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Determination of nelfinavir, a potent HIV protease inhibitor, and its active metabolite M8 in human plasma by high-performance liquid chromatography with photodiode-array detection

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Abstract

We developed and characterized a high-performance liquid chromatographic assay for the determination of nelfinavir (NFV), a potent HIV protease inhibitor, and its active metabolite M8 in human plasma. Extraction of the internal standard, M8 and NFV from the plasma buffered at pH 9.5 was achieved by a liquid–liquid extraction with a mixture of methyl-*tert*.-butyl ether and hexane. Following two washes of the reconstituted sample with hexane, separation was achieved on an octadecylsilyl analytical column with a mobile phase containing 0.1% trifluoroacetic acid–acetonitrile–methanol (51:46:5, v/v). Detection was performed using an ultraviolet photodiode-array detector. The signal was monitored at a wavelength of 220 nm. The assay was found to be linear and has been validated over the concentration range of 25 to 3000 μg/l for M8 and 25 to 6000 μg/l for NFV, from 500 μl of plasma. Recoveries were 98.9% (SD 8.9%), and 100.2% (SD 11.7%) for M8 and NFV, respectively. Concentrations that gave a signal-to-noise ratio of three (15 μg/l for both M8 and NFV) were selected to determine the limit of detection. The lower limit of quantification (25 μg/l for both M8 and NFV) was defined as the concentration for which the relative standard deviation and the percent deviation from the nominal concentration were lower than 20%.

Keywords: Nelfinavir; M8

1. Introduction

Nelfinavir mesylate (AG1343, Viracept®, NFV) {[3*S*-(3*R**,4a*R**,8a*R**, 2′*S**,3′*S**)]-2-[2′-hydroxy-3′-phenylthiomethyl-4′-aza-5′-oxo-5′-(2″-methyl-3″-hydroxyphenyl)pentyl] - decahydroisoquinoline - 3*N*-*tert*.-butylcarboxamide methanesulfonic acid salt} (Fig. 1) is a potent and selective human immunodeficiency virus 1 (HIV-1) protease inhibitor (PI) (Ki=2 n*M*) [1]. In vivo, NFV has been shown to significantly reduce viral load and to increase CD_4^+T cells in adults and children infected with HIV infection especially when administered in association with others anti-HIV agents, nucleoside analogues

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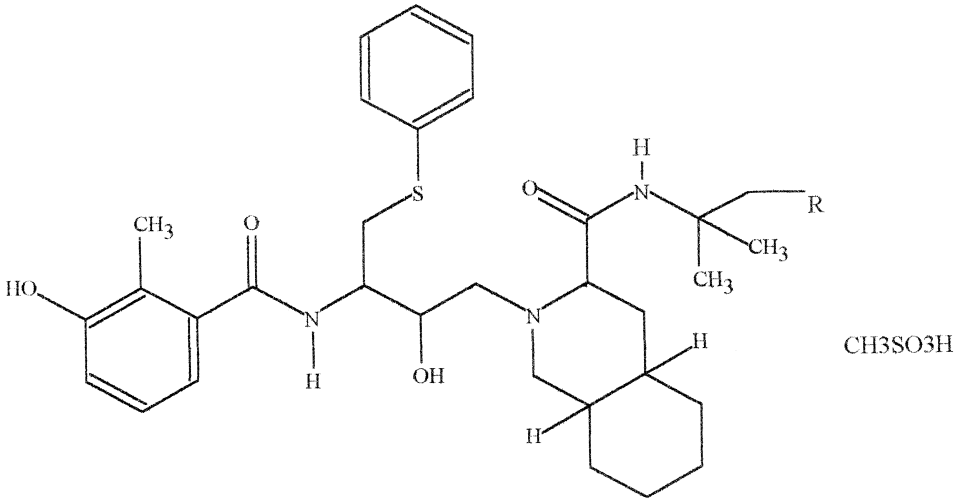

Fig. 1. Molecular structures of nelfinavir (R=H) and its hydroxy-*tert*.-butylamide metabolite M8 (R=OH).

(NRTIs), non nucleoside analogues of reverse transcriptase inhibitors (NNRTIs) or protease inhibitors (PIs) [2]. NFV metabolism is mediated by cytochrome P450 (CYP). Isoenzymes involved in the in vitro metabolism of NFV are primarily CYP3A4 (more than 50%), CYP2C19, CYP2D6 and CYP2C9 [3–5]. Two of the multiple metabolites of NFV have demonstrated an antiviral potency in vitro. Thirty percent of the major metabolite of NFV, the hydroxy-*tert*.-butylamide metabolite M8 (Fig. 1), is produced from the parent compound. Thus, M8 contributes substantially to improve NFV antiviral efficacy after administration of Viracept® in HIV-infected patients [6].

Despite a widely reported inter-individual variability concerning the metabolism of HIV PIs in patients, determination of plasma concentrations of M8 allows one to appreciate this variability, and to individualize NFV doses for each patient. Moreover, because of a probable auto-induction of the metabolism of NFV, determination of plasma concentrations of NFV and especially of its metabolite M8 could reflect patients adherence [7].

Moreover, since antiviral activity and PI plasma concentrations seem to be highly correlated in clinical studies, monitoring both NFV and M8 plasma concentrations clearly appears of major importance to evaluate therapeutic efficacy and clinical implications of drug–drug interactions in patients treated with NFV [2,8,9].

Several high-performance liquid chromatography (HPLC) assays have been described for determination of HIV PIs in human plasma. They included solid–liquid extraction or liquid–liquid extraction procedures coupled or not to mass spectrometry (MS). The latest developed assays aimed to monitor PIs simultaneously: ritonavir and saquinavir [10], or ritonavir, saquinavir, amprenavir, indinavir and NFV [11]. Solid–liquid extractions have been used for such determinations. Chromatographic conditions are determined as a compromise of optimal conditions for all compounds. Limits of quantification, precision and accuracy could not be optimized for all compounds in an unique assay.

Two methods have been reported for the analysis of NFV in human plasma: the first one analyzed NFV without metabolites or other PIs [12], while the second one analyzed NFV and other PIs [11]. Their limits of quantification for NFV were 50 and 30 μg/l, respectively, but both of them failed to measure the metabolites. One method has been reported for the determination of NFV and its active metabolite M8 in plasma. It involved LC–MS, but no details were given about its analytical characteristic [6]. Such procedures are time-consuming, and include expensive equipment, which is not adapted for drug monitoring. To our knowledge, no HPLC assay for NFV and M8 has been reported to date.

We describe the development and validation of a simple, sensitive, accurate and precise HPLC assay

coupled to an ultraviolet (UV) detection for the determination of NFV and its active metabolite M8 concentrations in human plasma. This assay allows to assess pharmacokinetic parameters and to monitor the efficacy of treatments based on NFV.

2. Experimental

2.1. Reference compounds

Nelfinavir mesylate (AG1343) (Lot. N960172), and metabolite M8 mesylate (AG1402) (Lot. 1258-157-1) were a kind gift of Agouron Pharmaceuticals (San Diego, CA, USA). The internal standard (I.S.), *N*-[2(*R*)-hydroxy-1(*S*)-indanyl]-5-[2(*S*)-*tert*.-butylaminocarbonyl]-4-{3-[4-methylpyridylmethyl]}piperazino]-4(*S*)-hydroxy-2(*R*)-phenylmethyl-pentanamide (L-738,804) (Lot. 000K-006) was kindly provided by Merck-Sharp and Dohme-Chibret (Clermont-Ferrand, France).

2.2. Chemicals

All substances used were of analytical grade, all solvents were HPLC grade. Acetonitrile and methanol were from Carlo Erba (Val de Reuil, France). Methyl-*tert*.-butyl ether (MTBE) was from Sigma–Aldrich (Steinheim, Germany). Hexane and trifluoroacetic acid were from Merck (Darmstadt, Germany). Ammonium acetate was from Prolabo (Paris, France). Sodium borate was supplied by Sigma (Buchs, Switzerland). Drug-free bovine serum Biotrol® (Merck, Chennevières Les Louvres, France) was used to prepare calibration standards and quality controls. Distilled water (Aguettant, Lyon, France) was used throughout.

2.3. Instrument parameters

The chromatographic system (Waters, Milford, MA, USA) consisted of a pump (Model 510), an automatic injector (Wisp 717 plus), a UV photodiode-array detection system (PDA 996), and a Millennium 2020 chromatography manager. Separation was achieved on an octadecylsilyl (C_{18}) analytical column (250×4.6 mm I.D., 5 μm particle size) (Beckman Instruments, Fullerton, CA, USA) protected by a C_{18} guard column (10×4.6 mm I.D., 5 μm particle size) (Hypersil, Thermoquist, Les Ulis, France). The signal was monitored at 220 nm on a UV PDA system, recorded and integrated using Waters software version 5.11 (Millipore, Milford, MA, USA). Peak purity tests were carried out continuously during the batch analysis. Eighty percent of the surface of the peak was analyzed for purity. A peak controlled spectrum recording was selected with a range of 190–400 nm and a step width of 2 nm. Peak spectrums were compared with that of a reference standard stored in a library previously recorded in the computer.

The mobile phase consisted of 0.1% trifloroacetic acid–acetonitrile–methanol (49:46:5, v/v). It was filtered and adjusted to pH 5 with 1 m*M* ammonium acetate before use. The flow-rate was 1.5 ml/min.

2.4. Preparation of standards and quality control samples

Stock solutions of M8 and NFV were prepared by dissolving separately in a volumetric flask, 58.2 mg of M8 mesylate (50 mg of M8 base equivalent) and 117 mg of NFV mesylate (100 mg of NFV base equivalent) precisely weighed, in 100 ml methanol to yield a concentration of 0.5 g/l for M8 and 1 g/l for NFV base, respectively. Similarly, a stock solution of the I.S. was prepared by dissolving a precisely weighed amount of the drug in a volumetric flask. The solutions were stored in polypropylene tubes at −80°C.

For the preparation of calibration standards, working solutions of M8 and NFV base were produced by diluting appropriate volumes of M8 and NFV base stock solutions with methanol. Calibration concentrations of 25 to 3000 μg/l of M8 and 25 to 6000 μg/l of NFV base were obtained by spiking drug-free bovine serum with diluted stock solutions.

Second stock solutions, weighed separately, were used for the preparation of quality control samples yielding concentrations of 25, 100, 500 and 2500 μg/l of M8, and 50, 200, 1000 and 5600 μg/l of NFV base and in drug-free bovine serum.

2.5. Preparation of patient samples

Blood samples from patients receiving Viracept 750 mg three times daily (tid) were collected predos-

ing and 3 h after dosing on heparinized tubes and centrifuged (5 min, 3500 rpm, 4°C) as soon as possible after collection. Plasma were aliquoted into polypropylene tubes, immediately frozen and stored at −20°C until analysis.

2.6. Sample extraction procedure

A 500-μl volume of calibration standard, quality control or plasma sample was placed into a 5-ml polypropylene tube, to which were added 100 μl of a 10 mg/l solution of I.S. in acetonitrile. After vortexing for 15 s, 500 μl of 0.1 *M* borate buffer, pH 9.5 was added. The tubes were mixed again using a vortex Reax 2000 (Heidolph, Kelheim, Germany). A 4-ml volume of a mixture of MTBE–hexane (90:10, v/v) was added to each tube. The tubes were capped and tumble mixed on a reciprocating shaker Reax 2 (Heidolph) at 1500 rotations per minute (rpm) for 10 min, and centrifuged at 3500 rpm for 10 min using a centrifuge (Jouan, Saint-Nazaire, France). The organic layer was separated by snap freezing the aqueous layer at −80°C. It was then transferred into new 5-ml polypropylene tubes, and evaporated to dryness under reduced pressure at 43°C in a Speed Vac (Model AES 2010, Bioblock Scientific, Illkirch, France). The residue was reconstituted in 300 μl of mobile phase. Each reconstituted sample was washed twice with 3 ml of hexane to remove the co-extracted contaminants. After vortexing for 5 s, the tubes were centrifuged as described above, and the hexane layer was removed by aspiration. The remaining aqueous phase was transferred to auto-sampler vials for HPLC analysis. A 100 μl injection volume was used throughout the analytical validation and batch analysis.

2.7. Validation tests

2.7.1. Calibration and calculation procedures

Data from four complete analytical batches were used to validate this assay. Each validation batch was comprised of two complete sets of calibration standards, and quality control samples in replicates of six for the within-batch validation, and in replicates of 12 for the between-batch validation [13]. Daily standard curves were constructed using a weighted ($1/\text{concentration}^2$) least-squares linear regression analysis of the observed peak area ratios of M8, NFV and the I.S. The concentrations of the standard were back-calculated using these standard curve parameters, and compared to expected concentrations. The unknown concentrations were calculated from the weighted linear regression equation of the peak area ratio against concentrations of the calibration curve.

2.7.2. Chromatography and detection

Different chromatographic conditions were examined. The variations of retention times and absorbance intensity of NFV and M8 versus pH were investigated by adjusting the pH of the mobile phase to values of 4.0, 5.0 and 6.0 by titration with ammonium acetate. At each pH value, M8 (5 mg/l) and NFV (10 mg/l) in solution in methanol or in the mobile phase, and I.S. (10 mg/l) in solution in acetonitrile or in the mobile phase were injected into the column. The retention times and the spectra of the different compounds were recorded. The capacity factors (k') of I.S., M8, and NFV were calculated.

Variations in the retention times of the compounds versus the composition of the mobile phase in acetonitrile, 0.1% trifluoroacetic acid and methanol were studied.

2.7.3. Specificity and selectivity

The interference of endogenous compounds was investigated by analysis of plasma samples of six individuals who did not receive NFV, and of a drug-free bovine serum sample. Due to the wide variety of co-administered drugs in patients infected with HIV, we investigated a large number of compounds for interference with the analytical method. These compounds were injected in solution in water or in methanol at a concentration of 10 mg/l, or treated according to the extraction procedure (including the sample pretreatment).

2.7.4. Accuracy, precision, linearity and recovery

Accuracy was calculated on the basis of the differences in the mean of a set of results and the nominal value, and expressed in percent.

The within-day and between-day precision was assessed both within and between-batches at con-

centrations of 25, 100, 500 and 2500 μg/l for M8, and 50, 200, 1000 and 5600 μg/l for NFV [14]. Precision was assessed by analysis of variance (ANOVA) for each validation control concentration percent using the analytical batch as the grouping variable. It is expressed in terms of the standard deviation (SD) and the relative standard deviation (RSD). Each calibration batch contained eight calibration standards in duplicate (25, 37.5, 125, 250, 600, 1500 and 3000 μg/l for M8, and 25, 75, 250, 500, 1200, 3000, 6000 μg/l for NFV) [13]. Standard curves were constructed using the ratios of the observed peak areas of NFV or M8 and of the I.S.

The linear relationship between concentrations of M8 and NFV and response is demonstrated by calculation of r^2. Linearity of all calibration curves was tested with the F-test for lack-of-fit, using a weight factor of $(1/\text{concentration}^2)$ for NFV and for M8 [14].

Recovery is reported as a percentage of the original drug carried through the sample purification steps of the method. The recovery of NFV, M8, and I.S. was evaluated under both acid and alkaline extraction conditions.

2.7.5. Limits of detection and quantification

The limit of detection of the assay was defined as the smallest concentration that can be differentiated from the baseline. Concentration that gave a signal-to-noise ratio of three was selected to determine the limit of detection. The lower limit of quantification was defined as the concentration for which the RSD and the percent deviation from the nominal concentration were lower than 20% [14,15].

2.7.6. Biological matrices

In order to assess absence of matrice effects, two complete sets of calibration standards using drug-free bovine serum and human plasma as matrices in replicate of two were analyzed during a single batch. The recoveries of the response were calculated and compared.

2.8. Statistics

Statistical analysis was performed using Stat View software (Abacus Concepts, Berkeley, CA, USA). Correlation was considered statistically significant when calculated P values were 0.05 or less.

3. Results

3.1. Chromatographic characteristics

Fig. 2 shows chromatograms of an extracted drug-free bovine serum sample, an extracted bovine serum sample containing 3333 μg/l I.S., an extracted bovine serum sample containing 3333 μg/l I.S., 2500 μg/l M8 and 5600 μg/l NFV, and an extracted plasma sample of a patient receiving 750 mg Viracept three times daily. Representation of the calculated capacity factors (k') of the compounds of interest shows that k' of I.S. slightly decreased with rising pH of the mobile phase, whereas those of M8 and especially NFV increased by a factor of 1.55, and 1.88, respectively (Table 1).

The UV spectra of 5.82 mg/l M8, and 10 mg/l NFV in solution in methanol were also affected by the pH of the mobile phase. For both compounds, maximal absorbance was found at pH 5. The influence of the amounts of acetonitrile and methanol in the mobile phase on retention times of compounds of interest is presented Table 1. Increasing amounts of acetonitrile in the mobile phase increased the elution of all compounds. Addition of methanol increased the selectivity between the compounds, which interfered and the measured drugs. The retention times of I.S., M8 and NFV were 4.00, 5.50 and 11.71, respectively, allowing a running time of 15 min. These performances were reproducible when the C_{18} guard column was changed after 1000 sample analysis. In the same way, no significant loss of resolution was observed after 1500 sample analysis.

3.2. Extraction procedure

Extraction of the compounds was achieved through a liquid–liquid extraction. Plasma samples were buffered to a pH value of 9.5, and extracted with MTBE. Interfering peaks were co-eluted in the extracts reconstituted with the mobile phase. To remove these lipophilic co-eluted contaminants, as well as to quantify the compounds in the low range, washes with hexane were necessary. The cleanest baseline was obtained after two washes with hexane.

The recovery following the extraction procedure was determined by comparing peak areas of working solutions of I.S., M8 and NFV in the mobile phase

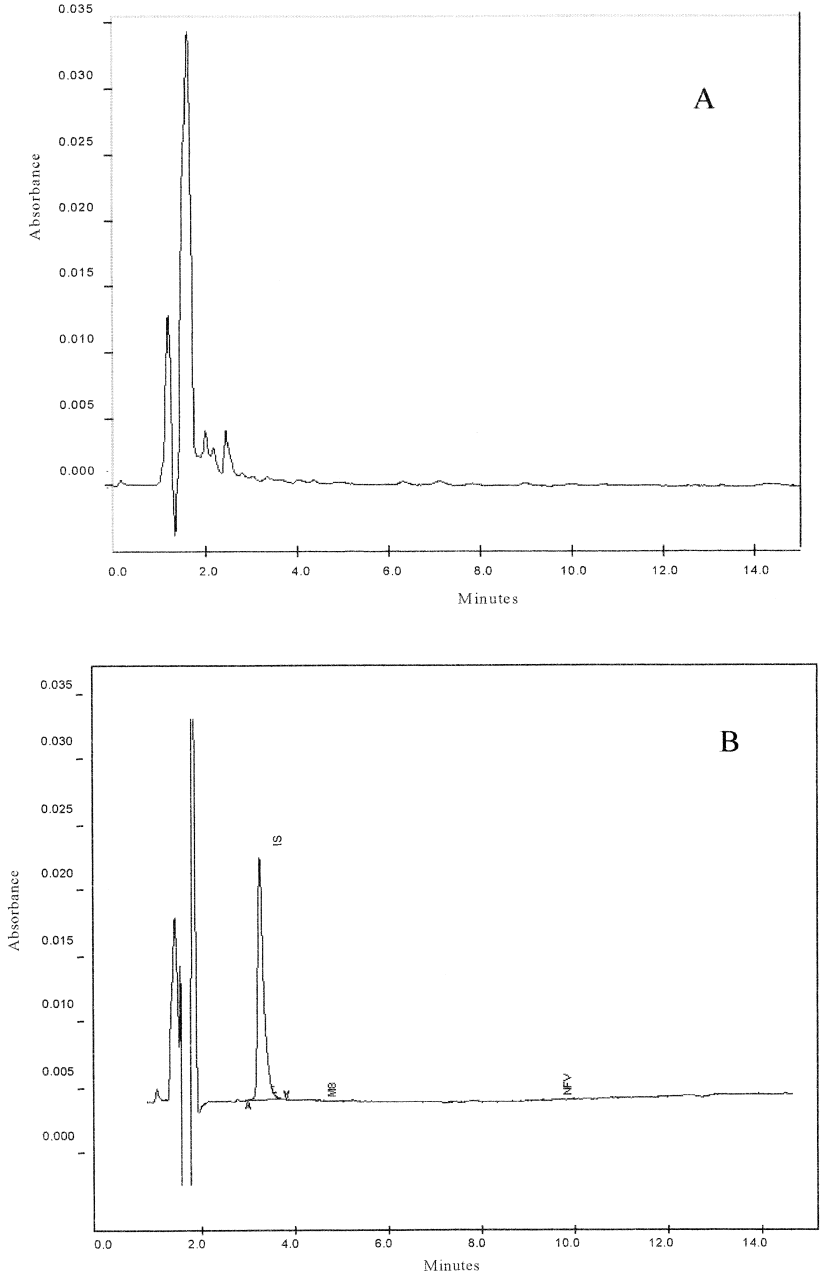

Fig. 2. Representative chromatograms of extracted samples. (A) Drug-free bovine serum sample, (B) bovine sample spiked with 3333 μg/l I.S., (C) bovine serum spiked with 3333 μg/l I.S., 2500 μg/l M8, and 5600 μg/l NFV (D) plasma from a patient receiving 750 mg of Viracept® three times daily collected 3 h after dosing.

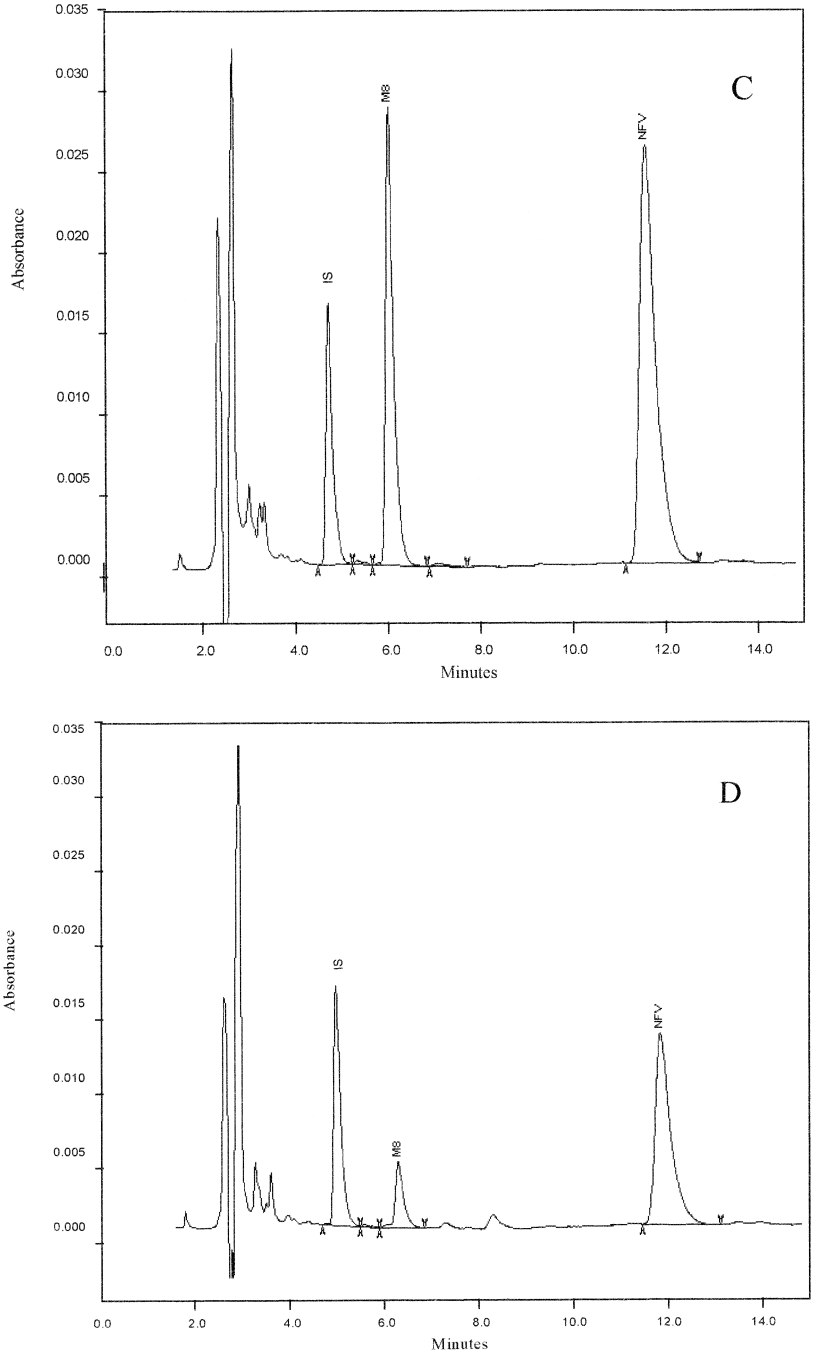

Fig. 2. (*continued*)

Table 1
The capacity factors (k') of I.S., M8 and NFV as a function of different mobile phase composition adjusted to pH 5, or with varying pH of the mobile phase containing 0.1% trifluoroacetic acid–acetonitrile–methanol (49:46:5, v/v/v)

	Compound		
	I.S.	M8	NFV
Composition of the mobile phase at pH 5 (0.1% trifluoroacetic acid–acetonitrile–methanol, v/v)			
50:50:0	2.47	2.79	3.20
49:46:5	2.23	3.71	9.88
55:45:0	2.61	4.44	12.06
pH of the mobile phase			
4	2.59	3.74	9.08
5	2.23	3.71	9.88
6	2.07	5.76	18.65

directly injected into the system, with those of extracted plasma. The mean recovery was 102.6±2.8% (2.7%), 98.9±8.9% (9.0%) and 100.2±11.7% (11.7%) for I.S., M8 and NFV, respectively ($n=14$) (mean±SD, RSD).

No significant difference between the extraction recoveries from spiked serum and spiked human plasma were shown.

3.3. Linearity

A weighting factor of ($1/\text{concentration}^2$) was used to construct calibration curves of NFV and M8 resulting in less than 10% variation between the nominal standard and the experimental back-calculated standards for both compounds. Calibration curves were linear ($r^2=0.997$ and $r^2=0.996$, for NFV and for M8, respectively) ($n=4$) in the range of 25 to 6000 μg/l for the parent drug and 25 to 3000 μg/l for M8 ($n=8$). Within these ranges, the calibration curves of both compounds were shown to be linear using the F-test for lack-of-fit as an indicator of linearity for each regression model.

3.4. Limits of detection and quantification

The limit of detection was 15 μg/l for both M8 and NFV using 500 μl of plasma. The limit of quantification in the described chromatographic system was 25 μg/l for NFV, and 25 μg/l for M8. At concentrations lower than 25 μg/l for NFV and for M8, the percent deviation of the nominal concentration was greater than 20%.

3.5. Specificity and selectivity

No endogenous peak was found to interfere with either I.S., NFV or M8. Most of the co-administered drugs or metabolites tested had retention times that were different from the analytes of interest, or were not eluted with the described analytical method (Table 2). Indinavir, amprenavir and 25-*O*-desacetylrifabutin, and rifabutin are eluted with retention times close to those of I.S., M8 and NFV, respectively. However, separation can be achieved. The specificity of the assay is further illustrated by the fact that all plasma samples from subjects receiving NFV, and monitored in our laboratory were free of interfering peaks. The analysis of a plasma sample from an HIV-infected patient treated with 750 mg of Viracept® three times daily (tid) is presented Fig. 2D.

3.6. Assay precision and accuracy

The method proved to be accurate and precise at four concentrations of both analytes M8 and NFV (Tables 3 and 4). The between-day precision ranged from 4.68 to 6.24% and from 1.57 to 3.98% for M8 and NFV, respectively ($n=12$). The within-day precision range was 1.24 to 5.59% and 0.84 to 3.07% for M8 and NFV, respectively ($n=4$). At the lower level of the calibration curves, RSD was 7.5% for NFV, and 8.4% for M8 for the between-day assay. Between-day accuracy ranged from 97.82 to 101.73% and 99.25 to 103.75% for M8 and NFV, respectively.

Table 2
Drugs tested for interferences with NFV, M8 and I.S.[a]

Compound	Retention time (min)
Internal standard	4.00
M8	5.50
Nelfinavir	11.71
Saquinavir	8.66
Indinavir	3.72
Ritonavir	13.47
Amprenavir	5.43
Efavirenz	NE
Nevirapine	NE
Delavirdine	5.43
N-Deisopropyl delavirdine	NE
Zidovudine	NE
5-Glucuronide zidovudine	NE
Stavudine	NE
Didanosine	NE
Lamivudine	NE
Rifabutin	12.20
25-*O*-Desacetyl rifabutin	5.71
Rifampicin	3.12
Isoniazid	NE
Pyrazinamide	NE
Itraconazole	NE
Hydroxy-itraconzaole	10.00
Fluconazole	NE
Ketoconazole	NE
Metronidazole	NE
Sulfamethoxazole	NE
N-Acetyl sulfamethoxazole	NE
Trimethoprim	NE
Atovaquone	NE
Dapsone	NE
Pyrimethamine	2.87
Aciclovir	NE
Ganciclovir	NE
Zolpidem	2.89

[a] Each compound was injected into the chromatographic system in solution in either methanol or distilled water at a concentration of 10 mg/l. All compounds were extracted and eluted under the method conditions except rifampicin, which was not extracted. Amprenavir was not eluted after extraction at clinically relevant plasma concentrations. NE designates compounds, which are not eluted.

close to those of NFV, M8 was extracted in the same way. In a first step, plasma samples were alkalinized,

3.7. Assay application

Peak plasma levels of NFV were reached approximately 3 h after dosing at steady state [16]. One hundred and twenty-nine plasma samples were analyzed in our laboratory. Plasma concentrations of M8 and NFV were assessed in patients at steady state predosing (C_{min}) and 3 h later (C_{max}). Patients receiving 750 mg of Viracept® three times daily showed M8 and NFV plasma concentrations ranging from 350±450 μg/l to 630±680 μg/l, and 1810±1620 μg/l to 3130 to±2030 μg/l for C_{min} and C_{max}, respectively (n=129).

4. Discussion

This is the first HPLC assay with UV detection for the simultaneous determination of NFV and M8 in human plasma samples. The method was specific and reproducible for the determination of NFV and M8 in plasma samples of patients receiving Viracept®. To sustain antiviral efficacy and to delay the emergence of resistance, levels of NFV in plasma should always exceed the 95% inhibitory concentration (IC_{95}) ranging from 40 to 74 μg/l as determined in vitro against laboratory and clinical strains of HIV [1,17,18]. Since M8 presented the same antiviral activity as NFV [6], it appears that only the determination of the pharmacologically active fraction of NFV, corresponding to the addition of M8 and NFV plasma concentrations, seems clinically relevant.

The choice of liquid–liquid extraction was guided by the physicochemical properties of NFV. Its pK_a values are 6.00 and 11.06, and its partitioning coefficient log $P_{octanol-water}$ is 4.1 for neutral species at pH 7.4 (Fig. 3). We previously developed a method involving a solid-phase extraction with C_{18} cartridges. However this technique required frequent changes of the guard column (after 60 injections) and of the chromatographic column (after 500 injections). Moreover, we observed a lack of sensitivity, and it was not possible to extract the metabolite M8 with a good yield. Also, we preferred a liquid–liquid extraction.

M8 has been described as a hydroxylated metabolite of NFV. Since its physicochemical properties are

Table 3
Between-day precision and accuracy data for NFV and M8 added to drug-free bovine serum (mean±SD, $n=24$)

Added concentration (μg/l)	Observed concentration (μg/l)	RSD (%)	Min (μg/l)	Max (μg/l)	Accuracy (%)
NFV					
50	51.18±2.04	3.99	45.02	54.72	102.35
200	200.03±3.15	1.57	193.51	207.03	100.02
1000	1048.58±34.71	3.31	947.43	1100.52	104.86
5600	5634.72±176.02	3.12	5224.97	5866.34	102.45
M8					
25	25.02±2.47	9.87	18.06	27.68	100.10
100	100.25±6.26	6.24	87.56	110.95	100.25
500	506.58±23.18	4.58	474.91	549.17	101.32
2500	2452.07±134.31	5.48	2250.31	2774.17	98.08

and extracted with MTBE. Resulting chromatograms presented endogenous interfering peaks co-eluting with the analytes of interest. These peaks could not be separated from M8 and NFV by only changing the composition of the mobile phase. Therefore, we developed two extraction strategies: (i) successive extraction steps of a buffered plasma sample into MTBE, back extracting into acid, and re-extraction into MTBE; (ii) extraction into MTBE, as described above, evaporation to dryness, and washes of the reconstituted extracts with hexane to remove contaminants. In both cases, the resulting chromatograms were free of interfering peaks. However, recovery using the first method was only 50 and 11% for M8 and NFV, respectively. These low recoveries probably resulted from the successive extractions. Moreover, washes with hexane increased the selectivity of the assay by removing the interfering peaks. In our approach, the washes with hexane did not involve the formation of any emulsion, and were performed by removing hexane by aspiration. The use of a snap-freeze step to remove hexane was time-consuming, difficult to execute routinely, and was unsuccessful as it asked the question of the stability of M8 and NFV in the aqueous phase after two freeze–thaw cycles. This method provided high recoveries for M8 and NFV in presence or absence of washes with hexane, showing that the analytes of interest were not eliminated through the washes with hexane.

During the optimization of the composition of the mobile phase, we observed that the pH was an influential parameter: NFV and M8 capacity factors decreased with the pH of the mobile phase between 6

Table 4
Within-day precision and accuracy data for NFV and M8 added to drug-free bovine serum (mean±SD, $n=12$)

Added concentration (μg/l)	Observed concentration (μg/l)	RSD (%)	Min (μg/l)	Max (μg/l)	Accuracy (%)
NFV					
50	50.67±1.47	2.90	49.06	53.90	101.34
200	201.32±5.21	2.59	192.83	212.04	100.66
1000	1068.83±32.82	3.07	996.07	1118.64	106.88
5600	5846.33±49.38	0.84	5729.86	5881.29	106.30
M8					
25	25.81±1.09	4.22	24.00	27.67	103.26
100	100.66±5.63	5.59	93.60	110.58	100.66
500	475.17±10.41	2.19	461.86	492.72	95.03
2500	2473.64±30.75	1.24	2416.70	2529.57	98.95

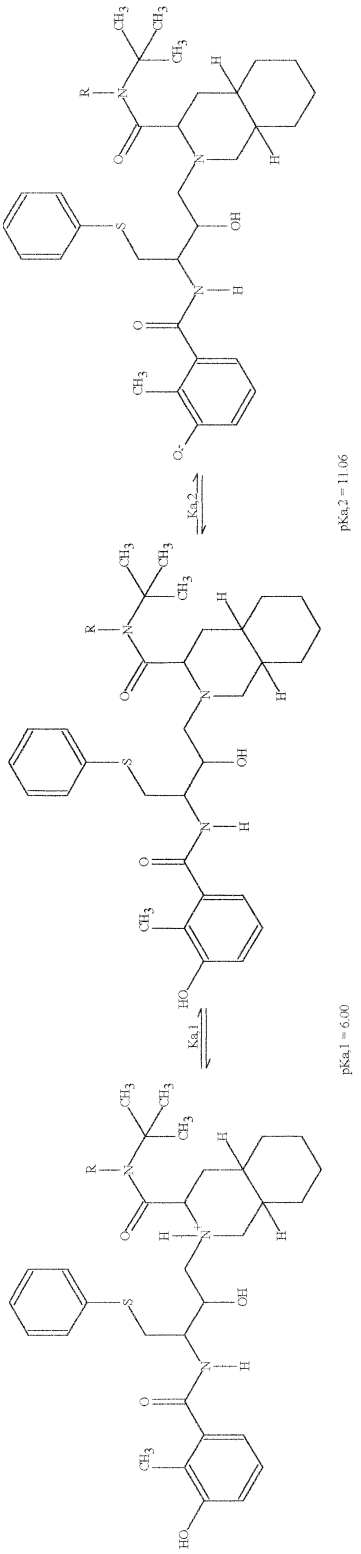

Fig. 3. Ionization scheme for NFV free base [19].

and 4. Moreover, spectral properties of M8 and NFV were highly dependant on the pH: the response of M8 and NFV in solution in methanol or in the mobile phase changes significantly. At 220 nm, we observed a maximal absorbance at pH 5, probably resulting from the ionization of M8 and NFV which is dependent on the pH (Fig. 3). This property guided our choice of the pH of the mobile phase.

Some compounds (indinavir, amprenavir, 25-*O*-desacetyl-rifabutin) are eluted at retention times close to the components of interest. Under the chromatographic conditions previously described, decreasing the amounts of acetonitrile in the mobile phase enhanced the resolution of all peaks. However, the running time of the assay was significantly increased. PDA, which allows one to match spectra of the eluted peaks, was also used.

This was of particular interest as it allowed us to explain a lack of adherence, drug–drug interactions, or to reveal unexpected compounds, in the context of drug monitoring.

The present assay reached a limit of quantification of 25 μg/l for both NFV and M8, which is consistent with the determination of the efficacy of the compounds, since the 95% inhibitory concentration (IC_{95}) determined in vitro in human T cell lines with acute HIV-1 infection ranged from 40 to 74 μg/l, and the minimal inhibitory concentration in vivo was estimated to 1000 μg/l in human plasma.

5. Conclusion

This is an original HPLC assay for the determination of both NFV and its active metabolite M8 in plasma. The method was found to be highly specific, accurate and sensitive. One hundred and twenty-nine clinical samples were successfully analyzed in our laboratory. It could be used for drug monitoring in every laboratory, and could be applied for the determination of pharmacokinetic parameters. Since it involves liquid–liquid extraction, it can be applied to biological matrices other than plasma like cerebrospinal fluid, saliva, urine or crushed tissue.

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