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Determination of indinavir, an HIV-protease inhibitor, in human plasma by reversed-phase high-performance liquid chromatography

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Abstract

A sensitive high-performance liquid chromatographic assay has been developed to determine the concentrations of the HIV-protease inhibitor indinavir in human plasma. The sample pretreatment involved a protein precipitation procedure using 100 μl of human plasma and 400 μl of acetonitrile. Chromatography was carried out on an Octadecyl column using a mobile phase of acetonitrile–water (40:60, v/v). The water phase contained 50 m*M* phosphate buffer pH 6 and 4 g/l tetramethylammoniumchloride. Ultraviolet detection at 210 nm was used. The method has been validated with regard to specificity, detection limit, lower and upper limit of quantitation, recovery, accuracy, and inter- and intra-assay precision. Stability tests under various conditions were performed. The bioanalytical assay is now in use for the determination of indinavir in several clinical pharmacokinetic studies in HIV-infected patients. © 1997 Elsevier Science B.V.

Keywords: Indinavir; Enzyme inhibitors; Human immunodeficiency virus protease inhibitor

1. Introduction

In 1996 the human immunodeficiency virus (HIV)-protease inhibitor indinavir (Fig. 1) was licensed in the European Community and the United States for the treatment of HIV infection in combination with nucleoside analogs. Together with the other approved HIV-protease inhibitors saquinavir and ritonavir, indinavir has markedly improved the treatment of HIV infection. Until recently, antiretroviral drug therapy was limited to the use of dideoxynucleoside analogs, such as zidovudine (AZT), didanosine (ddI) and zalcitabine (ddC). Since mid-1996, the armamentarium of antiretroviral drugs has been extended to eight (European Community) or eleven (US) agents. Treatment of HIV infection nowadays consists of triple drug therapy, because it

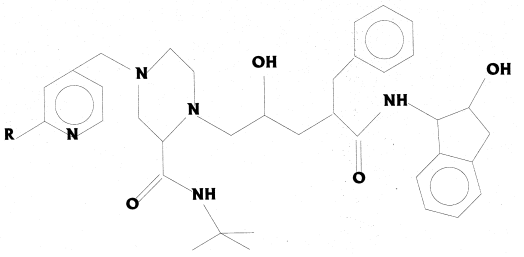

Fig. 1. Chemical structures of indinavir (R=H) and internal standard (I.S.; R=CH_3).

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has been demonstrated that the combination of two nucleoside analogs with one protease inhibitor results in a stronger and more sustained antiviral effect [1].

Indinavir is one of the protease inhibitors that is frequently used as part of triple drug therapy. Its antiretroviral activity appears to be stronger than that of saquinavir (in its current formulation), while its tolerability is better than that of ritonavir. In addition, the potential of drug interactions is smaller for indinavir than for ritonavir.

At the University Hospital of Nijmegen, Netherlands, indinavir was chosen as the subject of extensive clinical pharmacokinetic research. It was therefore necessary to develop a bioanalytical method for the determination of indinavir in human plasma. The assay has been validated according to standard operation procedures used at our laboratory for the validation of bioanalytical methods.

2. Experimental

2.1. Chemicals

Indinavir·1H_2O and the internal standard (I.S.) methylindinavir (Fig. 1) were obtained from Merck (Rahway, NJ, USA). Super gradient acetonitrile and HPLC quality methanol were purchased from Labscan Analytical Sciences (Dublin, Ireland). All other reagents were obtained from Merck (Darmstadt, Germany) and were of analytical grade. Demineralized water was used throughout. The drugs that were investigated for possible interference with the assay were obtained from Sigma (St. Louis, MO, USA) or were extracted from commercial products.

2.2. Stock solutions

Stock solutions of indinavir (250 μg/ml, calculated on the anhydrous base) and the I.S. (125 μg/ml) were prepared by dissolving the appropriate amount of drug, accurately weighed. Indinavir was dissolved in acetonitrile to which an equal volume of water was added. The I.S. was dissolved in acetonitrile. The stock solutions were kept at 4°C. Different stock solutions were used for the construction of the calibration lines and for the assessment of the accuracy and the precision.

2.3. Equipment

The HPLC system consisted of a Spectra Physics Analytical P4000 pump, a Kratos Analytical Spectroflow 757 UV detector, a Spark Holland Separations Marathon autosampler and a Merck–Hitachi D2500 integrator. The analytical column was a Chrompack Inertsil ODS-2 C_{18} column (5 μm particle size; 150×4.6 mm). The pre-column was a Chrompack SS 75×2 mm Valco reversed-phase column. Ultraviolet spectra of indinavir in eluent were recorded with a Perkin Elmer Lambda2 UV–Vis spectrophotometer.

2.4. Sample preparation

For the preparation of the standard samples, stock solutions of indinavir were diluted with acetonitrile–water (50:50, v/v). To achieve calibration concentrations of 50 to 12 500 ng/ml, appropriate amounts of the various diluted stock solutions of indinavir were added to blank plasma. 100 μl of each sample were transferred to an Eppendorf cup of 1.5 ml. To each cup 10 μl of the I.S. solution and 400 μl of acetonitrile were added. Each cup was vortexed for 1 min and subsequently centrifuged for 5 min at 10 500 *g*. The supernatant was transferred to a clean Eppendorf cup and evaporated to dryness under a nitrogen stream at 40°C. Next, the residue was dissolved in 150 μl of eluent, mixed and brought into autosampler vials for injection into the HPLC system.

2.5. Chromatography

Chromatographic analysis was performed at ambient temperature with a mobile phase composed of acetonitrile–water (40:60, v/v). The water phase contained 50 m*M* phosphate buffer pH 6 and 4 g/l tetramethylammoniumchloride. Ultraviolet detection was monitored at 210 nm. Flow-rate was maintained at 1 ml/min. Aliquots of 20 μl were injected into the chromatograph.

2.6. Validation procedures

The specificity of the assay was investigated by analyzing blank plasma samples of six different individuals who did not use indinavir. No interfering

peaks with the peak of indinavir or the peak of the I.S. were allowed. In addition, a large number of drugs were chromatographed which are frequently combined with indinavir by HIV-positive patients. Interference by metabolites from indinavir was not investigated as these substances were not available.

The limit of detection was defined as the lowest concentration that reliably could be differentiated from background levels. The difference between a spiked sample and a background sample was tested with a paired *t*-test. Significance was considered if *p* was less than 0.05. Concentrations that gave a signal-to-noise ratio of 3 were first selected to determine the limit of detection.

The lower limit of quantitation was determined as the concentration for which the relative standard deviation and the percentual deviation from the nominal concentration were both less than 20%. Samples were assayed as five replicates.

Accuracy and precision were determined in three separate runs. Five replicates of three different concentrations of indinavir (75, 1000 and 3750 ng/ml) were determined. The accuracy was calculated as the average percentage of the nominal concentration. Analysis of variance (ANOVA) was used to calculate the inter- and intra-assay variation.

The inter-assay precision was calculated as follows:

$$\frac{\{(\text{Day mean square} - \text{Error mean square})/n\}^{0.5}}{\text{Grand mean}} \cdot 100\%$$

The intra-assay precision was calculated as follows:

$$\frac{(\text{Error mean square})^{0.5}}{\text{Grand mean}} \cdot 100\%$$

in which day mean square, error mean square and grand mean are terms from ANOVA and *n* is the number of replicates within each day (five) per concentration. In cases were error mean square is greater than the day mean square, the resulting variance estimate is negative. This implies that no significant additional variation is observed as a result of performing the assay in different runs.

Recovery was determined by calculating the quotient of the slopes of the calibration lines in plasma and eluent, respectively. This quotient was calculated for the three different runs and subsequently averaged to determine the recovery.

2.7. Stability

The stability of indinavir was investigated for a number of conditions that are relevant for clinical pharmacokinetic research. Plasma was spiked with two concentrations of indinavir (1401 and 5176 ng/ml) and subsequently stored at 20°C (7 days), 4°C (7 days) and −20°C (1 month, only for the highest concentration). A sample of blank blood was also spiked with these concentrations of indinavir and kept at 20°C for three days.

2.8. Pharmacokinetic experiments

Four HIV-infected persons gave informed consent to participate in a clinical pharmacokinetic experiment for which the local Ethics Committee of the University Hospital Nijmegen had given approval. Plasma was collected for 0–8 h after the ingestion of 800 mg of indinavir (two capsules of Crixivan 400 mg). Pharmacokinetic analysis was performed by noncompartmental methods.

3. Results

3.1. Development phase

During the development phase a method was sought that was easier to perform than the method reported by Woolf et al. [2]. First, a UV spectrum of indinavir was recorded. Maximal absorbance was found at 262 nm with a molar absorbance coefficient of 3800 M^{-1} cm^{-1}. This value was in our opinion not high enough for the detection of indinavir concentrations at the ng/ml range. Subsequently, 210 nm was chosen because the molar absorbance coefficient at that wavelength was more than eight-times greater (30 700 M^{-1} cm^{-1}). However, it was anticipated that interference with other (endogenous) compounds was likely to occur because this wavelength is non-specific.

Subsequently, the retention of indinavir and the I.S. were investigated on an Inertsil ODS C_{18}

column because this column was also used by Woolf et al. [2] and because it gave a better separation than Spherisorb CN and Chrompack Hypersil ODS columns. An optimal separation of indinavir and the I.S. was reached by varying the amounts of acetonitrile and water in the mobile phase. The optimal strength of the phosphate buffer was found to be 50 m*M*, so that the mobile phase was composed of acetonitrile–water (40:60, v/v), where the water phase contained 50 m*M* phosphate buffer pH 6. Tetramethylammoniumchloride (TMACl) was added as a modifier in order to avoid tailing on the chromatogram. A number of other modifiers were rejected because they showed strong absorbance at 210 nm. The retention times of indinavir and the I.S. were 6.7 and 8.0 min, respectively.

100 μl of plasma was spiked with indinavir and I.S. and mixed with different organic solvents. The supernatant was subsequently isolated, evaporated and the residue was redissolved in the eluent. The largest recovery and the cleanest extraction procedure was obtained with acetonitrile. Extraction with ether, methanol, or hexane resulted in smaller recoveries and/or more interfering peaks in the chromatogram.

A representative chromatogram of a plasma sample containing 1250 ng/ml of indinavir is given in Fig. 2.

3.2. Validation phase

Blank plasma of six different individuals, who did not use indinavir, did not contain interfering compounds that co-eluted with indinavir or the I.S. The following drugs, which are frequently used by HIV-infected patients, did not gave interference with the analytical method: acyclovir, amphotericin B, amoxycillin, atovaquone, calcium folinate, azithromycin, clarithromycin, clindamycin, caffeine, dapsone, didanosine, erythromycin, ethambutol, famotidine, fluconazole, folic acid, ganciclovir, isoniazid, itraconazole, ketoconazole, lamivudine, methadone, nevirapine, oxazepam, paracetamol (acetaminophen), pentamidine, phenytoin, pyrazinamide, pyrimethamine, rifabutin, rifampin, ritonavir, saquinavir, stavudine, sulfamethoxazole, sulfametrol, trimethoprim, zalcitabine, zidovudine.

The detection limit was 12.5 ng/ml, using 100 μl of plasma. Because at this concentration, and also at 25 ng/ml, the percent deviation of the nominal concentration was greater than 20%, these concentrations could not be considered as the lower limit of quantitation. At 50 ng/ml, the percentual deviation of the nominal concentration and the relative standard deviation (of five replicates) were both less than 20% and, therefore, this concentration was considered to be the lower limit of quantitation.

The results of the determination of the accuracy and the precision of the assay are listed in Table 1. The average recovery (determined in three separate runs) was 101.3±3.6%. The calculation of indinavir concentrations from the calibration curve was performed with linear regression after logarithmic transformation of the data. This transformation is necessary because of the large range of concentrations (50 to 12 500) in the calibration curve. As can be seen in Table 1, the inter-assay variation of the lowest indinavir concentration appears to be somewhat higher than the variation in the other concentrations.

The stability experiments demonstrated that indinavir is stable under the investigated conditions (Table 2).

3.3. Pharmacokinetic experiment

The plasma concentration vs. time curves of the four HIV-infected patients have been analyzed (Fig. 3). The most relevant patient characteristics and the calculated pharmacokinetic parameters are listed in Table 3. The available data from the literature are included in this table. For all four patients, the bioanalytical method appeared to be suitable for the determination of indinavir plasma concentrations.

4. Discussion

In this paper, the development, validation, and application of a bioanalytical method for the determination of the HIV-protease inhibitor indinavir has been described. So far, only one other paper has dealt with the determination of this new compound in biological matrices [2]. This method, which has been developed by Woolf et al. from the laboratories of the manufacturer of indinavir (Merck), contains some disadvantages which made that method less

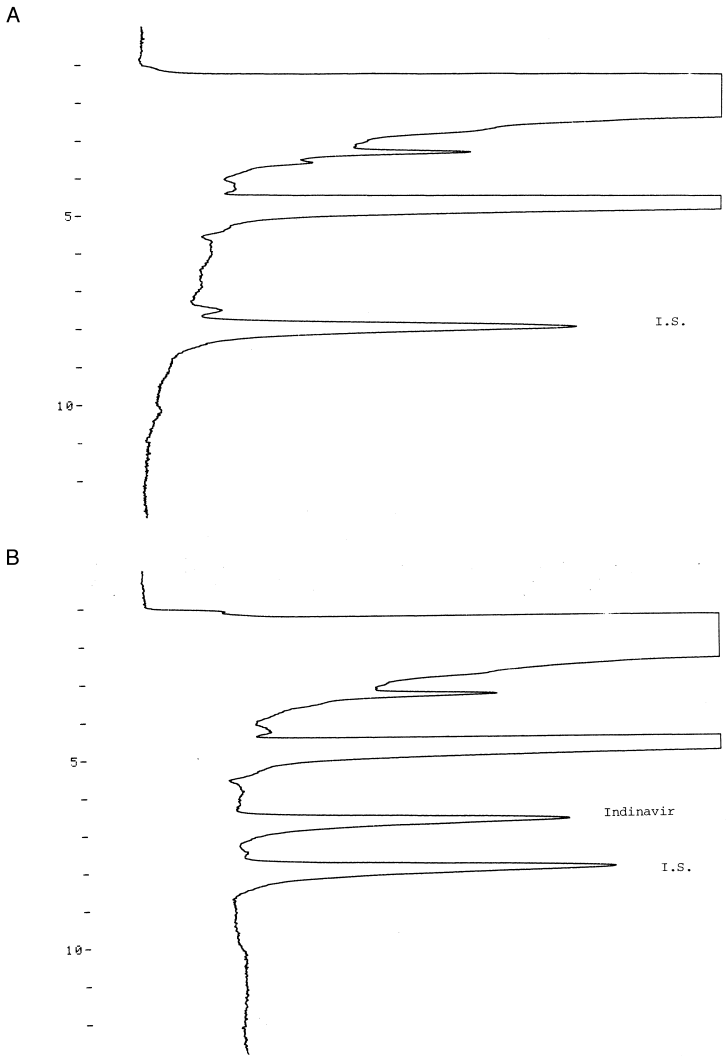

Fig. 2. (A) Chromatogram of a blank plasma sample containing 1250 ng/ml of I.S. (1). (B) Chromatogram of a plasma sample containing 1250 ng/ml of I.S. and 1250 ng/ml of indinavir.

Table 1
Accuracy and precision data

Indinavir concentration (ng/ml)	Accuracy (%)	Inter-assay precision (%)	Intra-assay precision (%)
75	88.5	16.7	7.8
1000	97.7	3.1	4.8
3750	97.9	4.6	9.6

Table 2
Stability experiments

Concentration (ng/ml)	Condition	Matrix	Time interval	Concentration found (%)
1401	25°C	Plasma	7 days	111.3
	25°C	Blood	3 days	97.8
	4°C	Plasma	7 days	92.1
5176	25°C	Plasma	7 days	100.6
	25°C	Blood	3 days	102.6
	4°C	Plasma	7 days	93.4
	−20°C	Plasma	1 month	107.2

attractive for us. First, Woolf et al. applied an extensive sample clean-up, consisting of several extraction procedures, in which subsequently organic solvent, acidic solvent, alkaline solvent, and again organic solvent were used. Large quantities of a derivative of ether were used as the organic solvent, which is not ideal from an environmental point of view. Furthermore, a relatively large volume of plasma was used (1 ml), which is not always available. Finally, a column-switching technique was used for chromatography, which is not available in every laboratory.

For the above reasons, we have tried to develop a bioanalytical assay that would overcome the above-mentioned problems. The advantages of our method are: easier and faster sample clean-up, a smaller volume of plasma (100 μl), and a less complicated chromatographic system. The lower limit of quantitation of our method is higher than that of the method described by Woolf et al. (50 vs. 5 ng/ml [2]).

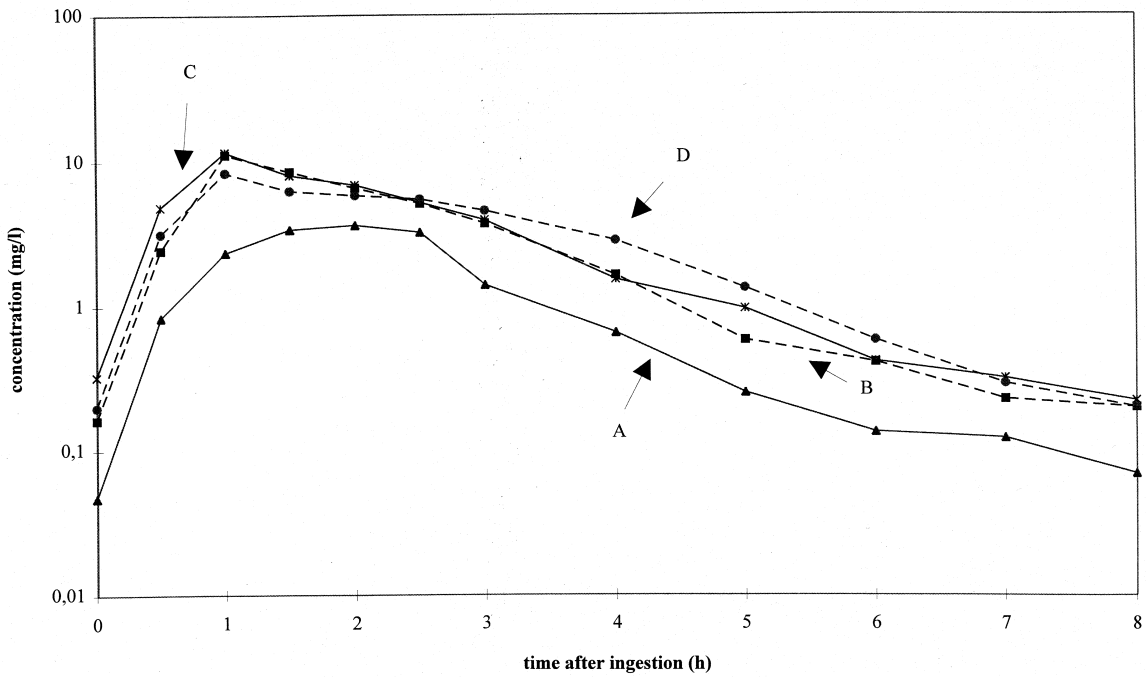

Fig. 3. Plasma concentration vs. time curves of 4 HIV-infected patients who ingested 800 mg of indinavir.

Table 3
Pharmacokinetic experiment

Patient code	A	B	C	D	
Patient characetristics					
Gender (M/F)	M	M	M	M	
Age (year)	43	43	36	49	
Length (cm)	180	183	160	197	
Weight (kg)	73	73	61	65	
Dose of indinavir (mg)	800	800	800	800	
Co-medication	Didanosine Stavudine Oxazepam Clindamycin Clomipramine Folic acid Pyrimethamin Ganciclovir	Zidovudine Lamivudine Valaciclovir Pentamidine	Zidovudine Didanosine Co-trimoxazole Clarithromycin Ethambutol	Stavudine Lamivudine	
Pharmacokinetic parameters					Ref. [3]
T_{max} (h)	2.0	1.0	1.0	1.0	0.8
C_{max} (mg/l)	3.6	11.2	11.6	8.4	7.7
C_{min} (mg/l)	0.06	0.18	0.27	0.20	0.15
AUC_{0-8} (h mg/l)	9.0	22.7	24.7	23.2	18.8
Cl/F (l/h)	88	34	32	34	
Vd/F (l)	148	91	64	50	
$T_{1/2}$ (h)	1.2	1.8	1.4	1.0	2

However, because we only use 100 μl instead of 1 ml of plasma, the absolute amount that is detected is similar. In addition, preliminary results from extractions using larger volumes of plasma than 100 μl suggest that a lower limit of quantitation is achievable. However, indinavir plasma levels in the clinical setting are usually higher than 50 ng/ml. For our purpose, it is not necessary to modify our sample clean-up.

Both methods use UV detection at 210 nm. The measurement at this non-specific wavelength bears the risk of interference with co-eluting substances from endogenous or exogenous origin. However, we have not found any interference among the many tested drugs or in the blank plasma of six different individuals.

The pharmacokinetic parameters of the four HIV-infected patients who ingested 800 mg of indinavir were in agreement with the sparse data from the literature [3]. Currently, our laboratory is participating in several clinical pharmacokinetic investigations with indinavir. The described method is suitable for the determination of the plasma concentrations of indinavir in these trials.

Acknowledgements

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