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Determination of an in vivo metabolite of a human immunodeficiency virus protease inhibitor in human plasma by high-performance liquid chromatography with tandem mass spectrometry

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Abstract

A method for the determination of a metabolite of the human immunodeficiency virus protease inhibitor indinavir, in human plasma is described. Isolation of the analyte and the internal standard from plasma was achieved via liquid–liquid extraction with a mixture of isopropanol–chloroform (5:95, v/v). The analytes were chromatographed under reversed-phase conditions on a Waters Symmetry C_8 column. A Sciex API III$^+$ tandem mass spectrometer equipped with a heated nebulizer was used as a detector and was operated in the positive ion mode. Multiple reaction monitoring using the precursor→product ion combinations of m/z 523.4→273.4 and 512.4→345.2 was used to quantify analyte and internal standard, respectively. The method was validated in the concentration range of 5–500 ng/ml plasma with adequate assay precision and accuracy. The assay was used to analyze samples collected during drug interaction studies of indinavir.

Keywords: Human immunodeficiency virus; Indinavir; Enzyme inhibitors

1. Introduction

Human immunodeficiency virus (HIV) has been identified as the causative agent of acquired immune deficiency syndrome (AIDS) [1–4]. Indinavir (I) (Fig. 1) has been found to be a potent and specific in vitro inhibitor of the human immunodeficiency virus Type 1 (HIV-1) encoded protease [5,6]. HIV-1 protease is utilized during the viral replication cycle to cleave a polyprotein into individual functional proteins, thus transforming the virus into an infectious form. In vivo, administration of I has been shown to cause significant decreases in the level of circulating virus in individuals infected with HIV [7,8]. Indinavir has recently been approved for marketing in the USA, for the treatment of HIV infection, under the trade name Crixivan.

The cytochrome P_{450} 3A4 (CYP3A4) enzyme system has been shown, in vitro, to be responsible for the metabolism of indinavir [9]. Compound II (Fig. 1) has been identified as one of the CYP3A4 mediated metabolites of indinavir in man [10]. Many drugs, such as ketoconazole, that are taken by AIDS patients to treat opportunistic infections, are known to be inhibitors or inducers of the CYP3A4 system [11]. An assay to quantify II in human plasma was required in order to determine whether the metabolism of I was affected by the coadministration of known CYP3A4 inhibitors.

The combination of HPLC with atmospheric pressure chemical ionization (APCI) tandem mass spectrometry (MS–MS) detection is becoming increas-

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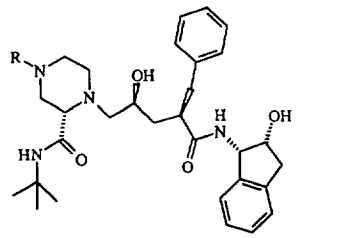
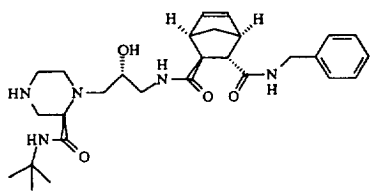


Compound I: R =

Compound II: R = H



Compound III

Fig. 1. Chemical structure of compounds I (indinavir), II and III (internal standard).

ingly popular as an effective and convenient method for the quantitation of drugs in biological fluids [12–19], especially for molecules such as II, which are difficult to detect by more conventional means. The application of this technique, as pioneered by Henion and his co-workers [12,13], involves several steps. First, sample molecules are ionized at atmospheric pressure using a corona discharge; ionization at atmospheric pressure is highly efficient owing to high collision frequencies between molecules [20]. Following ionization, the resulting pseudo-molecular ions enter the first quadrupole (Q1) of the mass spectrometer. The pseudo-molecular ions of the analyte molecules are passed by Q1 into a collision cell (Q2), where they collide with neutral gas (argon) molecules and fragment into product ions. Finally, the product ions are separated based on their mass to charge (m/z) ratio by the third quadrupole (Q3) and detected by an electron multiplier. In the multiple reaction monitoring (MRM) mode, precursor → product ions are monitored while the sample is eluting, thus making APCI-MS–MS a highly specific detection technique, ideally suited for compounds such as II. A number of recent examples from our laboratories demonstrating the applicability of this method for the quantitative determination of various drug candidates have been described [16–19]. The development of an HPLC–MS–MS assay for the determination of II in human plasma samples is the subject of this paper.

2. Experimental

2.1. Materials

Compounds II and III (internal standard) (Fig. 1) were prepared in the Medicinal Chemistry Department of Merck Research Laboratories (West Point, PA, USA). Acetonitrile and isopropanol (Omnisolve HPLC grade) were from EM Science (Gibbstown, NJ, USA). Chloroform (ethanol added as preservative) was purchased from Fisher Scientific (Springfield, NJ, USA). Drug-free human plasma was obtained from Sera-Tech Biologicals (New Brunswick, NJ, USA).

2.2. Instrumentation

The HPLC system consisted of a Perkin-Elmer (Norwalk, CT, USA) model 250 pump, a Waters (Milford, MA, USA) WISP 715 autosampler, and an API III$^+$ triple quadrupole tandem mass spectrometer (PE-Sciex, Thornhill, Canada) equipped with a heated nebulizer interface.

2.3. Chromatographic conditions

The HPLC mobile phase consisted of a mixture of acetonitrile water (40:60, v/v), to which ammonium acetate was added to give a final concentration of 2 mM in the total mobile phase volume. The mobile phase was filtered through a nylon membrane (0.20 μm) prior to use. Mobile phase was delivered at a flow-rate of 1.0 ml/min through a Waters Symmetry C_8 column (5 μm silica, 50×3.9 mm). The sample injection volume was 50 μl, while the total run time was 5 min.

2.4. Mass spectrometric conditions

The mass spectrometer was interfaced to the HPLC system via a heated nebulizer probe that was maintained at 500°C. Nebulizer (nitrogen) pressure was set at 80 p.s.i. (1 p.s.i.=6894.76 Pa). Nebulizer and curtain gas (nitrogen) flows were each set at flow at 0.6 l/min. Positive chemical ionization was effected by a corona discharge needle (+4.5 μA) and the sampling orifice potential was set at +75 V. The first quadrupole, Q1, was set to monitor the protonated molecules $(M+H)^+$ at m/z 523 for II and m/z 512 for III with collision-induced fragmentation at Q2 (collision gas argon, 260×10^{12} atoms cm^{-2}), and monitoring of the product ions via Q3 at m/z 273 and 345 for II and III, respectively. The electron multiplier setting was −4.0 KV and detector electronics were set to counts of ten. The dwell time was 400 ms with a 30 ms pause time between scans.

2.5. Data acquisition and analysis

Data acquisition and analyses were performed using RAD and MacQuan software (PE-Sciex). Unknown sample concentrations were calculated from the equation $y=mx+b$, as determined by the weighted $(1/y)$ linear least-squares regression of the calibration line constructed from the peak area ratios of drug to internal standard versus drug concentration.

2.6. Preparation of standards

A 20 μg/ml stock solution of II was prepared by weighing 1.0 mg of reference material into a 50 ml volumetric flask, dissolving the compound in 25 ml of acetonitrile, and filling the flask to volume with water. A 2.0 μg/ml stock solution was prepared by diluting 5 ml of the 20 μg/ml solution to 50 ml with acetonitrile–water (50:50, v/v).

Working standards of 10, 8, 4 and 2 μg/ml were prepared by dilution of the 20 μg/ml stock with acetonitrile–water (50:50, v/v). Working standards of 1, 0.4, 0.2 and 0.1 μg/ml were prepared by dilution of the 2.0 μg/ml stock with acetonitrile–water (50:50, v/v).

Analysis standards were prepared by adding 50 μl of each working standard to 1 ml of drug-free plasma. The resulting standards ranged in concentration from 5 to 500 ng/ml.

2.7. Sample preparation procedure

A 1 ml volume of plasma (sample or standard) was pipetted into a 15 ml disposable glass centrifuge tube (Kimble, Vineland, NJ, USA). A 50 μl volume of a 1 μg/ml solution of internal standard (III) in acetonitrile–water (50:50, v/v) was added and the contents of the tube were vortex mixed. A 1 ml volume of 0.1 *M* pH 9.5 borate buffer was added, the tube contents were vortexed, and 8 ml of extraction solvent (isopropanol–chloroform, 5:95, v/v) were added. The tube was sealed with a PTFE-lined screw cap (Qorpak No. 5201; Fisher Scientific) and the tube contents were mixed for 15 min on a flat-bed shaker set at a speed of 60 excursions per min. Following mixing, the tube was centrifuged at 1500 *g* for 5 min to affect a phase separation of the contents. After phase separation, the upper aqueous layer was aspirated to waste and the remaining organic layer was decanted into a clean 16×100 mm glass culture tube. The tube containing the organic layer was placed in a Turbo-Vap evaporator (Zymark, Hopkinton, MA, USA) set at 42°C and the solvent was evaporated under a stream of nitrogen. The resulting residue was reconstituted in 1 ml of mobile phase, and 500 μl were transferred to a polymethylpentene autosampler vial prior to injection (50 μl) into the HPLC–MS–MS system.

3. Results

3.1. Assay specificity

Fig. 2 shows chromatograms of extracted control plasma, a plasma standard containing both II (20 ng/ml) and III (50 ng/ml) and a plasma sample taken from a subject after receiving a 400 mg dose of indinavir. A comparison of Fig. 2A with Fig. 2B illustrates that no endogenous peaks co-elute with either II or III. The specificity of the method is further illustrated by the fact that all pre-dose plasma samples from subjects involved in clinical trials were free of interfering peaks.

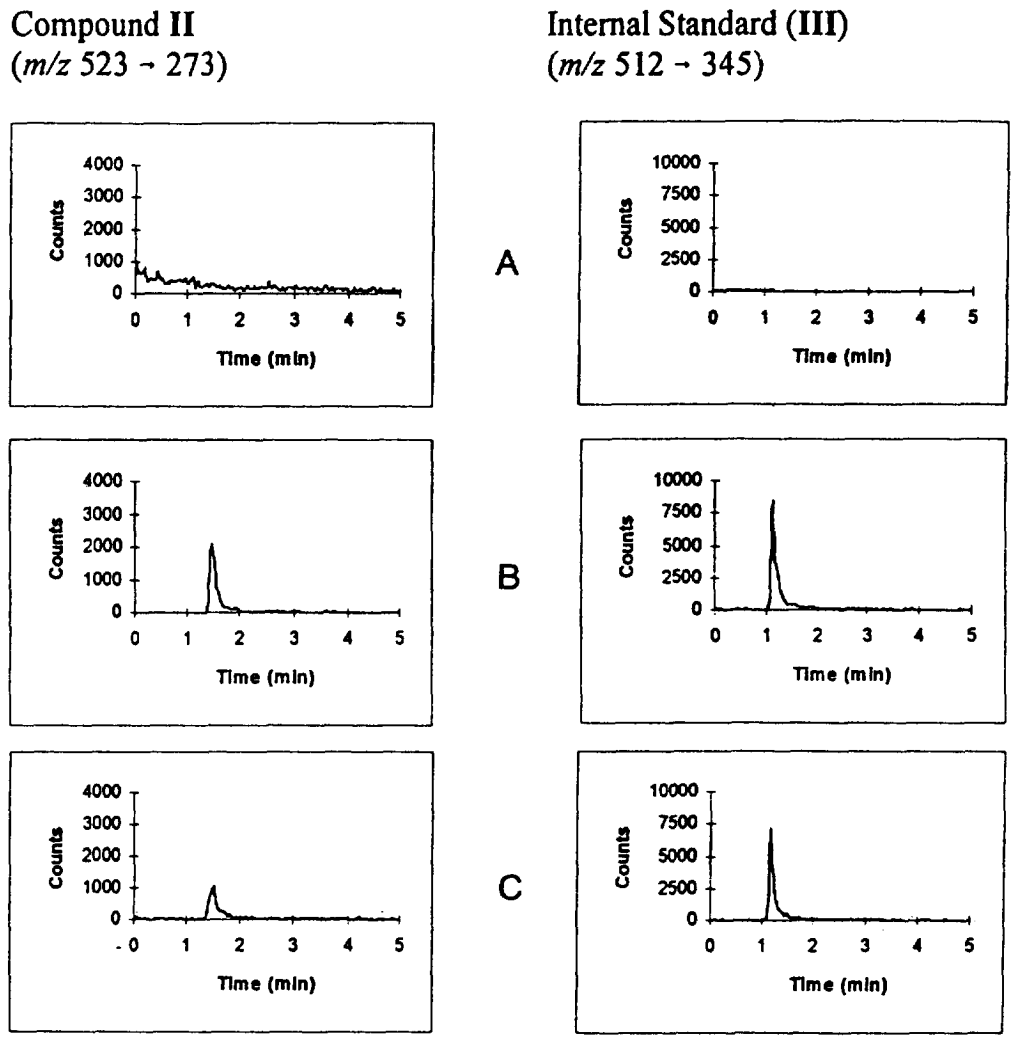


Fig. 2. Representative chromatograms of plasma. (A) Control human plasma, (B) plasma spiked with 20 ng/ml II and internal standard III (50 ng/ml), (C) plasma sample from human subject 2.0 h after administration of 400 mg indinavir (I) to which 50 ng/ml of internal standard (III) has been added. Sample diluted 1:4 prior to addition of internal standard. The concentration of II is equivalent to 55.2 ng/ml.

3.2. Linearity

Weighted (weighting factor=$1/y$ where y=peak area ratio) least-squares regression calibration curves, constructed by plotting the peak area ratio of II/III versus standard concentration of II yielded coefficients of regression typically greater than 0.99 over the concentration range of 5–500 ng/ml of II. The use of the weighted least-squares regression resulted in less than a 10% deviation between the nominal standard concentrations and the experimentally determined standard concentrations calculated from the regression equation.

3.3. Extraction recovery

The recovery of the extraction procedure was determined by comparing the responses of working standards of II injected directly into the HPLC system with those of extracted plasma standards. The results indicate that the recovery of the extraction procedure was 88.5%, 90.8%, and 90.4% for con-

centrations of II of 10, 100, and 500 ng/ml, respectively. The extraction recovery of the internal standard, III, at a concentration of 50 ng/ml in plasma was 86.1%.

3.4. Assay precision and accuracy

Replicate standards ($n=5$) were analyzed to assess the within-day variability of the assay. The mean assayed concentrations as well as the mean accuracy and relative standard deviations (R.S.D.s, %) of the analyses are shown in Table 1.

Quality control samples (QCs) containing concentrations of 15 and 350 ng/ml II in plasma were prepared and frozen (−20°C) in 1 ml volumes. Following initial replicate ($n=5$) within-day analysis, the quality controls were analyzed several ($n=7$) times over a 2 week period to assess the inter-day variability of the assay. The results (Table 2) indicate that the inter-day variability of the assay, as measured by the R.S.D. is under 7%. The results also indicate that frozen plasma samples containing II appear stable for at least 2 weeks.

3.5. Limit of quantification

The limit of quantification of the assay, defined as the lowest concentration that yielded an within-day R.S.D. of less than 10% and an within-day accuracy of between 90 and 110% of nominal concentration was 5 ng/ml.

Table 1
Intra-day precision and accuracy data for the determination of II in plasma as assessed by the replicate ($n=5$) analysis of standards

Nominal standard concentration (ng/ml)	Mean ($n=5$) calculated standard concentration (ng/ml)	Accuracy[a] [%]	R.S.D.[b] [%]
5.0	4.99	99.8	7.7
10.0	9.94	99.4	7.5
20.0	19.44	97.2	5.4
50.0	49.74	99.5	8.7
100.0	104.92	104.9	4.2
200.0	208.36	104.2	5.5
400.0	405.43	101.4	6.8
500.0	486.71	97.3	4.9

[a] Expressed as [(mean observed concentration)/(nominal concentration)].
[b] Relative standard deviation of the ratio of the compound II peak area to that of III.

Table 2
Initial within-day analysis of quality control samples and inter-day variability of the assay as assessed by R.S.D.s of low and high quality control samples

	Q.C. Concentration (ng/ml)	
Nominal Concentration:	15.0	350.0
Initial mean ($n=5$) assayed concentration	16.10	338.89
S.D.	0.42	6.86
R.S.D. (%).	2.6	2.0
Daily Results:[a]		
	17.27	385.82
	15.95	352.89
	15.95	338.40
	15.21	357.40
	17.87	375.62
	15.21	379.44
	15.94	330.96
Mean ($n=7$)	16.20	360.08
S.D.[b]	1.01	21.04
R.S.D. (%)	6.22	5.84

[a] Average of two values for each daily QC result listed.
[b] S.D.=Standard deviation.

4. Discussion

Compound II was found to lack significant UV absorption at wavelengths greater than 220 nm. Additionally, the molecule was not found to be fluorescent. Thus, its quantitation at ng/ml levels in plasma utilizing conventional detection was difficult. Although we have quantitated indinavir (I) in plasma using HPLC with UV detection at 210 nm, such an approach required a relatively complex column switching system [21], and development of a similar method for II would require significant method development time. In order to avoid potential difficulties associated with the development of an HPLC–UV assay for II, an alternative detection method based on HPLC with MS–MS detection was evaluated.

Full-scan positive-ion spectra of both II and III yielded predominantly the protonated molecular ions at m/z 523 and 512, respectively. The product ion mass spectra of these protonated molecular ions (Fig.

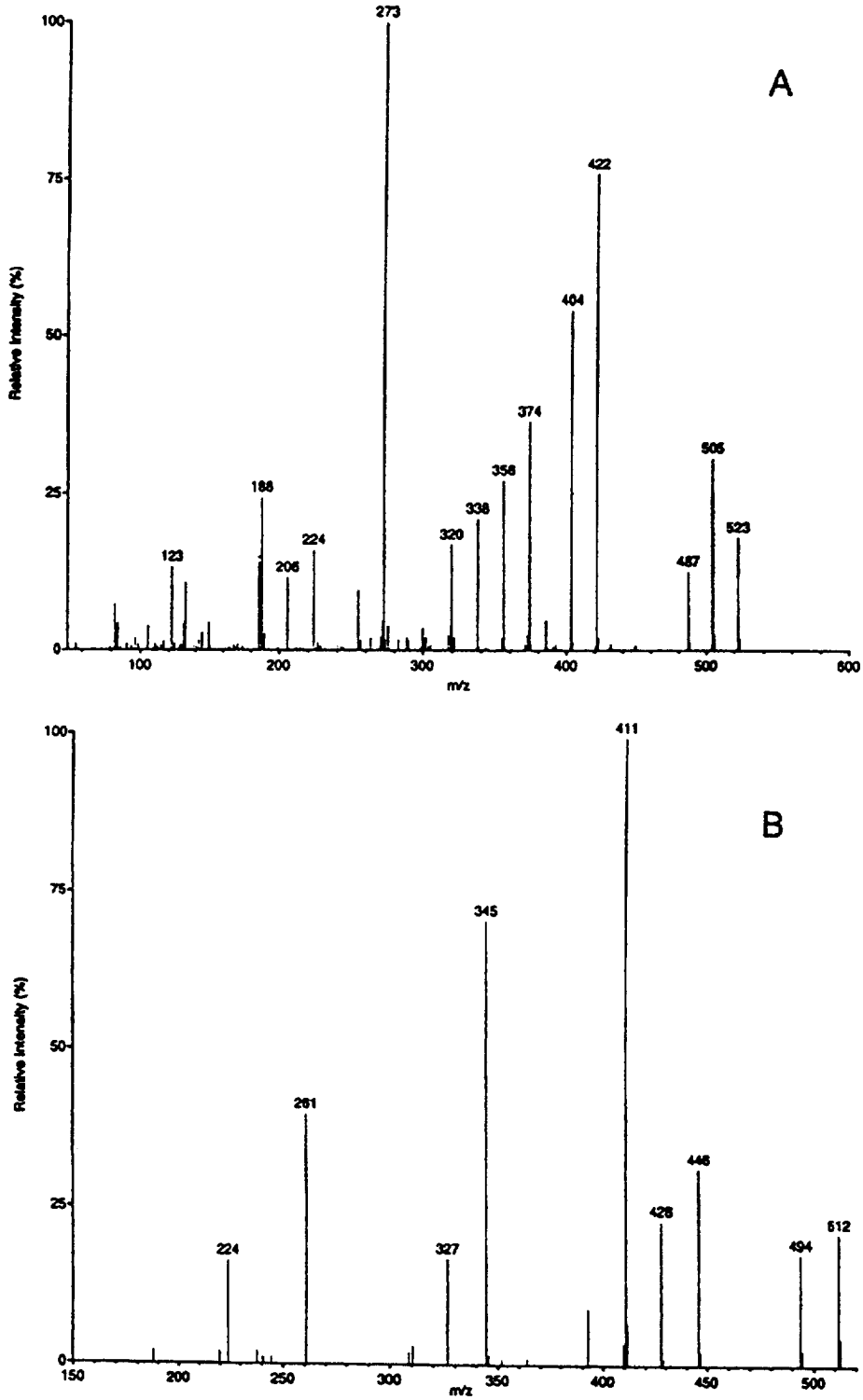


Fig. 3. Positive product ion mass spectra of the protonated molecular ions of II (m/z 522, A) and internal standard, III (m/z 512, B).

3) indicated the presence of numerous ions. The most intense product ions of II were found at m/z 422 and 273, while for III, the major product ions were at m/z 411 and 345. The proposed fragmentation pattern for the protonated molecule of II into major fragments at m/z 422 and 273 is presented in Fig. 4.

In order to determine which precursor→product ion combinations were preferable for the analysis of plasma samples, a suitable analyte isolation procedure was required. Previous work had shown that indinavir could be extracted from buffered (pH 9.5) plasma using methyl *tert.*-butyl ether (MTBE) as the extraction solvent [21]. Attempts to use the same conditions to extract II resulted in a recovery of only about 55%. At pH 9.5, the piperazine ring of II was expected to be fully un-ionized, allowing high extractability of the compound into the organic phase. Further optimization of pH using MTBE as an extraction solvent failed to improve the extraction efficiency of II.

In contrast with the good recovery of indinavir (I) at pH 9.5 using MTBE as solvent, the poor extractability of II was probably due to the fact that II was considerably more polar than indinavir (I) and probably required a more polar extraction solvent. Substituting ethyl acetate for MTBE improved the extraction efficiency of II from buffered aqueous samples to 90%, while with chloroform, the extraction efficiency was 95%. The addition of a small amount of isopropanol to the chloroform further improved the efficiency of extraction of II from aqueous samples to approximately 100%. Under these conditions, the extraction efficiency of II and III from plasma was about 90%. Although chloroform is a hazardous substance to work with, no other solvent yielded a comparable recovery.

The precursor→product ion combinations of 523→422 and 523→273 were evaluated for the detection of II in plasma extracts, while the combinations of 512→411 and 512→345 were studied for the detection of III. Best results, in terms of lowest background noise and the absence of interferences, were obtained using the precursor→product combinations of 523→273 for the analysis of II and 512→345 for the analysis of III. Thus, these precursor→product ion combinations were used for quantitation in the MRM mode.

The assay has been used for the determination of II in plasma samples obtained during a drug interaction study between indinavir and ketoconazole. Fig. 5A shows the mean plasma concentration-time

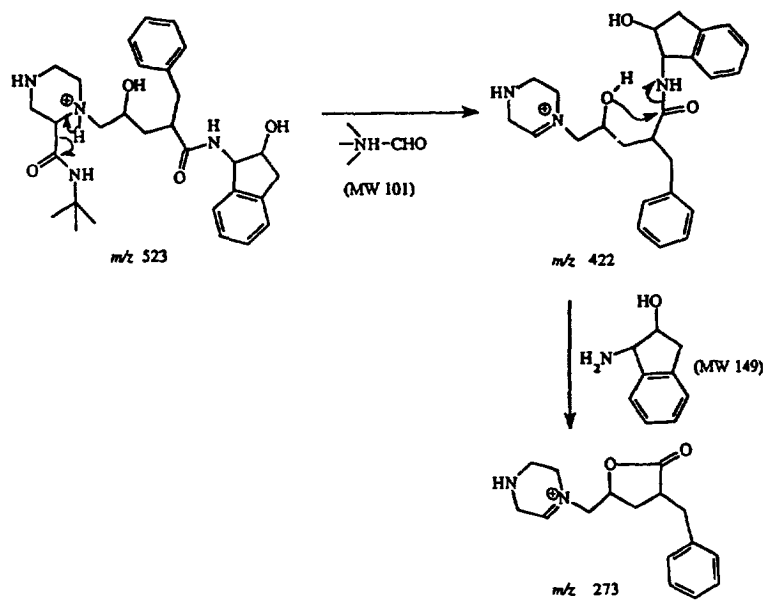


Fig. 4. Proposed fragmentation of the protonated molecule of II into major fragments at m/z 422 and 273.

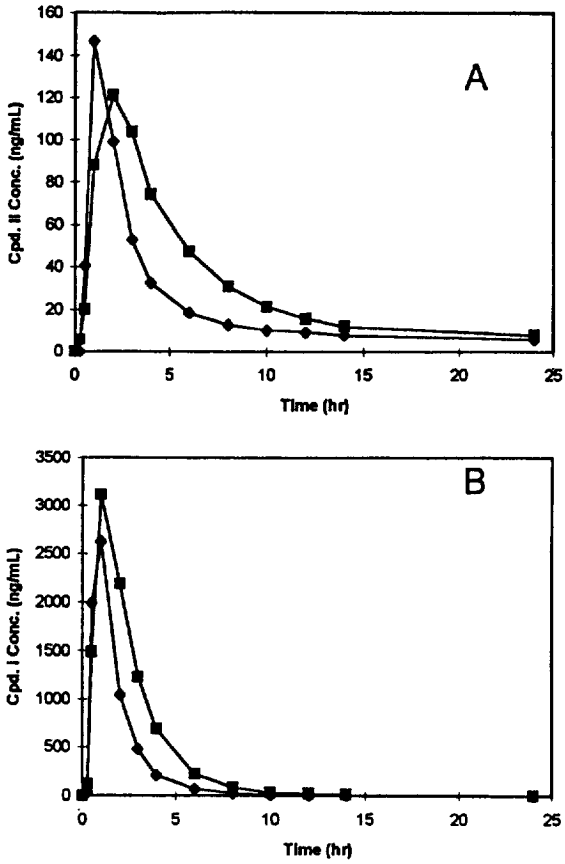


Fig. 5. Mean plasma concentrations of II (A) and indinavir (B) following oral administration of 400 mg indinavir alone (♦) or in combination with 400 mg ketoconazole (■) in ten subjects.

profile for II during both study treatments. The corresponding levels of indinavir, as determined by the HPLC–UV-column switching assay [21], are shown in Fig. 5B. Concentrations of indinavir (Fig. 5B) were slightly higher when administered with ketoconazole, a known inhibitor of CYP3A4. Surprisingly, concentrations of II (Fig. 5A), a CYP3A4 mediated metabolite of indinavir, were also generally higher when indinavir was administered with ketoconazole. The increased levels of II may be due to the inhibition of the presystemic metabolism of indinavir by ketoconazole and/or the inhibitory effect of ketoconazole on the secondary metabolism of II. Based on these results, it was recommended that physicians consider reducing the dose of indinavir from 800 mg every 8 h to 600 mg every 8 h in patients concomitantly receiving ketoconazole.

5. Conclusions

An assay using HPLC with tandem mass spectrometry detection has been developed for the determination of II in human plasma samples. The precision and accuracy of the assay make it suitable for the analysis of clinical samples. Utilization of data generated by the analysis of clinical samples has helped to elucidate the mechanism of the indinavir-ketoconazole interaction in man.

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