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Development and validation of a quantitative assay for the measurement of two HIV-fusion inhibitors, enfuvirtide and tifuvirtide, and one metabolite of enfuvirtide (M-20) in human plasma by liquid chromatography–tandem mass spectrometry

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Abstract

A method for the quantification of two peptide HIV-1 fusion inhibitors (enfuvirtide, T-20 and tifuvirtide, T-1249) and one metabolite of enfuvirtide (M-20) in human plasma has been developed and validated, using liquid chromatography coupled with electrospray tandem mass spectrometry (LC–MS/MS). The analytes were extracted from plasma by solid-phase extraction (SPE) on vinyl-copolymer cartridges. Chromatographic separation of the peptides was performed on a Symmetry 300 C_{18} column (50 mm × 2.1 mm I.D., particle size 3.5 μm), using a water–acetonitrile gradient containing 0.25% (v/v) formic acid. The triple quadrupole mass spectrometer was operated in the positive ion-mode and multiple reaction monitoring (MRM) was used for peak detection. Deuterated (d60) enfuvirtide and (d50) tifuvirtide were used as internal standards. The assay was linear over a concentration range of 20–10,000 ng/ml for enfuvirtide and tifuvirtide and of 20–2000 ng/ml for M-20. Intra- and inter-assay precisions and deviations from the nominal concentrations were ≤13%. Stability of the analytes was tested under all relevant conditions for sample handling. The method was capable to measure concentrations of enfuvirtide and its metabolite in plasma samples of human immunodeficiency virus type-1 (HIV-1) infected patients treated with the drug.

Keywords: HIV-fusion inhibitor; Enfuvirtide; Tifuvirtide; Peptide; LC–MS/MS

1. Introduction

Enfuvirtide (Fuzeon®, T-20) and tifuvirtide (T-1249) both belong to a novel class of antiretroviral agents for the treatment of human immunodeficiency virus type-1 (HIV-1): the HIV-1 fusion inhibitors. Enfuvirtide has successfully completed phase III clinical trials and is commercially available worldwide. The mechanism of action, pharmacokinetics, therapeutic efficacy, tolerability and availability of enfuvirtide are discussed in several articles [1–8]. Tifuvirtide is a second-generation fusion inhibitor and has shown to retain activity against enfuvirtide resistant virus [9–11]. However, development of tifuvirtide has been suspended due to production difficulties [12].

Both compounds block viral fusion by inhibiting the interaction of the gp41 transmembrane glycoprotein of HIV-1 with the host cell. Enfuvirtide is a 36 amino acid peptide with a molecular weight of 4492 Da and corresponds to a part of the second heptad repeat region (HR2) of HIV-1 gp41. Tifuvirtide consists of 39 amino acids with a molecular weight of 5037 Da and is derived from different sections of the HIV-1, HIV-2 and simian immunodeficiency virus (SIV) gp41 transmembrane glycoprotein [13–15]. Fig. 1 depicts the amino acid sequences of both peptides.

Enfuvirtide is administered subcutaneously and the recommended dose is 90 mg twice daily. A metabolite (M-20) is formed by deamidation at the C-terminus. Except for the hydroxylated phenylalanine C-terminus, M-20 has the same amino acid sequence as enfuvirtide and has a molecular weight of 4493 Da. M-20 has approximately 20% of in vitro activity of the parent compound. The areas under the plasma concentration–time

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						1					6				
Enfuvirtide					Ac	Tyr	Thr	Ser	Leu	Ile	His	Ser	Leu	Ile	
		11					16					21			
	Glu	Glu	Ser	Gln	Asn	Gln	Gln	Glu	Lys	Asn	Glu	Gln	Glu	Leu	
			26					31					36		
	Leu	Glu	Leu	Asp	Lys	Trp	Ala	Ser	Leu	Trp	Asn	Trp	Phe	Am	
			1					6					11		
Tifuvirtide		Ac	Trp	Gln	Glu	Trp	Glu	Gln	Lys	Ile	Thr	Ala	Leu	Leu	
				16					21					26	
	Glu	Gln	Ala	Gln	Ile	Gln	Gln	Glu	Lys	Asn	Glu	Tyr	Glu	Leu	
					31					36					
	Gln	Lys	Leu	Asp	Lys	Trp	Ala	Ser	Leu	Trp	Glu	Trp	Phe	Am	

Fig. 1. Amino acid sequences of enfuvirtide and tifuvirtide. N-termini are acetylated (Ac) and C-termini are amidated (Am). Similarities in amino acids are shown in grey.

curve (AUC) of the metabolite range from 2.4 to 15% of the enfuvirtide AUC values [16,17].

A 14-day phase 1/2 study of tifuvirtide showed very potent antiretroviral responses after once daily doses of 144 and 192 mg by subcutaneous injection [9].

For pre-clinical and clinical investigations of drug concentrations in plasma a quantitative bioanalytical method is required. Quantification of large peptides, however, remains difficult, mainly because of interferences from biological matrices. Therefore, highly selective, robust and accurate methods are required, in which sample extraction techniques play an important role in order to remove interfering matrix components.

Conventionally, immunoassays have been widely employed for the quantification of peptides and proteins. However, accurate quantification is limited with this technique since cross-reactivity can occur when the used antibodies cannot discriminate structurally related compounds. Nowadays, liquid chromatography–tandem mass spectrometry (LC–MS/MS) has become the preferred technique for sensitive and selective quantification in biological matrices. LC–MS/MS assays for the quantification of peptides in biological samples have been described in combination with different sample extraction techniques, such as protein precipitation [18,19], liquid–liquid extraction [20,21] and solid-phase extraction (SPE) [22–25].

For enfuvirtide a quantitative HPLC method with fluorescence detection has been described [26]. More recently, Chang et al. [27] reported a bioanalytical method for enfuvirtide and its metabolite (M-20) using LC–MS/MS. In both methods, sample preparation was performed by protein precipitation. For the quantification of another fusion inhibitor, the 36 amino acid peptide sifuvirtide, a LC–MS/MS method was developed with a more selective on-line SPE procedure [28].

Besides the quantification of enfuvirtide and its de-amidated metabolite, the LC–MS/MS assay described in this report is applicable for the quantification of a second fusion-inhibitor, tifuvirtide. Two stable isotopically labelled internal standards were used, with d60-enfuvirtide as internal standard for the quantification of both enfuvirtide and M-20 and with d50-tifuvirtide as internal standard for the quantification of tifuvirtide.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile (gradient grade), methanol (HPLC grade), formic acid (analytical grade) and LC–MS grade water were from Biosolve Ltd. (Valkenswaard, The Netherlands). Ammonium hydroxide (A.C.S. reagent) and trifluoroacetic acid (99^{+}%, spectrophotometric grade) were obtained from Sigma–Aldrich Inc. (Steinheim, Germany). Drug free human plasma was obtained from the Sanquin Bloedbank (Utrecht, The Netherlands). Enfuvirtide was supplied by Cook Pharmaceutical Solutions (Bloomington, IN, USA) with 98.6% purity. M-20 was kindly supplied by Hoffmann-La Roche (Nutley, NJ, USA) with 81.4% purity. Tifuvirtide and stable isotopically labelled internal standards (IS) for enfuvirtide and tifuvirtide were synthesised and kindly supplied by H. Hilkmann (The Netherlands Cancer Institute, Amsterdam, The Netherlands). The peptides were produced by solid phase peptide synthesis on a Syro II peptide synthesizer (MultiSynTech GmbH, Germany), using Fmoc chemistry with PyBop and Dipea as activators. For synthesis of the stable isotopically labelled ISs all leucine residues in the molecule were replaced by d10-leucine. Purities of tifuvirtide, d60-enfuvirtide and d50-tifuvirtide were 48.9, 63.3 and 5.7%, respectively and were determined according to the Edelhoch method with enfuvirtide as a reference [29,30].

2.2. Equipment

HPLC was performed using a Shimadzu system (Shimadzu, Kyoto, Japan) consisting of a SCL-10Avp system controller, a LC-10Advp-μ pump, a SIL-HTC autosampler, a CTO-10Avp column oven, a DGU-14A degasser and a SPD-10Avp UV-VIS detector. Both analytical column (Symmetry 300 C_{18}, 50 mm × 2.1 mm I.D., 3.5 μm particle size) and pre-column (Symmetry 300 C_{18} pre-column, 10 mm × 2.1 mm I.D.) were from Waters (Waters Chromatography, Milford, MA, USA). The HPLC system was connected to a Finnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer (Thermo Electron Inc., San Jose, CA, USA) equipped with an electrospray source. Data were processed using Xcalibur Software from Thermo Electron. Oasis HLB 1 ml extraction car-

tridges (Waters), containing 30 mg of poly(divinylbenzene-co-*N*-vinylpyrrolidone) copolymer, were used for sample pretreatment by SPE.

2.3. LC–MS/MS conditions

A gradient HPLC method was employed for separation. Mobile phase A consisted of 0.25% (v/v) formic acid in water and mobile phase B consisted of 0.25% (v/v) formic acid in acetonitrile. Eluent composition raised linearly after injection from 35 to 45% of eluent B in 5 min and then linearly to 75% B during 2.5 min. After maintaining at 75% B for 1.5 min the eluent composition returned to 35% B for re-equilibration during 2 min. Total run time was 11.0 min. The flow rate was 0.2 ml/min, except between 5 and 9 min when the rate was 0.4 ml/min. The analytical column was placed in an isolated box with melting ice, to achieve a column temperature of 0 °C. The autosampler temperature was set at 15 °C.

The ESI source of the mass spectrometer was operated in positive ion mode, with the nitrogen sheath, ion sweep and auxiliary gasses set at 49, 50 and 24 arbitrary units (AU), respectively. The capillary temperature was maintained at 242 °C and the capillary voltage was set at 4.5 kV. Resolutions of Q1 and Q3 quadrupole were set at 1.4 FWHM, to measure all the different natural isotopes of the peptides. Argon was used as collision gas and the collision energy and collision pressure were set at 18 eV and 1.5 mTorr, respectively. Individual parameters for the different compounds are listed in Table 1.

For stability determinations of the stock and working solutions and for purity determinations with the Edelhoch method UV detection was used at a wavelength of 280 nm.

2.4. Preparation of standards and quality control samples

All stock and working solutions were prepared in methanol: 0.1% (v/v) formic acid in water, 1:1 (v/v), except for the stock solution of M-20, which was, because of solubility, prepared in methanol: 0.4% (w/v) ammonium hydroxide in water, 1:1 (v/v). Stock solutions of enfuvirtide and tifuvirtide were prepared at a concentration of 1 mg/ml and stock solutions of M-20 were prepared at a concentration of 0.2 mg/ml. These stock solutions were combined and further diluted to obtain working solutions with concentrations of 300/300/60 and 100/100/100 μg/ml of enfuvirtide, tifuvirtide and M-20, respectively.

Plasma was spiked with appropriate volumes of working solutions to provide plasma samples of 7500/1500 and 500/500 ng/ml enfuvirtide and tifuvirtide/M-20. These samples were diluted with blank human plasma to obtain quality control samples of 7500/1500; 1950/390; 50/50 and 20/20 ng/ml enfuvirtide and tifuvirtide/M-20.

Independently prepared stock solutions were combined and diluted similarly to obtain a working solution with a concentration of 250/250/50 μg/ml of enfuvirtide, tifuvirtide and M-20, respectively, for the preparation of calibration standards. Calibration standards were obtained by serial dilution prior to pretreatment with blank human plasma to obtain calibration standards of 20, 100, 200, 500, 1000, 2000, 5000 and 10,000 ng/ml for enfuvirtide and tifuvirtide and of 4, 20, 40, 100, 200, 400, 1000 and 2000 ng/ml for M-20. Stock solutions of the deuterated ISs (0.5 mg/ml) were combined and diluted to a concentration of 50 μg/ml. All solutions were stored at −20 °C.

Table 1
MS/MS-parameters for all analytes

Compound	Tube lens off set	Parent ion (*m/z*)	Product ion (*m/z*)
Enfuvirtide/M-20	124	1124.0	1343.5
Tifuvirtide	102	1008.4	1219.0
d60-Enfuvirtide	131	1139.0	1363.5
d50-Tifuvirtide	113	1018.4	1231.5

2.5. Sample preparation

Oasis HLB solid-phase extraction cartridges were conditioned with 1 ml methanol and 1 ml 0.1% trifluoroacetic acid in water. A 500 μl aliquot of plasma, to which 10 μl of IS working solution was added, was loaded onto the cartridge. The SPE cartridge was washed with 1 ml of 0.1% (v/v) trifluoroacetic acid and 1 ml of acetonitrile:water:trifluoroacetic acid 20:80:0.1 (v/v/v), respectively. The analytes were eluted with 1 ml of acetonitrile:water:trifluoroacetic acid 60:40:0.1 (v/v/v). The eluate was transferred to an injection vial and 10 μl was injected onto the LC–MS/MS system.

2.6. Validation

Validation was based on the FDA guidelines for Bioanalytical Method Validation [31].

2.6.1. Linearity

Calibration standards ranging from 20 to 10,000 ng/ml for enfuvirtide and tifuvirtide and from 4 to 2000 ng/ml for M-20 were analysed in five separate runs. Ratios of analyte versus IS 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.

2.6.2. Precision and accuracy

The precision and accuracy of the method were determined by analysis of quality control samples at four different concentration levels in three separate runs ($n = 6$ at each level). The MQC concentration is defined to be in the middle range of the standard curve [31]. However, for a better reflection of the total calibration range by the QC samples, a more logarithmic distribution is chosen with the MQC concentration at about 20% of the upper limit of quantification (ULQ). Deviations of the accuracies were expressed as [((overall mean concentration − nominal concentration)/nominal concentration) × 100%]. Average intra-assay precision was defined as the average relative standard deviation of the three runs and inter-assay precision as the relative standard deviation of the overall measured concentrations ($n = 18$). Accuracy should be within 15% and precision should be less than 15%. At the lower limit of quantification (LLOQ) accuracy should be within 20% and precision should be less than 20%.

Accuracy and precision of samples with a concentration above the ULQ (15,000 ng/ml enfuvirtide and tifuvirtide; 3000 ng/ml M-20) were validated by analysing the samples after a five-fold dilution with blank human plasma ($n = 6$; one run).

2.6.3. Specificity and selectivity

Specificity and selectivity of the assay were assessed by analysing six blank plasma samples and six plasma samples at LLOQ level from six different sources. To determine whether co-medication interferes with the method, six different mixtures of common co-medication used during anti-HIV treatment were added to LLOQ and blank plasma samples. The tested co-medication mixtures were: tenofovir, delaviradine, methadone, fluconazole, nevirapine, pyrimethamine (mix 1); abacavir, lamivudine, morphine, pyrazinamide, 3′amino-3′-deoxythymidine, nelfinavir (mix 2); amprenavir, stavudine, caffeine, itraconazole, efavirenz (mix 3); ritanovir, zalcitabine, paracetamol, trimethoprim, rifampicin (mix 4); saquinavir, zidovudine, oxazepam, ganciclovir, sulfamethoxazole (mix 5); indinavir, didanosine, ranitidine, folic acid and zidovudine-glucuronide (mix 6). Concentrations of co-medications were 5 μg/ml, except for morphine (18 μg/ml), amprenavir (2.5 μg/ml), caffeine (7.5 μg/ml) and paracetamol (10 μg/ml).

Peak areas of endogenous and exogenous compounds co-eluting with the analytes or internal standards should be less than 20% of the peak area of the LLOQ standard and less than 5% of the response of the IS. The deviations of the nominal concentrations for the LLOQ samples should be within ±20%.

2.6.4. Recovery and ion suppression

The extraction recovery was determined by comparing the peak areas of blank human plasma samples spiked before extraction with blank human plasma samples spiked after extraction at three different concentration levels (low, mid and high QC). The peak areas of plasma samples spiked before extraction and plasma samples spiked after extraction were also compared to the peak areas of reference samples in elution solvent to determine total recovery and ion suppression in the ESI source, respectively. All samples were analysed in triplicate.

2.6.5. Stability

The stability of enfuvirtide, tifuvirtide and M-20 was examined at various concentrations during all stages of the method. Stability of stock and working solutions were determined by comparing UV peak areas of stored samples with peak areas of freshly prepared solutions. The stability of the three analytes in plasma was tested under various conditions, including 24 and 8 h at ambient temperature, three additional freeze-thaw cycles and storage at −20 °C for 3 months. To examine the stability in the final extract, the concentrations in the extracts after 24 h storage in the autosampler (15 °C) was determined by comparison with freshly prepared calibration standards. For all stability tests QC samples at three concentration levels (high, mid and low) were analysed in triplicate and deviations from the initial concentrations were determined in relation to freshly prepared samples. The analytes are considered stable in plasma when 85–115% of the initial concentration is found. Stability in stock and working solutions is accepted when 95–105% of the initial concentration is found.

2.7. Analysis of patient samples

The described assay was used to determine enfuvirtide and M-20 concentrations in plasma from HIV-1 infected patients to show the applicability of the method. These patients received enfuvirtide as part of their anti retroviral regimen. As enfuvirtide is used for the treatment of highly resistant viruses, patients received a range of other anti retroviral drugs (including a nucleoside backbone and a boosted protease inhibitor).

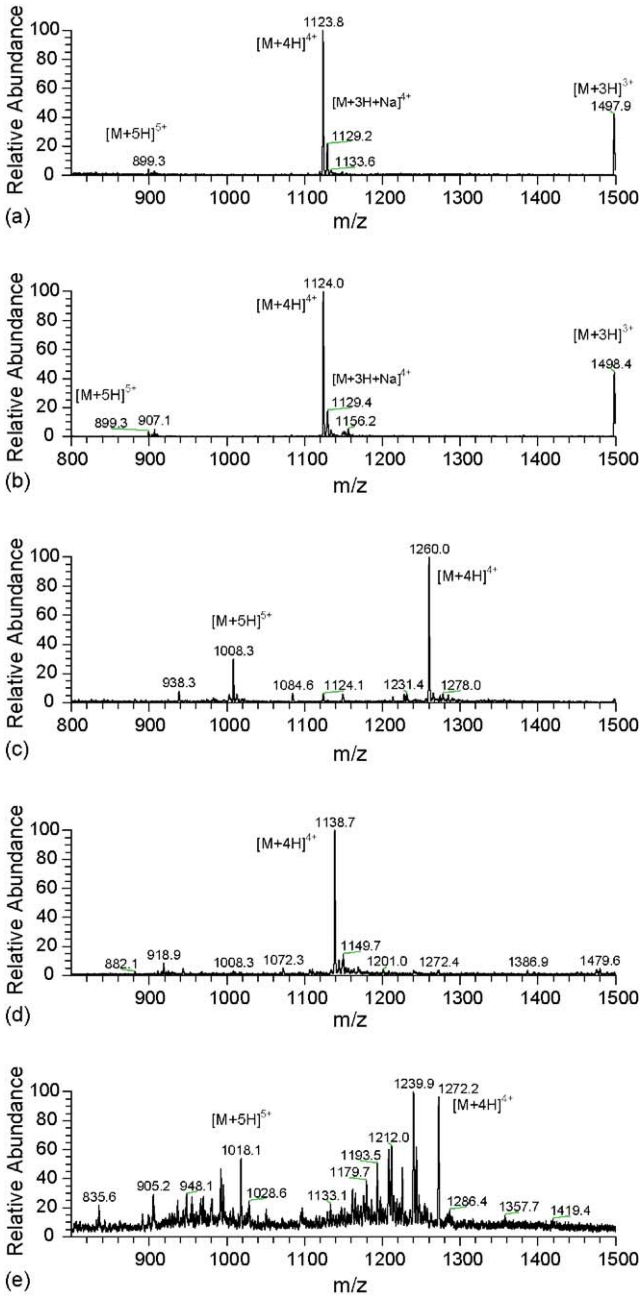

Fig. 2. Mass spectra of (a) enfuvirtide; (b) M-20; (c) tifuvirtide; (d) d60-enfuvirtide and (e) d50-tifuvirtide. Q1 resolution was 0.70 FWHM.

3. Results and discussion

3.1. Method development

3.1.1. Sample pre-treatment

In earlier studies of enfuvirtide protein precipitation has been used as sample pre-treatment technique [26,27]. However, in our laboratory these techniques were either too laborious or resulted in strong interferences from the sample matrix. Therefore, SPE was selected as sample pre-treatment technique for the isolation of the investigated peptides. SPE can offer additional separation of the analyte from possible interfering matrix components, since changing sorbent materials and solvent composition can optimise its selectivity.

Different types of SPE columns were evaluated for the extraction of enfuvirtide and tifuvirtide from plasma. These included silica based extraction columns with C_2, C_8, C_{18} and CN sorbent material, a mixed mode column with C_8 sorbent and anion exchange properties and Oasis HLB columns containing copolymer. Recovery of enfuvirtide was 60% on the C_{18} column and about 80% for all the other silica-based extraction columns. The difference with the recovery on Oasis HLB columns (85%) was

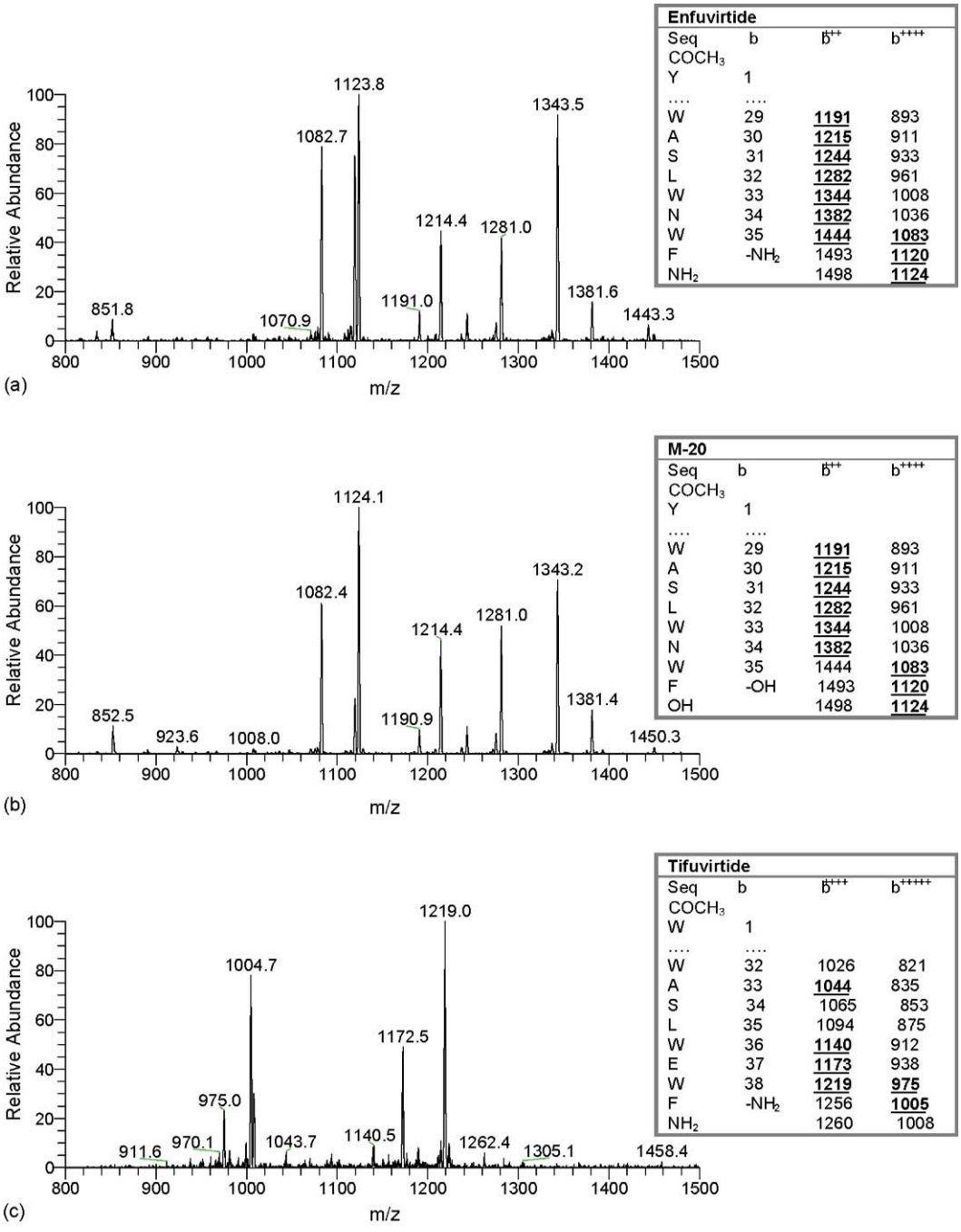

Fig. 3. Product ion spectra of (a) enfuvirtide; (b) M-20 and (c) tifuvirtide at a collision energy of 21 eV and a collision pressure of 1.5 mTorr. Precursor ions were *m/z* 1124.0 for enfuvirtide and M-20 and 1008.4 for tifuvirtide. Q1 and Q3 resolutions were 1.4 FWHM. Inlets show corresponding peptide fragments.

relatively small. However, for tifuvirtide the difference in recovery on the copolymer based columns (±80%) compared to all the silica-based columns (±50%) was much higher. Recoveries of M-20 and the ISs were only compared to the recovery of the mixed-mode column, but showed similar improvements. Different conditioning, washing and elution solvents were tested to further optimise the procedure. Addition of 0.1% TFA to all solvents showed a beneficial effect for the recovery of the peptides.

The eluate was directly injected into the LC–MS/MS system, since drying and concentrating did not lead to reproducible results, probably caused by adhesion of the peptides to container surfaces. Moreover, omission of a time-consuming evaporation step results in a more simple and rapid sample pre-treatment procedure.

3.1.2. MS/MS optimisation

Parent ion spectra of enfuvirtide, M-20, tifuvirtide and ISs are shown in Fig. 2. For enfuvirtide and M-20 the most abundant ions were the $[M+4H]^{4+}$ ions at *m/z* 1123.8 and 1124.0, respectively. The mass spectrum of tifuvirtide shows two abundant peaks, corresponding to the $[M+5H]^{5+}$ ion and the $[M+4H]^{4+}$ ion. Fragmentation of the $[M+4H]^{4+}$ ion of tifuvirtide resulted in very low abundant product ions, whereas fragmentation of the $[M+5H]^{5+}$ ion yielded several useful transitions (Fig. 3). The product ion spectra of enfuvirtide and M-20 show the same fragmentation pattern (Fig. 3). The transitions 1124.0 → 1343.5 for enfuvirtide and M-20 and 1008.4 → 1219.0 for tifuvirtide were selected, since they showed the highest selectivities and signal-to-noise ratios. Product ion spectra of d60-enfuvirtide and d50-tifuvirtide are shown in Fig. 4.

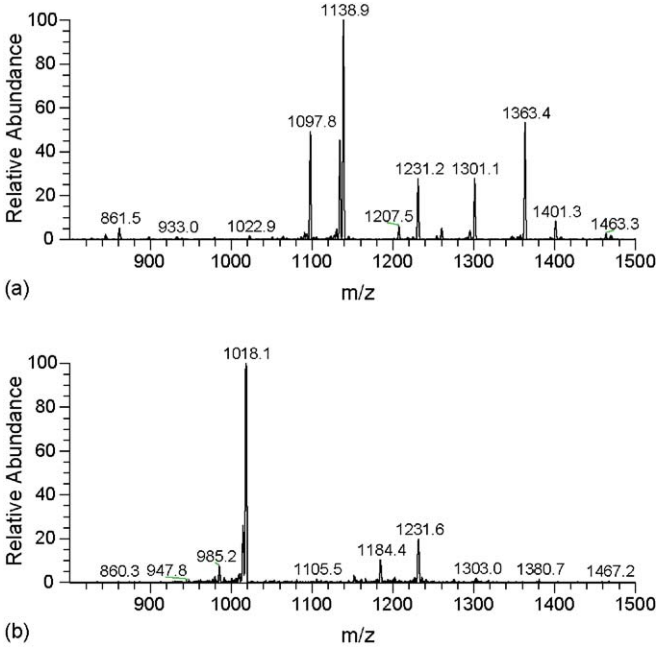

Fig. 4. Product ion spectra of (a) d60-enfuvirtide and (b) d50-tifuvirtide at a collision energy of 21 eV and a collision pressure of 1.5 mTorr. Precursor ions were *m/z* 1139.0 for d60-enfuvirtide and 1018.4 for d50-tifuvirtide. Q1 and Q3 resolution were 1.4 FWHM.

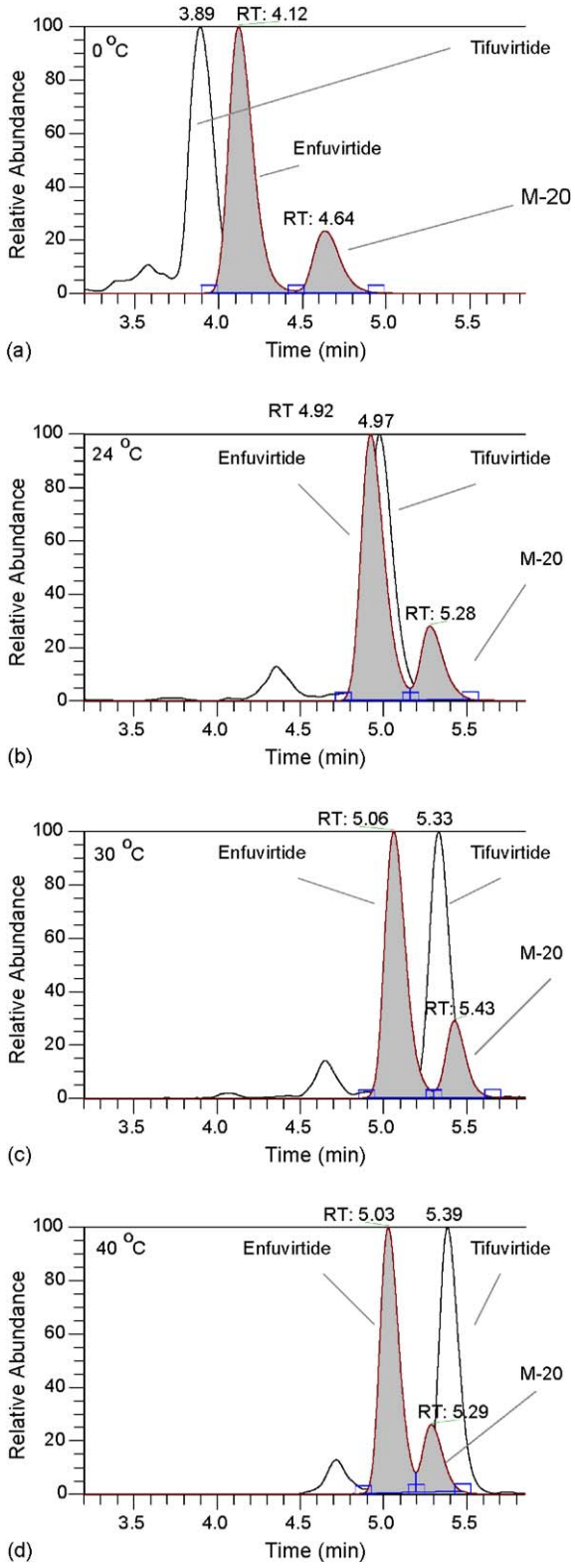

Fig. 5. Influence of column temperature on chromatographic behaviour of tifuvirtide, enfuvirtide and M-20. (a) 0 °C; (b) 24 °C; (c) 30 °C and (d) 40 °C. Eluent composition increased from 35% solvent B with 2% B/min at a flow rate of 2 ml/min. Chromatographic resolutions for enfuvirtide and M-20 are (a) 1.53; (b) 1.31; (c) 1.31 and (d) 0.98.

3.1.3. Liquid chromatography

Because enfuvirtide and M-20 have the same MRM transition it was important to obtain sufficient chromatographic resolution between these analytes. By decreasing column temperature from 40 to 0 °C, separation between tifuvirtide and enfuvirtide and its metabolite was improved. Furthermore, decreasing column temperature led to improved separation of enfuvirtide and M-20 and lower retention times of all analytes (Fig. 5). As lowering column temperature usually leads to increased retention, the decrease in retention time was not expected. However, this "reverse effect" has been previously observed in other studies and can appear when the eluent pH is close to the pK_a of the analytes [32]. Under these circumstances pK_a shifts caused by changing temperature can have a drastic effect on the ratio of charged and uncharged amino acid residues in the molecule and thus on its retention behaviour. For tifuvirtide, containing one more acidic amino acid residue than enfuvirtide, this effect would be more severe, corresponding to the experimental results. With the use of an isolated box with melting ice the column temperature remained 0 °C overnight (>16 h).

For good chromatographic peak shape an ion-pairing agent was needed. Trifluoroacetic acid is the ion-pairing agent most widely used in the separation of peptides by reversed-phase HPLC [33]. However, trifluoroacetic acid causes significant ion suppression when used with electrospray ionisation [34]. Formic acid and acetic acid are alternatives to trifluoroacetic acid, allowing a compromise between a sensitive mass detection and an efficient chromatographic separation [35]. The effect of trifluoroacetic acid, formic acid and acetic acid on peak shape and mass spectrometric sensitivity was investigated. A concentration of 0.25% (v/v) formic acid was found as an optimal compromise for both good chromatographic peaks and sensitivity.

A representative MRM-chromatogram of a human plasma sample spiked at the LLOQ level is shown in Fig. 6. The signal-to-noise ratio at the LLOQ level was approximately 10 for tifuvirtide and about 50 for enfuvirtide and M-20.

3.1.4. Internal standards

The deuterated IS of enfuvirtide was used for quantification of both enfuvirtide and M-20. The small differences in molecular structure and retention time of M-20 compared to enfuvirtide and d60-enfuvirtide did not have any influence on the suitability of d60-enfuvirtide as an internal standard for M-20. For tifuvirtide a deuterated IS was necessary to correct for its different extraction and retention behaviour, compared to enfuvirtide. Unfortunately, the synthesized d50-tifuvirtide was contaminated and had a purity of only 5.7%.

Internal standard interferences were examined by introducing samples containing only IS. The peak areas in the MRM chromatograms at the expected retention times of the analytes were compared to the peak areas of the analytes at the LLOQ level. Furthermore, the responses of the analytes in the presence of ISs were compared to the responses of the analytes without ISs. Since no internal standard interferences and no additional ion suppression were observed, the ISs could be used as intended.

Table 2
Concentrations of calibration standards back-calculated from the nominal concentrations

Nominal concentration (ng/ml)	Measured concentration (ng/ml)	Precision (%)	Deviation (%)
Enfuvirtide			
20.2	20.2 ± 0.3	1.3	−0.15
101	104 ± 6	6.0	3.3
202	194 ± 12	6.2	−4.0
505	489 ± 43	8.8	−3.1
1010	1006 ± 31	3.1	−0.4
2019	2090 ± 157	7.5	3.5
5049	5313 ± 323	6.1	5.2
10097	9981 ± 744	7.5	−1.2
Tifuvirtide			
19.6	19.6 ± 0.3	1.4	−0.39
98.1	101 ± 5.6	5.6	3.2
196	191 ± 12	6.5	−2.6
491	496 ± 22	4.4	1.1
981	962 ± 50	5.2	−2.0
1962	1990 ± 73	3.7	1.4
4906	4937 ± 169	3.4	0.6
9811	9682 ± 458	4.7	−1.3
M-20			
20.9	20.4 ± 1.4	6.9	−2.5
41.7	42.4 ± 3.0	7.1	1.5
104	100 ± 4.8	4.8	−4.3
209	201 ± 30	14.9	−3.8
417	413 ± 16	4.0	−1.1
1044	1051 ± 68	6.5	0.7
2087	2157 ± 166	7.7	3.3

Table 3
Intra- and inter-assay performance data of the analytes at five concentration levels ($n = 18$)

Nominal concentration (ng/ml)	Found concentration (ng/ml)	Intra-assay precision (%)	Inter-assay precision (%)	Deviation (%)
Enfuvirtide				
20.51	20.46 ± 2.4	9.2	11.4	−0.3
51.27	51.36 ± 4.7	10.1	10.2	0.2
2000	1842 ± 91	4.8	4.9	−7.9
7691	7327 ± 300	3.5	4.0	−4.7
15382[a]	13487 ± 346	2.6	–	−12.3
Tifuvirtide				
20.51	21.92 ± 2.3	12.2	13.1	6.9
51.26	54.06 ± 5.2	9.8	10.1	5.5
1999	1867 ± 85	4.0	4.7	−6.6
7690	7278 ± 424	4.8	6.4	−5.4
15379[a]	13582 ± 450	3.3	–	−11.7
M-20				
19.40	17.77 ± 2.2	12.2	13.4	−8.4
48.50	44.55 ± 3.8	9.6	9.3	−8.1
378	367 ± 18	6.3	6.9	−3.0
1455	1497 ± 88	5.0	5.9	2.9
2910[a]	2992 ± 189	6.8	–	2.8

[a] Above ULQ, quantified after five-fold dilution with blank human plasma ($n = 6$).

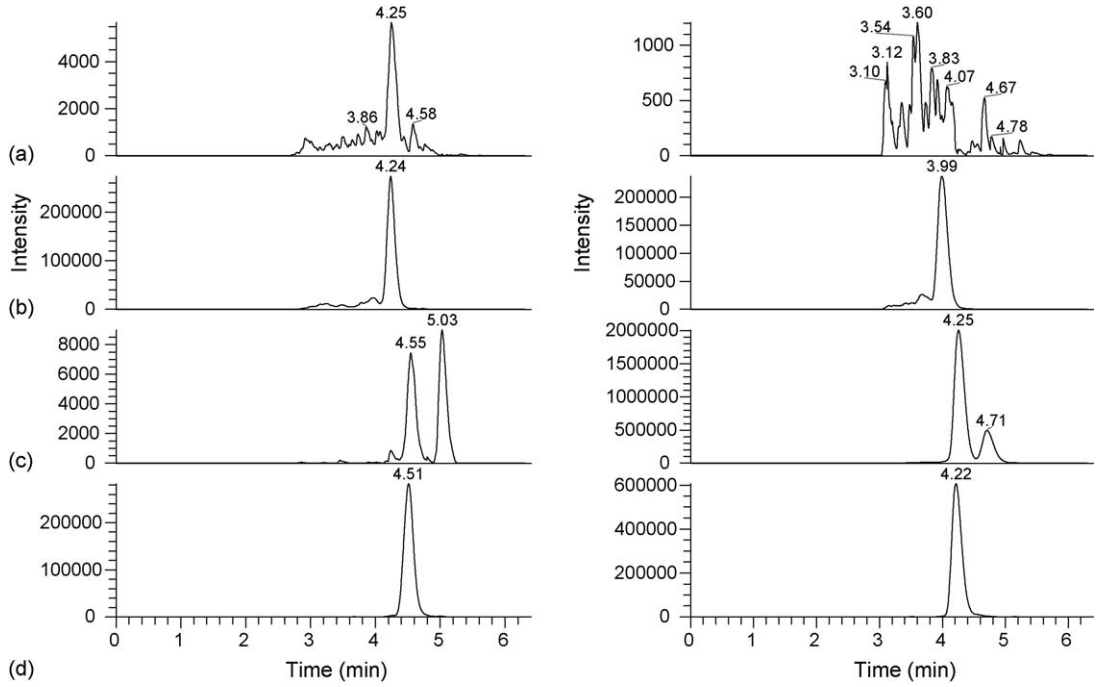

Fig. 6. MRM chromatograms of a LLOQ sample (left) and a patient sample (right). (a) Tifuvirtide; (b) d50-tifuvirtide; (c) enfuvirtide and M-20; and (d) d60-enfuvirtide.

Fig. 4 illustrates the specificity of the product ion spectra of the internal standards.

3.2. Method validation

3.2.1. Linearity

The assay was linear over a concentration range from 20 to 10,000 ng/ml for enfuvirtide and tifuvirtide and from 20 to 2000 ng/ml for M-20. Correlation coefficients (R^2) ranged from 0.9868 to 0.9993, 0.9958 to 0.9997 and 0.9932 to 0.9989 for enfuvirtide, tifuvirtide and M-20, respectively. The calibration curves were defined by slopes of 0.0017 (±0.0002) ml/ng, 0.0013 (±0.0003) ml/ng and 0.0018 (±0.0004) ml/ng and intercepts of −0.0003 (±0.0045), 0.0001 (±0.0068) and −0.0035 (±0.0033) for enfuvirtide, tifuvirtide and M-20, respectively.

The calibration standard with the lowest concentration was not taken into account for M-20, since its concentration (4 ng/ml) was below the LLOQ of M-20. Calibration standards were analysed in five separate runs and concentrations were back-calculated from nominal concentrations (Table 2). Deviations from the nominal concentrations over five runs were less than ±5.2, ±3.2 and ±4.3% for enfuvirtide, tifuvirtide and M-20, respectively.

3.2.2. Precision and accuracy

Assay performance data are presented in Table 3. For the lowest QC sample (20 ng/ml) the accuracies and precisions were within the ±20% range. This value can therefore, be used as the lower limit of quantitation (LLOQ). Intra- and inter-assay precisions for the other concentrations were less than 10.1 and 9.6%, respectively. Deviations from the nominal concentrations were less than ±12.3% for all analytes at all concentration levels.

3.2.3. Specificity and selectivity

Peak areas of co-eluting endogenous and exogenous compounds did not exceed 20% of the peak area response of the LLOQ samples. Plasma samples spiked at LLOQ level either from six different batches or spiked with different mixes of co-medication were accurate and precise.

3.2.4. Recovery and ion suppression

Extraction recoveries and ion suppression of the analytes are shown in Table 4. Both small losses during sample preparation and ionisation in the electrospray source result in reproducible mean recoveries of 78, 76 and 88% for enfuvirtide, tifuvirtide and M-20, respectively. Total recoveries of ISs were 88 ± 13% for d60-enfuvirtide and 81 ± 10% for d50-tifuvirtide ($n = 9$). The mean total recoveries calculated with the peak area ratio of the analytes and the ISs were 90, 101 and 94% for enfuvirtide, tifuvirtide and M-20, respectively. These results indicate that the two ISs are well suited to compensate for any changes in recoveries of the three analytes.

It should be mentioned that plasma from only one source was used. However, during specificity and selectivity examinations six different plasma sources were used and the absence of significant differences between the absolute responses of the analytes and the ISs indicates that suppression is not influenced by the plasma source.

Table 4
Recovery data for the analytes ($n = 3$)

Nominal concentration (ng/ml)	Extraction recovery (%)	Ion suppression (%)	Total recovery (%)
Enfuvirtide			
51.27	88.5 ± 9.9	−7.2 ± 0.9	82.2 ± 4.9
2000	89.2 ± 9.7	−10.8 ± 1.3	79.6 ± 6.9
7691	87.1 ± 3.1	−15.6 ± 0.6	73.5 ± 2.7
Tifuvirtide			
51.26	94.3 ± 16.1	−11.4 ± 2.1	83.6 ± 13.8
1999	82.8 ± 8.9	−8.5 ± 1.1	75.8 ± 8.0
7690	81.4 ± 3.1	−16.6 ± 0.6	67.8 ± 2.4
M-20			
19.40	91.5 ± 11.2	−3.2 ± 0.5	88.6 ± 9.8
378	96.6 ± 11.6	−7.9 ± 1.2	88.9 ± 12.4
1455	103.0 ± 5.0	−16.7 ± 1.0	85.8 ± 4.3

3.2.5. Stability

The results of stability experiments are reported in Table 5. In stock and working solutions all compounds were stable for at least 3 months. Stability of all the analytes in plasma was limited at room temperature. After 24 h at room temperature, plasma samples showed a significant loss of all analytes at all concentration levels. Stability of the analytes in plasma samples at room temperature is guaranteed for a maximum of 8 h, which is sufficient for routine use of the assay. All analytes were stable in plasma during three freeze-thaw cycles and for at least 3 months at −20 °C. The peptides in the final extracts were not stable during 24 h at 15 °C in the autosampler (more than 15% deviation from the nominal values), compared to freshly prepared QC samples and calibrations standards. The same deviations were observed after storage of the final extracts for 24 h at 4 °C and ambient temperature. Since this deviation seemed to be caused by an increase of the analyte responses rather than a decrease of the IS responses, it seems to be more likely an inter-assay variability problem than an instability matter.

Re-injection after 24 h is therefore, possible with sufficient accuracy and precision, since stored calibration standards do not exhibit the inter-assay variability.

3.3. Analysis of patient samples

Concentrations of enfuvirtide and M-20 in plasma were analysed in samples from HIV-1 infected patients. Fig. 7 shows concentration versus time data of enfuvirtide and its metabolite. Plasma samples were collected from different patients at various time points ($n = 7$) and during one dosing interval after two-fold subcutaneous administration of 90 mg enfuvirtide. These data show the applicability of the assay in these heavily treated patients.

Table 5
Stability data of enfuvirtide (Enf), tifuvirtide (Tif) and M-20 under different conditions

Condition	Matrix	Initial concentration (μg/ml)			Dev (%)			R.S.D. (%)			*n*
		Enf	Tif	M-20	Enf	Tif	M-20	Enf	Tif	M-20	
−20 °C 3 months	Stocks	1025	1025	194	4.9	0.95	2.1	2.6	3.8	3.4	3
−20 °C 3 months	Working solutions	263	255	54	4.8	0.26	−4.3	4.6	6.9	12.3	3
		103	103	97	0.64	−3.9	−2.3	12.1	15.4	15.2	3
Ambient, 24 h	Working solutions	308	308	58	−10	−9	−10	–	–	–	1
		263	255	54	0	2	1	–	–	–	1
		103	103	97	−6	−6	−6	–	–	–	1
Three freeze (−20 °C) thaw cycles	Plasma	0.05	0.05	0.05	−1	−1	−1	13.9	9.2	13.6	3
		2.00	2.00	0.38	1	−4	−2	7.9	11.3	10.0	3
		7.69	7.69	1.46	3	1	2	3.2	0.8	5.3	3
Ambient, 24 h	Plasma	0.05	0.05	0.05	−9	−17	−22	29.2	12.0	13.2	3
		2.00	2.00	0.38	−9	−6	5	7.1	7.3	26.7	3
		7.69	7.69	1.46	−22	−18	−16	–	–	–	2
Ambient, 8 h	Plasma	0.05	0.05	0.05	11	1	3	15.5	17.1	12.6	3
		2.00	2.00	0.38	−5	−3	−7	3.5	9.4	11.0	3
		7.69	7.69	1.46	−10	−0.1	−6	7.0	6.9	8.3	3
−20 °C 3 months	Plasma	0.05	0.05	0.05	−14	−7	−10	15.3	16.9	22.1	3
		2.00	2.00	0.38	5	2	9	6.7	8.9	6.9	3
		7.69	7.69	1.46	−1	2	7	5.6	4.2	17.2	3
Autosampler, 15 °C, 24 h	Elution solvent	0.05	0.05	0.05	−7	9	−4	6.0	14.8	7.9	3
		2.00	2.00	0.38	12	19	15	7.3	6.1	8.8	3
		7.69	7.69	1.46	10	20	13	5.9	4.1	6.6	3
Re-injection, autosampler, 24 h	Elution solvent	0.05	0.05	0.05	1	1	8	8.6	14.0	12.3	6
		2.00	2.00	0.38	−2	1	−2	6.3	6.8	9.8	6
		7.69	7.69	1.46	2	−1	−5	4.1	5.9	6.0	6

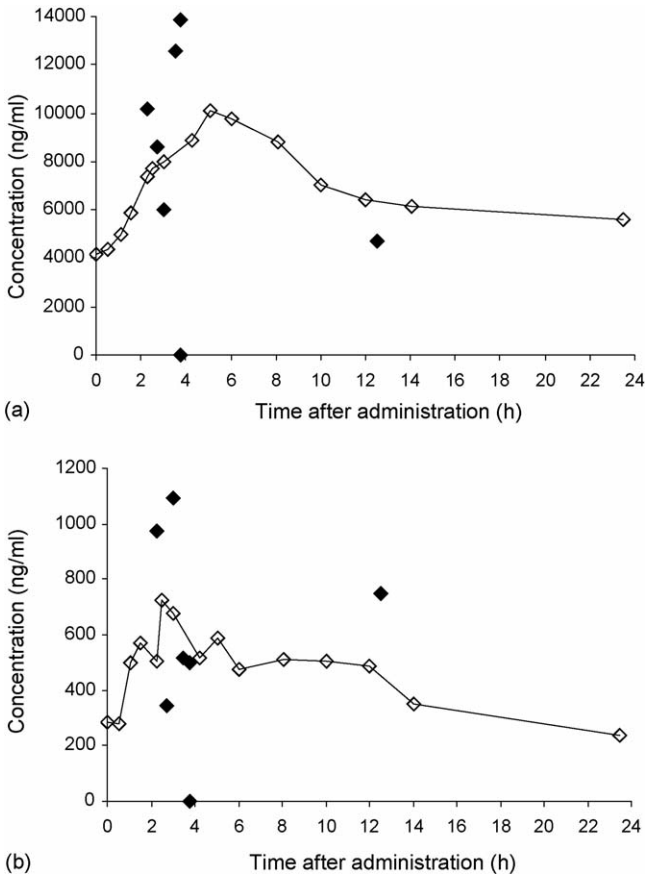

Fig. 7. Concentration of enfuvirtide (a) and its metabolite M-20 (b) in plasma vs. time after administration. Plasma samples are collected during a dosing interval (◊) and at various time points (♦).

4. Conclusions

An LC–MS/MS assay for the simultaneous quantitation of two peptide HIV-1 fusion inhibitors, enfuvirtide and tifuvirtide, and a metabolite of enfuvirtide (M-20) in human plasma has been successfully developed and validated. The assay provided accurate and precise results and has demonstrated it usefulness in the analysis of plasma samples of HIV-1 infected patients. The method will be used for therapeutic drug monitoring of enfuvirtide. In addition, the method will be relevant when development of tifuvirtide continues and clinical studies will require a bioanalytical assay.

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