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Simultaneous quantification of the new HIV protease inhibitors atazanavir and tipranavir in human plasma by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry

K.M.L. Crommentuyn [a,*], H. Rosing [a], M.J.X. Hillebrand [a], A.D.R. Huitema [a], J.H. Beijnen [a,b]

[a] *Department of Pharmacy and Pharmacology, Slotervaart Hospital, Louwesweg 6, 1066 EC Amsterdam, The Netherlands*
[b] *Department of Biomedical Analysis, Faculty of Pharmaceutical Sciences, Division of Drug Toxicology, P.O. Box 80.082, 3508 TB Utrecht, The Netherlands*

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Abstract

We have developed and validated an assay, using liquid chromatography coupled with electrospray tandem mass spectrometry (LC–MS/MS), for the quantification of the novel protease inhibitors (PIs) atazanavir and tipranavir. The sample pre-treatment consisted of protein precipitation with a mixture of methanol and acetronitrile using 100 μl plasma for atazanavir and 50 μl for tipranavir. Chromatographic separation was achieved on an Inertsil ODS3 column (50 mm × 2.0 mm i.d., particle size 5 μm), with a quick stepwise gradient using an acetate buffer (pH 5) and methanol, at a flow rate of 0.5 ml/min. The analytical run time was 5.5 min. The triple quadrupole mass spectrometer operated in the positive ion-mode and multiple reaction monitoring (MRM) was used for drug quantification. The assay was linear over a concentration range of 0.05–10 μg/ml for atazanavir and 0.1–75 μg/ml for tipranavir. Saquinavir-d5 was used as internal standard. The intra- and inter-day coefficients of variation were less than 3.8% for atazanavir and less than 10.4% for tipranavir. Accuracies were within ±7.3 and ±7.2% for atazanavir and tipranavir, respectively. Both drugs were stable under various relevant storage conditions. The validated concentration ranges proved to be adequate to measure concentrations of human immunodeficiency virus type-1 (HIV-1)-infected individuals. The developed method could easily be combined with a previously developed LC–MS/MS assay for the quantification of protease inhibitors.

Keywords: Atazanavir; Tipranavir

1. Introduction

Atazanavir and tipranavir are two novel human immunodeficiency virus type-1 (HIV-1) protease inhibitors (PIs), which were designed to have a more beneficial pharmacodynamic and/or pharmacokinetic profile compared to the currently licensed PIs [1,2].

Atazanavir (Fig. 1A) is an azapeptide PI with a pharmacokinetic profile that allows once-daily oral administration [1]. It has a distinct resistance pattern and has shown efficacy in treatment-naive as well as in experienced patients. The recommended non-boosted dosage of atazanavir is 400 mg once-daily, if desired in combination with saquinavir. When co-administered with efavirenz or other interacting drugs, it is recommended that atazanavir (300 mg) is boosted with ritonavir (100 mg) once-daily [1].

Tipranavir (Fig. 1B) is the first non-peptidic PI in development for the treatment of HIV-1-infected patients [2]. It has a markedly improved resistance profile compared with traditional, peptidic PIs, possibly requiring as many as 16–20 protease gene mutations, including at least three universal protease inhibitor resistance-associated mutations (PRAMs), to confer resistance. Sustained viral suppression has been demonstrated in phase II clinical trials involving patients with prior PI experience and resistance. Tipranavir requires co-administration with ritonavir to achieve adequate plasma

* Corresponding author. Tel.: +31-20-512-4481; fax: +31-20-512-4753.
E-mail address: apkcr@slz.nl (K.M.L. Crommentuyn).

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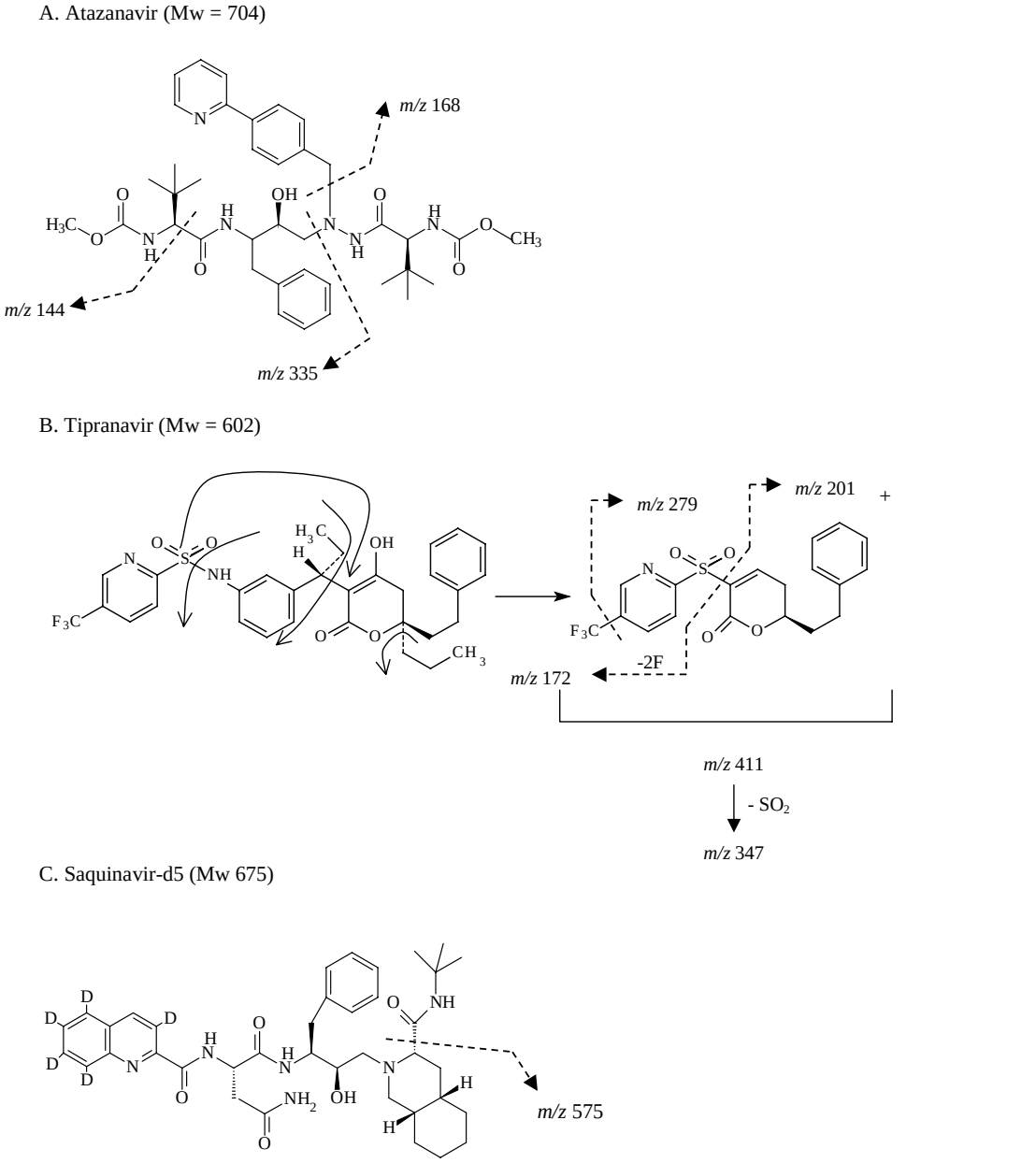

Fig. 1. Molecular formulas and proposed fragmentation patterns of atazanavir (A), tipranavir (B), and the internal standard saquinavir-d5 (C).

drug concentrations. It is currently under investigation in a dosage of 500 mg/200 mg tipranavir/ritonavir in two large phase III studies [2].

Atazanavir was approved in the US in June 2003 and approval in Europe is pending. In anticipation of approval, early access programs were developed for atazanavir as well as for tipranavir. To support the treatment of HIV-1-infected patients with these new promising drugs, we developed and validated a rapid and sensitive method to measure plasma concentrations of atazanavir and tipranavir using liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC–MS/MS). For atazanavir, assays measuring the drug in human plasma and in peripheral blood mononuclear cells (PBMC) have been published recently [3–5]. Tipranavir assays have not been published hitherto, as far as we know. We developed the assay for atazanavir and tipranavir so that it could be combined with our earlier described LC–MS/MS method for the quantification of the protease inhibitors amprenavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir, and the pharmacologically active nelfinavir metabolite M8 in human plasma [6]. The validated concentration ranges were based on the concentrations expected in routine clinical practice. The validation of the method has been executed based on the most recent international guidelines for bioanalytical validation [7].

2. Experimental

2.1. Chemicals

Atazanavir (Reyataz™, 200 mg per capsule) and tipranavir (PNU-140690, 250 mg per capsule) (Fig. 1A and B) originated from Bristol Myers Squibb, PRI, Wallingford, CT, USA and Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT, US, respectively. The internal standard d5-saquinavir-mesylate (saquinavir-d5) (Fig. 1C) was kindly provided by Roche Products, Research and Development (Welwyn Garden City, UK). Acetonitrile and methanol were HPLC-grade reagents and were obtained from Biosolve (Valkenswaard, The Netherlands). Ammonium acetate and glacial acetic acid of analytical grade were obtained from Merck (Amsterdam, The Netherlands). Distilled water was used throughout the analysis. Drug free human plasma originated from Sanquin (Amsterdam, The Netherlands).

2.2. Chromatographic and mass spectrometric conditions

An Agilent (Agilent Technologies, Palo Alto, CA, USA) HPLC system was used consisting of an 1100 series pump and cooled well plate autosampler (4 °C) that accommodates two 96-well plates. Separation was carried out on a reversed-phase system with an Inertsil ODS3 column (50 mm × 2.0 mm i.d., particle size 5 μm) (Chrompack, Middelburg, The Netherlands) protected with a Chromguard minibore reversed-phase precolumn (10 mm × 2.0 mm i.d., Chrompack) and an in-line filter (0.5 μm, Upchurch Scientific, Oak Harbor, WA, USA). A quick, stepwise gradient was used to elute the compounds from the column. At time zero, 100% eluent A (methanol—10 mM ammonium acetate buffer pH 5.0 (35:65, v/v)) was flushed through the column. After 0.2 min, 15% of eluent A was mixed with 85% of methanol and this mobile phase composition was maintained for 1.6 min. In 0.1 min, the mobile phase consisted of eluent A again and the column was re-conditioned in 3.5 min before the next injection. The flow rate was 0.5 ml/min. The column outlet was connected to the electrospray sample inlet (Sciex, Thornhill, ON, Canada) through a post column splitter (1:4) (ICP-04-20-CR, LC packings, Amsterdam, The Netherlands) and a divert valve (Valco Instruments, Houston, TX, USA). The latter was used to direct the first 1.5 min of the eluent flow to waste to prevent the introduction of endogenous compounds into the mass spectrometer. The source temperature was held constant at 350 °C. Ions were created at atmospheric pressure and were transferred to an API 3000 triple quadrupole mass spectrometer (Sciex). The nebulizer (1.8 ml/min) and turbo (7.0 l/min) gases were zero air, while the curtain (1.3 l/min) and collision activated dissociation gas (240×10^{12} molecules/cm^2) consisted of nitrogen (grade 5.0). The electrospray voltage was +4 kV and the dwell time was 50 ms with a 5 ms pause between scans. Q1 and Q3 were operating at unit mass resolution. Multiple reaction monitoring (MRM) was used for drug quantification. Precursor ions of analytes and internal standards were determined from spectra obtained during the infusion of standard solutions using an infusion pump connected directly to the electrospray source. Only singly charged molecular ions were observed. Each of the precursor ions was subjected to collision induced dissociation to determine the product ions. The transitions of the protonated precursor/product ion pairs that were used for recording the selected-ion mass chromatograms are from mass-to-charge (*m*/*z*) 705 to 168 for atazanavir, *m*/*z* 603 to 411 for tipranavir and *m*/*z* 676 to 575 for the internal standard saquinavir-d5. Data were processed by Analyst™ software (Version 1.2, Sciex).

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

Two stock solutions of each analyte were prepared independently in methanol at a concentration of approximately 2 mg/ml for atazanavir and 1 mg/ml for tipranavir. One solution was used to spike the plasma calibration samples and the other was used to prepare the quality control (QC) samples. Both stock solutions were diluted further with methanol to obtain working solutions with concentrations of 1 mg/ml (atazanavir), and 100, 10 and 1 μg/ml, respectively. The stock and working solutions were stored at −20 °C. Aliquots of the stock solutions were stored at −70 °C for stability experiments.

A stock solution of the internal standard saquinavir-d5 was prepared in methanol at a concentration of approximately 400 μg/ml. The stock solution was diluted to a concentration of approximately 1.5 μg/ml in a mixture of methanol and acetonitrile (1:1, v/v). This solution was used as 'protein precipitation' reagent.

Separate calibration standards (at seven concentrations) and QC samples (at the lower limit of quantification (LLQ), low, medium, high and above the upper limit of quantification (ULQ) concentration levels) in plasma were prepared for atazanavir and tipranavir by diluting various volumes of the working solutions in control human plasma. The following ranges were validated: 0.05–10 μg/ml for atazanavir and 0.1–75 μg/ml for tipranavir. The choice for these ranges was based on the expected concentrations in patients in the daily practice of therapeutic drug monitoring.

2.4. Sample processing

For atazanavir, the sample processing was equal to the previously described procedure for PIs [6]. To 100 μl of plasma, 200 μl of protein precipitation reagent (including the internal standard) was added (to the double blank sample of the calibration curve 200 μl of protein precipitation reagent without internal standards was added). After vortex mixing for 30 s, the samples were centrifuged at 23,100 × *g* for 15 min. A volume of 150 μl of supernatant was then transferred to a 96-well plate. The extracts were diluted by

adding 150 μl of the dilution reagent (50 mM ammonium acetate buffer, pH 5) with a multichannel pipette (Eppendorf, Hamburg, Germany). The 96-well plate was shaken for 20 min on a Heidolph shaking device (Emergo, Landsmeer, The Netherlands). The sample processing for tipranavir was slightly divergent. To 50 μl of plasma, 600 μl of protein precipitation reagent (including the internal standard) was added (to the double blank sample of the calibration curve 600 μl of protein precipitation reagent without internal standards was added). After vortex mixing for 30 s, the samples were centrifuged at 23,100 × *g* for 15 min. A volume of 150 μl of supernatant was transferred to a 96-well plate and the procedure was proceeded as described before. For both compounds, a volume of 10 μl of each sample was injected onto the analytical column.

2.5. Validation procedures

The validation of the assay was based on the FDA guidelines for Bioanalytical Method Validation [7].

2.5.1. Linearity

Calibration standards were prepared and analyzed in triplicate in three independent runs. Calibration curves (area ratio with the internal standard versus nominal analyte concentration) were fitted by least-squares linear regression without weighting and using 1/concentration and 1/concentration^2 as weighting factors. In order to establish the best weighting factor, back-calculated concentrations were determined. The model with the lowest total bias and the most constant bias across the concentration range was considered to be the best fit. To assess linearity, deviations of the mean calculated concentrations over three runs should be within ±15% from nominal concentrations for the non-zero calibration standards. At the LLQ level a deviation of ±20% was permitted.

2.5.2. Accuracy and precision

Accuracy, intra- and inter-assay precision of the method were determined by assaying five replicates of each of the spiked QC samples with analyte concentrations around the LLQ, and in the low, mid and high concentration range in three separate analytical runs. Accuracy was measured as the percentage deviation from the nominal concentrations. The intra- and inter-assay precision should not exceed 15% coefficient of variation (CV) except for the LLQ, were it should not exceed 20% CV.

To validate the accuracy and precision of the analysis of samples originally above the upper limit of quantification, a QC sample 1.5 (atazanavir) and 1.3 (tipranavir) times higher than the upper limit of the calibration curve was diluted five-fold with control human plasma and processed and analyzed according to the described method.

2.5.3. Recovery

To determine the amount of ion suppression, the analytical results of spiked plasma extracts were compared with those for diluted working solutions. The protein precipitation recovery of the analytes (at three concentrations) was determined by comparing the analytical results for extracted samples with those for the spiked plasma extracts. Total recovery was determined by comparing the analytical results for extracted samples with those for diluted working solutions.

2.5.4. Selectivity and specificity

Out of six batches control human plasma double blank-, blank- and LLQ samples were prepared, processed, and analyzed to determine whether endogenous plasma constituents interfered with the assay.

To investigate the potential interference of co-medication with the quantification of the analytes, the co-medicated drugs were added to LLQ samples. The samples were then processed and assayed according to the described method. The following drugs were tested: abacavir, caffeine, co-trimoxazole, didanosine, efavirenz, enfuvirtide, fluconazole, folinic acid, ganciclovir, itraconazole, lamivudine, methadone, nevirapine, oxazepam, paracetamol, pyrazinamide, pyrimethamine, ranitidine, rifampin, stavudine, tenofovir, zalcitabine, zidovudine, and zidovudine-glucuronide in a final concentration of 20 μg/ml. In addition, the potential interference of the ULQ concentration of the protease inhibitors amprenavir (10 μg/ml), indinavir (10 μg/ml), lopinavir (20 μg/ml), nelfinavir (10 μg/ml), ritonavir (10 μg/ml), saquinavir (10 μg/ml), and the pharmacologically active nelfinavir metabolite M8 (5 μg/ml) was tested by spiking these compounds to control human plasma and to plasma containing both analytes at the LLQ level. Areas of peaks co-eluting with the analytes should not exceed 20% of the area at the LLQ level. The deviation of the nominal concentration for the LLQ samples should be within ±20%.

2.5.5. Stability

The stability of the analytes was investigated at various concentrations during all steps of the analysis, which includes the stability in the stock solutions, in plasma under various conditions, and in the final extract. The stability in the stock solutions at −20 °C was determined in relation to a portion of the stock solutions that was kept at −70 °C. Freshly prepared QC samples at the low and high concentration levels were stored for 24 h at ambient temperature, 9 days at 4 °C and underwent three freeze-thaw cycles. The long-term stability at −20 °C in plasma was determined for a minimum of 2 months. For each of the storage conditions three replicates were analyzed at two concentration levels (QC low and high). The concentrations of the analytes were related to the initial concentration as determined for the samples that were freshly prepared and processed immediately.

The processed sample stability of all analytes in the final extract was studied at three concentrations. Hereto, the measured concentrations in a final extract of QC samples prepared 4 days earlier (three for tipranavir), were related to the measured concentrations of the same QC samples

immediately after processing. Moreover, the re-injection reproducibility was studied over a period of 4 days for atazanavir and 3 days for tipranavir.

The analytes were considered to be stable in the biological matrix and in the final extract when 85–115% of the initial concentration was found. For stability in stock solutions these figures were 95–105%.

2.6. Analysis of patient samples

Concentrations of atazanavir and tipranavir in plasma were analyzed with the currently reported method in samples from HIV-1-infected patients to assess the applicability of the method. Blood samples were drawn in heparinized tubes. Plasma was isolated by centrifugation during 10 min at 900 × *g* and stored at −20 °C until analysis.

3. Results and discussion

Positive-ion electrospray Q1 mass spectra and the MS/MS product-ion spectra of atazanavir and tipranavir are shown in Figs. 2 and 3, respectively. The proposed fragmentation patterns of the analytes and the internal standard resulting in the formation of the product ions used in MRM analysis are shown in Fig. 1. For atazanavir, the most intense signal in the Q1 spectrum was seen at mass-to-charge (*m*/*z*) ratio 705, corresponding with the $[M + H]^+$ ion. In the product ion mass spectrum the most intense fragments were seen at *m*/*z* 335, 168, and 144. The proposed structure of the fragments was adapted from Schuster et al. [3] and shown in Fig. 1. The fragment with highest intensity at *m*/*z* 168 was used for MRM analysis.

In the Q1 spectrum of tipranavir, two predominant peaks at *m*/*z* 625 and 585 were present besides the $[M+H]^+$ ion at *m*/*z* 603, corresponding with the molecular ion plus sodium, and minus H_2O, respectively. The product ion mass spectrum showed intense fragments at *m*/*z* ratios of 585 (corresponding with the molecular ion $-H_2O$) and 411. The fragment at *m*/*z* 411 was used in the MRM analysis. Fragments with less intensity were seen at *m*/*z* ratios of 279, 201, and 172. To elucidate the putative structure of the product ion used in MRM analysis, a MS/MS spectrum of *m*/*z* 411 was recorded, in which a peak at *m*/*z* ratio 347 was shown in addition to the fragments at *m*/*z* ratios of 279, 201, and 172. From this information a structural, speculative, proposal was formulated in which the sulphonamide bond was cleaved and the remaining trifluoromethyl-2-pyridinesulphon group merged with the 6-(2-phenylethyl)-dyhydropyrone moiety with

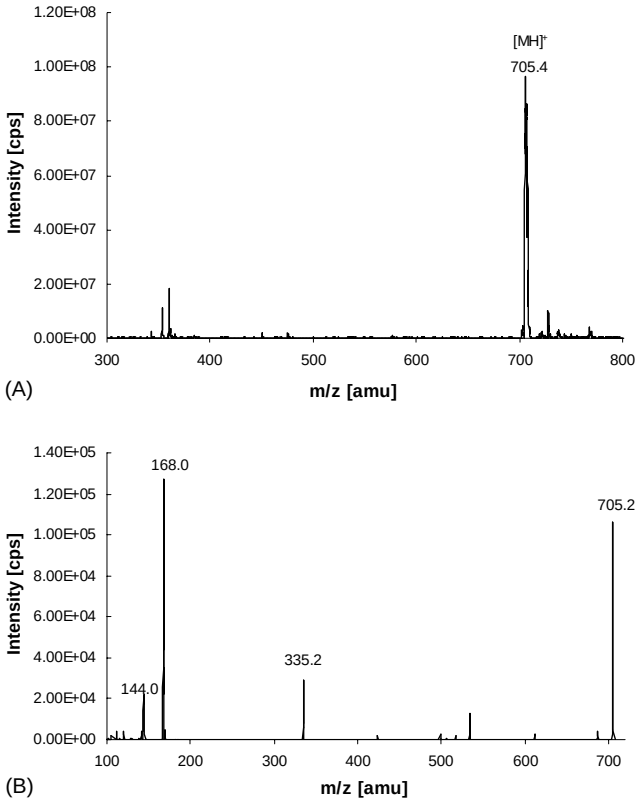

Fig. 2. Positive-ion electrospray Q1 mass spectrum (A) and MS/MS product-ion spectrum of *m*/*z* 705 (B) for atazanavir.

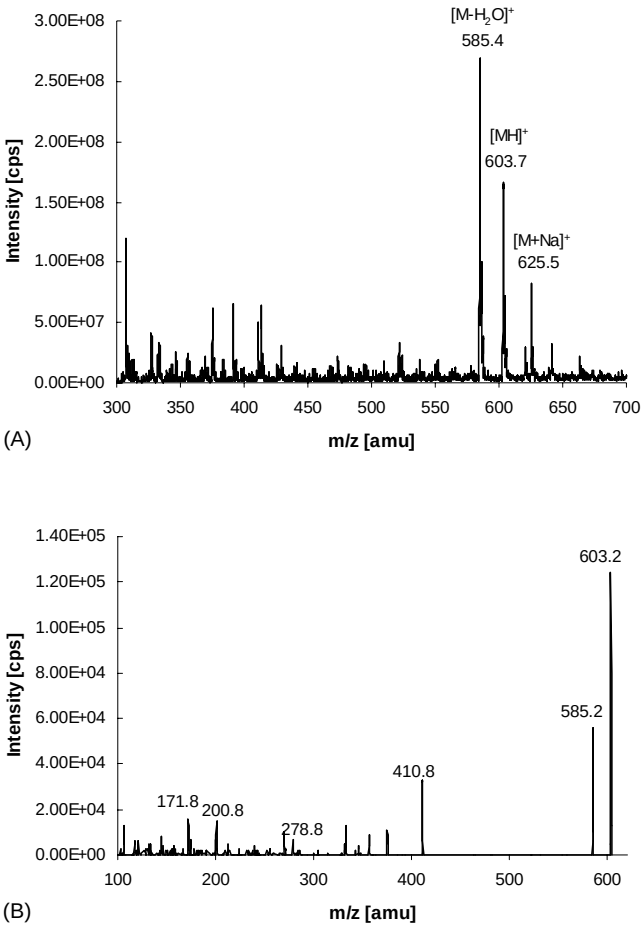

Fig. 3. Positive-ion electrospray Q1mass spectrum (A) and MS/MS product-ion spectrum of *m*/*z* 603 (B) for tipranavir.

exclusion of 2-propylaniline (Fig. 2). Information about the fragmentation patterns of sulphonamides was derived from Klagkou et al. [8].

The sample pre-treatment and liquid chromatography part of the assay was derived from the previously developed PI assay [6]. It was attempted to maintain identical procedures so that the analysis of the two new PIs could be fully integrated in the existing assay. The attempt was successful for atazanavir. For tipranavir, however, a calibration range of 0.1–75 μg/ml was preferred, which was based on the relatively high concentrations expected in routine clinical practice. With the previously developed pre-treatment procedure, this resulted in concentrations exceeding the linear dynamic range of the detector. Therefore, the sample pre-treatment procedure was adjusted to comprise a larger dilution factor. To 50 μl of plasma, 600 μl of protein precipitation reagent (including the internal standard) was added. After vortex mixing and centrifugation, a volume of 150 μl of supernatant was transferred to a 96-well plate and diluted by adding 150 μl of the dilution reagent. This adjusted procedure reduced the tipranavir amount that was introduced into the mass spectrometer by approximately nine-fold and resulted in linear calibration curves. The signal-to-noise ratio at the LLQ level was in agreement with the requirements set by the FDA guidelines. The chromatographic procedure remained identical to the previously developed procedure, hence tipranavir containing samples could be analyzed in the same run, although not simultaneously with samples containing the other PIs. Retention times were approximately 3.3, 3.5, and 3.4 min for atazanavir, tipranavir, and the internal standard saquinavir-d5, respectively. Representative chromatograms of control human plasma samples and spiked samples at the LLQ level are shown in Fig. 4.

3.1. Validation procedures

The assay was linear over the validated concentration ranges of 0.05–10 μg/ml for atazanavir and 0.1–75 μg/ml for tipranavir. The best fit for the calibration curves was obtained by using a weighting factor of 1/concentration^2 for

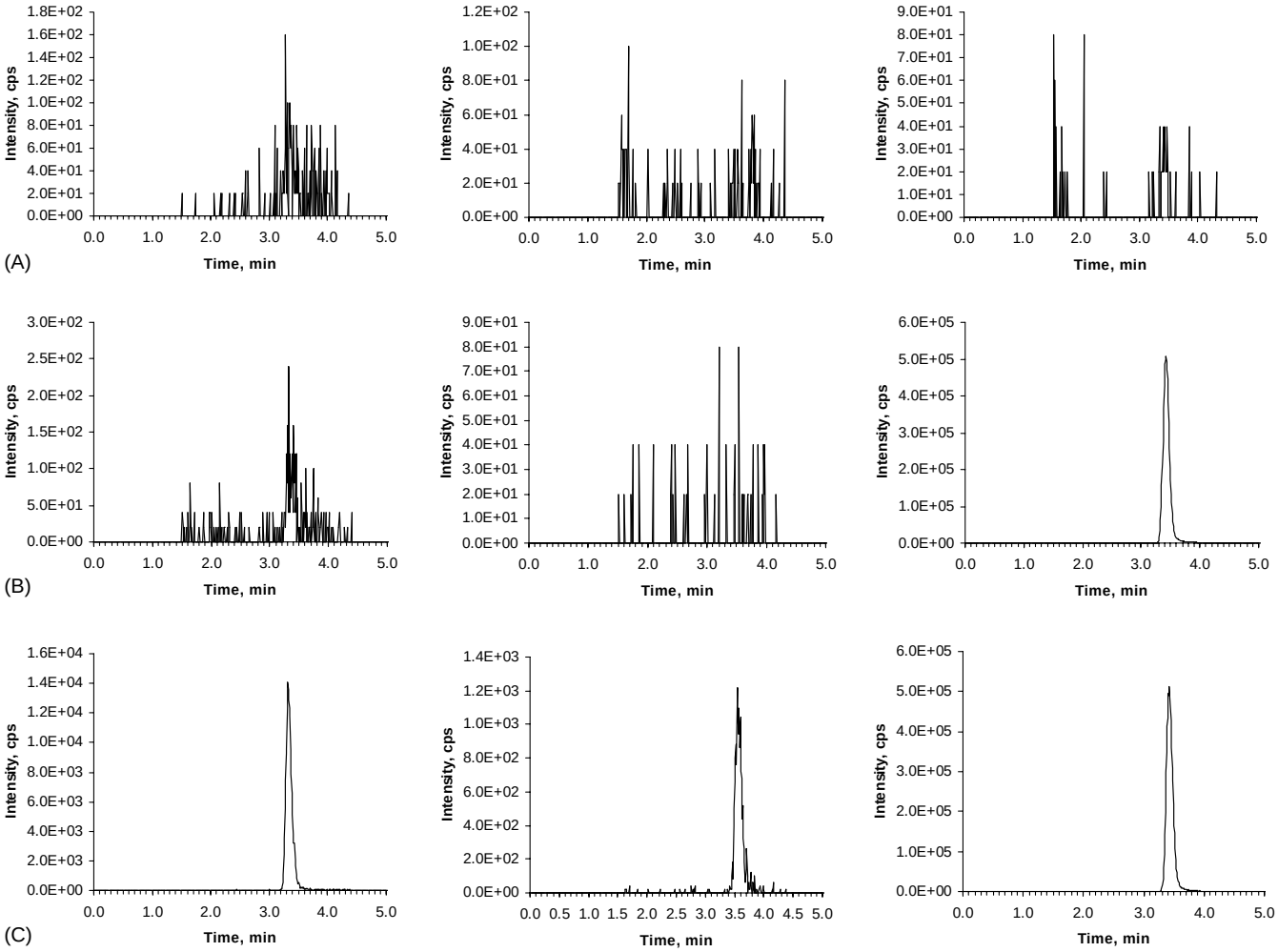

Fig. 4. MRM chromatograms of atazanavir (left), tipranavir (middle), and the internal standard saquinavir-d5 (right), of a double blank sample (A), a blank sample (B), and an LLQ sample (C).

Table 1
Intra- and inter-assay performance data of the analytes at five concentration levels ($n = 15$)

Nominal Concentration (μg/ml)	Measured concentration (μg/ml)	Accuracy (%)[a]	Intra-assay precision (%)	Inter-assay precision (%)
Atazanavir				
0.0550	0.0510	−7.3	2.5	3.5
0.165	0.169	2.4	1.8	3.1
1.10	1.09	−0.9	0.9	2.0
8.25	8.07	−2.2	2.5	3.8
16.5[b]	15.6	−5.3	2.2	2.5
Tipranavir				
0.100	0.0928	−7.2	7.6	10.4
0.300	0.294	−2.0	2.5	3.9
10.0	9.63	−3.7	2.8	3.1
65.0	65.2	0.3	2.8	4.4
100.0[b]	95.9	−4.1	4.0	6.4

[a] Accuracy = [(measured concentration − nominal concentration)/nominal concentration] × 100.
[b] Samples originally above the ULQ, quantified after five-fold dilution with control human plasma.

Table 2
Recovery data for the analytes ($n = 9$)

Nominal concentration (μg/ml)	Ion suppression (%)	Protein precipitation recovery (%)	Total recovery (%)
Atazanavir			
0.165	−1.0	82.9	83.9
1.10	−2.5	84.3	86.8
8.25	2.0	95.6	93.6
Tipranavir			
0.300	−0.5	87.1	87.6
10.0	1.7	93.1	91.4
65.0	2.6	92.6	90.0

both analytes. The deviations from the nominal concentration were less than 8% for atazanavir and less than 7% for tipranavir at all concentrations (including the LLQ). Correlation coefficients were at least 0.997.

The intra- and inter-assay performance data are presented in Table 1. Accuracies were −7.3 and −7.2% for the LLQ and within ±2.4 and ±3.7% for the low, mid, and high concentrations, for atazanavir and tipranavir, respectively. Inter-assay precision was less than 3.8% for atazanavir and less than 10.4% for tipranavir at all tested concentrations. The mean intra-assay precision did not exceed 2.5 and 7.6% for atazanavir and tipranavir, respectively. Samples originally above the ULQ could be quantified with acceptable accuracy and precision after a five-fold dilution with control human plasma. Measured accuracies were −5.3 and −4.1% for atazanavir and tipranavir, respectively.

The recovery data for the analytes are listed in Table 2. Total recovery values were between 84 and 94% for atazanavir and between 88 and 91% for tipranavir. The degree of ion suppression was marginal.

3.1.1. Selectivity and specificity

MRM chromatograms of six batches of control human plasma contained no endogenous peaks co-eluting with any of the analytes. LLQ samples, prepared in these six batches of human plasma, could be quantified within the required 20% deviation from the nominal concentration. No chromatographic interferences were found from the tested drugs in control human plasma.

3.1.2. Stability

Atazanavir and tipranavir were stable in the stock solution at −20 °C for at least 2 months (data not shown). The internal standard stock solution (saquinavir-d5) was stable for at

Table 3
Stability data for atazanavir and tipranavir in spiked human plasma ($n = 3$)

Storage condition	Nominal concentration (μg/ml)	Mean concentration at $t = 0$ (μg/ml)	Mean concentration recovered (μg/ml)	Deviation (%)	CV (%)
Atazanavir					
24 h at 25 °C	0.165	0.164	0.167	1.8	0.5
	8.25	8.49	8.03	−5.4	0.6
9 days at 4 °C	0.165	0.167	0.167	0.0	0.7
	8.25	7.97	7.89	−1.0	1.1
Three freeze-thaw cycles	0.165	0.167	0.170	1.8	1.1
	8.25	8.01	7.86	−1.9	0.6
3 months at −20 °C	0.165	0.166	0.166	0.0	4.2
	8.25	7.86	7.91	0.6	6.2
Tipranavir					
24 h at 25 °C	0.300	0.313	0.320	2.2	0.5
	65.0	61.4	59.8	−2.6	0.5
9 days at 4 °C	0.300	0.276	0.271	−1.8	0.8
	65.0	66.2	64.2	−3.0	1.2
Three freeze-thaw cycles	0.300	0.320	0.320	0.0	1.5
	65.0	55.6	58.0	4.3	1.5
2 months at −20 °C	0.300	0.307	0.333	8.5	5.2
	65.0	65.7	64.7	−1.5	3.4

CV: coefficient of variation.

Table 4
Processed sample stability of the analytes at 4 °C ($n = 3$)

Nominal concentration (μg/ml)	Mean concentration at $t = 0$ days (μg/ml)	Mean concentration at $t = 3$ days/4[a] days (μg/ml)	Deviation (%)	CV (%)
Atazanavir				
0.165	0.160	0.167	4.4	2.3
1.10	1.040	1.075	3.4	1.5
8.25	7.83	7.67	−2.0	0.8
Tipranavir				
0.300	0.287	0.279	−2.8	2.6
10.0	9.55	9.84	3.0	0.9
65.0	62.9	62.8	−0.2	1.2

[a] $t = 4$ days for atazanavir and $t = 3$ days for tipranavir.

least 22 months. The percentage of none-deuterium-labeled saquinavir present in the stock solutions was less than 0.1%. Atazanavir and tipranavir were stable in plasma for at least 24 h at ambient temperature, 9 days at 4 °C, 3 (atazanavir) and 2 months (tipranavir) at −20 °C, and after three freeze-thaw cycles (Table 3). The stability of atazanavir and tipranavir in the final extract at 4 °C was guaranteed for at least 4 and 3 days, respectively (Table 4). Re-injection of processed samples was feasible for the same periods of time (data not shown).

3.2. Analysis of patient samples

Concentrations of atazanavir and tipranavir in plasma were analyzed with the currently reported method in samples from HIV-1-infected patients. Fig. 5A shows concentration versus time data of atazanavir from various subjects ($n = 8$) at different time points. In addition, a full pharmacokinetic curve during a dosing interval is shown. This HIV-1-infected female received atazanavir boosted with ritonavir in a dosage of 300 mg/100 mg once-daily, in combination with 600 mg of efavirenz and 245 mg tenofovir disoproxil once-daily. Fig. 5B shows the plasma concentration versus time curve of an HIV-1-infected male, treated with ritonavir-boosted tipranavir in a dosage of 500 mg/200 mg twice-daily in combination with didanosine 250 mg and tenofovir disoproxil 245 mg once-daily.

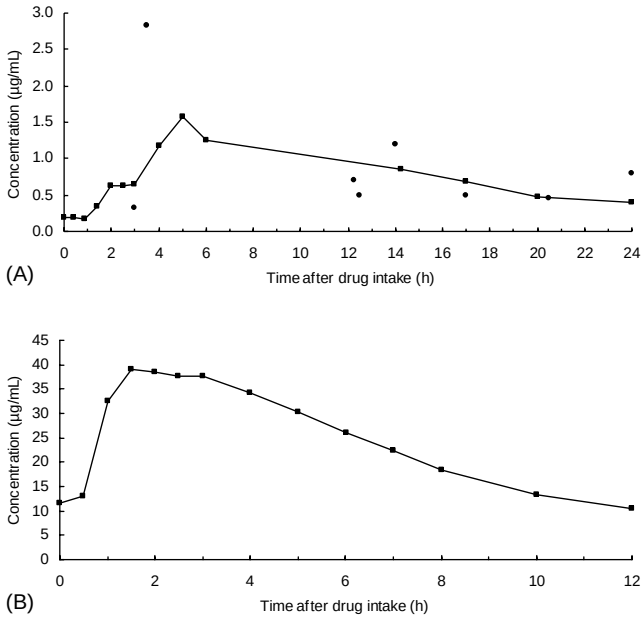

Fig. 5. Concentration vs. time data of atazanavir (A) and tipranavir (B) in plasma during one dosing interval (—) and at various time points (●).

4. Conclusion

A simple and rapid assay was developed and validated for the determination of two novel protease inhibitors atazanavir and tipranavir in human plasma using HPLC-tandem mass spectrometry. The validation was based on current FDA guidelines for bioanalytical method validation [7].

The analysis of atazanavir and tipranavir containing samples can be easily combined with the analysis of other PIs in the previously developed assay [6]. This is especially useful since concomitant administration of other PIs will be frequent in the population of heavily pre-treated patients. The applicability of the method and the appropriateness of the validated concentrations ranges are demonstrated in the analysis of plasma samples of HIV-1-infected subjects.

The assay proved to be accurate and precise and will be used for therapeutic drug monitoring and pharmacokinetic research in our hospital.

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