We have developed an enzyme immunoassay to measure atazanavir (ATV) levels in plasma and cells. Anti-ATV polyclonal antibodies were raised in rabbits by using a synthetic ATV derivative coupled to bovine serum albumin as the immunogen, and the enzyme tracer was prepared by chemically coupling the ATV derivative with acetylcholinesterase. These reagents were used to develop a sensitive competitive enzyme immunoassay performed in microtiteration plates, and the lowest limit of quantification was 150 pg/mL, which is about 10 times more sensitive than previously published techniques. The plasma assay was performed, after a simple methanol extraction, with a minimum of 30 μL of plasma. This assay showed good precision and efficiency, since the rates of recovery from human plasma and cell extracts spiked with ATV ranged from 93 to 113%, with coefficients of variation of less than 10%. ATV concentrations were measured in peripheral blood mononuclear cells incubated with various ATV concentrations and in CEM cells in the absence or presence of antiretroviral drugs and drug transporter inhibitors. The results indicated a dose-dependent uptake (intracellular concentration/extracellular concentration ratio range, 0.04 to 19). A significant increase in the accumulation of ATV was noticed in the presence of P-glycoprotein and MRPI inhibitors (dipyridamole, inter alia). Interestingly, efavirenz significantly increased the baseline accumulation of ATV, whereas nevirapine induced a marked reduction. This new enzyme immunoassay for measuring plasma and intracellular ATV levels was fully validated and provides an inexpensive and useful tool for routine therapeutic drug monitoring. Moreover, in vitro results suggested the implication of drug transporters and interactions with other antiviral drugs that should be further explored in human immunodeficiency virus-infected patients.

The introduction of protease inhibitors (PIs) as treatment against human immunodeficiency virus (HIV) infection has led to a marked increase in the potency of antiretroviral therapy and therefore has reduced the rates of morbidity and mortality (22). Atazanavir (ATV; Reyataz) is the first azapeptide inhibitor of HIV type 1 (HIV-1) protease approved for treatment and has a half-life that allows once-daily dosing. ATV is currently used to develop a sensitive competitive enzyme immunoassay performed in microtiteration plates, and the lowest limit of quantification was 150 pg/mL, which is about 10 times more sensitive than previously published techniques. The plasma assay was performed, after a simple methanol extraction, with a minimum of 30 μL of plasma. This assay showed good precision and efficiency, since the rates of recovery from human plasma and cell extracts spiked with ATV ranged from 93 to 113%, with coefficients of variation of less than 10%. ATV concentrations were measured in peripheral blood mononuclear cells incubated with various ATV concentrations and in CEM cells in the absence or presence of antiretroviral drugs and drug transporter inhibitors. The results indicated a dose-dependent uptake (intracellular concentration/extracellular concentration ratio range, 0.04 to 19). A significant increase in the accumulation of ATV was noticed in the presence of P-glycoprotein and MRPI inhibitors (dipyridamole, inter alia). Interestingly, efavirenz significantly increased the baseline accumulation of ATV, whereas nevirapine induced a marked reduction. This new enzyme immunoassay for measuring plasma and intracellular ATV levels was fully validated and provides an inexpensive and useful tool for routine therapeutic drug monitoring. Moreover, in vitro results suggested the implication of drug transporters and interactions with other antiviral drugs that should be further explored in human immunodeficiency virus-infected patients.

Indeed, only the fraction reaching the intracellular compartment is expected to have antiviral activity; therefore, antiviral drugs need to penetrate the cell at a concentration high enough to inhibit viral replication in order to be effective. Failure to do so may result in the establishment of a sanctuary for the virus. The accumulation of the drug within a target cell is controlled by influx and efflux processes (9). Most PIs are lipophilic and are assumed to enter cells by passive diffusion; moreover, a number of drug transporter proteins have been identified to expel drugs out of cells, including P-glycoprotein (P-gp) (15), multidrug resistance-associated proteins (MRPs) (9, 12), breast cancer resistance protein (9, 12), and organic anion transporters (OATs) (24). Thus, the intracellular concentration of the protease inhibitor ATV should be influenced by these processes, and an assay that enables determination of the concentration of the drug in cells may help provide an understanding of the mechanisms of intracellular accumulation. Moreover, the intracellular pharmacokinetics of the drug would be important for the better optimization of dosing regimens.

Several high-performance liquid chromatographic (HPLC) assays combined with UV detection (6, 7, 18, 25) or liquid
chromatography with tandem mass spectrometry (LC-MS-MS) (5, 8, 11, 21) have been described for the quantitative determination of ATV in plasma. Only a few of these assays have been validated for use for the measurement of intracellular concentrations (5, 11), and all of them involve the use of LC-MS-MS; but LC-MS-MS systems are not available in all routine laboratories that perform therapeutic drug monitoring and require expensive equipment. However, no immunoassay with a sensitivity, rapidity, and cost-effectiveness superior to those of LC-MS-MS has been published to date.

In this report we describe the development and application of a competitive enzyme immunoassay (EIA) for the quantification of ATV in plasma and cells. This new assay is based on the use of specific anti-ATV polyclonal antibodies raised in rabbits and an enzyme tracer, prepared from a synthetic derivative of ATV. We took advantage of the high sensitivity of the assay to measure and compare intracellular ATV accumulation and the effects of drug transporter proteins and other antiretroviral drugs on its accumulation.

MATERIALS AND METHODS

Reagents. Unless otherwise stated, all reagents and solvents were of analytical grade and were from Sigma (St. Louis, MO). Acetylcholinesterase (ACHE; EC 3.1.1.7), extracted from the electric organ of the Electrophorus electricus eel, was purified by affinity chromatography, as reported previously (3). Modified Eilman’s reagent was a solution of 7.5 × 10⁻³ M acetylthiocholine iodide (enzyme substrate) and 5 × 10⁻⁴ M 5, 5-dithiobis-2-nitrobenzoic acid (chromogen) in 0.1 M phosphate buffer (pH 7.4).

All reagents used for the immunoassays were diluted in the following buffer (EIA buffer): 0.1 M potassium phosphate (pH 7.4) containing 0.1% NaCl, 0.1% bovine serum albumin (BSA), and 0.01% sodium azide. The washing buffer was 10 mM phosphate (pH 7.4) containing 0.05% Tween 20.

Apparatus. Solid-phase EIA was performed in 96-well microtiter plates (Immunoplate Maxisorb with certificate; Nunc, Roskilde, Denmark) with specialized microtitration equipment, a washer (Atlantic+; ASYSSHitc, Engelendorf, Austria), and an automatic plate reader (GENios Pro, Tecan, Switzerland). HPLC experiments were performed with an apparatus from Waters (St. Quentin en Yvelines, France), including an HPLC Alliance 2605 996-photodiode-array detector, a Millennium chromatographic manager, and a fraction collector (Retriever IV; Isco, France).

Immunogen preparation and immunization. After chemical modification to introduce an arm spacer bearing an amino function, ATV was coupled to BSA and administered to rabbits in order to induce the synthesis of antibodies, as follows.

ATV (300 mg, 0.42 mmol) and 4-tert-butyloxybenzoylamino-butyric acid (94 mg, 0.46 mmol) were dissolved in anhydrous dichloromethane (5 ml) under nitrogen. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (97 mg, 0.52 mmol) and 4-dimethylaminopyridine (62 mg, 0.5 mmol) was added. The solution was stirred for 20 min at room temperature, the thioester obtained was hydrolyzed in the presence of hydroxylamine (0.25 M) and the thioldated product was purified by adsorption on a Sep-Pak C₁₈ cartridge. Conjugation of the ATV spacer with the enzyme was obtained by mixing the thioldated ATV spacer (1 nmol) with 0.1 nmol of ACHE-SMCC. After reaction overnight at 4°C, the conjugate was purified by molecular sieve chromatography with a Bio-Gel A 1.5-m column (90 by 1.5 cm; Bio-Rad) and was stored at −20°C until use.

Clinical sample preparation. Blood samples were collected in heparinized tubes and centrifuged (1,800 g for 10 min). Plasma was removed and stored at −80°C until analysis. Heat-inactivated plasma samples (60°C for 40 min; ATV proved to be stable under these conditions [6]) were extracted with methanol (4 volumes of methanol for 1 volume of sample) and then centrifuged (12,000 × g for 10 min). The supernatant was dried under vacuum with a Speed Vac concentrator. The final product was characterized by nuclear magnetic resonance (NMR) imaging and mass spectrometry: for 1H NMR (CDCl₃, 200 MHz) δ = 8.74 (t, J = 8.2 Hz, 1H), 8.33 (d, J = 8.2 Hz, 1H), 8.12 (m, 2H), 8.38 (t, J = 8.2 Hz, 1H), 8.26 (d, J = 8.2 Hz, 1H), 8.76 (d, J = 8.2 Hz, 1H), 7.77 (m, 1H), 7.34 (m, 1H), 7.27 (m, 3H), 7.12 (d, J = 9.4 Hz, 1H), 7.08 (d, J = 9.3 Hz, 1H), 4.10 (m, 1H), 4.02 (m, 2H), 3.97 (m, 1H), 3.75 (m, 2H), 3.61 (s, 3H), 3.55 (s, 3H), 2.96 (m, 2H), 2.85 to 2.81 (m, 2H), 2.54 to 2.44 (m, 2H), 2.01 to 1.91 (m, 2H), 0.91 (s, 9H), 0.75 (s, 9H) and for 13C NMR (CDCl₃, 75 MHz) δ = 26.3, 26.7, 33.3, 33.6, 34.1, 37.7, 41.3, 51.4, 51.6, 60.7, 61.0, 61.3, 63.2, 68.3, 124.2, 124.8, 126.2, 127.5, 128.2, 129.1, 129.3, 131.3, 139.3, 141.6, 144.2, 147.4, 152.3, 156.5, 170.1, and 172.8. Mass spectrometry: C₂₆H₂₄N₇O₇ [M+H]⁺ = 676.3.

The ATV spacer was covalently coupled to BSA by reacting glutaraldehyde with primary amino groups of the carrier protein and the ATV spacer. Briefly, the ATV spacer (5.2 mg, 5.8 μmol) was reacted with BSA (20 mg, 0.29 μmol) and glutaraldehyde (25%) in water (25 μl) in 10 ml of 0.1 M phosphate buffer (pH 7.4). The reaction mixture was stirred overnight at room temperature in the dark. The immunogen was then extensively dialyzed against 0.1 M phosphate buffer (pH 7.4), aliquoted, and kept frozen at −20°C until use. Matrix-assisted laser desorption ionization–time of flight analysis showed that 11 atazanavir spacers were coupled per BSA.

Rabbits (Blanc du Bouscat, Éric, France) were immunized with 1 mg of immunogen by using complete Freund’s adjuvant and multiple subcutaneous injections. Booster injections (1 mg of immunogen in complete Freund’s adjuvant) were repeated every 2 months for 8 months. Rabbits were bled from the central ear artery 1 week after each booster injection. The blood was centrifuged, and the sera were stored at 4°C in the presence of sodium azide (final concentration, 0.01%).

Enzymatic tracer preparation. The tracer was obtained by covalently coupling the ATV spacer to ACHE by using the heterofunctional reagent succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylic acid (SMCC). Briefly, 10 μl of N-succinimidyl 3-acylthiohydacetate was added to the ATV spacer (385 μg, 1 μmol), which was dissolved in 0.1 M borate buffer (pH 9). After 30 min of reaction at room temperature, the thioester obtained was hydrolyzed in the presence of hydroxylamine (0.25 M) and the thiolated product was purified by adsorption on a Sep-Pak C₁₈ cartridge. Conjugation of the ATV spacer with the enzyme was obtained by mixing the thiolated ATV spacer (1 nmol) with 0.1 nmol of ACHE-SMCC. After reaction overnight at 4°C, the conjugate was purified by molecular sieve chromatography with a Bio-Gel A 1.5-m column (90 by 1.5 cm; Bio-Rad) and was stored at −20°C until use.

Enzyme immunoassay. Enzyme immunoassays were performed in 96-well microtiter plates coated with a goat anti-rabbit monoclonal anti-rabbit immunoglobulin (Jackson, West Grove, PA), as described previously (3). After the coating microtiter plate was washed, the assay was performed in a total volume of 150 μl. The following were successively added to each well: 50 μl of calibrator, 50 μl of tracer enzyme (2 Ellman units/ml) and 50 μl of diluted antiserum. The optimal working dilution for the different rabbit antiserum was previously determined by serial dilution experiments (data not shown). After 12 h of immunoreaction at 4°C, the plate was washed and 200 μl of modified Eilman’s reagent was added to each well. After 2 h of gentle shaking in the dark at room temperature, the absorbance at 410 nm (with the reference filter at 570 nm) was measured in each well. The results are expressed as (B/B₀) × 100 as a function of the concentration (logarithmic scale), where B and B₀ represent the bound enzymatic activity in the presence and absence of the competitor, respectively. A linear log-log transformation was used to fit the calibration curve. All measurements for the calibrators (78.125, 156.25, 312.5, 625, 1,250, 2,500, 5,000, and 10,000 pg/ml) and the samples were done in duplicate, and all measurements for B₀ values were done in quadruplicate.

Validation studies. The specificity of the anti-ATV antibody was determined by evaluating its ability to bind to various compounds likely to be present with ATV in treated human subjects. The percent cross-reactivity of each compound was calculated as the ratio of the ATV and the tested compound at Bₐ/Bo equal to 50% of the accuracy and precision of the assay were determined by using quality control samples (drug-free human plasma spiked with ATV) and clinical samples previously quantified by HPLC-UV in six replicates. One-way analysis of variance (ANOVA) of the results was performed. The lower limit of quantitation (LLOQ) is defined as the lowest concentration on the standard curve that can be
were processed by the protocols described above before ATV quantification. The samples were bated for 2 h at 37°C in the absence or presence of probenecid, verapamil, or atazanavir. Furthermore, all the CEM cell pellets were extracted twice, and no ATV was detected in the second extraction. The cell density was adjusted to 1 × 10^6/ml; and 5 ml of the suspension was centrifuged (2,000 × g for 5 min at 4°C), and with a Mallowe cell before a final extraction in 1 ml of a methanol-H_2O mixture (90/10; vol/vol). The samples were then centrifuged (12,000 × g for 10 min), and the supernatant was dried under vacuum by using a Speed Vac apparatus. The dry residue was dissolved in 1 ml of EIA buffer and assayed for its ATV content.

Antibody production and assay development. In order to achieve a covalent link with proteins, ATV was converted into a primary amino derivative through its hydroxyl function (see Materials and Methods). This provides a useful way to couple ATV with BSA, which is an obligatory step for specific antibody production, as well as with AchE for the preparation of a tracer. A very good antibody titer (greater than 1/100,000) was achieved at a flow rate of 1 ml/min with a linear gradient from 10% to 100% of solvent B (solvent A was acetonitrile) in 30 min (solvent A = H_2O and 0.1% trifluoroacetic acid). One-milliliter fractions were collected, dried under vacuum, and resuspended in 1 ml of EIA buffer before the assays for ATV were performed.

Cell cultures in presence of atazanavir. CEM CCL-19 cells (ATCC, Manassas, VA) and PBMCs prepared as described above were grown at 37°C in RPMI 1640 medium supplemented with 0.2 mM sodium pyruvate, 0.2 mM glutamine, 10% heat-inactivated fetal cell serum, and antibiotics in a 5% CO_2 humidified atmosphere. The cell density was adjusted to 1 × 10^6/ml; and 5 ml of the suspension was incubated with 0.2, 2, and 20 μg/ml of atazanavir for 24 h at 37°C. Control experiments showed that these concentrations were not toxic for the cells. The cells were washed three times in ice-cold phosphate-buffered saline (PBS), centrifuged (2,000 × g for 5 min at 4°C), and counted with a Mallowe cell before a final extraction in 1 ml of a methanol-H_2O mixture (90/10; vol/vol). The samples were then centrifuged (12,000 × g for 10 min), and the supernatant was dried under vacuum by using a Speed Vac apparatus. The dry residue was dissolved in 1 ml of EIA buffer and assayed for its ATV content.

RESULTS

Antibody production and assay development. In order to allow a covalent link with proteins, ATV was converted into a primary amino derivative through its hydroxyl function (see Materials and Methods). This provides a useful way to couple ATV with BSA, which is an obligatory step for specific antibody production, as well as with AchE for the preparation of a tracer. A very good antibody titer (greater than 1/100,000) was measured by EIA for the two immunized rabbits from the body production, as well as with AchE for the preparation of a tracer. A very good antibody titer (greater than 1/100,000) was selected through its hydroxyl function (see Materials and Methods). This provides a useful way to couple ATV with BSA, which is an obligatory step for specific antibody production, as well as with AchE for the preparation of a tracer.

Drug accumulation was assessed at 37°C. CEM cell suspensions were incubated for 2 h at 37°C in the absence or presence of probenecid, verapamil, or dipryridamole (50 μM each); efavirenz (EFZ; 24 μM); nevirapine (NVP; 28 μM); ritonavir (RTV; 2.3 μM); or ATV (7 μM, which corresponds to the mean maximum concentrations observed with boosted ATV treatment). The samples were processed by the protocols described above before ATV quantification. Intracellular concentrations were calculated by assuming cell volumes of 1 pl for each CEM and 0.4 pl for each PBMC.

Validation studies. The specificities of the polyclonal anti-ATV antibodies were first analyzed by using antiviral agents commonly used during HIV treatment. No interference, as characterized by a cross-reactivity of <0.001%, occurred with drugs structurally unrelated to ATV, such as the nucleoside reverse transcriptase inhibitors zidovudine, lamivudine, dideoxynucleoside, dideoxyinosine, and abacavir and the non-nucleoside reverse transcriptase inhibitors NVP and EFZ. Others licensed PIs, such as nelfinavir, saquinavir, indinavir, and RTV, were not recognized by the antibodies (cross-reactivities, less than 0.01%).

Since the main known metabolites of ATV (Reyatz, Summary of product characteristic, Bristol-Myers Squibb, 2004) were not available for use as pure chemical compounds, the potential recognition by the antibodies of ATV metabolites or endogenous compounds in plasma samples from HIV-positive patients was evaluated by HPLC fractionation coupled with EIA detection. As shown in Fig. 2, we observed that immunoreactive material in both types of samples eluted as a single homogeneous peak corresponding to the elution volume of true ATV.

Assay precision and accuracy were evaluated with human plasma spiked with known concentrations of ATV and patient plasma, and the values were compared to those obtained by HPLC. When assaying pure plasma samples, we observed a matrix effect, which led to changes in the sensitivity and precision characteristics of the assay. However, the original sensitivity of the assay could be recovered by performing a simple extraction procedure with methanol (1 volume of plasma and 4 volumes of methanol), which allows the precipitation of most of the plasma proteins and their elimination by centrifugation. One-way ANOVA of all results for the quality control samples and the clinical samples established that the method has good accuracy and precision. As shown in Table 1, the recovery of ATV ranged from 93 to 113% in samples, with coefficients of variation between 1.7 and 4.9%. The LLOQ was then similar to those observed in EIA buffer.

For intracellular quantification, the LLOQ is dependent on the cell number from which the cellular extract is obtained. On
the basis of an average number of $2 \times 10^6$ cells (the minimum number of cells which allows good precision and accuracy), the LLOQ is close to 150 pg/ml.

**EIA-HPLC correlation.** ATV concentrations were assessed in plasma samples from HIV-infected patients ($n = 23$) receiving ATV (Reyataz) therapy by both our immunoassay and a classical HPLC method routinely used for monitoring in hospitals. Figure 3 shows that the values obtained by the two techniques were highly correlated ($r^2 = 0.97$). The regression equation was $y = 0.9508 \pm 0.03459x + 0.05441 \pm 0.1075$ µg/ml (ATV concentration range examined, 0.06 to 8 µg/ml). The 95% confidence interval of the y intercept included zero. A comparison of the two methods by use of the Pearson correlation coefficient was highly significant ($P < 0.0001$). However, because correlation coefficients are measures of the association between two methods and not the agreement between them, the degree of agreement was assessed by the Bland-Altman graphical technique (graph not shown). For our study, the bias was $-3.1\%$, which indicated that the results of the immunoassay were in concordance with those of the HPLC method, with a slight underestimation; and all the values were inside the limits of agreement, i.e., between the bias and $\pm 2$ standard deviations.

**Cell studies.** The immunoassay proved to be sensitive enough to measure the intracellular concentrations of ATV. This was performed with CEM cells and PBMCs cultured in the presence of three different concentrations of ATV (0.2, 2, and 20 µg/ml) for 24 h. The results, summarized in Table 2, demonstrated a concentration-dependent uptake of ATV into CEM cells and PBMCs. The intracellular ATV concentrations represented only 4 to 45% of the extracellular concentrations, showing that CEM cells did not accumulate ATV, whereas in the case of the PBMCs, the intracellular concentration/extracellular concentration ratios ranged from 0.6 to 19.

In order to study the effects of drug efflux inhibitors and

![FIG. 2. Typical immunochromatographic profiles of plasma extracts from a drug-free patient spiked with ATV (A) and an HIV-infected patient (B). The elution time of ATV spiked in drug-free plasma was 15 to 18 min.](image)

![FIG. 3. Correlation between the immunoassay and the HPLC methods with plasma from HIV-infected patients. The scatter plot shows plasma ATV levels determined by EIA versus those quantified by HPLC analysis. The solid line is the line of identity. The dashed line indicates the slope of the linear regression analysis.](image)

### TABLE 1. Validation parameters for EIA of ATV in plasma

<table>
<thead>
<tr>
<th>Sample</th>
<th>ATV concn (µg/ml)</th>
<th>Accuracy (%)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20</td>
<td>93</td>
<td>2.45</td>
</tr>
<tr>
<td>Plasma 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2</td>
<td>106</td>
<td>1.7</td>
</tr>
<tr>
<td>Plasma 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.001</td>
<td>108</td>
<td>4.9</td>
</tr>
<tr>
<td>Plasma 4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.00015</td>
<td>95</td>
<td>6.3</td>
</tr>
<tr>
<td>Plasma of patient 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.261</td>
<td>113</td>
<td>1.8</td>
</tr>
<tr>
<td>Plasma of patient 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.376</td>
<td>102</td>
<td>1.65</td>
</tr>
<tr>
<td>Plasma of patient 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.38</td>
<td>99</td>
<td>2.13</td>
</tr>
</tbody>
</table>

<sup>a</sup> Human plasma spiked with ATV at the indicated concentrations, as follows: a known quantity of ATV was added to 1 ml of drug-free human plasma, and after 1 h of incubation at 37°C, the samples were treated as described in Materials and Methods.

<sup>b</sup> HIV-infected plasma.

<sup>c</sup> Recovery of added ATV and comparison with the values obtained by HPLC in the intraday experiment ($n = 6$).

<sup>d</sup> CV, coefficient of variation ($n = 6$).
other antiviral drugs on accumulation, CEM cells were cultured in the presence or absence of probenecid, verapamil, and dipyridamole (50 μM each); EFZ (24 μM); NVP (28 μM); RTV (2.3 μM); and ATV (7 μM). The data for the controls and the drug-treated samples were compared; a one-way ANOVA followed by Dunnett’s correction was applied. In each case, a significant difference between the means for the control and drug-treated samples was assumed if \( P \) was <0.05.

As shown in Fig. 4, verapamil increased the level of accumulation of ATV up to 70%. Probenecid had less of an effect on the accumulation and increased the concentration of ATV up to 25%. Dipyridamole significantly increased the level of accumulation of ATV up to 125% \((P < 0.01)\). Similarly, EFZ coinubated with ATV in the same ratio used in HAART (Reyataz, Summary of product characteristic, Bristol-Myers Squibb, 2004) \((i.e., \text{ATV}/\text{EFZ} = 4/6 \ [\text{wt/wt}])\), significantly increased the accumulation of ATV (up to 125%). In contrast, NVP coinubated with ATV in the same ratio as EFZ had a negative effect on the level of accumulation of ATV (up to 50%). RTV, also given in the same ratio used in HAART \((i.e., \text{ATV}/\text{RTV} = 1/3 \ [\text{wt/wt}])\), had a nonsignificant effect on the level of accumulation of ATV.

**DISCUSSION**

This paper reports on the development of a new competitive enzyme immunoassay for ATV that allows measurements of its concentrations in biological fluids and cell extracts. The polyclonal anti-ATV antibodies raised in rabbits are specific for ATV since no interference due to other anti-HIV drugs (cross-reactivities, <0.01%) or endogenous plasma or cell compounds was recorded. The present assay was also highly sensitive, with an LLOQ of 150 pg per ml, which compares favorably with those close to 50 ng/ml (5, 7, 18, 25) and above 1 ng/ml (8, 11, 21) previously reported for the LC-UV and LC-MS-MS methods, respectively. Attempts to obtain direct measurements of the concentrations in plasma were not successful probably due to the binding of the drug to plasma proteins, in particular, α-1-acid glycoprotein and albumin (levels of binding, 89% and 86%, respectively) (Reyataz, Summary of product characteristic, Bristol-Myers Squibb, 2004). However, after a simple methanol extraction, the recovery and precision of EIA were good.

As a first application of this assay, we analyzed a limited number of plasma samples from HIV-infected patients to establish the reliability of the assay. The comparison study showed a very good concordance between the results of our immunoassay and those of the HPLC method, with a high correlation \((r^2 = 0.97; P < 0.0001)\), a regression line very close to the line of identity, and a favorable Bland-Altman assessment of the agreement of the data. These data strongly support the specificity of the assay. Thus, this method provides a useful tool that can be used to study the relationships between plasma ATV concentrations and antiretroviral effects and drug toxicities. Moreover, this analytical method can be carried out in less than 24 h in any conventional laboratory.

To illustrate better the value of the method, we measured the intracellular uptake of ATV. Cells are considered a major site of the virus-host interaction (28), and knowledge of intracellular penetration is crucial for a better understanding of the development of drug resistance and the failure of antiretroviral therapy. The intracellular accumulation of drugs is a dynamic process that is not only a function of drug concentration but also of drug permeability, drug uptake, cell volume, and the intracellular distribution of the drug. To measure the intracellular concentration of ATV, we developed a novel competitive enzyme immunoassay and those of the HPLC method, with a high correlation \((r^2 = 0.97; P < 0.0001)\), a regression line very close to the line of identity, and a favorable Bland-Altman assessment of the agreement of the data. These data strongly support the specificity of the assay. Thus, this method provides a useful tool that can be used to study the relationships between plasma ATV concentrations and antiretroviral effects and drug toxicities. Moreover, this analytical method can be carried out in less than 24 h in any conventional laboratory.

To illustrate better the value of the method, we measured the intracellular uptake of ATV. Cells are considered a major site of the virus-host interaction (28), and knowledge of intracellular penetration is crucial for a better understanding of the development of drug resistance and the failure of antiretroviral therapy. The intracellular accumulation of drugs is a dynamic process that is not only a function of drug concentration but also of drug permeability, drug uptake, cell volume, and the intracellular distribution of the drug. To measure the intracellular concentration of ATV, we developed a novel competitive enzyme immunoassay and those of the HPLC method, with a high correlation \((r^2 = 0.97; P < 0.0001)\), a regression line very close to the line of identity, and a favorable Bland-Altman assessment of the agreement of the data. These data strongly support the specificity of the assay. Thus, this method provides a useful tool that can be used to study the relationships between plasma ATV concentrations and antiretroviral effects and drug toxicities. Moreover, this analytical method can be carried out in less than 24 h in any conventional laboratory.

To illustrate better the value of the method, we measured the intracellular uptake of ATV. Cells are considered a major site of the virus-host interaction (28), and knowledge of intracellular penetration is crucial for a better understanding of the development of drug resistance and the failure of antiretroviral therapy. The intracellular accumulation of drugs is a dynamic...
process influenced by cellular influx and efflux, plasma protein binding, and intracellular trapping. The first experiments performed with CEM cells and primary cultured PBMCs demonstrated the capacity of the immunoassay described here to measure the intracellular ATV content accurately. The uptake seems to be dependent on the concentration of ATV present in the cell culture medium. The same trend was observed for both cell lines, although the range was more important with PBMCs.

At a low concentration of ATV, the intracellular concentration/extracellular concentration ratio increased by 10- to 30-fold compared to those obtained with high concentrations (Table 2). One explanation could be that ATV is secreted by an active process. Indeed, it has been shown in other studies (16, 20) that ATV is either an inhibitor and or an inducer of P-gp and MRP, depending on its concentration. A high concentration of ATV is reported to induce the function of P-gp, which may explain the low ratio observed.

Moreover, xenobiotic transporters play an important role in drug interactions and may be involved in the selection of HIV-resistant mutants. Therefore, the study of transport phenomena could be of value in attempts to address these issues. Even though PIs must passively diffuse through the lipid membrane due to its lipophilicity, several studies have shown that P-gp and MRP take part in the intracellular accumulation of PIs (9).

However, to date, no study has focused on the intracellular concentration of ATV. In the current study, we demonstrated marked differences in the intracellular accumulation of ATV in vitro in the presence of drug transport inhibitors and non-nucleoside reverse transcriptase inhibitors. Caution must be exercised when our findings are interpreted, since they may not directly translate into differences in clinical efficacy. Despite these reservations, we noticed that specific and relatively specific inhibitors of P-gp, MRP1, MRP2, and OAT influenced the accumulation of ATV in CEM cells (Fig. 4). Verapamil (4), a well-known nonspecific P-gp inhibitor, induced moderate but significant accumulation, whereas probenecid (10), an inhibitor of MRP2 and OAT, had no significant effect. Dipyridamole, a clinically used antiplatelet drug, has been shown to inhibit P-glycoprotein in vitro (13) and has recently been listed as a drug candidate potentially responsible for P-glycoprotein-dependent drug interactions (27). In our case, it significantly increased the level of accumulation of ATV (up to 125%), which strengthens the possibility that P-gp are involved in ATV efflux.

The use of antiretroviral drugs together as part of a potent regimen for the treatment of HIV infection or in combination with other therapies can result in a number of (sometimes unpredicted) drug-drug interactions. These interactions may influence treatment outcomes. However, few in vivo or in vitro data regarding this problem are available, especially data on interactions at the intracellular level.

RTV is widely used in clinical practice to boost the plasma concentration of another PI due to its high inhibitory effect on cytochrome P450. It has been shown to abrogate the functions of P-gp (26) and MRP1 (19). However, in our case no significant effect was observed. Conflicting data are available for NVP. While NVP induces P-gp expression in the intestinal cell lines at 30 μM (23), it was not the case in PBMCs in vitro at 10 μM (4). More recently, another study (1) suggested that NVP may up-regulate P-gp expression in PBMCs in vivo and is a substrate for lymphocyte efflux transporters, possibly P-gp (2) or MRP1. The latter results would be in agreement with our data, since the level of accumulation of ATV was divided by two in the presence of NVP, suggesting an interaction with efflux transporters. Interestingly, even though EFZ has not been clearly determined to be an inhibitor of P-gp, its coadministration with ATV at a ratio similar to that used in HAART dramatically increased the level of accumulation of ATV (up to 125%). These results suggest an intriguing drug-drug interaction between the nonnucleoside reverse transcriptase inhibitor efavirenz and the protease inhibitor ATV, insofar as EFZ is often coadministered with ATV.

These data strongly suggest that drug efflux transporters are involved in the transport of ATV and emphasize the need for complementary intracellular studies, especially with HIV-infected patients, of drug-drug interactions. This assay provides the opportunity to easily explore the relationship between the intracellular concentration of ATV and both its efficacy and toxicity. Besides the potential advantages of an enzyme immunoassay for ATV in intracellular quantification studies, as demonstrated here, the technique should also be useful in the clinical laboratory for the monitoring of patients during HAART.

ACKNOWLEDGMENTS

This work was supported in part by the Conseil General 06, Ensemble contre le Sida (SIDACTION), and the Caisse d’Assurance Maladie des Professions Libérales Provinces. C. Roucairol is a recipient of a Ph.D. fellowship from CNRS and region PACA.

REFERENCES


