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Short communication

# Determination of the novel non-peptidic HIV-protease inhibitor tipranavir by HPLC–UV after solid-phase extraction

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**Abstract**

An HPLC method previously described for the assay of amprenavir (APV), ritonavir (RTV), indinavir (IDV), saquinavir (SQV), nelfinavir (NFV), lopinavir (LPV), atazanavir (ATV), nevirapine (NVP) and efavirenz (EFV) can be also conveniently applied, with minor gradient program adjustment, for the determination of the novel non-peptidic HIV protease inhibitor tipranavir (TPV) in human plasma, by off-line solid-phase extraction (SPE) followed by HPLC coupled with UV–diode array detection (DAD). After viral inactivation by heat, the plasma is diluted with phosphate buffer (pH 7), and subjected to a SPE on a C18 cartridge. Matrix components are eliminated with a solution of 0.1% $H_3PO_4$ solution neutralised to pH 7, and TPV is eluted with MeOH. The resulting eluate is evaporated and reconstituted in 100 μl MeOH/$H_2O$ 50/50. A 40 μl volume is injected onto a Nucleosil C18 AB column and TPV is analysed by UV detection at 201 nm using a gradient elution program constituted of MeCN and phosphate buffer adjusted to pH 5.12 and containing 0.02% sodium heptanesulfonate. The calibration curves are linear up to 75 μg/ml, with a lower limit of quantification of 0.125 μg/ml. The mean absolute recovery of TPV is 77.1 ± 4.0%. The method is precise with mean inter-day coefficient of variations (CVs) within 2.2–3.4%, and accurate (range of inter-day deviations from 0.7 to 1.2%). The method has been validated and is currently applied to the monitoring of TPV plasma levels in HIV patients.


*Keywords:* Tipranavir; Protease inhibitors; Therapeutic drug monitoring; Ion pair HPLC; Solid-phase extraction

## 1. Introduction

Tipranavir (TPV, Fig. 1) constitutes a novel class of non-peptidic protease inhibitor (PIs) with activity against both wild-type virus and variants resistant to current PIs [1], and is also characterized by a high genetic barrier [2]. Chemically, TPV was developed from coumarin and sulfonamide templates, and thus markedly departs structurally from all peptido-mimetic PIs developed at present [3]. The approved dose of TPV is 500 mg in association with 200 mg of ritonavir (TPV/r) taken twice daily as part of combination antiretroviral therapy for HIV-1 in heavily pre-treated patients having HIV-1 strains resistant to multiple PIs.

TPV is predominantly metabolized by, and is at the same time an inducer of cytochrome P450 3A4 (CYP3A4) enzyme. TPV is also a P-glycoprotein (P-gp) substrate, a weak P-gp inhibitor, and appears to be also a potent P-gp inducer, resulting in a net P-gp inducting effect on TPV/r at steady state [4]. As a result, TPV/r has a somewhat more complex pattern of drug interactions than other PIs. Combining amprenavir (APV), lopinavir (LPV) or saquinavir (SQV) with TPV/r is currently not recommended, because a clinically significant decrease in the co-administered drugs exposure was observed [4].

Since increasing clinical evidences indicate that levels of systemic exposure to PIs and non-nucleoside reverse transcriptase inhibitors (NNRTIs) correlate with efficacy as well as with some of their adverse effects, Therapeutic drug monitoring (TDM) of PIs and NNRTIs may be useful to optimise antiretroviral treatment. Numerous high performance liquid chromatography methods have been developed to quantify simultaneously the HIV PIs (i.e. indinavir (IDV), APV, SQV, ritonavir (RTV), nelfinavir (NFV), lopinavir (LPV), atazanavir (ATV)) and NNRTIs (efavirenz (EFV) and nevirapine (NVP))

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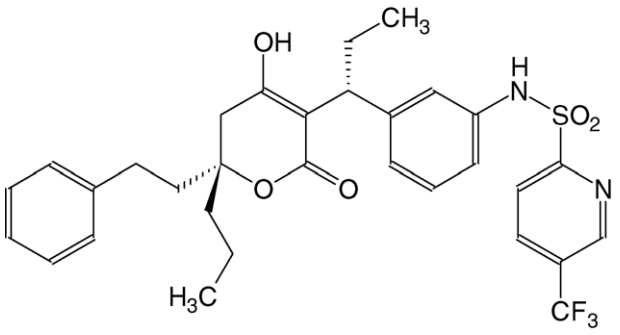


Fig. 1. Tipranavir chemical structure.

[5–13] (list not exhaustive). To date however, two assays only have been reported for the quantification of TPV, one using the LC tandem MS technology [14], and just recently another using by HPLC–UV after liquid–liquid extraction [15].

We report here a simple and robust method for the assay of TPV in human plasma, by offline solid-phase extraction (SPE) followed by HPLC coupled with UV–diode array detection (DAD), using the sample processing previously proposed for the first six marketed PIs, for EFV and NVP [5,6,16]. This method provides an excellent separation of TPV from the previously marketed HIV PIs and NNRTIs, enabling the accurate measurement of TPV in any concomitant antiretroviral treatment. The HPLC method for TPV has the required level of sensitivity and reproducibility for routine clinical application such as TDM.

## 2. Materials and methods

### 2.1. Chemicals

Tipranavir standard substance was kindly provided by Boehringer Ingelheim (Ridgefield, CT, USA); in initial development experiments, TPV stock solution (5 mg/ml) was obtained by extraction of Aptivus™ capsules (250 mg of TPV) with MeOH (in a 50 ml-volumetric flask), in which TPV is reported to be freely soluble [17]. Clozapine (internal standard, I.S.) stock solution (250 μg/ml) was obtained by extraction with MeOH of Leponex® (Novartis, Basel, Switzerland) tablet. This solution was diluted down to 45 μg/ml before use. Acetonitrile (MeCN), methanol (MeOH), 100% acetic acid (AcOH) and 85% phosphoric acid ($H_3PO_4$) were from E. Merck (Darmstadt, Germany). Sodium hydroxide puriss p.a. and sodium heptanesulfonate p.a. were purchased from Fluka (Buchs, Switzerland). All other chemicals were of analytical grade and used as received. Ultrapure water was obtained from a Milli-Q® UF-Plus apparatus (Millipore).

### 2.2. Chromatographic system

The chromatographic system consisted of a HP 1090 Series II (Agilent, formerly Hewlett-Packard, Germany) pump equipped with a spectrophotometric UV–DAD detector set at 201 nm. The separation was performed at room temperature (RT) on a ChromCart® cartridge column (125 × 4 mm I.D.) filled with Nucleosil 100–5 μm C18 AB (Macherey-Nagel, Düren, Germany) and equipped with a guard column (8 mm × 4 mm I.D.) filled with the same packing material.

Table 1
Gradient elution program

| Time (min) | Solvent A (%) | Solvent B (%) | Solvent C (%) |
|---|---|---|---|
| 0 | 15 | 85 | – |
| 2 | 30 | 70 | – |
| 8 | 32 | 68 | – |
| 10 | 35 | 65 | – |
| 12 | 40 | 60 | – |
| 34 | 60 | 40 | – |
| 35 | 100 | – | – |
| 36 | 10 | – | 90 |
| 40 | 10 | – | 90 |
| 41 | 33 | 34 | 33 |
| 42 | 46 | 54 | – |
| 42.5 | 15 | 85 | – |
| 50 | 15 | 85 | – |

Solvent (A) MeCN; solvent (B) $H_3PO_4$ 0.1% + 0.2 g/l sodium heptanosulfonate (pH 5.12); solvent (C) MeCN + 0.3% AcOH.

The mobile phase was delivered at 1 ml/min and the gradient program conditions are given in Table 1. Solvent A consisted of pure MeCN. Solution B was prepared by adding 11.8 ml $H_3PO_4$ 0.1% and 0.2 g sodium heptanesulfonate to 988.2 ml $H_2O$ with pH carefully adjusted to 5.12 with NaOH 10 N. The solvent C was 0.3% AcOH in MeCN. Solutions B and C were prepared prior to each series of analysis.

The HP-ChemStation A.06.03 software loaded on a Compaq DeskPro EP 6/400c was used to pilot the HPLC instrument and to process chromatographic data (area integration, calculation and plotting of chromatograms). In addition, the full-scan UV spectrum of TPV, recorded on-line by diode array detection, was carefully checked in all chromatograms from patients samples to verify that no co-eluting peak are present at TPV retention time.

### 2.3. Stock solution, working solution, plasma calibration and control samples

Stock solution of TPV (5 mg/ml) was further diluted with MeOH/$H_2O$ 50:50 for the preparation of working solutions. Plasma calibration samples at 1.875, 7.5, 18.75, 37.5, 60 and 75 μg/ml, together with plasma quality control (QC) samples at 5.625, 22.5 and 67.5 μg/ml, were prepared by dilution of working solutions by blank plasma from outdated transfusion bags (total added volume ≤10% of the biological sample volume), in accordance with the recommendations on bioanalytical method validation [18]. The buffer used for the dilution of plasma samples was prepared by mixing 82.6 ml of $KH_2PO_4$ 1/15M (9.97 g/l) and 117.4 ml of $Na_2HPO_4{\cdot}2H_2O$ 1/15 M (11.9 g/l) (=buffer D). The solid-phase extraction (SPE) cartridge conditioning (see below) was performed with a solution of 0.1% $H_3PO_4$ neutralized with NaOH to pH 7 (=buffer E).

The calibration standards and QC samples were prepared in batches at the same occasion and they were stored at −20 °C after viro-inactivation (see below).

### 2.4. Samples collection and HIV inactivation

Blood samples (5 ml) were collected as previously described [5,6,16]. After centrifugation at 1850 × *g* (3000 rpm) for 10 min at +4 °C (Beckmann Centrifuge, Model J6B), the plasma was heated at 60 °C for 60 min in a thermostated water bath (Memmert® WB 7, Schwabach, Germany) for viro-inactivation [19–22]. The processed plasma samples were stored at −20 °C until analysis.

The sample preparation and clean-up procedure is the same as that previously described for the former PIs and NNRTIs [5,6,16]. Briefly, 600 μl plasma samples (calibration, QC, patients) were diluted with 500 μl of solution D and 100 μl of I.S. solution in an Eppendorf microvial. The resulting solution was vortexed and centrifuged (5.0 min, 20,000 × *g*, 4 °C). After conditioning of C18 cartridges, an aliquot (1000 μl) of the diluted plasma sample was applied onto the cartridge and drawn through completely under vacuum. The cartridge was washed twice and TPV was desorbed with MeOH. The eluted solutions were evaporated and the residue reconstituted in 100 μl of MeOH:$H_2O$ 50:50. After centrifugation (20,000 × *g* for 5 min at 4 °C), the supernatants were introduced into 200 μl HPLC microvials and a volume of 40 μl was used for HPLC analysis.

### 2.5. Calibration curves

Quantitative analysis of TPV was performed using the I.S. method (I.S. = clozapine). The TPV calibration curve was established over the range 1.875–75 μg/m, and was fitted by least-squares linear regression using 1/concentration$^2$ ($1/x^2$) as weighting factor of the peak-area ratio of TPV to I.S. versus the ratio of the injected amount of the respective TPV to I.S., in each standard samples. The QC levels of TPV were selected to encompass the range of concentrations expected in patients plasma samples.

### 2.6. Analytical method validation

The validation of the method was based on FDA guidelines [23] and on the standard analytical method validation recommendations [18,24]. Each level of the calibration curve was measured with two sets of calibrators: one at the beginning and the second at the end of the run. QC samples were assayed throughout patient sample analysis.

Replicate analysis ($n = 6$) of QC samples were used for the precision and accuracy determination. Precision being calculated as the coefficient of variation (CV%) within a single run (intra-assay) and between different assays (inter-assays), and the accuracy as the percentage of deviation between nominal and measured concentration.

Both experimental lower limit of quantification (LLOQ) and limit of detection (LOD) were determined by diluting the calibration samples. The LLOQ for TPV in plasma was experimentally chosen as the minimal concentration in plasma samples which could be confidently determined in accordance with analytical method validation guidelines [18,23,24], recommending that the deviation between measured and nominal concentration at LLOQ should not deviate more than ± 20%. LLOQ was assessed by analysing triplicate TPV samples between 0.075 and 1.875 μg/ml. The limit of detection (LOD) was considered as the concentration of TPV that provides a signal corresponding to three times the HPLC background signal.

### 2.7. Stability of TPV

The stability of TPV in plasma under the thermisation process (60 °C for 60 min) was assessed as follows: two series of QC samples in duplicate were analysed in parallel. One was heated, while the thermisation procedure was omitted in the second. Similarly, a calibration curve in duplicate was also heated. All the samples were submitted simultaneously to SPE extraction according to the procedure described above. The variation of TPV concentrations were expressed in percentage of the levels of samples not subjected to thermisation.

Freeze–thaw stability was determined by assaying duplicate QC samples over two freeze–thawing cycles, according to the previously described methodology [16]. The stability of processed samples (i.e. reconstituted in MeOH:$H_2O$ 50:50) into HPLC vials was assessed with duplicated TPV QC samples. These latter were analysed either immediately after preparation, or after being left 120 h at RT in the auto-sampler rack. The results were compared.

The short-term temperature stability at RT was determined as follow: triplicate aliquots of plasma spiked with TPV at 1.875, 7.5, 18.75, 37.5, 60 and 75 μg/ml were thawed and the volume was divided into two tubes (A and B). Tubes A were kept at RT for 24 h, which corresponds to the maximal duration that samples may be left in this condition. Tubes B were stored for 24 h at 4 °C. The slopes of TPV calibration curves in both groups were compared (paired Student's *t*-test).

### 2.8. Recovery and selectivity

The efficiency of the solid phase extraction was determined with triplicate QC samples. The determination of the absolute recovery of drug from plasma was performed as previously described [16].

The selectivity of the proposed analytical method was determined by injecting onto the HPLC column drugs currently prescribed to HIV-infected patients at our hospital: stavudine (Zerit®), zidovudine/lamivudine (Combivir®), didanosine (Videx®), abacavir (Ziagen®), tenofovir (Viread®), EFV (Stocrin®), NVP (Viramune®), SQV (Invirase®, Fortovase®), RTV (Norvir®), IDV (Crixivan®), NFV (Viracept®), M8 (pharmacologically active metabolite of NFV), fos-/APV (Telzir®/Agenerase®), LPV/r (Kaletra®), ATV (Reyataz®), atorvastatine (Sortis®), loperamide (Imodium®), trimethoprim/sulfamethoxazole (Bactrim®), levofloxacine (Tavanic®), azithromycine (Zithromax®), piperacilin/tazobactam (Tazobac®), ceftazidine (Fortam®), meropenem (Meronem®), cefepime (Maxipime®), imipenem/cilastatine (Tienam®), itraconazole (Sporanox®), amoxicillin/clavunalic acid (Augmentin®), azathioprine (Imurek®), rifampicin (Rimactan®), sulfasalazine (Salazapyrin®), fluconazole

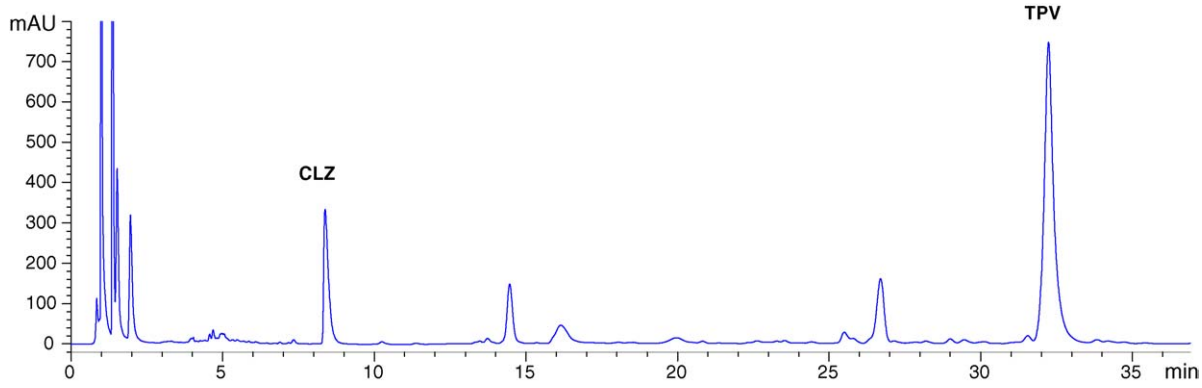


Fig. 2. Chromatographic profile of a calibration sample of TPV (37.5 μg/ml) spiked with I.S. (CLZ).

(Diflucan®), ciprofloxacine (Ciproxine®), vancomycine (Vancocin®), voriconazole (Vfend®), acetylsalicylic acid (Aspirin®), mefenamic acid (Ponstan®), acenocoumarol (Sintrom®), esomeprazole (Nexium®), amlodipine (Norvasc®), candesartan (Atacand®), prednisone (Prednisone®), pancreatine (Creon®), tramadol (Tramal®), residronate (Actonel®), spironoloctone/furosemide (Lasilactone®), torasemid (Torem®), atenolol/chlortalidone (Tenoretic®), bromazepam (Lexotanil®), oxazepam (Seresta®), cisapride (Prepulsid®), diazepam (Valium®), ciclosporin (Sandimmun®), mycophenolate (Cellcept®) and tacrolimus (Prograf®).

## 3. Results and discussion

### 3.1. Chromatograms

The proposed HPLC method enables the measurement of TPV in plasma with UV detection at 201 nm, with clozapine and TPV retention times of 8.3 and 32.2 min, respectively (Fig. 2). Fig. 3 shows a TPV QC sample at 22.5 μg/ml onto which all marketed PIs/NNRTIs have been added, demonstrating the absence of chromatographic interferences with TPV. Fig. 4 shows a plasma chromatographic profile from a patient receiving a regimen of TPV 500 mg BID, LPV 400 mg BID, RTV 100 mg BID, lamivudine 150 mg BID and tenofovir 300 mg QD. The level of TPV measured 6 h 30 min after the Aptivus™ intake is 35.6 μg/ml and the LPV level is 3.8 μg/ml.

### 3.2. Calibration curves

As previously reported [16], the model with the lowest total bias and the most constant bias across the concentration range was considered to be the best fit. Visual inspection of the plot of residuals of the $1/x^2$ weighted regression indicates that there is no trend in variability throughout the delineated range of concentrations. Over the concentration range from 1.875 to 75 μg/ml for TPV, the regression coefficient $r^2$ of the calibration curves remained excellent, always greater than 0.999.

### 3.3. Validation of the HPLC method: precision, accuracy, LLOQ/LOD and recovery

The method is precise with mean inter-day CVs for the 3 plasma QC samples within 2.2–3.4%, and accurate (range of inter-day deviations from 0.7 to 1.2%).

The LLOQ for TPV was experimentally found to be 0.125 μg/ml (with of precision and deviation of 11 and 19%, respectively), and the LOD was 0.075 μg/ml.

The mean absolute recovery of TPV measured with the three QC levels was 77.1 ± 4.0%. The clean-up procedure by SPE was

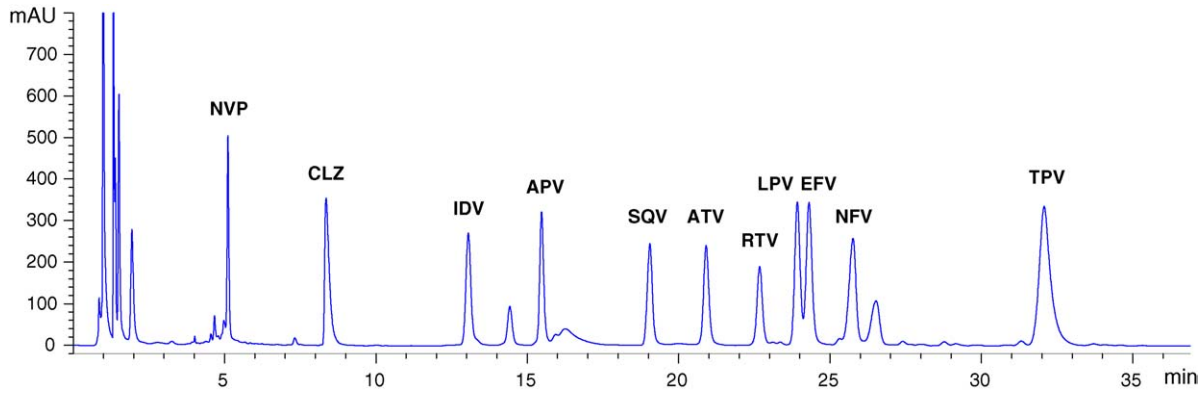


Fig. 3. Chromatographic profile of a plasma control sample of TPV (22.5 μg/ml) spiked with I.S. (CLZ) and with all PIs/NNRTIs available at present.

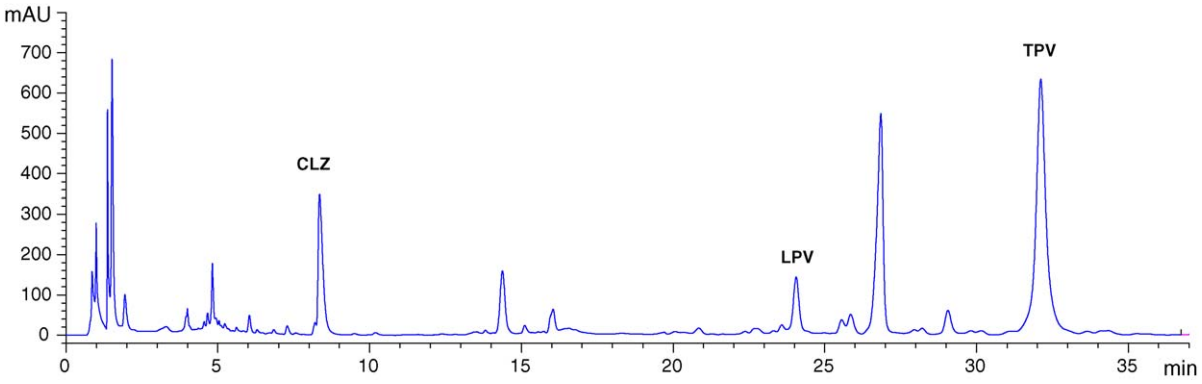


Fig. 4. Plasma chromatogram of one HIV-patient receiving TPV, LPV, RTV, lamivudine and tenofovir (details in the text).

found to be a reliable way of eliminating interfering material from plasma, with low recovery variability.

### 3.4. Selectivity

At 201 nm, the large series of tested drugs gave no interfering peaks at the retention time of TPV, except for tacrolimus, which appears nearby TPV as a broad peak at a retention time at 33.9 min.

### 3.5. Samples stability

#### 3.5.1. Stability during thermisation (HIV inactivation)

Back-calculated values of both thermised and non-thermised QCs samples using calibration curves established with thermised samples had deviations from nominal concentration always lower than 5 and 12%, respectively. Considering the experimental variability, and by comparison with stability studies performed with previous PIs subjected to the same procedure [5,6,16], these results indicate that such a procedure does not affect, or only slightly, TPV concentrations, within the considered concentrations range. Nevertheless, and for the sake of standardisation, all calibration and control samples were treated similarly and heated at 60 °C for 60 min.

#### 3.5.2. Stability of plasma samples at room temperature

The mean slope of calibration curves established with samples left 24 h at RT ($m = 1.590$, $n = 3$) was not different than the slope of calibration curves calculated with samples stored during the same time at 4 °C ($m = 1.596$; $n = 3$), ($p = 0.9$, paired Student's *t*-test), indicating a good stability of TPV in plasma at RT.

#### 3.5.3. Stability of plasma samples after one and two freeze–thaw cycles

The variation of TPV concentration was expressed as percent of the initial TPV concentration (no thaw–freeze cycle). At the three QC levels, the percent decrease in initial TPV concentration was lower than 4 and 15%, for one and two freeze–thaw cycles, respectively. Taking into account the analytical variability, this indicates that no significant loss of TPV is to be expected at least after one freeze–thaw cycle.

#### 3.5.4. Stability of extracts samples at room temperature

Plasma extracts (i.e. reconstituted in MeOH:$H_2O$ 50:50) left at RT for 5 days (120 h), showed a TPV variations (expressed in percentage of change of the starting levels, i.e. after immediate analysis) lower then $-1.9 \pm 1.1$%. These results indicate that the processed samples stability is excellent throughout the HPLC run performed over one day.

### 3.6. Clinical applications

This HPLC assay is currently used for the analysis of samples collected for the Therapeutic Drug Monitoring of TPV in HIV-positive patients, mainly in conjunction with the *Swiss Tipranavir Compassionate Use Program*, for assessing drug exposure and evaluating drug–drug interactions and ascertaining short term compliance. A number of drug–drug interactions has been documented for TPV and the FDA has recently issued warnings about this problem [25]. In addition, safety studies reported by the Tipranavir Review Team [26] have found a relationship between TPV trough concentration and the probability of having elevated liver enzymes. Similarly, Giraud et al. have recently reported the case of one patient who had a TPV trough concentration of 50.8 μg/ml and who developed a few days later an hepatic cytolysis [15]. If these observations are confirmed, the monitoring of TPV plasma concentrations may thus play an important role in the management of patients.

## 4. Conclusions

This HPLC method provides a robust and convenient procedure for determining TPV in plasma by using samples processing, column type and solvent mixture previously proposed for the previous PIs and NNRTIs [5,6,16], and run routinely in our laboratory for many years. This is a clear advantage for a laboratory where large numbers of samples containing multiple analytes are to be analysed on a routine basis.

To the best of our knowledge, this is the first report describing an assay of TPV by HPLC–UV at 201 nm, after SPE from plasma. Just recently, Giraud et al. has reported an assay for TPV by HPLC–UV at 260 nm after liquid–liquid extraction [15]. At this wavelength however, their method does not allow the simultaneous measurement of IDV. Our method enables the analysis

of the eight PIs marketed to date (TPV, ATV, SQV, RTV, IDV, NFV, APV and LPV) and two NNRTIs (NVP and EFV). It can be used conveniently on a routine basis, with minor adjustments, with one single sample processing procedure followed by a chromatographic step on an identical column type, using the same solvent mixture, changing only the elution gradient program for NVP, LPV [6] and TPV. Our approach represents therefore a useful and convenient tool contributing to the optimal follow-up of HIV patients through TDM.

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