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Determination of protease inhibitors using liquid chromatography–tandem mass spectrometry

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Abstract

A method for the analysis of six protease inhibitors and one metabolite has been developed and validated. Amprenavir, ritonavir, saquinavir, lopinavir, indinavir, nelfinavir, and an active metabolite of nelfinavir (M8) are quantitated using reversed-phase liquid chromatography coupled to tandem mass spectrometry, equipped with an electrospray ionization source (ESI-LC–MS–MS). The validation data presented here shows that the method allows the rugged analysis of these species from one aliquot. The evolution of complex drug interactions assessments and the clinical use of therapeutic drug monitoring for these antiretrovirals will be a potential immediate application of this method.

Keywords: Protease inhibitors

1. Introduction

Protease inhibitors are a class of compounds used in the treatment of human immunodeficiency virus (HIV) infection. This class of drugs inhibits the HIV-1 protease, which acts to process viral proteins essential for the completion of the viral life cycle and subsequent infection of other cells [1]. Since these drugs are typically part of multi-drug regimens [2], there is a need for specific methods to assess pharmacokinetic parameters in combination therapy to identify complex drug interactions and as well, the emerging role of therapeutic drug monitoring (TDM). The proposed role for TDM of protease inhibitors is that it may allow practitioners to better determine and maintain appropriate plasma concentrations, identify interactions with other medications, and assess medication adherence. With a greater number of drugs being co-administered, more data is needed to elucidate this information. Thus, the development of efficient analytical techniques capable of determining information about multiple species is desired.

The use of liquid chromatography coupled to tandem mass spectrometry (LC–MS–MS) has emerged as the developmental method of choice in supporting clinical and pre-clinical pharmacokinetic studies [3]. This is based on the ability of this technique to provide superior specificity, speed and sensitivity, as compared to commonly used high-pressure liquid chromatography with ultra-violet absorbance detection (HPLC–UV) methods [3]. The

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quantitation of some of the protease inhibitor species using LC–MS–MS has been documented previously [4–9], although HPLC has been more commonly used [2,10–15]. Multi-analyte analysis of these species using liquid chromatography coupled to mass spectrometry is less common [7,9]. When comparing these two techniques, LC–MS–MS is able to analyze more compounds in less time, with a lower limit of quantitation.

However, with ESI-LC–MS–MS, several issues must be considered. One in particular is unstable instrument response due to ion-suppression. Ion suppression arises when co-eluting ions within a matrix reduce the ion intensity of the analytes, thus affecting quantitative reproducibility [6,16,17]. The protease inhibitor, IDV, has been shown to be subject to such matrix effects [6]. Matuszewski et al. [3] experimentally demonstrated the need for a chromatographic separation of samples in order to decrease ion suppression and provide reproducibility in quantitative assays in 1998. For reliable quantitation, the belief that very little, if any, sample preparation is needed is typically untrue [18]. Therefore, it is critical that any method developed by LC–MS–MS for quantitation of clinical samples be thoroughly characterized, especially for accuracy in various matrices. This is increasingly important as a greater number of analytes are included in one analysis

Our recent objectives have been to develop a method for the analysis of all currently approved HIV-1 protease inhibitors including indinavir (IDV), nelfinavir (NFV), an active metabolite of nelfinavir (M8), amprenavir (APV), saquinavir (SQV), ritonavir (RTV), and lopinavir (LPV) in plasma using ESI-LC–MS–MS (see Fig. 1). The use of LC–MS–MS allowed for a greater number of analytes to be assayed within less time than with conventional HPLC–UV detection coupling. The validated assay is currently being used for the support of clinical investigations studying these drugs. Through our work, we have determined the method to be rugged, reliable, and more sensitive than our other HPLC–UV methods [10]. In addition, the same sample preparation described here can be used to prepare samples for the analysis of efavirenz, a non-nucleoside reverse transcriptase inhibitor commonly administered in combination with protease inhibitors. For the determination of efavirenz, a second injection is made into the chromatographic system and the LC–MS is operated using negative ionization. This method is not presented here, but has also been validated for use in our laboratory.

2. Experimental

2.1. Chemicals

Analyte drugs were generously donated from several pharmaceutical companies. Indinavir sulfate was provided by Merck (Whitehouse Station, NJ, USA) nelfinavir, and M8 were obtained from Agouron Pharmaceuticals (San Diego, CA, USA.). Ritonavir, lopinavir, and a-86093 (ritonavir internal standard, RIS) were obtained from Abbott Laboratories (Abbott Park, IL, USA). Saquinavir powder was obtained from Roche Pharmaceuticals (Welwyn Garden City, UK). Finally, amprenavir was obtained from GlaxoSmithKline (Research Triangle Park, NC, USA).

Water, acetic acid, methanol, sodium hydroxide, hexanes, and ethyl acetate were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Acetonitrile was obtained from VWR (South Plainfield, NJ, USA). All solvents used in sample preparation and chromatographic separations were of HPLC grade. Plasma for preparation of standards and quality controls was obtained from The Interstate Blood Bank (Memphis, TN, USA).

2.2. Instrumentation

The LC–MS–MS system consisted of a Perkin-Elmer Series 200 auto sampler (Norwalk, CT, USA), two Perkin-Elmer Series 200 LC pumps, and an Applied Biosystems PE/Sciex, API 3000 mass spectrometer (Foster City, CA, USA) equipped with a Turbo-ionspray source. The system was controlled through Analyst Software, version 1.1 from Applied Biosystems.

2.3. Separation conditions

Analytes were separated on a Waters Symmetry C_{18} column (Milford, MA, USA), which was 30× 2.1 mm I.D. packed with 3.5-μm particles, preceded

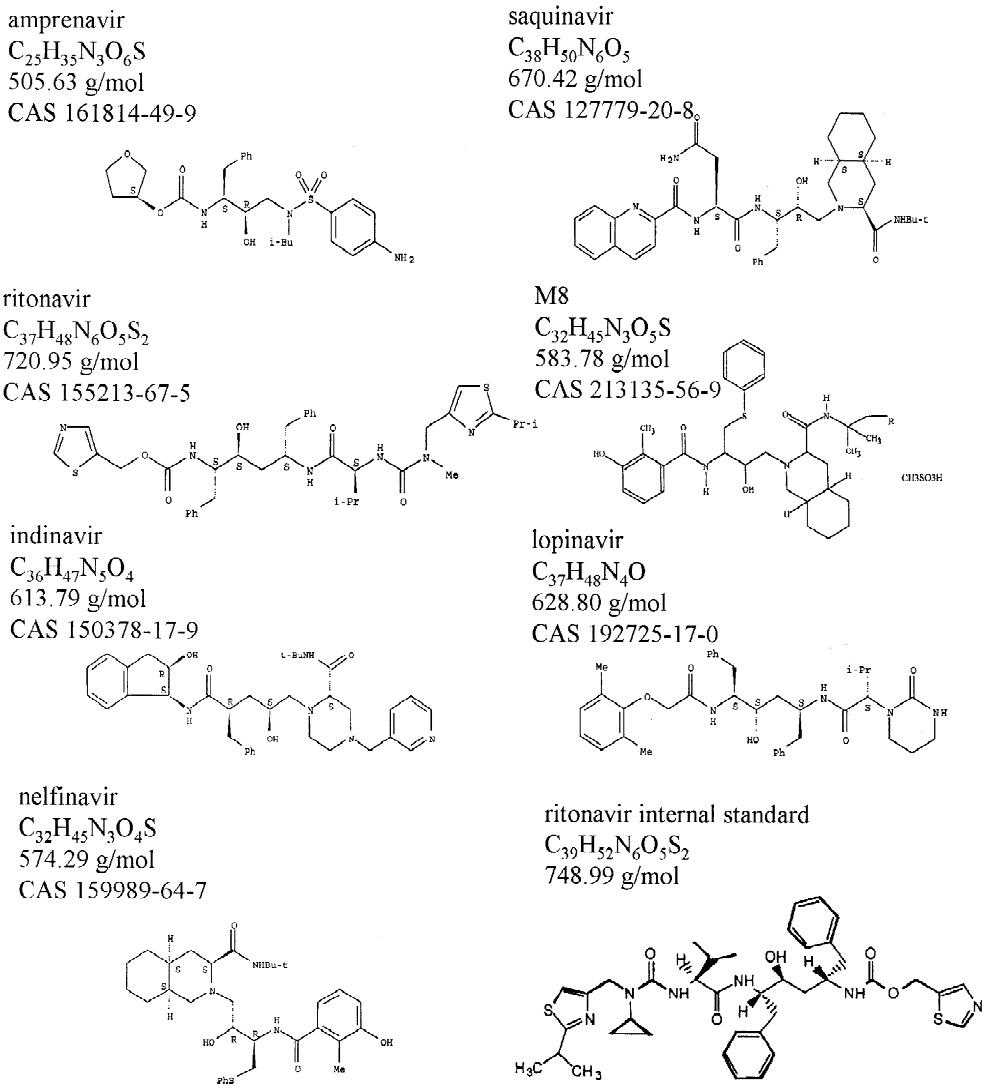

Fig. 1. Structures of the protease inhibitors and internal standard [19,20].

by a Waters Symmetry Shield Guard column, 2.1× 10 mm. The injection volume was 20 μl. Gradient elution was used to separate the analytes. The mobile phase consisted of acetonitrile and 5 m*M* acetate buffer, pH 3.25. The mobile phase gradient progressed from 75% acetate buffer to 20% in 4 min, using a nonlinear gradient. The flow-rate was 350 μl/min. Prior to entering the electrospray source housing, the flow was split 1:1 using a PEEK tubing splitter (Upchurch Scientific, Oak Harbor, WA, USA), with the one split line directed to waste and the other to the Turbo-ionspray source.

2.4. Preparation of stocks, standards, control, and internal standard solutions

Two 1 mg/ml stock solutions of each analyte and internal standard were made in methanol. Stock solutions were protected from light and stored at −70 °C for up to 6 months. Standards were made by

combining one set of stock solutions and diluting serially in methanol. Six to eight concentrations were used for each drug. Control solutions were made from the other set of stock solutions in a plasma matrix previously tested for presence of analyte interference. Standards (in methanol) and control solutions (in plasma) were made ahead of time and stored at −70 °C for up to 3 months. A 2-μg/ml internal standard solution was diluted from the 1-mg/ml stock solution in methanol. The internal standard solution was stored at −20 °C for up to 3 months.

2.5. Preparation of samples

On the day of analysis, 50 μl of each standard was diluted in 250 μl of plasma, and 50 μl of methanol was added to 250 μl of each control and unknown sample. All samples, now in plasma–methanol matrices, were then prepared using a liquid–liquid extraction method. After 100 μl of 2 μg/ml internal standard was added to 250 μl of each of the plasma samples, the samples were made basic through the addition of 250 μl of 1 *M* NaOH. Next, 3 ml of hexane–ethylacetate (1:1, v/v) was added. Each sample was shaken on high speed using a shaker (Eberbach Instruments, Ann Arbor, MI, USA) for approximately 25 min. Samples were then centrifuged for 15 min at 3000 *g*, leaving two layers within each test tube. The organic layer was removed, placed into a clean test tube and evaporated to dryness with air using a Zymark Turbo Vap LV (Hopkinton, MA, USA). Samples were reconstituted in 1 ml of 5 m*M* acetate buffer, pH 3.25–acetonitrile (75:25, v/v) and injected into the LC–MS–MS system for analysis.

2.6. Optimization of MS–MS detection parameters

Experiments were conducted to discern the optimized detection parameters for MS–MS detection of the analytes. Each of the drugs was dissolved in 5 m*M* acetate, pH 3.25–acetonitrile (75:25, v/v) at a concentration of 1 μg/ml. To observe how the potential settings affect primary and fragment ions, analytes were directly infused into the instrument using a syringe pump. The Analyst software ''Quantitative Optimization'' wizard was used to discern the optimal parameters.

2.7. Calibration procedures, accuracy and precision

Calibration curves were constructed on a daily basis using an internal standard (RIS). Unknown and control samples were quantitated using a linear regression of the calibration samples, as calculated by the Analyst program. For all of the analytes, calibration curves were weighted by a factor of $1/(\text{analyte concentration})^2$. To accept the day's calibration, two criteria had to be satisfied. First, it was required that at least five standard concentrations be included within the calibration curve (Any back-calculated standards that did not fall within 15% of the nominal value were excluded and the curve was recalculated). Second, at least two thirds of the standard concentrations' back-calculated values were required to be with 15% of their nominal values. For example, working curves with eight standard concentrations were only acceptable if six of those eight met the criteria for accuracy. Otherwise, the entire analysis was repeated. Calibration samples were randomized throughout the batch of injections and peak areas were used for all measurements.

For each analyte, the within and between-day precision was determined using six replicate samples of each control concentration. For most of the analytes, three control concentrations were used. For analytes that utilized a greater working curve range, four control concentrations were used. Control samples were prepared and analyzed repeatedly on six different days, allowing for a total of 36 samples. In each case, the lowest control sample concentration was a maximum of three times the lowest calibration concentration. For the assay to be considered valid, at least two thirds of the samples of each control concentration had to be within 15% of the target value. Control samples were randomized throughout the batch of injections.

2.8. Lowest limit of quantitation and limit of detection

The lowest limit of quantitation (LOQ) was defined as the concentration for which drug species could be determined reproducibly within 15% of the targeted value. In addition, at the LOQ, we required the signal-to-noise ratio to be at least 5. Five to six

analyses were completed for each analyte at the LOQ. To be considered acceptable as the LOQ, all of the samples were required to produce a result within 15% of the target value. The limit of detection was defined as the concentration that produced a signal that was three times the noise level of a blank preparation.

2.9. Reliability of the method in various matrices

Due to the variability of plasma drawn from different individuals, the effect of varying matrices on quantitation is critical, especially where ion-suppression is a possibility. One way to accomplish this is to compare post-extraction spiked standard samples to pre-extraction spiked standard samples for demonstration of such matrix effects [3,18]. Each analyte was added to each of six lots of independent blank matrix and assayed for precision and accuracy. Five repetitions were prepared in each matrix. For comparison, a blank of each matrix was also analyzed. It should be noted that heparin was used as an additive in all of the plasma matrices.

2.10. Stability and recovery

Repeat analysis of clinical samples is anticipated, for various reasons. For example, an instrument or human error could cause the loss of the prepared sample. This means that samples may potentially be frozen and thawed more than once. To prepare for such occurrences, the stability of the samples after three repeated freeze–thaw cycles was determined to assess the integrity of the analytes if analysis had to be conducted up to three times. To do this, control samples, which were analyzed after freezing and thawing once, were compared to control samples, which were frozen and thawed three times. After thawing, samples were allowed to sit at room temperature for at least 4 h; samples were then frozen for at least 12 h. Then samples were thawed for analysis. Six replicate samples of each control were used for the comparison.

The recovery of the analytes from the plasma matrix was also determined. First, blank plasma was prepared by the typical preparation procedures described above. However, rather than adding the control before extraction, it was added prior to evaporating the sample to dryness. Additionally, samples of the same concentration were prepared routinely. By comparing the samples that underwent extraction to the control samples that did not, the recovery of each of the species could be calculated.

2.11. Analysis of standard samples of unknown concentration

For the determination of accuracy, we assayed three samples containing known amounts of NFV, IDV, SQV, and RTV added to plasma by another laboratory. For these samples, the plasma matrix included ethylenediaminetetraacetic acid (EDTA) as an additive. In addition, we analyzed six LPV containing samples supplied by Abbott Laboratories. We were unable to obtain samples containing the metabolite M8, or APV at the time of this validation.

3. Results

3.1. Optimization of MS–MS detection parameters

For optimization of detection potential settings for each drug, analytes were infused directly into the mass spectrometer. Sequentially ramping each potential allows for identification of the appropriate settings for the precursor and product ions. For all of the analytes, positive ionization proved to be the best in terms of detectability. The resulting optimized source and detection parameters are shown in Table 1.

3.2. LC–MS–MS chromatographic characteristics

Through the use of tandem mass spectrometry, we were able to deconvolute co-eluting species. Nonetheless, the chromatography does provide several advantages. First, through appropriate retention of the analytes, the separation allowed the diversion of unretained species to waste, helping to safeguard the mass spectrometer. In addition, separation of any residual matrix components left after extraction minimizes matrix effects, which can affect quantitation [18]. A chromatogram of a standard solution extracted from a plasma matrix is shown in Fig. 2.

It is interesting to note that for M8, two peaks are

Table 1
Optimized detection parameters for each analyte precursor and fragment ion

Analyte	Mass/charge: precursor/fragment	Declustering Potential (V)	Focusing potential (V)	Excitation potential (V)	Collision Energy (V)	Collision cell exit potential (V)
Amprenavir	506.3/245.2	56.00	280.00	−10.00	26.00	14.00
Nelfinavir	568.4/330.2	66.00	280.00	−10.00	47.00	19.00
Indinavir	614.6/421.2	58.00	260.00	−10.00	47.00	25.00
M8	584.4/330.0	58.00	260.00	−10.00	47.00	20.00
Ritonavir	721.6/296.2	56.00	260.00	−10.00	27.00	17.00
Saquinavir	671.5/570.3	66.00	320.00	−10.00	47.00	32.00
Lopinavir	629.4/447.6	46.00	230.00	−10.00	31.00	28.00
RTVIS	747.3/322.2	61.00	260.00	−10.00	37.00	19.00

observed in the chromatogram (second peak indicated with an arrow). Since the use of MS–MS detection provides good selectivity, it stands to reason that another component of the mixture with the same precursor and fragment weight is being detected. Because of the separation, the two are resolved effectively and do not interfere. It should be noted that this peak is only seen for plasma containing samples (not for standards diluted in methanol).

3.3. Assay calibration, accuracy and variation

Calibrations for each analyte of six separate assays produced a range of correlation coefficients (r^2) from 0.981 to 1.000, and an average r^2 value of 0.995 (0.37% RSD, $n=35$). The calibration range for each analyte is shown in Table 2. As can be seen in Fig. 1, two internal standards were originally used in the separation, one that elutes early (peak 1, IDVIS) and one that elutes later (peak 9). This measure was taken in order to determine which of the internal standards best reflects the drugs' behavior throughout sample preparation and LC–MS–MS analysis. Through validation of the method, it became clear that both internal standards were suitable. As a convention, RIS is used.

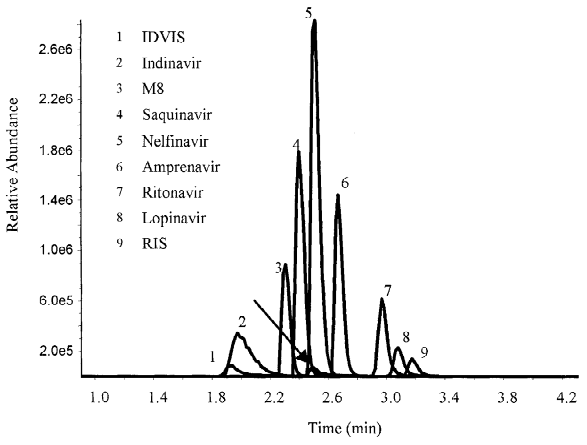

Fig. 2. Chromatogram of a standard sample added to a plasma matrix. APV, NFV, IDV, SQV, and LPV are at a concentration of 2.5 μg/ml; RTV and M8 are at a concentration of 1.25 μg/ml.

For evaluation of accuracy and variation, control samples were run on each analysis day. For the validation, six replicates of each control were used. Table 2 shows a summary of six assays of calibration and control samples. Also represented is the within and between-assay variation over these 6 days. Means of all control samples were within 15% of the target values. Also, at least four of each set of six replicates were acceptable on all 6 days. Within and between-day accuracy and precision was under 12% for all analytes on all 6 days.

3.4. Limits of detection and quantitation

Results for the limits of detection and quantitation are shown in Table 3. The lowest limit of detection was obtained for amprenavir, at 380 pg/ml, while the lowest limit of quantitation was 8.19 ng/ml, obtained for M8. For both the limit of detection and quantitation average errors and precision were below 13% for all analytes. For our current clinical studies, this level of quantitation is sufficient. Although, these limits may be further reduced if the volume of mobile phase used to reconstitute samples after evaporation is decreased. Currently, the method has

Table 2
Summary of assay accuracy and variability

Analyte	Calibration range (μg/ml)	APV, NFV, IDV, SQV, LPV: 0.0480 μg/ml M8: 0.0240 μg/ml			APV, NFV, IDV, SQV, LPV: 0.240 μg/ml RTV, M8: 0.120 μg/ml			APV, NFV, IDV, SQV, LPV: 1.20 μg/ml RTV, M8: 0.600 μg/ml			APV: 6.00 μg/ml RTV, M8: 3.00 μg/ml		
		Mean	% Error	RSD (%)	Mean	% Error	RSD (%)	Mean	% Error	RSD (%)	Mean	% Error	RSD (%)
Within-assay variability													
APV	0.0163–10.0	0.0490	2.08	9.00	0.248	3.33	5.56	1.30	8.33	1.44	6.19	3.17	2.88
IDV	0.0163–4.00	0.0481	0.21	10.2	0.237	−1.25	6.31	1.34	11.7	1.97			
LPV	0.0163–4.00	0.0490	2.08	9.54	0.235	−2.08	3.52	1.16	−3.33	1.18			
NFV	0.0163–4.00	0.0508	5.83	6.73	0.253	5.41	2.67	1.18	−1.67	2.28			
M8	0.00819–5.00	0.230	−4.17	10.8	0.122	1.67	7.65	0.654	9.00	3.33	3.25	8.33	4.04
RTV	0.0512–5.00				0.128	6.67	4.85	0.643	7.17	5.33	2.97	−1.00	5.95
SQV	0.0163–4.00	0.0491	2.29	9.83	0.234	−2.50	3.45	1.14	−5.00	1.72			
Between-assay variability ($n = 36$)													
APV	0.0163–10.0	0.0487	1.46	11.4	0.237	−1.11	9.06	1.21	0.42	6.57	5.89	−1.81	6.32
IDV	0.0163–4.00	0.0503	4.79	10.8	0.234	−2.56	8.37	1.30	8.43	10.1			
LPV	0.0163–4.00	0.0488	1.67	9.76	0.234	−2.50	10.3	1.15	−4.17	7.74			
NFV	0.0163–4.00	0.0498	3.75	10.8	0.244	1.67	11.2	1.23	2.50	6.78			
M8	0.00819–5.00	0.0247	2.92	11.5	0.123	2.50	8.57	0.643	7.17	5.16	3.09	3.00	6.50
RTV	0.0512–5.00				0.126	5.00	11.8	0.602	0.330	8.62	2.75	0.207	7.51
SQV	0.0163–4.00	0.0496	3.33	10.4	0.234	−2.50	8.32	1.18	−1.67	5.75			

Table 3
Limits of detection and quantitation

Analyte	Limit of detection	Limit of quantitation (ng/ml)	Accuracy at limit of quantitation [average % error (RSD)]
Amprenavir	380 pg/ml	16.3	−3.19 (12.4)
Nelfinavir	330 pg/ml	16.3	−4.91 (4.76)
Indinavir	1.50 ng/ml	16.3	12.5 (5.51)
M8	550 pg/ml	8.19	10.6 (7.98)
Ritonavir	650 pg/ml	51.2	−7.62 (6.57)
Saquinavir	780 pg/ml	16.3	−2.33 (4.52)
Lopinavir	750 pg/ml	16.3	−6.13 (7.48)

been validated using a reconstitution volume of 1 ml. However, only 20 μl is needed for injection. If the reconstitution volume were to be reduced, further experiments would need to be conducted to assure that a more concentrated matrix would not affect quantitation.

3.5. Accuracy and precision in various matrices

The accuracy and precision of the method was tested in six different matrices to demonstrate utility in the quantitation of an unknown clinical sample. Results are shown in Table 4. Precisions in all matrices were less than 7%. In addition, blank preparations of each matrix showed no appreciable interfering signals.

3.6. Stability after repeated freeze–thaw

The results for the recovery and stability of the analytes after three freeze–thaw cycles is shown in Table 5. Overall, recoveries were stable at each concentration for each analyte, with relative standard deviations between 1.8 and 7.9%. Recovery was the overall lowest for RTV, with values at each concentration below 70%. There was no statistical difference in the recoveries between the three concentrations of any one analyte, as determined by a *t*-test. All of the analytes could be quantitated within 15% error after three freeze–thaw cycles.

3.7. Analysis of samples of unknown concentration

Analysis of samples from an external source allows us to evaluate the accuracy of our assay without any bias. Results from such samples are shown in Table 6. The actual values were provided to our laboratory after analysis was complete. As shown in Table 6, all of the analytes were determined within 15% of the actual values, except for sample 5 for LPV. Reanalysis of this sample produced the same result. It should be noted that the content of sample 1 for IDV was out of the range of our working curve. Finally, an example chromatogram from a human subject is included in Fig. 3. This sample was taken following the individual's oral consumption of APV, RTV and LPV.

4. Conclusions

We have developed and validated a method for the

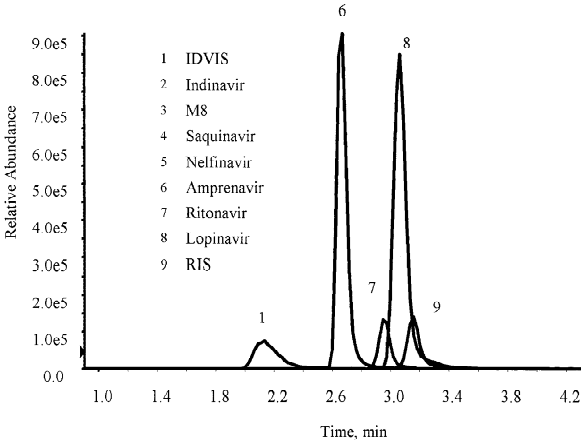

Fig. 3. Chromatogram of sample taken from a human subject after dosing with APV, RTV and LPV. Concentrations of APV, RTV and LPV were determined to be 7.20, 10.1 and 1.28 μg/ml, respectively.

Table 4
Summary of data for analytes in different matrices with corresponding relative standard deviations for five measurements of each

Analyte	Expected value (μg/ml)	Matrix 1		Matrix 2		Matrix 3		Matrix 4		Matrix 5		Matrix 6	
		Conc. (μg/ml)	RSD (%)	Conc. (μg/ml)	RSD (%)	Conc. (μg/ml)	RSD (%)	Conc .(μg/ml)	RSD (%)	Conc. (μg/ml)	RSD (%)	Conc. (μg/ml)	RSD (%)
Amprenavir	1.00	1.02	2.52	0.99	3.99	0.93	2.20	0.99	2.58	1.03	2.75	0.88	5.11
Indinavir	1.00	1.05	2.41	1.03	2.89	0.92	1.97	1.05	4.30	1.05	2.28	0.98	4.66
Lopinavir	1.00	1.07	1.40	1.05	1.35	1.02	0.49	1.04	2.52	1.03	1.65	1.01	1.77
Nelfinavir	1.00	1.10	2.17	1.10	2.27	1.09	2.66	1.00	4.34	1.00	2.22	0.97	1.72
Nelfinavir's metabolite	0.50	0.56	2.70	0.56	2.48	0.54	3.03	0.51	3.06	0.51	3.83	0.50	2.33
Ritonavir	0.50	0.51	4.94	0.47	1.99	0.52	2.39	0.48	3.06	0.54	6.16	0.53	3.31
Saquinavir	1.00	1.10	3.50	1.05	3.59	1.00	2.04	1.01	3.53	0.99	3.19	0.95	2.41

Table 5
Freeze–thaw stability and recovery of analytes

Analyte	Recovery (% RSD)			Quantitation after three freeze–thaw cycles [μg/ml (% RSD)]			
	0.160	0.630	2.50	0.0480	0.240	1.20	6.00
Amprenavir	80.7	88.0	82.1	0.0478	0.247	1.25	6.44
	(5.9)	(1.8)	(5.9)	(9.10)	(8.90)	(7.22)	(3.17)
Nelfinavir	95.9	95.1	83.2	0.0521	0.272	1.28	
	(6.1)	(3.2)	(6.3)	(4.12)	(2.42)	(1.33)	
Indinavir	97.1	95.3	70.5	0.0482	0.250	1.20	
	(7.9)	(4.5)	(7.5)	(8.52)	(5.75)	(3.70)	
Lopinavir	85.1	84.0	85.7	0.0490	0.266	1.30	
	(5.3)	(7.8)	(4.6)	(2.96)	(1.60)	(4.04)	
Saquinavir	95.7	89.9	82.3	0.0533	0.264	1.25	
	(3.5)	(5.8)	(5.1)	(5.91)	(4.21)	(4.86)	
	0.0800	0.320	1.25	0.0240	0.120	0.600	3.00
Ritonavir	58.3	67.1	59.0		0.125	0.625	2.82
	(7.7)	(5.6)	($n=2$)		(5.66)	(9.35)	(4.03)
M8	87.7	91.9	78.0	0.0249	0.128	0.643	3.23
	(4.9)	(4.3)	(5.2)	(6.66)	(4.85)	(3.12)	(2.64)
Internal standard (2 μg/ml)		82.9 (1.08)					

Table 6
Analysis of standard samples of unknown concentration

Analyte	True concentration (μg/ml)	Analysis result (μg/ml)	% Error
Indinavir			
Sample 2	1.19	1.09	−6.83
Sample 3	0.180	0.189	5.00
Nelfinavir			
Sample 1	7.50	7.30	−2.67
Sample 2	0.24	0.264	10.0
Sample 3	1.50	1.63	8.67
Ritonavir			
Sample 1	0.210	0.204	−2.86
Sample 2	1.50	1.55	3.33
Sample 3	12.64	11.00	−12.97
Saquinavir			
Sample 1	0.238	0.235	−1.26
Sample 2	4.11	4.05	−1.46
Sample 3	1.10	1.20	9.09
Efavirenz			
Sample 1	0.51	0.58	13.7
Sample 2	3.04	3.29	8.22
Sample 3	7.10	7.52	5.92
Lopinavir			
Sample 1	0.0252	0.0249	−11.9
Sample 2	0.0525	0.0565	7.62
Sample 3	0.158	0.176	11.4
Sample 4	0.630	0.701	11.3
Sample 5	1.26	1.51/1.50	*19.8/19.0*
Sample 6	2.52	2.54	0.79

analysis of six protease inhibitors and one active metabolite using ESI-LC–MS–MS. Solid phase extraction was used to isolate analytes from the samples; recoveries ranged from 59 to 97%. Through validation, the method proved to be accurate and reliable. Using 250 μl of plasma sample volume, a limit of quantitation of 8–50 ng/ml could be achieved. In the future, this could be improved by decreasing the reconstitution volume prior to injection. Validation included testing the accuracy in various matrices, freeze–thaw stability testing, within and between-day reliability, reproducibility of standard curves, quantitation of unknowns, and quantitation at the lower limit of detection. Accuracies and coefficients of variation were acceptable for all validation tests performed.

Prior methods have reported some of the currently approved HIV-1 protease inhibitors using LC–MS–MS [4–9], although HPLC has been more commonly used [2,10–15]. We report a method that incorporates all currently approved HIV-1 protease inhibitors as well as the active metabolite of nelfinavir. The evolution of complex drug interactions assessments and the clinical use of therapeutic drug monitoring for these antiretrovirals will be a potential immediate application of this method.

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