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Bioanalytical method development and validation for a large peptide HIV fusion inhibitor (Enfuvirtide, T-20) and its metabolite in human plasma using LC–MS/MS

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Abstract

A method for measuring a human immunodeficiency virus (HIV) cell membrane fusion inhibitor (T-20/Ro 29-9800) and its metabolite (M-20/Ro 50-6343) in human plasma by liquid chromatography tandem mass spectrometry (LC–MS/MS) was developed. The relatively large peptide analytes and their corresponding deuterated (d_{10}) peptides used as internal standard were isolated from plasma by protein precipitation with two volumes of acetonitrile to plasma. A large pore size reversed-phase C_{18} column was employed to elute the peptides. A triple quadrupole mass spectrometer with electrospray interface operating in positive ion and multiple reaction monitoring modes with transitions m/z 1124 → 1343 for both T-20 and M-20 was utilized for peak detection. The advantages of the method were a simple sample preparation, specific and sensitive MS/MS detection, and a wide dynamic range of 10–2000 ng/ml for T-20. The method was validated and used for analyzing samples from clinical studies to provide pharmacokinetic profiles of the HIV fusion inhibitor peptide drug and its metabolite.

Keywords: HIV fusion inhibitor; Enfuvirtide; T-20; LC–MS/MS; Polypeptide bioanalysis; gp41

1. Introduction

Enfuvirtide (T-20), also known as Ro 29-9800, is currently in Phase III clinical development as a human immunodeficiency virus (HIV) fusion inhibitor [1,2]. It is a 36-amino acid synthetic peptide with molecular weight of 4492 Da. The primary sequence was derived from a naturally occurring motif within the gp41 transmembrane glycoprotein of HIV-1. The N-terminus was acetylated and the C-terminus was amidated. T-20 exhibits potent and selective inhibition of virus infection by binding to a critical region of gp41 which regulates the fusion of HIV-1 to host cell membranes. A metabolite, M-20, produced by deamidation at the C-terminus was detected in rat plasma and after incubation of T-20 with rat or human liver microsomes.

Liquid chromatography tandem mass spectrometry (LC–MS/MS) has been used extensively as a quantitative technique in the analysis of drugs in biological matrices with high sensitivity, selectivity, and speed [3]. Most LC–MS/MS assays are for small drug molecules. Quantitation of peptides of similar size to T-20 using selective ion monitoring (SIM) mode was recently reported for insulin [4], insulinotropins [5], and endothelins [6]. Mass spectrometric detection in the SIM mode provided poor selectivity for these peptides. Complex sample cleanup using solid phase extraction or immunoprecipitation and high sample volumes were employed to improve selectivity. In the T-20 assay, a triple quadrupole mass spectrometer in the multiple reaction monitoring (MRM) mode and selected specific fragment ions for T-20 were used to provide excellent selectivity and sensitivity.

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A simple protein precipitation was sufficient for plasma sample cleanup.

Several challenges exist for quantitation of large peptides by LC–MS/MS. Multiple charges on a large peptide, sample extraction, poor chromatography, and sticking to tubing and container walls present challenges to development of methods to quantitate large peptides in plasma. Bioanalytical methods for these compounds usually rely on immunoassays. Selectivity and interference from metabolites in the incurred samples could be a potential problem with immunoassays. Most immunochemical methods quantify only one analyte at a time, and additional time and efforts would be needed for method development and sample analysis to obtain data for the drug metabolite(s).

A simultaneous assay was developed for a large peptide (T-20) and its C-terminus de-amidated metabolite (M-20) through a limited acetonitrile (ACN) protein precipitation under acidic conditions, chromatography on a wide pore C-18 column, and analysis of a multiply charged ion on a tandem mass spectrometer. The assay for T-20 and M-20 was validated in the range of 10–2000 and 10–500 ng/ml, respectively, and used for analysis of samples from clinical trials [7]. The formula of T-20 is $C_{204}H_{301}N_{51}O_{64}$, MW 4492. The peptide sequence is: Ac-Tyr Thr Ser Leu Ile His Ser Leu Ile Glu Glu Ser Gln Asn Gln Gln Glu Lys Asn Glu Gln Glu Leu Leu Glu Leu Asp Lys Trp Ala Ser Leu Trp Asn Trp Phe-NH_2. The empirical formula of M-20 is $C_{204}H_{300}N_{50}O_{65}$, MW 4493. The sequence of M-20 is the same as T-20 except the C-terminus is Phe-OH.

2. Experimental

2.1. Reagents and chemicals

All the chemicals were HPLC grade or reagent grade unless otherwise stated. Acetonitrile, methanol, glacial acetic acid, and ammonium hydroxide (30%) were from Fisher Scientific (Fair Lawn, NJ, USA). Trifluoroacetic acid (TFA) was from Pierce (Rockford, IL, USA). De-ionized water was from in-house Milli-Q UF Plus. T-20 was supplied by Trimeris, Inc. (Durham, NC, USA) with 92.5% purity. The de-amidated metabolite M-20 was supplied by Hoffman LaRoche (Nutley, NJ, USA) with 81.1% purity. Stable isotope labeled internal standards (I.S.) for both T-20 and M-20 were labeled with 10 deuterium atoms introduced at the fourth amino acid of leucine. d_{10} T-20 and d_{10} M-20 were supplied by Trimeris, Inc. (Durham, NC, USA) with purity of 88.8 and 86.1%, respectively. Human control EDTA plasma from healthy volunteers and HIV^+ control blank EDTA plasma were purchased from BioChemed (Winchester, VA, USA).

2.2. Equipment

LC–MS/MS analyses were performed using Jasco PU-980 pumps (Tokyo, Japan) connected to a Micromass Ultima triple quadrupole mass spectrometer (Micromass UK Limited, Manchester, UK). The CTC HTS PAL autosampler was from Leap Technologies (Carrboro, NC, USA). The analytical column was a Keystone BioBasic C_{18}, 50 mm × 2 mm, 300 Å with 5 μm particle size (PN 055721-2, Keystone Scientific, Bellefonte, PA, USA). A Phenomenex Widepore C_{18}, 4 mm × 2 mm i.d. cartridge (PN AJO-4320), was used as a guard column. Data acquisition and processing were accomplished by MassLynx 3.4 (Micromass). The Quadra 96 automatic work station was from Tomtec (Hamden, CT, USA), and the Turbo Vap 96 evaporator was from Zymark (Hopkinton, MA, USA). The microcentrifuge was Micromax RF from International Equipment Company (Needham Heights, MA, USA).

2.3. Liquid chromatographic conditions

Mobile phase A was water:acetic acid:TFA, 100:0.2:0.02 (v/v/v), and mobile phase B was ACN:methanol:acetic acid:TFA, 85:15:0.2:0.02 (v/v/v/v). A linear gradient with flow rate at 0.4 ml/min was ramped up from 45% B to 54% B in 3 min, and then to 75% B in 2.5 min at a flow rate of 1.0 ml/min, and returned to 45% B for re-equilibration at 0.4 ml/min. The total run time was 7.5 min. The injection syringe was washed three times with methanol:water, 1:1 (v/v), followed by three times with water. The detailed mobile phase gradient is shown in Table 1. The gradient conditions could be modified slightly if necessary with each lot of chromatographic packing material using a post-column infusion test to separate the analytes from signal suppressing matrix components [8].

2.4. Mass spectrometer conditions

The mass spectrometer was operated in positive ion electrospray ionization mode. The capillary voltage was set at 4.0 kV, cone voltage at 70 V, and collision potential difference set at 20 V. The source block temperature was set at 130 °C and the desolvation temperature was at 400 °C. The transitions for multiple reaction monitoring were *m/z* 1124.0 → 1343.5 for T-20 and M-20, 1126.5 → 1346.8 for corresponding deuterated I.S. The dwell time was 0.700 s for

Table 1
T-20 mobile phase gradient elution program

Time (min)	Flow (ml/min)	Linear gradient		Description
		A (%)	B (%)	
Initial	0.4	55	45	Flow to MS
3.0	0.4	46	54	Gradient change
4.0	1.0	46	54	Flow rate change, flow to waste
6.5	1.0	25	75	Gradient change
6.6	0.6	25	75	Flow rate change
6.7	0.6	55	45	Gradient change
7.2	0.4	55	45	Flow rate change
7.5	0.4	55	45	Total time

the analytes and 0.300 s for the I.S. The acquisition time was 5.5 min.

2.5. Preparation of standards and quality controls

To prepare T-20 and M-20 stock solutions, approximately 10 mg of the reference standard was accurately weighed into a sealable, polypropylene test tube and dissolved in an appropriate volume of stock solution solvent (ACN:0.2% ammonium hydroxide in water, 20:80, v/v) to make 1 mg/ml stock. The stock was further diluted to substock solutions with the same solvent. The appropriate amounts of the stock or substock solutions were spiked into human control plasma to prepare standards and quality control (QC) samples. T-20 stock solution of 100 μg/ml or higher could be stored at −20 °C for up to 6 weeks. All M-20 solutions and the combined substock solutions at lower concentration were prepared and used immediately to avoid potential degradation or adsorption problems. Concentrations of parent peptide solutions were 10, 25, 50, 100, 200, 1000, and 2000 ng/ml for standards, and 15, 150, 1500, and 15,000 ng/ml for QCs. Concentrations of metabolite solutions were 10, 25, 50, 100, 200, 400, and 500 ng/ml for standards, and 15, 75, 375, and 3000 ng/ml for QCs. The highest concentration QC was diluted 10-fold with blank control plasma before analysis. Calibration standards and QC samples were pipetted as 250 μl aliquots into polypropylene tubes and stored frozen at −70 °C until analysis. Deuterated I.S. working solution at 2.5:1.0 μg/ml of parent peptide:metabolite was prepared in the stock solution solvent. System suitability solutions were used to monitor the performance of the LC–MS/MS systems. Working solutions of system suitability solutions at concentrations of 20:5 μg/ml (parent peptide:metabolite) and at 100 ng/ml of both analytes were spiked into processed control blank plasma that had gone through the sample cleanup processing steps.

2.6. Sample processing

Plasma sample processing was by protein precipitation. One hundred microliters of study samples, calibration standards, or QCs was pipetted into a 96-well plate. The internal standard, 25 μl, and 20 μl of 1.0 mol/l HCl were introduced and mixed gently. ACN, 0.2 ml, was added to all samples in the matrix tubes in a 96-format plate using the Quadra 96®. The tubes were capped, and vortex mixed for 5 min, and centrifuged at 19,600 × *g* for 3 min in a microcentrifuge. 0.20 ml of the supernatant was transferred to another set of 96-format tubes with the Quadra 96® and evaporated to dryness using the Zymark Turbo Vap 96® system. All samples were reconstituted in 150 μl reconstitution solution (ACN:water:acetic acid:TFA, 30:70:0.14:0.014, v/v/v/v), capped, and vortex mixed for 7 min. The reconstituted samples were centrifuged at 19,600 × *g* for 10 min, the supernatant was transferred to an autosampler vial and 15–40 μl was injected onto the LC–MS/MS system.

2.7. Analytical data treatment

Chromatograms were integrated using MassLynx software, and raw data were subsequently transferred into the OpenVMS® on AlphaServer® Systems Oracle® database. A weighted [$(1/x^2)$ where x: analyte concentration] linear regression was used to determine slopes, intercepts, and correlation coefficients. The resulting parameters were used to calculate concentrations:

$$\text{concentration} = \frac{\text{ratio} - (y\text{-intercept})}{\text{slope}}$$

where "ratio" is the ratio of the compound peak area to the internal standard peak area.

3. Results and discussion

3.1. Method development

3.1.1. MS/MS optimization

The mass spectrometer was operated in the positive ion multiple reaction monitoring mode in the LC–MS/MS analysis. The mass spectrum of T-20 (Fig. 1) shows a prominent ion at m/z 1124.0 ($z = 4$). The mass spectrum of M-20 (Fig. 2) shows a major ion at m/z 1124.2 ($z = 4$). The product ion scans of both T-20 (Fig. 3) and M-20 (Fig. 4) show that fragmentation of T-20 and M-20 would yield several useful transitions. Transitions to low molecular mass product ions with a singly charged fragment at m/z 159 (Fig. 5), the immonium ion of tryptophan, were initially considered for the analyses. While this fragment with the transition m/z of 1124 → 159 was more abundant with high collision energy 200 eV, resulting in high signal intensity, there was interference from the endogenous substances of the control plasma. This was probably due to the large number of residual proteins or peptides in the plasma extract containing tryptophan in their amino acid sequences. Therefore, transitions to low molecular mass singly charged fragments were not considered to be suitable for a selective assay. The transition m/z 1124 → 1343, representing a triply charged ion fragment generated from the quadruply protonated molecular ion (m/z 1124, $[M + 4H]^{4+}$), was then selected for chromatographic peak detection and quantitation. The triply charged fragment ion m/z 1343 was a large b_{33} fragment (MW = (1343 × 3) − 3 = 4026 Da), which was more specific to T-20 and M-20, providing excellent selectivity and signal-to-noise ratio for the analysis.

3.1.2. Sample processing and liquid chromatography

There were limited options in sample cleanup for a relatively large peptide because both solid phase and liquid/liquid extraction using solvents of a high organic content would not be able to separate T-20 and M-20 peptides from other biopolymers in plasma. Protein precipitation to remove the macromolecule plasma proteins was chosen as a convenient and simple sample cleanup method for peptide analytes.

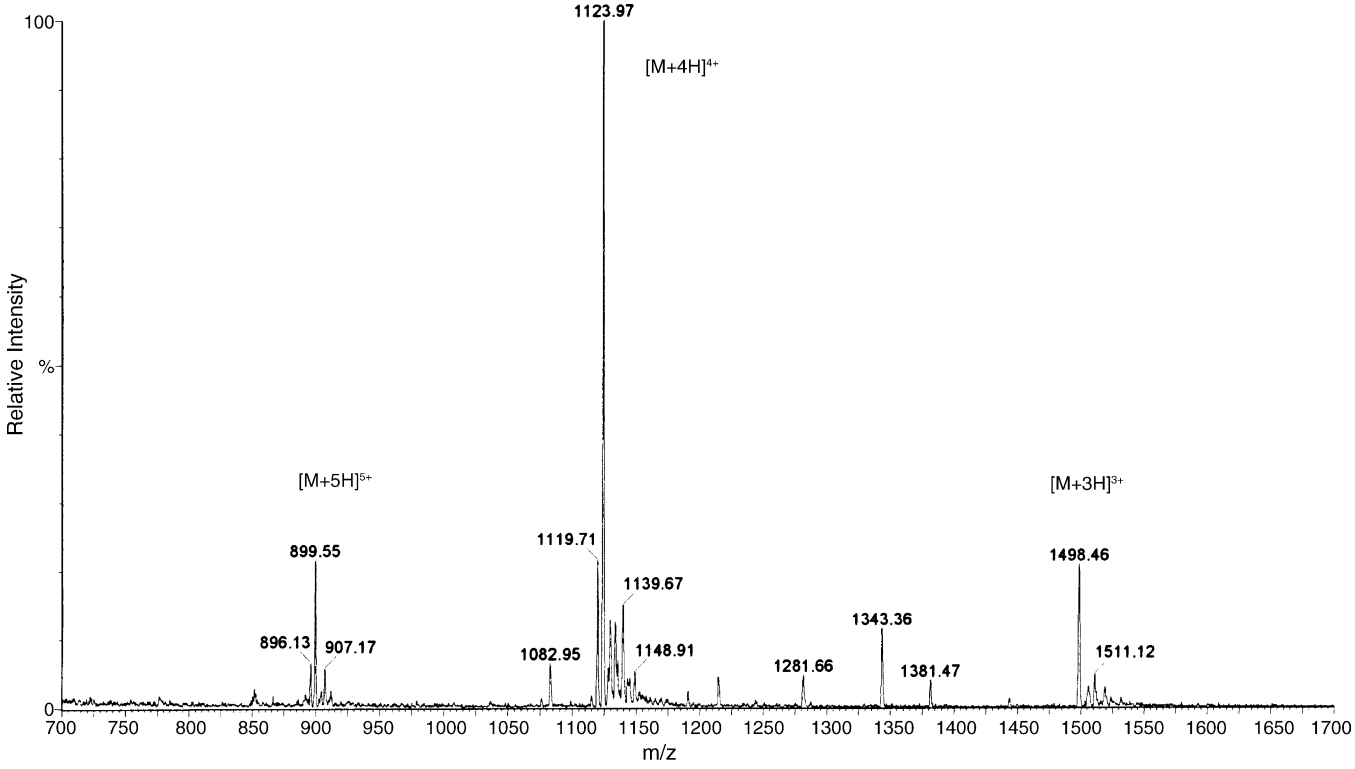

Fig. 1. Mass spectrum of T-20.

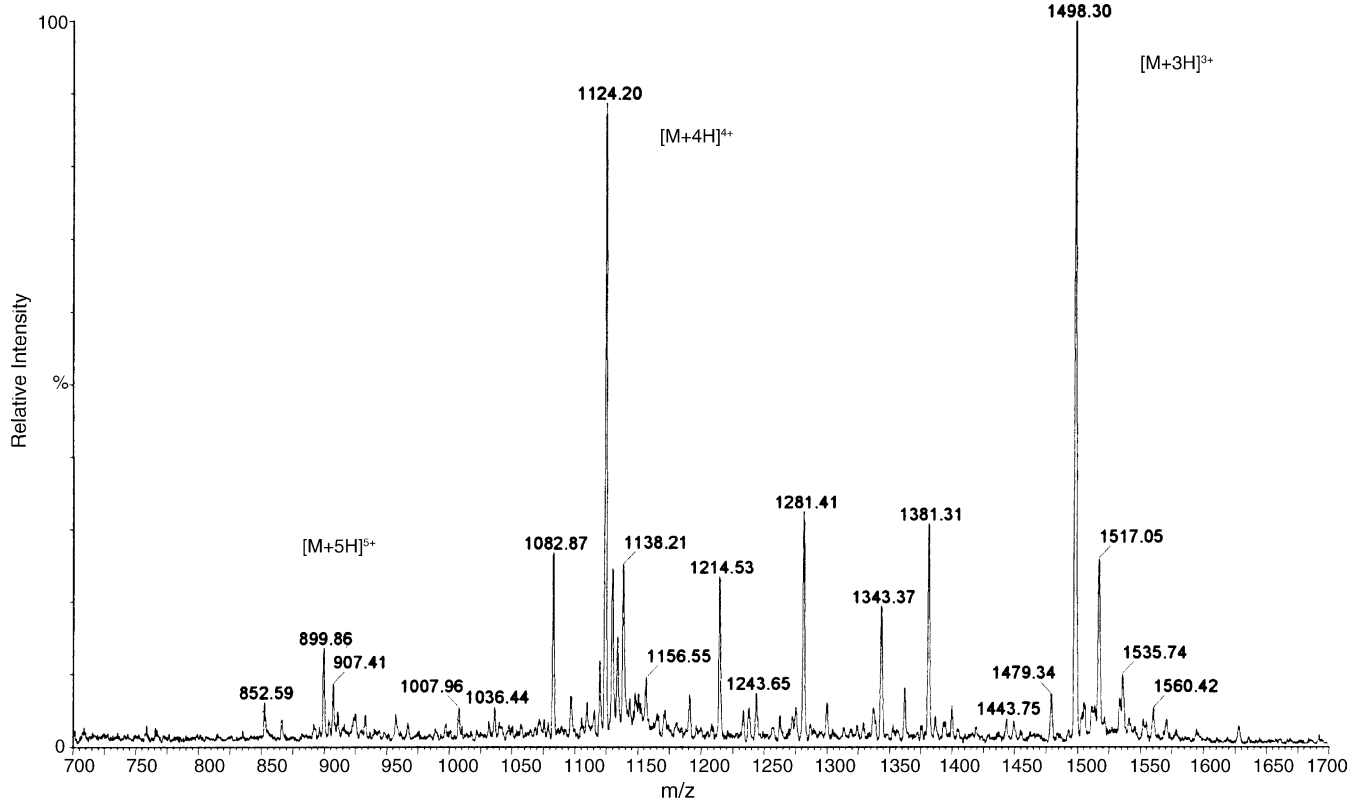

Fig. 2. Mass spectrum of M-20.

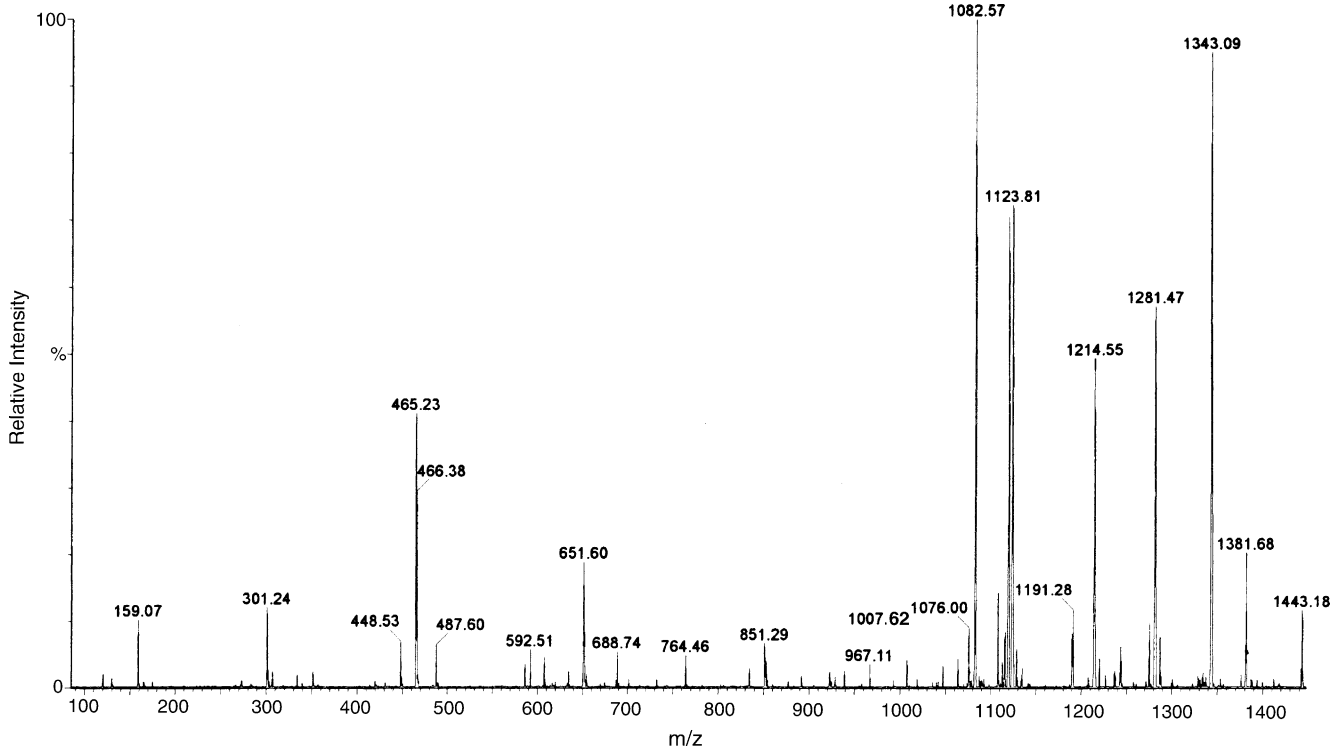

Fig. 3. Product ion spectrum of T-20 from *m/z* 1124 at collision energy of 80 eV (20 V collision potential difference).

Initially, four volumes of ACN to one volume of plasma sample were used to precipitate the plasma proteins. This high ACN to plasma volume ratio is commonly used for separating plasma proteins from small drug molecules. This process usually results in clean samples in the supernatant. Because of the relatively large molecular size, some of the analyte peptides were found to precipitate with the plasma proteins with the high organic solvent. When the ratio was reduced to

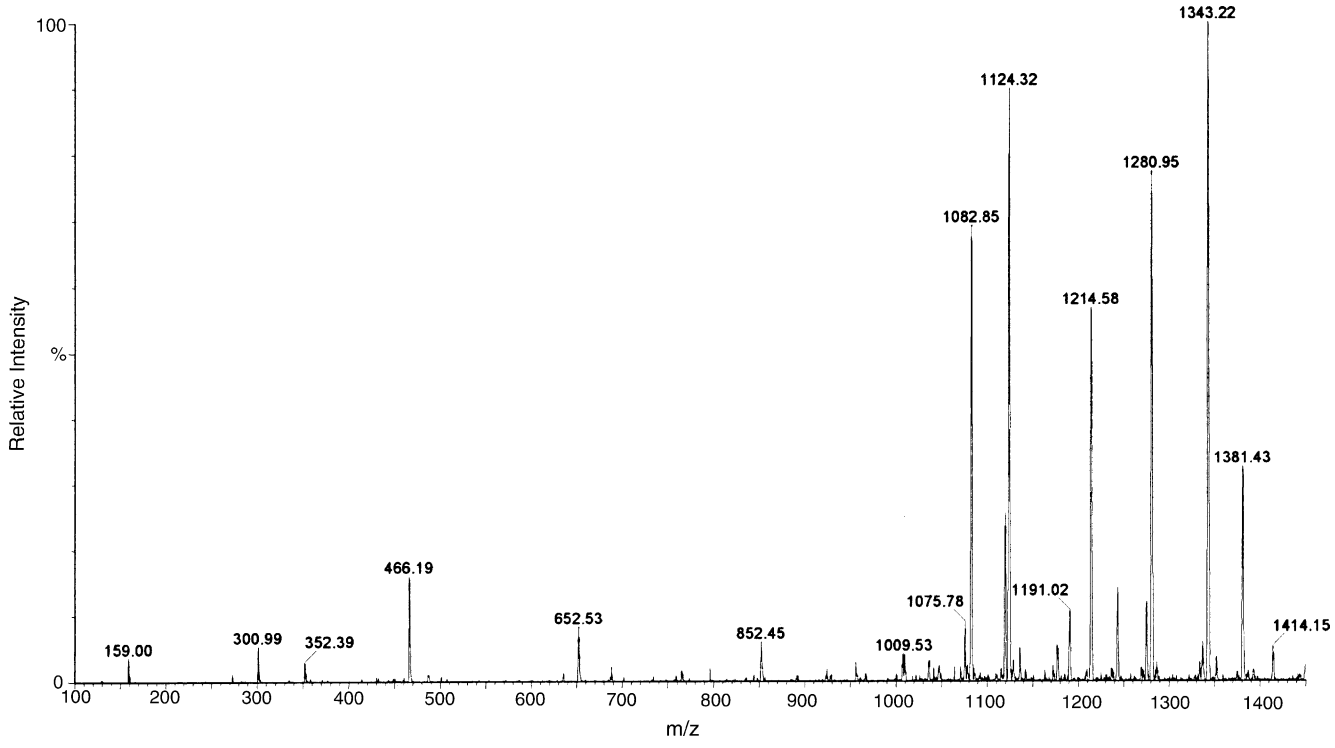

Fig. 4. Product ion spectrum of M-20 from *m/z* 1124 at collision energy of 100 eV (25 V collision potential difference).

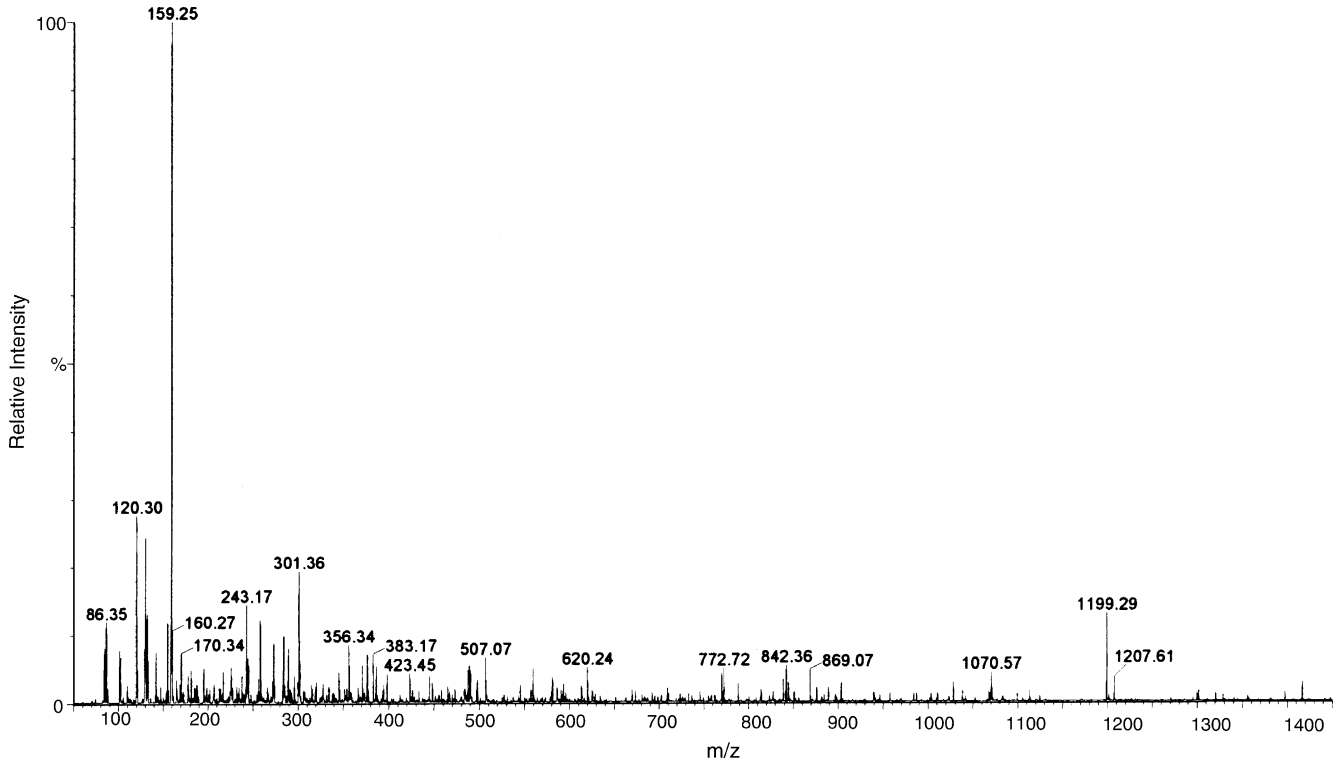

Fig. 5. Product ion spectrum of T-20 from *m/z* 1124 at collision energy of 200 eV (50 V collision potential difference).

2:1 of ACN:plasma, the results were satisfactory with consistently good recovery and relatively clean supernatant after centrifugation at high speed. Table 2 shows the recovery of T-20, which was 116%, and 105% for the I.S. The recovery of M-20 was 124%, and 110% for the I.S. Recoveries of greater than 100% may be due to losses of analyte by non-specific binding to surfaces during preparation of the post-extraction spiked samples. The high recoveries are not due to matrix effects, since matrix was present in the extracted sample and in the sample spiked after extraction. The recovery was consistent over the entire concentration range of both analytes and their corresponding I.S.

During method development, various analytical columns of different lengths and bonded phases were evaluated. The

Table 2
Recovery of analytes

	Peak area					
	100 ng/ml		2000 ng/ml		I.S.	
	REX	EXT	REX	EXT	REX	EXT
(a) T-20						
Mean	41747	27872	808740	545354	204297	124490
C.V. (%)	3.1	2.6	4.6	6.2	8.2	6.5
N	6	6	6	6	18	18
Recovery (%)	115		116		105	
Mean recovery (%)			116			
(b) M-20						
Mean	45515	30337	195227	149161	76354	48791
C.V. (%)	3.3	3.9	4.2	4.8	8.2	6.9
N	6	6	6	6	18	18
Recovery (%)[a]	115		132		110	
Mean recovery (%)			124			

[a] Extraction recoveries were calculated by using the equation (EXT/0.58REX × 100) where EXT is the peak area of analyte added to plasma prior to the extraction and REX is the peak area of analyte added to plasma after extraction. The post-extraction samples (REX) were prepared by reconstituting an extract of blank matrix in a solution of analyte prepared in reconstitution solvent. The factor 0.58 was included to compensate for the partial transfer of supernatant (200/345 μl = 0.58) of microcentrifuged EXT samples during sample processing (see Section 2.6).

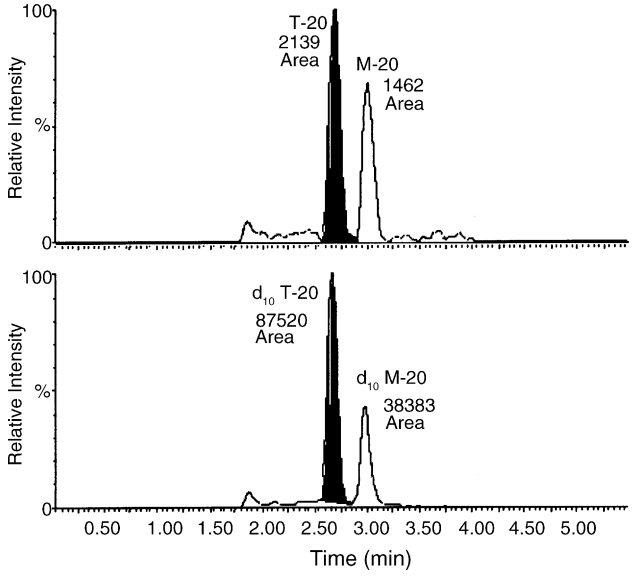

Fig. 6. Chromatograms of plasma sample with 10 ng/ml of T-20, 10 ng/ml of M-20, 625 ng/ml of d_{10} T-20, and 250 ng/ml of d_{10} M-20. The upper chromatogram is the MRM channel for T-20/M-20, and the lower chromatogram is the MRM channel for I.S.

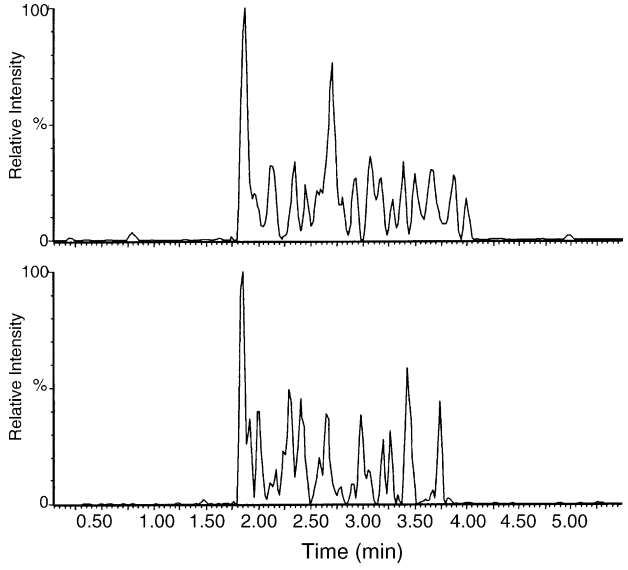

Fig. 7. Chromatograms of blank control plasma sample without the analytes and I.S. The upper chromatogram is the MRM channel for T-20/M-20, and the lower chromatogram is the MRM channel for I.S.

wide pore (300 Å) Keystone Bio Basic C_{18} (2 mm × 50 mm, 5 μm) column provided fast elution time for the large peptides with good peak shape. TFA in the mobile phase usually enhances sharpness of the peptide chromatographic peak but causes signal suppression in the electrospray ionization, reducing MS–MS sensitivity [9]. Mobile phases containing 0.02% TFA and 0.2% acetic acid provided a good compromise of maintaining peak sharpness without sacrificing sensitivity. Fig. 6 shows the chromatogram of the analytes at the low limit of quantification (LLOQ) of 10 ng/ml. The retention time of T-20 was at 2.70 min, which was well separated from its de-amidated metabolite at a retention time of 3.02 min. It was important to have chromatographic resolutions of the two analytes (and the corresponding I.S.) because the metabolite (or the corresponding I.S.) has the same MRM transition as parent peptide. Figs. 7 and 8 are the chromatograms of blank control plasma extracts without and with the I.S. The signal-to-noise ratios of both analytes are about 10, dependent on the different blank control lots, recovery, matrix effect, and LC–MS/MS platform conditions. There were no obvious differences in the background noise between the control plasma lots from the HIV^+ patients from those of healthy volunteers.

3.1.3. Internal standards

Deuterated I.S. of T-20 and M-20, each containing 10 deuterium atoms on the molecule, were synthesized and applied to the LC–MS/MS method. In addition to the matrix effect and recovery variations, there is ion distribution shift for multiple charged peptide ions during LC–MS/MS acquisition. It is necessary to use stable labeled internal standard to track analytes for peptide quantitation. The results were satisfactory for both analytes in our study using deuterated internal standards. The transition *m*/*z*'s of the d_{10}-I.S. were three atoms heavier than those of the corresponding analyte. Due to the hydrogen-containing impurity in the deuterated internal standard, a small signal in the blank control sample spiked with the d_{10}-I.S. appeared in the corresponding analyte channel, as shown in Fig. 8 comparing to Fig. 7 with no d_{10}-I.S. present. However, this signal contribution by the d_{10}-I.S. was very small relative to the lower limit of quanti-

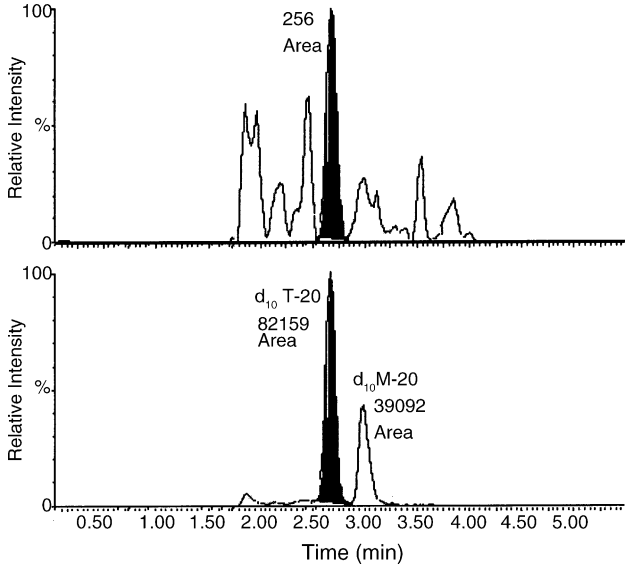

Fig. 8. Chromatograms of plasma sample with 0 ng/ml of analytes, 625 ng/ml of d_{10} T-20, and 250 ng/ml of d_{10} M-20. The upper chromatogram is the MRM channel for T-20/M-20, and the lower chromatogram is the MRM channel for I.S.

Table 3
Sensitivity and selectivity

Lot no.	Peak area		A[a] (10.0 ng/ml)		B (10.0 ng/ml)	
	Blank	LLOQ	Calculated concentration	Deviation (%)	Calculated concentration	Deviation (%)
(a) T-20						
1	0	1592	9.33	−6.7	9.69	−3.1
2	15	1707	8.93	−10.7	9.69	−3.1
3	0	1627	9.52	−4.8	8.60	−14.0
4	0	2023	10.8	+8.4	10.9	+8.6
5	0	1894	9.92	−0.8	8.49	−15.1
6	0	2009	9.91	−0.9	10.2	+1.8
Mean			9.74		9.59	
C.V.%[b]			6.7		9.5	
R.E.%[c]			−2.6		−4.1	
N			6		6	
(b) M-20						
1	31	830	8.54	−14.6	8.90	−11.0
2	0	1120	10.8	+8.4	10.5	+5.0
3	0	1133	10.3	+2.5	8.58	−14.2
4	0	1283	10.1	+1.2	11.3	+12.5
5	0	1110	9.47	−5.3	10.2	+1.5
6	0	898	8.57	−14.3	9.67	−3.3
Mean			9.63		9.84	
C.V.%[b]			9.8		10.2	
R.E.%[c]			−3.7		−1.6	
N			6		6	

[a] To evaluate sensitivity and selectivity, six lots of EDTA plasma were spiked with the analyte at 0 and 10 ng/ml. Column A: analyte 10 ng/ml, one replicate in six lots of EDTA plasma. Column B: analyte 10 ng/ml, six replicates in a single lot of EDTA plasma.
[b] C.V.%, coefficient of variation = (S.D./mean) × 100%.
[c] R.E.%, percent relative error = [(mean/nominal) − 1] × 100%.

tation signal in Fig. 6. It had no effect on the linearity of the standard curve or the accuracy of the LLOQ quantification as shown in the data in Section 3.2.1. In order to minimize the I.S. contribution to the analyte signal while maintaining adequate I.S. response, the d_{10}-I.S. were kept at a modest amount, not to exceed the mid-standard concentrations of the analytes.

3.1.4. Adsorption problem

Peptides are known to adhere to glass, plastic, and metal surfaces. When injected as a neat solution (without plasma matrix extracts) into the LC–MS/MS, the chromatographic peaks of the peptide analytes were small. The adhesion problem was more severe at low concentrations because the adsorption loss to the vessel walls would be a substantial fraction of the total. To minimize adsorption during standard curve and QC preparations, aliquots of the stock solutions were spiked promptly into the control blank plasma to prepare the standards and QCs. Once the compounds were in an environment of protein solutions, the adsorption problem was alleviated. The de-proteinized extracted samples did not show the severe adhesion problem as the analyte in neat solution as reflected by the stability data of the extracts. The chromatograms (Figs. 6–8) show that there was very little tailing of the compound peaks, indicating that they were not adhering to the LC–MS/MS system.

3.2. Method validation

3.2.1. Sensitivity and selectivity

To evaluate sensitivity and selectivity, the signals at the LLOQ of 10 ng/ml of each analyte were evaluated against those of control plasma from healthy volunteers. Table 3 shows the plasma data from healthy donors. There was no significant interference at the retention time for either analyte from all plasma lots tested. The typical signal-to-noise ratio at the LLOQ was 11 for T-20 and 8 for M-20, as shown in chromatograms for the LLOQ and their blank controls in Figs. 6–8. Table 3 shows that the LLOQ intra-assay accuracy and precision was −4.1% R.E. and 9.5% C.V. for T-20, and −1.6% R.E. and 10.2% C.V. for M-20 from six determinations from a single plasma lot in column B of the table.

To show that there was no lot-to-lot variability due to matrix effect, the analytes at LLOQ were spiked into control plasma from six individual healthy volunteers. The data were compared to those from a single donor in Table 3. The C.V.% from the multiple lots was either lower or very similar to that of the single lot (column B versus column A in Table 3). Selectivity against blank control plasma from the healthy volunteers was therefore established. Selectivity and the lack of lot-to-lot matrix variability were also established in plasma from 10 individual lots from HIV^+ patients. T-20 was spiked at 15, 150, and 1500 ng/ml concentrations to the plasma, the

Table 4
Between-batch variability of calibration standards from five validation batches

	10.0 ng/ml	25.0 ng/ml	50.0 ng/ml	100 ng/ml	200 ng/ml	1000 ng/ml	2000 ng/ml
(a) T-20							
Mean (ng/ml)	10.2	23.7	50.9	99.8	198	1005	2048
C.V.%	7.1	9.4	7.4	5.1	6.0	6.2	4.9
R.E.%	+0.2	−5.2	+1.8	−0.2	−1.0	+0.5	+2.4
N	10	10	10	10	10	10	10
(b) M-20							
Mean (ng/ml)	9.95	24.7	52.0	101	203	398	481
C.V.%	15.8	8.9	5.4	4.5	5.3	4.2	5.5
R.E.%	−0.8	−1.2	+4.0	+1.0	+0.5	−0.5	−3.8
N	10	10	10	10	10	10	10

R.E.% was 2.5, 2.7, and 15.5, and the C.V.% was 8.6, 6.6, and 6.1, respectively.

3.2.2. Precision and accuracy

Method validation was performed in multiple validation batches to obtain statistical data on precision and accuracy. Each batch consisted of two sets of calibration standards, one situated at the front and the other at the back of the batch run. At least six replicates of QCs at each level and other test samples were scattered in between the standard sets to mimic the conditions of clinical sample analysis. The statistical data for the standards and QCs are presented in Tables 4 and 5. Linearity of the standards was established by the low R.E.% of all the standards. The highest deviation from the nominal value was 5% for T-20, and 4% for M-20. The overall mean of the correlation coefficient was 0.9964 and 0.9949 for T-20 and M-20, respectively. In addition to the three levels of QCs at the low, middle, and high concentrations of the standards dynamic range, a QC that exceeds the highest standard concentration was prepared to mimic clinical samples at high levels. The accuracy and precision of all QCs are shown in Table 5. The values were acceptable according to the current FDA guidance [10] and the recent Crystal City Conference report recommendation [11].

3.2.3. Analyte stability

The stability of the analytes in the plasma matrix from healthy donors (QCs) was tested after exposure to room temperature (benchtop stability) for 23 h, six cycles of freezing and thawing, and long-term storage at −70 °C for 33 weeks and at −20 °C for 8 weeks. The results in Table 6 showed that the peptides were stable under these conditions compared to control samples that had not been subjected to the exposure. The storage stability was determined by comparison of QC results from the initial validation batch to results of the same QCs determined by freshly prepared calibrators after the given time. It appeared that the plasma hydrolytic enzymes had no effect on these peptides in EDTA plasma and there was no need to add hydrolytic enzyme inhibitors to maintain sample integrity. Since the clinical samples were to be heat-treated to inactivate viral activity before analysis, analyte stability in HIV^+ plasma was tested after heat treatment at 56 °C for 1 h. Ten lots of HIV^+ EDTA plasma were spiked with the analytes in triplicate at 15, 150, and 1500 ng/ml and were heat-treated for 1 h at 56 °C. The mean values were compared to the theoretical values. Data in Table 7 show that the heat treatment did not affect quantification of the peptides.

Table 5
Between-batch variability of quality control samples from five validation batches

	15.0 ng/ml	150 ng/ml	1500 ng/ml	15000 ng/ml
(a) T-20				
Mean (ng/ml)	14.5	152	1527	14571
C.V.%	9.8	5.3	4.8	4.8
R.E.%	−3.3	+1.3	+1.8	−2.9
N	30	30	30	30
(b) M-20				
Mean (ng/ml)	14.7	71.6	346	2693
C.V.%	14.9	5.3	6.5	7.4
R.E.%	−2.2	−4.5	−7.7	−10.0
N	30	30	30	30

3.2.4. Method ruggedness

Ruggedness of the established method was extensively evaluated. The number of samples in validation batches was similar to the number of samples in the anticipated clinical

Table 6
Analyte stability

	Period	Control (%)	
		T-20	M-20
In the biological matrix			
Benchtop	23 h at RT under white light	99–105	96–107
Freeze/thaw	6 cycles	95–98	96–104
Heat treated HIV^+ plasma	56 °C for 1 h	93–100	88–96
Long-term storage	33 weeks at −70 °C	93–104	102–105
	8 weeks at −20 °C	90–99	94–103
In the processed sample			
Re-injection/ refrigeration	94 h	99–117	97–100

Table 7
Analyte stability in HIV^+ plasma after heat treatment[a]

	Nominal concentration (ng/ml)		
	15	150	1500
(a) T-201			
Mean	13.9	142	1505
C.V.%	9.5	9.8	6.8
% of nominal	92.9	94.7	100
(b) M-20			
Mean	13.2	66	361
C.V.%	12.6	6.9	7.1
% of nominal	88.1	88.4	96.2

[a] To evaluate heat treatment stability, 10 lots of HIV^+ EDTA plasma were spiked with the analytes in triplicate at 15, 150, and 1500 ng/ml and were heat-treated for 1 h at 56 °C. The means of all values were then compared to the theoretical values.

sample run of about 96 injections. At least three different Micromass Ultima triple quadrupole mass spectrometers were tested. Seven lots of analytical columns were used during the course of method development and validation. A slight difference in retention time was observed and minor adjustment in the gradient program was required for certain lots to separate matrix effect components. At least three analysts contributed to the validation data of this work to show good method repeatability.

3.3. Method application

The LC–MS/MS method has been applied to sample analysis in phase 3 clinical studies of T-20 [7]. The method provided reliable and simultaneous quantification of T-20 and its de-amidated metabolite. Pharmacokinetic profiles were generated readily for both compounds in the HIV^+ patients. The turnaround time was fast with this relatively simple and selective method. Analysis of clinical samples also showed that the current assay is too sensitive for most of the samples which routinely required dilution. Experiments are currently planned to modify the assay range with a ten fold higher LLOQ and upper limit of quantitation (ULOQ) for both analytes.

4. Conclusion

A novel LC–MS/MS method was developed for the quantitation of a large peptide, T-20, and its metabolite in human plasma. The multiply charged ions of T-20 and M-20 and production of suitable fragment ions enabled detection and accurate quantification of these analytes. In this method, a simple protein precipitation procedure followed by a gradient HPLC with MS/MS detection offered sufficient quantification and selectivity for analysis of clinical samples. The assay has a wide calibration range of 10–2000 ng/ml for T-20 and a smaller range of 10–500 ng/ml for M-20. The chromatography allowed simultaneous quantitation of the de-amidated metabolite.

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References

[1] C. Wild, T. Greenwell, T. Matthews, AIDS Res. Hum. Retroviruses 9 (1993) 1051–1053.
[2] J.M. Kilby, S. Hopkins, T.M. Venetta, B. DiMassino, G.A. Cloud, J.Y. Lee, L. Alldredge, E. Hunter, D. Lambert, D. Bolognesi, T. Matthews, M.R. Johnson, M.A. Nowak, G.M. Shaw, M. Saag, Nat. Med. 4 (1998) 1302–1307.
[3] T.R. Covey, E.L. Lee, J. Henion, Anal. Chem. 58 (1986) 2453–2460.
[4] S.M. Darby, M.L. Miller, R.O. Allen, M. LeBeau, J. Anal. Toxicol. 25 (2001) 8–14.
[5] R. Wolf, F. Rosche, T. Hoffmann, H.-U. Demuth, J. Chromatogr. A 926 (2001) 21–27.
[6] M. Carrascal, K. Schneider, R.E. Calaf, S. van Leeuwen, D. Canosa, E. Gelpi, J. Abian, J. Pharm. Biomed. Anal. 17 (1998) 1129–1138.
[7] X. Zhang, K. Nieforth, A. Rodwell, J.M. Lang, R. Rouzier-Panis, J. Reynes, A. Dorr, S. Kolis, M.R. Stiles, T. Kinchelow, I.H. Patel, Third International Workshop on Clinical Pharmacology of HIV Therapy, Washington, DC, April 11–13, 2002.
[8] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkie, Rapid Commun. Mass Spectrom. 13 (1999) 1175–1185.
[9] A. Apffel, S.M. Fischer, G. Goldberg, P.C. Goodley, F.E. Kuhlmann, J. Chromatogr. A 712 (1995) 177–190.
[10] FDA Guidance for Industry, Bioanalytical Method Validation, May 2001.
[11] V.P. Shah, K.K. Midha, J.W.A. Findlay, H.M. Hill, J.D. Hulse, I.J. McGilveray, G. McKay, K.J. Miller, R.N. Patnaik, M.L. Powell, A. Tonelli, C.T. Viswanathan, A. Yacobi, Pharm. Res. 17 (2000) 1551–1557.