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High Performance Liquid Chromatographic Method for the Determination of HIV-1 Protease Inhibitor Tipranavir in Plasma of Patients during Highly Active Antiretroviral Therapy

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Abstract
A new high-performance liquid chromatographic method for the determination of tipranavir in human plasma is described.

Quantitative recovery following liquid – liquid - extraction with diethylether from 100 µl of human plasma was achieved. Subsequently, the assay was performed with 67 mM potassium dihydrogen phosphate-acetonitrile as a mobile phase, a Phenomenex C 18 column and UV detection at 255 nm. Linear Standard curves were obtained for concentrations ranging from 2.5 to 400 µg/ml. The calculated intra- and inter-day coefficents of variation were below 7%.

Keywords: HPLC, tipranavir, drug monitoring

1. Introduction

Tipranavir is a novel, nonpeptidic protease inhibitor with activity against wild-type and multi-drug resistant HIV-1. In a clinical study, tipranavir/ritonavir 500mg/200mg twice daily in combination with an optimized background regimen was more effective than a ritonavir-boosted comarator PI plus an optimized background regimen [1, 2]. Tipranavir is a substrate and inducer of cytochorme P450 3A4 isoenzyme, thus predisposed to interactions with other agents that are substrates, inducers or inhibitors of the enzyme family. Significant drug-drug interactions have been reported with coadministration of tipranavir/ritonavir and other PIs. Concomitant adminsitration with low dose ritonavir significantly increases tripranavir plasma concentrations.

Severe adverse events that require close monitoring include hepatotoxicitiy and lipid abnormalities. Tipranavir retains activity in many highly treatment experienced patients with a large number of protease mutations.

Therefore, therapeutic drug monitoring may be warranted to manage a patient`s medication regimen.

The aim of the present study was to develop a valid, reliable and convenient HPLC-based method for the determination of tipranavir in human plasma samples.

2. Experimental

2.1. Chemicals and Reagents

Tipranavir (PNU 140690), was kindly provided by Boehringer-Ingelheim/ Ridgefield USA. The internal standard (A-86093) was kindly supplied by Abbott Inc. (Abbott Lab. North Chicago, USA).

Diethylether, acetonitrile, methanol, distilled water, potassium dihydrogen phosphate, disodium hydrogen phosphate dihydrate and sodium hydroxide were products of Merck (Darmstadt, Germany). Blank, drug free plasma was obtained from Sigma-Aldrich Chemie (Deisenhofen, Germany).

All chemicals were of analytical reagent grade and all solvents were of HPLC grade.

2.2. Chromatographic Equipment and Conditions

The high-performance liquid chromatography (HPLC) system consisted of a Beckman System Gold (Beckman Coulter Krefeld, Germany) equipped with a 126 solvent delivery module, a 508 autoinjector, a 168 UV-Vis photodiode-array detector and a Beckman System 32 Karat Gold software for peak identification and integration.

The analytical column was a XTerra™ RP-C18 column (2.1x150 mm I.D., particle size 5 µm; Waters Corporation, Eschborn, Germany) protected with a security guard C 18 (4 x 2.0 mm I.D.; Phenomenex Inc. Hösbach, Germany). The signal was monitored at 255 nm. Peak purity tests were carried out continuously during the batch analysis. A peak controlled spectrum recording was selected with a range of 190 – 300 nm and a peak purity method represents the storage of one spectral scan at peak upslope, apex and downslope.

The mobile phase, delivered at a flow rate of 0.2ml/min at room temperature, was a linear gradient of solution A acetonitrile in 67mM potassium dihydrogen phosphate (adjusted to pH 6.3 with sodium hydroxide, 32%) at a ratio of 60 : 40 (v/v) and solution B acetonitrile in 67mM potassium dihydrogen phos-

phate (adjusted to pH 6.3 with sodium hydroxide, 32%) at a ratio of 70 : 30 (v/v) programmed as follows: 60% solution A during 6min degreased to 20% in a time range of 10 min and maintained until reaching 2 min. The total run time to reached the original conditions was 26 min.

2.3. Solutions and Standard Preparation

Stock solution (10mg/100ml) of tipranavir was prepared in methanol and was appropriately diluted for the preparation of working solutions at concentrations of 1.25 – 400 μg/ml.

The internal standard (A-86093) working solution was prepared at 10mg/100ml in a mixture of 67 mM potassium dihydrogen phosphate in methanol 50 : 50 (v/v) . Each solution was stored at -20°C and stable for at least three months.

For preparation of the plasma standard samples, an appropriate amount of the stock and working solution of TPV and the internal standard (25 μg/ml) were added to blank drug-free plasma to achieve the above mentioned calibration concentrations.

2.4. Sample Preparation

A 100μl aliquot of plasma was mixed with 500 μl phosphate buffer (67mM potassium dihydrogen phosphate (41.3 parts) in 67 mM disodium hydrogen phosphate dihydrate (58.7parts) pH 7.0), furthermore an aliquot of 25 μl solution of internal standard (A-86039) was added to a 10 ml glass tube. After vortexing for 10 s the tubes were capped and extracted twice with 3 ml dietyl ether for 5 min, followed by centrifugation at 3000 g (4°C) for 5 min . The organic layers were transferred to a glass centrifuge tube and evaporated to dryness at 37°C with a gentle stream of nitrogen.

After evaporating, the residue was resolved in 300 μl 0.67 mM potassium dihydrogen phosphate in methanol 50 : 50 (v/v). and 20 μl of this solution was injected into the HPLC system.

2.5 Selectivity and Specificity

To evaluate selectivity and specificity of the method, drug-free plasma was spiked with therapeutic concentrations of the following compounds: abacavir, adefovir, amprenavir, atazanavir, delavirdine, didanosine, efavirenz, fluconazole, folinic acid, ganciclovir, itraconazole, lamivudine, methadone, methotrexate, nelfinavir, M8-metabolite of nelfinavir, nevirapine, oxazepam, pyrazinamide, pyrimethamine, ranitidine, rifampin, saquinavir, stavudine, sulfamethoxazole, sulfadoxin, tenofovir, trimethoprim, zalcitabine, and zidovudine, followed by extraction and analysis as described above. No interfering peaks were observed at the retention time for the TPV and IS peaks.

2.6. Sensitivity

The detection limit (LOD) was defined as the lowest concentration level resulting in a signal-to-noise ratio of three (S/N = 3).

For the concentration to be accepted as the lower limit of quantitation (LOQ), the measure of accuracy (percent deviation from the nominal concentration) and precision (relative standard deviation) are to be less than 20%. All samples were assayed in triplicates.

The upper limit of quantitation (ULQ) was arbitrarily set at 400 μg/ml.

2.7. Accuracy, Precision, Linearity, Recovery and Stability

Intra-day accuracy and precision of the method were determined by measuring 16 replicates at three different concentrations of tipranavir (5, 50 and 100 μg/ml) on the same day.

To obtain the inter-day accuracy and precision, four replicates of each concentration were analyzed at eight different days as described above.

Accuracy was calculated as the relative error of the nominal concentration. Precision was expressed in terms of relative standard deviation and obtained by analysis of variance (ANOVA) for each test concentration using the analytical run as the grouping variable.

Daily standard curves were evaluated by duplicate analysis of eight spiked drug free plasma samples for tipranavir in the range of 1.25 to 400 μg/ml.

A linear weighted [1/concentration squared] least squares regression analysis to plot the observed peak area/internal standard ratio of tipranavir was performed. Linearity and assay reproducibility were determined by measuring the standard concentration in five separate assay runs on five separate days.

The linearity of five calibration curves was tested with the F-test for lack of fit, using a weight factor of [1/conc].

The absolute recovery of tipranavir was calculated by comparing the detected concentrations of tipranavir (5 μg/ml, 10 μg/ml, 50 μg/ml, 100 μg/ml) in four extracted spiked drug-free plasma samples to those of non-processed standard solutions.

Stability studies were performed after 30 days at – 20 °C, 7 days at -20 °C including 3 freeze and thawed cycles, 7 days at 4 °C, 24 h at room temperature and 60min at 56 °C. The concentration of TPV after each storage period was related to the initial concentration as determined for the samples that were freshly prepared.

2.8. Analysis of Patient Samples

Plasma samples derived from 35 HIV-infected patients during antiretroviral therapy with combination of different NRTI zidovudine, lamivudine, tenofovir, emitricitabine or NNRTI efavirenz or viramune and tipranavir were taken 12 hours after the ingestion of 500 mg tipranavir with 200mg ritonavir. Plasma samples of all patients were obtained by a standardized procedure. Plasma was separated by centrifugation at 3000g for 10 min at 4°C and was immediately stored at -20°C until further analysis.

2.9. Calculation and Data Analysis

All statistical calculations were performed with the Statistical Product and Service Solutions (SPSS) for

Windows, version 12.0.1. (SPSS, Chicago, IL, USA). Correlation was considered statistically significant if the calculated value of p was 0.05 or less.

3. Results and Discussion

3.1. Chromatography and Detection

Sample preparation by protein precipitation with acetonitrile using a volume of 500 µl plasma or 250 µl plasma was described previously [3, 4, 5]. We use a 100 µl aliquot of plasma following liquid-liquid extraction with diethyl ether. By this extraction procedure, an even baseline and a good sensitivity of our assay was reached. Liquid-liquid extraction further resulted in less residue in the column after each run. Therefore, a high number of runs were possible in one column. Compared to solid-phase extraction, liquid-liquid extraction procedure seems to be more cost sparing [4, 5]. The use of an internal standard (ISTD) makes our

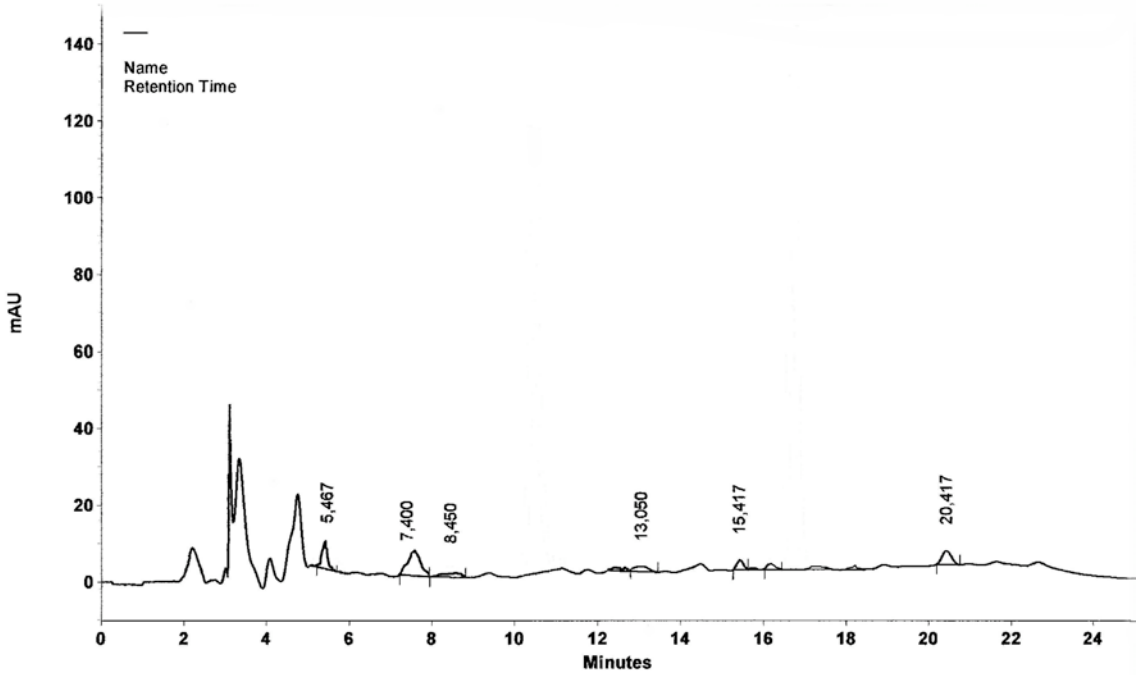

Fig. 1. Chromatogram of a blank human plasma sample UV- detection at 255 nm.

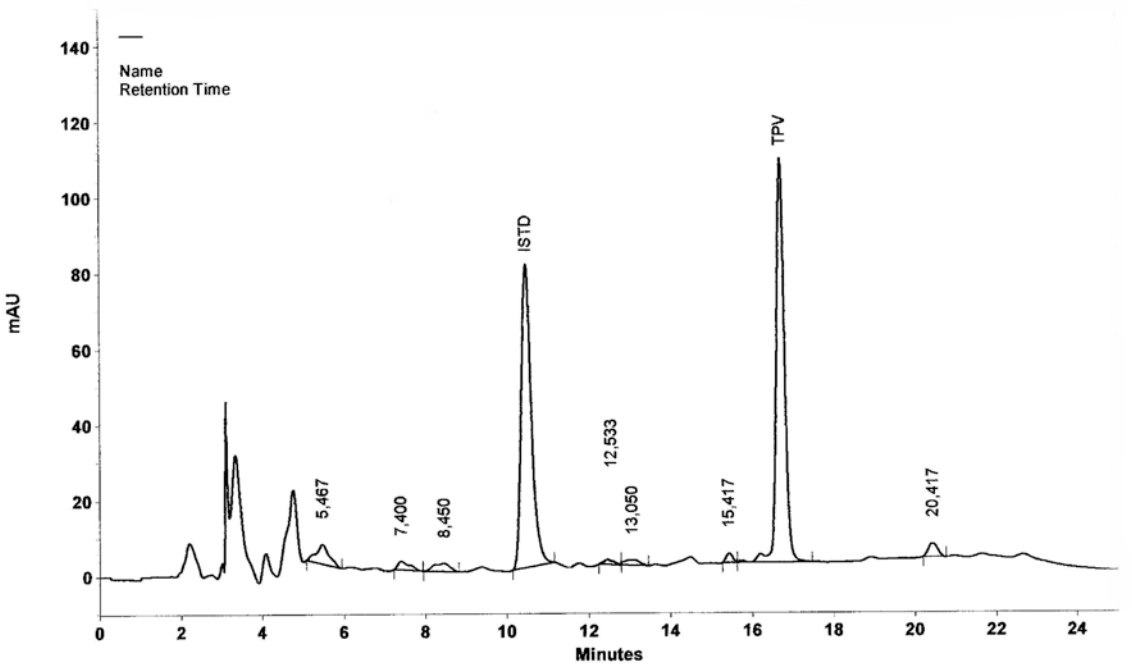

Fig. 2. Chromatogram of a blank human plasma sample spiked with 25µg/ml internal standard A-86093 (10.5min) and tipranavir 37.8µg/ml (16.7min) UV-detection at 255nm

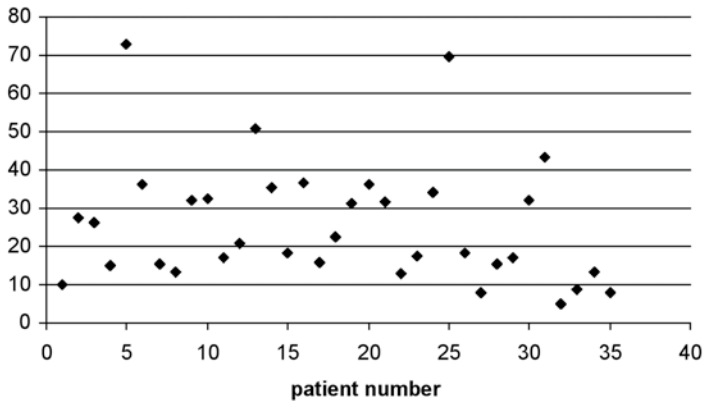

Fig. 3. Tipranavir plasma concentrations from 35 HIV-infected patients during antiretroviral therapy. Plasma samples were taken 12 hours after the ingestion of 500 mg tipranavir/200 mg ritonavir.

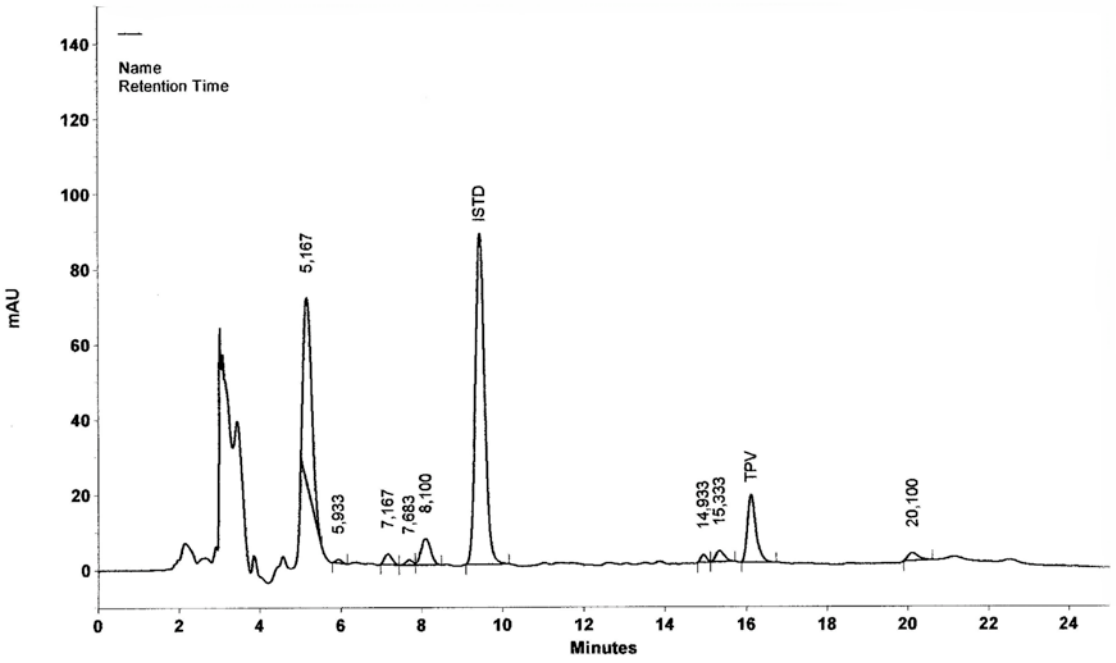

Fig. 4. Chromatogram of a patient plasma sample, receiving emitricitabine, tenofovir and tipranavir/ritonavir (500mg/200mg bid). The retention time of tipranavir was 16.7min. A trough plasma concentration of tipranavir from 8.1μg/ml was detected.

assay more reliable than a previously described method [3, 4, 5 6, 7, 8, 9]. The use of A-86093 as an internal standard with a retention time of 10.5 min was found to be convenient.

Peak shape, separation from endogenous compounds and separation from other antiretrovirals especially PI were therefore optimized by using a XTerra™ RP 18 column (2.1x150 mm I.D., particle size 5 μm; Waters Corporation, Eschborn, Germany) protected with a security guard C18 (4 x 2.0 mm I.D.; Phenomenex Inc. Hösbach, Germany). Under these conditions with a linear gradient of the mobile phase an adequate peak separation is reachable.

A chromatogram of a blank plasma sample (Fig.1) showed no interfering endogenous peaks. A representative HPLC run of a spiked plasma sample with 37.8 μg/ml tipranavir is shown in Figure 2.

A HPLC run of a plasma sample derived from a patient receiving a therapeutic regimen of emitricitabine, tenofovir and tipranavir/ritonavir (500mg/200mg bid) is shown in Figure 4. The retention time of tipranavir was 16.7 min. A trough plasma concentration of tipranavir from 8.1 μg/ml was detected.

3.2. Specificity and Selectivity

Drug-free plasma samples obtained from healthy individuals were devoid of interference near the retention time of tipranavir and the internal standard.

The analysis of spiked drug-free plasma samples containing abacavir, adefovir, amprenavir, atazanavir, delavirdine, didanosine, folinic acid, fluconazole, ganciclovir, indinavir, itraconazole, lamivudine, methadone, methotrexate, nelfinavir, M8 metabolite of nelfinavir, nevirapine, oxazepam, pyrazinamide, pyrimethamine, ranitidine, rifampin, stavudine, sulfamethoxazole, sulfadoxin, trimethoprim, zalcitabine, or zidovudine showed no interference with the extraction procedure nor with the analytical method.

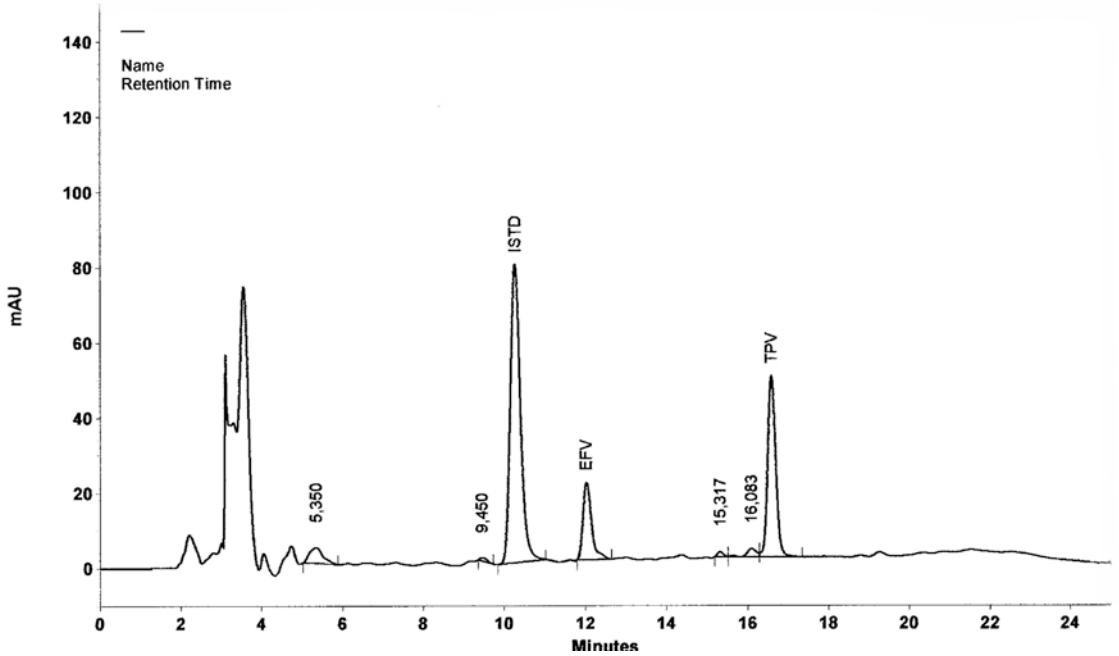

Fig. 5. Chromatogram of a patient receiving efavirenz 600mg qd and tipranavir/ritonavir (500mg/200mg bid). The retention time of tipranavir was 16.7 min. A trough plasma concentration of tipranavir from 17.3 μg/ml was detected. Efavirenz trough plasma level was 3.420 ng/ml at a retention time of 12.0 min.

Efavirenz is detectable in the described conditions at a retention time of 12.0min . In Figure 5 a HPLC run of a patient receiving 600mg efavirenz qd. and tipranavir 500mg bid is shown. Efavirenz trough level is detected 3420ng/ml at a retention time of 12.0 min and tipranavir trough level is 17.3 μg/ml at a retention time of 16.7 min.

3.3. Limit of Quantitation

The detection limit of tipranavir in plasma was determined at 32ng/ml. The lower limit of quantitation was reached at a concentration of 0.13 μg/ml. The upper limit of quantitation was arbitrarily set at 400 μg/ml.

3.4. Accuracy, Precision, Linearity and Recovery of the Assay

The intra-day accuracy and precision of the method was determined in 16 analytical runs including three different concentrations. Precision ranged from 1.31% to 2.90%. Intra-day accuracy was 0.21% to 5.57% . The results of intra-day validation are presented in Table 1. The inter-day accuracy and precision of tipranavir as shown in Table 2 were below 7%.

Using the ratios of the observed peak areas for tipranavir and the internal standard in eight spiked plasma samples analyzed in duplicate, the standard curves showed a correlation coefficient of 0.997 (range 1.25μg/ml to 400μg/ml) as determined by least-square analysis. All calibration curves proved to be linear in the respective ranges listed above in the F-test for lack of fit, which was performed to assess the linearity of the regression model. The CV% of five slopes was 3%.

Table 1. Intra-day accuracy and precision for the analysis of tipranavir (TPV) in spiked plasma samples: Precision (C.V. %) and accuracy (R.E. %).

Sample concentration (μg/ml)	5	50	100
Concentration found (μg/ml)			
mean	4.72	49.42	99.79
± S.D.	0.14	1.25	1.31
C.V. (%)	2.90	2.53	1.31
R.E. (%)	5.57	1.17	0.21
n =	16	16	16

Table 2. Inter-day accuracy and precision for the analysis of tipranavir (TPV) in spiked plasma samples: Precision (C.V. %) and accuracy (R.E. %).

Sample concentration (μg/ml)	5	50	100
Concentration found (μg/ml)			
mean	4.94	50.15	02.63
*S.D.	0.30	1.54	6.99
C.V. (%)	6.02	3.07	6.81
R.E. (%)	1.25	-0.30	-2.63
n =	8	8	8

The recovery of tipranavir was estimated by comparison of peak areas in extracted spiked drug free plasma with those of standard solutions. Best recovery

Table 3. Stability of tipranavir (TPV) in spiked plasma samples precision (C.V. %) and accuracy (R.E. %).

Sample concentration (μg/ml)	5	50	100
Concentration found (μg/ml)			
30 days at -20°C			
mean	5.00	51.00	106.10
*S.D.	0.00	2.00	3.00
C.V. (%)	4.50	4.02	3.17
R.E. (%)	1.50	-2.50	-6.00
7 days at -20°C with 3 freeze/ thawed cycles			
mean	5.00	52.00	107.20
*S.D.	0.00	1.04	1.00
C.V. (%)	2.48	1.57	0.54
R.E. (%)	-1.50	-4.00	-6.50
7 days at 4°C			
mean	4.90	52.30	109.03
*S.D.	0.38	2.20	6.99
C.V. (%)	7.82	4.22	1.01
R.E. (%)	2.00	-4.50	-8.75
24 h at room temperature			
mean	5.01	50.15	99.63
*S.D.	0.30	2.04	1.09
C.V. (%)	1.72	4.00	1.42
R.E. (%)	4.05	0.00	1.00
60 min at 56°C			
mean	4.94	50.05	101.63
*S.D.	0.30	1.54	1.99
C.V. (%)	1.66	0.07	0.81
R.E. (%)	2.05	-0.50	-0.63

(n = 4)

was found at the pH of 7.0 to be 96.2% ± 1.0% (n = 4) . If extraction conditons was variated e.g. at pH 9.4, recovery was only 76.1% ± 2.2% (n = 4).

Stability studies of tipranavir are shown in Table 3. Stability could be shown after 30 days at – 20° C, 7days at -20°C after 3 freeze and thawed cycles, 7 days at 4°C, 24h at room temperatue and 60 min at 56°C.

3.5 Analysis of Patient Samples

Plasma samples derived from 35 HIV-infected patients during antiretroviral therapy with a combination of different NRTI zidovudine, lamivudine, stavudine or didanosine and/or protease inhibitor and tipranavir were taken 12 hours after the ingestion of 500 mg tipranavir.

Plasma concentrations of tipranavir were 25.76 ± 15.96 μg/ml. The presented results, as shown in Figure 4, demonstrate the applicability of the assay for therapeutic drug monitoring in HIV-infected patients.

4. Conclusion

We present a validated, reliable and convenient assay for the determination of tipranavir in human plasma. The described HPLC assay can readily be used in a standard hospital laboratory.

In our hands, the described procedure was most suitable. Calibration curves for tipranavir from 1.25 μg/ml to 400 μg/ml are appropriate for clinical drug monitoring, and are especially suitable for assessment of drug levels in the salvage patients`s situation with multiresistant virus and a lot of drug-drug interactions.

The practicability of the assay is demonstrated by plasma levels of 35 patients regularily seen in our outpatient care unit.

This HPLC method is preferentially used for drug monitoring in patients treated with tipranavir in combination with other antiretroviral agents. This type of drug monitoring may be essential for the estimation of drug plasma level in patients with loss of antiretroviral efficacy caused of interaction with other hepatically metabolized co-medications, noncompliance or resorption deficite of tipranavir in contrast to viral resistance.

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