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An LC-MS-MS method for the determination of indinavir, an HIV-1 protease inhibitor, in human plasma

Anura L. Jayewardene [a], Brian Kearney [b], Judith A. Stone [a], John G. Gambertoglio [a], Francesca T. Aweeka [a,*]

[a] *Department of Clinical Pharmacy, Drug Research Unit, School of Pharmacy, University of California, San Francisco, CA 94143-0622, USA*

[b] *Gilead Sciences Inc., Biopharmaceutics, 333 Lakeside Drive, CA 94404, USA*

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Abstract

A method for the determination of indinavir (IDV) (L-735 524) in human plasma by LC-MS-MS is discussed, and the validation data is presented. The analyte and internal standard are isolated from plasma by a simple acetonitrile precipitation of plasma proteins followed by centrifugation. LC-tandem mass spectrometry in positive ion, multiple reaction monitoring mode used pairs of ions at m/z of 614/421 for indinavir and 628/421 for internal standard, respectively. The calibration curve had a linear range from 3.0 to 12320 ng/ml when linear least square regression weighing $1/x$ was applied to the concentration versus peak area plot. The advantages of this method are the fast sample preparation, wide dynamic assay range and quick analysis taking only 5 min for each sample run. The robust nature of this assay has been further verified during routine use over several months involving multiple analysts.
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Keywords: HIV-1; Indinavir; LC-MS-MS; Protease inhibitor

1. Introduction

Indinavir (IDV) (Fig. 1) is a potent inhibitor of human immunodeficiency virus (HIV) proteases [1,2] now prescribed in combination therapy potentially with another protease inhibitor and one or more nucleoside analogs or non-nucleoside reverse transcriptase inhibitors or with two reverse transcriptase inhibitors. Determination of the indinavir concentrations in body fluids including serum, plasma and cerebrospinal fluid is of importance in conducting clinical studies of this drug with regard to efficacy, toxicity and dose ranging. Modern pharmacokinetic studies require parts per billion characterization and quantification, as well as, the ability to provide analytical results with rapid turn around from large batches of samples [3].

In recent years the use of high performance liquid chromatography (HPLC) with ultraviolet

* Corresponding author. Tel.: +1-415-4760339; fax: +1-415-4760307.

E-mail address: aweeka@itsa.ucsf.edu (F.T. Aweeka).

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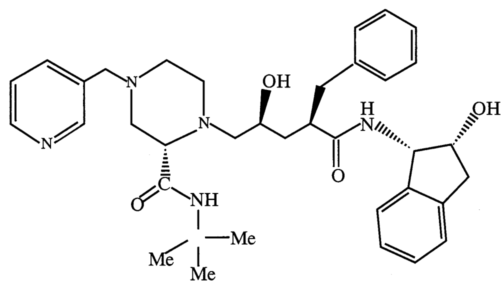
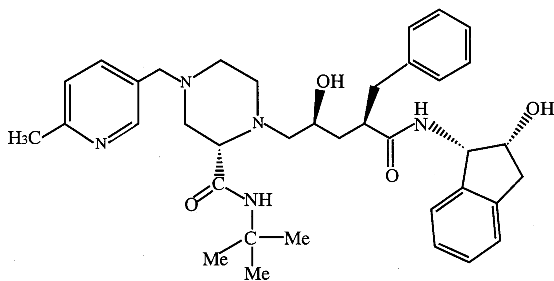
(UV) detection, mass spectrometric detection (MSD) and tandem mass spectrometry (MS-MS) have been found to be ideally suited for the determination of analytes in diverse biomatrices. Several HPLC methods have been published for quantitatively determining indinavir levels in animal and human plasma [4–9]. These methods use isocratic and gradient HPLC or HPLC with column switching and liquid chromatography (LC) mass spectrometry. Some of these assay methods are relatively time-consuming while others have poor lower limits of quantitation. Sample preparation by liquid–liquid extraction was another time consuming step in these methods. Therefore, our goal was to develop a relatively rapid, high sensitivity method for indinavir in human plasma with a very low limit of quantitation, using a newly acquired LC-MS-MS instrument.



INDINAVIR [L 735.524]
$C_{36}H_{47}N_5O_4$ M.W. 613.8



Methyl derivative of INDINAVIR [L 738.804]
$C_{37}H_{49}N_5O_4$ M.W. 627.8

Fig. 1. Chemical structure of indinavir and internal standard (methyl indinavir).

This paper describes the method validation of this rapid, liquid chromatography-tandem mass spectrometric (LC-MS-MS) assay for indinavir using an internal standard. The sample preparation is simple and consists of precipitating plasma proteins with pure acetonitrile by vortex mixing and spinning down the protein to a pellet. The clear supernatant is directly injected into a short LC column and eluted with a linear gradient of 10 mM ammonium formate buffer (pH 4.10) and acetonitrile. The initial acetonitrile concentration of 20% was ramped to 80% and then decreased to 20% over 4 min. The broad calibration curve range from 3.0 to 12 320 ng/ml was adequate to handle most pharmacokinetic samples. Some samples were obtained at extended sampling intervals beyond the usual 12 h, and also to measure samples from peak concentrations without dilution.

2. Experimental

2.1. Chemicals/consumables

All the chemicals were HPLC grade or reagent grade unless otherwise ststed. Acetonitrile, ammonium formate, formic acid (99–100%), ammonium hydroxide solution and HPLC-water were from Fisher Scientific (Fair Lawn, NJ, USA). Indinavir sulfate (Crixivan, L-735 524) Lot #001J-113 and its methylated derivative L-738 804, Lot #000K-006 (internal standard (IS), Fig. 1) were supplied by Merck Research Laboratory (Rahway, NJ, USA). Drug free human plasma was obtained from the Long Hospital Blood Bank (University of California at San Francisco, San Francisco, CA, USA). Color-coded, 1.5 ml, polypropylene, snap cap micro centrifuge tubes were from Fisher Scientific.

2.2. Chromatography/mass spectrometry equipment

Perkin-Elmer Biosystems, Norwalk, CT, supplied the PE Biosystems 200 series autosampler and twin PE Biosystems series 200 micro HPLC pumps. Perkin-Elmer-Sciex, Concord, Ontario,

Canada provided the PE-Sciex API 2000 triple quadrupole Mass Spectrometer with TurboIon spray sample inlet.

2.3. MS-MS parameters

Specific MS-MS parameters are as follows. The ionization was via a TurboIon spray inlet in the positive ion mode. All gases were ultrahigh purity nitrogen (99.999%). The nebulizer, auxiliary, and curtain gas pressures were 60, 60 and 30 psi, respectively. The interface temperature was at 90°C and the heated nebulizer was set at 300°C. The mass scanning mode was by multiple reaction monitoring (MRM) with a parent/daughter ion pair for IDV of 613.8–421.2 *m/z* and for methyl indinavir (IS) of 628.8–421.2 m/z. 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, Experiment editor and sample editor were installed on a Macintosh OS 8.5 platform with 64 MB of RAM and a 16 GB hard disk.

2.4. Liquid chromatography parameters

The specific liquid chromatographic (LC) parameters for the assay are as follows. The column was a Zorbax XDB-C8 reverse phase column, 2.1 mm i.d. × 50 mm, with 5 µm particle size packing and a Zorbax-Eclipse polymeric 2.1 × 12.5 mm guard column. The mobile phase flow rate was kept constant at 400 µl/min. Mobile phase A was 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 (v/v) over 1.00 min, held at 20:80 for 1.0 min, and then returned to 80:20 over 1.0 min. This condition was held for a further 1.8 min prior to the injection of another sample. Mobile phase A was prepared by dissolving 1.28 g ammonium formate in 2.0 l HPLC-grade water, adjusting pH to 4.1 with formic acid prior to filtration through a 0.22 µm membrane, followed by sonication under vacuum to degas. Mobile phase B was a 0.1% (v/v) solution of pure formic acid in HPLC-grade acetonitrile which was mixed well, filtered and degassed under vacuum. The volume of injection was 10 µl through a 50 µl loop.

2.5. Preparation of standards and controls

2.5.1. Indinavir stock solutions

To prepare indinavir stock solutions, 5.79 mg IDV sulfate was weighed accurately and dissolved in a 10 ml volumetric flask with 50% acetonitrile in water, to generate a concentration of 500 µg/ml of IDV base. This was appropriately diluted to lower concentrations for spiking the calibration standards. These were prepared by spiking 5 ml aliquots of drug-free plasma with the indinavir working standard solutions to give a range of concentrations from 3.0 to 12 000 ng/ml. Frozen quality control (QC) pools were prepared at four different concentrations of indinavir using a separate stock solution of indinavir containing 1000 µg/ml as the base in aqueous acetonitrile. The IS (methyl indinavir), was prepared in aqueous acetonitrile (50:50 v/v) at a concentration of 100 µg/ml and further diluted 1:100 v/v to a working concentration of 1.0 µg/ml with aqueous acetonitrile (75:25 v/v). Aliquots (200 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, QC samples and calibration standards were thawed as needed. The same procedure was followed for all samples. Exactly 50 µl of internal standard solution was added to aliquots of 200 µl of plasma from deactivated study samples, calibrators and QC in 1.5 ml microcentrifuge tubes, and mixed gently. Acetonitrile 400 µl was added to each tube and vortexed for 20 s at high speed. The tubes were 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.

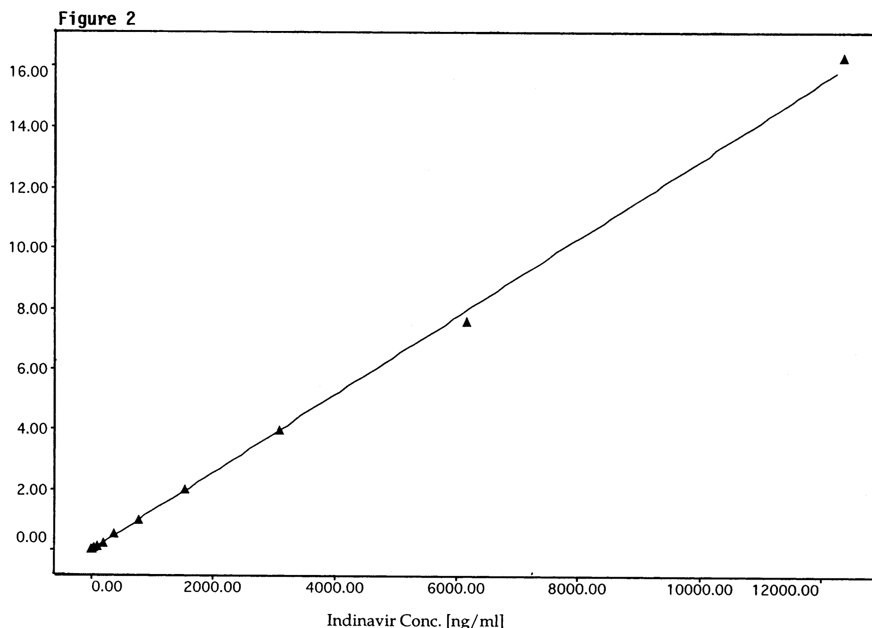


Fig. 2. Indinavir calibration curve (peak area ratio versus concentration). Regression is least squares with 1/x weighting.

2.7. Data analysis

Calibration standards in plasma containing 3.0, 6.1, 12.2, 24.4, 48.8, 96.6, 193.2, 385, 770, 1540, 3080, 6160 and 12 320 ng/ml were used to establish a single calibration curve with $1/x$ weighted linear regression (Fig. 2). Indinavir concentrations versus peak area ratios were plotted in Turboquan, a separate quantitation software program supplied with the mass spectrometer.

2.8. Inter-assay and intra-assay precision

Inter-assay and intra-assay precision was evaluated using previously frozen controls at four concentrations of 70, 400, 4000 and 9000 ng/ml designated as low, medium, high and extra high. For inter-assay precision, six samples of each concentration, for a total of 24, 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 (C.V.%) for the four different levels ($n = 36$ for each) were determined (Table 1). For intra-assay precision, ten control samples from each of four concentrations were assayed with a single calibration curve and coefficients of variation for the calculated drug concentrations were determined (Table 2). The accuracy was calculated using the equation.

$$\text{Accuracy\%} = \left[\frac{\text{Calculated concentration}}{\text{Nominal concentration}}\right]100$$

2.9. Limit of quantitation

The intra-assay lower limit of quantitation was verified by adding indinavir to six aliquots of blank plasma at a concentration of 3 ng/ml and assaying them with a set of calibration standards. The mean value of the lower limit of quantitation was determined with the standard deviation (S.D.) and the C.V. %.

2.10. Recovery of indinavir

Recovery of IDV from plasma following sample preparation was assessed by comparing the concentration of drug from plasma samples to the concentration of drug spiked into mobile phase at

the same concentration as in the plasma samples. In order to avoid the loss of internal standard (IS) during sample preparation, the IS was added after plasma samples were precipitated and centrifuged. Mean recovery was calculated as,

$$\text{Mean recovery} = \frac{\text{Mean calc. plasma IDV conc.}}{\text{Mean calc. buffer IDV conc.}} 100$$

3. Results

Extracted ion chromatograms from a patient sample with indinavir and added internal standard and a drug free plasma blank are illustrated in Figs. 3 and 4. Calibration standards in human plasma containing 3.0–12 320 ng/ml of indinavir were used to establish calibration curves for assay validation. Linear regression of concentration ver-

Table 1
Indinavir in plasma inter-assay precision and accuracy

Replicate set[a] number	Mean concentration of indinavir (ng/ml) over days 1–6			
	Low (70 ng/ml)	Medium (400 ng/ml)	High (4000 ng/ml)	Extra high (9000 ng/ml)
1	73	422	4239	8722
2	74	400	4189	8634
3	72	413	4260	8858
4	72	402	4339	8985
5	71	416	4262	8602
6	68	427	4119	8739
Mean	72	413	4235	8757
S.D.	1.9	10.8	74.5	143.6
R.S.D.%	2.62	2.60	1.76	1.64
Accuracy%	102.4	103.4	105.9	97.3

[a] Each set is 6 aliquots

Table 2
Indinavir in plasma intra-assay precision and accuracy

Sample number	Mean concentration of indinavir (ng/ml)			
	Low (70 ng/ml)	Medium (400 ng/ml)	High (4000 ng/ml)	Extra-high (9000 ng/ml)
1	71	406	4119	9019
2	75	408	3890	9305
3	66	389	3788	9147
4	73	413	4101	8881
5	72	421	4054	9103
6	70	424	4120	9096
7	71	362	3948	8693
8	72	413	4123	8787
9	75	371	4074	9307
10	69	416	4137	8816
Mean	71	402	4035	9015
S.D.	2.6	21.4	119	215
R.S.D.%	3.7	5.3	3.0	2.4
Accuracy%	101.7	100.6	100.9	100.2

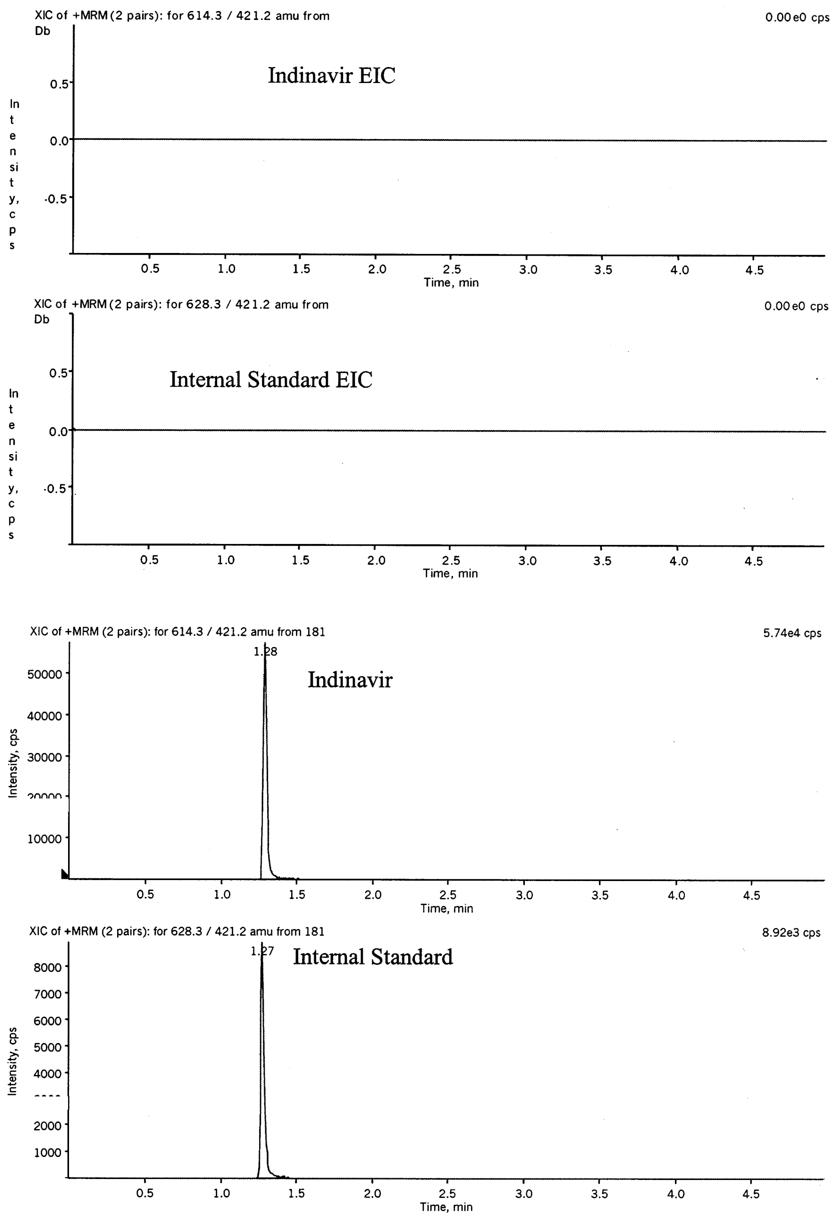


Fig. 3. Extracted ion chromatograms of plasma blank and a patient plasma sample with indinavir and internal standard.

sus peak area ratio plots resulted in coefficients of determination (r^2) consistently greater than 0.997. The reproducibility of the calibration standards over 6 days of assay are indicated by regression parameters, with a mean slope of 0.0155 ± 0.0638, the mean intercept 0.0019 ± 0.0041 and the coefficient of determination 0.9985 ± 0.0016 for a C.V. of 0.16% over 6 days. As a measure of goodness of fit, coefficients of variation of the mean concentrations of indinavir were calculated. They ranged from 2.8 to 11.6% and accuracy ranged from 97.3 to 112.8% of the nominal concentrations of the calibrators.

Intra-assay precision ($n = 10$) ranged from 2.4

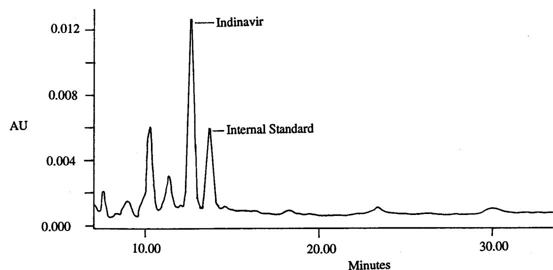


Fig. 4. An LC-UV chromatogram of indinavir and internal standard in plasma.

to 5.3% and the accuracy was within 100.2–101.7% of the nominal concentration of the QC samples. Inter-assay precision ($n=36$) ranged from 5.3 to 9.5% and accuracy was within 97.5–105.9% of the nominal concentration of the QC samples.

3.1. Limit of quantitation

The lower limit of quantitation for indinavir by this method was 3.0 ng/ml with a C.V. of 6.6% (six replicates) and a mean accuracy of 100.4%. For the interassay LOQ over 6 days the C.V. was 19.9% and accuracy was 113.3%.

3.2. Recovery of indinavir from plasma

Overall recovery of IDV was found to be 94.6%, S.D. 4.3 and C.V. of 4.6% over a concentration range of 100–9000 ng/ml (Table 3).

3.3. Stability of indinavir after extraction

The stability of the analyte after extraction from plasma was evaluated by re-injecting the calibration standards at the end of a 12 h run of IDV samples. The mean change in the concentration of the calibrators was −3.6%.

4. Discussion

Quantitative analysis of compounds with high selectivity and sensitivity by LC-MS-MS uses the technique of selected reaction monitoring (SRM). In this mode only a selected MS-MS or collision induced dissociation (CID) transition needs to be monitored. This is now considered to be the best analytical approach for accurate and highly selective quantitative measurement of drugs and metabolites in complex matrices such as biological

Table 3
Recovery of indinavir from plasma

Nomial concentration of indinavir (ng/ml)	Concentration of indinavir (ng/ml)				
	Buffer solution	Plasma extract	Buffer mean ($n=3$)	Plasma mean ($n=3$)	Recovery (%)
100	124	123			
100	128	122	125	122	97.2
100	128	120			
400	456	422			
400	501	456	519	458	88.2
400	599	495			
4000	3690	3683			
4000	4128	3675	3739	3638	97.3
4000	3398	3557			
9000	9019	9433			
9000	10446	9400	9711	9306	95.8
9000	9667	9085			
Mean overall recovery					94.6
S.D.					4.3
C.V.%					4.6

samples [10]. In this method the unique pair of ions with m/z 614.8 and m/z 421.2, for IDV and m/z 628.4 and m/z 421.2 for the internal standard, respectively, constitute the parent/daughter ion pairs which are scanned alternately every 200 ms with 5 ms delay between scans. It is this capability of the SRM 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 indinavir concentrations as low as 3 ng/ml in plasma can be measured quantitatively in 200 μl of plasma. For an HPLC-UV method, the best lower limit of quantitation (LOQ) for a similar sample size was 15 ng/ml, while another LC–UV method, which used 1.0 ml samples and a column switching mode resulted in an LOQ of 5 ng/ml [7,5]. Sensitivity associated with LC-MS-MS is especially useful for quantitating antiretroviral drug levels at peripheral tissue sites of interest. Anti-HIV compounds should be available at sites such as lymph node peripheral blood mononuclear cells and the central nervous system. But estimates of exposure at these sites require sensitive methods, such as, those described herein.

The absence of interference from residual endogenous plasma components or from other concomitantly administered drug analytes is another valuable 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 drug analytes was clearly seen in the analysis of an indinavir plasma sample, which was spiked with three other protease inhibitors (nelfinavir, ritonavir and saquinavir), two nucleoside analogues (ZDV, 3TC) and nevirapine. Both the UV detector and the TMS are considered to be universal detectors as far as small bio-molecules. However, if chromatograms from an LC–UV assay and an LC-MS-MS assay 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 analyte peak in one panel and the internal standard 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 [7]. 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 advantages of small sample aliquots required for LC-MS-MS, there are considerable savings in time and materials as well. A comparative study in this laboratory, showed that liquid–liquid extraction and HPLC-UV analysis of 40 samples of indinavir in plasma takes about 12–14 h as compared with 4.5 h by the LC-MS-MS method discussed above. For nelfinavir and its metabolite in human plasma a similar study indicated very similar savings in time and materials [11]. The savings in reagents and consumables are also comparable.

The robust nature of this assay was further verified when the method was put into routine use. During a 5-month period, over 500 samples were processed by several analysts. Despite these additional variables (multiple batches, time interval and analyst) the precision of the method was excellent with C.V. less than 14% for the low control (70 ng/ml) and less than 8% for all the other controls.

LC-TMS is a highly selective, sensitive and accurate bio-analytical technique that can maximize sample throughout resulting in much reduced method development and sample analysis time.

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