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Short communication

High-performance liquid chromatography assay for the determination of the HIV-protease inhibitor tipranavir in human plasma in combination with nine other antiretroviral medications

Sun Ok Choi *, Naser L. Rezk, Angela D.M. Kashuba

Clinical Pharmacology/Analytical Chemistry Core, Center for AIDS Research, University of North Carolina, 3323A Kerr Hall, CB# 7360, Chapel Hill, NC 27599, USA

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Abstract

An accurate, sensitive and simple reverse-phase (RP) high-performance liquid chromatography (HPLC) assay has been developed and validated for the simultaneous quantitative determination of tipranavir with nine other antiretroviral drugs in plasma. A liquid–liquid extraction of the drugs in *tert*-butylmethylether (TBME) from 200 μL of plasma is followed by a reversed phase gradient HPLC assay with UV detection at 210 nm. The standard curve for the drug was linear in the range of 80–80,000 ng/mL for tipranavir; 10–10,000 ng/mL for nevirapine, indinavir, efavirenz, and saquinavir; and 25–10,000 ng/mL for amprenavir, atazanavir, ritonavir, lopinavir, and nelfinavir. The regression coefficient (r^2) was greater than 0.998 for all analytes. This method has been fully validated and shown to be specific, accurate and precise. Due to an excellent extraction procedure giving good recovery and a clean baseline, this method is simple, rapid, accurate and provides excellent resolution and peak shape for all analytes. Thus this method is very suitable for therapeutic drug monitoring.
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Keywords: Tipranavir; High-performance liquid chromatography (HPLC); Antiretroviral drug; Protease inhibitor; Therapeutic drug monitoring

1. Introduction

Tipranavir (TPV; 2-pyridinesulfonamide, *N*-[3-[1*R*)-1-[(6*R*)-5,6-dihydro-4-hydroxy-2-oxo-6-(2-phenylethyl)-6-propyl-2*H*-pyran-3-yl]propyl]phenyl]-5-(trifluoromethyl) is a novel non-peptidic protease inhibitor (PI) used for the treatment of human immunodeficiency virus type-1 (HIV-1) and type-2 (HIV-2) infection (Fig. 1) [1–4]. Chemically, TPV was developed from coumarin and sulfonamide templates and has been shown to have antiviral activity against viral strains cross-resistant to other marketed protease inhibitors (PIs). These results have been confirmed clinically [2].

Since co-administration of ritonavir (RTV) with TPV substantially increases the steady-state exposure to TPV [5,6], it is currently co-administered with low-dose RTV. The recommended oral dose of TPV is 500 mg, co-administered with RTV 200 mg, twice daily in combination with other antiretroviral agents. TPV is predominantly metabolized by, and is an inducer of, the cytochrome P450 3A4 (CYP3A4) enzyme. TPV is also a P-glycoprotein (P-gp) substrate, a weak P-gp inhibitor, and appears to be a potent P-gp inducer. Taken together, this results in a complex pattern of drug interactions [2].

Therapeutic drug monitoring (TDM) of PIs and non-nucleoside reverse transcriptase inhibitors (NNRTIs) may be useful tool to optimize antiretroviral treatment. To quantify the HIV PIs and NNRTIs simultaneously, several high-performance liquid chromatography (HPLC) methods have been developed [7–11]. To date, several methods to quantify TPV have been published [4,12–16]. However, some of these methods are somewhat cumbersome, and have limited selectivity. Also, there is no known efficient method which can simultaneously quantitate multiple antiretroviral agents with tipranavir yet. We therefore developed an HPLC assay with UV detection for the simultaneous quantification of TPV and nine other antiretroviral medications.

* Corresponding author. Tel.: +1 919 843 2791; fax: +1 919 962 0644.
E-mail address: sochoi@email.unc.edu (S.O. Choi).

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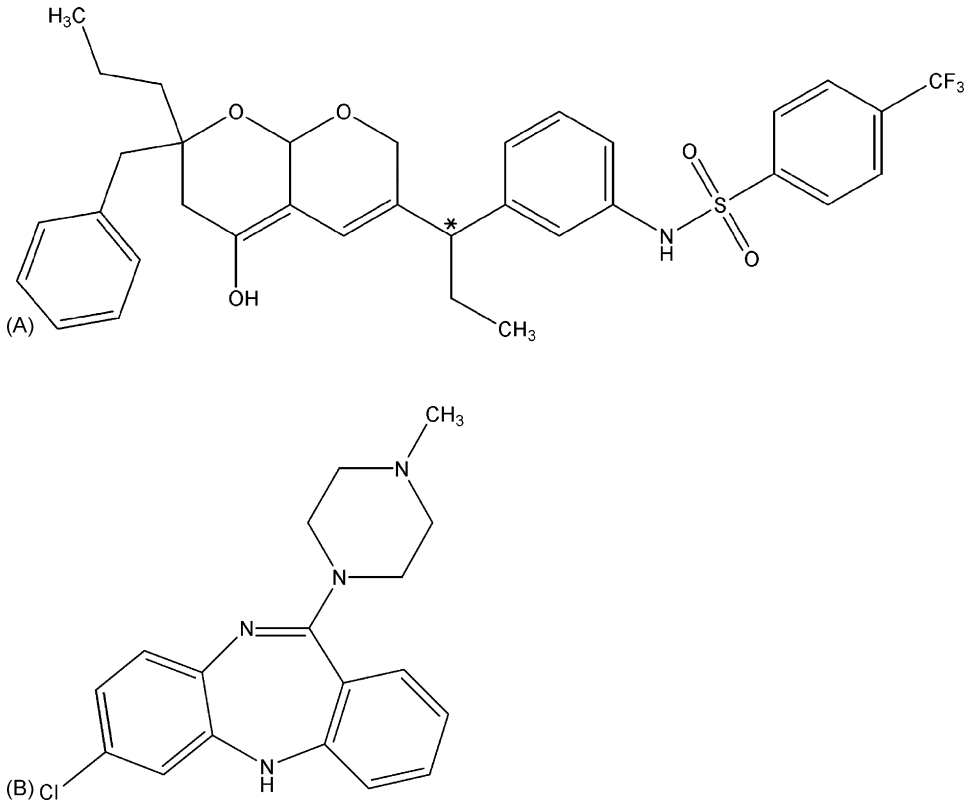

Fig. 1. The chemical structure of Tipranavir (A) and IS clozapine (B).

2. Experimental

2.1. Chemicals

Clozapine was purchased from Sigma Chemical Company (St. Louis, MO, USA). Its structure is shown in Fig. 1. Tipranavir (TPV) was kindly obtained from the NIH AIDS Research & Reference Reagent Program (McKesson HBOC BioServices, Rockville, MD, USA). The origin of other compounds has been previously reported [11]. The origin of plasma was human NaEDTA blank plasma.

2.2. Preparation of standard solutions

Individual clear 1 mg/mL stock solutions of ATV, IDV, APV, NFV, EFV, RTV, SQV, LPV, and NVP were prepared, while TPV was prepared as a 4 mg/mL stock solution. Five milligrams of each analyte (20 mg for TPV) were accurately weighed and dissolved with 50% methanol in water for ATV, IDV, APV, EFV, RTV, SQV, NVP; 70% methanol in water for LPV; and 80% methanol in water for NFV and TPV. The master stock solution was prepared as a composite of all analytes (100 μg/mL standard solution for nine analytes and 800 μg/mL standard solution for TPV). This master stock solution was then used to prepare eight working standard solutions (10, 25, 50, 100, 500, 1000, 5000 and 10,000 ng/mL for nine analytes and 80, 200, 400, 800, 4000, 8000, 40,000 and 80,000 ng/mL for TPV) in drug-free pooled plasma. Plasma quality control (QC) samples at 60, 600 and 6000 ng/mL for nine analytes and 480, 4800 and 48,000 ng/mL for TPV were prepared from a separate master stock solutions.

2.3. Internal standard (IS) solution

Clozapine (5 mg) was weighed and dissolved in methanol to achieve a final concentration of 1.0 mg/mL stock solution. The internal standard working solution was prepared by diluting an aliquot from the stock solution in 50% methanol solution to a final concentration of 2.5 μg/mL (working solution).

2.4. Sample preparation and liquid–liquid extraction procedure

Two hundred microliters blank plasma and spiked plasma standard working solutions and QC solutions were combined with 50 μL of internal standard solution in a 2.0 mL conical polypropylene Eppendorf tube. The other procedures are already reported [11].

2.5. Equipment, chromatographic conditions

All of these have been previously reported [11]. During development of this method, we experimented with how much time the HPLC took to re-equilibrate, and used 2–8 min. According to our experiments, a 3-min re-equilibration time was sufficient to regenerate a good baseline for this method.

2.6. Validation of the method

Validation of the analytical method for nine analytes plus TPV was performed. The limit of quantification, selectivity, linearity, accuracy, precision, recovery and stability of TPV in plasma samples were evaluated. Precision was quantified as the coefficient of variation (CV %) within a single run (intra-assay) and between different runs (inter-assays). Accuracy was quantified as the percentage of deviation between nominal and measured concentrations. Lower limit of quantification was determined as a signal to noise ratio of 5:1.

During this validation, only TPV stability testing was performed, as all other analytes had been evaluated in prior validation processes [17]. Short term stability of TPV in plasma was evaluated by leaving samples at room temperature for 24 h prior to extraction, and long term stability was evaluated after placing samples in freezer at −70 °C for 3 months. TPV's stability during sample handling was evaluated by subjecting samples to three freeze–thaw cycles, in addition to heat inactivation in a heating block at 60 °C for 1 h. Finally, testing was performed to verify TPV's stability when held in an autosampler for 72 h. Quality control (QC) samples at two concentrations (48,000 and 480 ng/mL) were used for all stability tests.

2.7. Analysis of clinical samples

We examined the applicability of the method by analyzing plasma samples collected from 10 HIV-negative subjects taking TPV/RTV for a drug interaction study.

3. Results and discussion

3.1. Linearity

The peak area ratios of the calibration standards to the internal standard were proportional to the concentration of each drug in plasma over the range tested. Weighed linear regression was used to generate the standard curves. To calculate concentrations of analytes in clinical samples and QC samples, calibration curves were obtained by weighed $1/x^2$ least squares linear regression analysis. The linear range for each analyte was as follows. 10–10,000 ng/mL for NVP, IDV, EFV and SQV; 25–10,000 ng/mL for APV, ATV, RTV, LPV and NFV; and 80–80,000 ng/mL for TPV [11]. The regression coefficients (r^2) were all greater than 0.998 for each analyte repeated daily for 6 days. Calibration parameters of slope, intercept, standard error, including their standard deviation for nine antiretroviral drugs were reported [11]. Weighed linear regression parameters for tipranavir curve was: intercept = 0.005 ± 0.059; slope = 0.2663 ± 0.05; S.E. = 0.041 ±0.03, S.D. = 0.03 ± 0.052.

3.2. Selectivity

Fig. 2 shows a representative chromatogram of blank plasma (A) and an extracted sample containing 80 ng/mL for TPV, and 10 ng/mL for the nine other analytes at the lower limit of quantification (B). The approximate retention times for all analytes are shown in Table 1. Other potential drugs of interference were not tested during this validation, as they had been previously evaluated under the same chromatographic conditions [7,11].

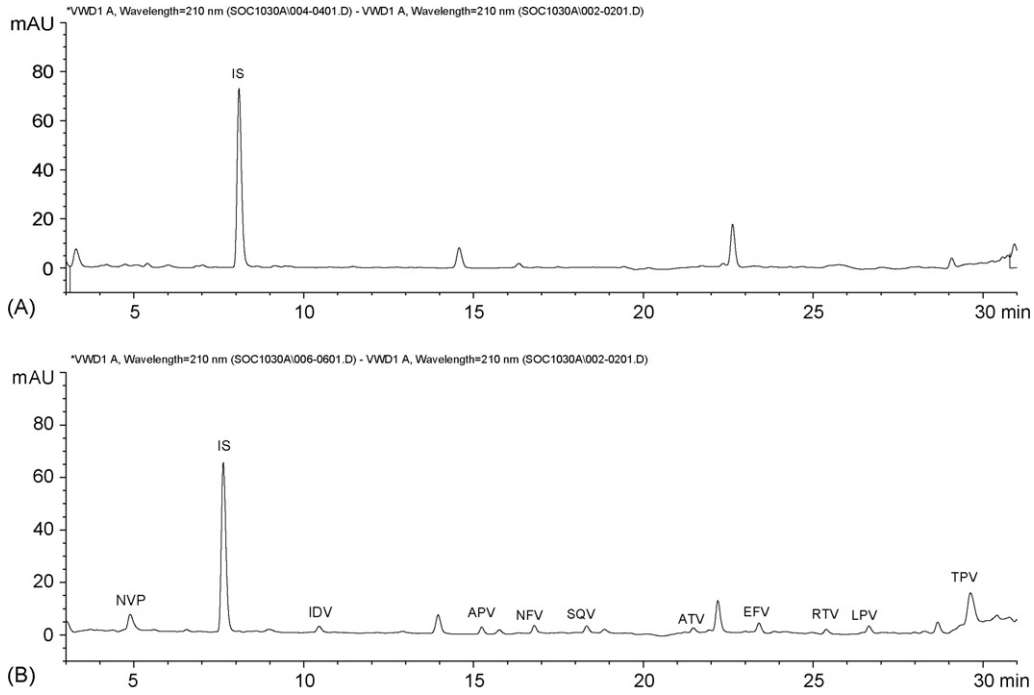

Fig. 2. Representative HPLC chromatograms of tipranavir and other nine anti-HIV drugs in plasma samples: (A) blank plasma and (B) 80 ng/mL of tipranavir and 10 ng/mL of nine other anti-HIV drugs at the lower limit of quantification in plasma (tipranavir, TPV; atazanavir, ATV; indinavir, IDV; amprenavir, APV; nelfinavir, NFV; efavirenz, EFV; ritonavir, RTV; saquinavir, SQV; lopinavir, LPV; nevirapine, NVP; clozapine, IS).

Table 1
Retention times of anti-HIV drugs

Drug name	Retention time (min)
Nevirapine	5.5
Clozapine (IS)	8.0
Indinavir	10.4
Amprenavir	15.5
Nelfinavir	16.7
Saquinavir	18.2
Atazanavir	21.2
Efavirenz	23.7
Ritonavir	25.6
Lopinavir	26.7
Tipranavir	29.4

3.3. Accuracy and precision

The intra- and inter-day accuracy and precision of TPV are listed in Table 2. The accuracy of TPV ranged from 98.3 to 106.0% with a mean of 101.4%. Throughout the concentration range of the control samples, the mean intra- and inter-assay precision for TPV remained less than 6.8%. All the accuracies and precisions of the nine analytes were within the acceptable range according to bioanalytical method guidelines [17]. Precision for all nine analytes was less than 9.7%.

3.4. The limit of quantification

The low limit of quantification for TPV was 80 ng/mL. The lower limit of quantification for NVP, IDV, EFV, and SQV was 10 ng/mL, and for all other analytes it was 25 ng/mL. Accuracy and precision at the lower limit of quantification were less than 7.4% for all analytes, and therefore within the acceptable range [17].

3.5. Recovery

The absolute recovery of TPV from plasma extracted with TMBE was calculated by comparing peak areas of extracted QC samples to peak areas of directly injected standards having equivalent concentrations. This extraction method resulted in a clean baseline (Fig. 2A), with a mean ± S.D. recovery of 73.7% for TPV. Recoveries for other analytes were >89.0% and that of internal standard was >61.3%.

Table 3
Stability of tipranavir in human plasma[a]

Test of stability	Accuracy of tipranavir (%)	
	Low QC[b]	High QC[c]
Freeze and thaw	98.2 ± 5.3	96.5 ± 2.0
Short-term, temperature[d]	91.0 ± 5.4	89.2 ± 2.0
Long-term, temperature[e]	90.0 ± 5.1	87.2 ± 2.1
Post-preparative	97.2 ± 7.7	95.3 ± 6.0
Heat[f]	94.7 ± 3.2	94.6 ± 3.0

[a] Data are mean values ± S.D. ($n = 3$).
[b] Low QCs of low quality control samples are 480 ng mL^{-1}.
[c] High QCs of high quality control samples are 48,000 ng mL^{-1}.
[d] Short-term stability was tested after 24 h at room temperature.
[e] Long-term stability was tested after 3 months at −70 °C.
[f] Heat stability was tested after 1 h at 60 °C.

3.6. Stability

Drug stability in biological fluids is a function of the sample storage conditions, the chemical properties of the drug, the matrix and the container system [17]. The stability of TPV was evaluated under conditions reflecting situations likely to be encountered during actual sample handling and analysis. TPV was found to be stable during sample collection and handling, after HIV heat inactivation procedures, after short and long-term storage, after three freeze–thaw cycles, and during the analytical process. Table 3 demonstrates that all results in the stability testing of TPV were within the acceptance range of ±15% deviation from the nominal concentration.

3.7. Method development

TPV/RTV has recently been approved by the US FDA for use as part of combination antiretroviral treatment in HIV-1 infected adults with evidence of viral replication who are highly treatment-experienced or who have multiple PI-resistant virus [2,3]. TPV's pharmacologic and pharmacokinetic properties have been previously reviewed [2]. As well as the interaction with RTV, which is used to increase the bioavailability of TPV, TPV has the potential to interact with other drugs. Therefore, a simple and sensitive method for measuring TPV plasma concentrations would be most beneficial for clinical studies and for therapeutic drug monitoring [2,3].

Six assays [4,12–16] have been previously published for measuring the plasma concentration of TPV. Crommentuyn et al.

Table 2
Accuracy and precision for the determination of tipranavir in human plasma

Nominal TPV concentration (ng mL^{-1})	Intra-day			Inter-day		
	Measured TPV concentration (ng mL^{-1})[a]	Accuracy (%)	Precision CV (%)	Measured TPV concentration (ng mL^{-1})[a]	Accuracy (%)	Precision CV (%)
80	84.8 ± 5.70	106.0 ± 0.071	6.8	79.3 ± 3.70	99.1 ± 0.046	4.6
400	418 ± 8.10	104.6 ± 0.020	1.9	400 ± 23.8	99.9 ± 0.060	5.9
4,000	3930 ± 59.2	98.3 ± 0.015	1.5	4040 ± 199	101.0 ± 0.050	4.9
40,000	41,600 ± 1070	103.9 ± 0.027	2.6	39,400 ± 2350	98.6 ± 0.059	6.0

[a] Data are mean values ± S.D. ($n = 6$).

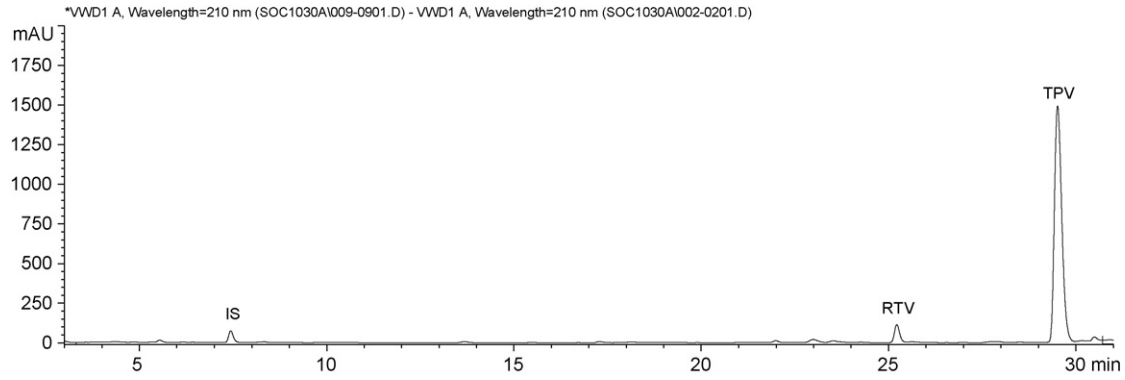

Fig. 3. Representative HPLC chromatogram of a healthy volunteer administered multiple doses of tipranavir/ritonavir 500/200 mg (6 h post-dose), along with a cytochrome P450 phenotyping cocktail consisting of caffeine 200 mg, warfarin 10 mg, Vitamin K 10 mg, omeprazole 40 mg, dextromethorphan 30 mg and IV midazolam 2 mg.

[12] proposed an assay based on mass spectrometry detection using equipment that is not readily available in all laboratories. Recently, methods using UV detection were developed by Giraud et al. [4], Colombo et al. [13], Dailly et al. [14], Sparidans et al. [15] and Keil et al. [16]. However, the first two methods are time consuming and yield suboptimal peak shapes, and the other methods are limited to quantifying TPV only.

To the best of our knowledge, this is the first report describing an assay of TPV by HPLC-UV at 210 nm that also simultaneously quantitates nine other antiretroviral drugs using clozapine as an internal standard after a simple liquid–liquid extraction procedure from plasma. This HPLC method is a robust and convenient procedure for determining TPV in plasma. We modified the previously validated method [11] to efficiently analyze tipranavir in addition to nine antiretroviral drugs. The NFV M8 peak was not interfered with any other analyte of interest [11], and by comparing the relative retention time of peaks found in 50 patients taking EFV, no interfering peaks were found at the elution time of TPV.

This method is specific, sensitive, accurate, precise and reproducible. This method has lower LLOQ for TPV than the previous methods [4,13–16]. Sensitivity can be an issue for pediatric samples, or other drug compartments of particular clinical interest, such as breast milk, cerebro spinal fluid (CSF) and genital tract secretions. Therefore, this method has clear advantages for laboratories where large numbers of samples containing multiple analytes are to be analyzed on a routine basis, and also for sensitivity issued samples.

3.8. Application of the method

Fig. 3 shows a representative chromatogram from a clinical sample 6 h after administration of TPV/RTV 500/200 mg. This subject was had also taken caffeine, warfarin, omeprazole, dextromethorphan, midazolam, and digoxin as part of a drug interaction study. No other interfering peaks were found.

3.9. Result of the proficiency testing (PT)

The results of PT from the AIDS clinical trial group (ACTG) pharmacology quality assurance program for therapeutic drug monitoring [18] demonstrated the robustness of our method. Accuracies ranged from 96.1% to 103.6% for six samples.

4. Conclusion

A liquid–liquid extraction method followed by an automated and optimized HPLC method has been developed for the analysis of tipranavir and nine other antiretroviral drugs in human plasma. This is a rapid and simple method that is sensitive, accurate, and precise. This analytical method will be useful for clinical studies and therapeutic drug monitoring.

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