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Simultaneous determination of five HIV protease inhibitors nelfinavir, indinavir, ritonavir, saquinavir and amprenavir in human plasma by LC/MS/MS

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Abstract

A sensitive and rapid liquid chromatography tandem mass spectrometry (LC–MS–MS) method has been developed to measure the levels of five HIV protease inhibitors nelfinavir (NFV), indinavir (IDV), ritonavir (RTV), saquinavir (SQV) and amprenavir (APV) in human plasma. The analytes and internal standard are isolated from plasma by a simple acetonitrile precipitation of plasma proteins followed by centrifugation. LC–MS–MS in positive mode used pairs of ions at m/z of 568.4/330.0, 614.3/421.2, 720.9/296.0, 671.1/570.2 and 505.9/245.0 for NFV, IDV, RTV, SQV and APV, respectively and 628/421 for the internal standard. Two $1/x$ weighted linear calibration curves for each analyte were established for quantitation with the low curve ranging from 5 to 1000 ng/ml and while the high curve ranging from 1000 to 10,000 ng/ml. Mean inter- and intra-assay coefficients of variation (CVs) over the ranges of the standard curves were less than 10%. The overall recovery of NFV, IDV, RTV, SQV and APV were 88.4, 91.4, 92.2, 88.9 and 87.6%, respectively.

Keywords: Nelfinavir; Indinavir; Ritonavir; Saquinavir; Amprenavir; LC/MS/MS system

1. Introduction

Nelfinavir (NFV), indinavir (IDV), ritonavir (RTV), saquinavir (SQV) and amprenavir (APV) are the first five approved protease inhibitors (PIs) (Fig. 1) for the treatment of human immunodeficiency virus (HIV) infected patients [1]. All are reported to produce marked reductions in plasma viral load, particularly when used in combination with other antiviral agents [2]. Nowadays, multiple protease inhibitors are often used in the combination in order to improve the limited bioavailability of the protease inhibitors and protect or delay the occurrence of viral resistance for

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protease inhibitors [3]. Numerous HPLC analytical methods with UV detection, mass spectrometry detection and tandem mass spectrometry have already been described to quantify single [4–22] and combined [23–31] anti-HIV agents in biological matrices. Some of these assay methods had either long run time with low throughput or poor lower limits of quantitation. Sample preparation by solid extraction or liquid–liquid extraction was another labor-intensive and time-consuming step in these methods. Our goal was to develop a relatively rapid, high sensitivity method for five multiple PIs in human plasma using a liquid chromatography-tandem mass spectrometric (LC–MS–MS) instrument.

We have developed a LC–MS–MS method to analyze five protease inhibitors of NEF, IDV, RTV, SQV and APV in one run using an internal standard. The sample preparation is simple and consists of precipitating plasma proteins with ace-

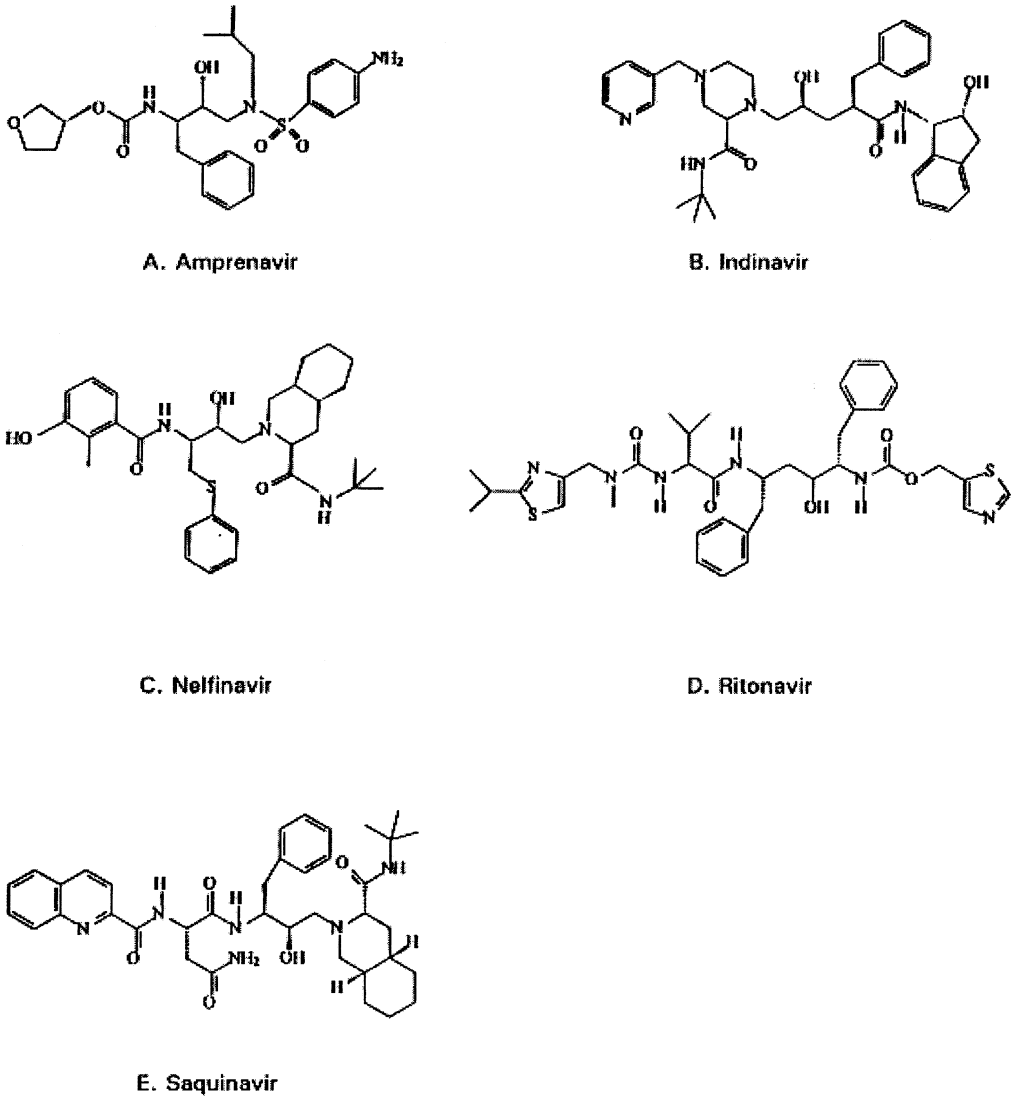

Fig. 1. Molecular structures of APV (A), IDV (B), NFV (C), RTV (D), and SQV (E).

tonitrile by vortex mixing and spining down the protein to a pellet. The clear supernatant is injected onto a short LC column and eluted with a linear gradient of 10 mM ammonium formate buffer (pH 4.10) and acetonitrile. This simple method allows to save time and decrease cost compared with that of six different assays. The broad calibration curve range from 5.0 to 10,000 ng/ml for each analyte was adequate to handle most pharmacokinetic samples.

2. Experimental

2.1. Materials

All chemicals were HPLC grade or reagent grade unless otherwise noted. Acetonitrile, ammonium formate, formic acid (99–100%), ammonium hydroxide solution and HPLC-water were from Fisher Scientific (Fair Lawn, NJ, USA). NFV was obtained from Agouron Pharmaceuticals, Lajolla, CA. IDV and methyl IDV (internal standard, IS) from Merck Research Laboratories, Rahway, NJ. RTV from Abbot, Abbot Park, IL. SQV from Roche Products Ltd., Herts, UK. APV from Glaxowellcome, Research Triangle Park, NC. Drug free human plasma was obtained from the Long Hospital Blood Bank (University of California at San Francisco, San Francisco, CA, USA). 1.5 ml snap cap micro centrifuge tubes from Fisher Scientific.

2.2. Apparatus

The PE Sciex API 2000 triple quadrupole mass spectrometer with turboIon spray sample inlet was purchased from. Applied Biosystems (Concord, ON, Canada). Perkin–Elmer, Norwalk, CT supplied the PE Biosystems 200 series Autosampler and twin PE Biosystems series 200 micro HPLC pumps.

2.3. MS–MS parameters

Mass spectral analyses were accomplished on a Model API 2000 triple quadrupole mass spectrometer, fitted with an API Turboion spray source and operated in the positive ionization mode. Nebulizer, turbo and curtain gases used ultra high purity nitrogen (99.999%) delivered at settings of 45, 55 and 30 psi, respectively. Nitrogen was also used as the collision gas at a setting of four, which produced an overall analyzer pressure of approximately 2.6×10^{-5} torr. The Turboion spray temperature was maintained at 495 °C. The collision energy settings for NFV, IDV, RTV, SQV and APV were -54, -45, -32, -44, and -31 V respectively. Tuning parameters were optimized individually for each drug in order to get the best signal to noise (S/N) ratio for NFV, IDV, RTV, SQV and APV. The mass spectrometer was operated in MS–MS mode using multiple reaction monitoring (MRM) to detect specific precursor ion to product ion transitions for each analytes. Following chromatographic separation, the protonated ion ($M + H^+$) for each analyte was selected by the first quadrupole (Q1) and focused into the collision cell (Q2) where it fragmented into product ions. For each $M + H^+$ precursor ion, a distinct product ion was selected by the third quadrupole (Q3). The precursor ion intensity was monitored and subsequently stored by computer system of the mass spectrometer. These mass spectral Q1/Q3 transitions, monitored for NFV, IDV, RTV, SQV, APV and IS, were 568.4/330.0, 614.3/421.2, 720.9/296.0, 671.1/570.2, 505.9/245.0 and 628/421 (m/z), respectively. A 5 ms delay between scans was found to be adequate for eliminating potential cross talk. The control software, including LC Tune, Multiview, Turboquan, Method Editor and Experiment Editor was installed on a Macintosh OS 8.5 platform with 64 MB of RAM and a 16 GB hard disk.

Instrument tuning parameters were optimized using 1000 ng/ml solutions of each analytes and IS prepared in acetonitrile–ammonium formate buffer (pH 4, 10 mM) (50:50, v/v). These solutions were infused separately at 10 μl/min into the LC/MS/MS interface using a built-in syringe pump.

2.4. Liquid chromatography parameters

The specific liquid chromatographic parameters for the assay were as follows: the column was Zorbax XDB-C8 reverse phase column, 2.1 mm i.d. × 50 mm, with 5 μm particle size packing and a Zorbax XDB-C8, 5 μm, 2.1 × 12.5 mm guard column. The flow rate was kept constantly at 400 μl/min. Mobile phase A was a buffer and mobile phase B was acetonitrile. The flow gradient was initially 80:20 v/v of A:B for 0.20 min, linearly ramped to 20:80 over 0.6 min, held at 20:80 for 2.7 min, and then returned to 80:20 over 0.3 min. This condition was held for a further 1.4 min prior to the injection of another sample. The volume of injection was 20 μl through 100 μl loop.

Mobile phase A was prepared by dissolving 1.28 ammonium formate in 2.0 l HPLC-grade water, adjusting pH to 4.1 with formic acid prior to filtration through a 0.2 μm membrane This was followed by sonication under vacuum to degas. The mobile phase B was a 0.1% v/v solution of pure formic in HPLC-grade acetonitrile which was mixed well, filtered and degassed under vacuum.

2.5. Preparation of standards and controls

To prepare stock solutions of NFV, IDV, RTV, SQV and APV, 5 mg of each analyte was accurately weighed and dissolved in a 5 ml volumetric flask with 60% acetonitrile in water to generate a concentration of 1000 μg/ml of each analyte. One milliliter of each standard solution were mixed together with 5 ml of 35% acetonitrile in water to produce a final concentration of 100 μg/ml per analyte. This solution was diluted appropriately to lower concentrations for spiking the calibration standards. These were prepared by spiking 5 ml aliquots of drug-free plasma with the working standards solutions to give a range of concentrations from 25.0 to 10,000 ng/ml. Frozen quality control (QC) pools were prepared at five different concentrations of each analyte using a separately weighed stock solution containing 100 μg/ml of each analyte in aqueous acetonitrile. The IS was prepared in aqueous acetonitrile (50:50 v/v) at a concentration of 100 μg/ml and further diluted 1:200 v/v to a working concentration of 500 ng/ml with 35% acetonitrile in water. Aliquots (100 μl) of calibration standards and QC samples were pipetted into 1.5 ml polypropylene snap cap tubes and stored frozen at −20 °C until required for analysis.

2.6. Processing of plasma samples

Frozen plasma samples from study subjects, quality control samples (QC) and calibration standards were thawed as needed. The same procedure was followed for all samples. Two hundred microliter of internal standard solution was added to aliquots of 100 μl of plasma from deactivated study samples, calibrators and QC in 1.5 ml microcentrifuge tubes, and mixed gently. Four hundred microliter, acetonitrile was added to each tube and vortexed for 20 s at high speed. The tube was centrifuged at 12,000 × g (11,000 rpm) for 5 min to pellet the precipitated proteins and give a clear supernatant. These clear extracts were transferred to vial inserts and placed in the autosampler tray for injection onto the LC column.

2.7. Data analysis

Calibration standards in plasma containing 5, 25, 100, 250, 500, 1000, 2500, 5000, 7500, and 10,000 ng/ml of each analyte were used to establish two calibration curves (low curves 25–1000 ng/ml, high curves 1–10 μg/ml) with $1/x$ weighted linear regression. For each standard analyte concentrations versus peak area ratios were plotted in Turboquan, a separate quantitation software program supplied with the mass spectrometer.

3. Results

3.1. Linearity

Extracted ion chromatograms from a standard sample with NFV, IDV, RTV, SQV, APV and added internal standard and drug-free plasma blank are shown in Fig. 2. The retention times of NFV, IDV, RTV, SQV, APV and IS were 2.21,

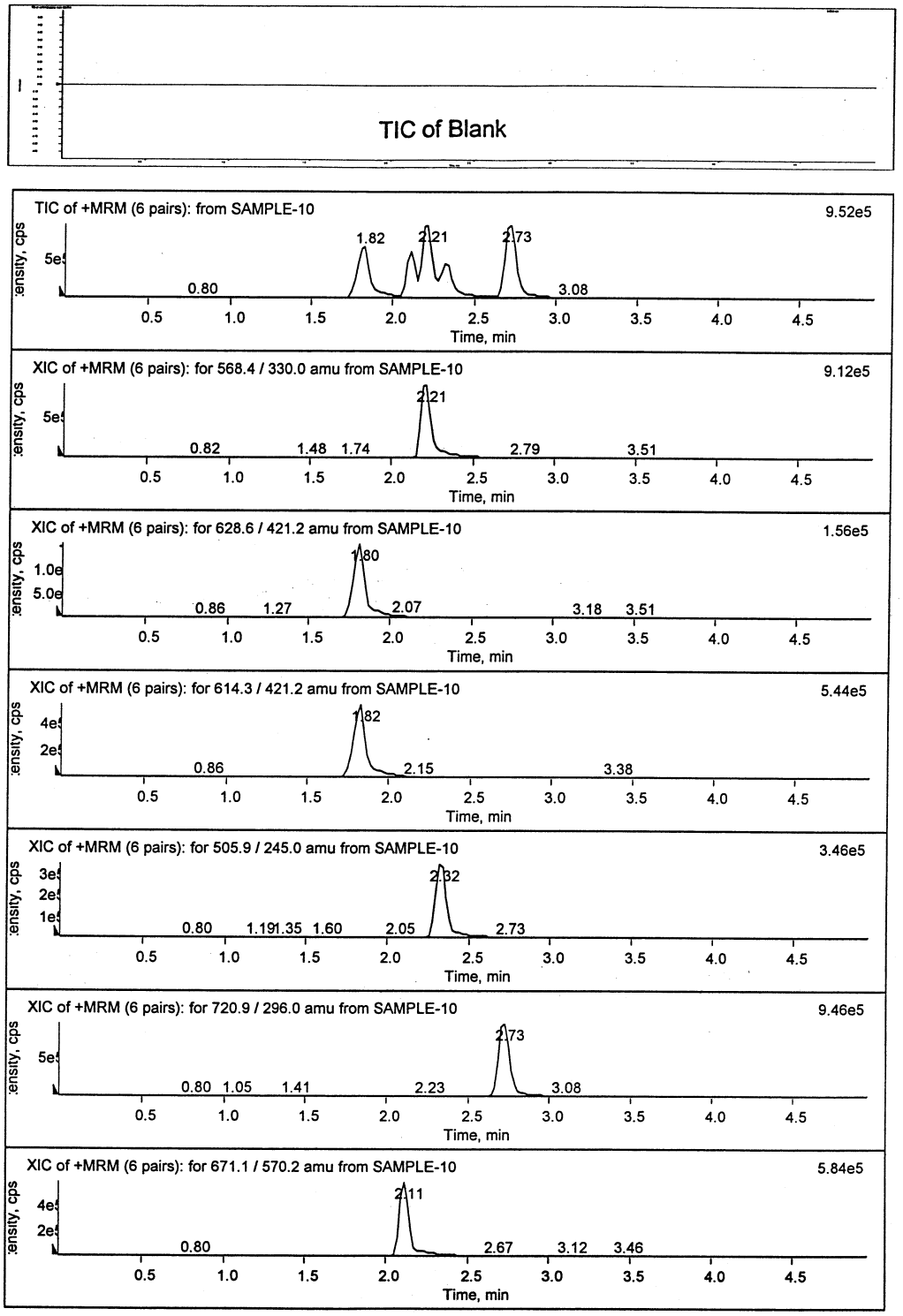

Fig. 2. Chromatograms of blank plasma and drug-free plasma spiked with 1000 ng/ml of NEF, IDV, RTV, SQV and APV standards and 500 ng/ml of the internal standard.

Table 1
Equation of calibration curves of NEF, IDV, RTV, SQV and APV

	Low curve[b]		High curve[c]	
	Mean equation[a]	$R2$	Mean equation[a]	$R2$
NEF	$y = 0.00226x$	0.9992	$y = 0.00158x + 0.1404$	0.9987
IDV	$y = 0.00139x$	0.9997	$y = 0.00126x + 0.1405$	0.9996
RTV	$y = 0.00215x$	0.9995	$y = 0.00171x + 0.4901$	0.9994
SQV	$y = 0.00117x$	0.9995	$y = 0.00091x + 0.2568$	0.9989
APV	$y = 0.00084x$	0.9998	$y = 0.00081x + 0.00446$	0.9997

[a] n, 6 days.
[b] Curve fit: linear thourgh zero, weighting: $1/x$.
[c] Curve fit: linear through, weighting: $1/x$.

1.82, 2.73, 2.11, 2.32 and 1.80 min, respectively. Calibration standards in human plasma containing 5–10,000 ng/ml of each analyte were used to establish calibration curves for assay validation. Linear regression of concentration versus peak area ratio plots resulted in coefficients of determination (r^2) consistently greater than 0.995 for each analyte. The mean equations of all analytes calibration curves over 6 days using linear regression analysis are given in Table 1.

3.2. Precision and accuracy

Inter- and intra-assay precision was evaluated at five concentrations of 15, 300, 1500, 3000 and 6000 ng/ml. For inter-assay precision, six samples of each concentration were assayed on 6 different days using six sets of standard curves. Means and standard deviations were obtained for the calculated drug concentrations over all 6 days and coefficients of variation (CV%) for the five different levels ($n = 36$ for each) were determined. For intra-assay precision, 10 control samples from each of five concentrations were assayed with a set of calibration curve and coefficients of variation for the calculated analyte concentrations were determined. The inter- and intra-assay data for the determination of NFV, IDV, RTV, SQV and APV is shown in Table 2.

3.3. Limits of quantitation and recoveries of NFV, IDV, RTV, SQV and APV

The lower limits of quantitation (LOQs) for NFV, IDV, RTV, SQV and APV by this method were all 5.0 ng/ml. For the intra-assay LOQs (10 replicates), the CVs of NFV, IDV, RTV, SQV and APV were 7.2, 5.5, 7.6, 4.3 and 9.2% with the mean accuracy of 111.1, 106.0, 100.3, 115.8, and 98.3%, respectively. For the inter-assay LOQs of NFV, IDV, RTV, SQV and APV over 6 days, the CVs were 6.1, 5.1, 4.9, 7.3 and 2.9% with the accuracy of 107.0, 108.9, 104.0, 104.6 and 103.3%, respectively.

Recoveries of NFV, IDV, RTV, SQV and APV from plasma following sample preparation were assessed by comparing the concentration of analyte from plasma samples to the concentration of drug spiked into the mobile phase at the same concentration as in the plasma samples. Overall mean recoveries of NFV, IDV, RTV, SQV and APV were found to be 88.4 ± 3.7, 91.4 ± 1.7, 92.2 ± 5.3, 88.9 ± 4.5 and 87.6 ± 6.3%, respectively over concentration ranges of 5–10,000 ng/ml.

3.4. Stability

3.4.1. Freeze/thaw stability

Stability of NFV, IDV, RTV, SQV and APV in human plasma during repeated three freeze/thaw cycles was evaluated with QC samples at five concentrations of 15, 300, 1500, 3000 and 6000 ng/ml. The calculated concentrations after repeated freezing and storage at -20 °C were compared to the calculated concentrations of freshly spiked plasma at same concentrations. The mean overall changes of NFV, IDV, RTV, SQV and APV in concentration from cycles 1 to 3 were -1.6, 1.6, 5.7, 0.2, and 2.5%, respectively.

3.4.2. Stability at room temperature

Frozen controls of NFV, IDV, RTV, SQV and APV in plasma at the five concentrations were thawed and left on the bench at room temperature for 24 h. These samples, a set of freshly thawed samples, and a set of calibration standards were analyzed and the concentrations of each analyte were compared to determine the stability of NFV, IDV, RTV, SQV. During 24 h storage at room temperature, the mean overall changes of NFV, IDV, RTV, SQV and APV in concentration were 0.1, −1.6, −2.2, 1.3, and 0.2%, respectively.

3.4.3. Storage stability of NFV, IDV, RTV, SQV and APV at −70 °C freezer

A set of six each of frozen controls at five concentrations of 15, 300, 1500, 3000, and 6000 ng/ml and a set of calibration standards were analyzed while these samples were fresh and also after storage at −70 °C freezer for 1, 2, and 6 months. Concentrations of NFV, IDV, RTV, SQV and APV were compared to determine the stability of each analyte during storage at −70 °C temperature. All five analytes were stable up to 6 months at temperature of −70 °C.

3.5. Application

This method has been used for the measurement of NFV and IDV concentrations in patient plasma. Fig. 3 shows plasma concentration versus time curves of IDV (800 mg) and NFV (750 mg)

Table 2
Intra- and inter-assay precision (RSDs) and accuracy data for the determination of NEF, IDV, RTV, SQV and APV in plasma

Analyte	Actual value (ng/ml)	Observed value (ng/ml)		CV (%)	
		Intra	Inter	Intra	Inter
NEF	15	16.40 ± 0.7	16.18 ± 0.7	4.0	4.2
	300	310 ± 8.2	316.5 ± 6.9	2.6	2.2
	1500	1505 ± 7.5	1544 ± 83.5	7.5	5.4
	3000	3395 ± 186.0	3248 ± 128.0	5.5	3.9
	6000	5822 ± 407.5	5784 ± 206.2	7.0	3.6
IDV	15	15.51 ± 0.5	15.41 ± 0.8	3.4	5.4
	300	305.3 ± 4.7	301.8 ± 11.6	1.5	3.8
	1500	1511 ± 19.6	1528 ± 61.7	1.3	4
	3000	3042 ± 52.4	3049 ± 87.9	1.7	2.9
	6000	5580 ± 312.6	5603 ± 172.5	5.6	3.1
RTV	15	14.18 ± 0.5	14.66 ± 0.8	3.8	5.5
	300	282.1 ± 6.1	293.4 ± 14.5	2.2	4.9
	1500	1423 ± 74.1	1452 ± 69.5	5.2	4.8
	3000	3211 ± 135.3	3118 ± 126.5	4.2	4.1
	6000	5768 ± 198.3	5668 ± 163.8	3.4	2.9
SQV	15	17.14 ± 0.8	15.56 ± 1.2	4.8	7.9
	300	330.6 ± 5.2	314.4 ± 13.7	1.6	4.4
	1500	1641 ± 78.1	1556 ± 58.2	4.8	3.7
	3000	3387 ± 142.2	3197 ± 141.5	4.2	4.4
	6000	5700 ± 312.7	5694 ± 142.5	5.5	2.5
APV	15	15.71 ± 0.8	15.27 ± 0.81	5.1	5.3
	300	305.4 ± 8.0	305.7 ± 11.3	2.6	3.7
	1500	1435 ± 63.6	1497 ± 58.7	4.4	3.9
	3000	3116 ± 128.2	3091 ± 104.4	4.1	3.4
	6000	5675 ± 205.5	5681 ± 244.3	3.6	4.3

The intra- and inter-assay data represent the mean ± SD of 10 and 36 observation, respectively.

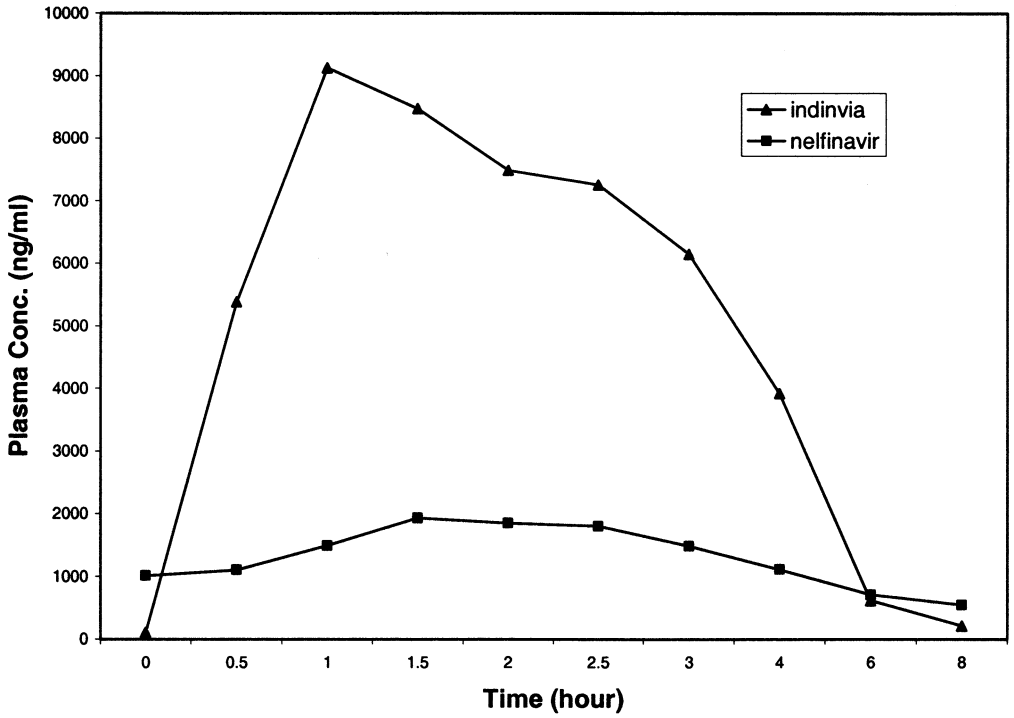

Fig. 3. Plasma concentration versus time curves of IDV (800 mg) and NFV (750 mg) after oral administration to an HIV-1 patient, respectively.

after oral administration to an HIV-1 patient, respectively. The close results were obtained from this method, which was compared with other two methods set up in this lab for the separate measurement of NFV and IDV.

4. Discussion

Quantitative analysis with high selectivity and sensitivity by LC–MS–MS uses the technique of selected MRM. In this mode only a selected MS/MS or collision induced dissociation (CID) transition needs to be monitored. This is now considered the best analytical approach for accurate and highly selective quantitative measurement of drugs and metabolites in complex matrices such as biological samples. In this method the unique parent/daughter ion pairs of NFV, IDV, RTV, SQV, APV and IS are 568.4/330.0, 614.3/421.2, 720.9/296.0, 671.1/570.2 and 505.9/245.0, respectively. These parent/daughter ion pairs are scanned alternatively every 200 ms with 5 ms delay between scan. It is the capability of the MRM mode of LC/MS/MS that allows a significant gain in signal strength over noise and permits the tandem mass spectrometer (TMS) to detect very small quantities of analytes. This is well illustrated by the results presented in this paper where five PI concentrations as low as 5 ng/ml can be measured quantitatively in 100 μl of plasma by deproteinizing plasma samples with acetonitrile. In HPLC–UV determinations, the range of the lower limit of quantitation (LOQ) for the five PIs was 10–50 ng/ml in sample size of 250–1000 μl with the time-consuming sample preparation procedures (solid phase extraction or liquid–liquid extraction). Further there was no interference observed from residual endogenous plasma components and from other concomitantly administered drug analytes (see Fig. 2 blank chromatography), which is another valu-

able aspect of LC–MS–MS. This is facilitated by scanning the unique ion pairs in preference to single ion monitoring available with LC–MS. The absence of interference from other antiviral agents was clearly seen in the analysis of the buffer sample of spiked with zidovudine (ZDV), nevirapine, lamivudine (3TC) and stavudine (d4T). Both the UV detector and the TMS are considered to be universal detectors as far as small biomolecules. However, if chromatograms from LC–UV and LC–MS–MS assays of the same sample are compared, the striking difference in the number of peaks mostly due to endogenous components is very clear. The MS–MS extracted ion chromatograms will show the each analyte peak in one panel and the internal standard peak in the next panel; whereas, in an LC–UV chromatogram there will be numerous peaks very near the origin, followed by the analyte and internal standard peaks at longer retention times. Since most patients are on more than one medication, frequent interferences from concomitant drugs or from metabolites can be observed in UV assays. MS–MS detection on the other hand collects signals only from specific parent/daughter ion pairs, thus eliminating all interferences. The turboion spray sample inlet has the capability to introduce higher liquid volumes containing larger proportions of water. This is important for introducing polar hydrophilic substances into the mass spectrometer.

In addition to the advantage of small sample aliquots required for LC–MS–MS, there are considerable saving in time that can maximize sample throughput resulting in much reduced sample analysis time.

Stability studies showed that all the analyes were found to be stable during repeated three freeze/thaw cycles, 24 h storage at room temperature and 6 months storage at temperature of -70 °C.

Two linear calibration curves were established for quantitation of NFV, IDV, RTV, SQV and APV with the low curve ranged from 5 to 1000 ng/ml and the high curve ranged from 1000 to 10,000 ng/ml. It was not possible to obtain a single linear curve for each analyte with such broad range of 5 to 10,000 ng/ml using the LC–MS–MS method. The accurate results were obtained with two calibration curves as shown in Table 2. The broad calibration curve range from 5 to 10,000 ng/ml was adequate and convenient to handle most pharmacokinetic samples.

5. Conclusion

Among the significant advantages of this method are high selectivity and sensitivity, good accuracy and recovery with low LOQ for each analyte. Furthermore, its rapid and simple sample preparation, short run time and small sample size are added advantages.

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