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RESEARCH ARTICLE

Pharmacokinetics of sildenafil and its metabolite, *N*-desmethylsildenafil, in rats with liver cirrhosis and diabetes mellitus, alone and in combination

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Abstract

1. Pharmacokinetics of sildenafil and its metabolite, *N*-desmethylsildenafil, in humans and rats with liver cirrhosis (LC) and diabetes mellitus (DM), alone and in combination (LCD) did not seem to be reported.
2. Sildenafil was administered intravenously (10 mg/kg) and orally (20 mg/kg) to control, LC, DM, and LCD rats. Expression of intestinal CYP isozymes in those rats was also measured.
3. In LC, DM, and LCD rats, the areas under the curve (AUCs) of intravenous sildenafil were significantly greater (by 195%, 54.2%, and 127%, respectively) than controls. In LC and LCD rats, AUCs of oral sildenafil were significantly greater (3010% and 2030%, respectively) than controls.
4. In LC, DM, and LCD rats, significantly greater AUCs of intravenous sildenafil were due to the slower hepatic extraction of sildenafil (because of decrease in the protein expression of hepatic CYP2C11 and 3A subfamily in LC and LCD rats, and CYP2C11 in DM rats). In LC and LCD rats, greater magnitude of increase in AUCs of oral sildenafil than those after the intravenous administration could be mainly due to the decrease in the intestinal extraction of sildenafil (because of decrease in the protein expression of intestinal CYP2C11 in LC and LCD rats).

Keywords: Sildenafil and N-desmethylsildenafil, pharmacokinetics, rats with liver cirrhosis and/or diabetes mellitus, hepatic and intestinal CYP2C11 and 3A subfamily

Introduction

Sildenafil (a weak basic compound with a pK_a of 6.5; Figure 1) is an inhibitor of the cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase type 5 (PDE5) found in the human corpus cavernosum. Sildenafil citrate (Viagra®) is an effective oral agent for the treatment of male erectile dysfunction. The following pharmacokinetics of sildenafil has been reported in male Sprague-Dawley rats (Shin et al. 2006). The total areas under the plasma concentration–time curve (AUCs) of intravenous sildenafil (10, 30, and 50 mg/kg) from time 0 to infinity were dose-proportional. However, its oral AUCs increased more than proportional to the dose increases (10, 30, and 100 mg/kg). After the intravenous, oral, intraportal, intragastric, or intraduodenal administration of sildenafil (30 mg/kg), the unabsorbed fraction up to 24 h

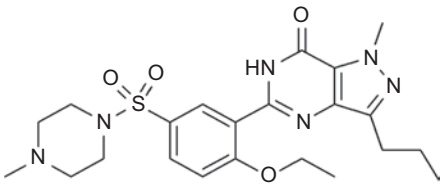

Figure 1. Chemical structure of sildenafil.

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was 0.00626 of the oral dose, its extent of absolute oral bioavailability (F) was 0.146, its hepatic first-pass extraction ratio after absorption into the portal vein was 0.49, and its intestinal first-pass extraction ration was 0.71 of the oral dose. The hepatic first-pass extraction ratio of 0.49 after absorption into the portal vein was equivalent to 0.137 of the oral dose considering that orally administered sildenafil was first undertaken (0.71 of the intestinal first-pass extraction ratio) before reaching the liver.

Sildenafil (UK-92,480) is metabolized to *N*-desmethylsildenafil (UK-103,320; M10) via piperazine *N*-demethylation (11% in rats), UK-150,564 (M9) via piperazine *N,N*-deethylation (16% in rats), UK-95,340 via pyrazole *N*-demethylation (20% in rats), UK-331,849 (M8A) via ring open of piperazine (12% in rats), and others (Walker et al. 1999). It has been reported that sildenafil was metabolized via hepatic cytochrome P450 (CYP) 2C11 and 3A1/2, and *N*-desmethylsildenafil was formed via hepatic CYP2C11 in male Sprague-Dawley rats (Bae et al. 2009).

In rats with liver cirrhosis (LC) induced by *N*-dimethylnitrosamine (LC rats), the protein expression of hepatic CYP2C11 and 3A subfamily significantly decreased (by 83.1% and 75.8%, respectively) compared with controls (Ahn et al. 2009). In rats with diabetes mellitus (DM) induced by streptozotocin (DM rats), the protein expression of hepatic CYP2C11 and 3A subfamily significantly decreased (by 28.8%) and increased (by 23.0%), respectively, compared with controls (Ahn et al. 2009). Similar results on CYP2C11 (Thummel and Schenkman 1990; Kim et al. 2005), 3A subfamily (Barnett et al. 1990), 3A1 (Kim et al. 2005), and 3A2 (Thummel and Schenkman 1990) have also been reported. In rats with LC with DM induced by *N*-dimethylnitrosamine and streptozotocin (LCD rats), the protein expression of hepatic CYP2C11 and 3A subfamily significantly decreased (by 32.8% and 52.9%, respectively) compared with controls (Ahn et al. 2009). However, in DM, LC, and LCD rats, the changes in the protein expression of intestinal CYP2C11 and 3A subfamily using western blot analysis compared with controls did not seem to be reported.

In patients with type I DM, the increase in CYP2E1 in peripheral blood mononuclear cells (Wang et al. 2003) and CYP1A2 activity (Matzke et al. 2000) has been reported. In insulin-dependent adults with untreated type I DM, antipyrine metabolism (markers of hepatic CYP1A2, 2B6, and 2C and 3A subfamilies) increased (Sotaniemi et al. 2002). In patients with LC, the mRNA levels of hepatic CYP1A2 and CYP1A immunoreactive proteins, mRNA levels of CYP2C9 and CYP2C proteins, and mRNA and protein level of CYP2E1 were reduced compared with control subjects (George et al. 1995). In patients with LC, mRNA level of CYP3A4 also decreased (by 68.8%) in the blood compared with control subjects (Horiike et al. 2005). However, in patients with LC and DM, the hepatic CYP isozyme changes did not seem to be reported.

The association between liver disease and DM is well known (Vidal et al. 1994; Kwon 2003; Moscatiello et al. 2007); the overall prevalence of DM in patients with LC is significantly higher than that expected. The erectile dysfunction has been reported in patients with LC (Toda et al. 2005) and DM (Musicki and Burnett 2007). The pharmacokinetic studies of chlorzoxazone (Ahn et al. 2008) and oltipraz (Ahn et al. 2009) in rats with LC and DM, alone and in combination (LCD) have been reported. However, no pharmacokinetic studies on sildenafil and *N*-desmethylsildenafil in patients and rats have yet been reported except in patients with LC (Muirhead et al. 2002). Thus, in the present study, sildenafil was selected and the pharmacokinetics of sildenafil and *N*-desmethylsildenafil were evaluated after the intravenous and oral administration of sildenafil to LC, DM, and LCD rats. The protein expression of intestinal CYP isozymes using western blot analysis was also examined.

Materials and methods

Chemicals

Sildenafil citrate, *N*-desmethylsildenafil (purity; 98%), and DA-8159 (purity; 99.5%) [Udenafil; Zydena®; internal standard for the liquid chromatography–tandem mass spectrometry (LC/MS/MS) analysis of sildenafil and *N*-desmethylsildenafil] were products from APIN Chemical (Oxfordshire, UK), Toronto Research Chemicals (North York, ON, Canada), and Dong-A Pharmaceutical Company, Ltd. (Yongin, South Korea), respectively. The reduced form of β-nicotinamide adenine dinucleotide phosphate (NADPH; as a tetrasodium salt), ethylenediaminetetraacetic acid (EDTA; as a disodium salt), streptozotocin, tris[hydroxymethyl]aminomethane (Tris®)-buffer, β-actin, primary monoclonal antibody for β-actin, and Kodak X-OMAT film were all purchased from Sigma-Aldrich Corporation (St. Louis, MO). Monoclonal anti-rat CYP1A1/2, 2B1/2, 2C11, CYP2D subfamily, 2E1, and 3A subfamily antibodies were products from Oxford Biomedical Research (Metamora, MI) and Detroit R&D (Detroit, MI). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies, and enhanced chemiluminescence reagents were purchased from Bio-Rad Laboratories (Hercules, CA) and Amersham Life Science Inc. (Piscataway, NJ), respectively. *N*-Dimethylnitrosamine was a product from Tokyo Kasei Kogyo Company (Tokyo, Japan). Other chemicals were of reagent or high-performance liquid chromatographic (HPLC) grade.

Animals

The protocols for the animal study were approved by the Animal Care and Use Committee of College of Pharmacy, Seoul National University, Seoul, South Korea. Male Sprague-Dawley rats (4–6 weeks old, weighing 160–210 g) were purchased from Charles River Company Korea (Orient, Seoul, South Korea). The procedures used for

housing and handling the rats were similar to the reported methods (Bae et al. 2004, 2009; Ahn et al. 2008, 2009).

Induction of LC

Freshly prepared *N*-dimethylnitrosamine (diluted in 0.9% NaCl-injectable solution) was injected intraperitoneally at a dose of 0.01 mg (1 mL)/kg on three consecutive days per week for 4 weeks (Ohara and Kusano 2002; Bae et al. 2004; Ahn et al. 2008, 2009). On Day 29, one dose (1 mL/kg) of citrate buffer (pH 4.5) was injected via the tail vein. On Day 36, the rats were treated with sildenafil citrate. Laboratory rats with *N*-dimethylnitrosamine-induced LC have clinical features similar to those of human with LC, such as increasing mortality, destruction of hepatic parenchymal cell, formation of connective tissue, and regeneration of nodule (Kang et al. 2002). Liver cirrhosis in LC rats was evident by liver histological analysis (Ohara and Kusano 2002). It has been reported that *N*-dimethylnitrosamine-induced LC in rats was reproducible (Jézéquel et al. 1987; Kang et al. 2002).

Induction of DM

A 0.9% NaCl-injectable solution was injected intraperitoneally (1 mL/kg) on three consecutive days per week for 4 weeks. On Day 29, one dose [45 mg (1 mL)/kg] of freshly prepared streptozotocin [dissolved in citrate buffer (pH 4.5)] was administered via the tail vein (Kim et al. 2005; Ahn et al. 2008, 2009). On Day 36, the rats were treated with sildenafil citrate. Diabetes mellitus in DM rats was evident by higher blood glucose level, greater 24-h urine output, and decrease in body weight gain (Ahn et al. 2008).

Induction of LCD

LC was induced by intraperitoneal injection of *N*-dimethylnitrosamine as described above. Then, on Day 29, DM was induced by injection of streptozotocin via the tail vein as described earlier. On Day 36, the rats were treated with sildenafil citrate. The presence of LC and DM in LCD rats was evident based on the blood chemistry data as well as liver histological analysis (Ahn et al. 2008).

Control rats

Rats were injected intraperitoneally with 0.9% NaCl-injectable solution (1 mL/kg) on three consecutive days per week for 4 weeks. On Day 29, one dose (1 mL/kg) of the citrate buffer (pH 4.5) was administered via the tail vein. On Day 36, the rats were treated with sildenafil citrate.

During the pretreatment, food and water were available *ad libitum* to all rats. Immediately before the experiment, blood glucose levels in all rats were measured using the Medisense Optium kit (Abbott Laboratories, Bedford, MA), and rats with blood glucose levels higher than 250 mg/dL were chosen as being diabetic (DM and LCD rats).

Preparation of hepatic microsomes

The procedures used for preparation of hepatic microsomes form control, LC, DM, and LCD rats ($n = 4$–6, each) were similar to the reported methods (Ahn et al. 2008, 2009; Choi et al. 2008). Then, the hepatic microsomes were stored at −70°C (Revco ULT 1490 D-N-S; Western Mednics, Ashville, NC) until use. Protein contents in hepatic microsomes were measured using a reported method (Bradford 1976).

Measurement of V_{max}, K_m, and CL_{int} for the disappearance of sildenafil in hepatic microsomes

The procedures used were similar to the reported methods (Ahn et al. 2008, 2009). The V_{max} (maximum velocity) and K_m (the apparent Michaelis–Menten constant; the concentration at which the rate is one-half of the V_{max}) for the disappearance of sildenafil in control, LC, DM, and LCD rats ($n = 5$–6, each) were determined after incubating the above microsomes (equivalent to 0.2 mg protein), 5 μL of methanol containing final sildenafil base (the same solution used in the intravenous study) concentrations of 1, 2, 4, 10, 20, 40, 100, and 200 μM, and 50 μL of 0.1 M phosphate buffer (pH 7.4) containing 1 mM NADPH. The volume was adjusted to 0.5 mL by adding 0.1 M phosphate buffer (pH 7.4), and the components were incubated at 37°C using a thermomixer (Eppendorf, Hamburg, Germany) at a rate of 50 oscillations/min (opm). Incubation times were 1, 5, 15, 30, and 60 min, respectively, in the preliminary study. All of the above microsomal incubation conditions were within the linear range of the reaction rate. The reaction was terminated by addition of 1 mL of acetonitrile after 5-min incubation. The kinetic constants (K_m and V_{max}) for the disappearance of sildenafil were calculated using a nonlinear regression method (Duggleby 1995). The intrinsic clearance (CL_{int}) for the disappearance of sildenafil was calculated by dividing the V_{max} by K_m.

Immunoblot analysis of intestinal CYP1A1/2, 2B1/2, 2C11, 2D subfamily, 2E1, and 3A subfamily

The procedures used for preparation of intestinal microsomes and immunoblot analysis of intestinal CYP isozymes were similar to a reported method (Lee and Lee 2008; Ahn et al. 2009). Intestinal microsomes were resolved by sodium dodecyl sulfated-polyacrylamide gel electrophoresis (SDS-PAGE) on a 7.5% gel (10 μg protein per lane; $n = 3$, each). Proteins were transferred to a nitrocellulose membrane (Pall Corporation, Ann Arbor, MI) that was then blocked for 2 h in the Tris®-buffered saline containing 0.1% (v/v) Tween 20 (TBS-T). For immunodetection, blots were incubated overnight at 4°C with anti-rat CYP isozyme antibodies (diluted 1:10,000 in TBS-T containing 5% bovine serum albumin), followed by incubation for 2 h at room temperature with a secondary antibody conjugated to horseradish peroxidase (diluted 1:10,000 in TBS-T containing 5% milk powder). The protein expression of the CYP isozymes were detected by enhanced chemiluminescence on Kodak

X-OMAT film and quantitated by densitometry with a microcomputer imaging device (model M1; Imaging Research, St. Catharines, Ontario, Canada). The β-actin was used as a loading control.

Intravenous and oral studies

The procedures used for pretreatment of rats including the cannulation (early in the morning on Day 36) of the jugular vein (for drug administration in the intravenous study) and the carotid artery (for blood sampling) were similar to the reported methods (Kim et al. 1993; Ahn et al. 2008, 2009).

Sildenafil base (sildenafil citrate was dissolved in distilled water in a minimum amount of 10 N NaOH) at a dose of 10 mg (2 mL)/kg was manually infused over 1 min via the jugular vein of rats in each group (n = 9, 10, 9, and 8 for control, LC, DM, and LCD rats, respectively). Blood samples (~0.12 mL, each) were collected via the carotid artery at 0 (control), 1 (end of infusion), 5, 15, 30, 60, 90, 120, 180, and 240 min after the start of the intravenous infusion of sildenafil citrate. A heparinized 0.9% NaCl-injectable solution (20 units/mL; 0.3 mL) was used to flush the cannula immediately after each blood sampling to prevent blood clotting. Each blood sample was immediately centrifuged and 50 μL of each plasma sample was stored at –70°C until used for the LC/MS/ MS analysis of sildenafil and *N*-desmethylsildenafil. The procedures used for preparation and handling of 24-h urine sample ($Ae_{0-24\ h}$) and gastrointestinal (GI) tract (including its contents and faeces) sample at 24 h ($GI_{24\ h}$) were similar to the reported methods (Kim et al. 1993; Ahn et al. 2008, 2009).

Sildenafil base (the same solution used in the intravenous study) at a dose of 20 mg (2 mL)/kg was administered orally using a gastric gavage tube to rats in each group (n = 8, 9, 10, and 10 for control, LC, DM, and LCD rats, respectively). Blood samples were collected at 0, 5, 15, 30, 45, 60, 90, 120, 180, and 240 min after the oral administration of sildenafil citrate. Other procedures for the oral study were similar to those for the intravenous study.

Measurement of rat plasma protein binding of sildenafil using equilibrium dialysis

Protein binding values of sildenafil to fresh plasma from control, LC, DM, and LCD rats (n = 4, each) were measured using equilibrium dialysis (Shim et al. 2000). One millilitre of plasma was dialyzed against 1 mL of isotonic Sørensen phosphate buffer (pH 7.4) containing 3% (w/v) dextran ("the buffer") to minimize volume shift (Boudinot and Jusko 1984) in a 1-mL dialysis cell (Fisher Scientific, Fair Lawn, NJ) using a Spectra/Por 4 membrane (molecular weight cutoff 12–14 kDa; Spectrum Medical Industries Inc., Los Angeles, CA). To reduce equilibrium time of sildenafil between "the buffer" and plasma compartments, sildenafil was spiked into the plasma side (Øie and Guentert 1982). An initial concentration of sildenafil spiked into the plasma compartment was 5 μg/mL. After 24-h incubation, two 50 μL were collected from each compartment and stored at –70°C until used for the LC/ MS/MS analysis of sildenafil.

LC/MS/MS analysis of sildenafil and *N*-desmethylsildenafil

Concentrations of sildenafil and *N*-desmethylsildenafil were determined using a LC/MS/MS method (Bae et al. 2009). In brief, to 50 μL of a sample, 200 μL of acetonitrile containing 250 ng/mL of DA-8159 (internal standard) was added. After vortex-mixing and centrifugation (9000 *g* for 10 min), the supernatant was transferred to another Eppendorf tube and a 6 μL was directly injected onto a reversed-phase HPLC column (Luna C_{18}; 50 mm, *l* × 2.0 mm, i.d.; particle size, 3 μm; Phenomenex, Torrance, CA). The mobile phase, 10 mM ammonium acetate (pH 5.2):acetonitrile (25:75, v/v), was run at a flow rate of 0.2 mL/min using an Agilent 1200 series HPLC system (Wilmington, DE). The column and autosampler temperatures were maintained at 40°C and 4°C, respectively. A LC/MS/MS analysis was performed using a PE SCIEX API4000 LC/MS/MS system (Applied Biosystems, Foster City, CA), equipped with an electrospray ionization interface used to generate positive ions, $[M+H]^+$. The optimized ion spray voltage and temperature were set at 5500 V and 500°C, respectively. The typical ion source parameters, viz., declustering potential, collision energy, entrance potential, and collision cell exit potential were 60, 35, 10, and 14 V, respectively. Nitrogen gas was used for the nebulizer (NEB) gas, curtain (CUR) gas, and collision-activated dissociation (CAD) gas. Quantification was performed by multiple reactions monitoring of the protonated precursor ion and the related product ion for sildenafil and *N*-desmethylsildenafil, using the internal standard method with peak area ratios and a weighing factor of $1/x^2$. The mass transitions used for sildenafil, *N*-desmethylsildenafil, and internal standard were m/z 475.3 → 100.1, 460.9 → 283.2, and 517.2 → 283.1, respectively, with a dwell time of 150 msec per transition. Quadrupoles Q1 and Q3 were set on unit resolution. The analytical data were processed by analyst software (version 1.4.1; Applied Biosystems). The retention times of sildenafil, *N*-desmethylsildenafil, and DA-8159 (internal standard) were approximately 1.2, 0.97, and 0.93 min, respectively. This assay was linear over concentration ranges from 5 to 1000 ng/mL with a lower limit of quantification of 5 ng/mL for both sildenafil and *N*-desmethylsildenafil. The coefficient of variation for the assay precision was less than 14.7%, and the accuracy was greater than 94.2%.

Pharmacokinetic analysis

Standard methods (Gibaldi and Perrier 1982) were used to calculate the following pharmacokinetic parameters using a non-compartmental analysis (WinNonlin®; Pharsight Corporation, Mountain View, CA): the AUC (Chiou 1978), time-averaged total body and renal clearances (CL and CL_R, respectively), terminal half-life ($t_{1/2}$), mean residence time (MRT), apparent volume of distribution at steady

state (V_{ss}), and F (Kim et al. 1993). The peak plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) were directly read from the experimental data.

Statistical analysis

A P-value <0.05 was considered to be statistically significant using a Duncan's multiple range test of Statistical Package for the Social Sciences (SPSS) *posteriori* analysis of variance (ANOVA) among the four means for the unpaired data. All data are expressed as mean ± standard deviation (SD) except median (range) for T_{max}.

Results

V_{max}, K_m, and CL_{int} for the disappearance of sildenafil in hepatic microsomes

The V_{max}, K_m, and CL_{int} for the disappearance of sildenafil in hepatic microsomes from four groups of rats are listed in Table 1. In LC, DM, and LCD rats, the V_{max}s were significantly slower (by 93.0, 65.3, and 94.1%, respectively) than controls. This suggests that in LC, DM, and LCD rats, the maximum velocity for the disappearance (primarily metabolism) of sildenafil was slower than controls. In LC, DM, and LCD rats, the K_ms were significantly lower (by 82.5, 42.7, and 92.4%, respectively) than controls. This suggests that in LC, DM, and LCD rats, the affinity of enzyme(s) for the sildenafil increased compared with controls. Since the total liver weight and total liver proteins were different among four groups of rats, the CL_{int}s were calculated based on the mL/min/whole liver; the values in LC (by 71.1%; $P<0.05$), DM (by 34.5%; $P=0.134$), and LCD (by 35.5%; $P=0.193$) rats were slower than controls. This suggests that in LC, DM, and LCD rats, formation of metabolites of sildenafil could be decreased compared with controls.

Protein expression of intestinal CYP1A1/2, 2B1/2, 2C11, 2D subfamily, 2E1, and 3A subfamily

The protein expression of intestinal CYP1A1/2, 2B1/2, 2C11, 2D subfamily, 2E1, and 3A subfamily in control, DM, LC, and LCD rats is shown in Figure 2. In LC rats, the protein expression of CYP2B1/2, 2C11, 2D6, 2E1, and 3A subfamily significantly decreased (by 89.4%, 94.9%, 86.3%, 67.3%, and 52.4%, respectively), but CYP1A1/2 was not altered compared with controls. In DM rats, CYP1A1/2 increased (by 1380%), 2C11 and 2D subfamily decreased (by 91.9% and 14.5%, respectively), but others were not altered compared with controls. In LCD rats, CYP1A1/2 increased (by 1510%), 2C11 decreased (by 94.6%), but others were not altered compared with controls.

Rat plasma protein binding of sildenafil

The binding values of sildenafil to fresh plasma from four groups of rats were 78.9 ± 5.89% (control rats), 72.7 ± 4.13% (LC rats), 63.7 ± 11.9% (DM rats), and 70.5 ± 5.76% (LCD rats); the value in DM rats was significantly smaller (by 19.3%) than controls.

It has been reported that the binding values of sildenafil in 4% human serum albumin, similar to the ratio of albumin in rat plasma (Mitruka and Rawnsley 1981), were constant, 95.0%, at sildenafil concentrations ranging from 0.01 to 10 μg/mL (Walker et al. 1999). Thus, a sildenafil concentration of 5 μg/mL was used in this plasma protein binding studies.

Pharmacokinetics of sildenafil and *N*-desmethylsildenafil after single intravenous administration of sildenafil

After the intravenous administration of sildenafil to control, LC, DM, and LCD rats, the mean arterial plasma concentration–time profiles of sildenafil and *N*-desmethylsildenafil are shown in Figure 3A and 3B, respectively. The relevant pharmacokinetic parameters are listed in Table 2. In LC rats, the AUC, CL, MRT, and V_{ss} of sildenafil were significantly greater (by 195%), slower (by 64.4%), longer (by 345%), and larger (by 44.8%), respectively; AUC, C_{max}, and T_{max} of *N*-desmethylsildenafil were significantly smaller (by 39.1%), lower (by 74.7%), and longer (by 500%), respectively; and metabolite ratio ($AUC_{N\text{-desmethylsildenafil}}/AUC_{sildenafil}$) was significantly smaller (by 78.1%) than controls. In DM

Table 1. V_{max}, K_m, and CL_{int} for the disappearance of sildenafil in hepatic microsomes from control, liver cirrhosis (LC), diabetes mellitus (DM), and liver cirrhosis with diabetes mellitus (LCD) rats.

Parameter	Control ($n=6$)	LC ($n=5$)	DM ($n=4$)	LCD ($n=5$)
V_{max} (nmol/min/mg protein)[a]	13.4 ± 6.95	0.933 ± 0.569	4.65 ± 3.01	0.792 ± 0.410
K_m (μM)[b]	52.9 ± 22.0	9.28 ± 5.60	30.3 ± 13.9	4.00 ± 1.85
CL_{int} (mL/min/mg protein)[c]	0.254 ± 0.0868	0.108 ± 0.0707	0.144 ± 0.0515	0.235 ± 0.130
CL_{int} (mL/min/whole liver)[c]	22.0 ± 8.35	6.35 ± 5.27	14.4 ± 4.37	14.2 ± 8.92
Total protein (mg/whole liver)[d]	86.1 ± 9.45	62.8 ± 10.8	103 ± 15.9	62.9 ± 11.3
Liver weight (% of body weight)[e]	3.48 ± 0.396	2.68 ± 0.389	3.92 ± 0.274	3.14 ± 0.237

Notes: Data are the mean ± standard deviation (SD).
CL_{int}, Intrinsic clearance; DM, diabetes mellitus; K_m, apparent Michaelis–Menten constant; LC, liver cirrhosis; LCD, liver cirrhosis with diabetes mellitus; V_{max}, maximum velocity.
[a]Control group was significantly different ($P<0.05$) from other three groups.
[b]Control group, DM group, and LC and LCD groups were significantly different ($P<0.05$).
[c]Control group was significantly different ($P<0.05$) from LC group.
[d]Control and DM groups were significantly different ($P<0.05$) from LC and LCD groups.
[e]Control and DM groups were significantly different ($P<0.05$) from LC group, and DM group was significantly different ($P<0.05$) from LCD group.

rats, the AUC and CL of sildenafil were significantly greater (by 54.2%) and slower (by 34.8%), respectively; C_{max} of *N*-desmethylsildenafil was significantly lower (by 35.5%); and metabolite ratio was significantly smaller (by 42.9%) than controls. In LCD rats, the AUC, CL, MRT, and V_{ss} of sildenafil were significantly greater (by 127%), slower (by 54.9%), longer (by 239%), and larger (by 51.9%), respectively; AUC, C_{max}, and T_{max} of *N*-desmethylsildenafil were significantly smaller (by 51.5%), lower (by 73.1%), and longer (by 300%), respectively; and metabolite ratio was significantly smaller (by 77.1%) than controls (Table 2). The $Ae_{0\text{-}24\,h}$ and $GI_{24\,h}$ (expressed in terms of the percentage of the intravenous sildenafil dose) of both sildenafil and *N*-desmethylsildenafil were below the detection limit in all rats studied. Note that in LC, DM, and LCD rats, body weight gain decreased (by 56.3, 22.6, and 55.8%, respectively) compared with controls as reported from other studies (Ahn et al. 2008, 2009).

Pharmacokinetics of sildenafil and *N*-desmethylsildenafil after single oral administration of sildenafil

After the oral administration of sildenafil to control, LC, DM, and LCD rats, the mean arterial plasma

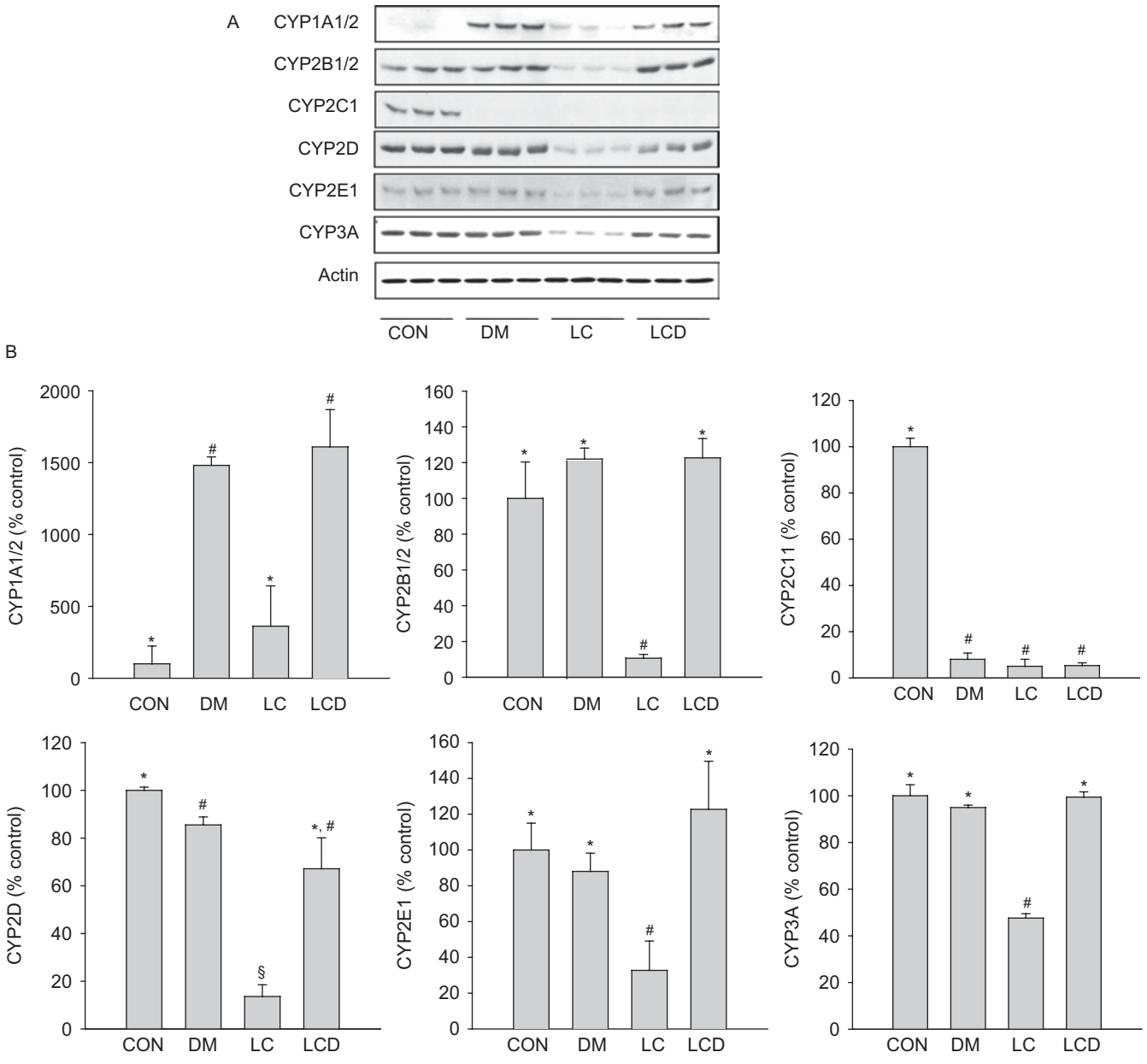

Figure 2. Immunoblotting of intestinal CYP1A1/2, 2B1/2, 2C11, 2D subfamily, 2E1, and 3A subfamily in control (CON), liver cirrhosis (LC), diabetes mellitus (DM), and liver cirrhosis with diabetes mellitus (LCD) rats (A). The protein expression was expressed in terms of % of control rats, 100% (B). The β-actin band was used as a loading control. Data are the mean ± standard deviation (SD). CYP1A1/2; DM and LCD groups (#) were significantly different ($P<0.05$) from control and LC groups (*); CYP2B1/2, CYP2E1, and CYP3A subfamily; LC group (#) was significantly different ($P<0.05$) from control, DM, and LCD groups (*); CYP2C11; DM, LC, and LCD groups (#) were significantly different ($P<0.05$) from control group (*); CYP2D subfamily; LC group (§) was significantly different ($P<0.05$) from other three groups (* and #), and DM group (#) was significantly different ($P<0.05$) from control group (*).

concentration–time profiles of sildenafil and *N*-desmethylsildenafil are shown in Figure 4A and 4B, respectively. The relevant pharmacokinetic parameters are listed in Table 3. In LC rats, the AUC, terminal $t_{1/2}$, and C_{max} of sildenafil were significantly greater (by 3010%), longer (by 33.0%), and higher (by 833%), respectively; AUC, C_{max}, and T_{max} of *N*-desmethylsildenafil were significantly smaller (by 61.5%), lower (by 84.6%), and longer (by 200%), respectively; and metabolite ratio was significantly smaller (by 97.1%) than controls. After the oral administration of 50-mg sildenafil to male patients with biopsy-proven LC, the AUC and C_{max} of sildenafil were also greater (by 85.0%) and higher (by 47.1%), respectively, but AUC of *N*-desmethylsildenafil was greater (by 155%) than control subjects (Murihead et al. 2002). In DM rats, the C_{max} of *N*-desmethylsildenafil was significantly lower (by 23.9%); and metabolite ratio was significantly smaller (by 63.6%) than controls. In LCD rats, the AUC, C_{max}, and T_{max} of sildenafil were significantly greater (by 2030%), higher (by 696%), and longer (by 50%), respectively; AUC, C_{max}, and T_{max} of *N*-desmethylsildenafil were significantly smaller (by 61.3%), lower (by 83.2%), and longer (by 400%), respectively; and metabolite ratio was significantly smaller (by 97.2%) than the controls. In LC, DM, and LCD rats, the *F* values were greater (by 953%, 101%, and 839%, respectively) than controls. The $Ae_{0\text{-}24\ h}$ and $GI_{24\ h}$ of sildenafil were almost negligible (<2.56% of the oral dose of sildenafil) and those of *N*-desmethylsildenafil (expressed in terms of the percentage of the oral dose of sildenafil) were below the detection limit for all rats studied.

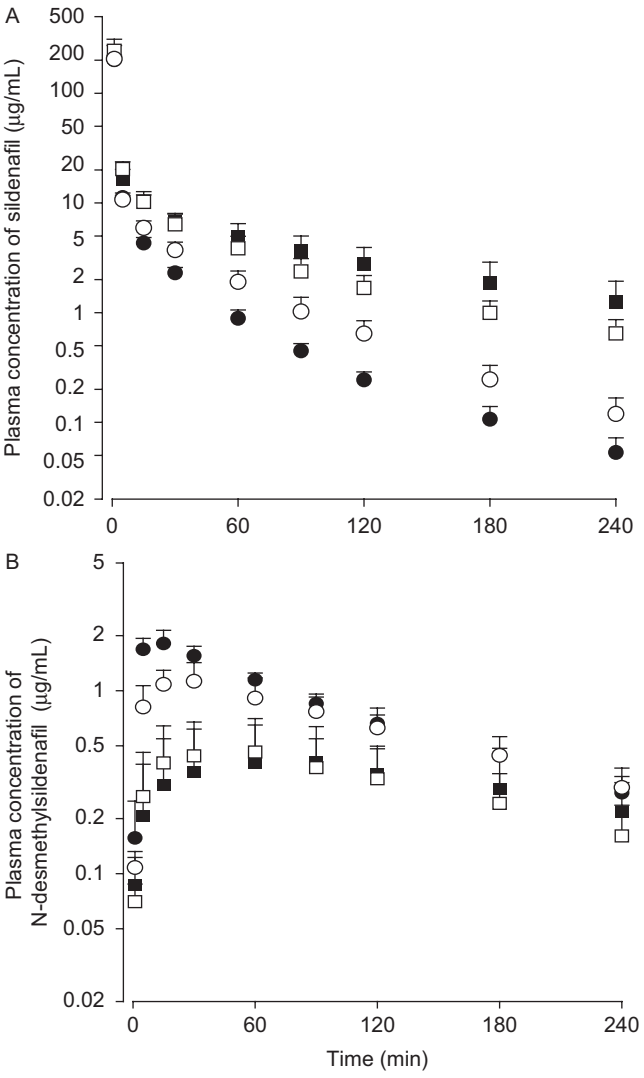

Figure 3. Mean arterial plasma concentration–time profiles of sildenafil (A) and *N*-desmethylsildenafil (B) after intravenous infusion of sildenafil at a dose of 10 mg/kg in control (solid circles; $n=9$), liver cirrhosis (LC) (solid squares; $n=10$), diabetes mellitus (DM) (open circles; $n=9$), and liver cirrhosis with diabetes mellitus (LCD) (open squares; $n=8$) rats. Data are the mean ± standard deviation (SD).

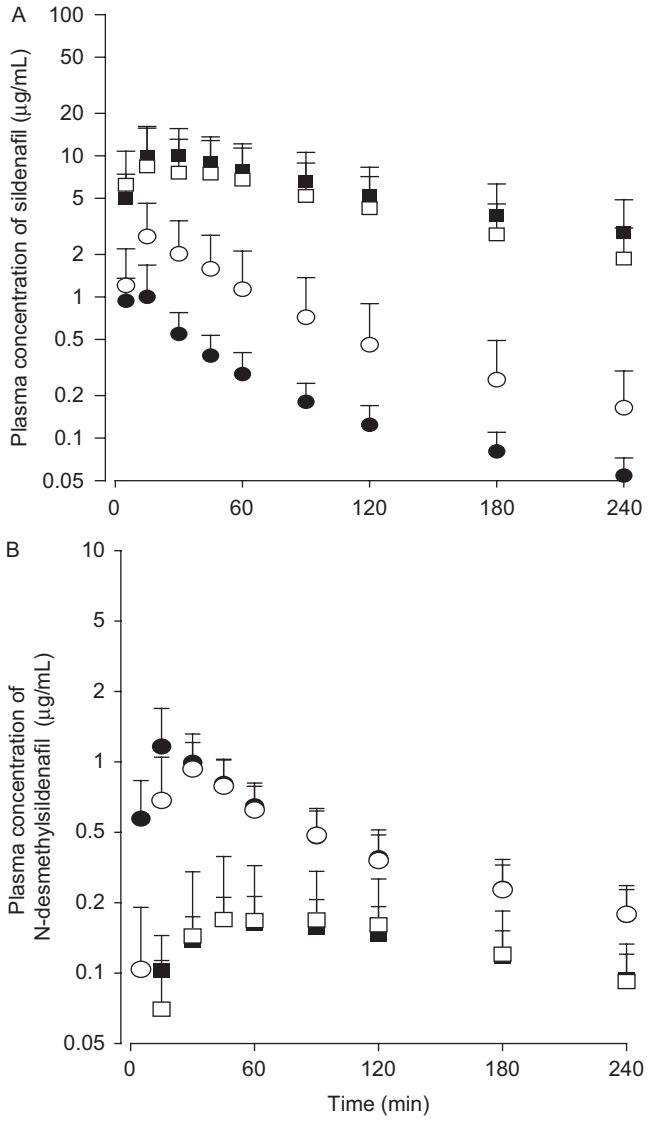

Figure 4. Mean arterial plasma concentration–time profiles of sildenafil (A) and *N*-desmethylsildenafil (B) after the oral administration of sildenafil at a dose of 20 mg/kg in control (solid circles; $n=8$), liver cirrhosis (LC) (solid squares; $n=9$), diabetes mellitus (DM) (open circles; $n=10$), and liver cirrhosis with diabetes mellitus (LCD) (open squares; $n=10$) rats. Data are the mean ± standard deviation (SD).

Discussion

The hepatic CL_{int}s for the disappearance of sildenafil based on the mL/min/whole liver were slower than controls in the order of DM, LCD, and LC rats (Table 1). This could have possibly been due to the differences in the magnitude of changes in the hepatic CYP isozymes compared with controls: in LC rats, the protein expression of hepatic CYP2C11 and 3A subfamily significantly decreased (by 83.1% and 75.8%, respectively); in LCD rats, they were also significantly decreased (by 32.8% and 52.9%, respectively), but the magnitude was smaller than that in LC rats (Ahn et al. 2009). However, in DM rats, the CYP2C11 and 3A subfamily were significantly decreased (by 28.8%) and increased (by 23.0%), respectively (Ahn et al. 2009).

Streptozotocin has "direct" (streptozotocin *per se*) effects on the pharmacokinetic parameters of drugs due to its hepatotoxicity (Ioannides 1996). Approaches have been proposed for ensuring that observed pharmacokinetic effects in DMIS rats are due to the "indirect" (changes in CYP isozymes due to diabetes-related changes) effect of streptozotocin and not streptozotocin *per se* (Ioannides 1996). Considering the very short $t_{1/2}$ of streptozotocin (about 15 min), it is unlikely that its "direct" effects could be sustained for 7 days after administration of streptozotocin. Streptozotocin had no effect on the metabolism of model substrates *in vitro* (Ackerman and Leibman 1977), and analogues of streptozotocin, devoid of diabetogenic activity, failed to influence the metabolism of various substrates (Reinke et al. 1978). The above data suggest that the effect of streptozotocin on Day 7 occurred through its "indirect," and not "direct," mechanism. Metabolic $t_{1/2}$ of *N*-dimethylnitrosamine was also short; <10 min in rodents and about 20 min in non-human primates (George et al. 2001). Therefore, it is also unlikely that its "direct" effects could be sustained for more than a week after administration of *N*-dimethylnitrosamine. However, the acute toxic effects of streptozotocin and *N*-dimethylnitrosamine could not be totally ruled out.

The contribution of GI (including biliary) excretion of unchanged sildenafil to its CL did not seem to be considerable; the $GI_{24\,h}$ values of sildenafil were below the detection limit (Table 2). Sildenafil was stable in buffer solutions having pH of 3, 7, and 11 up to 12-h incubation (>98% of the spiked amounts of sildenafil were recovered) (Wang et al. 2008). Moreover, the 24-h biliary excretion of sildenafil after its intravenous administration (20 mg/kg) to three rats with bile duct cannulation was only 0.142 ± 0.0321% of the dose in the present study. The $Ae_{0-24\,h}$ was also below the detection limit for all rats studied (Table 2). The above data suggest that intravenous sildenafil was almost completely metabolized and the CLs of sildenafil listed in Table 2 could represent its metabolic clearances.

Table 2. Pharmacokinetic parameters of sildenafil and *N*-desmethylsildenafil after the intravenous administration of sildenafil at a dose of 10 mg/kg to control, liver cirrhosis (LC), diabetes mellitus (DM), and liver cirrhosis with diabetes mellitus (LCD) rats.

Parameter	Control ($n=9$)	LC ($n=10$)	DM ($n=9$)	LCD ($n=8$)
Initial body weight (g)	178 ± 9.72	186 ± 14.5	179 ± 12.4	183 ± 14.4
Final body weight (g)[a]	377 ± 11.5	273 ± 16.2	333 ± 21.4	271 ± 16.6
Sildenafil				
AUC (μg min/mL)[b]	577 ± 34.9	1700 ± 399	890 ± 92.8	1310 ± 229
CL (mL/min/kg)[b]	34.8 ± 2.12	12.4 ± 3.37	22.7 ± 2.47	15.7 ± 2.58
Terminal $t_{1/2}$ (min)	49.1 ± 5.74	91.6 ± 23.6	49.8 ± 11.6	69.6 ± 30.8
MRT (min)[c]	17.4 ± 2.87	77.5 ± 33.2	22.9 ± 3.66	58.9 ± 7.48
V_{ss} (mL/kg)[d]	603 ± 94.6	873 ± 218	515 ± 64.7	916 ± 140
$Ae_{0-24\,h}$ (% of sildenafil dose)	BD[e]	BD	BD	BD
$GI_{24\,h}$ (% of sildenafil dose)	BD	BD	BD	BD
N-Desmethylsildenafil				
AUC (μg min/mL)[d]	233 ± 15.0	142 ± 87.8	206 ± 47.6	113 ± 45.9
Terminal $t_{1/2}$ (min)	95.7 ± 16.6	186 ± 77.9	113 ± 13.8	145 ± 79.1
C_{max} (μg/mL)[a]	1.86 ± 0.292	0.471 ± 0.326	1.20 ± 0.262	0.500 ± 0.159
T_{max} (min)[d]	15 (5–15)	90 (15–120)	15 (5–30)	60 (5–60)
$Ae_{0-24\,h}$ (% of sildenafil dose)	BD	BD	BD	BD
$GI_{24\,h}$ (% of sildenafil dose)	BD	BD	BD	BD
$AUC_{N\text{-desmethylsildenafil}}/AUC_{sildenafil}$ (%)[a]	40.6 ± 3.92	8.91 ± 6.61	23.2 ± 4.93	9.29 ± 3.96

Notes: Data are the mean ± standard deviation (SD) except for T_{max}, which is median (range).

$Ae_{0-24\,h}$, Percentage of the dose excreted in the 24-h urine; AUC, area under the plasma concentration–time curve; C_{max}, peak plasma concentration; DM, diabetes mellitus; $GI_{24\,h}$, percentage of the dose recovered from the gastrointestinal tract (including its contents and faeces) at 24 h; LC, liver cirrhosis; LCD, liver cirrhosis with diabetes mellitus; MRT, mean residence time; T_{max}, time to reach C_{max}; V_{ss}, volume of distribution at steady state.

[a]Control group, DM group, and LC and LCD groups were significantly different ($P<0.05$).

[b]Each group was significantly different ($P<0.05$).

[c]Control and DM groups, LCD group, and LC group were significantly different ($P<0.05$).

[d]Control and DM groups were significantly different ($P<0.05$) from LC and LCD groups.

[e]Below the detection limit.

In LC, DM, and LCD rats, the AUCs of intravenous sildenafil were significantly greater than controls, possibly as a result of the significantly slower CLs of sildenafil than controls (Table 2). Because sildenafil is a drug with an intermediate hepatic extraction ratio [hepatic first-pass extraction ratio of 0.49 after absorption into the portal vein (Shin et al. 2006)], its hepatic clearance depends on the *in vitro* hepatic CL_{int} for the disappearance of sildenafil, the free (unbound to plasma proteins) fraction of sildenafil in the plasma, and the hepatic blood flow rate in rats (Wilkinson and Shand 1975). The significantly slower CL of sildenafil in LC rats (Table 2) could have been supported by the significantly slower hepatic CL_{int} (mL/min/whole liver) for the disappearance of sildenafil (Table 1) and slower hepatic blood flow rate than controls, because the free fraction of sildenafil in the plasma was comparable with controls. Goeting et al. (1986) reported that the hepatic blood flow rate was slower in rats with LC induced by carbon tetrachloride. In LC rats, the slower hepatic CL_{int} (Table 1) could have been due to the decrease in the protein expression of both hepatic CYP2C11 and 3A subfamily compared with controls (Ahn et al. 2009). In DM rats, the slower CL of sildenafil (Table 2) could have been due to the significantly slower CL_{int} (mL/min/whole liver) (Table 1) because the free fraction of sildenafil in the plasma was significantly greater and the hepatic blood flow rate was faster (Sato et al. 1991) than controls. In DM rats, the slower CL_{int} could be due to the decrease in the protein expression of CYP2C11 because CYP3A subfamily increased in DM rats compared with controls (Ahn et al. 2009). In LCD rat, the significantly slow CL of sildenafil (Table 2) could at least partly have been due to the slower hepatic CL_{int} (mL/min/whole liver) (Table 1) because the free fraction of sildenafil in the plasma was comparable with controls. In LCD rats, the hepatic blood flow rate changes did not seem to be reported. In LCD rats, the slower CL_{int} (Table 1) could have been due to the decrease in the protein expression of both CYP2C11 and 3A subfamily compared with controls (Ahn et al. 2009). In LC, DM, and LCD rats, the significantly smaller metabolite ratios (by 78.1, 42.9, and 77.1%, respectively) than controls suggest that formation of *N*-desmethylsildenafil decreased compared with controls. This could at least partly have been due to the significant decrease in the protein expression of hepatic CYP2C11 compared with controls (Ahn et al. 2009).

In LC and LCD rats, the V_{ss}s of intravenous sildenafil were significantly larger than controls (by 44.8% and

Table 3. Pharmacokinetic parameters of sildenafil and *N*-desmethylsildenafil after the oral administration of sildenafil at a dose of 20 mg/kg to control, liver cirrhosis (LC), diabetes mellitus (DM), and liver cirrhosis with diabetes mellitus (LCD) rats.

Parameter	Control ($n=8$)	LC ($n=9$)	DM ($n=10$)	LCD ($n=10$)
Initial body weight (g)	169 ± 9.04	167 ± 7.55	164 ± 11.5	165 ± 7.45
Final body weight (g)[a]	328 ± 12.3	279 ± 17.2	299 ± 12.0	273 ± 32.4
Blood glucose (mg/dL)[b]	91.4 ± 8.05	103 ± 8.20	284 ± 32.1	318 ± 69.6
Sildenafil				
AUC (μg min/mL)[c]	64.7 ± 22.4	2010 ± 1300	202 ± 151	1380 ± 909
Terminal $t_{1/2}$ (min)[d]	106 ± 31.3	141 ± 44.0	85.2 ± 13.9	104 ± 26.6
C_{max} (μg/mL)[c]	1.20 ± 0.617	11.2 ± 5.64	2.76 ± 1.85	9.55 ± 7.67
T_{max} (min)[e]	15 (5–15)	15 (5–45)	15 (15–45)	22.5 (5–45)
CL_R (mL/min/kg)	0.484 ± 0.294	0.317 ± 0.199	0.293 ± 0.120	0.511 ± 0.635
$Ae_{0\text{-}24\,h}$ (% of sildenafil dose)	0.151 ± 0.105	2.56 ± 2.12	0.242 ± 0.103	2.44 ± 2.33
$GI_{24\,h}$ (% of sildenafil dose)	BD[f]	BD	BD	BD
F (%)	5.61	59.1	11.3	52.7
N-Desmethylsildenafil				
AUC (μg min/mL)[c]	155 ± 50.5	59.7 ± 16.7	141 ± 36.2	60.0 ± 32.5
Terminal $t_{1/2}$ (min)	144 ± 43.8	182 ± 39.9	141 ± 29.4	184 ± 81.5
C_{max} (μg/mL)[g]	1.34 ± 0.514	0.207 ± 0.0614	1.02 ± 0.291	0.225 ± 0.173
T_{max} (min)[c]	15 (5–45)	45 (15–120)	22.5 (15–45)	75 (15–120)
$Ae_{0\text{-}24\,h}$ (% of sildenafil dose)	BD	BD	BD	BD
$GI_{24\,h}$ (% of sildenafil dose)	BD	BD	BD	BD
$AUC_{N\text{-desmethylsildenafil}}/AUC_{sildenafil}$ (%)[g]	243 ± 38.3	7.08 ± 10.4	88.5 ± 42.2	6.75 ± 7.10

Notes: Data are the mean ± standard deviation (SD) except for T_{max}, which is median (range).
AUC, Area under the plasma concentration–time curve; $Ae_{0\text{-}24\,h}$, percentage of the dose excreted in the 24-h urine; C_{max}, peak plasma concentration; CL_R, renal clearance; DM, diabetes mellitus; *F*, extent of absolute oral bioavailability; $GI_{24\,h}$, percentage of the dose recovered from the gastrointestinal tract (including its contents and faeces) at 24 h; LC, liver cirrhosis; LCD, liver cirrhosis with diabetes mellitus; T_{max}, time to reach C_{max}.
[a]Control group was significantly different ($P<0.05$) from DM and LCD groups.
[b]Control and LC groups were significantly different ($P<0.05$) from DM and LCD groups.
[c]Control and DM groups were significantly different ($P<0.05$) from LC and LCD groups.
[d]LC group was significantly different ($P<0.05$) from other three groups.
[e]Control group was significantly different ($P<0.05$) from LCD group.
[f]Below the detection limit.
[g]Control group, DM group, and LC and LCD groups were significantly different ($P<0.05$).

51.9%, respectively; Table 2). However, this was not likely due to the increase in the free fraction of sildenafil in plasma compared with controls; the free fractions were comparable among three groups of rats. Similar results have also been reported in LC rats that the V_{ss} of theophylline was slightly larger although the protein binding value was comparable with controls (Nam et al. 1997). The exact reason for this is unclear and more studies are required.

In LC and LCD rats, the AUCs of oral sildenafil were also greater than controls (by 3010% and 2030%, respectively) (Table 3). However, this was not likely due to the increased GI absorption of sildenafil compared with controls. For comparison, the "true" fractions of the oral dose of sildenafil unabsorbed ("F_{unabs}") were estimated using the reported equation (Lee and Chiou 1983). The "F_{unabs}" values thus estimated were almost negligible, suggesting almost complete absorption of sildenafil for all groups of rats. As mentioned earlier, the intestinal and hepatic first-pass extraction ratios of sildenafil were 0.71 and 0.137 of the oral dose, respectively, in rats (Shin et al. 2006). Thus, the contribution of intestinal first-pass extraction (0.71 of the oral dose) to the significantly greater AUC of oral sildenafil was greater than that of the hepatic first-pass extraction (0.137 of the oral dose). In LC and LCD rats, the magnitude of the increase in AUCs of oral sildenafil (by 3010% and 2030%, respectively) was considerably greater than 195% and 127% increase, respectively, after the intravenous sildenafil (Table 2). Thus, only inhibition of hepatic metabolism of sildenafil could not fully explain the considerable increase in AUCs of oral sildenafil (Table 3). This could mainly be due to the decrease in the intestinal metabolism of sildenafil because of decrease in the protein expression of intestinal CYP2C11 in LC and LCD rats (Figure 2). This could be supported by the greater magnitude in the smaller metabolite ratios in LC and LCD rats after the oral administration (by 97.1% and 97.2%, respectively) (Table 3) than that after the intravenous administration (by 78.1% and 77.1%, respectively) (Table 2). Therefore, in LC and LCD rats, the significantly greater AUCs of oral sildenafil were mainly due to the decrease in intestinal metabolism of sildenafil in addition to decrease in hepatic metabolism compared with controls. The above results could explain the greater *F* values in LC and LCD rats than controls (Table 3).

In conclusion, in LC, DM, and LCD rats, the CLs (AUCs) of intravenous sildenafil were significantly slower (greater) and the metabolite ratios were significantly smaller than controls. This could have mainly been due to the slower hepatic metabolism of sildenafil than controls. In LC and LCD rats, the magnitudes of increase in the AUCs of oral sildenafil and decrease in metabolite ratios were greater than those after the intravenous administration. This could have mainly been due to the decrease in intestinal extraction of sildenafil because of decrease in the protein expression of intestinal CYP2C11 in LC and LCD rats. The changes in hepatic CYP2C and 3A subfamilies in patients with DM alone and with LC did not seem to be reported. Moreover, the pharmacokinetic studies on sildenafil in patients with diabetes with and without LC also did not seem to be reported. In patients with LC, the changes in AUC of *N*-desmethylsildenafil (Murihead et al. 2002) were different from those in the present LC rats. Thus, the present experimental data in rats should be extrapolated with care to the human situation.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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