Quantification of 8 HIV-Protease Inhibitors and 2 Nonnucleoside Reverse Transcriptase Inhibitors by Ultra-Performance Liquid Chromatography with Diode Array Detection

Laure Elens,1* Sophie Veriter,1 Vincent Di Fazio,2 Roger Vanbinst,2 Daniel Boesmans,1 Pierre Wallenacq,2 and Vincent Haufroid1,2
1 Industrial Toxicology and Occupational Medicine Unit, Université Catholique de Louvain, Brussels, Belgium; 2 Clinical Chemistry Department, University Hospital St. Luc, Brussels, Belgium; * address correspondence to this author at: 53.02, Avenue E. Mounier, 1200 Bruxelles, Belgium. Fax +32 (0)2/764.53.38; e-mail laure.elens@uclouvain.be.

BACKGROUND: Most HPLC-UV methods for therapeutic drug monitoring of anti-HIV drugs have long run times, which reduce their applicability for high-throughput analysis. We developed an ultra-performance liquid chromatography (UPLC)–diode array detection method for the simultaneous quantification of the HIV-protease inhibitors (PIs) amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir, and tipranavir (TPV), and the nonnucleoside reverse transcriptase inhibitors (NNRTIs) efavirenz and nevirapine.

METHODS: Solid-phase extraction of 1 mL plasma was performed with Waters HLB cartridges. After 3 wash steps, we eluted the drugs with methanol, evaporated the alcohol, and reconstituted the residue with 50 μL methanol. We injected a 4-μL volume into the UPLC system (Waters ACQUITY UPLC BEH C8 column maintained at 60 °C) and used a linear gradient of 50 mmol/L ammonium acetate and 50 mmol/L formic acid in water versus acetonitrile to achieve chromatographic separation of the drugs and internal standard (A-86093). Three wavelengths (215, 240, and 260 nm) were monitored.

RESULTS: All drugs were eluted within 15 min. Calibration curves with concentrations of 0.025–10 mg/L (1.875–75 mg/L for TPV) showed coefficients of determination ($r^2$) between 0.993 and 0.999. The lower limits of quantification were well below the trough concentrations reported in the literature. Inter- and intraassay CVs and the deviations between the nominal and measured concentrations were <15%. The method was validated by successful participation in an international interlaboratory QC program.

CONCLUSIONS: This method allows fast and simultaneous quantification of all commercially available PIs and NNRTIs for therapeutic drug monitoring.

Despite recent advances in HIV therapy with the introduction of highly active antiretroviral (ARV) therapy, the rate of therapeutic failure remains too high. This failure rate is ascribed in part to suboptimal concentrations of ARV drugs selecting for the emergence mutant viral strains or to the occurrence of toxicity at high drug concentrations (1). There is, therefore, a need to monitor the blood concentrations of ARV drugs in each patient by means of therapeutic drug monitoring. A reliable and fast analytical method for measuring ARV drugs in the blood is an important prerequisite.

Several HPLC methods for simultaneously quantifying ARV drugs in human plasma have been described. The most commonly used techniques are liquid chromatography–tandem mass spectrometry (LC-MS/MS) and reversed-phase HPLC with UV detection. The great advantages of LC-MS/MS methods are not only their low limits of quantification, high selectivities, and high specificities, but also their short running times because these methods do not require a complete separation of all chromatographic peaks. One major limitation associated with LC-MS/MS analysis is its susceptibility to matrix effects. By contrast, UV detection is less prone to matrix effects. Furthermore, UV detection remains an available option for many laboratories. Unfortunately, analysis times are usually very long with conventional HPLC columns (up to 70 min) (2–17) because UV detection requires complete resolution of all chromatographic peaks to avoid interferences. Faster and higher-resolution separation can be achieved with ultra-performance liquid chromatography (UPLC) technology. We describe a novel, fast approach for measuring 10 ARV drugs in plasma that uses UPLC coupled with diode array detection to achieve good recovery at low drug concentrations.

The drugs investigated were amprenavir, atazanavir, efavirenz, indinavir, lopinavir (LPV), nelfinavir, nevirapine (NVP), ritonavir, saquinavir, and tipranavir (TPV). We used substance A-86093 (Abbott Laboratories; see Fig. 1 in the Data Supplement that accompanies the online version of this Brief Communication.

3 Nonstandard abbreviations: ARV, antiretroviral; LC-MS/MS, liquid chromatography–tandem mass spectrometry; UPLC, ultra-performance liquid chromatography; LPV, lopinavir; NVP, nevirapine; TPV, tipranavir; LLOQ, lower limit of quantification; PI, HIV-protease inhibitor.
HPLC-grade acetonitrile and methanol were purchased from Biosolve. We obtained ammonium acetate, acetic acid, and 13.4 mol/L ammonia solution from Merck, formic acid from Sigma–Aldrich, and Oasis HLB cartridges from Waters Corporation.

We serially diluted calibrator solutions in methanol to obtain 8 working concentrations (100, 50, 10, 5, 2.5, 1, 0.5, and 0.25 mg/L), except for TPV, for which we used 6 calibrators (750, 375, 187, 75, 37.5, and 18.75 mg/L). We used 9-point calibration curves for all compounds except TPV (7 points). These solutions were prepared by diluting 100 μL of each calibrator with 900 μL of ARV drug–free heparinized plasma (including a blank) provided by the blood bank of Saint-Luc Hospital, Brussels, Belgium.

For sample preparation, 1.0 mL of plasma calibrator or sample was diluted with 0.6 mL of 150 mmol/L sodium acetate buffer (pH 3.8) containing 5.33 mg/L internal standard. The samples were vortex-mixed for 15 s and centrifuged at 20 000g at room temperature for 8 min. We then transferred the supernatants (2 × 800 μL) onto conditioned Oasis HLB columns (1 mL, 30 mg) for extraction and washed the columns as described by Poirier et al. (10). After vacuum suction for 15 s, elution with methanol was performed in 2 steps (2 × 600 μL). The methanol was then evaporated to dryness under continuous nitrogen flow. The dried residues were reconstituted in 50 μL methanol, and 4 μL was injected into the chromatographic system.

The UPLC unit consisted of a Waters ACQUITY UPLC® instrument coupled with an ACQUITY UPLC® Photodiode Array Detector. Separation was performed on an ACQUITY UPLC BEH C8 column (2.1 × 100 mm). The sample manager was thermostated at 10 °C, and Waters Empower 2® Software was used for data management. Eluant A was an aqueous solution of 50 mmol/L ammonium acetate and 50 mmol/L formic acid, and eluant B was acetonitrile. At time zero, a mixture of 95% eluant A and 5% eluant B was flushed through the column at a constant flow rate of 0.450 mL/min. From 0–14 min, a linear gradient was set up to reach 75% B and 25% A at 14 min. The initial conditions were then restored by a 0.2-min linear gradient and then maintained for 0.8 min. The column temperature was set at 60 °C. Spectra were acquired for all peaks from 205–350 nm. LPV absorbance was monitored at 215 nm; NVP, saquinavir, atazanavir, efavirenz, and ritonavir were monitored at 240 nm; and indinavir, amprenavir, nelfinavir, and TPV were monitored at 260 nm.

Calibration curves were constructed by calculating the ratio of the peak area for each compound to the peak area for the internal standard and comparing the concentration against the nominal concentration of the sample. Curves were fitted by least-squares linear regression with a 1/x weighting factor. Over the concentration range, the coefficients of determination (r²) were 0.993–0.999 (Table 1). The slopes of the calibration curves were linear and were stable throughout the validation procedure (Table 1). The use of higher

### Table 1. Retention times, LLOQs, limits of detection (LODs), and regression coefficients for all ARV drugs.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time, min</th>
<th>LLOQ, mg/L</th>
<th>LOD, mg/L</th>
<th>Linearity (r²)</th>
<th>Slope±</th>
<th>y Intercept, mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>NVP</td>
<td>4.89</td>
<td>0.050</td>
<td>0.006</td>
<td>0.995 (0.006)</td>
<td>0.0009 (0.00005)</td>
<td>0.12</td>
</tr>
<tr>
<td>IDV</td>
<td>8.04</td>
<td>0.050</td>
<td>0.025</td>
<td>0.999 (0.0003)</td>
<td>0.0002 (0.000007)</td>
<td>0.0035</td>
</tr>
<tr>
<td>SQV</td>
<td>9.52</td>
<td>0.025</td>
<td>0.006</td>
<td>0.999 (0.0005)</td>
<td>0.0024 (0.00008)</td>
<td>0.033</td>
</tr>
<tr>
<td>NFV</td>
<td>10.47</td>
<td>0.050</td>
<td>0.025</td>
<td>0.999 (0.0002)</td>
<td>0.0004 (0.00002)</td>
<td>–0.0056</td>
</tr>
<tr>
<td>APV</td>
<td>8.61</td>
<td>0.050</td>
<td>0.003</td>
<td>0.999 (0.0002)</td>
<td>0.0013 (0.00004)</td>
<td>0.020</td>
</tr>
<tr>
<td>ATZ</td>
<td>10.38</td>
<td>0.025</td>
<td>0.025</td>
<td>0.999 (0.0004)</td>
<td>0.0006 (0.00002)</td>
<td>0.0021</td>
</tr>
<tr>
<td>RTV</td>
<td>10.91</td>
<td>0.050</td>
<td>0.050</td>
<td>0.999 (0.0001)</td>
<td>0.0005 (0.00001)</td>
<td>0.008</td>
</tr>
<tr>
<td>IS</td>
<td>11.50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.56</td>
</tr>
<tr>
<td>LPV</td>
<td>11.01</td>
<td>0.250</td>
<td>0.100</td>
<td>0.993 (0.005)</td>
<td>0.0009 (0.00007)</td>
<td>0.56</td>
</tr>
<tr>
<td>EFV</td>
<td>10.67</td>
<td>0.025</td>
<td>0.003</td>
<td>0.999 (0.0001)</td>
<td>0.0016 (0.00004)</td>
<td>0.016</td>
</tr>
<tr>
<td>TPV</td>
<td>12.12</td>
<td>0.235</td>
<td>0.235</td>
<td>0.994 (0.007)</td>
<td>0.0002 (0.00004)</td>
<td>–0.028</td>
</tr>
</tbody>
</table>

* LLOQ is defined as the lowest measurable concentration with a CV of <20%.

± Data are expressed as r² (SD).

± Data are expressed as the mean (SD) (n = 15).

IDV, indinavir; SQV, saquinavir; NFV, nelfinavir; APV, amprenavir; ATZ, atazanavir; RTV, ritonavir; IS, internal standard (substance A-86093; Abbott Laboratories); EFV, efavirenz.

at http://www.clinchem.org/content/vol55/issue1) as the internal standard.
Fig. 1. Chromatograms of blank plasma pool spiked with all drugs at 1 mg/L [except TPV, 75 mg/L, and the internal standard (IS), 5.33 mg/L].

Measurements were at 215 nm for LPV quantification (A); at 240 nm for NVP, saquinavir (SQV), atazanavir (ATZ), efavirenz (EFV), ritonavir (RTV), and IS quantification (B); and at 260 nm for indinavir (IDV), amprenavir (APV), nelfinavir (NFV), and TPV quantification (C). AU, absorbance units.
Brief Communications

pressures in the UPLC system reduced the total run time to 15 min, with retention times ranging from 4.89–12.12 min (Table 1). The 4-μL injection volume provided sharper peaks than obtained for HPLC chromatograms (Fig. 1).

Inter- and intra-run imprecision and recovery values were calculated for 3 different concentrations (0.5, 1, and 5 mg/L) for all drugs except TPV, for which higher concentrations (7.5, 18.75, and 37.50 mg/L) were selected. The inter- and intra-run imprecision (CV) values and the differences between nominal and measured concentrations were <15% (see Table 1 in the online Data Supplement).

The method was also validated through the analysis of external controls and successful participation in the KKGT (Stichting Kwaliteitsbewaking Klinische Geneesmiddelanalyse en Toxicologie) international interlaboratory QC program for therapeutic drug monitoring in HIV infection. For all ARV drugs except TPV, the deviations from the target concentrations ranged from −18% (LPV) to +19% (nelfinavir) (see Table 2 in the online Data Supplement). Because TPV was not included in the KKGT program, we obtained 2 different concentrations of other external controls from Dr. J. Droste (Clinical Department of the University Medical Centre of Nijmegen, Nijmegen, The Netherlands). The method for TPV quantification showed a deviation of <15%.

Values for the lower limit of quantification (LLOQ), defined as the lowest concentration of the calibration curve for which the CV did not exceed 20%, and the limit of detection, defined as the concentration that provided a signal-to-noise ratio of 3, are reported in Table 1. The chromatograms at the LLOQ for each analyte are shown in Fig. 2 in the online Data Supplement. With the exception of LPV, for which the LLOQ seems to be slightly higher (0.25 mg/L) than previously reported LPV LLOQs (from 0.025–0.2 mg/L), the LLOQs agree with those previously reported. All values are well below the trough concentrations reported in the recent literature (18).

The drugs quantified with the method we have described include the relatively new HIV- protease inhibitor (PI), TPV. Only 2 published HPLC-UV methods (3, 4) have quantified TPV simultaneously with other PIs and nonnucleoside reverse transcriptase inhibitors, and these methods featured longer running times (30 min and 50 min, respectively). Moreover, both of the methods used clozapine as the internal standard, which is a commercial drug that in clinical practice could be coadministered with one of the ARV drugs. Our analytical tool for TPV monitoring could be beneficial in the follow-up of TPV-treated patients, because excessive TPV plasma concentrations have been associated with hepatic cytolysis and increased aminotransferases (19, 20). Furthermore, TPV is prone to many drug interactions (20).

With solid-phase extraction, our recoveries for most of the drugs were >87.5% and were quite stable across the calibration interval. For TPV and NVP, extraction recoveries were <50%, but the SDs were low for both substances, indicating that the extractions were reproducible (see Table 3 in the online Data Supplement). These lower extraction recoveries, however, had no negative impact on assay performance and do not preclude the clinical applicability of TPV and NVP assays, because the currently reported concentrations of these drugs [3.4 mg/L for NVP (21) and 25 mg/L for TPV (19)] are well above our LLOQs of 0.05 mg/L for NVP and 0.235 mg/L for TPV.

The chromatogram obtained with pooled blank plasma showed no interfering peaks at retention times corresponding to those of ARV drugs (see Fig. 3 in the online Data Supplement). Furthermore, solutions of currently prescribed drugs for HIV patients (4) did not interfere with the tested ARV drugs (see Table 4 in the online Data Supplement). Finally, it must be stressed that we systematically perform peak-purity testing and library matching with the Empower 2 Software, which has the potential to detect any coeluting impurity.

We did not test the stability of the ARV drugs in plasma and methanol, because stability assessments for the drugs we tested have largely been performed in other studies (3, 4, 6, 13–15), and these studies have shown the drugs to be stable, even after heat inactivation of the virus.

In conclusion, we have developed a rapid and specific UPLC-UV assay with low limits of detection for the simultaneous quantification of efavirenz, NVP, and all of the currently prescribed PIs, including the relatively new PI, TPV.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors’ Disclosures of Potential Conflicts of Interest: Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honorary: None declared.
Research Funding: This research was supported by the Fonds Pierre et Colette Bauchau (UCL, Belgium) and the Fonds National de la Recherche Scientifique (FNRS, Belgium). L. Elens is a research fellow with the FNRS (FC 75 424).
Expert Testimony: None declared.
Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

Acknowledgments: Amprenavir was kindly provided by GlaxoSmithKline Research and Development, Middlesex, UK; atazanavir was a gift of Bristol-Myers Squibb, New Brunswick, NJ; nelfinavir and saquinavir were gifts of Roche Diagnostics, Mannheim, Germany; indinavir and efavirenz were gifts of Merck, Whitehouse Station, NJ. LPV, ritonavir, and the internal standard were gifts of Abbott Laboratories, Abbott Park, IL; and NVP and TPV were kindly provided by Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT. The authors thank Dr. J. Droste from the Clinical Department of the University Medical Centre of Nijmegen, the Netherlands, for the kind gift of control plasma samples.

References


DOI: 10.1373/clinchem.2008.108647