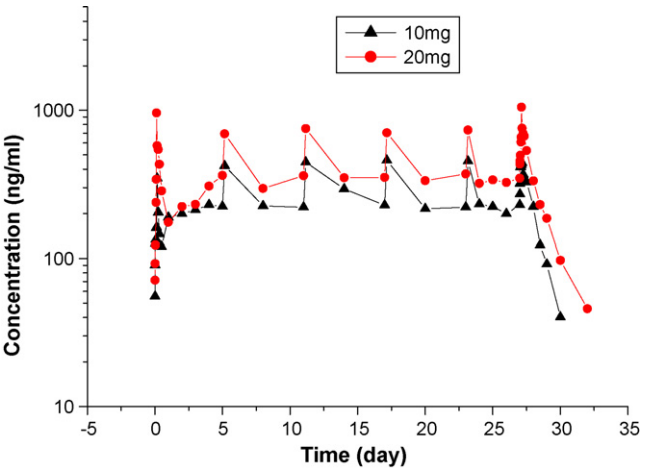

Fig. 5. Concentration vs. time curve of continuous sc 10 mg/day × 28 day (A) sifuvirtide of one patient and 20 mg/day × 28 day (B) sifuvirtide of one patient.

Table 3
Summary of stability of sifuvirtide in human plasma.

	QC1	QC2	QC3
Nominal concentration (ng/ml)	9.75	312.5	5000
Room temperature (12 h)			
Mean found concentration (ng/ml)			
$n = 5$	9.54	326.60	4730.00
CV (%)	4.95	3.86	6.15
Bias (%)	−2.11	4.51	−5.40
4 °C (48h)			
Mean found concentration (ng/ml)			
$n = 5$	9.708	295.8	4958
CV (%)	6.58	8.15	7.31
Bias (%)	−0.43	−5.34	−0.84
Freeze–thaw (3 times)			
Mean found concentration (ng/ml)			
$n = 5$	9.11	295.8	4958
CV (%)	10.41	8.15	7.31
Bias (%)	−6.56	−5.34	−0.84
HIV virus inactivation			
Mean found concentration (ng/ml)			
$n = 5$	9.26	316.8	5058
CV (%)	3.62	4.56	2.47
Bias (%)	−5.1	1.38	1.16
Long term (30 days)			
Mean found concentration (ng/ml)			
$n = 5$	9.21	314.60	5018.00
CV (%)	10.1	6.16	4.80
Bias (%)	3.09	0.67	0.36

Table 4
The main pharmacokinetic parameters of sifuvirtide.

The administration time	PK parameter	Unit	10 mg/day	20 mg/day
The first administration	$AUC_{(0-24h)}$	ng h ml^{-1}	3906	7784
	C_{max}	ng ml^{-1}	347	959
	T_{max}	h	4	3
The 28th administration	$AUC_{(0-24h)}$	ng h ml^{-1}	7718	13,025
	$AUC_{(0-72h)}$	ng h ml^{-1}	12,665	25,726
	$AUC_{(72h-\infty)}$	ng h ml^{-1}	1163	2357
	$AUC_{(0-\infty)}$	ng h ml^{-1}	13,827	28,084
	MRT	h	22	32.0
	CL	ml h^{-1} kg^{-1}	12.1	11.9
	Vss	ml kg^{-1}	267	380
	$t_{1/2}$	h	20.0	35.7
	K	1/h	0.0346	0.0194
	C_{max}	ng ml^{-1}	441	1050
	T_{max}	h	4.0	3.0
	C_{min}	ng ml^{-1}	226	340
	C_{av}	ng ml^{-1}	322	543
	DF	%	67	131

and C_{max} after the first and those after the 28th administration. This suggested there was no accumulative toxicity for sifuvirtide.

4. Conclusions

A novel sensitive LC–MS/MS method was developed for quantitation of sifuvirtide, a new HIV fusion inhibitor, in HIV$^+$ human plasma. The sample preparation was optimized. The HIV virus of plasma was treated by solvent/detergent method, not heat treatment as for enfuvirtide [8,9]. Various methods for protein removal were compared. In this method, a simple and safe protein precipitation procedure followed by a gradient HPLC with MS/MS detection offered sufficient selectivity for analysis of clinical samples. The method is sensitive with LLOQ of 9.75 ng ml^{-1}, which was similar to enfuvirtide (10 ng ml^{-1}) [9]. The assay has a wide calibration range of 9.75–5000 ng ml^{-1} for sifuvirtide. The method has successfully been used for the analysis of thousands of samples from clinical studies of sifuvirtide. This method appears to be the first method for quantitation of sifuvirtide in HIV$^+$ human plasma, which used S/D method and protein precipitation as sample processing procedure.

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