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High-performance liquid chromatographic method for the determination of nelfinavir, a novel HIV-1 protease inhibitor, in human plasma

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Abstract

Nelfinavir mesylate, a potent and orally bioavailable inhibitor of HIV-1 protease (K_i=2 n*M*), has undergone Phase III clinical evaluation in a large population of HIV-positive patients. A high-performance liquid chromatography analytical method was developed to determine the pharmacokinetic parameters of the free base, nelfinavir, in these human subjects. The method involved the extraction of nelfinavir and an internal standard, 6,7-dimethyl-2,3-di-(2-pyridyl)quinoxaline, from 250 μl of human plasma with a mixture of ethyl acetate–acetonitrile (90:10, v/v). The analysis was via ultraviolet detection at 220 nm using a reversed-phase C_{18} analytical column and a mobile phase consisting of 25 m*M* monobasic sodium phosphate buffer (adjusted to pH 3.4 with phosphoric acid)–acetonitrile (58:42, v/v) that resolved the drug and internal standard peaks from non-specific substances in human plasma. The method was validated under Good Laboratory Practice (GLP) conditions for specificity, inter- and intra-assay precision and accuracy, absolute recovery and stability. The mean recovery ranged from 92.4 to 83.0% for nelfinavir and was 95.7% for the internal standard. The method was linear over a concentration range of 0.0300 μg/ml to 10 μg/ml, with a minimum quantifiable level of 0.0500 μg/ml for nelfinavir.

Keywords: Nelfinavir

1. Introduction

Nelfinavir mesylate (VIRACEPT®, formerly AG1343) ([3*S*-(3*R**,4*aR**,8*aR**,2′*S**,3′*S**)]-2-[2′-hydroxy- 3′ -phenylthiomethyl-4′-aza-5′-oxo-5′-(2″-methyl - 3″ - hydroxyphenyl)pentyl] - decahydroiso - quinoline-3-N-tertiary-butylcarboxamide methanesulfonic acid salt; Fig. 1), with pK_a values of 6.00 and 11.06, was designed by protein structure-based drug design techniques [1–3] to inhibit the replication of human immunodeficiency virus type 1 (HIV-1). Nelfinavir mesylate has demonstrated effective in vitro anti-viral activity against a number of laboratory and clinical HIV-1 and HIV-2 strains in acute

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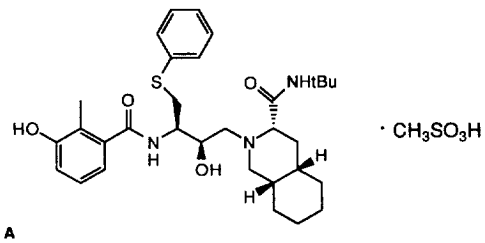

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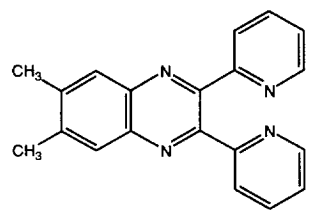

B

Fig. 1. Chemical structures for (A) nelfinavir mesylate (VIRACEPT, formerly AG1343) ([3*S*-(3*R**,4*aR**,8*aR**,2′*S**,3′*S**)]-2-[2′hydroxy-3′-phenylthiomethyl-4′-aza-5′-oxo-5′ - (2″-methyl-3″-hydroxy-phenyl)pentyl]-decahydroiso-quinoline-3-N-*t*-butylcarboxamide methanesulfonic acid salt) and (B) internal standard, 6,7-dimethyl-2,3-di-(2-pyridyl)-quinoxaline.

and chronic infection models (effective dose that inhibits 50% of the virus, ED_{50}=9 to 60 n*M*) [4], and has shown substantial oral bioavailability coupled with safety in animal models [5]. This potent inhibitor of HIV-1 protease (K_i=2 n*M*) [4] has undergone efficacy testing in Phase III clinical evaluations in a large population of HIV-positive patients. Results indicated that nelfinavir mesylate is well-tolerated and demonstrates robust, prolonged, dose-dependent antiviral activity when used alone and in combination with some reverse transcriptase inhibitors, the nucleoside analogs [6–8].

To investigate the clinical pharmacokinetics of nelfinavir mesylate, the development of a suitable analytical method for monitoring nelfinavir concentrations in human plasma was necessary. Described here is the development and Good Laboratory Practice (GLP) validation of a sensitive and reproducible high-performance liquid chromatography (HPLC) method for the analysis of the free base, nelfinavir, in human plasma.

2. Experimental

2.1. Materials and reagents

Nelfinavir mesylate (Batch No. 5A0017Z) was synthesized at Agouron Pharmaceuticals (San Diego, CA, USA). The internal standard, 6,7-dimethyl-2,3-di-(2-pyridyl)quinoxaline (ALD-12646, Lot No. 04902HT), was purchased from the Aldrich (Milwaukee, WI, USA). Reagents used in the extraction and HPLC analysis of samples were as follows: sodium phosphate, monobasic monohydrate, reagent-grade (J.T. Baker, Phillipsburg, NJ, USA); phosphoric acid, 85% (Mallinckrodt Specialty Chemicals, Paris, KY, USA); acetonitrile and methanol, both high purity (Burdick and Jackson, Muskegon, MI, USA); Milli-Q water (Millipore, Milford, MA, USA); 30% ammonium hydroxide (Baxter Scientific Products, Charlotte, NC, USA). Blank human plasma (heparinized) used to prepare standards and quality control samples was purchased from Biological Specialties (Colmar, PA, USA).

2.2. Instrumentation and mobile phase

The HPLC system consisted of a Waters Model 501 solvent delivery pump, a Waters Model 712 plus Wisp autosampler, a Waters Model 486 UV spectrophotometric detector. Data acquisition was done using Nelson 2600 chromatography software on a personal computer. Samples (100 μl) were injected at ambient temperature onto a Waters Symmetry C_{18} analytical column (5 μm, 250×4.6 mm I.D.) (Millipore, Milford, MA, USA), preceded by a Waters Nova-pak C_{18} guard column (Millipore, Milford, MA, USA). The absorbance of the effluent was monitored using a variable-wavelength UV detector set at 220 nm. The mobile phase consisted of 25 m*M* monobasic sodium phosphate buffer (adjusted to pH 3.4 with phosphoric acid)–acetonitrile (58:42, v/v), and was deaerated with helium gas. The elution conditions were isocratic, and the mobile phase flow-rate was 1.3 ml per min.

2.3. Preparation of standard solutions and quality control samples in plasma

A stock solution (1.0 mg/ml) of nelfinavir was

prepared by dissolving an accurately weighed amount of the drug in methanol in a volumetric flask. Similarly, a stock solution of the internal standard was prepared by dissolving an accurately weighed amount of the drug in methanol in a volumetric flask. Appropriate dilutions of this solution were made with methanol to achieve a final internal standard working solution concentration of 100 μg/ml. All stock solutions were stored tightly capped at 4°C when not in use.

Human plasma calibration standards (CAL) were prepared at final nelfinavir concentrations of 0.0300 to 10.0 μg/ml. The highest calibration standard pool (10.0 μg/ml) was prepared from the stock nelfinavir solution (1.0 mg/ml) and the remaining calibration pools were dilutions of this standard. After thorough mixing, the calibration standards were separated into daily aliquots and stored in polypropylene tubes at −20°C.

Human plasma quality control (QC) pools were prepared at final nelfinavir concentrations of 0.0300 to 25.0 μg/ml. The 7.50 μg/ml QC sample pool was prepared from a separate weighing of the nelfinavir stock solution, and the remaining lower concentration quality control samples were dilutions of this pool. The highest concentration QC sample pool (25.0 μg/ml) was also prepared from a separate weighing of the drug, and these samples were used only for dilution validations. After thorough mixing, the quality control samples were separated into daily aliquots and stored in polypropylene tubes at −20°C.

2.4. Sample preparation

All plasma samples were removed from the freezer, thawed in cool tap water, and vortexed briefly before processing by solvent extraction. Aliquots (250 μl) of the plasma blank, calibration standard, quality control samples, or plasma samples were placed into 13×100 mm borosilicate glass culture tubes. A 250-μl aliquot of the internal standard (1.0 μg/ml) was added to all tubes except the reagent blank and plasma blank tubes. After vortexing briefly, a 500-μl aliquot of 0.1 *M* NH_4OH (pH 10.5) was added to each tube, and the tubes were vortexed again for one min using a multi-vortex mixer (Fisher Scientific, Pittsburgh, PA, USA). A 2-ml aliquot of the extraction solvent (ethyl acetate–acetonitrile, 90:10, v/v) was then added to each tube and the tubes were capped. Following vortexing for 4 min, all samples were centrifuged for 5 min at ambient temperature at 2060 *g* (3000 rpm) using a Beckman Model GS-6R swinging bucket centrifuge (Fullerton, CA, USA). The organic layer from each tube was then carefully transferred into a 12×75 mm borosilicate tubes and evaporated at 50°C under nitrogen on a Techne Model DB-3 Dri-Block sample concentrator (Princeton, NJ, USA). The dried samples were reconstituted in 150 μl of mobile phase and vortexed for 2 min. The clear supernatant from each tube was transferred to autosampler vials with glass inserts for the HPLC analysis of nelfinavir using an injection volume of 100 μl.

2.5. Determination of standard curve and assay validation

The linearity of the standard curves was evaluated by analysis of nine calibration standards (in duplicate) over the range of 0.0300 μg/ml to 10.0 μg/ml using a linear weighted (1/concentration squared) least squares regression analysis to plot the peak height ratio of nelfinavir to internal standard versus nelfinavir concentration. Linearity and assay reproducibility were determined by assaying the standards in seven separate assay runs on five separate days. Intra- and inter-assay precision and accuracy were determined for four different plasma concentrations of nelfinavir (0.0300, 0.0750, 0.750 and 7.50 μg/ml).

2.6. Specificity, recovery and stability of nelfinavir

Human plasma samples from six different lots (6 different normal individuals) were extracted and analyzed for nelfinavir. Extraction efficiency was determined for the solvent extraction method by comparing the peak heights of three concentrations of nelfinavir in human plasma quality control samples that underwent liquid extraction to that of identical concentrations of nelfinavir prepared in mobile phase without extraction.

Freeze–thaw stability of nelfinavir was determined

at low (0.0750 μg/ml), medium (0.750 μg/ml) and high (7.50 μg/ml) drug concentrations via the analysis of quality control samples over three freeze–thaw cycles within three days. Samples remained unfrozen for a minimum of 2 h per cycle, and there were at least 20 h between cycles. Room temperature stability in human plasma was investigated by allowing a set of quality control samples to remain at room temperature for 4 h prior to extraction. The stability of extracted nelfinavir in mobile phase was determined by allowing the extract to remain at room temperature in the autosampler vials for at least 48 h. The stability of nelfinavir (0.075, 0.750 and 7.50 μg/ml) in human plasma stored at −20°C was studied by analyzing a set of samples at various time points for more than four months.

3. Results and discussion

The addition of 500 μl of 0.1 *M* NH_4OH (pH 10.5) to plasma samples adjusted the pH to approximately 10, thereby allowing nelfinavir (pK_a values of 6.00 and 11.06) to exist in an uncharged form in the medium and be more readily extracted by the extraction solvent. Specificity of the method was demonstrated by extracting and analyzing spiked nelfinavir from six different lots of normal human plasma; there were no interfering peaks demonstrated in these plasma samples. Typical retention times for nelfinavir and the internal standard, 6,7-dimethyl-2,3-di-(2-pyridyl)quinoxaline, were 8.2 and 9.9 min, respectively. There was a small plasma endogenous peak near the retention time of nelfinavir; however, it was found that slight mobile phase modifications during analytical runs could effectively separate the endogenous peak from the nelfinavir peak. The plasma peak had an average response less than 50% of the average response of the limit of quantitation standard (0.0300 μg/ml) during the validation and was shown not to affect the quantitation of nelfinavir. Though the limit of quantitation (the lowest non-zero human plasma concentration level that could be accurately and reproducibly quantitated) was validated at 0.0300 μg/ml, it was raised to 0.0500 μg/ml during analysis of actual patient samples due to the presence of other interfering endogenous peaks. There were no significant interferences at the retention time of the internal standard. A typical HPLC chromatogram of 0.0300 μg/ml and 10.0 μg/ml nelfinavir and internal standard in human plasma is shown in Fig. 2.

The assay was validated for day-to-day variability by analyzing nine nelfinavir concentrations in human plasma (in duplicate) on five separate days over seven separate assay runs. Precision was expressed as the % coefficient of variation (%C.V.) of each pool. The results of this study are presented in Table 1. The nine-point standard curve was linear over a concentration range of 0.0300 to 10.0 μg/ml, with a mean slope (±S.D.) of 0.356632 (±0.018213) and a mean *y*-intercept (±S.D.) of 0.001286 (±0.001268). Linearity was indicated by an average correlation coefficient of 0.9972 from seven standard curves. The average coefficient of variation for average results of back-calculated calibration standard concentrations compared to theoretical concentrations was 3.57%, and none were above 6.41%.

The absolute recovery of nelfinavir from human plasma was determined by comparing the peak heights of extracted samples at three nelfinavir concentrations to that of external samples at the same concentrations. The mean recovery was 92.4% for the 0.0750 μg/ml nelfinavir samples ($n=6$) with a coefficient of variation of 5.16%. The mean recovery for the 0.750 μg/ml nelfinavir samples ($n=6$) was 90.0% with a coefficient of variation of 4.73%. The 7.50 μg/ml nelfinavir samples ($n=6$) had a mean recovery of 83.0% with a coefficient of variation of 2.11%. The absolute recovery of the internal standard for the mid-concentration samples ($n=6$) was determined in a similar manner and was found to be 95.7% with a coefficient of variation of 4.16%.

The intra- and inter-assay precision and accuracy of the method were determined at four pre-selected plasma nelfinavir concentrations: 0.0300, 0.0750, 0.750 and 7.50 μg/ml, reflecting the minimum quantifiable level and the typical low, medium and high concentrations that were anticipated in patient samples. Accuracy was measured as the % difference from theoretical according to the equation:

$$\text{Accuracy}\ (\%) = [\text{conc.}_{\text{exp}}/\text{conc.}_{\text{theor}}] \cdot 100$$

The results of the intra-assay and inter-assay validation studies are shown in Tables 2 and 3,

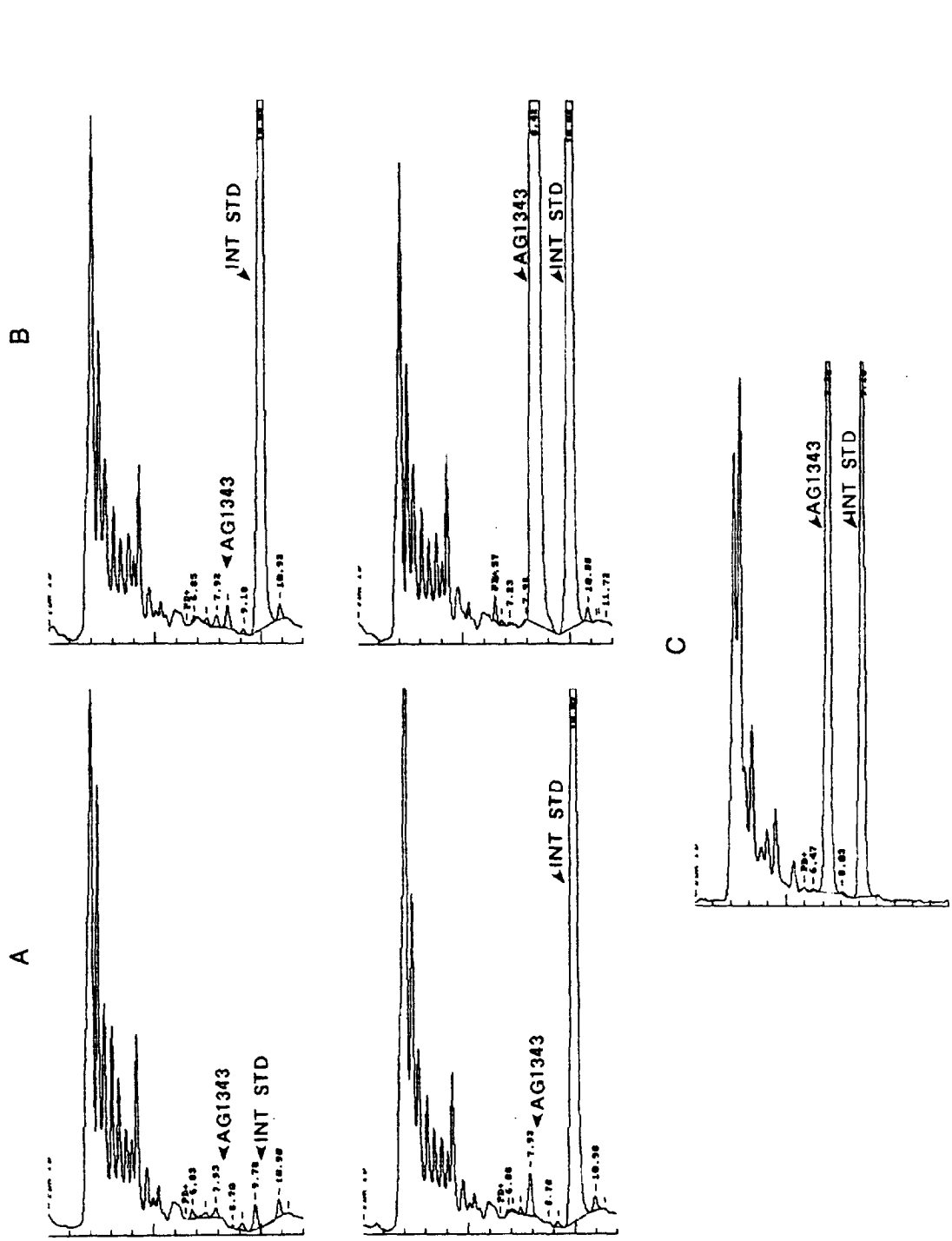

Fig. 2. Typical HPLC chromatograms showing nelfinavir (AG1343) and internal standard (INT STD) extracted from human plasma. (A) Blank normal human plasma (upper) and spiked with 1.0 μg/ml internal standard (lower). (B) Blank normal human plasma spiked with 1.0 μg/ml internal standard and 0.0300 μg/ml nelfinavir (upper) and 10.0 μg/ml nelfinavir (lower). (C) Chromatogram of a typical patient sample representing 3.32 μg/ml nelfinavir.

Table 1
Day-to-day variability of nine nelfinavir concentrations measured on five separate days in human plasma[a]

Nelfinavir concentration (μg/ml)		C.V. (%)
Theoretical	Experimental ($n=14$)	
0.0300	0.0294±0.00138[b]	4.68
0.0500	0.0501±0.00321[c]	6.41
0.100	0.103±0.00369	3.58
0.250	0.254±0.0114[c]	4.49
0.500	0.531±0.0119	2.23
1.00	1.04±0.0245	2.36
2.50	2.54±0.0522	2.05
5.00	4.79±0.139	2.89
10.0	8.93±0.309	3.47

Standard curve (generated over five separate days): slope (mean±S.D.)=0.356632±0.018213; y-intercept (mean±S.D.)=0.001286±0.001268; mean $r^2=0.9972$.
[a] Generated over seven separate assay runs.
[b] Value from $n=11$.
[c] Value from $n=13$.

Table 2
Intra-assay precision and accuracy for HPLC analysis of nelfinavir in human plasma ($n=6$)

Theoretical concentration (μg/ml)	Concentration found (mean±S.D.) (μg/ml)	C.V. (%)	Accuracy (%)
0.0300	0.0332±0.0014	4.36	110.7
0.0750	0.0810±0.00175	2.16	108.0
0.750	0.825±0.00788	0.956	110.0
7.50	6.95±0.173	2.49	92.7
25.0[a]	24.3±0.478	1.96	97.2
25.0[b]	25.8±0.346	1.34	103.2

[a] Original sample was diluted five-fold with human plasma prior to extraction.
[b] Original sample was diluted ten-fold with human plasma prior to extraction.

Table 3
Inter-assay precision and accuracy for HPLC analysis of nelfinavir in human plasma[a] ($n=15$)

Theoretical concentration (μg/ml)	Concentration found (mean±S.D.) (μg/ml)	C.V. (%)	Accuracy (%)
0.0300	0.0311±0.00329[b]	10.6	103.7
0.0750	0.0827±0.0110[c]	13.3	110.3
0.750	0.829±0.0164	1.98	110.5
7.50	7.01±0.304	4.34	93.5

[a] Generated over seven separate assay runs.
[b] Value from $n=11$.
[c] Value from $n=14$.

respectively. Intra-assay precision was evaluated for each quality control sample pool by multiple analyses of the pool on one assay day (Table 2). The limit of quantitation pool (0.0300 μg/ml nelfinavir) had an intra-assay coefficient of variation of 4.36%. The remaining quality control sample pools had intra-assay coefficients of variation ranging from 0.956 to 2.49%. To check for intra-assay precision and accuracy on diluted samples, the 25.0 μg/ml QC samples were assayed in replicate ($n=6$) as five-fold and ten-fold dilutions (Table 2). The coefficient of variation for these two dilutions were 1.96 and 1.34%, respectively. Inter-assay precision and accuracy were evaluated for each quality control sample pool by analysis of the pool on seven separate analytical runs (Table 3). The limit of quantitation pool (0.0300 μg/ml nelfinavir) had an inter-assay coefficient of variation of 10.6% and an accuracy of 3.5%. The remaining quality control sample pools had inter-assay coefficients of variation ranging from 1.98 to 13.3% and an accuracy within 10.5% of theoretical.

Stability studies of nelfinavir were conducted at three different concentrations at a storage temperature of −20°C. Nelfinavir stored in human plasma at −20°C in capped polypropylene tubes for more than four months showed little to no change in concentration. Recovery from stability samples at 0.075, 0.750 and 7.50 μg/ml ranged within 95.1 to 97.6% accuracy, suggesting that nelfinavir was stable for at least 142 days when stored at −20°C (Table 4). Nelfinavir was also shown to be stable in human plasma through three cycles of freeze–thaw conducted within three days. The mean concentrations for all three concentrations of the quality control samples were within 13.0% of theoretical following the third freeze–thaw cycle (Table 5). Moreover, nelfinavir was shown to be stable in human plasma at

Table 4
Nelfinavir stability after storage in human plasma for 142 days at −20°C ($n=6$)

Theoretical concentration (μg/ml)	Concentration found (mean±S.D.) (μg/ml)	C.V. (%)	Accuracy (%)
0.0750	0.0732±0.0112	1.53	97.6
0.750	0.721±0.00594	0.823	96.1
7.50	7.13±0.0953	1.34	95.1

Table 5
Freeze–thaw stability of nelfinavir in human plasma ($n=3$)

Theoretical concentration (μg/ml)	Concentration found (mean±S.D.) (μg/ml)	C.V. (%)	Accuracy (%)
Freeze–thaw cycle 1			
0.0750	0.0799±0.00275	3.45	106.5
0.750	0.802±0.00971	1.21	106.9
7.50	6.99±0.0300	0.429	93.2
Freeze–thaw cycle 2			
0.0750	0.0813±0.000850	1.05	108.4
0.750	0.794±0.00777	0.977	105.9
7.50	6.99±0.134	1.92	93.2
Freeze–thaw cycle 3			
0.0750	0.0848±0.00116	1.36	113.0
0.750	0.793±0.0254	3.20	105.7
7.50	6.89±0.0723	1.05	91.9

Table 6
4-hour bench top stability and 48-hour extract stability of nelfinavir in human plasma ($n=3$)

Theoretical concentration (μg/ml)	Concentration found (mean±S.D.) (μg/ml)	C.V. (%)	Accuracy (%)
4-h benchtop stability			
0.0750	0.0784±0.00309	3.95	104.5
0.750	0.820±0.0123	1.50	109.3
7.50	7.32±0.0739	1.01	97.6
48-h extract stability			
0.0750	0.0775[a]	NA	103.4
0.750	0.860±0.0119	1.39	114.6
7.50	7.54±0.357	4.74	100.5

[a] $n=2$; value deleted from calculations due to interference at the retention time of nelfinavir.
NA = calculation not applicable.

room temperature for at least 4 h prior to extraction (Table 6), and the extracted drug was demonstrated to be stable in mobile phase for at least 48 h (Table 6).

Nelfinavir has been investigated in Phase III clinical trials. The drug was orally administered at 500, 750 and 1000 mg t.i.d. alone or concomitantly with other anti-HIV therapeutics such as nucleoside analogs. Serial blood samples were collected over a period of 8 h after dosing. Typical mean plasma concentration–time profiles for nelfinavir from three dose levels (500, 750 and 1000 mg t.i.d.) in three groups of patients are shown in Fig. 3. There appears to be significant variability in the plasma nelfinavir concentration between patients being dosed with the

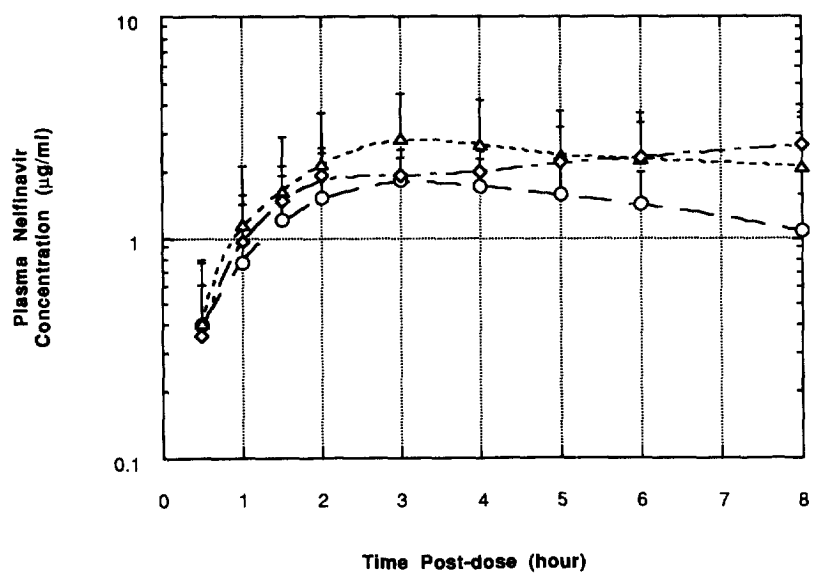

Fig. 3. Typical mean plasma concentration–time profiles of nelfinavir in HIV-positive patients ($n=5$ at each dose) following oral t.i.d. doses of (○) 500 mg, (△) 750 mg and (◇) 1000 mg of nelfinavir mesylate on day 1 of dosing.

same regimen. Plasma nelfinavir concentrations, determined using the method described above, thus far have ranged from less than 0.0300 μg/ml (the original limit of quantitation) to greater than 7.50 μg/ml, and have been used to calculate the pharmacokinetics of nelfinavir in these clinical studies.

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