Pharmacokinetics of Human Immunodeficiency Virus Protease Inhibitor, Nelfinavir, in Poloxamer 407-Induced Hyperlipidemic Model Rats

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The effect of hyperlipidemia (HL) on the pharmacokinetics of nelfinavir (NFV), a human immunodeficiency virus protease inhibitor, was investigated, focusing on the change of NFV distribution in plasma using poloxamer 407-induced HL model rats (HL rats). The plasma unbound fraction (fu) in HL rats (0.20—0.39%) was significantly lower than the control (0.92—1.47%). Lipoprotein level in HL rats was about 5 times higher and low- and very low-density lipoproteins ratio were 1.7—4.5 times higher than the control. NFV recovery in the triglyceride-rich lipoprotein such as chylomicron, low- and very low-density lipoproteins fractions of HL rats were significantly higher. The area under the plasma concentration–time curve (AUC) of NFV after intravenous (i.v.: 5 mg/kg) and intraduodenal (i.d.: 30 mg/kg) administration to HL rats (i.v.: 6.12±0.48, i.d.: 24.4±2.2 µg·h/ml) were higher than the control (i.v.: 1.62±0.21, i.d.: 5.00±0.36 µg·h/ml). The steady state volume of distribution (Vdss) in HL rats (0.60±0.07 l/kg) was lower than the control (6.25±0.55 l/kg). Systemic availability (F) in HL rats (66.6%) was higher than the control (51.4 %). Directly absorbed NFV from the gastrointestinal tract to the lymphatic system in HL rats was about 2 times higher than the control. From the results of this study, it was concluded that the increase of AUC was caused by decreasing fu and Vdss due to the increase of the triglyceride-rich lipoprotein level. In addition, it was suggested that the increase of absorbed NFV through the lymphatic system, which did not receive the first-pass effect, was one reason for the increase of F in HL rats.

Key words nelfinavir; hyperlipidemia; pharmacokinetics; protein binding; lipoprotein; human immunodeficiency virus protease inhibitor

The combined use of nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors and protease inhibitors (PIs) is recommended as the initial regimen for managing human immunodeficiency virus (HIV). PIs are among the most potent anti-HIV therapies and have contributed significantly to the reduction in mortality among patients with HIV. The pharmacological and adverse effects of PI correlate with its plasma concentration; therefore, therapeutic drug monitoring is useful for anti-HIV drug therapy.

It has been reported that PI often induces hyperlipidemia (HL) as a side effect. Furthermore, it has been reported that the lipoprotein binding function of high lipophilic drugs increases when lipoprotein increases in HL, and that the clearance of cyclosporine A (CyA), a high lipophilic drug, decreased due to the increase of the lipoprotein level, as they are the major complexing constituents for CyA. In our clinical study of CyA in renal transplant recipients, similar results were obtained; therefore, investigation of the relationship between the change of binding characteristics to serum protein (focusing on lipoproteins) and pharmacokinetics (PK) behavior in HL is important.

On the other hand, an HIV protease inhibitor, nelfinavir (NFV), which is a high lipophilic drug as well as CyA, has been widely used for HIV infection. NFV has high binding characteristics to serum protein (about 99%), therefore, changes of the protein binding ratio may strongly affect the PK of NFV, such as hepatic clearance and tissue distribution. NFV, which is a basic drug, is well bound to α1-acid glycoprotein (AAG) and albumin (ALB); however, it has been reported that the free fraction of NFV was not affected by drugs that bind extensively to AAG or ALB when these drugs were added to plasma, suggesting a compensatory effect of alternate binding proteins. It is anticipated that NFV also binds to lipoproteins for its high lipophilicity. In addition, it has been reported that NFV induces HL as a side effect, as described above; however, there is no information regarding the binding characteristics to lipoproteins of NFV in plasma and the effect of lipoproteins on pharmacokinetics of NFV as far as we know.

In this study, to investigate the effect of the change of plasma lipids on protein-binding characteristics, distribution in plasma components, and PK properties of NFV in detail, we quantified the protein binding ratio and NFV distribution in plasma components such as lipoproteins in poloxamer 407 (P407)-induced hyperlipidemic model rats (HL rats), moreover, we performed PK studies of NFV using HL rats.

MATERIALS AND METHODS

Materials NFV and atazanavir were extracted from commercial tablets and the crude extract was purified by a preparative HPLC method. Atazanavir was used as an internal standard for LC-MS method as described below. Propylene glycol and acetonitrile (HPLC grade) were purchased from Wako Pure Chemical Industries (Osaka, Japan). P407 (Pluronic F-127®) was obtained from Sigma (St. Louis, MO, U.S.A.). All other reagents were of analytical grade and were used without further purification.

Animal Preparation All animal experiments were performed in accordance with the Guidelines for Animal Experimentation of Kyoto Pharmaceutical University. Male Wistar rats (weighing 280—320 g) of 10 weeks old were obtained from Nippon SL Co., Ltd. (SLC, Hamamatsu, Japan). Rats had free access to food and water, and were maintained in a temperature-controlled facility with a 12 h light/dark cycle for at least 5 d before use. HL rats were prepared by intraperitoneal administration of P407 solution (0.1 g/ml in

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saline) at a dose of 1 g/kg to rats. Control rats received the same volume of vehicle without P407. All experiments were performed 36 h after P407 administration. There were no differences in body weight between HL and control rats just before experiments.

Determinations of biochemical parameters such as high-density lipoprotein ratio (HDL%), low-density lipoprotein ratio (LDL%), very low-density lipoprotein ratio (VLDL%), HDL-cholesterol level (HDL-ch) and LDL-cholesterol level (LDL-ch) were performed by a commercial laboratory, Kyoto BIKEN (Kyoto, Japan).

**Pharmacokinetic Studies of NFV in HL Rats** HL and control rats were fasted overnight prior to the administration of NFV with free access to water. Under anesthesia by an intraperitoneal injection of sodium pentobarbital, 32 mg/kg, these rats were placed in a supine position on a heating pad under a surgical lamp to maintain body temperature during the experiment.

For the intravenous (i.v.) study, NFV test solution (5 mg/ml in propylene glycol) was administered to both HL model rats (n=6) and control rats groups (n=6) at a dose of 5 mg/kg under an abdominal sham operation. Blood samples were withdrawn into heparinized microcentrifuge tubes at 0, 5, 10, 15, 30, 60, 90, 120, 240 and 360 min after drug administration. Blood samples (200 µl) were collected from the external left jugular vein.

For the intraduodenal (i.d.) study, NFV test solution (10 mg/ml in propylene glycol) was administered into the duodenum (through a pore made in the stomach using a polyethylene cannula; 0.5 mm inner diameter (ID), 0.8 mm outer diameter (OD) of both HL model rats (n=6) and control rats (n=6) at a dose of 30 mg/kg under an abdominal operation. After administration, the pore made in the stomach was closed with a drop of tissue cement. Blood samples were withdrawn into heparinized microcentrifuge tubes at 0, 30, 60, 90, 120, 180, 240, 360 and 540 min after drug administration. Blood samples (200 µl) were collected from the external left jugular vein.

To investigate NFV absorption to the lymphatic system in HL rats, we used a thoracic duct cannulated rat model previously established in our laboratory.21 Thoracic ducts were cannulated with a heparin-filled flexible vinyl catheter (ID 0.5 mm, OD 1.2 mm; Dual Plastics) and fixed with a drop of tissue cement (Aron Alpha A®, Sankyo Co., Tokyo, Japan). After administration of NFV test solution to both HL and control rats (i.v.; 5 mg/kg, i.d.; 30 mg/kg), the continuous output of lymph from the thoracic duct was collected at 30-min intervals in microtubes until 6 h, and sample volumes were determined gravimetrically.

In each PK study, after collecting blood or lymph samples, supernatants were obtained by centrifuging each sample at 9000 g for 10 min, and immediately frozen at −80 ºC until analysis of NFV.

To investigate the distribution of NFV to the liver, the amount of NFV after i.v. administration in the liver was measured. After 0.5, 1, 2 h of i.v. bolus infusion of NFV test solution (5 mg/kg) to HL and control rats, the animals were killed by cervical dislocation, and their livers were perfused with phosphate-buffered saline (PBS) to remove blood using an infusion pump. After the livers were blotted using filter paper, the tissue samples were weighed and homogenized in PBS (5-fold volume of tissue weight) using a glass homogenizer. The supernatant fractions obtained after the removal of cell debris by centrifugation at 1000 g for 5 min were stored at −80 ºC until analysis of NFV.

**Pharmacokinetic Analysis** Noncompartmental pharmacokinetic analysis was applied to the plasma concentration–time data using a computer program, WinHARMONY.23 The terminal elimination rate constant (λz) was determined by linear regression of at least three data points from the terminal portion of the plasma concentration–time plots. The area under the plasma concentration–time curve (AUC) was calculated using the linear trapezoidal rule up to the last measured plasma concentration (Cp(last)), and extrapolated to infinity using a correction term, namely Cp(last)/λz. The area under the first-moment curve to the last measured plasma concentration (AUMC) was also calculated using the linear trapezoidal rule and the addition of the concentration term after the last measured point (t(last)) to infinity, namely, t(last)Cp(last)/λz + Cp(last)/λz2. The terminal elimination half-life (t1/2) was determined by dividing ln 2 by λz. The mean residence time (MRT) was calculated by dividing AUMC by AUC. Total body clearance (CLtot) was calculated by D/F/AUC, where D and F represent the dose administered and bioavailability, respectively. The steady-state volume of distribution (Vdss) was calculated by multiplying CLtot by MRT. Peak time (Tmax) and Peak plasma concentration (Cmax) were from actual values. Systemic availability (F) was calculated by dividing the AUC after i.d. administration (AUC(i.d.) obtained from each animal by the mean AUC after i.v. injection (AUC(i.v.)) corrected for the dose of NFV.

**In Vitro Protein Binding Study** A preliminary experiment showed that the degree of adsorption of NFV on to the membranes used in ultrafiltration and equilibrium dialysis devices was very high. In addition, the ultracentrifugation method may be impractical due to lipoprotein contamination of the plasma water supernatant.24 Therefore, the erythrocyte vs. buffer or plasma partitioning method25 was used to determine the unbound NFV concentration in rat plasma. Briefly, HL and control rats were anesthetized, and blood was collected into centrifuge tubes by cardiac puncture. The blood was split equally into two tubes. Plasma was separated from blood cells by centrifugation of whole blood at 2500 g for 10 min at 25 ºC. After removal of the plasma and Buffy-coat layers, blood cells were washed in an equal volume of phosphate-buffered saline containing 25 mM glucose (PBS-Glu, pH 7.4), followed by centrifugation at 2500 g for 8 min at 25 ºC. This washing procedure was repeated twice. After the third wash, the volume of total erythrocytes was noted in each tube, and either PBS-Glu (pH 7.4) or undiluted plasma was added to make a hematocrit (HCT) of 0.3. NFV methanic solution was added to buffer- and plasma-containing erythrocyte mixtures to make final concentrations of 1, 5, or 10 µg/ml for both HL and control rat groups (n=5, respectively). This corresponded to the approximate value observed in plasma in this PK study. The total methanol concentration was 0.4% (it has been reported that a methanol concentration of up to 4% in plasma is permissible for binding studies26). Buffer- and plasma-containing erythrocyte mixtures were incubated for 1 h. After centrifugation at 9000 g for 10 min, the concentration of NFV in the supernatant was measured.
The calculation of \( f_u \) was described as follows. The erythrocyte concentration of NFV (\( C_E \)) was determined by the following equation:

\[
C_E = \frac{C_B - C_P (1 - HCT)}{HCT}
\]

where \( C_B \) is the concentration of NFV in the blood cell–plasma suspension and \( C_P \) is the concentration of NFV in the plasma.

To estimate the erythrocyte concentration (\( C_E \)) of NFV in erythrocyte-buffer samples, the concentrations of NFV in blood cell-buffer suspensions were substituted for \( C_B \), and the concentration of NFV in buffer was substituted for \( C_P \). The \( f_u \) values were determined by

\[
f_u(\%) = 100 \times \frac{P_b}{P_u}
\]

where partition coefficients for erythrocyte-plasma or buffer are represented by the terms \( P_p \) (\( C_E/C_{\text{plasma}} \)) and \( P_u \) (\( C_E/C_{\text{buffer}} \)), respectively.

**In Vitro Distribution Study in Rat Blood** NFV methanolic solution was added to drug-free blood samples obtained from both HL model rats and control rats at a total NFV concentration of 1.0, 5.0 and 10.0 \( \mu \)g/ml, and total methanol concentration of 0.4%, as described above. After incubation at 37 °C for 1 h, NFV concentrations in the mixtures were measured as the whole blood concentration. The remaining mixtures were centrifuged at 2500 \( g \), 25 °C for 10 min to obtain the plasma fraction, and NFV concentrations were measured as the plasma concentration. The whole blood–plasma concentration ratio (B–P ratio) was calculated by dividing the plasma concentration by the whole blood concentration.

**Plasma Lipoprotein Separation** Lipoproteins were isolated from NFV-spiked HL and control rat plasma (final concentration of 10 \( \mu \)g/ml) on the basis of their hydrated density with a single-step procedure using ultracentrifugation on a potassium bromide (KBr) gradient, as previously described by Terpstra et al.\(^\text{21,22}\). The main lipoprotein fractions were isolated using density ranges of <0.95 g/ml, 0.95—1.006 g/ml, 1.006—1.063 g/ml, 1.063—1.210 g/ml and >1.210 g/ml for chylomicron (CM), VLDL, LDL, HDL and lipoprotein-deficient fractions (LPDF), respectively. The fractions collected were frozen at –80 °C until analysis of NFV.

**Assay Procedure** NFV in plasma and the other samples in this study was assayed by the LC-MS method, developed by Gao et al.\(^\text{21}\). Briefly, 10 \( \mu \)l of atazanavir (ATV, internal standard, 100 \( \mu \)g/ml in methanol) and 100 \( \mu \)l of 2% \( \text{ZnSO}_4 \) in 50% methanol solution was added to aliquots of 100 \( \mu \)l plasma sample in a 1.5 ml microcentrifuge tube and vortexed vigorously for 15 s. Diethyl ether (1 ml) was then added to the tube, vortexed for 30 s, and centrifuged at 12000 \( g \) for 5 min. The aqueous phase in test tubes was frozen in a cold bath at –10 °C and the ether phase was transferred to HPLC sample vials. The organic phase was evaporated to dryness at 70 °C in a water bath with air flow. The residues were reconstituted with 100 \( \mu \)l of mobile phase and then 30 \( \mu \)l was injected into the LC-MS system (Shimadzu, Kyoto, Japan), which consisted of the following components: a SIL-10A system controller, LC-10ADvp pump, SPD-10A UV detector, SIL-10ADvp automatic injector, CTO-10A column oven and an LC-MS-QP8000a mass spectrometer equipped with a CLASS-8000 work station. The analytical column for the separation of NFV was a Quicksorb ODS (2.1 mm ID×150 mm, 5 \( \mu \)m size, Chemco, Osaka, Japan), and column temperature was maintained at 60 °C for all separations. Elution was carried out isocratically at a flow rate of 0.2 ml/min with 90% acetonitrile containing 1% acetic acid. Mass spectrometry was performed utilizing atmospheric pressure chemical ionization (APCI) in the negative mode. The voltages of the APCI probe and the curved desolvation line (CDL) were set at 5 kV and –30 V, respectively, and the flow rate of the nebulizing gas (\( N_2 \)) was set at 2.5 l/min. The temperatures of the APCI probe and CDL were set at 400 °C and 250 °C, respectively. The voltage of deflectors was set at –80 V. The peaks of ATV and NFV were detected as deprotonated ions at 705 and 568 m/z, respectively. NFV was quantified by calculating the peak area ratio of NFV against ATV. The detection limit of this assay method was 0.01 \( \mu \)g/ml from 100 \( \mu \)l of sample. The intra-day reproducibility of this assay method at concentrations of 0.01, 0.1 and 1.0 \( \mu \)g/ml ranged from 3.1 to 6.1% coefficient of variation (C.V.).

**Western Blot Analysis** Crude intestinal membrane and liver microsome fraction were prepared according to the manufacturer’s instructions with some modifications. Briefly, the luminal contents of the whole small intestine were isolated and flushed with phosphate-buffered saline. The intestine was opened and laid on a chilled glass plate. Intestinal tissue was collected by scraping with a slide glass. Collected intestinal and liver tissues were homogenized by adding T-PER (Pierce, Rockford, IL, U.S.A.) containing 1 mM phenylmethylsulfonylfluoride (PMSF) 20 ml/g tissue. Homogenates was centrifuged for 15 min at 3000 \( g \), and the resulting supernatant was centrifuged at 27000 \( g \) for 30 min. The pellets were resuspended in buffer containing 300 mm mannitol and 40 \( \mu \)g/ml PMSF (pH 7.5). Protein concentrations in each sample were determined by a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, U.S.A.) with bovine serum albumin as a standard. Samples were normalized to equal protein concentrations, loaded (20 \( \mu \)g/lane) onto 7.5% SDS-gel, electrophoresed at 200 V for 1 h, and then transferred onto polyvinylidene difluoride membranes at 100 V for 1 h. Blots were blocked for nonspecific binding by overnight incubation (4 °C) on a rotator in Tris–buffered saline buffer containing 0.1% Tween 20 and 5% dry fat milk. The polyvinylidene difluoride membranes were then washed three times with Tris–buffered saline containing 0.1% Tween 20, and then incubated with the primary antibody, C219 (anti P-gp monoclonal antibody; Calbiochemi, CA, U.S.A.) or anti-rat CYP3A2 (Daichi Pure Chemicals Co., Ltd., Japan) for 1 h at 37 °C. After washing three times with buffer, membranes were incubated for 45 min with secondary antibody (anti-mouse immunoglobulin G (IgG), Bio-Rad Lab., U.S.A.) or anti rabbit horseradish peroxidase (HRP)-linked IgG (Daichii Pure Chemicals Co., Ltd., Japan), and then washed three times and visualized by chemiluminescence with an Immune-Star HRP chemiluminescent kit (Bio-Rad Lab., U.S.A.). The stained bands were detected and quantified using a Dolphin-chemi (Waltect Co., GA, U.S.A.) to quan-
The plasma unbound fraction of NFV in HL and control rats is summarized in Table 1. The plasma unbound fraction in HL rats was significantly lower than that in control rats in each sample (19.8—26.5% of plasma unbound fraction in control rats). On the other hand, there were no differences between the plasma unbound fraction at concentrations of 1 μg/ml and 5 μg/ml in both control and HL groups; however, at a concentration of 10 μg/ml, the plasma unbound fraction slightly increased. Table 1 also shows the B–P ratio of NFV in HL and control rats. There were no differences in the B–P ratio between HL and control rats in each sample (1, 5, 10 μg/ml).

Table 2 shows biochemical parameters for plasma lipoproteins in HL and control rats. Both HDL-ch and LDL-ch levels in HL rats were about 5 times higher than in control rats. HDL% in HL rats was remarkably lower than in control rats. In contrast, LDL% and VLDL% were higher than in control rats.

NFV distribution in plasma lipoprotein fractions in HL and control rats is shown in Fig. 1. NFV recovery in CM, VLDL and LDL fractions was significantly higher than in control rats; however, NFV recovery in HDL and LPDF fractions was significantly lower than in control rats.

Mean plasma NFV concentration–time profiles after i.v. bolus injection of NFV test solutions at a dose of 5 mg/kg to HL and control rats are shown in Fig. 2. PK parameters of NFV for each group are shown in Table 3. Statistically significant differences were detected in AUC<sub>1→x</sub>, t<sub>1/2</sub>, CL<sub>tot</sub>, V<sub>ss</sub> and MRT. In particular, AUC<sub>1→∞</sub> in HL rats was approximately 5 times greater than in control rats.

Figure 3 shows NFV concentrations in the liver 0.5, 1, 2 h after i.v. bolus infusion of NFV test solutions at 5 mg/kg. In

Table 1: Plasma Unbound Fraction and Whole Blood–Plasma Concentration Ratio of NFV in HL and Control Rats

<table>
<thead>
<tr>
<th>Blood NFV conc. (μg/ml)</th>
<th>Plasma unbound fraction (%)</th>
<th>Whole blood–plasma concentration ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>HL rats</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.92±0.10</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.11±0.02</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.47±0.02</td>
</tr>
</tbody>
</table>

Blood used in both studies were collected at 36 h after intraperitoneal administration of P407 (1 g/kg, HL rats) or the same volume of vehicle without P407 (control). Values are the mean ± S.D. of 6 experiments. **p<0.01 compared with control.

Table 2: Biochemical Parameters for Serum Lipid in HL and Control Rats

<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>Control</th>
<th>HL rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL-ch (mg/dl)</td>
<td>53.0±7.7</td>
<td>253.5±10.4**</td>
</tr>
<tr>
<td>LDL-ch (mg/dl)</td>
<td>9.8±1.7</td>
<td>51.5±4.2**</td>
</tr>
<tr>
<td>HDL%</td>
<td>44.3±2.1</td>
<td>4.0±0.8**</td>
</tr>
<tr>
<td>LDL%</td>
<td>8.8±1.0</td>
<td>39.3±10.8**</td>
</tr>
<tr>
<td>VLDL%</td>
<td>34.5±3.7</td>
<td>56.8±11.1**</td>
</tr>
</tbody>
</table>

Determinations of these parameters were performed at 36 h after intraperitoneal administration of P407 (1 g/kg, HL rats) or the same volume of vehicle without P407 (control). Values are the mean±S.D. of 4 rats. **p<0.01 compared with control.

Table 3: Pharmacokinetic Parameters of NFV after i.v. Bolus Injection at a Dose of 5 mg/kg

<table>
<thead>
<tr>
<th>PK parameters</th>
<th>Control</th>
<th>HL rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC&lt;sub&gt;1→∞&lt;/sub&gt; (μg·h/ml)</td>
<td>1.62±0.21</td>
<td>6.12±0.48**</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>1.80±0.14</td>
<td>1.33±0.30*</td>
</tr>
<tr>
<td>CL&lt;sub&gt;tot&lt;/sub&gt; (l/h/kg)</td>
<td>3.13±0.43</td>
<td>0.82±0.07**</td>
</tr>
<tr>
<td>V&lt;sub&gt;ss&lt;/sub&gt; (l/kg)</td>
<td>6.25±0.55</td>
<td>0.60±0.13**</td>
</tr>
<tr>
<td>MRT&lt;sub&gt;1→∞&lt;/sub&gt; (h)</td>
<td>2.13±0.27</td>
<td>0.73±0.14**</td>
</tr>
</tbody>
</table>

Experiments were performed at 36 h after intraperitoneal administration of P407 (1 g/kg, HL rats) or the same volume of vehicle without P407 (control). **p<0.01 compared with control.
HL rats, NFV concentration in the liver was significantly higher than in control rats 0.5 and 1 h after drug administration; however, 2 h after drug administrations, NFV concentrations in the liver were almost the same.

Mean plasma NFV concentration–time profiles after i.d. administration of NFV test solutions at a dose of 30 mg/kg to HL and control rats are shown in Fig. 4. PK parameters of NFV after administration of NFV test solutions at a dose of 30 mg/kg to HL (1 g/kg, HL rats) or the same volume of vehicle without P407 (control). Values are the means ± S.D. of 6 experiments, *p<0.05, **p<0.01 compared with control.

The protein expression level of hepatic CYP3A in HL rats was 1.01 ± 0.03 fold compared with the control. In addition, the protein expression level of intestinal CYP3A and P-gp in HL rats were 1.04 ± 0.23 and 1.05 ± 0.24 fold, respectively, compared with the control. There were no differences in hepatic CYP3A expression and intestinal CYP3A and P-gp expression between HL and control rats.

Figure 5 shows the cumulative amount of NFV versus time profile in lymph after i.v. and i.d. administration. There was no difference in the cumulative amount of NFV until 6 h after i.v. administration between HL (1.57 ± 0.33 μg) and control rats (1.25 ± 0.35 μg). However, in HL rats, the cumulative amount of NFV until 6 h after i.d. administration was significantly increased compared with the control (HL rats; 11.00 ± 0.96 μg, control; 5.07 ± 0.54 μg). In addition, lymph concentration of NFV at 1.5—2.5 h fraction after i.d. administration was high in both HL and control rats. These results corresponded to the T_{max} in plasma after i.d. administration in HL rats. On the other hand, mean lymph flow in each interval was 0.12 ± 0.05 ml/h for control rats and 0.15 ± 0.05 ml/h for HL rats. No significant difference was observed between HL and control rats. Moreover, as an index of directly absorbed NFV from the gastrointestinal tract to the lymphatic system, c_{a_{i,x}} over c_{a_{i,d}} (c_{a_{i,d}}/c_{a_{i,x}}) was calculated by dividing the c_{a_{i,d}} obtained from each animal by the mean c_{a_{i,x}} value corrected for the dose of NFV (i.v.; 5 mg/kg, i.d.; 30 mg/kg). c_{a_{i,d}}/c_{a_{i,x}} in HL rats was about 2 times higher than in control rats.

DISCUSSION

P407, a nonionic surface active agent, which is nontoxic to cellular membranes, has been shown to cause a significant increase in circulating lipoproteins by decreasing lipoprotein hepatic lipase and increasing lecithin cholesterol acyl transferase and cholesterol ester transfer protein activities. In addition, P407-induced hyperlipidemia rat model has been used for several PK studies of drugs having high binding charac-
teristics to serum lipoproteins such as CyA,28,29 amiodaron30 or nifedipine31 due to its convenience, reproducibility and lack of undesirable underlying pathological conditions.32 Moreover, in the preliminary experiment, we checked the hepatic and renal function in P407-induced HL rat. There were no differences in plasma creatinine, and AST and ALT levels between HL and control rats; therefore, the P407-induced HL rat was suitable for the purpose of this study.

Although the plasma concentration of lipoprotein is relatively low, lipoprotein plays an important role as a drug-binding protein affecting the disposition of some drugs. For example, probucol, an anti-hyperlipidemia agent, can account for as much as 95% of total drug binding in plasma.33 Neutral and basic lipophilic drugs most commonly bind to lipoproteins; however, little attention has been given to the lipoprotein as a binding protein of NFV in plasma. Liposolubilization is probably the major mechanism for drug association with lipoproteins. It has been reported that the octanol-water partition coefficient (log P) of NFV was approximately 4.1. Gershkovich et al. reported that drug distribution to lipid-rich lipoproteins correlated with log P of the drug.34 In addition, it has been reported that the free fraction of NFV was not affected by drugs that bind extensively to AAG or ALB.35 Therefore, although the well-known binding proteins of NFV are AAG and ALB, it is anticipated that NFV also binds to lipoproteins for its high lipophilicity as described in the introduction.

On the other hand, the B-P ratio in each sample (1, 5, 10 µg/ml) in both HL and control rats was the same (about 1.0), suggesting no effect of HL on the B-P ratio in rats; therefore, in our protein-binding study, the total concentration in blood of 1, 5 and 10 µg/ml was almost equal to plasma concentration. In both HL and control rats, the non-linearity of protein binding may be slightly observed at 10 µg/ml of blood concentration; however, in our PK studies, plasma concentrations were almost always under 5 µg/ml; therefore, protein-binding saturation did not need to be taken into account.

In this study, NFV was highly bound to plasma protein in rats, and the unbound fraction of NFV in HL rats was significantly lower than the control. In HL rats, both HDL-ch and LDL-ch levels were 5 times higher than that of control rats; therefore, it was suggested that the increased lipoprotein level may be the cause of the decreasing unbound fraction.

In this study, HDL% in HL rats dramatically decreased compared to control rats in contrast to increasing LDL% and VLDL%. Moreover, NFV recovery in triglyceride-rich lipoprotein, such as VLDL and CM in HL rats, was greater than that of control rats; however, NFV recovery in HDL and LPDP in HL rats was smaller than that of control rats. In particular, NFV in the HDL fraction in HL rats was about one fifth of that in control rats. On the other hand, both HDL-ch and LDL-ch levels in HL rats were about 5 times higher than in control rats; therefore, it was suggested that NFV in the HDL fraction was approximately the same amount in both HL and control rats. These findings also suggested that triglyceride-rich lipoprotein was an important factor in NFV protein-binding characteristics in plasma. Ramaswamy et al. investigated the distribution of amphotericin B, an antifungal agent, in HL rabbit plasma, and obtained similar results.36 In addition, Bassissi et al. reported that moxidectin, a potent antiparasitic drug, well bound to HDL in normolipidemic human and rabbit plasma; however, in hyperlipidemic plasma, moxidectin shifted to the VLDL and LDL fraction with a subsequent alternation in PK properties.37 These findings also suggested that triglyceride-rich lipoproteins may be an important mediator of the disposition of a lipophilic drug such as NFV.

As shown in Table 3, Vdss after i.v. administration in HL rats was about 10 times higher than in control rats. In addition, plasma concentration of NFV at 5 min after i.v. administration in HL rats was also about 10 times higher than in control rats. These findings clearly show that NFV distribution from the blood to peripheral tissues was limited, caused by the large increase in the lipoprotein level resulting in a decrease of the unbound fraction in plasma. The marked decrease of Vdss was the primary reason for the significantly higher AUC of NFV in HL rats.

On the other hand, t1/2 in HL rats was shorter than in control rats, although the plasma unbound fraction of NFV in HL rats was only about 20% of control rats. However, it is difficult to consider the induction of enzyme by HL. In the present study, we performed Western blot analysis of hepatic CYP3A in P407-induced HL rats; however, induction by HL was not recognized. In addition, there was no difference in hepatic function (AST and ALT) between HL and control rats. Moreover, in the preliminary experiment, bile flow in HL and control rats was stable throughout the experiments. On the other hand, it has been reported that CyA concentrations in the kidney and liver after i.v. administration increased in HL rats; however, CyA concentrations in the spleen and heart decreased compared to control rats, so the expected decrease in hepatic clearance caused by the decrease of the protein-free fraction of the drug was compensated for by increased hepatic uptake.29 In the present study, NFV liver concentration in HL rats was significantly higher than in control rats; therefore, the shorter t1/2 in HL rats can be explained by the increased hepatic uptake of NFV.

In the present i.d. study, regarding Vdss/F, CLtot/F and t1/2, similar results to the i.v. study were obtained. In addition, although AUCτss of NFV in HL rats was 3.8 times higher than in control rats, AUCτss of NFV in HL rats was 4.1 times higher than in control rats; therefore, F of NFV in HL rats was significantly increased. In the present study, we also performed Western blot analysis of intestinal CYP3A and P-glycoprotein in HL rats; however, induction by HL was not recognized in both proteins. In addition, PK experiments were performed after overnight fasting; therefore, although more investigations are needed, it is difficult to consider that the ordinary absorption route of NFV was affected by HL. Therefore, the absorption route from the gastrointestinal tract through the lymphatic system, through which drugs can escape the hepatic first-pass effect, was investigated. In addition, this investigation may give us the interesting information for NFV medication, because the lymphatic system is an target of NFV. c.a.i.d./c.a.i.v. as an index of directly absorbed NFV from the gastrointestinal tract to the lymphatic system in HL rats was 2 times higher than in control rats. Gastrointestinal absorption of the drug through the lymphatic system did not undergo the first-pass effect; therefore, it was considered that the increase of absorbed NFV from the gastrointestinal tract to the lymphatic system was one reason for the
increase of $F$ in HL rats. In addition, HIV in the lymphatic system is an effective target of NFV; therefore, this result has led to increasing interest in NFV PK.

The results of this study also suggested that the increase of plasma NFV concentration in patients with high triglyceride-rich lipoprotein level does not induce a greater pharmacological effect because of lower unbound fraction of NFV. Therefore, the monitoring of triglyceride-rich lipoprotein, such as LDL, VLDL and CM are need for therapeutic drug monitoring of NFV. The results of this study provide useful information for the investigation of dose adjustment by therapeutic drug monitoring of NFV in patients with hyperlipidemia.

CONCLUSION

The plasma unbound fraction of NFV in HL rats was significantly lower than in control rats. In HL rats, the plasma lipoprotein level was significantly higher than in control rats, and triglyceride-rich lipoprotein, such as VLDL and CM, was remarkably increased. Moreover, NFV recovery in VLDL and CM in HL rat plasma was greater than in control rats; however, NFV recovery in HDL and LPDF in HL rat plasma was smaller than in control rats. Therefore, it was suggested that an increased lipoprotein level may be the cause of the decreasing unbound fraction, and that triglyceride-rich lipoproteins may be an important mediator of the disposition of a lipophilic drug such as NFV. AUC in HL rats after both i.v. and i.d. administration was remarkably greater than in control rats. In addition, $F$ of NFV after i.d. administration in HL rats was larger than that in control rats. The marked decrease of $V_d$ was the primary reason for the significantly higher $AUC$ of NFV in HL rats. The shorter $t_{1/2}$ in spite of the decrease of the plasma unbound fraction in HL rats can be explained by the increased hepatic uptake of NFV in HL rats. On the other hand, it was considered that the increase of absorbed NFV from the gastrointestinal tract to the lymphatic system, through which drugs did not undergo the first-pass effect, was one reason for the increase of $F$ in HL rats. The results of this study provide useful information for the investigation of dose adjustment by therapeutic drug monitoring of NFV in patients with hyperlipidemia.

REFERENCES