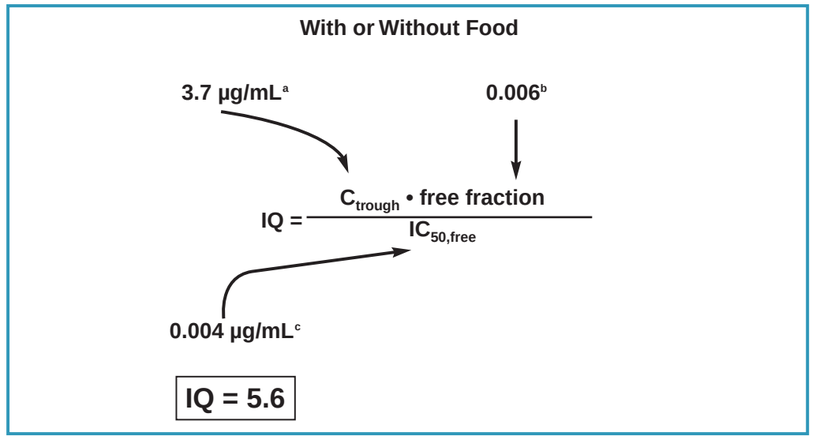
Figure 8. Calculation of RTV Inhibitory Quotient (IQ)



IQ based on IC_{50} determined in 10% FCS + 50% Human Serum:
(C_{trough} of 3.7 µg/mL/$IC_{50,10\%FCS50\%HS}$ of 0.96 µg/mL) = 3.9

[a]Reference [9]; [b]Reference [9] (0.6%); [c]Average $IC_{50,free}$ (Table 2, Figure 6)

IQ Calculated from $IC_{50,free}$ Are Similar to Previous Estimates Using $IC_{50,total}$ Determined in 10% FCS/50% HS

- The mean IQ for LPV in patients with wild-type HIV calculated by the $IC_{50,free}$ method was 67 and 52 with and without food, respectively (Figure 7), and the mean IQ for RTV (600 mg BID) in patients was 5.6 with or without food (Figure 8).
- LPV and RTV IQ based on IC_{50} determined in 10%FCS + 50% human serum was previously estimated as 78 and 3.9, respectively (Figure 7 and 8).
- Therefore these data indicate that the previous method using 10% FCS supplemented with 50% human serum to estimate virus $IC_{50,total}$ in plasma [1] was a reasonable and valid approach.

CONCLUSIONS

- Binding to fetal calf serum in the culture media can significantly impact the *in vitro* antiviral potency of HIV protease inhibitors.
- The determination of IC_{50}s and free fractions using varying amounts of FCS under identical conditions provides a quantitative estimate of the serum-free IC_{50}.
- The serum-free IC_{50} values for LPV and RTV using this standardized method (for the MT4-MTT tissue culture system) were 0.69 and 4.0 ng/mL, respectively.
- This method may be useful for assessing the inhibitory quotient for individual patients for whom the free fraction of PI is available. However, since IC_{50} and free fraction measurements are method-dependent, it is important that a standardized method be adopted for any comparison of multiple drugs.
- The average IQ values for wild-type HIV estimated for LPV and RTV by this method were similar to those previously estimated by a method that employed the IC_{50} directly determined in the presence of 50% human serum and 10% FCS.

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Poster #1740

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Estimation of the Serum-Free IC_{50} for the Protease Inhibitor Lopinavir

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INTRODUCTION

All currently marketed HIV protease inhibitors (PI), with the exception of indinavir, are bound extensively to plasma proteins, primarily albumin and/or α1-acid glycoprotein (AGP). Both lopinavir and ritonavir are bound to albumin and AGP. Protein-bound drug generally is considered to be too large to pass through most cell membranes to exert pharmacological actions. *In vitro*, the addition of human serum or serum proteins attenuates the antiviral activity of PIs. Clinical data also suggest that protein binding attenuates potency *in vivo*. *In vitro* tissue culture assays maximally tolerate *ca.* 50% human serum. Furthermore, the addition of purified serum proteins may under- or overestimate the attenuating effect of those proteins *in vivo*. Consequently, the antiviral potency of PIs can vary substantially in various *in vitro* systems. Furthermore, disease, age, and other altered physiological states can often result in altered plasma compositions, the effect of which may be underestimated *in vitro*.

Theoretically, determination of antiviral activity in a serum-free environment ($IC_{50,free}$), coupled with direct measurement of protein binding in patient plasma, should improve quantitative estimates of the IC_{50} to be used assessing *in vivo* potency. However, the $IC_{50,free}$ cannot be determined directly because PIs also can be substantially bound by fetal calf serum (FCS), which is required in the tissue culture medium to promote cell growth. Therefore we have investigated a novel approach for quantitatively estimating the $IC_{50,free}$ of lopinavir (LPV) and ritonavir (RTV). This approach requires the determination of both IC_{50} and free fraction at various concentrations of FCS with subsequent derivation of an $IC_{50,free}$ that is independent of protein binding. The $IC_{50,free}$ may serve as the basis for accurate estimates of *in vivo* IC_{50} and inhibitory quotient values.

OBJECTIVES

- To investigate the effect of FCS concentration on IC_{50} values and free fractions of LPV and RTV.
- To estimate the serum-free IC_{50} of LPV and RTV.
- To provide a new estimate of the IC_{50} for assessing LPV and RTV *in vivo* activity based on the above relationship.
- To compare the inhibitory quotient (IQ) of LPV and RTV based on this method with that calculated using an *in vitro* system containing 10% FCS+50% human serum.

MATERIALS AND METHODS

- RPMI 1640 medium and penicillin/streptomycin (PS) solution were purchased from Gibco Chemical Co.
- Fetal calf serum (FCS) was purchased from JRH Bioscience.
- FCS was combined with RPMI 1640/PS solution to prepare test media with 5, 10, 20 and 50% FCS exactly analogous to the antiviral assay.
- 0.02 M phosphate buffer, pH 7.4, containing 0.6% NaCl substituted for FCS in the dialyzing media.
- [^{14}C]Radio-labeled lopinavir and ritonavir were synthesized by John Uchic and Bruce Surber and purified (>99%) by Jia Du of Abbott's Drug Metabolism Department.
- Equilibrium Dialysis
  - Spectrum Equilibrium Dialysis System
  - 1 mL cells – Spectra/Por 2 membrane (12-14kD cut off)
  - Test medium with [^{14}C]Drug vs. dialysing medium at 37°C
  - Final 0.5% DMSO in test medium, analogous to virus assay
  - 3 hour equilibration (drug stability tested by radio-HPLC)
  - $\%\ \text{Bound} = \frac{\text{dpm/mL test - dpm/mL dialysing}}{\text{dpm/mL test}} \times 100\%$
- Analytical limitations would not allow determination of free fraction below 0.1 µg/mL for either drug.
- MTT-MT4 tissue culture system
  - Anti-HIV activity was assessed against the IIIB strain of wild-type HIV in MT4 cells in media containing 5, 10, 25 and 50% FCS.
  - Inhibition of HIV-induced cytopathic effect over a range of drug concentrations was monitored by uptake of MTT [1].
  - IC_{50} values for each of the four FCS concentrations were determined in two sets of six-replicate measurements.

RESULTS

- The protein binding of LPV and RTV to FCS was saturable (0.1 to 10 µg/mL) and dependent on FCS concentration (Figures 1 and 2).
- The IC_{50}s of both drugs increased incrementally as the free fraction of each decreased with higher proportions of FCS in the media (Figures 3 and 4). The free fractions for 0.1 µg/mL drug concentration are also shown in the accompanying graphs to depict the relationship among the three variables.

Figure 1. Effect of % FCS on the Free Fraction of [^{14}C]Lopinavir in RPMI 1640/PS

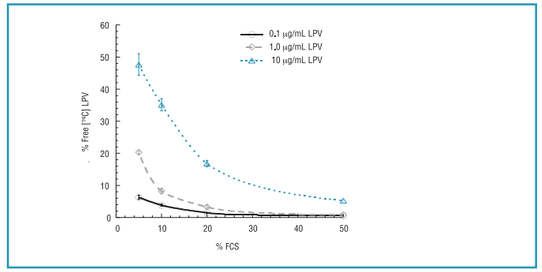


Figure 2. Effect of % FCS on the Free Fraction of [^{14}C]Ritonavir in RPMI 1640/PS

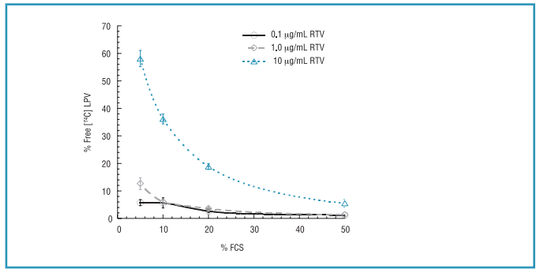


Figure 3. Effect of FCS on Mean Virus LPV IC_{50} and Mean % Free LPV (0.1 µg/mL) in RPMI1640/PS Media

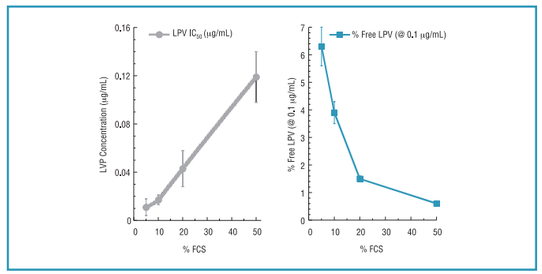
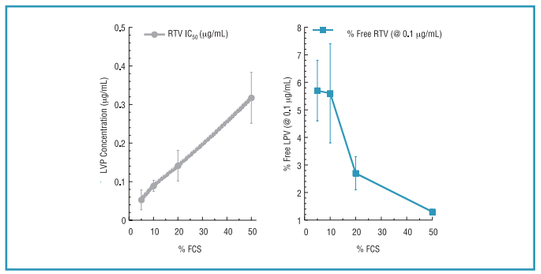


Figure 4. Effect of FCS on Mean Virus RTV IC_{50} and Mean % Free RTV (0.1 µg/mL) in RPMI1640/PS Media



- The mean measured IC_{50} ($IC_{50,total}$) of LPV and RTV, determined in the 5-50% FCS media, ranged from 0.011 to 0.119 and from 0.053 to 0.318 µg/mL, respectively (Tables 1 and 2).
- The protein binding values determined at 0.1 µg/mL were therefore used for estimation of the serum-free IC_{50} ($IC_{50,free}$).
- The free fractions of LPV and RTV in 5-50% FCS ranged from 6.3 to 0.6% and from 5.7 to 1.3%, respectively (Tables 1 and 2).

Table 1. Mean LPV $IC_{50,total}$, % Free LPV, and Derived LPV $IC_{50,free}$ in 5-50% FCS Media

FCS in Culture Medium (%)	LPV $IC_{50,total}$ (µg/mL)	LPV Free Fraction (%)[1]	LPV $IC_{50,free}$ (µg/mL)[2]
5	0.011 ± 0.007	6.3 ± 0.7	0.00069 ± 0.00044
10	0.017 ± 0.004	3.9 ± 0.4	0.00068 ± 0.00014
20	0.043 ± 0.015	1.5 ± 0.1	0.00064 ± 0.00023
50	0.119 ± 0.021	0.6 ± 0.1	0.00077 ± 0.00014

1 Free fraction at 0.1 µg/mL LPV for each FCS concentration.
2 Calculated as the product of LPV $IC_{50,total}$ and LPV free fraction.

Table 2. Mean RTV $IC_{50,total}$, % Free RTV, and RTV $IC_{50,free}$ in 5-50% FCS Media

FCS in Culture Medium (%)	RTV $IC_{50,total}$ (µg/mL)	RTV Free Fraction (%)[1]	RTV $IC_{50,free}$ (µg/mL)[2]
5	0.053 ± 0.026	5.7 ± 1.1	0.0030 ± 0.0015
10	0.089 ± 0.014	5.6 ± 1.8	0.0050 ± 0.0008
20	0.141 ± 0.040	2.7 ± 0.6	0.0038 ± 0.0011
50	0.318 ± 0.066	1.3 ± 0.1	0.0040 ± 0.0008

1 Free fraction at 0.1 µg/mL RTV for each FCS concentration.
2 Calculated as the product of RTV $IC_{50,total}$ and RTV free fraction.

- The serum-free IC_{50} for each set of *in vitro* conditions was calculated using the following equation:

$$IC_{50,free} = IC_{50,total} \bullet \text{free fraction}$$

- The serum-free IC_{50} for both drugs remained constant over a wide range (from 5 to 50%) of FCS concentrations (Figures 5 and 6).
- The average serum-free LPV IC_{50} for wild-type virus across 5 to 50% FCS was 0.00069 µg/mL (0.69 ng/mL).
- The average serum-free RTV IC_{50} for wild-type virus across 5 to 50% FCS was 0.0040 µg/mL (4.0 ng/mL).
- A more sophisticated approach utilizing non-linear curve fitting to extrapolate the free fraction of each drug to the concentration representing the $IC_{50,total}$ (i.e., beyond the analytical limitations of the protein binding experiments) yielded very similar estimates of the $IC_{50,free}$.
- The ratio of LPV $IC_{50,free}$/LPV K_i for purified wild-type HIV protease was similar to the ratio of RTV $IC_{50,free}$/RTV K_i (Table 3).

Figure 5. The Serum-Free IC_{50} of LPV Is Constant Under Varied Conditions of Protein Binding

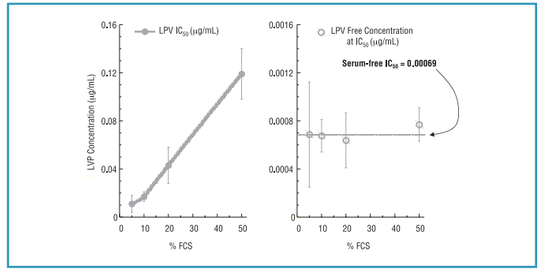


Figure 6. The Serum-Free IC_{50} of RTV Is Constant Under Varied Conditions of Protein Binding

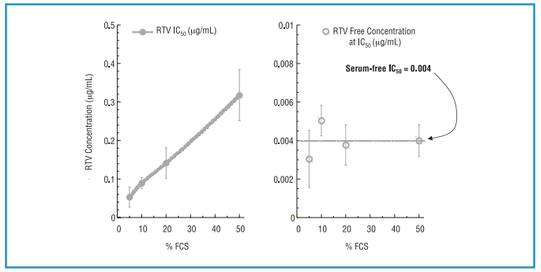


Table 3. Comparison of $IC_{50,free}$ and K_i Values for LPV and RTV

	LPV	RTV
IC_{50},free (pM)	1100	5500
K_i* (pM)	1.3	10
$IC_{50,free}/K_i$ Ratio	850	550

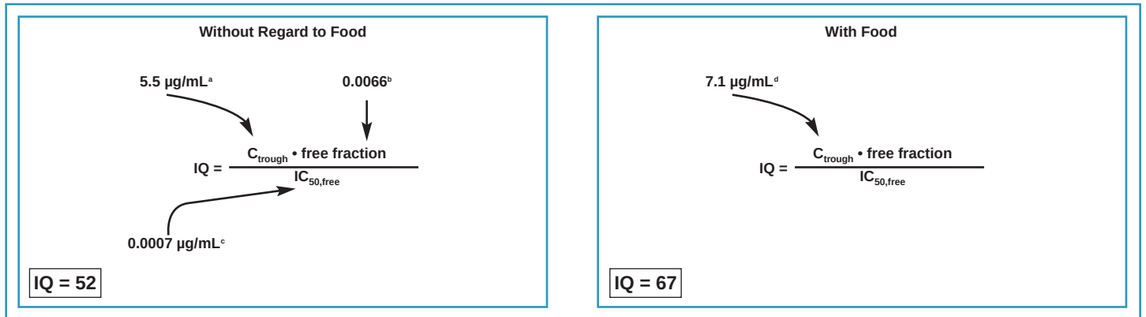
* Reference [2]

Calculation of the Inhibitory Quotient

- The IQ [3], defined as the C_{trough}/IC_{50} ratio, has been shown to be predictive of virologic response to antiretroviral therapy [4-6].
- Using the serum-free IC_{50}, the IQ is calculated by the following equation:

$$IQ = \frac{C_{trough} \bullet \text{free fraction}}{IC_{50,free}}$$

Figure 7. Calculation of LPV Inhibitory Quotient (IQ)



[a]Reference [7]; [b]Reference [7] (0.66%); [c]Average $IC_{50,free}$ (Table 1, Figure 5); [d]Reference [8]