The unbound percentage of saquinavir and indinavir remains constant throughout the dosing interval in HIV positive subjects

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Aims To measure the unbound plasma concentrations of saquinavir (SQV) and indinavir (IDV) and to relate them to the total plasma concentrations in order to establish the unbound percentage of protease inhibitors *in vivo* during a full dosage interval profile.

Methods HIV-infected subjects ($n = 35$; median CD4 cell count = 340×10^6 cells l^{-1}, range: 120–825; viral load < 50 copies ml^{-1} in 22/35) treated with SQV or IDV containing regimens were studied. Plasma drug samples were collected at 0, 2, 4, 8 and 12 h postdose for the twice daily regimens and 0, 1, 2, 4 and 8 h for the three times daily regimens. Ultra-filtration was used to separate unbound IDV and SQV in plasma and their respective concentrations were measured by a fully validated method using high performance liquid chromatography-mass spectometry (h.p.l.c.-MS/MS).

Results Based on the ratio $AUC_{unbound}/AUC_{total}$, the median unbound percentage (95% CI for differences) of SQV and IDV from all the samples studied was 1.19% (0.99, 1.58%) and 36.3% (35.1, 44.2%), respectively. No significant difference was seen in the percentage binding of SQV between patients receiving SQV alone (median = 1.49%) or with ritonavir (median = 1.09%; $P = 0.141$; 95% CI for difference between medians = –0.145, 0.937) over the pharmacokinetic profile. Similarly, no significant difference was seen in the percentage binding of IDV in patients receiving IDV alone (median 35.2%) or with ritonavir (median = 41.3%; $P = 0.069$; 95% CI for difference between medians = –0.09, 15.4). The unbound concentrations of SQV ($P < 0.0001$; 95% CI for $r^2 = 0.634$, 0.815) and IDV ($P < 0.0001$; 95% CI for $r^2 = 0.830$, 0.925) remained constant as a proportion of total concentration over the full dosing profile.

Conclusions These *in vivo* data confirm previously published *in vitro* measurements of SQV and IDV protein binding. The unbound percentage of both protease inhibitors remained constant over the dosing interval.

Keywords: indinavir, pharmacokinetics, protein binding, saquinavir, unbound percentage

Introduction

The use of antiretroviral agents in potent combination regimens controls viral replication and improves HIV-infected patients host defences [1, 2]. However, more than 30% [3] of patients fail to achieve maximal viral suppression with the recommended treatment. Reasons behind therapeutic failure include the emergence of resistant viral strains, incomplete adherence to therapy, and pharmacokinetic factors [4]. Understanding pharmacokinetic principles and determining pharmacokinetic parameters is important for the optimization of dosing regimens to ensure maximum therapeutic benefit. It has been widely shown that plasma concentrations of protease inhibitors correlate with the magnitude and durability of viral suppression [5–8].

Protease inhibitors, such as saquinavir (SQV) and indinavir (IDV) are large lipophilic weak basic molecules, which bind to plasma proteins, with α_1-acid glycoprotein (AAG) having the greatest affinity for these drugs [9].

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Therefore, plasma concentrations of protease inhibitors are a composite of both protein bound and unbound drug. The ratio of unbound to bound drug is mainly governed by drug and protein concentrations and binding constants. A knowledge of unbound concentrations may be useful in establishing the actual concentration necessary to achieve the maximum antiviral effect and in helping to develop important pharmacodynamic models to define better the *in vivo* potency of antiretroviral agents [10, 11]. For most protease inhibitors, total plasma trough concentrations are targeted to be above their 90% inhibitory concentrations (IC_{90}) measured *in vitro* for the wild type strains. However, little data are available for the unbound concentrations of protease inhibitors in plasma and their correlation with the antiviral response.

Therefore, the aim of this study was to measure over the dosing period, unbound fraction of SQV and IDV when these drugs were administered as the sole protease inhibitor or given with low dose ritonavir.

Methods

Patients

Patients ($n = 35$; 31 male and 4 female) treated with a SQV ($n = 18$; median duration of SQV intake 20 months, range 8–39) or an IDV ($n = 17$; median duration of IDV intake 16 months, range 6–33) regime took part in the study at the Department of Infectious Diseases at the University of Torino, Italy. Approval for the study was obtained from the local ethics commitee and patients gave their written consent.

The median age of the patients was 39 years (range: 29–55 years). Patients had a median CD4 cell count of 340×10^6 cells l^{-1} (range: 120–825) and 22 subjects had undetectable viral load in plasma (< 50 copies ml^{-1}; Roche Amplicor Ultrasensitive Assay; Roche, Basel, Switzerland). Median viral load among the remaining patients was 200 copies ml^{-1} (range: 69–8500).

Blood samples (21 ml) were taken following an overnight fast at 8 h after the night dose for patients taking IDV three times daily and at 12 h after the night dose for all other patients. Four samples (14 ml) were subsequently taken over the dosing schedule at 1, 2, 4 and 8 h from patients on three times daily regime and at 2, 4, 8 and 12 h from patients on the twice daily regime. Blood was collected in heparinized tubes and centrifuged immediately (1851 *g*; 10 min). Plasma was removed and stored at −20 °C until analysis. Serum AAG concentrations were measured in all patients on the day of the study (Behring Nephelometer Analyser, BN2, Marburg, Germany). Immediately prior to drug analysis, samples were heat inactivated (58 °C; 30 min) to reduce the infectivity of HIV.

Total and unbound drug separation

Ultra-filtration was used to separate unbound from bound SQV and IDV in plasma. After a 30 min equilibration at 37 °C, plasma (500 µl) was injected into an Amicon Centrifree Filter System (Millipore Corporation, Bedford, MA, USA) and samples were centrifuged (1500 *g*; 90 min; 37 °C – temperature was kept constant in order not to alter drug protein binding). Each sample provided approximately 200 µl of ultra-filtrate containing the unbound drug. Centrifree tubes were washed with methanol (100 µl) for samples containing SQV to prevent drug adsorption on the membrane.

SQV and IDV analysis

All samples were analysed in duplicate. Plasma (50 µl) and ultrafiltrate (50 µl) SQV and IDV concentrations were extracted using diethyl ether (3 ml for 30 min). Prior to extraction, samples were spiked with an internal standard (Ro 31–9564; 10 µl; 100 ng ml^{-1}). Following centrifugation (3291 *g*; 5 min) the organic layer was removed and evaporated to dryness. Extracts were reconstituted in mobile phase (150 µl for unbound fractions; 1.5 ml for total drug) prior to injection (20 µl) onto the h.p.l.c./mass spectrometer.

SQV and IDV were eluted on a Hypurity Elite $5C_{18}$ Column (5 µm: 250 × 4.6 mm) using a mobile phase of 20 mmol l^{-1} ammonium formate buffer-acetronitrile (30 : 70; v/v) at a flow of 1.2 ml min^{-1}.

SQV (retention time 4.2 min), IDV (retention time 2.9 min) and internal standard (retention time 8.0 min) were analysed by fragmentation of the parent compound and quantification of resulting fragment ions (monitoring of ions m/z SQV 671.4/570.3, 388.2; IDV 614.4/465.3, 596.3; internal standard 674.4/573.3, 388.2) using a mass spectrometer (electrospray ionization) and Xcalibur software. The lower limit of detection for SQV and IDV on column are both less than 5 pg [12]. These correspond to plasma concentrations of 375 pg ml^{-1}. The interassay coefficients of variation (CV) for SQV were 9.7 and 3.9% at concentrations of 100 ng ml^{-1} and 5 µg ml^{-1}, respectively. The intra-assay CV were 2.0 and 3.5% at the same concentrations. The interassay CVs for IDV were 6.9 and 1.5% at concentrations of 150 ng ml^{-1} and 3 µg ml^{-1}, respectively. The intra-assay CVs were 4.5 and 4.7% at the same concentrations.

Data analysis

The proportion of unbound drug was calculated by dividing the unbound drug concentration by the total drug concentration and expressed as a percentage. The area under the plasma concentration-time curve

(AUC(0,8 h) and AUC(0,12 h)) was calculated for both unbound and total SQV and IDV using the linear trapezoid rule (TOPFIT computer software, Gustav Fischer Verlag, Stuttgard, Germany).

Data on the binding of SQV and IDV with or without RTV over the dosing interval were subjected to analysis of variance (ANOVA). Correlations between unbound and total concentrations of SQV and IDV were investigated using linear regression (Pearson's correlation test). This regression analysis was also used to examine relationships between unbound drug percentages and CD4 cell count and viral load.

Results

The median total AUC of SQV was 7544 ng ml^{-1} h when given alone (range 546–28938 ng ml^{-1} h) and 11279 ng ml^{-1} h when coadministered with RTV (936–34816 ng ml^{-1} h). The median total AUC of IDV was 29175 ng ml^{-1} h when given alone (range 15742–57800 ng ml^{-1} h) and 28773 ng ml^{-1} h when coadministered with RTV (18424–115625 ng ml^{-1} h) (Figure 1).

Variability in the isolation of unbound percentages was assessed by repeated ultrafiltration ($n \geq 6$) of the same plasma sample. Coefficients of variation of 17% and 5% were obtained for SDV and IDV, respectively.

Based on the ratio of $AUC_{unbound}/AUC_{total}$ in the six profiles of patients treated with SQV alone, percentage unbound drug ranged from 1.1 to 2.4% (median 1.49%; 95% CI = 1.05, 2.06%). The median unbound percentage of IDV among the eight profiles of patients treated with IDV three times daily as the sole protease inhibitor was 49.2% (95% CI = 34.9, 51.7%) (Figure 2a,c).

The addition of ritonavir to therapy had no effect on either the unbound percentage of SQV or IDV. Among patients treated with SQV and ritonavir containing regimens, the percentage SQV unbound ranged from 0.55 to 2.7% (median 1.09%; 95% CI = 0.76, 1.55%) which was not significantly different from patients receiving SQV alone ($P = 0.141$; 95% CI = –0.145, 0.937). Similarly the percentage IDV unbound (median 35.2%; 95% CI = 31.4, 41.3%) was not significantly different ($P = 0.069$; 95% CI = –0.09, 15.4) to patients receiving IDV alone (Figure 2b,d).

The unbound percentage of SQV remained constant as a proportion of total concentration over the full dosing profile ($r^2 = 0.863$; $P < 0.0001$; 95% CI for $r = 0.796$–0.903; Figure 3a) with no significant difference at different time points in the pharmacokinetic profiles of patients receiving SQV alone ($P = 0.517$; ANOVA) or with ritonavir ($P = 0.492$; ANOVA). Similarly, the unbound percentage of IDV remained constant as a

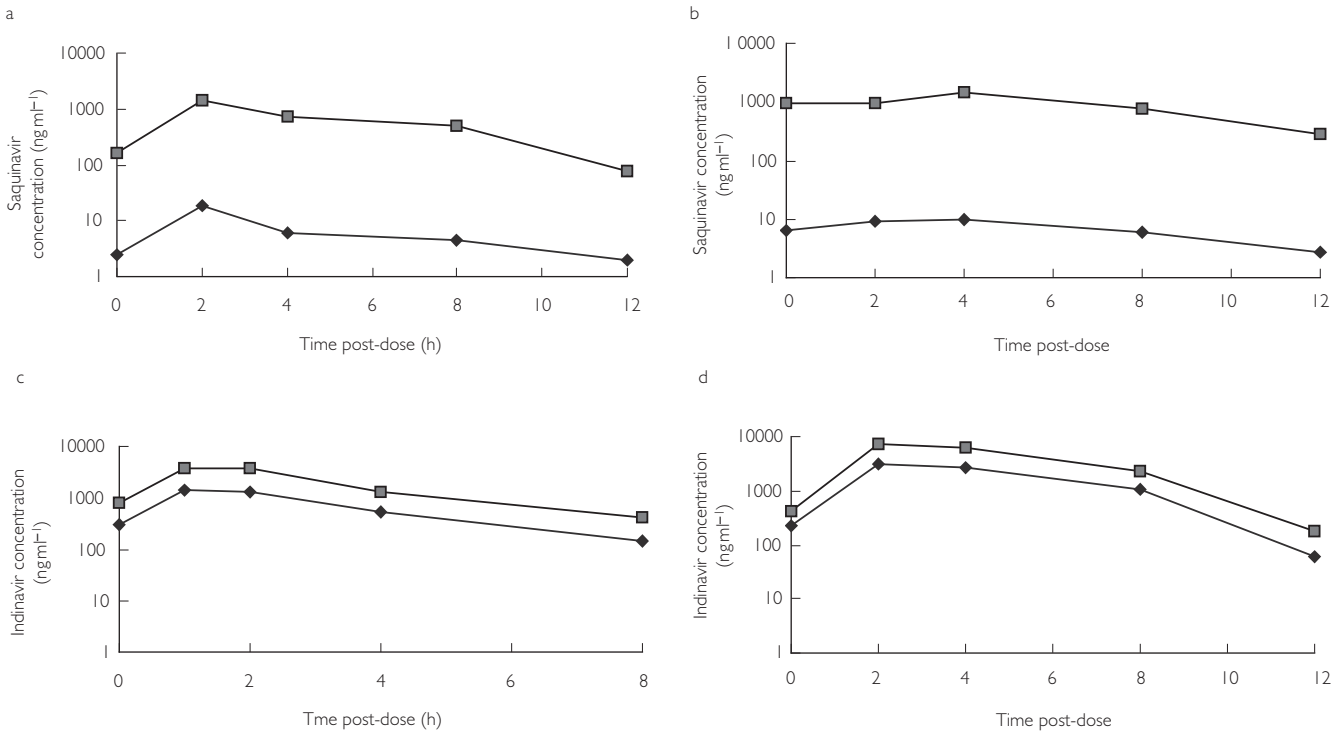

Figure 1 Median unbound and total concentration of (a) saquinavir when admistered as the only PI ($n = 6$), (b) saquinavir given with ritonavir ($n = 12$), (c) indinavir when administered alone ($n = 8$) and (d) indinavir given with ritonavir ($n = 9$), over the full dosing profile. Unbound concentration ◆; total concentration ■.

proportion of total concentration over the full dosing interval ($r^2 = 0.886$; $P < 0.0001$; 95% CI for $r = 0.911$, 0.962; Figure 3b) with the percentage binding of IDV not significantly changing with time in patients receiving IDV alone ($P = 0.975$; ANOVA) or with ritonavir ($P = 0.927$; ANOVA).

To investigate the relationship between disease state and the binding of SQV and IDV, we examined the effect of changes in the surrogate markers, CD4 cell count and HIV plasma viral load on the unbound percentage of these two PIs. No correlation was seen between CD4 cell count and the unbound percentage of SQV (AUC, $P = 0.714$; 95% CI for $r = -0.498$, 0.383; C_{min}, $P = 0.158$; 95% CI for $r = -0.143$, 0.701) or IDV (AUC, $P = 0.729$; 95% CI for $r = -0.418$, 0.596; C_{min}, $P = 0.286$; 95% CI for $r = -0.740$, 0.280). Although no relationship was seen between CD4 and both total and unbound concentration of SQV, weak relationships were seen between CD4 and both total and unbound concentration of IDV (unbound SQV AUC $r^2 = 0.01$, 95% CI for $r = -0.49$, 0.34 and C_{min} $r^2 = 0.09$, 95% CI for $r = -0.65$, 0.17; unbound IDV AUC $r^2 = 0.36$, 95% CI for $r = -0.87$, -0.07 and C_{min} $r^2 = 0.23$, 95% CI for $r = -0.82$, 0.10; total SQV AUC $r^2 = 0.03$, 95% CI for $r = -0.57$, 0.29 and C_{min} $r^2 = 0.11$, 95% CI for $r = -0.61$, 0.10; unbound IDV AUC $r^2 = 0.24$, 95% CI for $r = -0.81$, -0.09 and C_{min} $r^2 = 0.36$, 95% CI for $r = -0.89$, -0.11).

Patients with undetectable viral load showed no differences in the unbound percentage of SQV compared with those with detectable viral load (AUC, $P = 0.181$; 95% CI for difference between medians = -1.12, 0.11; C_{min}, $P = 0.566$; 95% CI for difference between medians = -1.36, 1.21) or IDV (AUC, $P = 0.871$; 95% CI for difference between medians = -17.87, 9.27; C_{min}, $P = 0.871$; 95% CI for difference between medians = -17.7, 15.9).

AAG concentrations were in the normal range for all patients studied (66–97 mg dl^{-1}) and were not significantly different among the four drug groups (Kruskal–Wallis, $P = 0.831$). No relationship was seen between AAG concentrations and the unbound percentage of either SQV ($P = 0.243$; 95% CI for $r = -0.638$, 0.192) or IDV ($P = 0.604$; 95% CI for $r = -0.579$, 0.369).

Discussion

Drug exists in plasma in a state of equilibrium between unbound drug and protein bound drug. Most pharmacokinetic studies of protease inhibitors have involved assessment of total drug concentrations [13]. However, pharmacological activity is dependent on unbound drug entering cells harbouring the virus.

The median unbound SQV percentage in plasma was 1.2% based on all samples taken from patients on different SQV containing regimens. The estimated bound percentage (median = 98.8%) confirmed *in vitro*-based data showing 98% SQV binding [14]. Variability in protein

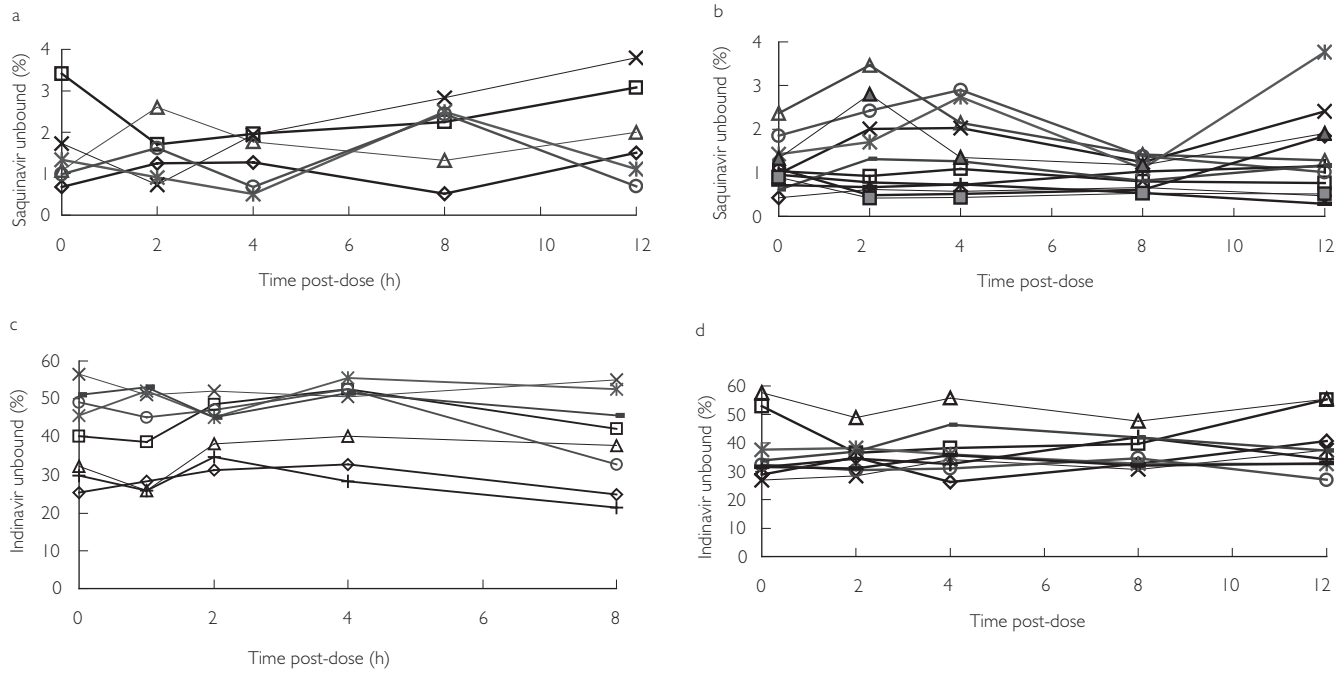

Figure 2 Unbound percentage of (a) saquinavir when admistered as the only PI ($n = 6$), (b) saquinavir given with ritonavir ($n = 12$), (c) indinavir when administered alone ($n = 8$) and (d) indinavir given with ritonavir ($n = 9$), over the full dosing profile.

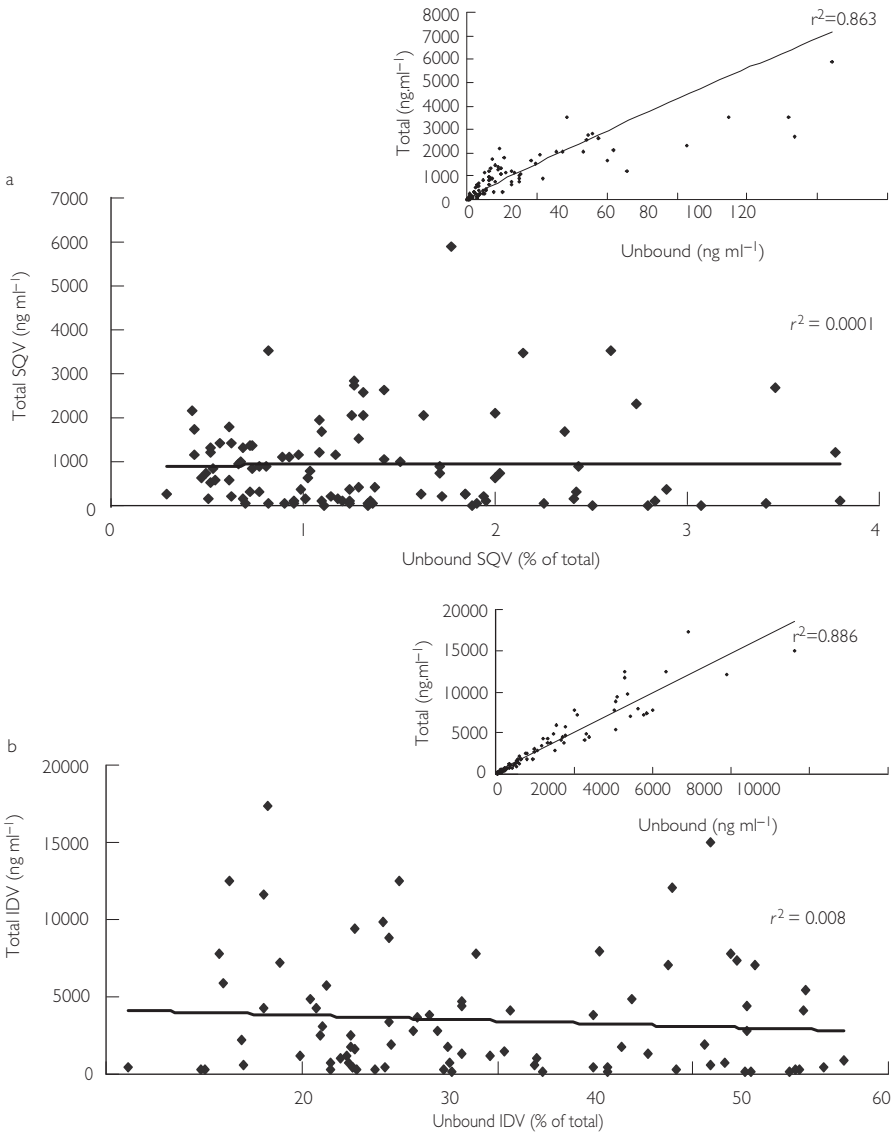

Figure 3 Linear regression analysis of the unbound percentage and the total drug concentration of (a) saquinavir (n = 18) and (b) indinavir (n = 17). Results shown are from all time points studied. Insets illustrate the unbound and total drug concentration. The area under the curve (AUC) was determined between 0 and 8 h for three times daily regimens and 0–12 h for twice daily regimens.

binding of SQV among all samples studied ranged between 97.3% and 99.5%.

The median percentage of unbound IDV in plasma was found to be 36% based on all samples taken from 17 patients on different IDV containing regimens. The bound percentage (median = 64%) confirmed *in vitro*-based data showing 60% IDV binding [15] and also agreed with previously published *in vivo*-data obtained from eight HIV positive men treated with two nucleoside reverse transcriptase inhibitors and indinavir who underwent a pharmacokinetic study characterized by methods similar to ours [16].

Variability in protein binding of IDV among all samples studied ranged between 49 and 70%. Previous *in vivo* data have also illustrated wide variability in the protein binding of IDV [16]. In most individuals AAG is a mixture of two or three genetic variants and its polymorphism is due to the presence of at least two different genes encoding the protein [17]. AAG also presents at least two separate drug binding sites with different binding specificities. These factors may account for the inter-individual differences and apparent bimodal distribution of drug binding with IDV. Indeed, large differences in the binding of other drugs have been shown between different variants [18, 19].

The unbound percentage of both SQV and IDV remained constant as a proportion of total concentration over the full dosing interval (Figure 3). Although, it is

possible that there may be a saturation in binding when total drug concentrations approach that of the AAG concentration in plasma. However, at the range of concentrations of SQV and IDV seen in the patients this would not be anticipated. This is in contrast to the findings of Anderson *et al.* who demonstrated that the unbound percentage of IDV changed over the 8 h dosing schedule [16].

Although there was no change in the unbound percentage of SQV and IDV with CD4 count, a weak relationship was seen with both total and unbound IDV concentration, as would be expected. Therapeutic drug monitoring of these drugs indicates that the total drug concentration of the protease inhibitors is indicative of virological (and probably immunological) response [13]. However, the unchanged unbound percentage suggests that saturation of binding to plasma proteins at concentrations seen *in vivo* does not occur. Ritonavir is also highly bound to plasma proteins (99%) [20]. However, no difference was seen in the unbound percentage of drug in regimens containing ritonavir suggesting no displacement of SQV or IDV binding. Nevertheless, displacement by other drugs, which bind similarly, may result in a drug interaction altering the equilibrium between plasma and tissue cells. *In vivo* studies with the aim of investigating interactions between protease inhibitors and other lipophilic drugs highly bound to AAG are also warranted, to establish if an increase in unbound percentage is associated with a better antiviral response.

HIV positive patients, even if treated with antiretroviral agents, are subject to a higher incidence of infections or other acute concurrent disease [21, 22]. During these events, concentrations of AAG, an acute phase reactant protein, may be increased [23–25], and the unbound percentage of highly bound drugs may consequently be decreased. However, none of the patients we studied had increased concentrations of AAG or evidence of infections. Secondly, we have no evidence to suggest that variability of AAG concentrations within the normal range is responsible for interpatient variability of protiein binding of the PIs investigated. However, larger studies are required to examine this relationship.

As cellular uptake of protease inhibitors *in vitro* [26–28] is limited by increasing concentrations of AAG, this may suggest that protein binding may play a role in decreasing antiviral activity. For the latter to occur, unbound drug is required to enter the cells and inhibit HIV protease, the extent of which seems to be associated with the concentration of the intracellular drug. Therefore, studies are required to investigate how unbound concentrations of antiretroviral protease inhibitors *in vivo* are altered in patients with AAG concentrations outside the normal range. Increased AAG may lead to a decreased percentage of unbound drug, and therefore, depending on the clearance of the drug, may alter efficacy.

The protease inhibitors are thought to be high clearance drugs in humans and their clearance may be limited by blood flow and less affected by protein binding. However, the influence of protein binding on clearance of the protease inhibitors requires further investigation. Studies in transgenic mice, with overexpression of plasma AAG, illustrated that SQV binding to plasma proteins was significantly raised and its unbound percentage decreased from 3.0% in control mice to 1.5% in transgenic mice. Systemic clearance and volume of distribution were significantly reduced in this model consistent with decreased systemic exposure to the drug [29]. The clearance of SQV in the mouse is unknown and further investigation in this *in vivo* model is warranted.

Therapeutic drug monitoring of protease inhibitors has been demonstrated to provide benefit in combination treatment of HIV patients [30]. However, in some patients therapeutic failure due to pharmacological reasons still occurs, even when total plasma protease inhibitor concentrations seem to be higher than minimum effective concentrations. As antiviral activity is in part determined by entry of unbound drug into the cell, it may be beneficial to measure this parameter in HIV patients. Indeed, pharmacologic exposure could be ideally expressed as the protein-free concentration of drug present over the dosing period. Furthermore analysis of unbound concentrations of protease inhibitors may be beneficial in determining pharmacodynamic models to fully quantify *in vivo* potency of these drugs [31].

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