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Enzymatic digestion as a tool for the LC–MS/MS quantification of large peptides in biological matrices: Measurement of chymotryptic fragments from the HIV-1 fusion inhibitor enfuvirtide and its metabolite M-20 in human plasma

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Abstract

The use of enzymatic digests of the peptide HIV-1 fusion inhibitor enfuvirtide as a tool for the absolute quantification of this polypeptide (MW 4492 Da) in human plasma by LC–MS/MS has been evaluated. Two different methods applying digestion of enfuvirtide with chymotrypsin after solid phase extraction (SPE) of the plasma samples have therefore been developed and validated. One method used a stable isotopically labeled analog of the complete peptide (d60-enfuvirtide) as internal standard (IS) and could use as much as four different chymotryptic fragments for the quantification of enfuvirtide in a range of 100–10,000 ng/ml. Intra- and inter-assay precisions and deviations from the nominal concentrations varied for the different fragments, but were below 9% when the four results were averaged. The other method used a stable isotopically labeled chymotryptic fragment of the peptide (d10-ASLW) as IS. Although this IS does not correct for variations in digestion recovery, it allows the selective quantification of enfuvirtide (100–10,000 ng/ml), besides the quantification of the sum of enfuvirtide and its de-amidated metabolite M-20 (120–12,000 ng/ml). Both methods were suitable for the absolute quantification of enfuvirtide and M-20 in plasma, but proper selection of the fragment(s) used for the quantification appeared crucial when the deuterated fragment was used as IS.
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1. Introduction

Despite the extensive use of mass spectrometric techniques such as LC–MS or LC–MS/MS for the quantification of small-to-medium size compounds (<2000 Da) from complex matrices, the quantification of large peptides and proteins in biological matrices remains a challenging task. Although the use of these techniques for quantitative bioanalytical assays of large peptides (>2000 Da) has recently been described more frequently [1–12], the application for absolute protein quantification (>6000 Da) is still not widespread [13–15]. However, with the increasing importance of peptides and proteins in different fields, e.g. medicines or proteomics, improvements and new analytical approaches in bioanalytical techniques are required.

While formerly immunoassays were mainly used for the quantification of peptides and proteins in biological matrices, LC–MS has nowadays become the preferred analytical technique. However, even with a very selective and sensitive detection technique such as (tandem) mass spectrometry, sensitivity for large peptides and proteins remains problematic. The difficulty of selectively extracting the peptide or protein of interest from the variety of proteins and other endogenous compounds in the matrix, results in the presence of interfering matrix components, suppressing the ionization of the analyte

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in the ion source. Furthermore, formation of multiple charged ions and/or adduct ions when performing mass spectrometry coupled to electrospray ionization (ESI-MS), results in distribution of the signal among more than one ion, thereby reducing the sensitivity of quantitative assays. Although these multiple charged ions allow the measurement of biomolecules with a high molecular weight in lower *m/z* ranges, ionization efficiency might be affected, especially for large molecules as polypeptides and proteins.

Another problem arising in the bioanalysis of peptides and proteins is the ability to find a suitable IS. Stable isotopically labeled ISs are most suitable for LC–MS quantification, but are, unfortunately, expensive, especially for large compounds. The use of structural analogs can be a very useful alternative [3,7,11], but a suitable structural analog of the peptide of interest may be hard to find. For the quantification of intact proteins it is even more difficult to find a suitable IS. An extensive amount of isotope label would be needed to distinguish the IS from the native protein, making the production of such an IS very complex and costly. An early LC–MS assay for the quantification of intact albumin in human serum with a biotinylated analog as IS is described by Bunk and Welch [13]. Ji et al. described the quantification of the intact protein rK5 (10464 Da) in human plasma with the use of a stable isotopically labeled [15] and structural [14] analog as IS. As far as we know, these two assays are the only ones employing selected reaction monitoring (SRM) for the absolute quantification of an intact protein, although SRM is widely used for the quantification of small molecules and peptides. SRM can greatly reduce background interference, as selection of a specific product ion formed after fragmentation of the parent ion, improves selectivity of the method.

The limitations in quantifying intact proteins have resulted in the development of alternative approaches. An example is the chemical or enzymatic cleavage of the complete protein into fragment peptides, allowing the measurement of a fragment and a labeled analog of the fragment as a representation of the concentration of the intact protein. This approach, protein cleavage coupled to isotope dilution mass spectrometry (PC-IDMS), was first described by Barr et al. [16] for the absolute quantification of apolipoprotein A-1 and has thereafter been used numerous times for the absolute quantification of other proteins. Although most of these methods are performed using pure reference solutions, some methods describe the absolute quantification of proteins from a complex mixture [17–21]. However, most of these methods are performed directly without purification of the sample or purification steps involve extensive sample preparation including immunoaffinity and/or size exclusion chromatography prior to addition of the IS without evaluation of the effect on sensitivity or reproducibility of the method. A quantitative LC–MS assay using labeled proteolytic peptides added prior to sample pre-treatment has recently been described by Aguiar et al., giving excellent analytical performance for C-reactive protein in urine [21].

In this article the LC–MS/MS quantification of the model peptide HIV-1 fusion inhibitor enfuvirtide (Fuzeon®, T-20) in human plasma after SPE and subsequent enzymatic cleavage with chymotrypsin is described. The use of a stable isotopi-

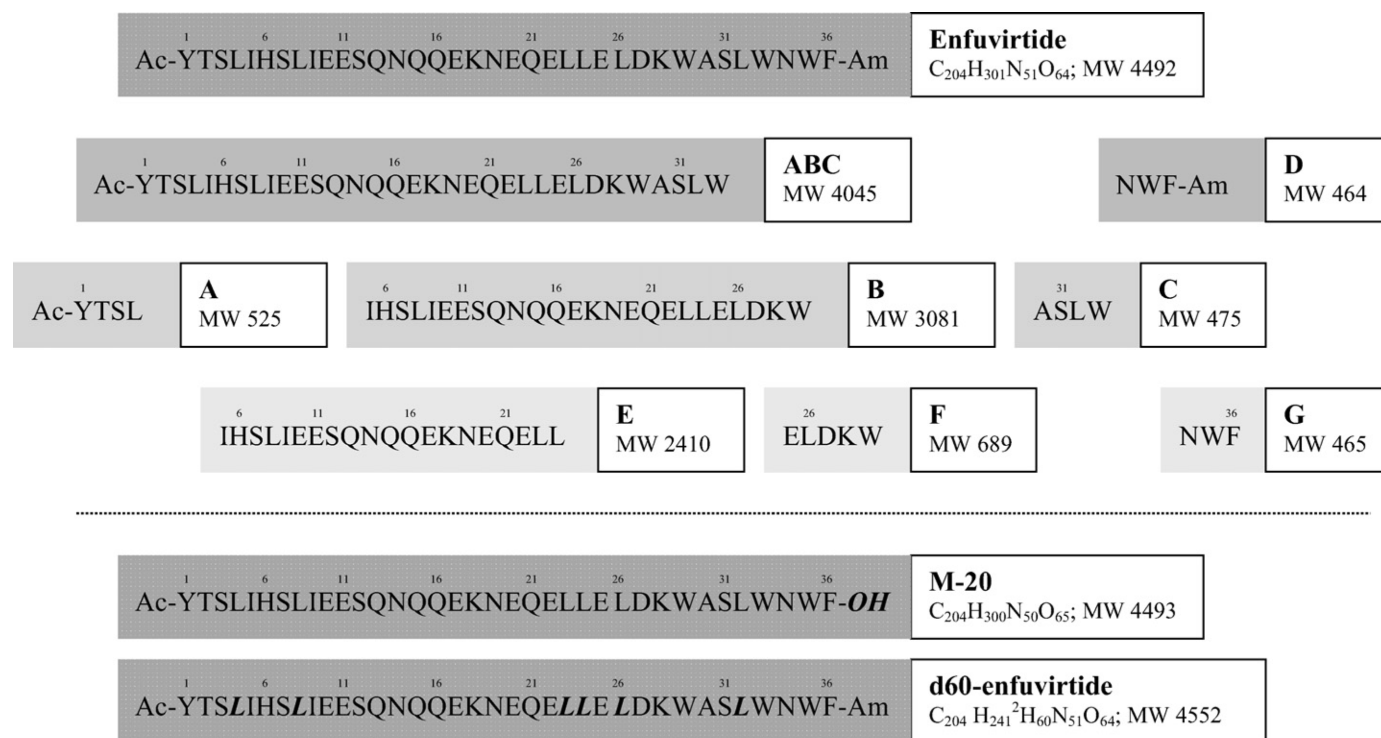

Fig. 1. Examples of (identified) fragments formed after digestion of enfuvirtide with chymotrypsin. The differences with M-20 and d60-enfuvirtide are shown underneath in italics/bold: *OH* indicates the hydroxylated C-terminus of M-20 and *L* indicates replacement of a leucine by d10-leucine amino acid residue in d60-enfuvirtide.

cally labeled analog of the peptide (d60-enfuvirtide) that is similarly digested has been compared to the use of a stable isotopically labeled fragment (d10-ASLW) as IS. This smaller stable isotopically labeled compound is less complex and less expensive to make than the complete stable isotopically labeled analog and offers the opportunity for the development of other similar quantitative bioassays for larger peptides and proteins. It also offers more information about the necessity of an (intact) IS correcting for losses during sample pre-treatment. In the procedure using d10-ASLW as IS, the de-amidated metabolite of enfuvirtide, M-20, is added to the plasma samples. M-20 only differs from enfuvirtide by the hydroxylated phenylalanine C-terminus (Fig. 1), complicating separate detection, especially as similar peptide fragments are expected.

Both methods have been validated and compared to a previously developed LC–MS/MS method for the quantification of intact enfuvirtide from human plasma [10]. Furthermore, the effect of measuring smaller compounds in comparison to the complete peptide on ESI-MS/MS detection has been studied. Several digestive enzymes have been tested and parameters influencing digestion efficiency have been described in detail.

2. Experimental

2.1. Chemicals and reagents

Methanol (HPLC grade), formic acid (analytical grade) and LC–MS grade water were from Biosolve (Valkenswaard, The Netherlands). Acetic acid (glacial), ammonium hydroxide (A.C.S. reagent), trifluoroacetic acid (99^{+}%, spectrophotometric grade), α-chymotrypsin from bovine pancreas and endoproteinase Glu-C from *Staphylococcus aureus* strain V8 were obtained from Sigma–Aldrich (Steinheim, Germany). Sequencing grade modified trypsin (porcine) was from Promega (Madison, WI, USA). Drug free human plasma was obtained from the Sanquin Bloodbank (Utrecht, The Netherlands). Enfuvirtide (98.6%) was supplied by Cook Pharmaceutical Solutions (Bloomington, IN, USA). M-20 (81.4%) was kindly supplied by Hoffmann-La Roche (Nutley, NJ, USA). Stable isotopically labeled peptides (d60-enfuvirtide and d10-ASLW) and the synthetic peptide fragments Ac-YTSL (**A**), IHSLIEESQNQQEKNEQELL (**E**), ELDKW (**F**) and ASLW (**C**) were synthesized and kindly supplied by H. Hilkmann (The Netherlands Cancer Institute, Amsterdam, The Netherlands).

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 the analytical column (Symmetry 300 C_{18}, 50 mm × 2.1 mm ID, 3.5 μm particle size) and pre-column (Symmetry 300 C_{18}, 10 mm × 2.1 mm ID) 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 cartridges (Waters), containing 30 mg of poly(divinylbenzene-co-*N*-vinylpyrrolidone) copolymer, were used for SPE.

2.3. LC–MS/MS

A gradient HPLC method was employed for separation of the peptide fragments. Mobile phase A consisted of 0.25% (v/v) formic acid in water and mobile phase B of 0.25% (v/v) formic acid in methanol. The gradient started with 25% of eluent B and linearly rose to 40% of eluent B in 6 min. Subsequently, the eluent composition linearly rose to 90% of eluent B in 2.5 min and maintained 90% for 2 min before it decreased to 25% eluent B for re-equilibration. The total run time was 13 min at a flow rate of 0.2 ml/min. The column oven was kept at 30 °C and the autosampler temperature was set at 5 °C.

Table 1
MS/MS settings during a quantitative chromatographic run

MS parameter		Segment 1 (0–3.8 min)	Segment 2 (3.8–6.3 min)			Segment 3 (6.3–10.0 min)	
Auxiliary gas (AU)		22	12			25	
Ion sweep gas (AU)		0	5			0	
Capillary temperature (°C)		225	236			251	
Collision gas pressure (mTorr)		1.5	1.5			2.6	
Resolution (FWHM)		0.7	0.7			1.0	
		F	**A**	**C**	**D**	**E**	Enfuvirtide M-20
Parent ion (*m*/*z*)	[a]	345.6	525.2	476.2	465.2	804.1	1124.0
	[b]	350.6	535.2	486.2	–	814.1	1139.0
Product ion (*m*/*z*)	[a]	197.1	376.1	188.0 205.0	273.0	1083.5	1343.5
	[b]	207.1	376.1	188.0 205.0	–	1088.5	1363.5
Tube lens off set		131	154	151	150	174	121
Collision energy (V)		19	18	25	20	25	18
Retention time (min)		3.1	4.9	5.7	5.2	7.6	9.2

[a] Unlabeled compound.
[b] Labeled compound.

The synthetic peptides of the chymotryptic fragments of enfuvirtide were used to optimize the MS settings for the different peptide fragments. The MS detection was divided into three segments, using the optimal MS settings of fragment **F**, **C** and **E**, respectively (Fig. 1). The different MS settings during these three segments as well as the transitions of the different peptide fragments used for SRM, their stable isotopically labeled analogs and enfuvirtide and d60-enfuvirtide are listed in Table 1. The capillary voltage was set at 5 kV and sheath gas pressure was 49 arbitrary units (AU) for all compounds. These settings were used during all experiments, including the validation procedure.

For the identification of the proteolytic fragments alternative conditions were used. With the same mobile phases, a gradient was applied that started at 10% eluent B rising linearly over 20 min to 90% eluent B. UV detection at 214 nm was used, as well as the MS full scan mode to identify molecular masses of the proteolysis products.

2.4. Preparation of standards and quality control (QC) samples

Stock solutions of M-20 (0.2 mg/ml) were prepared in methanol/0.4% (w/v) ammonium hydroxide in water, 1/1 (v/v). Stock solutions of enfuvirtide (1 mg/ml) and working solutions of enfuvirtide (250 μg/ml) and combined enfuvirtide/M-20 (250/50 μg/ml) were prepared in methanol/0.1% formic acid in water, 1/1 (v/v). Plasma was spiked with appropriate volumes of working solutions to provide QC plasma samples of 7500, 1950, 250 and 100 ng/ml enfuvirtide. When the combined enfuvirtide/M-20 working solution was used, the samples additionally contained 1500, 390, 50 and 20 ng/ml M-20, respectively. Independently prepared stock solutions of enfuvirtide and M-20 were combined and diluted similarly for the preparation of calibration standards. Calibration standards of 100, 200, 500, 1000, 2000, 5000, and 10000 ng/ml enfuvirtide and of 120, 240, 600, 1200, 2400, 6000, and 12000 ng/ml combined enfuvirtide and M-20 were obtained by serial dilution with blank human plasma. Stock solutions of d60-enfuvirtide and d10-ASLW were prepared in methanol/0.1% (v/v) formic acid in water, 1/1 (v/v) at concentrations of 1 and 0.1 mg/ml, respectively. The stock solution of d60-enfuvirtide was diluted with the same solvent to obtain a working solution of 250 μg/ml, whereas the stock solution of d10-ASLW was added to the SPE elution solvent to obtain a final concentration of 250 ng/ml.

Chymotrypsin solutions were prepared freshly prior to each analysis in 50 mM acetic acid at a concentration of 1.5 mg/ml. The solution was kept on ice during handling. All stock and working solutions were stored at −30 °C, except for the chymotrypsin solution that was stored at −80 °C.

2.5. Sample preparation

Enfuvirtide was extracted from plasma by SPE on Oasis HLB extraction cartridges, as previously described [10]. The samples analyzed with d10-ASLW were eluted with the elution solvent containing 250 ng/ml of the deuterated IS. The eluate was evaporated under a stream of nitrogen and the samples were reconstituted in 200 μl 100 mM Tris–HCl/2 mM $CaCl_2$ buffer (pH 8.6). Five microlitre of 1.5 mg/ml chymotrypsin solution were added to each sample. The mixtures were incubated for 30 min for the samples containing d10-ASLW and for 60 min for the samples containing d60-enfuvirtide at 37 °C in a shaking water bath. The digestion was ended by acidification with 4 μl of 100% acetic acid.

2.6. Quantification

Two different methods were used for the quantification of enfuvirtide in plasma after chymotrypsin digestion. In the first method a deuterated analog of the complete peptide (d60-enfuvirtide) was used as IS and samples containing only enfuvirtide were measured. The responses of four unlabeled chymotryptic fragments (**A**, **C**, **E**, and **F**) relative to their labeled analogs were used for the quantification of the intact peptide concentration in plasma. In the second method a deuterated analog of a specific chymotryptic fragment of enfuvirtide (d10-ASLW) was used as IS and samples containing both enfuvirtide and M-20 were measured. The response of the unlabeled fragment (**C**) as well as the response of one other chymotryptic fragment (**A**) relative to the response of the deuterated fragment was used to quantify the total amount of enfuvirtide and M-20 in plasma. The response of the chymotryptic fragment containing the C-terminus of the peptide (**D**) relative to the response of the deuterated fragment was used to specifically quantify the enfuvirtide plasma level. The concentration of M-20 could be determined by subtracting both found concentrations. The small difference in molecular weight between enfuvirtide and M-20 (<0.025%) was neglected and concentrations of both enfuvirtide and M-20 were expressed in ng/ml.

2.7. Recovery

To determine the overall recovery of the SPE and digestion procedures, plasma samples spiked with enfuvirtide before SPE and digestion were measured, as well as blank plasma samples spiked with the four synthetic peptides after SPE and digestion. The samples were prepared and analyzed in six-fold at three different concentration levels, corresponding to the molar concentrations of enfuvirtide in the low, mid and high QC samples. By comparing the response ratios of the different fragments in the samples initially spiked with enfuvirtide to the response ratios of the corresponding synthetic peptides in blank plasma samples, the overall recovery was determined. The same procedure was executed using either d60-enfuvirtide or d10-ASLW as IS. Recovery of the SPE, evaporation and reconstitution in buffer was assessed by comparing the MS response of enfuvirtide spiked to blank plasma before these sample handling steps with the MS response of enfuvirtide added to blank plasma after these sample handling steps. After correction of the overall recovery for the extraction recovery, the digestion recovery of the four fragments could be calculated.

Additional information about the digestion recovery of the different fragments was obtained using UV detection.

Therefore, buffer solution was spiked with enfuvirtide at three different concentrations: 5, 20 and 100 μg/ml. Five microlitre of 1 mg/ml chymotrypsin solution were added to 200 μl of enfuvirtide solution and the solution was maintained at 37 °C for 90 min. UV absorbance at 214 and 280 nm of enfuvirtide before digestion was measured, as well as UV absorbance of the fragments formed after digestion. Extinction coefficients at 280 and 214 nm of both enfuvirtide and the fragments were calculated according to Edelhoch [22] and were used to calculate the relative amount of formed fragment, according to the following equation: [%digestion = $(A_{\text{fragment}} \times \varepsilon_{\text{enfuvirtide}}/\varepsilon_{\text{fragment}} \times A_{\text{enfuvirtide}}) \times 100\%$]. In which A is the UV absorbance and ε is the extinction coefficient at a certain wavelength.

2.8. Validation

2.8.1. Linearity

For both methods, seven calibration standards were analyzed in three separate runs. The calibration standards analyzed with d60-enfuvirtide ranged from 100 to 10,000 ng/ml enfuvirtide. For the method using d10-ASLW, calibration standards contained both enfuvirtide and M-20 in a total concentration ranging from 120 to 12,000 ng/ml. Response ratios of the selected fragment relative to the corresponding IS were used to construct standard curves by least square linear regression analysis. A weighting factor of $1/x^2$, in which x is the concentration in ng/ml, was used for both methods.

2.8.2. Accuracy and precision

Accuracy and precision of the procedures with d60-enfuvirtide and the leucine deuterated fragment as IS were determined by analyzing QC samples at four different concentration levels in three separate analytical runs ($n = 6$ for each run). Deviations of the accuracy were expressed as: [((overall mean concentration − nominal concentration)/nominal concentration) × 100%]. The 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% [23].

2.8.3. Specificity and selectivity

Specificity and selectivity of the assay were assessed by analyzing six blank plasma samples and six plasma samples at the LLOQ level from six different sources. Furthermore, six double blank plasma samples with and without chymotrypsin digestion were analyzed. Peak areas of endogenous and exogenous compounds co-eluting with the fragments or ISs 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% [23].

2.8.4. Stability

Stabilities of the intact enfuvirtide and M-20 in different matrices under different conditions were already extensively explored [10]. Further stability assessments of both intact compounds included long term stabilities in plasma after 1 year storage at −30 °C and in-process stabilities in the dried extract and the reconstitution solvent and were performed with this previously described procedure, capable of quantifying intact enfuvirtide and M-20 separately. Additional in-process stabilities of the different chymotryptic fragments after digestion of enfuvirtide and acidification with acetic acid were assessed with both procedures described in this paper, using either d10-ASLW or d60-enfuvirtide as IS, after storage of the samples in the autosampler for 24 h. Furthermore, stability of the chymotrypsin solution was evaluated by comparing the digestion efficiency of a freshly prepared chymotrypsin solution to chymotrypsin solutions stored at −20 °C for 3 days, at −80 °C for 1, 3 and 60 days and for 1 h at room temperature or on ice. The absolute MS responses of the different fragments were measured and used as an indication of the digestion efficiency and therewith of the stability of chymotrypsin.

3. Results and discussion

3.1. Selection of proteolytic enzyme

For enzymatic digestion of enfuvirtide, three different proteolytic enzymes were tested: trypsin, endoproteinase Glu-C and chymotrypsin.

As trypsin specifically cleaves a protein at the C-terminus of arginine and lysine amino acid residues, digestion of enfuvirtide with trypsin was expected to yield three peptide fragments with different lengths (8, 10 and 18 amino acids). However, digestion was slow and cleavage did not occur at the C-terminus of K^{28}. Further optimization to yield three representative tryptic fragments only resulted in the formation of more semitryptic fragments and trypsin was therefore not found to be a suitable cleavage enzyme for evaluating the effect of measuring smaller peptides on sensitivity of MS detection.

Endoproteinase Glu-C specifically cleaves at the C-terminus of glutamic acid residues and digestion of enfuvirtide with this enzyme should theoretically lead to the formation of six fragments with different lengths. Unfortunately, many overlapping peptide fragments were formed and as digestion efficiency could not be satisfactorily optimized, cleavage experiments of enfuvirtide were not continued with this enzyme.

Chymotrypsin was the third proteolytic enzyme tested and was initially thought to be unsuitable since it primarily cleaves at the C-terminus of tryptophan, tyrosine and phenylalanine residues and secondarily at the C-terminus of especially leucine residues, cleaving enfuvirtide in either very large or very small fragments. However, chymotrypsin digestion of enfuvirtide in 50 mM Tris–HCl/1 mM $CaCl_2$ buffer resulted in the formation of five major products that could be identified as Y^1-L^4, I^5-L^{24}, E^{25}-W^{29}, A^{30}-W^{33} and N^{34}-F^{36}, named as **A**, **E**, **F**, **C** and **D**, respectively (Fig. 1). The fast digestion of enfuvirtide and the formation of several fragments with different lengths with chy-

motrypsin seemed most suitable for further evaluation of the effects of peptide digestion on quantification by LC–MS/MS. Fig. 2 shows three TIC chromatograms obtained by analyzing 50 μg/ml of enfuvirtide, d60-enfuvirtide and M-20 after digestion with chymotrypsin in buffer in the full scan mode in a range of *m*/*z* 280–1500. The generated full scan mass spectra with a Q1 resolution of 0.1 FWHM were used to identify the charge state and the molecular weights of the different ions, which were compared with the different possible chymotryptic cleavage fragments of enfuvirtide. Subsequently a full scan product ion scan was performed for the most abundant molecular ion of each detected peptide fragment to confirm the amino acid sequence of the proposed peptide fragment.

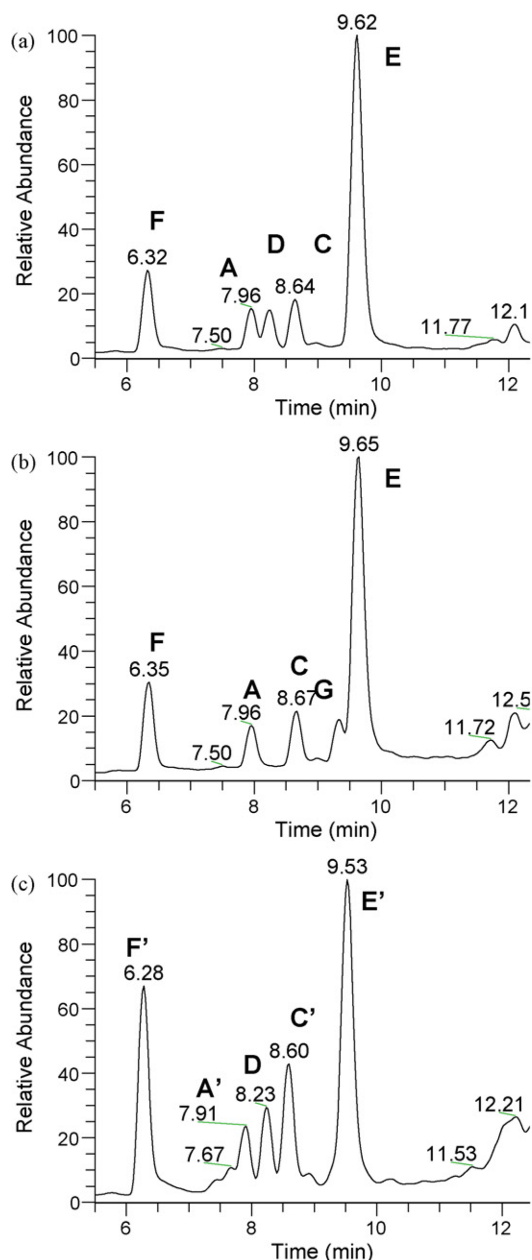

Fig. 2. TIC of (a) enfuvirtide; (b) M-20; and (c) d60-enfuvirtide (50 μg/ml) after chymotrypsin digestion in 50 mM Tris–HCl, 1 mM $CaCl_2$ buffer at 37 °C for 60 min. The conditions are reported in Section 2.3. The peaks are identified as the fragments named in Fig. 1. (') refers to the deuterium labeled analog.

3.2. LC–MS/MS

Improved resolution of the different fragments was achieved when methanol was used as organic modifier instead of acetonitrile. Representative SRM chromatograms of a LLOQ and a double blank plasma sample of enfuvirtide after digestion with chymotrypsin in the presence of d60-enfuvirtide and d10-ALSW are shown in Figs. 3 and 4, respectively. In addition to better chromatographic results, the use of methanol as organic modifier improved detection sensitivity of the fragments compared to the use of acetonitrile.

ESI-MS spectra of the peptide fragments, formed after chymotrypsin digestion of both enfuvirtide and M-20, are shown in Fig. 5. Similar spectra with respect to their mass differences were obtained for the fragments obtained after chymotrypsin digestion of d60-enfuvirtide. The ESI-MS spectra of the C-terminus fragments, formed after chymotrypsin digestion of either M-20 or enfuvirtide and d60-enfuvirtide, are shown in Fig. 6. These spectra are obtained from the TIC chromatograms shown in Fig. 2. Product ion spectra of the fragments were obtained by analyzing the same samples and recording a Q3 full scan after selection of the parent ion, and these spectra are shown in Fig. 7. Similar ESI-MS and ESI-MS/MS spectra were obtained after direct infusion of the synthetic deuterated chymotryptic fragment d10-ASLW and the synthetic peptides **A**, **C**, **E** and **F**, confirming the correct identities of these peptides.

The most abundant parent and product ions were selected for the SRM transitions, as listed in Table 1, except for **F** where the transition 345.6 → 197.1 gave the highest selectivity and S/N ratio. For **C** the sum of the two most abundant product ions is used as this gave more reproducible results than the use of a single ion.

LC–MS/MS analysis of the chymotryptic fragments involved measurements of smaller molecules compared to the intact enfuvirtide that has a molecular mass of 4492 Da. This could result in better ionization efficiency and therefore more sensitive detection of the entire peptide. In order to evaluate the effect of measuring smaller fragments on the sensitivity of mass spectrometric detection, standard solutions of enfuvirtide and the synthetic peptides were prepared at the same molarity in methanol/0.1% formic acid (1/1, v/v) and in blank plasma after extraction. Each peptide was analyzed under its own specific optimal conditions and absolute responses and signal-to-noise ratios (S/N) at different concentration levels were compared after both selected ion monitoring (SIM) and SRM analysis. For **A** and **C** the singly charged molecular ions were measured, whereas the $[M+2H]^{2+}$ (*m*/*z* 345.6), $[M+3H]^{3+}$ (*m*/*z* 804.1) and $[M+4H]^{4+}$ (*m*/*z* 1124.0) ions were monitored for **F**, **E** and enfuvirtide, respectively. Measuring singly charged ions did not result in higher absolute responses or S/N ratios, compared to measuring the multiply charged ions. Fragment **E** showed the highest absolute response and S/N ratio for both SIM and SRM analysis. The $[M+4H]^{4+}$ (*m*/*z* 1124.0) ion of enfuvirtide showed good absolute responses, but the S/N ratio for SIM analysis was very

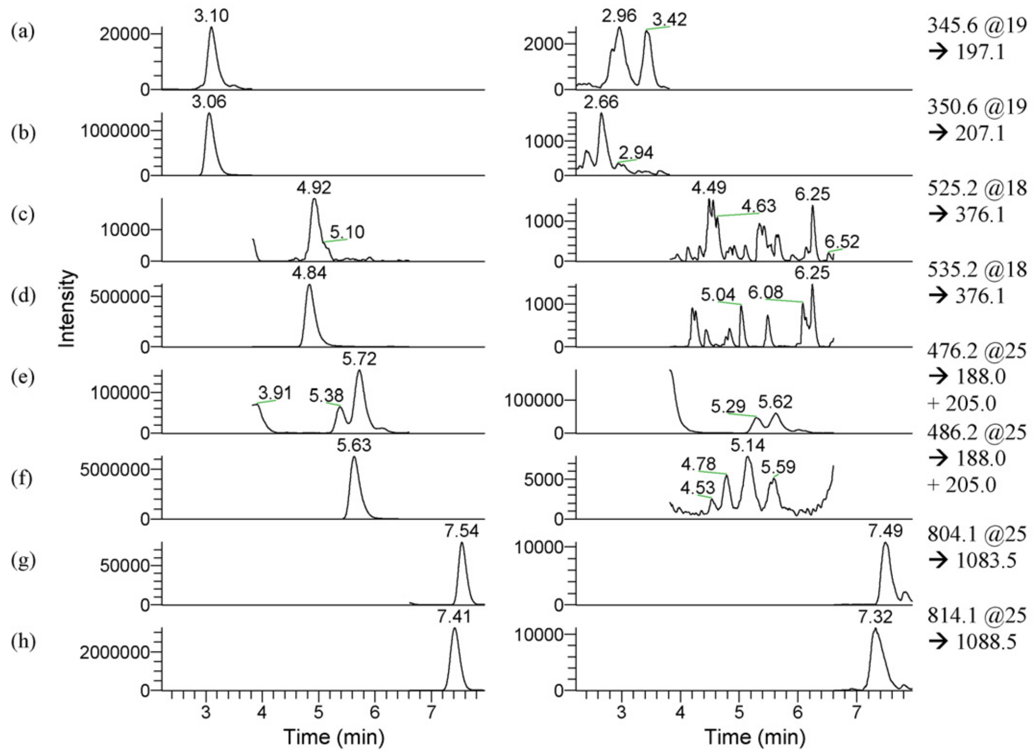

Fig. 3. SRM chromatograms of the fragments used for the quantification of enfuvirtide after digestion with chymotrypsin using d60-enfuvirtide as IS in a LLOQ (left), and double blank (right) plasma sample. (a) **F**; (b) **F**′; (c) **A**; (d) **A**′; (e) **C**; (f) **C**′; (g) **E;** and (h) **E**′.

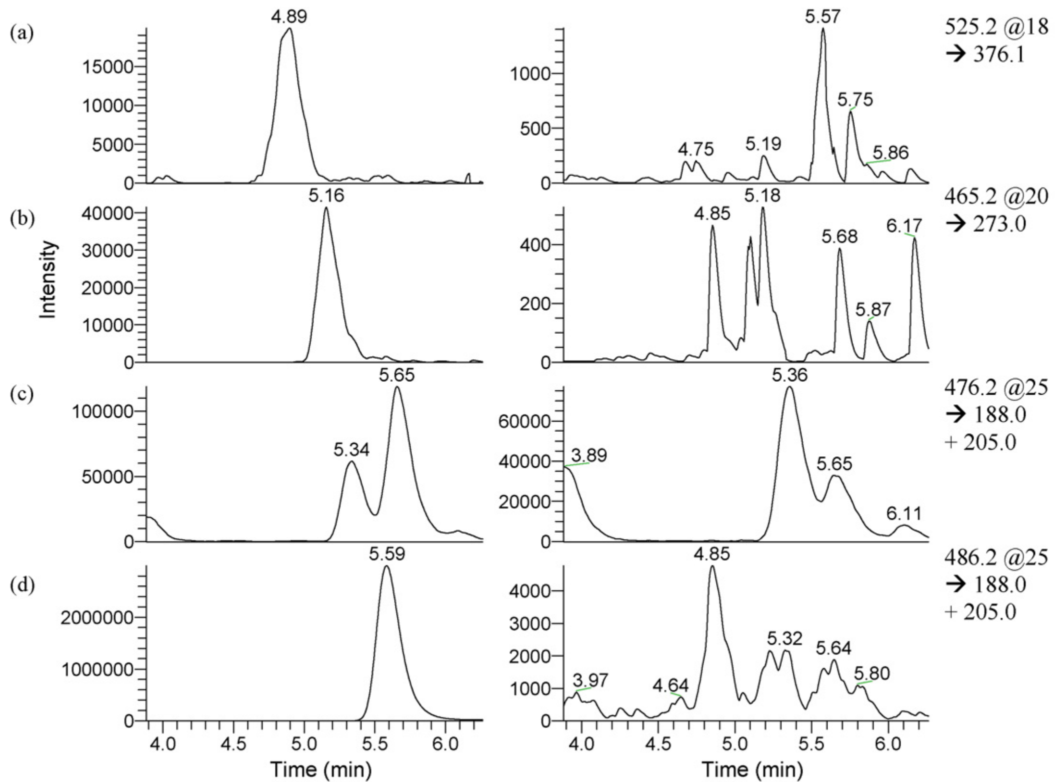

Fig. 4. SRM chromatograms of the fragments used for the quantification of enfuvirtide and M-20 after digestion with chymotrypsin using d10-ASLW as IS in a LLOQ (left), and double blank (right) plasma sample. (a) **A**; (b) **D**; (c) **C**; and (d) **C**′ (d10-ASLW).

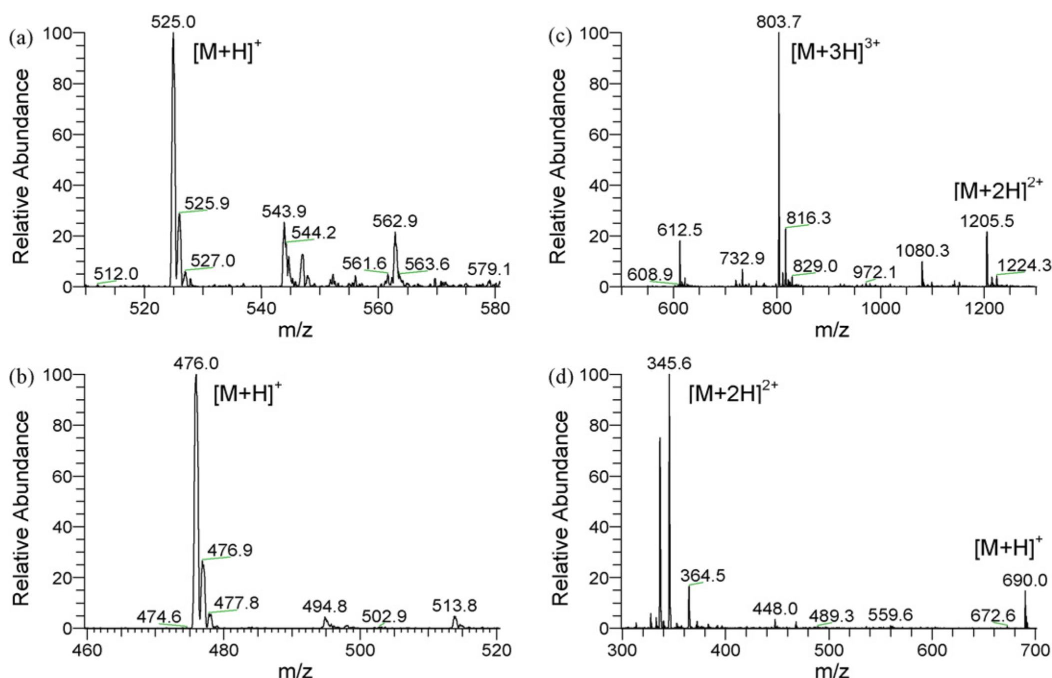

Fig. 5. ESI-MS spectra of four similarly formed fragments after chymotrypsin digestion of enfuvirtide and M-20: (a) **A**; (b) **C**; (c) **E** and (d) **F**. Q1 resolution was set at 0.70 FWHM.

poor, whereas S/N ratios after SRM analysis were much better. Figs. 8 and 9 show a SIM and SRM chromatogram of enfuvirtide and the four fragments at a concentration of 89 nM (≅400 ng/ml enfuvirtide) and 11 nM (≅50 ng/ml enfuvirtide), respectively. These analyses showed that sensitive MS measurement of the investigated peptides is not simply a matter of size or charge state of the peptide, but probably also depend on a peptide's specific characteristics.

3.3. Chymotrypsin digestion of enfuvirtide

The digestion of enfuvirtide with chymotrypsin is a continuous process: fragments are formed slowly or rapidly while further cleavages at less specific sites also occur. Enfuvirtide is rapidly cleaved at the C-terminus of W^{33} to form **D**. Cleavage at the C-termini of L^4 and W^{29} is also easily achieved to form **A** and **C**. Cleavage at the C-terminus of L^{24} occurs more slowly, resulting in formation of fragment **B** which is thereafter digested into **E** and **F**. Fragment **D** is further digested by cleavage at the C-terminus of F^{36}, resulting in the de-amidated form of fragment **D** (**G**). Since **C** was rapidly formed and was not further digested when digestion time was increased, a labeled analog of this specific fragment was synthesized. Determination of the digestion recoveries of the different fragments provided more detailed information about the proteolysis of enfuvirtide by chymotrypsin, as described in Section 3.5.

The metabolite and deuterated analog are digested similarly by chymotrypsin as enfuvirtide itself. Since M-20 only differs from enfuvirtide by de-amidation of the C-terminus, the only differing fragment after chymotrypsin digestion is the fragment containing the C-terminus, corresponding to the peak at 8.2 min for enfuvirtide (**D**; Fig. 2a) and at 9.3 min for M-20 (**G**; Fig. 2b). The isotopically labeled fragments formed after chymotrypsin digestion of d60-enfuvirtide (Fig. 2c) show a slightly lower retention time than the fragments formed after digestion of enfuvirtide, except for **D** that does not contain any leucine amino

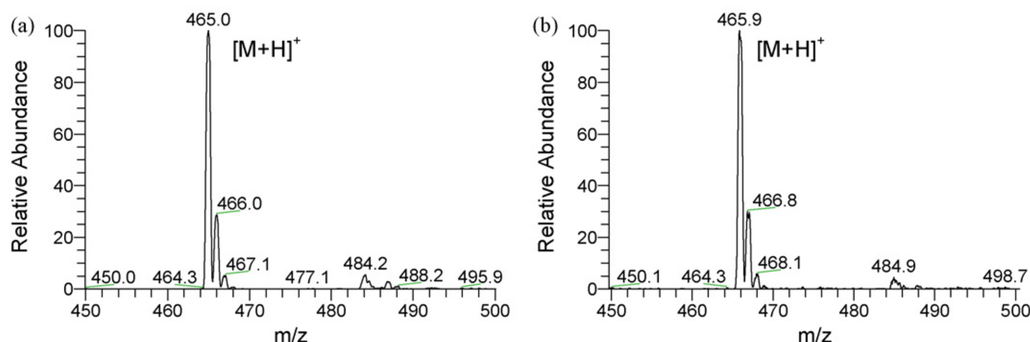

Fig. 6. ESI-MS spectra of the C-terminus fragment after chymotrypsin digestion of (a) enfuvirtide and d60-enfuvirtide (**D**) and of (b) M-20 (**G**).

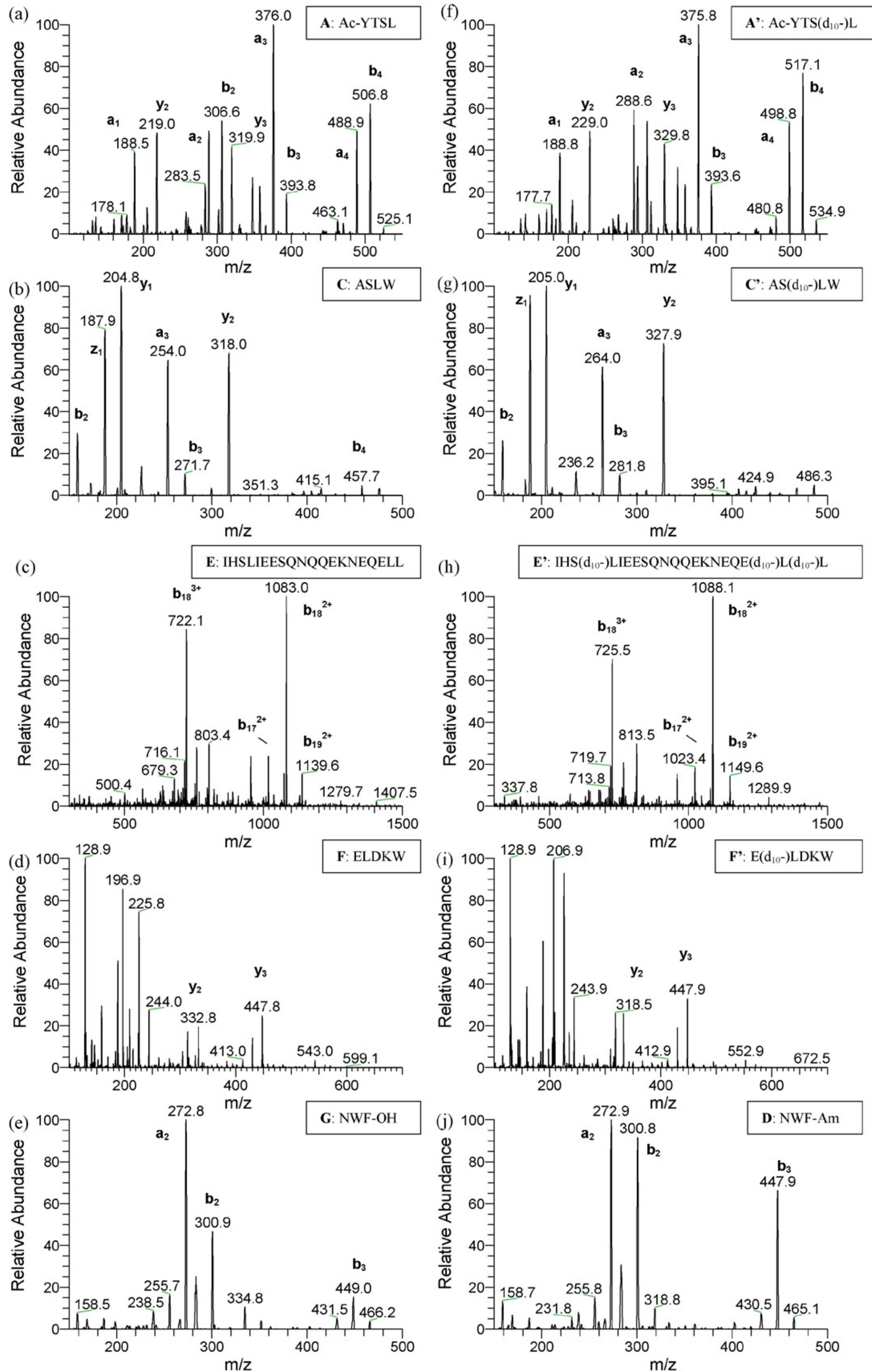

Fig. 7. Product ion spectra of the different chymotryptic fragments of M-20 (a–e), d60-enfuvirtide (f–j) and enfuvirtide (a–d, and j). (a) **A** (525.2 @ 18 V); (b) **C** (476.2 @ 25 V); (c) **E** (804.1 @ 25 V); (d) **F** (345.6 @ 19 V); (e) **G** (466.2 @ 20 V) (f) **A**′ (535.2 @ 18 V); (g) **C**′ (486.2 @ 25 V); (h) **E**′ (814.1 @ 25 V); (i) **F**′ (350.6 @ 19 V); and **D** (465.2 @ 20 V). Parent ion masses and collision energies are mentioned between brackets. Q1 and Q3 resolutions were set at 0.70 FWHM. Peptide fragment ions are identified according to a proposed fragmentation pattern and are indicated in the spectra by the Biemann notation for specific peptide fragments. The amino acid sequence of the parent ion peptide fragment is indicated in the boxes.

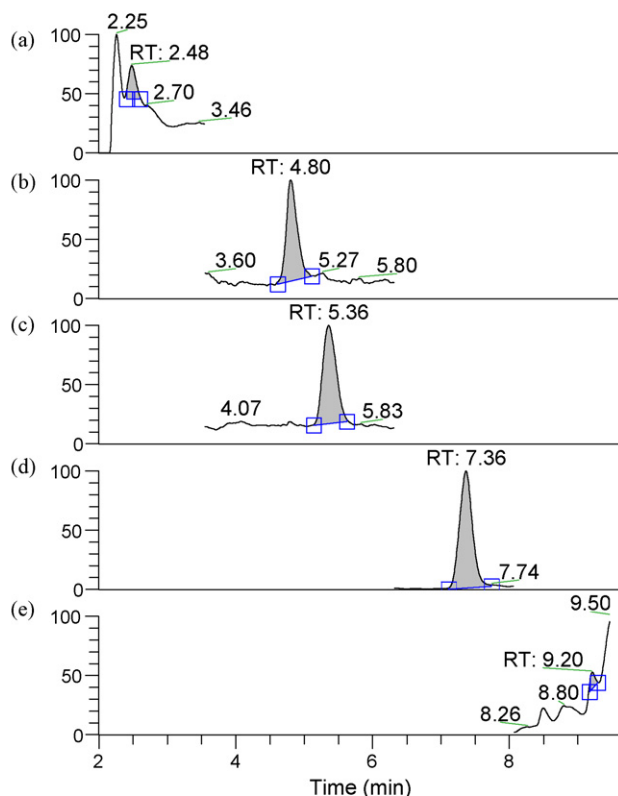

Fig. 8. SIM chromatograms of (a) **F**; (b) **A**; (c) **C**; (d) **E**; and (e) enfuvirtide at concentrations of 89 nM. Monitored ions were *m/z* 345.6, 525.2, 476.2, 804.1 and 1124.0 for **F**, **A**, **C**, **E** and enfuvirtide, respectively.

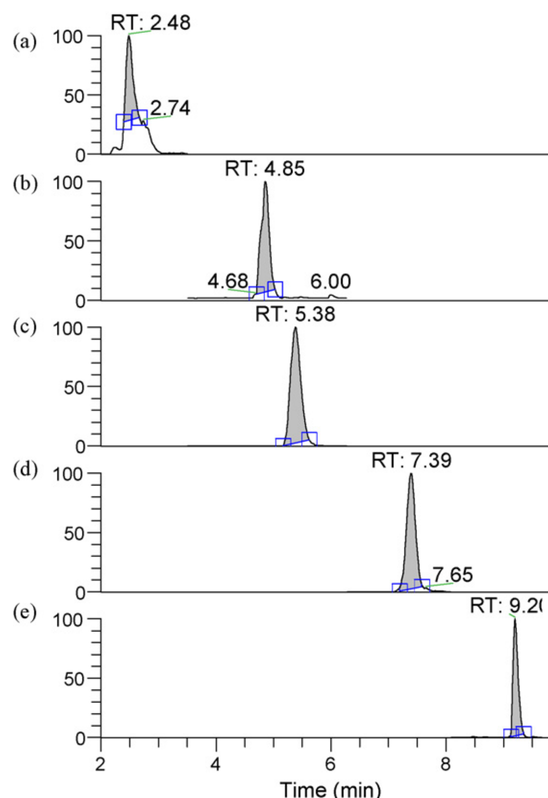

Fig. 9. SRM chromatograms of (a) **F**; (b) **A**; (c) **C**; (d) **E**; and (e) enfuvirtide at concentrations of 11 nM. Monitored ions were *m/z* 345.6 → 197.1 @19 V, 525.2 → 376.1 @ 18 V, 476.2 → 188.0 @ 18 V, 804.1 → 1083.5 @ 25 V and 1124.0 → 1343.5 @ 18 V for **F**, **A**, **C**, **E** and enfuvirtide, respectively.

acid residues and is similarly formed after proteolysis of both enfuvirtide and d60-enfuvirtide.

The digestion procedure of enfuvirtide extracted from plasma had to be carefully optimized. As digestion of enfuvirtide is performed after extraction from a complex matrix, the presence of remaining matrix components, e.g. other peptides or proteins, affects its reproducibility. A poor digestion was sometimes observed, in which only **D** was formed and only little further cleavage of the fragment Y^1-W^{33} occurred. Special attention to buffer strength and pH, amount of chymotrypsin and IS, and of digestion time was therefore required. Initial experiments were performed in 50 mM Tris–HCl/1 mM $CaCl_2$ buffer (pH 7.8), but the optimal buffer for chymotrypsin digestion of enfuvirtide after SPE was a 100 mM Tris–HCl/2 mM $CaCl_2$ buffer with a pH of 8.6. Increased buffer strength and higher pH to compensate for the presence of acids, such as remaining TFA, seemed beneficial for better reproducibility of the digestion. The optimal amount of chymotrypsin added to the buffer was 7.5 μg. The digestion time was also optimized individually for both procedures with d60-enfuvirtide and d10-ASLW as IS. For the procedure using d10-ASLW, the precise measurement of **D** was required to make distinctive quantification of enfuvirtide and M-20 possible. As **D** is rapidly formed and thereafter slowly transformed into **G**, the digestion time could not be too long. Therefore, **E** and **F** that are formed more slowly could not be used for quantification of enfuvirtide concentration with this procedure. For the highest recovery of **A**, **C** and **D** a digestion time of 30 min at 37 °C was optimal. With d60-enfuvirtide as IS, **D** could not be used and the digestion could be performed longer to obtain maximum recoveries of **A**, **C**, **E** and **F** after 60 min at 37 °C.

3.4. Quantification of enfuvirtide and M-20

Chymotryptic digestion of enfuvirtide and d60-enfuvirtide yield the same fragment at the C-terminus (**D**). Because enfuvirtide only differs from M-20 by the amide group at the C-terminus, this fragment will be crucial when both compounds should be quantified separately and the lack of a labeled analogous fragment therefore complicates the distinction of these two analytes. Moreover, since **D** is very slowly digested into the de-amidated M-20 specific fragment **G**, distinction between enfuvirtide and M-20 is impossible when d60-enfuvirtide is used as IS, i.e., when using the response of the M-20 specific fragment **G** relative to the response of the enfuvirtide and d60-enfuvirtide specific fragment **D** as a representation of the amount of M-20, an excess of d60-enfuvirtide is required. However, this excess of d60-enfuvirtide results in a relatively high formation of **G** in comparison to the amount formed by digestion of the metabolite, despite its slow formation.

When d10-ASLW is used as an IS, **D** is primarily originating from enfuvirtide. The response of the enfuvirtide specific fragment **D** relative to the response of d10-ASLW could therefore be used as a specific representation of the amount of enfuvir-

tide. Since the two other fragments (**A** and **C**) are a measure for the total amount of both enfuvirtide and M-20, a distinction between enfuvirtide and M-20 might be possible by this approach. Therefore, plasma samples were spiked with both enfuvirtide and M-20, whereas the plasma samples measured with d60-enfuvirtide as IS were only spiked with enfuvirtide.

However, although both enfuvirtide and combined enfuvirtide and M-20 concentrations could be accurately and precisely quantified, the concentration of M-20 could not be determined by subtracting both concentrations. Samples containing enfuvirtide and M-20 in a 10:2 ratio were used and the concentrations of enfuvirtide and combined enfuvirtide and M-20 therefore only slightly differed. As a result, large variations and negative values for the M-20 concentrations occurred within the accepted standard deviations. Separate quantification of enfuvirtide and M-20 was therefore not feasible with this method. For eventual future quantitative methods for large peptides or proteins from biological matrices using proteolytic digests, it is important to be aware of the possible co-quantification of similar proteolytic digests of metabolites or other similar compounds. If separate quantification of an analyte and its metabolites is required, peptide fragments that are similarly formed cannot be used.

When the method with a labeled peptide fragment as IS would be applied in a clinical setting, quantification of enfuvirtide alone using **D** would be preferred, as M-20 exposes approximately 20% of *in vitro* activity of enfuvirtide [24,25]. Moreover, formation of **D** was most easily achieved after chymotrypsin digestion and **D** showed very accurate and precise responses.

3.5. Digestion recovery

The recovery of enfuvirtide after SPE and subsequent evaporation and reconstitution in digestion buffer was determined to be ca. 80% at all concentration levels. The digestion recovery of **C** was calculated to be approximately 100% for both methods. Since the procedure measuring UV absorbance also showed a digestion recovery of about 100%, the digestion of enfuvirtide into **C** was assumed to be complete. Digestion recovery of **A** was around 40% using d60-enfuvirtide and around 55% using d10-ASLW as IS. This might indicate that longer digestion of enfuvirtide results in the further cleavage of **A**, probably after Y^1, although these fragments were never identified. With the experiment using UV absorption, a digestion recovery of about 80% was calculated for **A**. However, these UV experiments were performed in pure buffer and with higher enfuvirtide concentrations and these results can therefore only be interpreted as an indication of digestion performance.

The fragments **E** and **F** were formed slowly and these fragments could not be used for quantification of enfuvirtide after digestion with d10-ASWL for only 30 min. The digestion recoveries of these fragments using d10-ASLW were low and with high variations within and between the different concentration levels. The digestion recovery of **F** was around 65% for the procedure using d60-enfuvirtide, whereas digestion recovery was estimated at around 75% with the UV procedure. The digestion recovery of **E** was calculated to be around 30% for the low and mid QC sample and to be 100% for the high QC sample, merely because of a three fold increase in the absolute response of the synthetic peptide at the low and mid QC level. Since response ratios of **E** always showed very good linearity, this observation could not be explained and these results have to be interpreted cautiously. Digestion recovery could not be calculated from the UV experiment, because this fragment possesses little or no UV absorbance at 214 and 280 nm.

No synthetic peptide of **D** was available and the digestion recovery of **D** could therefore only be estimated by the UV-experiment, which gave a result of more than 100% recovery. It can therefore be assumed that cleavage of enfuvirtide at Trp^{33} is complete and **D** is almost 100% recovered, except for the small fraction that is further digested into the de-amidated fragment **G**.

Although complete digestion of the peptide into a specific fragment is preferable when this fragment is used as a representation of the concentration of the intact peptide, digestion recovery should mainly be reproducible and precise. When using d60-enfuvirtide as IS this was not as important as when the labeled fragment was used, since d60-enfuvirtide corrects for variations during the digestion procedure. Relative standard deviations (RSD) of the total recovery of **A** and **C** using d10-ASLW were below 11.3% and 6.8%, respectively.

3.6. Validation

3.6.1. Linearity

The assay using d60-enfuvirtide as IS was linear from 100 to 10,000 ng/ml enfuvirtide for each of the four different fragments. With d10-ASLW as IS, the assay was linear from 120 to 12,000 ng/ml enfuvirtide and M-20, using either **A** or **C**. Using **D**, enfuvirtide showed perfect linearity from 100 to 10,000 ng/ml. Linearity data obtained after analysis of the calibration standards using either d60-enfuvirtide or d10-ASLW are listed in Tables 2 and 3, respectively. With the procedure using d10-ASLW, **A** gave more precise results, possibly because of shorter digestion time.

3.6.2. Accuracy and precision

Assay performance data for both procedures are presented in Tables 4 and 5. For the lowest QC sample (100 ng/ml) the accuracies and precisions were within the ±20% range, except for the inter-assay precision using **C** and d10-ASLW as IS. With d60-enfuvirtide as IS, as much as four different fragments could be used for the quantification of the enfuvirtide concentration. When the results of the four fragments were averaged for each analysis, improvement of both accuracy and precision was observed (Table 4).

Table 2
Linearity data for four different chymotryptic fragments after analysis of seven calibration standards of enfuvirtide (250–10,000 ng/ml) in three runs

Fragment	Slope (ml/ng)	Intercept	R^2
A	0.0002 ± 0.0001	0.0066 ± 0.0068	0.9929 ± 0.0081
C	0.0002 ± 0.0001	0.0066 ± 0.0068	0.9954 ± 0.0035
E	0.0001 ± 0.0011	−0.0007 ± 0.0017	0.9951 ± 0.0030
F	0.0001 ± 0.00002	0.0002 ± 0.0022	0.9914 ± 0.0031

Table 3
Linearity data for three different chymotryptic fragments after analysis of seven calibration standards of enfuvirtide (250–10,000 ng/ml) and M-20 (50–2000 ng/ml) in three runs

Fragment	Slope (ml/ng)	Intercept	R^2
A	0.0001 ± 0.00002	−0.0006 ± 0.0010	0.9950 ± 0.0045
C	0.0002 ± 0.0001	0.0052 ± 0.0071	0.9949 ± 0.0025
D	0.0002 ± 0.00004	−0.0028 ± 0.0047	0.9981 ± 0.0014

The proteolyses of enfuvirtide were initially performed in order to investigate if MS measurements of smaller fragments could result in a more sensitive assay for the quantification of the complete peptide. Unfortunately, the LLOQ of enfuvirtide was higher when chymotryptic fragments were used. This was partially because the smaller fragments did not have better MS responses and partially because of higher variations due to more sample-handling steps and incomplete digestion. Initial validation of the procedure at 20 and 50 ng/ml enfuvirtide, showed the best results using **E**, although these results were not acceptable.

When d10-ASLW was used as IS, **D** could be measured. Not only the sensitivity of **D** was high, but also its digestion recovery was very reproducible, resulting in the most accurate and precise results at the lowest concentrations. During initial experiments a poor digestion was sometimes observed, in which **A** and **C** were not formed, despite complete formation of **D**. This problem could be solved by increasing the pH of the buffer and the buffer strength, but it indicates the differences in reproducibility using different proteolytic fragments. It can therefore be concluded that cleavage of a large peptide or protein results in the formation of fragments possessing different MS sensitivities, which might be better than the sensitivity of the intact compound. However, this improved sensitivity is not related to the smaller sizes of these peptides, merely to their specific characteristics. Alternatively, higher MS responses might not directly result in a more sensitive assay, as the more extensive sample pre-treatment procedure or irreproducible or incomplete digestion might complicate precise and accurate measurements at low concentrations.

Table 5 also shows the results of calculating the M-20 concentration from the enfuvirtide concentration, quantified with **D**, and the total concentration of enfuvirtide and M-20, quantified with **C**. These results reveal that despite accurate and precise quantification of enfuvirtide and the sum of enfuvirtide and M-20, specific quantification of M-20 is not useful with this procedure. Using the total concentration, quantified with either **A** or with the mean of **A** and **C**, resulted in even poorer accuracy and precision.

3.6.3. Specificity and selectivity

Since chymotrypsin is able to cleave all other peptides and proteins present in the extract in addition to enfuvirtide and its metabolite, it might be possible that similar cleavage products are formed. Furthermore, interfering (unlabeled) cleavage products could be formed from impurities in the IS. Analysis of double blank plasma samples that were not digested with chymotrypsin showed a co-eluting peak for **E**. However, after digestion of the double blank plasma samples, the peak area of this co-eluting peak never exceeded 13% of the peak area of **E** at the LLOQ level. For **C** a co-eluting peak that was not present in the blank plasma sample without chymotrypsin digestion could

Table 4
Assay performance data of QC samples of enfuvirtide with the use of four different chymotryptic fragments (**A**, **C**, **E**, **F** and average of **A**–**F**) and d60-enfuvirtide as IS ($n = 18$)

Nominal concentration of enfuvirtide (ng/ml)	Measured concentration (ng/ml)	Intra-assay precision (%)	Inter-assay precision (%)	Deviation (%)
A				
104	101 ± 18	18.0	18.2	−2.4
259	264 ± 24	8.4	8.9	1.9
2024	2019 ± 192	6.2	9.5	−0.2
7783	7396 ± 910	5.6	12.3	−5.0
C				
104	104 ± 12	9.9	11.4	0.1
259	266 ± 25	8.4	9.5	2.6
2024	1956 ± 229	3.3	11.7	−3.4
7783	7440 ± 867	6.2	11.7	−4.4
E				
104	103 ± 12	9.0	11.6	−0.7
259	256 ± 22	7.1	8.4	−1.2
2024	2077 ± 174	7.4	8.4	2.7
7783	7960 ± 551	5.4	6.9	2.3
F				
104	101 ± 11	9.4	11.2	−2.7
259	257 ± 23	6.3	12.9	−1.1
2024	1935 ± 212	5.3	11.0	−4.4
7783	7203 ± 797	4.5	11.1	−7.4
Mean				
104	103 ± 8	7.3	8.0	−1.2
259	261 ± 19	4.9	7.2	0.6
2024	1997 ± 164	3.0	8.2	−1.3
7783	7500 ± 663	2.9	8.8	−3.6

Table 5
Assay performance data of QC samples of enfuvirtide and M-20, with the use of three different chymotryptic fragments (**A**, **C** and **D**) and d10-ASLW as IS ($n = 18$)

	Measured concentration (ng/ml)	Intra-assay precision (%)	Inter-assay precision (%)	Deviation (%)
Nominal concentration of sum of enfuvirtide and M-20 (ng/ml)				
A				
124	113 ± 18	11.4	16.1	−8.9
309	263 ± 33	8.3	12.6	−15.0
2411	2339 ± 291	12.0	12.4	−3.0
9273	9527 ± 1012	7.6	10.6	2.7
C				
124	114 ± 24	12.2	21.4	−8.0
309	303 ± 28	7.9	9.4	−2.0
2411	2579 ± 316	11.7	12.3	7.0
9273	10062 ± 696	5.2	6.9	8.5
Nominal concentration of enfuvirtide (ng/ml)				
D				
104	105 ± 14	11.2	13.1	1.5
259	246 ± 35	12.9	14.2	−5.0
2024	2056 ± 214	7.2	10.4	1.6
7783	7485 ± 525	5.8	7.0	−3.8
Nominal concentration of M-20 (ng/ml)				
C-D				
20	9 ± 21	1256	243	−57
50	51 ± 40	83	78	3.1
387	325 ± 398	79	123	−16.2
1490	2618 ± 904	30	35	76

be observed after digestion of blank and double blank plasma samples, as shown in Figs. 6 and 7. The peak area of this co-eluting peak sometimes exceeded 20% of the peak area of **C** at the LLOQ level. This means that the selectivity of the assay can be affected by the formation of similar chymotryptic fragments of other plasma components when only **C** is used for the quantification of enfuvirtide.

3.6.4. Stability

The results of the stability experiments of either enfuvirtide, M-20 or the chymotryptic fragments in dried extract and in reconstitution buffer before and after digestion are shown in Table 6. Enfuvirtide and M-20 were stable in plasma after 1-year storage at −30 °C and in both the dried extract and the reconstitution solvent for 24 h at 5 °C. After digestion of the peptides with chymotrypsin, the possibility for delayed LC–MS/MS analysis depends on the stability of the different proteolytic fragments. When the deuterated analog of enfuvirtide is used as IS, all fragments showed a negative deviation, but only **F** showed a too large deviation at the LQC concentration of enfuvirtide. In the presence of d10-ASLW, **C** gave a too large deviation at the LQC concentration of enfuvirtide and M-20, and **D** at the MQC and HQC concentration of enfuvirtide. As these deviations were mainly caused by differences in the absolute responses of the fragments, the use of a labeled analog of the complete peptide seems to correct for their degradation, whereas the use of a labeled fragment does not.

Digestion with chymotrypsin solutions stored at −80 °C for 3 days or for 1 h at room temperature or at ice did not influence the digestion efficiency compared to digestion with a freshly prepared solution. However, absolute MS response of all fragments after using a solution that was stored at −30 °C for 3 days or at −80 °C for 3 months was about 60% of the absolute MS response after using a freshly prepared solution.

3.7. Internal standards

The use of a smaller and cheaper stable isotopically labeled IS might be advantageous, especially for quantitative measurements of larger peptides or proteins. However, since the labeled fragment will not posses the same characteristics as the analyte until after proteolysis, correction for SPE and digestion variabilities might be better for the deuterated analog of the complete peptide. The effect of a cleavable IS that possesses more similar cleavage characteristics than a non-cleavable IS on the accuracy and precision of the values obtained when performing PC-IDMS was already evaluated by Barnidge et al. [26]. For the digestion of enfuvirtide with d60-enfuvirtide or d10-ASLW as IS, both the effect on the digestion as well as the sample pre-treatment procedure of the plasma samples has been evaluated. Absolute quantification of enfuvirtide in plasma could be performed using both ISs. The use of the deuterated analog of enfuvirtide did not significantly improve the accuracy and precision of the quantification. However, since the digestion of enfuvirtide with chymotrypsin is a non-ending process, the use of d10-ASLW was more complicated. Less complicated proteolysis could have occurred with another proteolytic enzyme, but chymotrypsin was initially chosen because it produced many different fragments, which could be used to define detection characteristics of peptides with different lengths. However, small differences in amount or freshness of the chymotrypsin solution or the pH of the buffer could affect the proteolysis process, especially the formation of **A** and **C**. Fragment **D** was most easily and reproducibly formed and this fragment was always successfully used

Table 6
Stability of enfuvirtide and the chymotryptic fragments under different conditions ($n = 3$ at each concentration)

Analyte	Matrix	Conditions	Deviation (%)			RSD (%)		
			L	M	H	L	M	H
Enfuvirtide	Plasma	−30 °C, 1 year	−8.4	9.4	11.3	15.8	4.8	2.2
M-20			−8.6	13.2	11.0	22.0	6.8	8.5
Enfuvirtide	Dried extract	5 °C, 24 h	8.3	0.4	6.3	5.0	3.8	4.7
M-20			−12.4	8.9	−0.9	23.6	5.9	8.1
Enfuvirtide	Reconstitution solvent	5 °C, 24 h	1.6	−5.0	4.5	7.4	5.6	5.7
M-20			−4.7	10.2	4.7	23.6	6.7	7.9
A	Final extract in presence of d60-enfuvirtide	Autosampler 5 °C, 24 h	−12.4	−12.3	−12.6	10.3	11.4	8.0
C			−7.0	−6.9	−5.6	5.5	2.3	7.6
E			−2.6	−5.7	0.8	7.7	10.3	9.9
F			−18.3	−12.5	−9.8	5.0	9.3	3.7
Mean			−10.3	−9.4	−6.8	3.0	5.3	3.9
A	Final extract in presence of d10-ASLW	Autosampler 5 °C, 24 h	5.1	−9.4	−11.9	12.3	15.9	11.3
C			−19.3	−11.2	−0.4	5.8	16.6	8.7
D			−14.4	−18.8	−21.0	15.3	14.2	11.4

for the quantification of enfuvirtide. These observations showed the importance and necessity for the proper selection of the fragment that is used for the quantification of the intact peptide or protein. If that fragment is easily formed and digestion is reproducible the use of a small labeled fragment is beneficial towards the use of a labeled analog of the complete analyte.

4. Conclusions

The LC–MS/MS measurement of proteolytic fragments of the polypeptide enfuvirtide after chymotrypsin digestion has shown its potential for the absolute quantification of large peptides and proteins in biological matrices. Two different methods for the quantification of enfuvirtide in plasma have been developed and validated, measuring chymotryptic peptide fragments and using either a stable isotopically labeled analog of the peptide or a stable isotopically labeled chymotryptic fragment as IS.

The use of a stable isotopically labeled fragment as IS gave similar results as the use of a stable isotopically labeled analog of enfuvirtide. This offers the possibility of using similar assays for the absolute quantification of larger peptides and proteins, especially when the fragment is easily and reproducibly formed. Otherwise, the use of a cleavable labeled fragment that is smaller than the complete compound and larger than the proteolytic fragment, will be preferable, as it corrects for digestion variabilities.

Besides the use of a simpler IS, formation of proteolytic fragments might result in better ionization properties of one or more fragments and therefore in a more sensitive assay. For enfuvirtide at least one chymotryptic fragment offered more sensitive MS detection, but as digestion was not completed for this fragment the LLOQ could not be improved. The ionization properties of the fragments appeared to depend on the specific characteristics of the formed fragments rather than their smaller size or charge state.

When proteolysis of a peptide or protein is used for the quantification of the intact compound, it is especially important to select a proper proteolytic enzyme and to carefully optimize the digestion procedure. It is most important that a proteolytic fragment that is reproducibly and preferentially completely formed is chosen to be used for the quantification of the complete compound. Furthermore, attention should be paid to the formation of similar fragments by proteolysis of other matrix components and especially of potential metabolites. Similar peptide fragments from matrix components interfere with the quantification of the analyte, whereas similar peptide fragments from metabolites obstruct the ability of separate quantification of the analyte and its metabolites.

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