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Short communication

An improved on-line solid phase extraction coupled HPLC–MS/MS system for quantification of Sifuvirtide in human plasma

Qing-Qing Wang[a], Shen-Si Xiang[a], Yan-Bo Jia[a], Lun Ou[a], Fang Chen[a], Hai-Feng Song[a,*], Qing Liang[b], Dan Ju[b]

[a] Laboratory of Drug Metabolism and Pharmacokinetics, Beijing Institute of Radiation Medicine, Beijing 100850, China
[b] FusoGen Pharmaceuticals Inc., Tianjin 300051, China

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ABSTRACT

An improved liquid chromatographic method with on-line solid phase extraction (SPE) and tandem mass spectrometric detection was optimised for quantification of the anti-HIV peptide Sifuvirtide in human plasma. The SPE sorbents, loading buffer composition and other aspects of the on-line SPE column were investigated in detail for efficiently extracting the interesting peptides and simultaneously discarding the large amount of proteins. The gradient elution program was optimised on the analysis column to decrease the matrix effect and obtain excellent selectivity. The multiple charge ion at *m/z* 946.4 of Sifuvirtide was quantified by a linear ion trap mass spectrometer, operating in the positive mode, and selective reaction monitoring (SRM) acquisition. Method validation results demonstrated that the linear calibration curve covered a range of 6.1–6250 ng/mL, and the correlation coefficients (r^2) were above 0.992. The lower limit of detection (LLOD) with a signal-to-noise (S/N) ratio higher than 10 was 6.1 ng/mL. The accuracy ranged from −7.6 to 10.6%, and the intra- and inter-batch precisions were less than 8.7% and 5.5%, respectively. Finally, more than nine hundred of samples from a clinical trial was completely analyzed using this on-line SPE coupled HPLC–MS/MS system in one single week, due to the rapid run-time of individual sample (6.5 min).

1. Introduction

Peptides are emerging as a novel class of therapeutic agents in the pharmaceutical industry. With this growing interest in peptides, techniques for the quantification of these compounds in their biological environment are required. Although immunoassays are mainly used for the analysis of peptide drugs, high performance liquid chromatography–mass spectrometry (HPLC–MS) has now become the preferred analytical technique [1–3]. The HPLC–MS technique can discriminate co-eluting peptides with different masses, and by performing tandem mass spectrometry, more compound specific masses of fragments can be detected and used for quantification [4–6].

However, even with a highly selective and sensitive tandem mass spectrometry, quantification of large peptides (MW > 2000 Da) in biological matrices remains a challenging task [7,8]. The primary difficulty is selectively extracting the interesting peptide from the endogenous compounds in the matrices. Efficient and reproducible sample pretreatment is the key to successful quantification analysis. Solid phase extraction (SPE) is an especially useful extraction method due to its ability to perform selective separation both manually and on-line. More recently on-line SPE coupling with HPLC and multidimensional chromatography techniques have been demonstrated not only to increase the analytical selectivity but also to improve the repeatability and reliability of peptide quantification [9–11]. A successful example of an on-line two-dimensional chromatographic approach for peptide analysis in plasma was reported by Liu [12]. Reversed phase (RP) and hydrophilic interaction chromatography (HILIC) were coupled for the on-line extraction and quantitative analysis of 11 peptides. This system provided highly reproducible and robust results for over 300 sequential matrix injections and was applied to the quantification of peptide PTHrP (1–36) in rat plasma.

Sifuvirtide, a novel anti-HIV peptide, was designed based on the three-dimensional structure of the HIV-1 gp41 fusogenic core conformation. Biophysical analyses demonstrated that this peptide has a different mechanism of action than T20 [13,14]. In previous study, we developed an on-line SPE HPLC–MS/MS approach for determination of Sifuvirtide in monkey plasma [15]. Following a timed valve-switching event, the analyte was eluted on-line to a RP-HPLC column and subsequently introduced into the MS. However, even using the selective reaction monitoring (SRM) mode, about

* Corresponding author. Tel.: +86 10 6693 0259; fax: +86 10 6693 1230.
E-mail addresses: bapklab@yahoo.com, songhf@nic.bmi.ac.cn (H.-F. Song).

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80% matrix suppression effect was observed by co-eluting species in monkey plasma. A large volume of 100 μL monkey plasma and a lengthy run-time of 18 min were needed to conduct the quantification analysis for one single sample. Another off-line sample pretreatment coupled LC–MS system for quantification of Sifuvirtide in HIV infected human plasma was also reported [16]. Still, a time-consuming analysis turn-around (12 min) and a high consumption of plasma (500 μL) were required. Further, coefficient of variation (CV) for the high limit of quantification (HLOQ) went beyond the acceptable value (20%), even a structural analog was recruited as the internal standard (IS).

Thus, the goals of present study focused on improving the efficiency, cutting down the plasma sample consumption, and reducing the severe matrix effect of our previous on-line SPE HPLC–MS/MS system. The SPE sorbents, loading buffer composition, gradient elution programme and other aspects of the on-line SPE column were investigated and optimised in detail. Via the improved system, Sifuvirtide in human plasma could be accurately quantified using a volume of only 20 μL sample, and a better low limit of quantitation (LLOQ) of 6.1 ng/mL was obtained. Due to the use of an on-line extraction column and basic separation of the endogenous interferences from the analyte, there was no need for any complex sample pretreatment or the use of IS, the plasma can be directly injected into the system [5,17]. The total analysis turn-around time for one single sample, including on-line sample pretreatment and MS determination, was only 6.5 min. The rapid run-time of the system made it possible to fulfil the analysis of as many as 912 plasma samples from Chinese healthy male volunteers in a clinical trial in one single week.

2. Materials and methods

2.1. Drugs and reagents

The Sifuvirtide standard was provided by FusoGen Pharmaceuticals Inc. (Tianjin, PR China). Drug-free human plasma was obtained from the blood centre of 307 Hospital (Beijing, China). Acetonitrile (ACN) and other solvents were HPLC-grade and purchased from Fisher Scientific (Pittsburgh, PA, USA). Protease inhibitor cocktail tablets, Complete Mini (Roche Diagnostics, Mannheim, Germany) without ethylenediamine tetraacetic acid (EDTA), were used to prevent peptide degradation in the biological matrices. Formic acid (FA) was obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA). Ultrapure water was produced by a Millipore Simplicity 185 unit (Bedford, MA, USA).

2.2. Pharmacokinetic study and sample collection

Subjects recruited in phase I clinical trial were 24 healthy Chinese male volunteers. In a single dose cohort, 12 subjects were randomised to receive a single subcutaneous (*sc*) injection once daily at the dosage of 10, 20, or 30 mg Sifuvirtide followed a 3 × 3 cross-over design. In a multiple dose study, another 12 subjects received *sc* injection once daily at the dosage of 30 mg for 7 consecutive days.

Whole blood samples were collected at appointed time points and transferred into heparinised tubes, the protease inhibitor was then immediately added. After being placed on ice for 30 min, the samples were centrifuged at 10,000 × *g* for 10 min, the plasma samples were separated and stored at −20 °C until analysis.

2.3. On-line solid phase extraction and chromatography

The scheme of the on-line SPE HPLC–MS/MS system was similar to that in previous study [15,17] with minor modifications

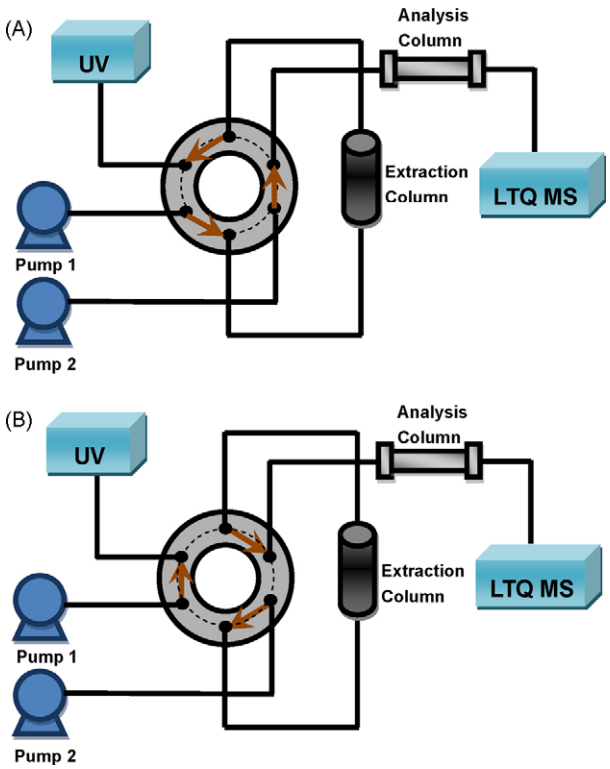

Fig. 1. The scheme of on-line solid phase extraction coupling with HPLC–MS/MS system. (A) The position of sample preparation and sample loading. (B) The position of sample elution and analysis.

(Fig. 1). Briefly, this system was composed of a one-column switching device using a six-port valve (Agilent Technologies, Waldbronn, Germany) and two solvent delivery units with an Agilent 1100 Binpump (Agilent Technologies, Waldbronn, Germany). One Binpump provided SPE column (50 mm × 2.1 mm i.d., 2 μm frits, Alltech Associates, Inc., Shanghai, China) with solvents for conditioning, sample application, cleanup, and equilibration. Another Binpump delivered solvents for elution and separation onto the analytical column (Grace Vydac C_8 column, 5 μm, 100 mm × 2.1 mm i.d., Hesperia, CA, USA).

In the sample pretreatment step, the valve stayed in position 1 (Fig. 1(A)), and a volume of 20 μL of the plasma sample was injected onto the extraction column, which was packed with RP materials based on octadecyl-bonded (Zorbax C_{18}, spherical, average particle size 50 mm, pore size 80 Å, Agilent Technologies, Palo Alto, USA). Then, protein and other interfering compounds were eluted with the wash solvent, while Sifuvirtide was retained on the extraction column. This course was monitored with UV detection at 280 nm.

In the elution and analysis step, the valve stayed in position 2 (Fig. 1(B)), and Sifuvirtide was flushed onto the analytical column for final separation and quantification. In the meantime, the extraction column was washed with a linear gradient at a flow rate of 0.2 mL/min to prepare for cleanup and recondition.

Finally, the switching valve was shifted to position 1 (Fig. 1(A)) again, which disconnected the extraction column and analytical column. The extraction column was successively washed with high ratio of organic solvents and equilibrated solution. The whole time schedule of the on-line SPE system is listed in Table 1.

2.4. Mass spectrometric conditions

A linear ion trap mass spectrometer (LTQ-MS, Finnigan, San Jose, CA, USA) equipped with an atmospheric pressure ionisa-

tion interface operating in electrospray ionisation (ESI) mode was applied. The spray voltage was 4.5 kV, and the temperature of the heated capillary was set at 200 °C. The flow rates of the sheath gas, auxiliary gas, and sweep gas were set (in arbitrary units/min) at 22, 5, and 2, respectively. Other parameters were optimised automatically by infusing the analyte in the mobile phase (acetonitrile/methanol/0.2% formic acid (15/15/70)) at a flow rate of 0.2 mL/min. The selective reaction mode (SRM) setup for the analyte Sifuvirtide was m/z 946.5 @ 35 → 871.8. Data were processed using LCQuan software (version 2.0). The computer was controlled by Xcalibur 1.4 software.

2.5. Stock solutions and calibration standard sample preparation

The stock solution of Sifuvirtide (1.0 mg/mL) was prepared in a solution of 10/90 (acetonitrile/water). Working stock solutions were prepared at 500 μg/mL by diluted (1:20) aliquots of the stock solution with the mobile phase of the analysis column (acetonitrile/methanol/0.2% formic acid (15/15/70). Calibration standards were prepared by spiking appropriate amounts of working stock solution to drug-free human plasma to make the concentrations of 6.1, 12.2, 48.8, 97.7, 195.3, 390.6, 1562.5, and 6250 ng/mL. The preparation procedures for quality control (QC) samples at concentrations of 15, 2000, and 5000 ng/mL were the same as that of the calibration standards. All of the standard stock, working stock, and calibration standard solutions were stored at −20 °C until use. Before the assay, the frozen samples were thawed at ambient temperature, vortex mixed and centrifuged at 10,000 × g for 10 min. For each sample, 50 μL aliquots of the supernatant were transferred to vials, and 20 μL aliquots of analyte were then introduced onto the SPE column for on-line preparation and LC–MS/MS analysis.

2.6. Validation of the method

The linear range of the calibration curve for Sifuvirtide was assessed by analysing series standard solutions by triplicate every day for three consecutive days. The regression of the plasma concentrations was weighted by 1/concentration. The LLOQ was defined as the lowest non-zero calibration standard yielding a precision of less than 20% and an accuracy of less than 20%. Intra- and inter-day precision were determined by calculating the CV of QC samples at low, medium, and high concentrations. The accuracy was assessed by calculating the relative error (RE), which compares the calculated and known concentrations of QC samples.

3. Results and discussion

3.1. The conditions of on-line SPE

3.1.1. The choice of the SPE sorbents

The greatest challenge for peptide quantification is that the approach should efficiently extract the interesting peptides and simultaneously discard the large amount of proteins. C_{18} sorbents are the most frequently used SPE materials in peptide extraction [2]. In a preliminary study, on-line SPE was carried out with the three kinds of C_{18} sorbents, including Zorbax C_{18} (Agilent, Palo Alto, USA), AcutBOND C_{18} (Agilent, Palo Alto, USA) and Sep-Vac C_{18} (Waters, Milford, USA). Independent of the pH value for sample loading and washing, the recoveries of the latter two sorbents were low, which was probably due to their asphericity and irregularity. Thus, the Agilent Zorbax spherical C_{18} sorbent was finally chosen for the on-line sample preparation.

3.1.2. Loading buffer composition

The sample injection composition often requires a relatively low organic content to achieve adequate focusing, especially when trying to maximise the injection volume and avoid peak symmetry issues. The percentage of acetonitrile in the loading buffer was evaluated from 10% to 60%. Peak splitting was observed when injected with 50% acetonitrile. Other researchers also observed the same phenomena when C_{18} sorbent was used as the SPE material [11]. The peptide solubility was explained with these results. With a high concentration organic solvents, the peptide was difficult to focus into one fraction and carry through the trap column without interacting with the stationary phase. Acetonitrile was set at 10% in the loading buffer to provide a balance between peptide retention and peptide solubility. Formic acid was also added for acidic conditions to enhance the trapping efficiency.

3.1.3. Other considerations

Some aspects that must also be taken into consideration were the loading capacity of the trap column, optimisation of column switch time and composition and flow rate of the eluting steps. Finally the elution of the analytes from the trap column was performed with an optimisation condition (Table 1) that provided the on-column stacking effect onto the analytical column.

3.2. The chromatographic conditions

The chromatographic conditions were optimised such that the analytes and matrix components were separated from each other as far as possible by changing the slope of the gradient on the anal-

Table 1
HPLC conditions for on-line SPE HPLC–MS/MS analysis.

Time (min)	Binary gradient for SPE column				Binary gradient for analysis column				Valve position
	A[a] (%)	B[b] (%)	Flow (μL/min)	Function	A (%)	C[c] (%)	Flow (μL/min)	Function	
0	90	10	1.0	Sample loading	70	30	0.2	Isocratic hold	A
1.5	90	10	1.0		70	30	0.2	Start gradient elution	
1.6	90	10	0.2	Flow decreasing	–	–	–	–	B
3.4	5	95	0.2	Wash preparation	–	–	–	–	
3.5	5	95	1.0	Start wash	20	80	0.2	End gradient elution	A
4.5	–	–	–		20	80	0.2	Isocratic hold	
4.6	–	–	–		70	30	0.3	Start equilibration	
5.0	5	95	1.0	End wash	–	–	–	–	
5.1	90	10	1.0	Recondition	–	–	–	–	
6.5	90	10	1.0		70	30	0.3	End equilibration	

[a] Solvent A: 0.2% formic acid in water.
[b] Solvent B: acetonitrile.
[c] Solvent C: acetonitrile/methanol = 50/50.

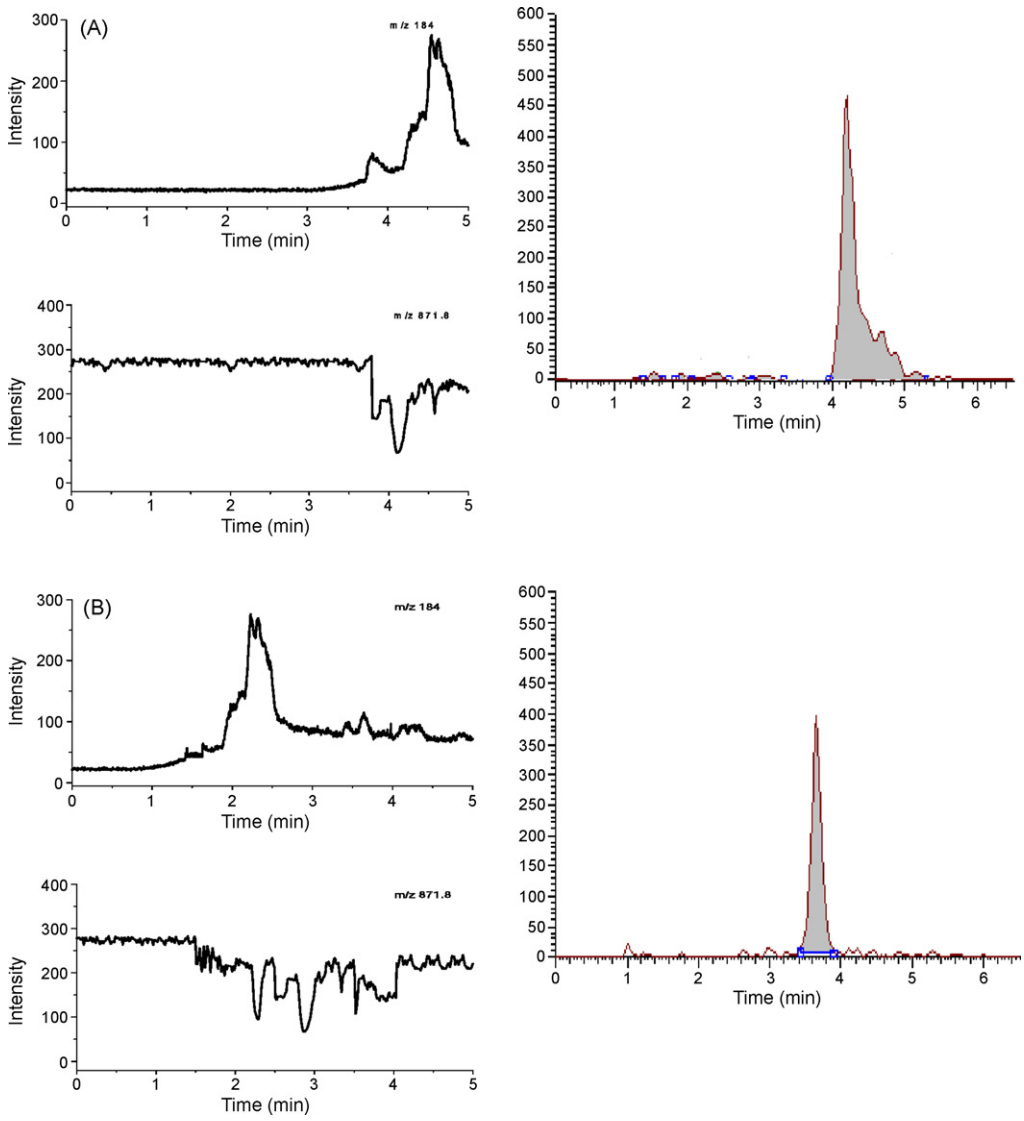

Fig. 2. Optimization of the chromatography conditions on analysis column by observing the peak of phospholipids (*m*/*z* 184). A blank human plasma sample was eluted from the trapping column to the analysis column with (A, left) isocratic or (B, left) gradient elution along with post-column infusion of Sifuvirtide (*m*/*z* 871.8). The retention time of Sifuvirtide was determined by directly injected the standard solution onto the trapping column and eluted at the same chromatography condition (A, right and B, right). HPLC condition: solvent A, 0.2% FA in water; solvent B, acetonitrile/methanol = 50/50; isocratic elution: 0–5 min, 70% A/30% B; gradient elution: 0 min, 70% A/30% B, 3.5 min, 20% A/80% B, 5 min, 20% A/80% B.

ysis column. The presence of ionisation suppression was confirmed by observing the peak of the phospholipids when running a blank plasma sample along with the post-column infusion of the analytes [9,18]. And the retention time of analyte was also determined by running sample without matrix at the same chromatographic conditions. The starting percentage of the organic phase was set at 30% for referencing the optimisation condition of the loading buffer composition on the SPE column. The retention time of Sifuvirtide was at about 4.4 min, co-eluting with the phospholipids (*m*/*z* 184) with isocratic elution (Fig. 2A). Finally, gradient elution was employed because it provided sufficient separation between endogenous interferences and Sifuvirtide (Fig. 2B) while still maintaining a good peak shape of the analyte.

After Sifuvirtide was eluted from the trap column, the six-port valve was switched back to the configuration of sample loading and sample preparation (Fig. 1A), and the ratio of acetonitrile in the trap column was rapidly increased to 95% to wash out contaminants and avoid carry-over effects in following runs. The total analysis time of one sample, with extraction, separation, MS analysis, and column re-equilibration was 6.5 min.

3.3. MS conditions

The mass spectrum was obtained by infusing 500 ng/mL solution of Sifuvirtide into LTQ using a syringe pump at a flow rate of 5 μL/min. ESI parameters such as capillary voltage, cone voltage and nebuliser gas were optimised to achieve a strong signal from multiple charged ions. The signal intensities and co-elution endogenous compound in blank plasma were compared in SRM LC–MS/MS mode. The three most intensive ions at *m*/*z* 946.4 ($[M+5H]^{5+}$), 1182.8 ($[M+4H]^{4+}$), and 1576.6 ($[M+3H]^{3+}$) were selected as the precursor ions to optimise the collision energy and helium gas flow. Finally, the fragment ion at *m*/*z* 871.8 ($b33^{5+}$) was selected as the product ion used in SRM acquisition regarding sensitivity and selectivity.

3.4. Validation of the on-line SPE coupled HPLC/MS/MS method

3.4.1. Assay specificity and matrix effect

Representative chromatography of drug-free plasma, plasma spiked with Sifuvirtide, and unknown plasma sample from vol-

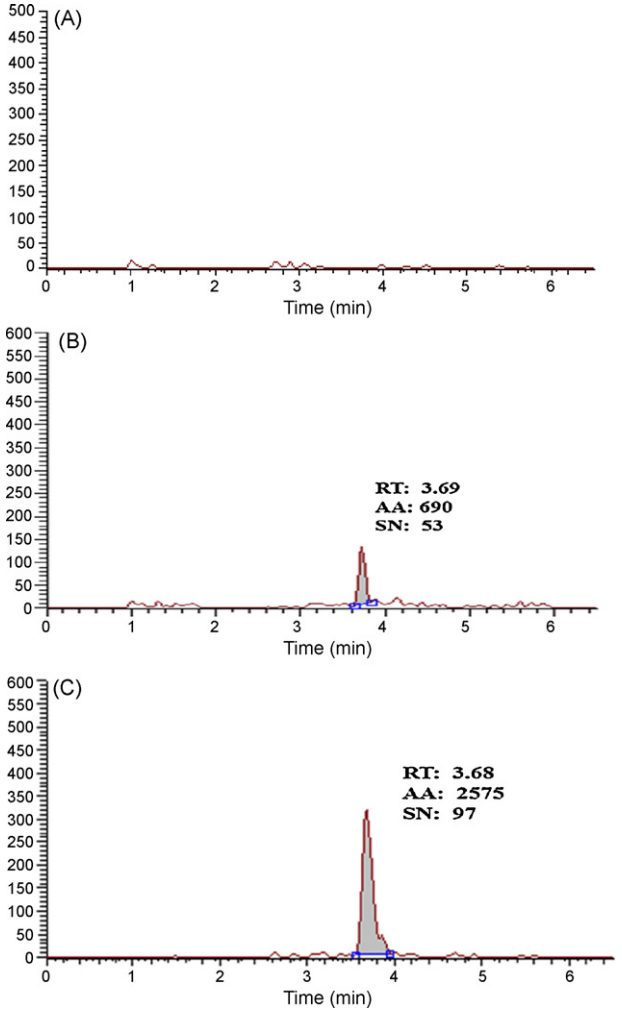

Fig. 3. Representative chromatograms of extracted blank human plasma (A), plasma spiked with 48.8 ng/mL Sifuvirtide (B), and test plasma at 4 h after *sc* injection of 20 mg Sifuvirtide (C).

unteers were acquired under the SRM operating in the positive mode (Fig. 3). It was demonstrated that no significant interferences were found at the retention time of the analyte. The matrix effect was estimated by comparing the response of sample spiked post-extraction with corresponding response of the standard prepared in the neat solution (Table 2).

3.4.2. Linearity and LLOQ

The linearity was evaluated in triplicate on three consecutive days using LCQuan quantitative analysis software and Finnigan LTQ Xcalibur software. The calibration curves were considered linear if the deviations from the nominal concentrations were below 20% at LLOQ and below 15% at all other levels. Linear regression with $1/x$ weighing was employed. The linear dynamic range of Sifuvirtide in human plasma was 6.1–6250 ng/mL ($R^2 > 0.992$), and the LLOQ with the signal-to-noise (S/N) ratio higher than 10 was 6.1 ng/mL.

3.4.3. Accuracy and precision

Inter-batch precision and accuracy were determined by analysis of five sets of QC samples (each $N = 1$) against five calibration curves in independent runs. Intra-batch data were obtained from 5-fold analysis of QC samples against one calibration curve in the same run. The results showed (Table 2) that the intra- and inter-batch CV (coefficient of variation) ranged from 1.3 to 8.7%, and the RE (relative error of the mean) ranged from −7.6 to 10.6%. These values indicated that the method was accurate, reliable and reproducible.

Table 2
Inter- and intra-day precision and accuracy of Sifuvirtide quality control samples in human plasma ($N = 5$).

	Concentration (ng/mL)		
	15	2000	5000
Intra-batch			
Mean ± S.D.[a] (ng/mL)	16.6 ± 1.4	1848.0 ± 70.2	4965.0 ± 64.5
CV[b] (%)	8.7	3.8	1.3
RE[c] (%)	10.6	−7.6	−0.7
Inter-batch			
Mean ± S.D. (ng/mL)	16.1 ± 0.9	1934.5 ± 85.1	4949.1 ± 143.5
CV (%)	5.5	4.4	2.9
RE (%)	7.1	−3.3	−1.0
Matrix effect[d]			
Mean ± S.D. (%)	91.2 ± 4.3	93.5 ± 3.1	95.1 ± 6.5

[a] S.D.: standard deviation.
[b] CV: coefficients of variation; RE: relative error.
[c] RE (%) = [(mean concentration − nominal concentration)/nominal concentration] × 100.
[d] Matrix effect (%) = [(mean peak area)$_{\text{post-extraction spike}}$/(mean peak area)$_{\text{neat}}$] × 100.

3.5. Application and column stability

As above mentioned, in the phase I clinical trial, a total of 912 plasma samples derived from Chinese healthy male volunteers were prepared, by using the improved on-line SPE coupled HPLC–MS/MS system optimised in present study. The quantification of all samples was successfully accomplished within one single week without loss the system performance.

For maintenance of the performance, the SPE column was conditioned with 1% FA in water/methanol/isopropanol (30/30/40) overnight at the end of every 200 injections. Furthermore, the back pressures of the SPE column and analysis column were also monitored at the start of each analysis. The results showed that if the pressure of the SPE column was same, the extraction efficiency was also similar. The variance of SPE pressure was within 2 bar in the whole duration of sample analysis.

4. Conclusion

In present study, an improved method of on-line SPE coupling with HPLC–MS/MS was developed and validated to ensure rapid and reliable determination of Sifuvirtide in human plasma. Compared with previous reports, the current improved system had some particular superiorities, such as (1) the short run-time of individual sample analysis, which gifted the rapid turn-around time of the experiment; (2) less plasma consumption, which enabled the method be more adaptable in some extreme cases, such as for analysis of samples from cachexia patients with AIDS or malignant tumors, or small animals with little blood volume for sampling; (3) matrix effect independence, which made present method be flexible for other biological matrix, such as urine or tissue samples, without major difficulties.

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