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Short communication

# A simple high performance liquid chromatography assay for monitoring plasma concentrations of tipranavir in HIV infected patients

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**Abstract**

A simple HPLC assay to determine plasma concentration of tipranavir is presented. A liquid/liquid extraction of the drugs in ethyl acetate/hexane from 250 μL of plasma is followed by a reversed phase isocratic HPLC assay with UV detection at 205 nm. The imprecision and inaccuracy are lower than 10%, the low limit of quantitation is 0.4 mg/L. Thus, this method can be used for therapeutic drug monitoring of tipranavir in HIV infected patients


*Keyword:* Tipranavir

## 1. Introduction

Numerous studies suggest improved management of antiretroviral therapy by monitoring plasma concentrations of protease inhibitors (PI) and non-nucleoside reverse transcriptase inhibitors (NNRTI) in human immunodeficiency virus (HIV) infected patients [1]. Consequently, several HPLC assays have been developed to measure plasma levels of PI and NNRTI [2–11]. However, few methods [12–14] determinate plasma concentration of the new protease inhibitor, tipranavir [15]. The aim of this study was to develop and validate a simple method using an isocratic HPLC system with a single wavelength for UV detection and requiring a low plasma volume for drug extractions to measure plasma concentrations of tipranavir.

## 2. Experimental

### 2.1. Chemicals

The internal standard (A 86093) (ABBOTT, IL, USA) and tipranavir (BOEHRINGER INGELHEIM, Ridgefield, CT, USA) were kindly provided by the pharmaceutical companies. Tetramethylammonium perchlorate was purchased from ACROS ORGANICS (Geel, Belgium), and trifluoroacetic acid was purchased from FLUKA CHEMIE AG (Buchs, Switzerland). Acetonitrile and ethyl acetate were from SDS (Peypin, France). Hexane and methanol (for analysis) were from MERCK (Darmstadt, Germany).

### 2.2. Equipment

The HPLC system consists of Agilent (Palo Alto, USA) 1100 Series components including a quaternary pump, degasser, autosampler, and a photodiode array detector. Chromatographic separations were achieved using a Waters (Milford, USA). Symmetry® 5 μm C18 column (250 mm × 4.6 mm I.D.) protected with a Waters Symmetry® 5 μM C18 pre-column (20 mm × 3.9 mm I.D.).

### 2.3. Chromatographic conditions

The mobile phase was composed of acetonitrile, methanol, 0.025 M tetramethylammonium perchlorate in 0.2% aqueous trifluoroacetic acid (21:53:26, v/v/v) and was delivered isocratically at 1 mL/min. Chromatographic separation was performed at ambient temperature and the UV detection was performed at 205 nm.

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### 2.4. Preparation of standards

The stock solution were prepared as follows: tipranavir at a concentration of 5000 mg/L (methanol) and internal standard (ethanol since the internal standard is weakly soluble in methanol) at a concentration of 1000 mg/L.

For the calibration samples, a working solution was prepared by diluting the stock solutions in a mixture methanol/water (1:1, v/v) to a final concentration of 800 mg/L. Known volumes of this working solution (5–800 μL) were diluted in methanol/water (1:1, v/v) to obtain a 1000 μL final volume. Twenty five microliters of these solutions were mixed with 225 μL of drug-free human plasma to prepare calibration samples.

For the quality controls, a high level solution was prepared by diluting the stock solutions in a mixture methanol/water (1:1, v/v) to a final concentration of 300 mg/L; a medium level solution was prepared by diluting the stock solutions in a mixture of methanol/water (1:1, v/v) to a final concentration of 150 mg/L; and a low level solution was prepared by diluting the stock solutions in a mixture of methanol/water (1:1, v/v) to a final concentration of 15 mg/L. These solutions (high, medium and low) were diluted with drug-free human plasma (1:9, v/v) to prepare the following quality control solutions: high level (30 mg/L); medium level (15 mg/L); low level (1.5 mg/L). These diluted solutions were distributed in tubes and stored at −20 °C.

For the internal standard, a solution was prepared by diluting the stock solution of internal standard in a mixture methanol/water to a final concentration of 20 mg/L.

### 2.5. Specificity

The specificity of the method was investigated using plasma spiked with antiretroviral drugs (zidovudine, stavudine, didanosine, lamivudine, tenofovir, abacavir, nevirapine, indinavir, nelfinavir and its active metabolite M8, amprenavir, saquinavir, ritonavir, atazanavir, lopinavir, and efavirenz) and possible co-administered drugs (itraconazole, sulfamethoxazole, trimethoprime, aciclovir, zolpidem, zopiclone, amitriptyline, bromazepam, alprazolam, tetrazepam, acetaminophen, aspirine, loperamide, phloroglucinol, omeprazole, naproxen, and ibuprofen).

### 2.6. Sample treatment

The blood samples with lithium heparinate as anticoagulant were centrifuged at 3000 rpm (1800 × *g*) for 10 min at +4 °C. A 250 μL aliquot of plasma (patient samples which were not heated, calibration samples, quality controls) was combined with 50 μL of the internal standard solution (20 mg/L), and 4 mL of a mixture of ethyl acetate/hexane (1:1, v/v). The tubes were shaken horizontally for 10 min followed by centrifugation at 3000 rpm (1800 × *g*) for 5 min at +4 °C. Three millilitres of the upper organic phase were evaporated to dryness under a gentle stream of nitrogen at +40 °C. The final residue was reconstituted by vortexing with 0.3 mL of mobile phase. Fifty microlitres of the solution were injected.

Table 1
Inter-day (ied) and intra-day (iad) imprecision and inaccuracy for tipranavir

| | Nominal plasma level (mg/L) | Average measured plasma level (imprecison % − inaccuracy%) | |
|---|---|---|---|
| | | iad ($n = 5$) | ied ($n = 15$) |
| Low level | 1.5 | 1.517 (5.64–1.16) | 1.475 (4.81–1.66) |
| Medium level | 15 | 15.783 (2.63–5.22) | 15.123 (3.79–0.82) |
| High level | 30 | 30.868 (2.64–2.89) | 29.69 (3.98–1.03) |

### 2.7. Calibration curves

The calibration curves were calculated by unweighted least squares linear regression. The range of tipranavir standard concentrations tested are from 0.4 to 64 mg/L.

## 3. Results

The imprecision, defined as the coefficient of variation of repeated measurements and inaccuracy, defined as the percent of deviation from the nominal level were investigated after repeated injections of high, medium and low level quality controls. For intra-day validation, five samples at each level of quality controls were analyzed within the same day. For inter-day validation, five samples of each level of quality controls were analyzed in three separate analytical runs. Theses results of imprecision and inaccuracy (Table 1) were less than 10%. The limit of quantitation, defined as the lowest concentration in a plasma sample such that the imprecision and inaccuracy are less than 20% is 0.4 mg/L. The recovery ratios, which were determined by comparing the peak areas of the quality controls samples after extraction with the peak areas of standard solutions at the same concentration and not extracted for the three levels of quality controls (1.5, 15, and 30 mg/L), are respectively 91.5, 89.5, and 93.7%. The linearity of the calibration curves (the correlation coefficients were higher than 0.99) was checked until at least 64 mg/L. The inter-day variability (mean ± standard deviation ($n = 5$)) of the slopes

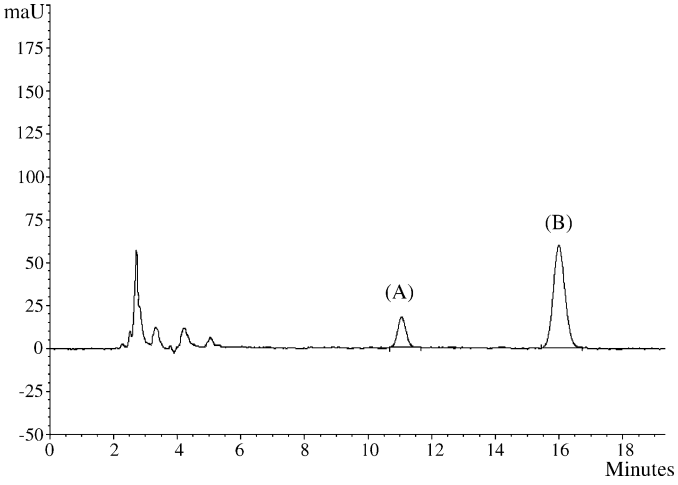


Fig. 1. Chromatogram of a spiked plasma sample: (A) A86093 internal standard (retention time = 11.0 min) and (B) tipranavir 16 mg/L (retention time = 15.9 min).

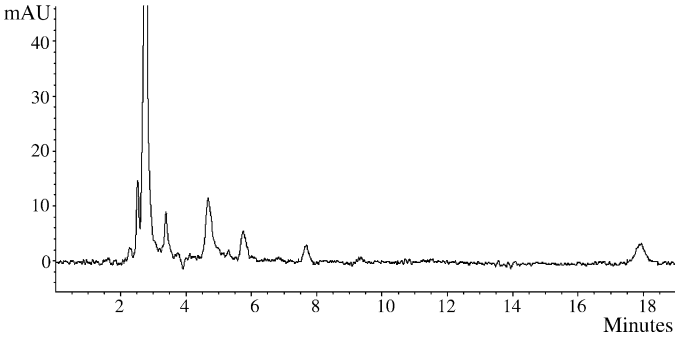


Fig. 2. Chromatogram of a free drug plasma sample.

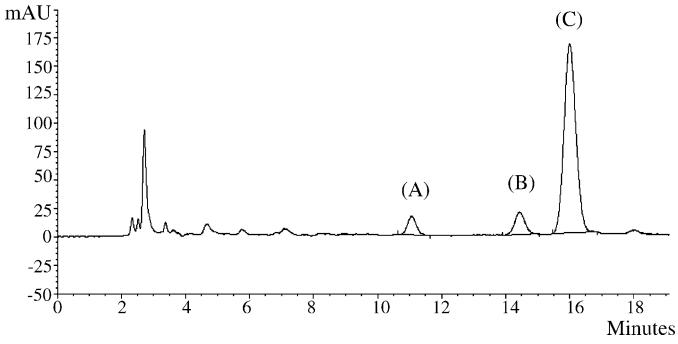


Fig. 3. Chromatogram of a plasma sample from a patient receiving tipranavir/ritonavir (500 mg/200 mg twice a day), lopinavir/ritonavir (533 mg/133 mg twice a day), enfuvirtide (90 mg twice a day), emtricitabine (200 mg once a day). The peaks A and B correspond, respectively to the internal standard A86093 (11.0 min) and lopinavir (14.4 min). The plasma concentration of tipranavir (C, 15.9 min) is 44.4 mg/L. The unidentified peaks are due to plasma interference.

($0.28865 \pm 0.01139$) and intercepts ($-0.00064 \pm 0.01845$) of the calibration curves is low. The stability of the frozen samples ($-20\,^{\circ}C$) was checked during 1 month. The retention times of the drugs listed in the paragraph 'specificity' were 3.18 min for nelfinavir, 3.76 min for atazanavir, 3.95 min for amprenavir, 4.54 min for saquinavir, 9.043 min for ritonavir, 9.85 min for efavirenz and 14.42 min for lopinavir. The other compounds were not detected. The chromatograms of the spiked plasma sample (16 mg/L for tipranavir), the blank plasma sample and the plasma sample from a patient who received tipranavir/ritonavir (500 mg/200 mg twice a day), lopinavir/ritonavir (533 mg/133 mg twice a day), enfuvirtide (90 mg twice a day), emtricitabine (200 mg once a day) are presented, respectively in Figs. 1–3. No interference was found with endogenous compounds up to a plasma concentration of 301, 181 μmol/L for, respectively creatinine and total bilirubin.

## 4. Discussion

A specific, accurate, precise, and reproducible HPLC method was developed to determine plasma concentration of tipranavir in HIV infected patients. The UV absorbance of tipranavir and the internal standard was monitored at 205 nm since these compounds demonstrated a high absorbance at 205 nm and the analysis of blank plasma monitored at 205 nm showed no endogenous peak at the retention time of tipranavir and the internal standard. Moreover, the limit of quantitation determined at 205 nm is consistent with target plasma trough concentrations of tipranavir (>9 mg/L) [16]. The separation of tipranavir and potentially co-administered antiretroviral drugs such as lopinavir was improved by adding a ion-paring agent, tetramethylammonium perchlorate, and trifluoroacetic acid in the mobile phase. Three previous assays [12–14] measured the plasma concentration of tipranavir. Crommentuyn et al. [12] proposed an assay based on mass spectrometry detection requiring sophisticated HPLC systems that are not available in all laboratories. Recently, methods using UV detection were developed by Giraud et al. [13] and Colombo et al. [14]. However, these methods are time consuming (the tipranavir retention times are 40 min [13] and 32 min [14] versus 15.9 min in our assay), prazepam [13] and clozapine [14] are used as internal standard despite the fact that prazepam and clozapine are market drugs (contrary to our internal standard) and can be potentially co-administered with tipranavir. Our limit of quantitation is lower than the value obtained by Giraud et al. [13] (2 mg/L versus 0.4 mg/L) and is consistent with the value obtained by Colombo et al. [14] (0.125 mg/L versus 0.4 mg/L) although the amount of plasma is lower in our assay (600 μL versus 250 μL).

In conclusion, our HPLC method requiring a low amount of plasma and a simple HPLC system (an isocratic mobile phase, a single wavelength UV detection) commonly available in laboratories allows a rapid, accurate and precise determination of tipranavir plasma concentrations in HIV infected patients and can be used for therapeutic drug monitoring of tipranavir in HIV infected patients.

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