Pharmacokinetic (PK) variability is important in relation to protease inhibitors (PIs), a group of drugs with considerable inter- and intra-individual variability in plasma concentrations and marked potential for drug interactions leading to decreasing or increasing PIs plasma levels. Combination antiretroviral therapy with two or more protease inhibitors has become the standard of care in the treatment of HIV infection (1, 2). Dual protease inhibitor therapy, such as lopinavir/ritonavir, are commonly used as initial PIs therapy. Alterations in the absorption and metabolism of PIs when co-administered with a cytochrome P450 enzyme inhibitor, such as low dose ritonavir, are reflected by impressive changes in pharmacokinetic parameters (3). The addition of ritonavir to saquinavir has been shown to increase saquinavir area under the concentration-time curve (AUC) by approximately 300-800% compared with saquinavir alone (4-6).

Because PIs are used in combination, it is important to develop assay, which it affords possibilities of determination of several drugs simultaneously. Currently antiretroviral plasma concentrations are determined either by high performance liquid chromatography (7-14) or liquid chromatography tandem mass spectrometry. The most of these methods required a costly chromatographic system or expensive cartridges for extraction. Therefore, this article describes an analytical method, which is simple and inexpensive, it may have applicability in developing countries with low resources. This procedure is modified method presenting by Yamada et al. in the Journal of Chromatography in 2001 (7).

Until now it is not clear which pharmacokinetic parameters, minimum plasma concentration (C_{min}), maximum plasma concentration (C_{max}) or area under the plasma concentration-time curve (AUC) should be monitored to maintain optimal viral suppression (15, 16). Therefore, in our study we estimated the value of three PK parameters: C_{min}, C_{max} and AUC_{0-24h}.

**EXPERIMENTAL**

**Materials, chemicals, apparatus**

Saquinavir (SQV), lopinavir (LPV), indinavir (IDV) and nelfinavir (NFV) pure substances were kindly provided by NIH AIDS Research and Reference Reagent Program (McKesson BioServices Corporation, Germantown, Maryland, USA). Diazepam was obtained from Sigma Aldrich and was used as an internal standard. Acetonitrile, methanol,
methyl tert-butyl ether and hexane were obtained from J.T. Baker. NH₄OH ammonium hydroxide, KH₂PO₄ potassium phosphate monobasic, Na₂HPO₄ sodium phosphate dibasic were obtained from Fluka.

The chromatographic system consisted of the following components: a LC-10Advp solvent delivery pump (Shimadzu Co., Tokyo), a manual sample injector – Rheodyne 7025; SPD-10Avp wavelength detector (Shimadzu Co., Tokyo).

Quantitative analysis of PIs was performed using the internal standard method. The calibration curves were obtained by unweighted least-squares linear regression of the peak-area ratio of SQV, LPV, IDV and NFV to diazepam (I.S.). The calibration was established over the range 0.1 – 15 µg/mL. The validation of the method was based on the recommendations published as a Conference Report of the Washington Conference on Analytical Methods Validation (17).

Each level of the calibration curve was measured with two sets of calibrations: one set at the beginning and a second set at the end of the HPLC run. Throughout patient sample analysis, control samples at three concentrations levels (low, medium and high, respectively, 1000, 2000 and 4000 ng/mL) were assayed every five samples.

Quality control samples were used for the precision and accuracy determination, the precision being calculated as the relative standard deviation (RSD) within a single run (intra-assay) and between different assays (inter-assays), and the accuracy as the percentage of deviation between nominal and measured concentration.

The limit of quantitation (LOQ) for PIs in plasma was experimentally chosen as the minimal concentration in plasma samples which could be confidently determined in accordance with the Conference Report on Analytical Method Validation recommending that the deviation between measured and nominal concentration should not deviate more than ± 20%. The LOQ values were used for the low concentration level of the standard curve.

Patients

Patients were recruited from the Hospital of Infectious Diseases in Warsaw, Poland and participated in an ongoing open-label, observational study to evaluate the safety and efficacy of combination antiretroviral therapy with protease inhibitors: lopinavir and saquinavir with ritonavir in HIV-infected adult patients. Local ethical committee in the Hospital of Infectious Diseases in Warsaw approval was obtained and all patients provided written informed consent before participation. Study design and conduct complied with the principles embodied in the Declaration of Helsinki.

Lopinavir was administered as tablets (Kaletra consisted from lopinavir/ritonavir) in a dosage of 800/200 mg QD. Lopinavir with saquinavir were administered in concomitant therapy in a dosage

Table 1. Patient characteristics.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>34</td>
<td>19 – 48</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>66</td>
<td>48 – 80</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>170</td>
<td>158 – 190</td>
</tr>
<tr>
<td>AST/ALT (U/L)</td>
<td>28/29</td>
<td>20 – 357 / 15 – 295</td>
</tr>
<tr>
<td>HIV-1-RNA (copies/mL)</td>
<td>443</td>
<td>97 – 8000</td>
</tr>
<tr>
<td>CD4+ lymphocytes (cells/µL)</td>
<td>355</td>
<td>17 – 649</td>
</tr>
</tbody>
</table>

Table 2. Precision and accuracy of the assay for protease inhibitors in plasma.

<table>
<thead>
<tr>
<th>PIs</th>
<th>Saquinavir</th>
<th>Lopinavir</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal conc. (ng/mL)</td>
<td>Concentration found (ng/mL)</td>
<td>Precision RSD (%)</td>
</tr>
<tr>
<td>Intra-assay n=5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>970 ± 60</td>
<td>6.2</td>
</tr>
<tr>
<td>2000</td>
<td>1930 ± 11</td>
<td>5.7</td>
</tr>
<tr>
<td>4000</td>
<td>3940 ± 15</td>
<td>3.9</td>
</tr>
<tr>
<td>Inter-assay n=10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>1050 ± 85</td>
<td>8.1</td>
</tr>
<tr>
<td>2000</td>
<td>2050 ± 60</td>
<td>2.9</td>
</tr>
<tr>
<td>4000</td>
<td>3850 ± 22</td>
<td>5.7</td>
</tr>
</tbody>
</table>
Table 3. Pharmacokinetic characteristics.

<table>
<thead>
<tr>
<th>Protease inhibitors</th>
<th>Pharmacokinetic parameters</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AUC_{0-24h} (µg·h/mL)</td>
<td>105</td>
<td>52.3 – 158.1</td>
</tr>
<tr>
<td>Lopinavir</td>
<td>C_{min} (mg/mL)</td>
<td>2.4</td>
<td>0.2 – 5.7</td>
</tr>
<tr>
<td></td>
<td>C_{max} (µg/mL)</td>
<td>7.15</td>
<td>3.8 – 10.2</td>
</tr>
<tr>
<td></td>
<td>Tmax (h)</td>
<td>4</td>
<td>1 – 7</td>
</tr>
<tr>
<td></td>
<td>AUC_{0-24h} (µg·h/mL)</td>
<td>47.5</td>
<td>18.5 – 91.7</td>
</tr>
<tr>
<td>Saquinavir</td>
<td>C_{min} (µg/mL)</td>
<td>1.6</td>
<td>0.5 – 7.85</td>
</tr>
<tr>
<td></td>
<td>C_{max} (µg/mL)</td>
<td>4.2</td>
<td>3.1 – 13.7</td>
</tr>
<tr>
<td></td>
<td>Tmax (h)</td>
<td>3</td>
<td>2 – 4</td>
</tr>
</tbody>
</table>

Figure 1. A. Chromatogram of a drug free plasma; B. Chromatogram of a spiked plasma sample containing 10 mg/mL of indinavir (IDV), saquinavir (SQV), lopinavir (LPV) and nelfinavir (NFV); C. Chromatogram of a spiked plasma sample containing 5 mg/mL of indinavir (IDV), saquinavir (SQV), lopinavir (LPV) and nelfinavir (NFV) with diazepam as internal standard (I.S.).
400/1000/100 mg BID lopinavir/saquinavir/ritonavir. The lopinavir is now routinely given in combination with low doses of the cytochrome enzyme inhibitor ritonavir, either in fixed or free combination at a dose of 100 mg twice daily. Ritonavir increases the bioavailability of the other PIs and prolongs their plasma elimination half-lives. Demographic characteristics of patients are shown in Table 1.

Pharmacokinetic sampling

After a minimum of 7 days on treatment with lopinavir and saquinavir the pharmacokinetic curve of LPV and SQV in plasma of 23 patients was assessed during 8 h dosing interval. A total of 11 heparinized blood samples were drawn during a dosing interval at the following times after drugs ingestion: 0 (trough) and 1, 2, 3, 4, 5, 6, 7, 8, 12, 24 h for the BID regimen. After isolation of the plasma by centrifugation on the same day, the samples were stored at -70°C until analysis.

Pharmacokinetic analysis and methods

Plasma concentration versus time data were analyzed by non-compartmental methods using WinNonlin program. The highest observed plasma concentration was defined as $C_{\text{max}}$, with the corresponding sampling time as $T_{\text{max}}$. The plasma concentration observed at the end of the dosing interval (12 or 24 h post ingestion for the every 12-h regimen) was defined as $C_{\text{min}}$. The area under the plasma concentration-time curve AUC$_{0-24\text{h}}$ was calculated using the trapezoidal rule from 0 to 24 h.

Lopinavir and saquinavir concentrations in plasma were quantified using a sensitive and validated isocratic, reversed-phase, high-performance liquid chromatographic assay with ultraviolet detection at 215 nm. Briefly, 0.5 mL of plasma was transferred to a 10-mL glass tube and heated for 30 min at 57°C to inactivate the HIV virus. 0.5 mL of plasma spiked with diazepam used as internal standard and 0.5 mL NH$_4$OH was extracted by 5 mL of methyl tert-butyl ether. After shaking for 10 min, the samples were centrifuged for 10 min at 3000 × g at 5°C. Four mL of organic phase was transferred to a clean 10-mL glass tube and evaporated at 57°C under a nitrogen stream. The residue was dissolved in 0.5 mL of mobile phase, and then the mixture was vortexed for 1 min. Two mL of $n$-hexane was added.
to the mixture and shaken for 5 min, then cen-
trifuged for 10 min at 3000 × g at 5°C. The upper
organic layer was discarded and the eluent was
transferred into manual injector for injection into the
chromatographic column. A 100 mL volume of each
sample was injected into the column Symmetry C18
(150 × 4.6 mm i.d., 5 mm). The mobile phase was a
mixture of acetonitrile and phosphate buffer pH 5.6
(43:57, v/v), filtered and degassed prior using, and
flowing at the rate of 2 mL/min.

RESULTS

Preliminary results of this study were published
as an abstract in Therapeutic Drug Monitoring dur-
ing 10th International Congress of Therapeutic Drug
Monitoring & Clinical Toxicology, which took place
in Nice, France in September 9-14, 2007 (18).

The chromatographic separation of mixture of
protease inhibitors (IDV, SQV, LPV and NFV) was
performed using isocratic elution. Figure 1 shows
chromatograms of blank drug-free plasma sample
[A], plasma spiked with the mixture of four PIs [B],
PIs with added diazepam as internal standard [C],
and chromatograms from patient samples with LPV
alone [D], LPV and SQV together [E]. The retention
times [min] were 1.77 for indinavir, 7.04 for
saquinavir, 9.25 for lopinavir, 8.29 for nelfinavir
and 3.24 for diazepam used as internal standard.
Drug-free plasma did not contain endogenous sub-
stances, which led to interfering peaks.

Standard curves data are summarized in Figure
2. The correlation coefficients ($r^2$) of the calibration
curves for each PIs were > 0.9960 as determined by
least-squares analysis over the concentration range
of 0.1 – 15 mg/mL. QC standards were assayed at 3
concentrations in 5 replicates on 10 different days to
evaluate accuracy and precision.

Recovery was measured by comparing the chro-
matographic responses of each compound, at one
level, with and without extraction steps. Recovery

Figure 2. Calibration curves for four protease inhibitors.

- Lopinavir $y = (1.079 \pm 0.0366)x - (0.0313 \pm 0.1112); r = 0.9960$,
- Indinavir $y = (0.9131 \pm 0.0237)x + (0.0279 \pm 0.0719); r = 0.9980$,
- Saquinavir $y = (0.3862 \pm 0.0141)x + (0.0805 \pm 0.0426); r = 0.9960$,
- Nelfinavir $y = (0.2975 \pm 0.0113)x - (0.0043 \pm 0.0034); r = 0.9960$.

Figure 3. Individual plasma concentrations versus time curves for HIV infected patient using saquinavir and lopinavir in concomitant
therapy. ……….. saquinavir, …….. lopinavir.
was measured by a ratio of peak height of extracted sample/peak height of non-extracted sample × 100%. Recoveries were 89%, 91%, 86% and 90% for IDV, SQV, LPV and NFV, respectively.

Intra- and inter-assay accuracy and precision over 3 concentrations are presented in Table 2. The mean intra-assay precision was always lower than 6.2, 5.7, 3.9% for SQV and 3.3, 1.5, 1.9% for LPV (1000, 2000, 4000 ng/mL). Overall, the mean inter-assay precision for SQV and LPV was similar, with mean RSDs within 2.9 – 8.1%.

Table 3 summarizes pharmacokinetic data for two PIs: lopinavir and saquinavir in concomitant therapy. The applicability of the assay for PK research in HIV-infected patients was demonstrated by analyzing ten patient curves of LPV and SQV.

Figure 3 illustrates individual pharmacokinetic profiles for saquinavir and lopinavir in concomitant therapy. We observed a wide interpatient range in LPV and SQV plasma levels. Pharmacokinetic profiles for 9 patients treated with lopinavir in monotherapy were presented in Figure 4.

Comparison of two PK profiles for lopinavir and saquinavir (as mean ± SD) for HIV-infected patients is presented in Figure 5.
DISCUSSION

The role of TDM in the routine management of HIV-infected patients is still discussed (19). The consensus of current international guidelines for the treatment of HIV infection is that data on therapeutic drug monitoring of protease inhibitors provide a framework for the implementation of TDM in certain defined scenarios in clinical practice (1, 20). The high plasma PI concentrations have been associated with renal/urological toxicity (indinavir), gastrointestinal disorders (ritonavir, lopinavir, saquinavir), elevated lipids (ritonavir, lopinavir) (1). On the other hand, the concentration of the active moiety of the anti-infective drug should remain well above the minimal concentration that can inhibit replication of the pathogen at the site of infection during the entire 24-h period (4). The pharmacokinetic characteristics of PIs make a once-daily regimen feasible. However, boosting with ritonavir may allow once-daily administration. Low doses of ritonavir combined with another PI will boost plasma concentrations of this PI, either by increasing its bioavailability by decreasing gut and liver first-pass effect (saquinavir or lopinavir) or by decreasing its hepatic clearance and increasing its half-life (indinavir). These higher plasma concentrations of the boosted PI allow a simplification of the PI regimen by decreasing pill count and administration frequency, and avoiding meal restrictions for some drugs.

Several studies with various PIs demonstrated a good relationship between drug exposure (AUC and Cmin levels) and virological response (3). Our median lopinavir Cmax and Cmin levels: 7.15 and 2.4 mg/mL, respectively, are within the reported range and similar to the results presented in previous publications (5-7). It is important, because exposure of a patient to sub-therapeutic levels of a PI may result in the stepwise accumulation of mutations in the HIV protease with subsequent resistance to both the prescribed drug and other members of the same class of drugs (15, 16, 20).

Several studies have found relationships between an inhibitory quotient and Cmin in the combination of lopinavir/ritonavir therapy. In some studies were defined a target value in heavily pre-treated patients. These investigators have shown that, in salvage therapy, a Cmin > 5.7 mg/mL is an independent predictor of response and defined efficacy threshold at 4.0 mg/mL for highly active antiretroviral therapy (HAART) – experienced patients (20).

Low-dosage ritonavir improves oral bioavailability of saquinavir and lopinavir, which undergo high-first pass metabolism. The ability of ritonavir to increase plasma Cmin of concomitantly administered PIs is perhaps the greatest clinical benefit of dual PI therapy (2). The Cmax increase is possibly achieved by ritonavir inhibiting P-glycoprotein transport and first-pass metabolism.

For saquinavir, many different dosage regimens are used in common practice, resulting in a wide range of concentrations encountered. Relationships between the exposure to saquinavir and efficacy has been described in various studies (1).

In a few studies PK data were collected from a cohort of patients receiving saquinavir and lopinavir/ritonavir BID. The median and interquartile range for LPV AUC0-12h, Cmax and Cmin were 88.0 (68.6-118.9) mg h/mL, 9.9 (7.9-12.5) mg/mL and 4.8 (3.9-5.6) mg/mL, respectively. The same parameters for SQV were 9.8 (8.3-24.5) mg h/mL, 1.6 (1.4-3.9) mg/mL and 0.40 (0.22-0.93) mg/mL, respectively (5). The authors observed that SQV AUC0-12h and Cmin were similar compared with values reported in the literature. In our study the median and interquartile range for LPV AUC0-12h, Cmax and Cmin were 105.0 (52.3-158.1) mg h/mL, 7.15 (3.8-10.2) mg/mL and 2.4 (0.2-5.7) mg/mL. The same parameters for SQV in our study were 47.5 (18.5-91.7) mg h/mL, 4.2 (3.1-13.7) mg/mL and 1.6 (0.5-7.85) mg/mL.

This modified HPLC method among the methods presented earlier in the literature is rather simple and rapid. There is no need for a gradient solution. The isocratic solution provides simple procedure for determining simultaneously in a single run the four currently used PIs in plasma within twenty minutes and SQV and LPV within 15 min. There are no need to wavelength and column switching. The methyl tert-butyl ether liquid-liquid extraction followed by hexane washing is a procedure already described in several assays for PIs. The step with hexane washing is useful to avoid interference.

Both precision and accuracy of the method were always < ± 15% in accordance with published recommendations (17).

This method has been used in determination of SQV and LPV for 1 year in our laboratory. Monitoring anti-HIV protease inhibitor concentrations identified clinically important information that could be used to improve individual patient care in adult HIV patients. Nevertheless, there has already been a discussion going on about the role of TDM in the treatment of HIV infection, about the relationship between plasma levels of individual drugs and their antiretroviral activity, and about variability of drug plasma samples.
As a conclusion, the present study has described the method that can be used in a hospital laboratory for therapeutic drug monitoring of the protease inhibitors in patient plasma and for pharmacokinetic studies of HIV-infected patients, thereby helping to improve their therapeutic success. Further studies are required to clarify the role and benefit of TDM in patients outcome.

Acknowledgments

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REFERENCES