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Quantification of tipranavir in human plasma by high-performance liquid chromatography with UV detection

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Abstract

A simple method for the quantification of tipranavir, a new non-peptidic protease-inhibitor, was developed. An internal standard, prazepam, was added to 100 μl of plasma before a liquid–liquid extraction by 3 ml of *tert*-butyl methyl ether. The extracts were evaporated to dryness and reconstituted with 100 μl of mobile phase before being injected in the chromatographic system. The separation was made on a C8 column using sodium acetate buffer (pH 5):methanol:acetonitrile (35:30:35, v/v/v) as mobile phase. The detection was performed at a wavelength of 260 nm. The method was linear and has been validated over a concentration range of 2–80 mg/l. The mean precision and accuracy of the method were respectively, 10.5 and −9.1%. The mean recovery was 70.8%.

Keywords: Tipranavir; Liquid chromatography; UV detection

1. Introduction

Tipranavir (TPV) is a novel non peptidic protease inhibitor (PI) used for the treatment of human immunodeficiency virus type-1 (HIV-1) infection.

In vitro studies have shown its efficacy on viral strains resistant to other PIs [1,2]. These results have been clinically confirmed by phase II studies as tipranavir treatment allowed sustained viral load suppression in heavily pretreated patients who previously experienced therapy failures with other PIs [3,4]. TPV is therefore recommended for the treatment of HIV-1-infected patients resistant to other PIs at the dose of 500 mg twice daily (bid) boosted with ritonavir 200 mg bid.

As it has been described for many PIs [5] a threshold through concentration is recommended for TPV. Indeed a target trough value of 20 μmol/l (i.e. 12 mg/l) corresponding to 10 times the IC90 of resistant HIV strains seems necessary to provide an optimal antiviral efficacy [1]. As tipranavir is extensively metabolised by hepatic cytochrome P450 3A4 [1], a metabolic pathway known for its high interindividual variability [6], some patients may not reach this 12 mg/l target value. This was confirmed in the phase II study cited above as 21% of the patients had a through concentration lower than 12 mg/l [7]. Therapeutic drug monitoring (TDM) and dose adjustment is commonly accepted as an important tool for antiretroviral therapy with PIs [8], as suboptimal PI concentration can lead to resistance emergence and therapy failure. TDM should be even more important during tipranavir therapy as almost no other treatment possibility remains in patients requiring this drug. An assay for TPV plasma concentration is therefore necessary to perform tipranavir TDM and to assess the possible drug interactions (patients being heavily treated). A dosage method has already been reported [9] using separation by HPLC and detection by electrospray ionisation tandem mass spectrometry. But the equipment used in this method is expensive. We therefore developed an assay using high performance liquid chromatography (HPLC) with UV detection. Such an assay could be used in every laboratory practising TDM for antiretroviral drugs.

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2. Experimental

2.1. Chemicals

Tipranavir was kindly supplied by Boehringer Ingelheim Pharmaceuticals (Ridgefield, CT, USA). The internal standard (IS) prazepam was obtained from Sigma chemical company (St Louis, MO, USA). The structure of TPV and the IS prazepam are described in Fig. 1.

Chromanorm® methanol for HPLC was purchased from Prolabo (Fontenay-sous-Bois, France). LiChrosolv® acetonitrile gradient grade for liquid chromatography, *tert*-butyl methyl ether (TBME) for spectroscopy and anhydrous sodium acetate GR were obtained from Merck (Darmstadt, Germany). Acetic acid (99–100%) came from Fischer scientific s.a. (Elancourt, France).

2.2. Chromatographic and detection conditions

The HPLC system consisted of a SpectraSYSTEM SCM 1000® degassing system, a SpectraSYSTEM P1000XR® pump, a SpectraSYSTEM AS3000® autosampler, a SpectraSYSTEM UV6000LP® UV detector and a SpectraPHORESIS SN4000® interface (ThermoFinnigan, Les Ulis, France).

The chromatographic separation was accomplished on a C8 plus Satisfaction column (250 mm × 3 mm, 5 μm; Cluzeau, Sainte-Foy la Grande, France) protected by a guard column (15 mm × 3 mm) of the same phase and a A-103× filter (Cluzeau).

The flow of the mobile phase which consisted of acetate buffer (pH 5.0):methanol:acetonitrile (35:30:35, v/v/v) was set at a flow rate of 0.5 ml/min. The acetate buffer was made with 1000 ml of a solution of sodium acetate 10 mM adjusted to pH 5 with 99–100% acetic acid and filtered on a 0.45 μm hydrophilic polypropylene filter.

2.3. Preparation of stock solutions, working solutions and quality control samples

Two independent stock solutions of TPV were prepared in methanol at a concentration of 1 and 0.5 mg/ml. The 0.5 mg/ml solution was used to spike the calibration standards and the 1 mg/ml solution was used to prepare the quality control (QC) samples. The stock solutions were stored at −30 °C.

A stock solution of IS was prepared in methanol at a concentration of 1 mg/ml. This stock solution was diluted in water to obtain a working solution of 10 mg/l.

QC samples were prepared at final TPV concentrations of 2, 4, 15, 60 and 80 mg/l by evaporating to dryness at 40 °C under a stream of nitrogen appropriate amounts of the 1 mg/l stock solution of TPV. The dry residue was then reconstituted with drug free human plasma. After 3 h of mixing, the QC samples were stored in glass tubes at −30 °C. The three concentration levels of the QC samples 4, 15 and 60 mg/l were used for routine analysis and the validation of the method, while the 2 and 80 mg/l were used only for the validation of the lower and upper limit of quantification.

2.4. Sample preparation

The calibration standards were obtained from appropriate dilutions of the stock solution in methanol/water (1:1, v/v).

One hundred microliters of each dilution was then evaporated to dryness at 40 °C under a stream of nitrogen. The dry residue was reconstituted with 100 μl of human blank plasma in order to obtain the final calibration standards (2, 5, 10, 20, 40 and 80 mg/l). Forty microliters of the IS working solution was added to plasma samples, QC and calibration standards. After 30 s of vortex, 3 ml of TBME was added for the extraction of TPV and prazepam from plasma. After an agitation of 10 min with a mechanical shaker, the tubes were centrifuged 5 min at 2200 × *g*. The supernatant was transferred in a glass tube and evaporated to dryness at 30 °C under a stream of nitrogen, the dry residue was reconstituted with 100 μl of the mobile phase. After mixing, the extracts were transferred into the autosampler vials and 60 μl of each sample was injected in the chromatographic system.

2.5. Validation procedures

2.5.1. Linearity

Complete standard curves (2, 5, 10, 20, 40 and 80 mg/l) were analysed in triplicate on 3 separate days. QC were assayed twice with each standard curve. A linear regression with a weighting factor of 1/(peak height ratio) was used to plot the peak height ratio of TPV to internal standard versus TPV concentration. Slope, *y*-intercept and correlation coefficient were calculated for each standard curve.

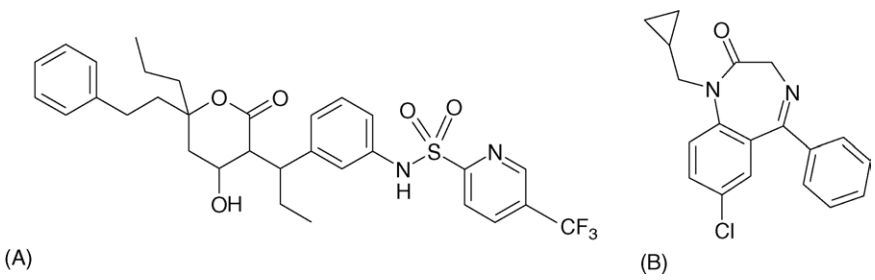

Fig. 1. Structure of TPV (A) and IS prazepam (B)

2.5.2. Accuracy and precision

Accuracy, within and between-day precision of the method were evaluated on the QC by multiple analysis, on the pool of 1 assay day and all the analytical runs, respectively. Accuracy was determined as the difference between back calculated concentration (C_{obs}) and theoretical concentration (C_{theo}) expressed in percent. The limit of quantification was set at the lowest calibration standard value (2 mg/l).

2.5.3. Recovery

The recovery of TPV was quantified at concentrations levels corresponding to the three routine QC values (4, 15 and 60 mg/l) analysed in triplicate. The peak height ratio of extracted samples containing both TPV and internal standard were compared to the ratio of samples containing the same amount of IS but no TPV which was added after the extraction step (on the dry tube). The same method was employed to study the recovery of the IS with TPV used as reference compound.

2.5.4. Selectivity and specificity

The specificity of the method was studied by injecting solutions of other ARVs in the chromatographic system. Furthermore the selectivity of the assay was assessed on the plasmas of six patients receiving other ARV's than TPV.

2.5.5. Stability

Freeze–thaw stability of TPV was determined by assaying the three routine QC samples in triplicate over three freeze–thawing cycles. The stability of TPV in plasma was investigated by assaying every other week the three concentration levels of routine QC samples stored at −30 °C for a 2 months period. Peak heights were compared with the one obtained from QC samples freshly made and concomitantly analysed. The stock solution of TPV stored at −30 °C was compared monthly to a stock solution freshly made during a 3 months period. The stability of TPV and IS in the extract was assayed by two ways. First, the dry residues of the extracts obtained from the three levels of routine QC samples analysed in duplicate were stored at −30 °C for a 2 weeks period and were reconstituted with 100 μl of the mobile phase before being injected in the chromatographic system. Second, reconstituted extracts of the three levels of the routine QC samples were left at room temperature in the autosampler for 3 days (i.e. approximately twice the time necessary for a routine analysis). For each experiment, peak heights were compared with the one obtained from QC samples freshly analysed.

3. Results

3.1. Validation procedures

3.1.1. Linearity

The nine standard curves were linear over a concentration range of 2–80 mg/l, with a mean slope (±S.D.) of 0.0812 ± 0.000530 and a mean *y*-intercept (±S.D.) of −0.014 ± 0.000534. The average correlation coefficient was 0.9993. Although the mean *y*-intercept was significantly different from zero it could be considered negligible since the average peak height ratio of TPV and IS for the first calibration standard concentration was 0.08.

3.1.2. Accuracy and precision

The average coefficient of variation of average results of back calculated calibration standard concentrations was 3.31% and the average coefficient of variation for the lower concentration (2 mg/l) was 4.19%.

The within-day precision, expressed by the coefficient of variation of observed concentrations of the routine QC was less than 7.3%, while the within-day accuracy, expressed by the calculated bias between observed and theoretical concentrations of the routine QC, ranged from −9.13 to −1.21%. The between-day precision and accuracy were respectively, less than 9.1 and −9.1%. The within-day precision for the 2 and 80 mg/l QC were 11.1 and 4.66%, respectively, whereas the within-day accuracy was −2.94 for the 2 mg/l QC and −4.29% for the 80 mg/l QC. The between-day precision was 10.5% for the 2 mg/L QC and 5.58% for the 80 mg/L QC, whereas the between-day accuracy were 7.44 and −9.08% for the 2 and 80 mg/l QC, respectively (Table 1). Representative chromatograms of a blank plasma and of the QC 2 mg/l corresponding to the limit of quantification are displayed in Figs. 2 and 3, respectively.

3.1.3. Recovery

The mean recovery of TPV was 69.8% for the 4 mg/l samples with a coefficient of variation of 2.59, 71.7 for the 15 mg/l samples with a coefficient of variation of 1.79 and 70.9% for the 60 mg/l samples with a coefficient of variation of 5.76%. The mean recovery of the internal standard was 80.3% with a coefficient of variation of 5.45%.

3.1.4. Specificity

No interference has been found between TPV or the IS and endogenous substances or ARV drugs (lopinavir, nelfinavir and its M8 metabolite, d4T, 3TC, ddI, AZT, efavirenz, indinavir, amprenavir, abacavir, nevirapine, saquinavir, atazanavir, tenofovir, emtricitabine, adefovir and ritonavir). Some of these drugs are not retained by the column and are therefore detected at the void volume time. Indinavir was not detected because its maximum of absorption wavelength (210 nm) is too different from

Table 1
Precision and accuracy of TPV determination in human plasma

Theoretical (mg/l)	Observed (mg/l)	Bias (%)	CV (%)
Within-day (n = 6)			
2	1.94	−2.94	11.1
4	3.84	−4.06	7.28
15	13.6	−9.13	3.13
60	59.3	−1.21	0.79
80	76.6	−4.29	4.66
Between-day (n = 18)			
2	2.15	7.44	10.5
4	3.64	−9.08	9.03
15	13.7	−8.79	2.46
60	58.3	−2.82	0.61
80	72.7	−9.08	5.58

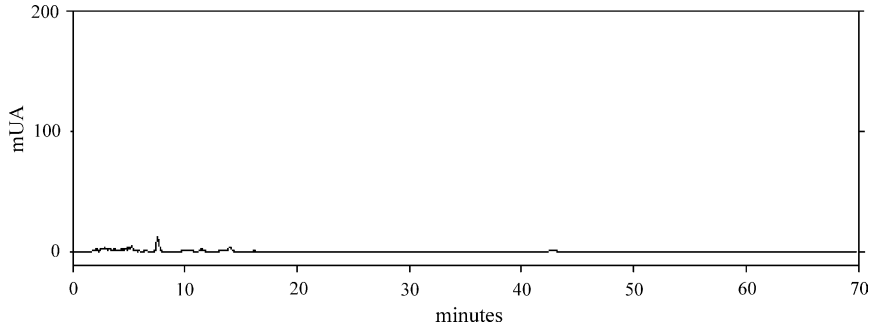

Fig. 2. Chromatogram of blank plasma.

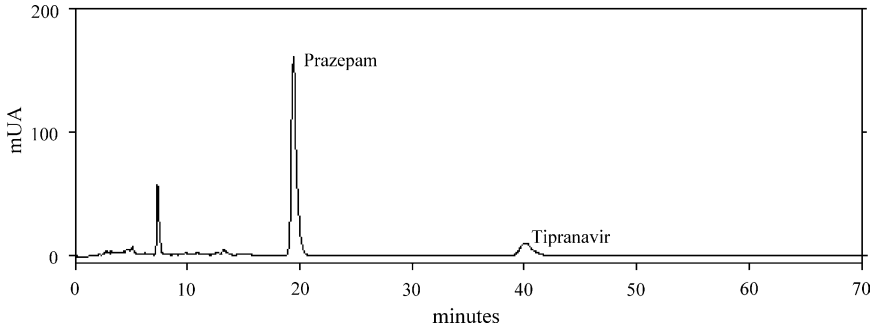

Fig. 3. Chromatogram of QC 2 mg/l.

Table 2
Retention times of other ARV drugs

Drug	$t'_r = (t_r - t_0)$ (min)
Tipranavir	37.8 = (40.2 – 2.4)
Prazepam (IS)	16.8 = (19.2 – 2.4)
Efavirenz	15.5 = (17.9 – 2.4)
Atazanavir	17.9 = (20.3 – 2.4)
M8-nelfinavir	19.4 = (21.8 – 2.4)
Ritonavir	23.0 = (25.4 – 2.4)
Saquinavir	30.3 = (32.7 – 2.4)
Lopinavir	30.5 = (32.9 – 2.4)
Nelfinavir	45.1 = (47.5 – 2.4)
Stavudine, lamivudine, didanosine, zidovudine, amprenavir, abacavir, nevirapine, tenofovir, emtricitabine, adefovir	Not retained by column
Indinavir	Not detected at 260 nm

t'_r, reduced retention time; t_r, retetion time; t_0, void volume time.

the wavelength used in the method. Retention times of which are listed in Table 2. The six samples of patients receiving other ARV's showed no interference with TPV or the IS. Representative chromatogram of a patient treated with TPV is displayed on Fig. 4.

3.1.5. Stability

Freeze–thaw stability of TPV was evaluated by the calculated bias between observed and theoretical concentrations (Table 3). These biases ranged from −2.25 to 20.0% for low control, from −3.80 to 9.61% for the medium control and from 3.25 to 13.7% for the high control. The assay of the stability of TPV in plasma for 2 months at −30 °C showed the absence of degradation of the product. The stock solution of TPV stored for 3 months at −30 °C was comparable to the stock solution freshly made (i.e. less than 5% difference). The assay of stability of the extracts showed a difference less than 5% for peak heights of both TPV and IS between the QC extracted the day of the injection and the

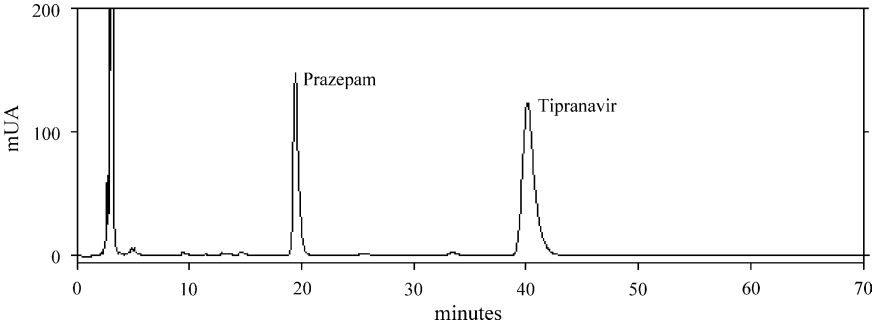

Fig. 4. Chromatogram of a patient plasma treated with tipranavir (concentration of TPV: 20.5 mg/l).

Table 3
Freeze–thaw stability of TPV in human plasma ($n = 9$)

Theoretical (mg/l)	Observed (mg/l)	Bias (%)	CV (%)
Freeze–thaw cycle 1			
4	4.35	8.62	9.28
15	16.4	9.61	9.10
60	67.1	11.8	1.34
Freeze–thaw cycle 2			
4	3.91	−2.25	2.53
15	14.4	−3.80	0.69
60	62.0	3.25	3.79
Freeze–thaw cycle 3			
4	4.80	20.0	6.43
15	15.7	4.53	2.98
60	68.2	13.7	1.51

extracts stored at −30 °C for 15 days as well as the extracts left at room temperature for 3 days.

4. Discussion

We developed a simple analytical method to quantify TPV in plasma. This method has been found accurate and precise enough to perform TDM in HIV-infected patients.

During the development an interference with lopinavir has been found with an acetate buffer (10 mM, pH 5.0):methanol:acetonitrile (30:35:35, v/v/v) mobile phase. This interference has been suppressed by the use of the mobile phase described in the chromatographic and detection conditions.

A problem we also had to deal with was the poor solubility of TPV in water. Indeed, the TPV stock solutions had to be diluted in a methanol:water (1:1, v/v) solvent in order to have linear calibration curves. In consequence, because of the presence of methanol, these dilutions had to be evaporated before adding the blank plasma in order to obtain limpid extracts.

Another point that could be noticed is that UV detection was saturated for TPV concentrations greater than 80 mg/l which made us limit our calibration standards to this maximum value.

The average through concentration (±S.D.) measured on 14 patients was 24.3 ± 16.6 mg/l. Three of these 14 patients had a through concentration smaller than 12 mg/l and could necessitate a dose increase. One patient presented a through concentration of 50.8 mg/l and developed a few days later an hepatic cytolysis.

These results seem to confirm the interest of TDM for TPV and the relevance of our calibration standard curve for the quantification of through concentration as all the measured values were comprised between the LOQ and the highest calibration standard (80 mg/l).

In conclusion this method using HPLC-UV detection can be used for the TDM of TPV.

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