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Simple high-performance liquid chromatographic determination of the protease inhibitor indinavir in human plasma

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Abstract

Indinavir is a member of a class of protease inhibitors that actively prevent the acquired immunodeficiency syndrome virion from maturing. A high-performance liquid chromatographic (HPLC) assay was developed and validated for the determination of indinavir in human plasma. Indinavir and the internal standard were isolated from the plasma by ether extraction. The residue after evaporation of ether was reconstituted with buffer and injected onto a C_4 reversed-phase column eluted isocratically with a mobile phase consisting of 35:65 (v/v) of acetonitrile and buffer. A wavelength of 210 nm was found to be optimum for detection. The calibration range of this assay was from 10 to 5000 ng/ml and coefficients of variation for the assay ranged from 4.6% to 11.0% for three different drug concentrations and the limit of quantitation was 10 ng/ml. During the validation, short-term stability of the drug in plasma, stability during heat deactivation and on repeated freezing and thawing of plasma was evaluated. The overall recovery of indinavir by the ether extraction method was 91.4%. This HPLC assay was found to be a simple and reproducible method for monitoring indinavir levels in human plasma obtained during clinical trials of the drug. © 1998 Elsevier Science B.V.

Keywords: Indinavir ; Protease

1. Introduction

Indinavir, N-(2(*R*)-hydroxy-1(*S*)-indanyl)-2(*R*)-(phenylmethyl) - 4(*S*) - hydroxy - 5 - [1-[4-(3-pyridyl - methyl)-2(*S*)-(N-*tert.*-butylcarbamoyl)piperazinyl]] - pentanamide sulfate hydrate (Fig. 1) is a member of a series of protease inhibitors used in the treatment of human immunodeficiency virus (HIV) infection [1]. Indinavir possesses a hydroxylamine-pentanamide transition state isostere. The incorporation of a basic amine into the backbone greatly improved the pharmacokinetic profile. [2,3]. Indinavir can efficiently inhibit the replication of HIV virus in vitro by blocking the activity of its protease enzyme. HIV protease acts like a ''molecular scissors'' to cleave the *gag* and *gag/pol* polyproteins into smaller functional proteins, thereby allowing the virion to mature [3]. Although its precise mechanism of action is yet unknown, indinavir appears to prevent the virions from maturing to their infectious state.

Several high-performance liquid chromatography (HPLC) methods have been reported to quantitate the determination of indinavir levels in animal and human plasma [4–8]. These methods use gradient HPLC or HPLC with column switching and liquid

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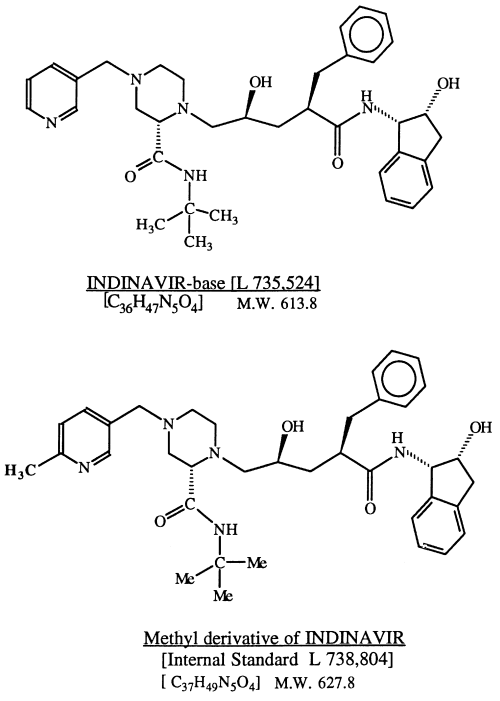

Fig. 1. Structural formulas of indinavir and its internal standard (methylindinavir).

chromatography–mass spectroscopy (LC–MS). These assay methods are relatively time-consuming or they use expensive instrumentation not readily accessible to many groups. Therefore, our goal was to develop a relatively rapid and less costly assay that could be performed at any regular laboratory with adequate HPLC instrumentation. All these methods have a small calibration range.

This paper describes a simple isocratic HPLC assay, using UV detection and a Delta-pak C_4 column. The sample preparation is easily performed in a fume hood and the HPLC run time for each sample is about 30 min. This is a first reported method for indinavir where column switching or LC–MS–MS is not required for quantitation. The calibration range from 10 to 5000 ng/ml is large enough to handle most pharmacokinetic samples without dilution. This method is also sensitive enough to detect clinically relevant concentrations with trough levels of >100 ng/ml usually desired with indinavir therapy.

2. Experimental

2.1. Chemicals

All chemicals were HPLC or reagent-grade unless otherwise noted. Acetonitrile, ammonium dihydrogen phosphate, and ammonium hydroxide solution, diethyl ether and 1-heptanesulfonic acid were obtained from Fisher (Fair Lawn, NJ, USA). Indinavir sulfate (Crixivan) Lot No. 001J-113 and its methylated derivative Lot No. 000K-006 (internal standard, Fig. 1) were supplied by Merck (Rahway, NJ, USA). Drug-free human plasma was obtained from the Long Hospital Blood Bank and the Irwin Memorial Blood Center (University of California San Francisco, San Francisco, CA, USA).

2.2. Chromatographic equipment and method

The Millennium-2010 ver. 2.15 chromatography manager software was used to control the HPLC system consisting of an M-510 HPLC pump, an M-717 autosampler, and a 996 PDA detector all from Waters (Milford, MA, USA). A Waters Delta-pak C_4 reversed-phase column, 150 mm×3.9 mm (I.D.)with 5 μm particle size packing was used. The 996 PDA detector was used to monitor the drug at a wavelength of 210 nm. The mobile phase flow-rate was 0.6 ml/min. and the volume of injection was 35 μl.

2.3. Mobile phase

The mobile phase consisted of 10 m*M* ammonium dihydrogen phosphate and 1 m*M* 1-heptanesulfonic acid sodium salt in water at pH 4.8, mixed with acetonitrile 65:35 (v/v). The buffer was prepared by dissolving 1.15 g of ammonium dihydrogen phosphate and 0.20 g of 1-heptanesulfonic acid sodium salt in 1.0 l of distilled water. Its pH was adjusted to 4.8 with ammonium hydroxide and 650 ml of this solution was mixed with 350 ml of acetonitrile. The mixture was passed through a 0.22 μm filter and degassed by sonication under a vacuum prior to use.

2.4. Preparation of standards and controls

The indinavir powder was weighed out and dissolved in acetonitrile–water (1:1, v/v) to obtain a solution of 1.0 mg/ml of the 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 10 ng/ml to 5000 ng/ml. Frozen quality assurance/quality control (QA/QC) samples were prepared at three different concentrations of indinavir using a separate stock solution of indinavir containing 500 μg/ml as the base in aqueous acetonitrile. The internal standard was prepared in aqueous acetonitrile at a concentration of 105 μg/ml and further diluted 1:10 (v/v) to 10.5 μg/ml with water. Aliquots of 300 μl of calibration standards and QA/QC samples were pipetted into screw-capped culture tubes and kept frozen at −20°C until required for analysis.

2.5. Processing of plasma

Frozen plasma samples from study subjects, QA/QC samples and calibration standards were thawed as needed. The same procedure was followed for all samples. Aliquots of 300 μl of ammonium dihydrogen phosphate buffer (50 m*M*, pH 9.0) and 30 μl of internal standard solution were added to each tube. Tubes were vortexed for 10 s, and 3 ml of diethyl ether was added to each tube. All tubes were then vortexed for 30 s and kept at −20°C for 30 min. The ether layer from each mixture was drawn out into clean labelled 13×100 mm glass tubes and evaporated under nitrogen at room temperature to dryness. The residue was reconstituted with 150 μl of 10 m*M*, pH 5.5 ammonium dihydrogen phosphate buffer and centrifuged for 5 min at 750 *g*. A 35 μl aliquot of each sample was injected onto the HPLC column for analysis at room temperature. The calibrators were injected once at the start and once at the end of each run. All samples with concentrations above the upper quantitation limit (UQL) were diluted with blank human plasma appropriately. Extrapolation of concentrations above UQL of the calibration curve was not done.

2.6. Data analysis

Calibration standards in plasma containing 10, 25, 100, 250, 500, 1000 and 5000 ng/ml of indinavir were used to establish pairs of low (10–250 ng/ml) and high (250–5000 ng/ml) calibration curves for assay validation and for clinical assays. Indinavir concentrations versus peak height ratio values were plotted and unweighted linear least-squares regression was performed to obtain the equation for the best-fit line. The equation from each curve was used to calculate the appropriate drug concentrations in pooled frozen controls and clinical samples using the peak height ratios obtained from the corresponding chromatograms.

2.7. Inter-assay and intra-assay precision

Interassay and intraassay precision were evaluated using previously frozen controls at three concentrations of 75 ng/ml, 600 ng/ml and 2500 ng/ml designated as low, medium, and high. For inter-assay precision, six samples of each concentration were assayed on five different days using five sets of standard curves. Means and standard deviations (S.D.) were calculated using the calculated drug concentrations over all five days and relative standard deviations (R.S.D.) for the three different levels were determined. For intra-assay precision, 12 control samples from each of three concentrations were assayed with a single calibration curve and R.S.D. for the calculated drug concentrations were determined. The accuracy was calculated using the equation,

$$\text{Accuracy\%} = [\text{Calc. Concentration}/\text{Nominal Concentration}] \times 100.$$

2.8. Limit of quantitation

The intra-assay lower limit of quantitation was determined by spiking six aliquots of blank plasma with indinavir at a concentration of 10 ng/ml and assaying them with a set of calibration standards. The mean value of lower limit of quantitation was determined along with the R.S.D.. Though this is not

the lowest point of reliable quantitation it was considered adequate for the clinical trials on indinavir as it is ten-times lower than the trough levels of the drug.

2.9. Recovery of indinavir

For assay recovery of indinavir, aqueous samples were spiked with the same drug concentrations as QA/QC plasma samples on the same day. The plasma samples alone were extracted by the standard procedure and then spiked with internal standard while the aqueous samples were not subjected to extraction. Assay recovery was determined by comparing peak height ratios from aqueous samples to the peak height ratios from the corresponding plasma QA/QC samples.

2.10. Stability of indinavir in plasma at room temperature

To assess the stability of indinavir at room temperature, a set of six each of plasma QA/QC frozen controls were thawed and stored at room temperature for 24 h. At the end of the respective time period, all QA/QC controls were assayed along with a set of six each freshly thawed controls. Stability at room temperature was evaluated by comparing the peak height ratio values of indinavir to determine the change of indinavir concentration over 24 h.

2.11. Freeze–thaw stability of indinavir in plasma

To determine freeze–thaw stability of indinavir, three sets of controls were thawed. One set was assayed while the remaining two sets were refrozen. These two sets were thawed on a subsequent day and one set was assayed while the other set was refrozen. The last set of controls was thawed on a third day and assayed. The calculated concentrations of three sets of QA/QC controls were compared to evaluate the stability of indinavir in plasma subjected to repeated thawing and freezing.

2.12. Stability on heat deactivation

To evaluate indinavir stability during heat deactivation of HIV-positive plasma samples, a set of QA/QC samples were placed in a water bath at a temperature of 57°C for 45 min. The heated samples were then processed along with a set of freshly thawed samples. The calculated indinavir concentrations of both sets were compared to determine any loss of indinavir during the heat deactivation.

3. Results

3.1. Data collection and calculation

Peak height ratio values of calibration standards were proportional to the concentrations of indinavir in plasma over the range tested. The calibration curves were fitted by linear least-squares regression and showed coefficients of determination greater than 0.999. The mean slope for the low curve (10–250 ng/ml) was 0.00093, with an S.D. of 0.00005 and an R.S.D. of 5.6%. The mean *y*-intercept for the low curve was 0.00441 and the mean coefficient of determination was 0.9999. The mean slope for the high curve (250–5000 ng/ml) was 0.00096, with an S.D. of 0.00010 and an R.S.D. of 10.2%. The mean *y*-intercept for the high curve was 0.00488 and the mean coefficient of determination was 0.9999. Microsoft Excel 5.0 software was used to calculate means, S.D. and R.S.D.% values

3.2. Selectivity

The assay was selective for indinavir in human plasma and other protease inhibitors nelfinavir, ritonavir and saquinavir (retention times between 22 and 27 min) and nucleoside analogue antivirals ddI, ddC, d4-T, 3-TC and ZDV did not cause interference. The retention time of indinavir was 13.2 ± 0.5 min, while the retention time of the internal standard (methylindinavir) was 14.4 ± 0.7 min. Chromatograms of drug-free human plasma did not show peaks at the retention time of indinavir and internal standard. Chromatograms of an extracted blank human plasma and one with internal standard added are shown in Fig. 2a,b. Four different samples of blank plasma were tested and found to be free of interference. A chromatogram of an extracted human

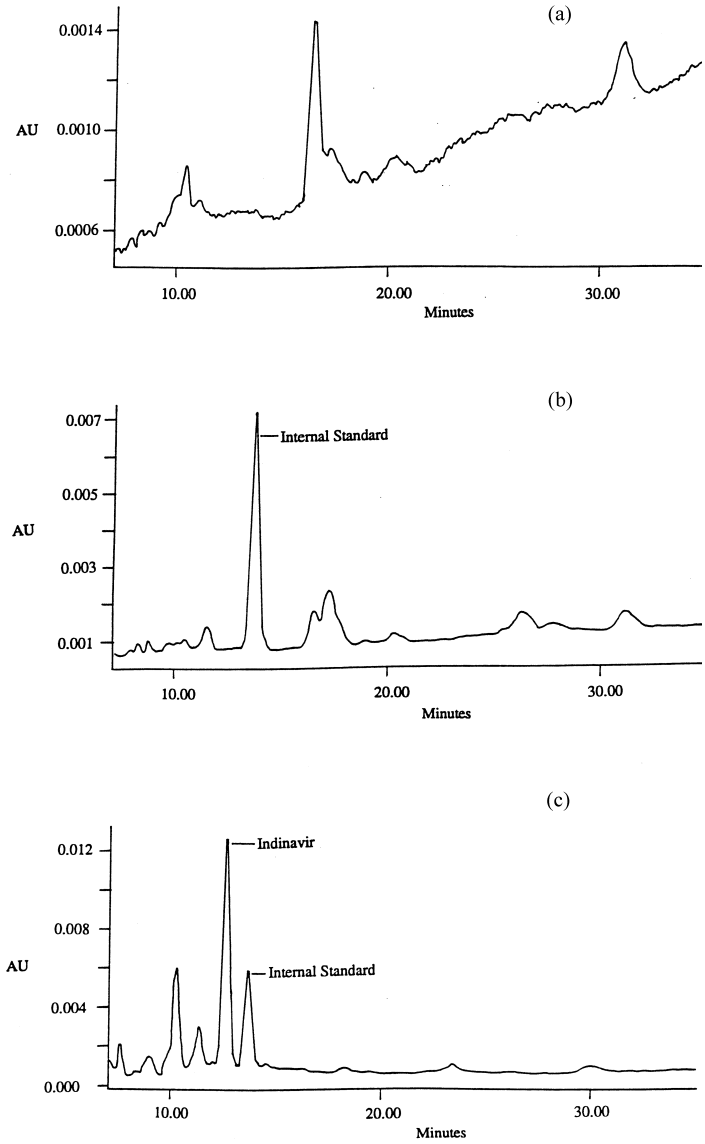

Fig. 2. Chromatograms of (a) blank plasma extract, (b) blank plasma extract with internal standard, and (c) plasma extract of a sample from a patient 3 h after an 800 mg oral dose of indinavir with a measured concentration of 2685 ng/ml indinavir. The coadministered drugs were ZDV and HBY-09, an experimental nonnucleoside reverse transcriptase inhibitor. For chromatographic conditions refer to Section 2. Blank and spiked plasma samples were extracted as described in Section 2.

plasma from a patient 3 h after being given an oral dose of 800 mg of indinavir is shown in Fig. 2c. The coadministered drugs were ZDV and HBY-097, an investigational nonnucleoside reverse transcriptase inhibitor. The measured plasma concentration of indinavir is 2685 ng/ml.

3.3. Inter-assay and intra-assay precision

For the inter-assay precision, the R.S.D. for calculated indinavir concentrations ranged from 4.6% to 11.0%. The mean accuracy for calculated indinavir concentrations ranged from 95.9% to 101.4%. For

Table 1
Indinavir in plasma: inter-assay precision and accuracy

Sample No.	Mean indinavir concentration[a] (ng/ml) days 1–5		
	Low (75 ng/ml)	Medium (600 ng/ml)	High (2500 ng/ml)
1	76	587	2395
2	71	623	2400
3	79	598	2402
4	76	599	2411
5	74	608	2394
6	70	636	2387
Mean	74	609	2398
S.D.	8	38	110
R.S.D.%	11.0	6.2	4.6
Accuracy %	99.2	101.4	95.9

[a] Each value represents mean of five days samples.

the intraassay precision, the R.S.D. for calculated indinavir concentration ranged from 4.2% to 11.5%. The mean accuracy for calculated indinavir concentration ranged from 102.8% to 108.0% (Tables 1 and 2.)

3.4. Limit of quantitation

The lower limit of quantitation at 10 ng/ml had an R.S.D. of 12.9%, and the mean accuracy was 117.5% of the target concentration in human plasma.

3.5. Recovery

The mean overall recovery of indinavir from human plasma during ether extraction prior to analysis was 91.4%. (Table 3.)

3.6. Stability of indinavir in plasma

The overall change of indinavir concentration in plasma samples during 24 h while standing at room

Table 2
Indinavir intra-assay precision

Number of Samples	Indinavir concentration (ng/ml)		
	Low (75 ng/ml)	Medium (600 ng/ml)	High (2500 ng/ml)
1	68	655	2665
2	79	766	2689
3	81	609	2677
4	84	740	2562
5	83	571	2658
6	77	542	2604
7	82	672	2327
8	92	575	2490
9	83	569	2531
10	71	700	2586
11	76	597	2594
12	91	600	2448
Mean	81	633	2569
S.D.	7	73	107
R.S.D. %	8.8	11.5	4.2
Accuracy %	108.0	105.5	102.8

Table 3
Recovery of indinavir from plasma and its stability on heat deactivation

Indinavir concentration (ng/ml)	[a]Mean peak height ratio (in plasma)	[a]Mean peak height ratio (in buffer)	Recovery (%)
Low – 75 ng/ml	0.0824	0.1111	74.4±26.2
Med – 600 ng/ml	0.8216	0.8421	97.5±9.0
High – 2500 ng/ml	3.7237	3.6391	102.4±7.0
Overall recovery of indinavir	91.4%		
Stability on heat deactivation	Low (75 ng/ml)	Medium (600 ng/ml)	High (2500 ng/ml)
Heated samples			
Mean concentration[a]	65	487	2058
S.D.	2	42	88
R.S.D.%	3.8	8.6	4.3
Fresh samples			
Mean concentration[a]	70	526	2242
S.D.	4	22	97
R.S.D.%	5.4	4.2	4.3
Loss of indinavir on heat deactivation (%)	6.2	7.4	8.2

[a] Each value represents mean of six samples.

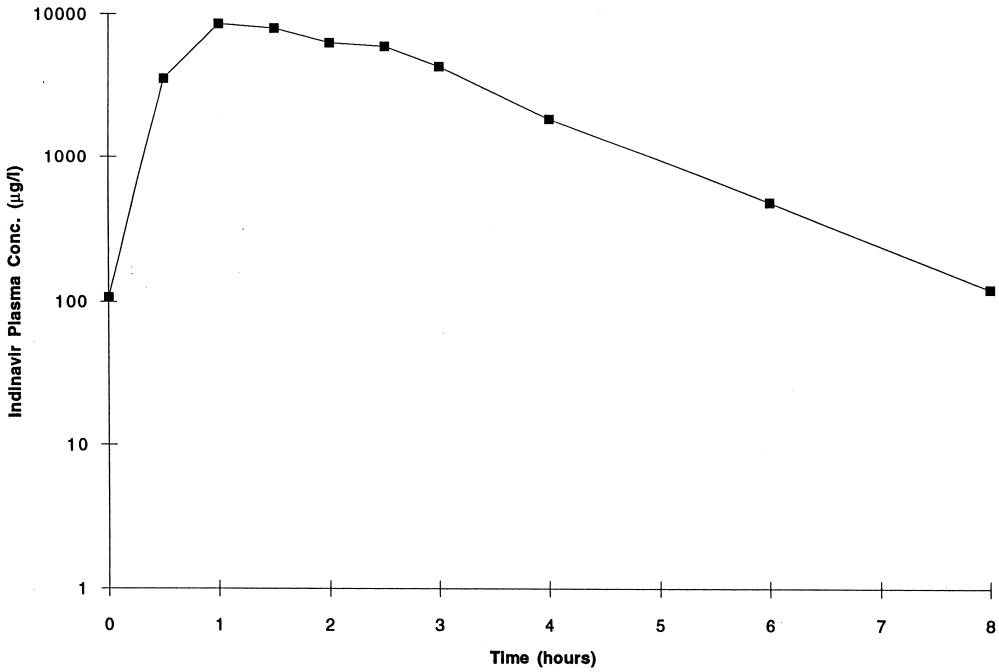

Fig. 3. Plasma concentration versus time curve from a patient after oral administration of 800 mg indinavir under steady state conditions. Experimental details are given in Section 2.

Table 4
Stability of indinavir in plasma at room temperature and after repeat freeze–thaw

Stability	Indinavir control sample concentration (ng/ml)		
	Low (75 ng/ml)	Medium (600 ng/ml)	High (2500 ng/ml)
At room temperature			
–Stored for 24 h			
Mean concentrationa[a]	74	574	2101
S.D.	5	19	22
R.S.D.%	6.1	3.3	1.0
–Fresh samples			
Mean concentration[a]	75	567	2371
S.D.	2	8	94
R.S.D.%	2.2	1.4	4.0
–Change in IDV concentration after 24 h (%)	−1.3	+1.2	−11.4
Overall change in IDV concentration after 24 h storage		−3.8%	
Freeze–thaw			
–First cycle			
Mean concentration[a]	73	545	2406
S.D.	4	20	153
R.S.D.%	5.3	3.7	6.4
–Second cycle			
Mean concentration[a]	78	594	2429
S.D.	6	33	105
R.S.D.%	7.5	5.5	4.3
–Third cycle			
Mean concentration[a]	72	573	2461
S.D.	2	25	69
R.S.D.%	3.4	4.5	2.8
Change in IDV concentration from Cycle 1 to 3 (%)	−1.4	5.1	2.3

[a] Each value represents mean of six samples.

temperature was −3.8%. (Table 4.) The percentage loss of indinavir concentration during heat deactivation ranged from 6.2% to 8.2%. (Table 3.) The change in indinavir concentration during three freeze–thaw cycles ranged from −1.4% to 5.1%. (Table 4.)

3.7. Applicability of the method

Fig. 3 depicts a plasma concentration–time curve of indinavir obtained after an HIV-positive patient at steady state was administered an oral dose of 800 mg of the drug. Plasma samples for pharmacokinetic analysis were taken at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0 and 8.0 h after the dose and a predose sample. ZDV and HBY-097 were coadministered with indinavir. The values for indinavir half-life and area under curve were 1.0 h and 37.3 $\mu M \cdot$h, respectively. It is noteworthy that these values are very similar to values obtained for the same set of samples when the indinavir concentrations were measured by LC–MS–MS. A paired *t*-test of the two sets of concentration data gave mean values±S.D. of 1183.6±1722 and 1198.9±1692 for the UV and MS–MS methods, respectively. These values were not significant with a *P*-value of 0.70.

4. Conclusions

An isocratic HPLC assay for indinavir in human plasma has been developed and validated. The assay is selective, precise, and linear over the ranges studied. It involves simple sample preparation. The

method has successfully been compared with the LC–MS–MS method for pharmacokinetic analysis. Even though this HPLC–UV method is less sensitive than the LC–MS–MS method, we have found that the limit of quantitation of 10 ng/ml is adequate for plasma pharmacokinetic analysis.

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