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Short communication

A simple and sensitive assay for determining plasma tipranavir concentration in the clinical setting by new HPLC method

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Abstract

A simple method for the quantification of tipranavir, the first non-peptidic HIV protease inhibitor, was developed and validated. Quinoxaline, as internal standard, was added to 50 μl of plasma before a liquid–liquid extraction by 600 μl of protein precipitation solution. The extracts were diluted before being injected in the chromatographic system. Chromatographic separation was made on a C18 column using potassium phosphate buffer (pH 3.2) and acetonitrile with gradient. Detection was performed by an UV detector at 260 nm. Relative error at three control quality concentrations ranged from −1.81 to 1.72%. Intra-day (CV%) and inter-day (CV%) precision ranged from 0.94 to 2.55% and from 3.07 to 4.24%, respectively. LOQ and LOD were 0.090 μg/ml and 0.035 μg/ml, respectively. Mean recovery was 87.1% ± 2.4%. Calibration curve was linear up to 180 μg/ml. Concentration range when optimized (0.703–180 μg/ml) proved to be adequate to measure tipranavir concentration in HIV-1-positive patients, therefore this method could be suitable for therapeutic drug monitoring of this drug.

Keywords: Tipranavir; UV detector; Liquid/liquid extraction; TDM

1. Introduction

Tipranavir (TPV) is the first non-peptidic protease inhibitor (PI) licensed for the treatment of HIV-1 infection. It showed to be active also in patients with extensive previous PI experience, who have accumulated broad cross-resistance to the compounds of this class [1]. It is recommended, at the dose of 500 mg boosted with ritonavir 200 mg twice daily (bid). Such boosting is essential to achieve appropriate TPV plasma concentrations. The selection of resistance to TPV requires the accumulation of a high number of mutations in the HIV protease gene, [2], therefore it can be still active as a component of many salvage regimens.

From a pharmocokinetic/pharmacodynamic viewpoint, TPV trough concentration of 12 μg/ml, 10-fold higher than the median IC90 of resistant HIV strains, was suggested as the minimum value associated with optimal antiviral efficacy [3]. As TPV is extensively metabolised by the isoform 3A4 of the hepatic cytochrome P450, a metabolic pathway known for high inter-individual variability, in the clinical setting some patients could not reach this target concentration value [4].

TPV is also a P-glycoprotein (P-gp) substrate, a weak P-gp inhibitor, and also a potent P-gp inducer, resulting in a net P-gp inducing effect of TPV/ritonavir (TPV/r) at steady state [5]. 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 of plasma levels of the co-administered drugs was observed [5].

Therapeutic drug monitoring (TDM) of anti-HIV drugs, especially of PIs and non-nucleoside reverse transcriptase inhibitors (NNRTIs), has been shown to be a useful tool for the management of antiretroviral therapy [6–8]. In this context, TDM should be even more important for TPV-containing salvage therapy, due to the crucial interplay between plasma concentrations and resistance mutations to achieve virological response [9], and to the potential for drug–drug interaction.

Some quantification methods for TPV, using separation by HPLC and detection by UV or electrospray ionisation tandem mass spectrometry, have already been described [10–13].

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Therefore, our aim was to develop a rapid, sensitive, precise, rugged and accurate HPLC method for measurement of TPV concentration in clinical plasma samples.

2. Materials and methods

2.1. Chemicals

TPV was kindly provided by Boehringer Ingelheim (Ridgefield, CT, USA). Methanol, quinoxaline, *ortho*-phosphoric acid and potassium dihydrogen phosphate were obtained from Sigma–Aldrich (Milan, Italy). Acetonitrile was obtained from J.T. Baker (Deventer, Holland). HPLC-grade water was produced with a Milli-DI system by Millipore (Milan, Italy). Plasma from healthy donors was kindly supplied by the Blood Bank of Maria Vittoria Hospital (Turin, Italy).

2.2. Materials and chromatographic conditions

The HPLC system used to assay TPV consisted of a pump model 515, an 717 autosampler, and 2487 UV detector (Waters, Milan, Italy). The detector was operated at a wavelength of 260 nm. A Pump Control Module (PCM) interface and Millenium^{32} software (Version 3.20, Waters) were used for management of the HPLC system.

Chromatographic separation was performed by a Luna 5 μ C18 column (150 mm × 4.6 mm i.d., Phenomenex, CA, USA), protected by a SecurityGuard with C18 (4.0 mm × 3.0 mm i.d., Phenomenex), at 45 °C using a column thermostat TS130 (Phenomenex).

The run was performed at 1 ml/min, the mobile phase was composed of Buffer A (KH_2PO_4 50 mM with *ortho*-phosphoric acid, final pH 3.23) and acetonitrile, and the gradient was: from 0.0 to 1.0 min 50% Buffer A and 50% acetonitrile, from 1.0 to 4.0 min there was a linear increment of acetonitrile to 70% and from 4.0 to 6.0 min to 75%. This condition was conserved until 9.5 min, then from 10.0 to 14.0 min in the initial condition.

2.3. Stock solutions, standards (STD) and quality controls (QC)

A stock solution of TPV was prepared in methanol:water (95:5) with a final concentration of 10 mg/ml and stored at −20 °C until use no more than 2 months [11]. The highest concentration point of the calibration curve was 180 μg/ml (STD 9) and was prepared by addition of 180 μl of the stock solution in 10 ml of drug-free plasma. Quality controls (QCs) were prepared in the same way, with concentrations of 100 (QC 1), 25 (QC 2) and 2.5 μg/ml (QC 3).

STDs were prepared by serial dilution from 180 (STD 9) to 0.703 μg/ml with drug-free plasma, to obtain 9 different spiked concentrations plus a blank sample.

STDs, QCs and patient samples underwent heat inactivation for HIV (35 min at 58 °C), stored at −20 °C until analyses, and used within 2 months [11]. In this condition, TPV was shown to be stable, also after samples inactivation [10,11].

The calibration curve included a wide range of TPV concentrations. The choice of this range was based on the values reported in previous clinical studies [5,14–16].

The quinoxaline stock solution was made in methanol:water (95:5) at a final concentration of 1 mg/ml and stored at 4 °C until use. The Internal Standard (IS) working solution was made in methanol:water (50:50) at final concentration of 7.5 μg/ml.

2.4. Clinical samples, standards and QCs processing

Blood samples, collected in lithium heparin tube (7 ml), were obtained from HIV-positive patients administered with TPV at standard doses. To avoid thawing cycles each patient plasma sample was aliquoted into two criovials of 300 μl.

Plasma was collected after centrifugation at 1400 × *g* (3000 rpm) for 10 min at +4 °C (Jouan Centrifuge, Model BR4i, Saint-Herblain, France) and then undergone heat inactivation, as described above.

Six hundred microlitres of protein precipitation solution (methanol:acetonitrile [50:50]) and 40 μl of IS working solution were added to 50 μl aliquot of plasma samples, in a PTFE microfuge tube. After vortexing for 30 s, the mixture was centrifuged at 12,000 rpm for 10 min at 4 °C. A volume of 300 μl of supernatant was transferred to a glass vial, diluted with 300 μl of water:acetonitrile (50:50) and then 100 μl were injected into the HPLC column. All analyses were performed in duplicate, and all procedure steps were carried out at room temperature.

2.5. Specificity and selectivity

Interference from endogenous compounds was investigated by the analysis of five different blank plasma samples. Potential interference by antiretroviral drugs concomitantly administered to the patients was also evaluated spiking plasma. These included: nucleoside or nucleotide reverse transcriptase HIV inhibitors (zidovudine, didanosine, stavudine, lamivudine, abacavir, tenofovir), non-nucleoside reverse transcriptase HIV inhibitors (nevirapine, efavirenz), fusion inhibitor (enfuvirtide) and protease inhibitors (saquinavir, nelfinavir and its active metabolite M-8, indinavir, amprenavir, atazanavir, ritonavir, lopinavir). Other concomitant drugs were also investigated (see Table 1).

2.6. Accuracy, precision, and limit of quantification

Intra-day and inter-day accuracy and precision of the method were determined by assaying 10 spiked plasma samples at three different concentrations (QCs). Accuracy was calculated as the percent deviation from the nominal concentration. Inter-day and intra-day precision were expressed as the standard deviation at each QC concentration.

The calibration curve was obtained using 9 calibration points in duplicate, ranging from 0.703 to 180 μg/ml, and considering analyte-to-IS area peaks ratio.

The limit of detection (LOD) in plasma was defined as the concentration that yields a signal-to-noise ratio of 3/1. Percent deviation from the nominal concentration (measure of accuracy)

Table 1
Concomitant drugs administered to the patients

Antitubercoulous	Antidepressant/benzodiazepines	Antiacid	Lipid lowering	Others	
Ethambutol	Alprazolam	Esomeprazole	Atorvastatin	Acetilsalicilic acid	Levotiroxine
Isoniazide	Bromazepam	Omeprazole	Bezafibrate	Amoxicillin	Loperamide
Piridoxin	Citalopram	Ranitidine	Fenofibrate	Atenolol	Metformin
Rifabutin	Clonazepam		Pravastatin	Atovaquone	Methadone
Rifampin	Clordemetildiazepam			Azitromicin	Metronidazole
	Desipramine			Benzil-penicillin	*N*-Acetil cystein
	Lorazepam			Candertesan cilexetil	Nimesulide
	Mirtazapine			Clavulanic acid	Paracetamol
	Sertraline			Co-trimoxazole	Phenobarbital
	Trazodone			Diclofenac	Pyrimethamine
	Triazolam			Ditiazem	Ramipril
	Zolpidem			Enalapril maleate	Repaglinide
				Folic acid	Tamsulosin
				Gabapentin	Telmisartan
				Ganciclovir	Ursodesossicolic acid
				Glybenclamide	Valacyclovir
				Iosartan	Valproate
				Lercanidipine	Warfarin

and relative standard deviation (measure of precision) of the concentration considered as the limit of quantification (LOQ) had to be <20%.

2.7. Recovery

Average recovery of TPV was determined by comparing the peak area of the drug extracted from spiked plasma samples (100, 25, and 2.5 μg/ml) with those obtained by direct injection of the same amount of drug.

2.8. Stability

Since other authors previously showed that TPV was stable up to 2 months, no stability study was conducted. All samples, STDs and QCs, were analysed within 2 months since their freezing [10–12].

3. Results

Retention times of IS and TPV were 5.6 (±0.1) and 10.0 (±0.1) min, respectively.

Representative chromatograms of the blank human plasma extract, LOQ and STD 7 are shown in Fig. 1. The calibration curve was linear up to 180 μg/ml, with mean $r^2 = 0.999$ ($n = 10$). Standard curves were calculated by the equation $Y = mX + c$ and resulting $Y = 0.0000292\ (\pm 0.0000001)X$, where "$c$" = 0 (curve forced through zero).

3.1. Specificity and selectivity

The assay did not show any significant interference by antiretroviral drugs, as listed in Section 2, and also by other concomitant drugs reported in Table 1.

There is no endogenous interference in blank plasma extract (see Fig. 1).

3.2. Accuracy, precision, and limit of quantification

Results of validation of the method are shown in Table 2.

The relative error at three concentrations ranged from −1.81 to 1.72%. Intra-day (CV%) and inter-day (CV%) precision ranged from 0.94 to 2.55% and from 3.07 to 4.24%, respectively.

LOQ and LOD were 0.090 and 0.035 μg/ml, respectively.

3.3. Recovery

Multiple aliquots ($n = 8$) at each different concentration were assayed. Mean recovery of tipranavir at 100, 25 and 2.5 μg/ml was 87.1 ± 2.4%.

3.4. Analysis of samples from patients

Four hundred and fifty three samples obtained from 55 patients were analysed in duplicate. Overall blood samples were obtained along the whole dosing interval (Fig. 2). Mean ± S.D.

Table 2
Summary of accuracy and precision during method validation at low, medium and high concentrations ($n = 10$)

Nominal concentration (μg/ml)	Measured concentration (μg/ml)	Accuracy (%)	Inter-day precision CV (%)	Intra-day precision CV (%)
2.500	2.476	−0.96	4.24	2.55
25.000	25.432	1.72	3.36	0.94
100.000	98.180	−1.81	3.07	1.93

The analyses were performed in 10 different days. CV: coefficient of variation.

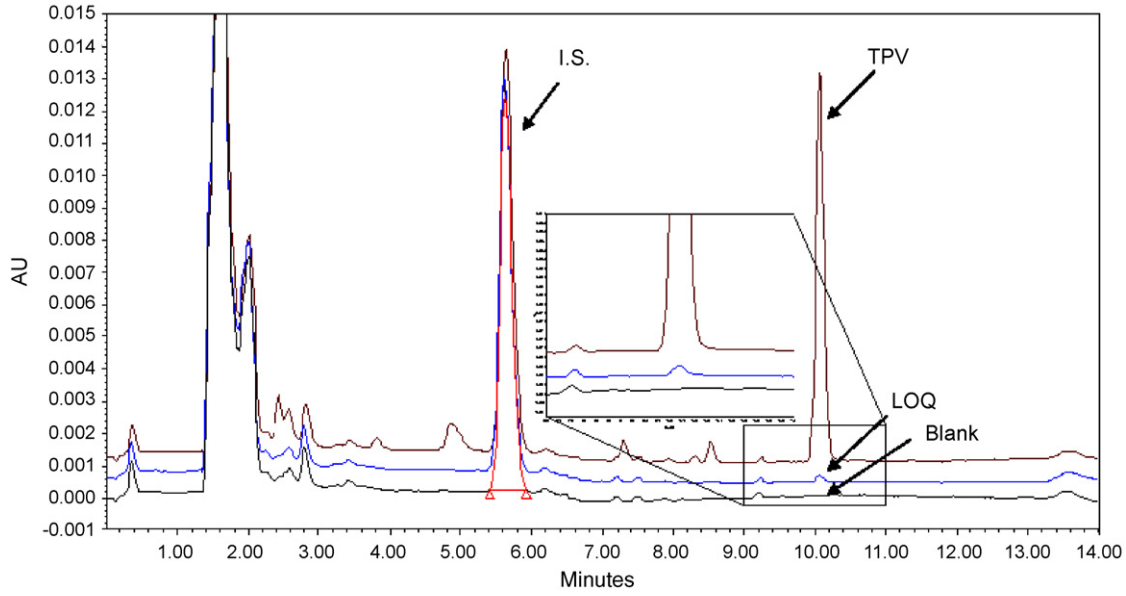

Fig. 1. Overlay chromatograms of a blank human plasma, LOQ level [0.090 μg/ml of tipranavir] and STD 7 [45 μg/ml of Tipranavir] (TPV).

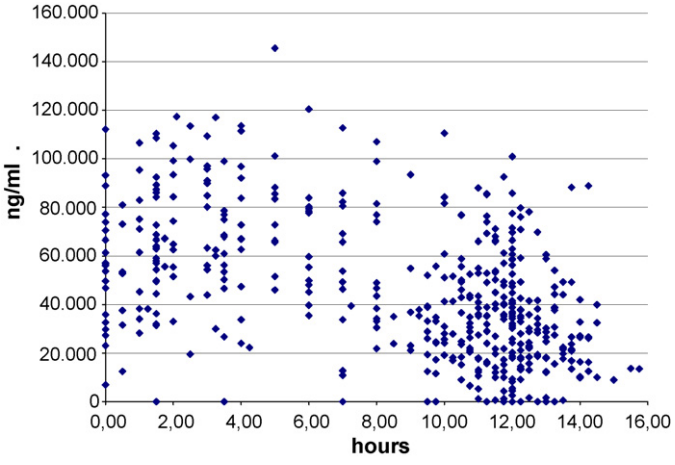

Fig. 2. Distribution of TPV concentrations over the dosing interval.

tipranavir plasma concentration was 44.909 ± 27.367 μg/ml. Maximum and minimum values were 145.618 and 0.598 μg/ml, respectively.

4. Discussion and conclusion

In our study, a new method to measure TPV plasma concentration was validated. As compared to previously published methods [10–13], our assay provides some technical and time saving advantages.

Firstly, one of the published method is based on solid phase extraction with C18 columns [10]. We also tried to use, during the development of the method, C18 columns (Lichrolut RP-18, 100 mg, 40–63 μm, Merck, Damstadt, Germany) with an extraction procedure similar to Colombo et al. [10], but we observed a lack of recovery (>20%, comparing SPE with liquid/liquid extraction) in 47% of patient samples. Possible reason of such occurrence was not investigated and this strategy was not further considered in our procedure. Our liquid/liquid extraction procedure did not compromise the specificity and selectivity, as shown by the lack of neither endogenous nor exogenous interferences on TPV retention time. Specificity was enhanced by detection at wavelength at 260 nm, avoiding also any possible interference. Moreover, this extraction procedure provided a good recovery from human plasma samples.

Another advantage related to the validation of our method was the short duration of extraction time (15 min) and analysis run time. In fact, IS and TPV peaks eluted in 5.6 and 10.0 min, respectively, and the system was ready for a new analysis after 14 min, including wash and conditioning steps. These retention times are shorter than the ones reported in the previously published methods [10,12,13]. Like Dailly et al., we preferred to not use a drug as IS. Prazepam and clozapine, used as IS by Giraud et al. and by Colombo et al., respectively, are licensed drugs and can be potentially co-administered with TPV [10,12,13]. Moreover, our IS is widely available and can be easily purchased.

The calibration curve of our method covered a wide range of TPV concentrations. It included the highest TPV levels observed in patients and LOQ can quantify the lowest C_{trough} values reported in the clinical setting [9,15,16]. In our patient samples, observed mean ± S.D. TPV plasma concentration was 44.909 ± 27.367 μg/ml, and maximum and minimum values were 126.634 and 0.598 μg/ml, respectively. Our measurements are slightly higher than few data previously obtained in clinical trials [16] and will be part of a pharmacokinetic publication. The other published methods have a very limited calibration range [10–13], ideally not able to cover the possible range of concentrations that can be found in the clinical setting.

Finally, our method produced good recovery, with low intra-day and inter-day CV% and the linearity of TPV standard curve is excellent. Moreover, in our procedure TPV measurements were performed in duplicate and results were validate with

RDS% <15%. A 50 μl aliquot of plasma was used, a really small amount as compared to other methods [10–13].

In conclusion, we validated a new liquid/liquid extraction method coupled with HPLC-UV detection for the measurement of TPV plasma concentrations. The method is simple to perform, accurate, cheap and rapid, warranting further evaluation as a tool for TDM of TPV in the clinical setting.

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References

[1] S. Rusconi, C.S. La Seta, P. Citterio, S. Kurtagic, M. Violin, C. Balotta, M. Moroni, M. Galli, A. D'Arminio-Monforte, Antimicrob. Agents Chemother. 44 (2000) 1328.
[2] G.L. Plosker, D.P. Figgit, Drugs 63 (2003) 1611.
[3] P. Yeni, J. Acquir. Immune Defic. Syndr. 34 (2003) 91.
[4] P. Yeni, T. MacGregor, J. Ghate, K. Arasteh, D. Jayaweera, J. Jemsek, T. Hawkins, W. Cameron, N. Bodsworth, S. McCallister, V. Kohlbrenner, A. Quinson, J. Leith, J. Sabo, D. Mayers, Proceedings of the 10th Conference on Retroviruses and Opportunistic Infections, Boston, MA, February 10–14, 2003.
[5] Food and Drug Administration, Tipranavir, label information (NDA 21–814), 2005.
[6] R.E. Aarnoutse, J.M. Schapiro, C.A. Boucher, Y.A. Hekster, D.M. Burgers, Drugs 63 (2003) 741.
[7] C. Chaix, C. Grenier-Sennelier, P. Clevenbergh, J. Durant, J.M. Schapiro, P. Dellamonica, I. Durand-Zaleski, J. Acquir. Immune Defic. Syndr. 24 (2000) 227.
[8] A.L. Rendón, M. Núñez, I. Jiménez-Nácher, D. González de Requena, J. González-Lahoz, V. Soriano, HIV Med. 6 (2005) 360.
[9] S. Bonora, D. Gonzalez de Requena, A. Calcagno, M. Milia, A. D'Avolio, M. Sciandra, S. Garazzino, M. Siccardi, A. Sinicco, G. Di Perri, 13th Conference on Retroviruses and Opportunistic Infections, Denver, CO, February 5–8, 2006 (poster no. 577).
[10] S. Colombo, A. Beguin, C. Marzolini, A. Telenti, J. Biollaz, L.A. Decosterd, J. Chromatogr. B 832 (2006) 138.
[11] K.M.L. Crommentuyn, H. Rosing, M.J.X. Hillebrand, A.D.R. Huitema, J.H. Beijnen, J. Chromatogr. B 804 (2004) 359.
[12] E. Giraud, E. Rey, J.M. Treluyer, G. Pons, V. Jullien, J. Chromatogr. B 830 (2006) 86.
[13] E. Dailly, V. Reliquet, C. Victorri-Vigneau, F. Raffi, P. Jolliet, J. Chromatogr. B 832 (2006) 317.
[14] Boehringer Ingelheim, Investigator's brochure, doc. no. U00-3106, Project no. 1182, Version no. 5, 2002.
[15] D. Gonzalez de Requena, A. Calcagno, S. Bonora, L. Ladetto, A. D'Avolio, M. Sciandra, M. Siccardi, O. Bargiacchi, A. Sinicco, G. Di Perri, 13th Conference on Retroviruses and Opportunistic Infections, Denver, CO, February 5–8, 2006 (poster no. 579).
[16] C.L. Yong, J.P. Sabo, C.G. Oksala, T.R. McGregor, V. Kohlbrenner, S. McCallister, J. Leith, D. Mayers, 12th Conference on Retroviruses and Opportunistic Infections, Boston, MA, February 22–25, 2005 (poster no. L-109).