In Vitro Evaluation of Inhibitory Effects of Antidiabetic and Antihyperlipidemic Drugs on Human Carboxylesterase Activities

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ABSTRACT:

Human carboxylesterase (CES) 1A is responsible for the biotransformation of angiotensin-converting enzyme (ACE) inhibitors such as imidapril and temocapril. Because antidiabetic or antihyperlipidemic drugs are often coadministered with ACE inhibitors in clinical pharmacotherapy, the inhibitory effect of these drugs on CES1A enzyme activity was investigated. In addition, the inhibitory effect on CES2 enzyme activity was evaluated to compare it with that on CES1A1. The inhibitory effects were evaluated with 11 antidiabetic and 12 antihyperlipidemic drugs. The imidapril hydrolyse activity by recombinant CES1A1 was substantially inhibited by lactone ring-containing statins such as simvastatin and lovastatin and thiazolidinediones such as troglitazone and rosiglitazone. The activity in human liver microsomes was also strongly inhibited by simvastatin and troglitazone ($K_i = 0.8 \pm 0.1$ and $5.6 \pm 0.2 \mu M$, respectively). However, statins containing no lactone ring such as pravastatin and fluvastatin did not show strong inhibition. 7-Ethyl-10-[4-(1-piperidono)-1-piperidono]carbonyloxycamptothecin hydrolase activity by recombinant human CES2 was substantially inhibited by fenofibrate ($K_i = 0.04 \pm 0.01 \mu M$) as well as by simvastatin ($0.67 \pm 0.09 \mu M$). Other fibrates such as clofibrate and bezafibrate did not show strong inhibition. Thus, the inhibitory effects of the thiazolidinediones and fenofibrate on CES1A1 and CES2 were different. Some statins such as simvastatin and lovastatin, thiazolidinediones, and fenofibrate might attenuate the drug efficacy of prodrugs biotransformed by CES1A and CES2.

Introduction

Human carboxylesterases (CESs) are members of the serine esterase superfamily and are responsible for the hydrolysis of a wide variety of xenobiotic and endogenous compounds. In humans, two CES families, CES1A and CES2, are known to be mainly involved in the biotransformation of a variety of clinically used drugs and prodrugs (Sato et al., 2002). CES1A is predominantly expressed in liver, but its expression in the gastrointestinal tract is markedly low (Schwer et al., 1997; Sato et al., 2002). In contrast, CES2 is expressed in both the liver and gastrointestinal tract (Xu et al., 2002). Human CES1A is classified into two isoforms, CES1A1 and CES1A2, which have high homology at the mRNA level (99.3%) (Fukami et al., 2008). Because only the signal peptide sequences of CES1A1 and CES1A2 are different, the mature proteins produced from both mRNAs are identical. CES1A is involved in the biotransformation of various angiotensin-converting enzyme (ACE) inhibitors to their pharmacologically active forms (e.g., imidapril, temocapril, and delapril) (Takai et al., 1997). Therefore, CES1A is considered to be one of the critical determinants of drug efficacy. ACE inhibitors are administered for the treatment of hypertension and congestive heart failure. However, because patients with diabetes and hyperlipidemia frequently have hypertension and heart failure, such patients are concurrently prescribed antihypertensive, antihyperlipidemic, and antidiabetic drugs. Fleming et al. (2005) found that mevastatin, which is an antihyperlipidemic drug, inhibits o-nitrophenyl acetate hydrolysis by CES1A ($K_i = 20.8 \mu M$). Thus, it is possible that antidiabetic or antihyperlipidemic drugs inhibit CES1A enzyme activity. If drugs coadministered with ACE inhibitors inhibit CES1A enzyme activity, the effectiveness of pharmacotherapy would be impaired. In the present study, we examined the inhibitory effects of various antidiabetic or antihyperlipidemic drugs on CES1A1 enzyme activity. In addition, the inhibitory effect on CES2 enzyme activity was evaluated to compare it with that on CES1A1.

Materials and Methods

Materials. Imidapril hydrochloride and imidaprilat were kindly supplied by Mitsubishi Tanabe Pharma Corporation (Osaka, Japan), Mitiglinide, clofibrate, niacin, rosiglitazone, and fenofibrate were kindly supplied by Kissei Pharmaceutical (Matsumoto, Japan), Dainippon Sumitomo Pharma Company (Osaka, Japan), Sanwa Kagaku Kenkyusho (Nagoya, Japan), and Kyorin Pharmaceutical (Tokyo, Japan), respectively. Acetohexamide, clofibrate, glibenclamide, gliclazide, lovastatin, metformin, pravastatin sodium, rosiglitazone, (±)-o-tocopherol nicotinate, tolbutamide, troglitazone, and p-nitrophenol were purchased from Wako Pure Chemi-
Expression of Human CES1A1 and CES2 in SF21 Cells. The expression of human CES enzymes using a Bac-to-Bac Baculovirus Expression System (Invitrogen, Carlsbad, CA) was performed according to the manufacturer’s protocol. Human CES1A1 and CES2 cDNAs were prepared by a reverse transcription-polymerase chain reaction technique using total RNA (Stratagene, La Jolla, CA) from human liver (CES1A1) and colon (CES2) with the following primer sets: CES1A1, CES1A1-S and CES1A-AS primers; CES2, CES2-S and CES2-AS primers (Table 1).The polymerase chain reaction products were first subcloned into pTARGET Mammalian Expression Vector (Promega, Madison, WI). The CES cDNA in the pTARGET vector was then transferred into the pFastBac1 vector using appropriate restriction enzymes. The pFastBac1 vector containing CES cDNA was transformed into DH10Bac competent cells, followed by transposition of the inserts into bacmid DNA. The sequences of the CES cDNAs were determined using a Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) with a Long-Read Tower DNA sequencer (GE Healthcare). Non-recombinant bacmid DNA (mock) was also prepared by the same procedures.

Spodoptera frugiperda SF21 cells (Invitrogen) were grown in SF-900 II SFM containing 10% fetal bovine serum at 27°C. The recombinant and mock bacmid DNAs were separately transfected into SF21 cells with Cellfectin Reagent (Invitrogen), and the virus was harvested by collecting the cell culture medium at 72 h after transfection. Cells were routinely harvested 72 h after the infection, washed twice with phosphate-buffered saline, and stored at 80°C until use. Cell homogenates were prepared by suspending in TGE buffer [10 mM Tris-HCl buffer (pH 7.4), 20% glycerol, and 1 mM EDTA (pH 7.4)] and by disrupting by freeze-thawing three times according to the method reported by Ren et al. (2000). Then, the suspensions were homogenized with a Teflon-glass homogenizer for 10 strokes. CES expression was confirmed by Western blotting according to a previous report (Watanabe et al., 2009). The protein concentrations were determined according to Bradford (1976).

Enzyme Activity. Imidapril, CPT-11, and p-nitrophenyl acetate hydrolysis activities were determined according to methods described previously (Takahashi et al., 2009; Watanabe et al., 2009; Maruichi et al., 2010).

Inhibition Analysis of CES Enzyme Activities. The inhibitory effects of 23 drugs and 3 metabolites on the imidapril and CPT-11 hydrolysis activities were investigated. Acetohexamide, tolbutamide, gliclazide, glibenclamide, nateglinide, mitiglizidine, pioglitazone, rosiglitazone, simvastatin, lovastatin, rosuvastatin calcium salt, clofibrate, clinofibrate, bezafibrate, fenofibrate, niconitol, and fenofibric acid were dissolved in DMSO. Metformin, bufornin, pravastatin sodium, fluvastatin sodium salt, simvastatin hydroxy acid ammonium salt, andLovastatin hydroxy acid sodium salt were dissolved in distilled water. Nicomol and (R)-/H9262-nicotinic acid was used as an inhibitor at a concentration of 100 μM because of the limited solubility.

For determination of the Kᵢ (inhibition constant) values for the imidapril hydrolysis activity, the concentrations of imidapril ranged from 0.5 to 5.0 mM. The concentrations of the inhibitors for the imidapril hydrolysis activity ranged as follows: simvastatin, 0.1 to 1.0 and 0.3 to 2.0 μM for recombinant CES1A1 and HLM, respectively; and troglitazone, 0.4 to 3.0 and 2 to 15 μM, respectively. For determination of the Kᵢ value for the CPT-11 hydrolysis activity, the concentrations of CPT-11 ranged from 1.0 to 12 μM for recombinant CES2 and from 2.5 to 15 μM for HLM and HJM. The concentrations of the inhibitors for the CPT-11 hydrolysis activity ranged as follows: simvastatin, 0.3 to 2.0 μM for recombinant CES2 and 1 to 10 μM for HLM and HJM; and fenofibrate, 0.02 to 0.10 μM for recombinant CES2, 0.5 to 2.0 μM for HJM, and 50 to 200 μM for HLM. The Kᵢ, Kᵢᵣ, and Vᵢᵣ max values and inhibition types were determined by fitting the kinetic data to a competitive, noncompetitive, uncompetitive, or mixed inhibition model by nonlinear regression analysis using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). The Kᵢ, Kᵢᵣ, and Vᵢᵣ max values represent the mean ± S.E.

For determination of the inhibitor concentration that caused 50% inhibition (IC₅₀), the p-nitrophenyl acetate hydrolysis activities by recombinant CES1A1 and CES2 at 200 μM were examined in the presence of the inhibitors. The concentrations of the inhibitors ranged as follows: simvastatin, 0.2 to 5.0 μM; troglitazone, 1 to 40 μM; and fenofibrate, 0.1 to 2.0 μM.

Results

Inhibitory Effects of 23 Drugs on Imidapril Hydrolysis Activities by Recombinant Human CES1A1. The inhibitory effects on the imidapril hydrolysis activity by human CES1A1 enzyme were investigated using 23 drugs (Fig. 1). The control activity by recombinant CES1A1 was 1.73 nmol/min/mg. It was confirmed that mock-transfected SF21 cell homogenates did not show imidapril hydrolysis activity. DMSO (1%) and ethanol inhibited the imidapril hydrolysis activity by CES1A1 by 30.2 and 46.5%, respectively (data not shown), and 24 mM HCl also inhibited the imidapril hydrolysis activity by CES1A1 by 4.0% (data not shown). If the drugs were dissolved in their solvents, the inhibition rate was calculated as percent inhibition of control activity by recombinant CES1A1 was 1.73 nmol/min/mg.
activity in the presence of the respective solvents. The activity by recombinant CES1A1 was strongly inhibited by glibenclamide (percentage of control: 18.4%), pioglitazone (17.0%), rosiglitazone (7.3%), troglitazone (0.9%), simvastatin (0.6%), and lovastatin (0.6%). Simvastatin and lovastatin are rapidly hydrolyzed to simvastatin hydroxy acid and lovastatin hydroxy acid, respectively, the active metabolites in vivo in humans (Duggan et al., 1989; Vickers et al., 1990). Therefore, the inhibitory effects of these metabolites on the activity by recombinant CES1A1 were examined, but they did not show potent inhibitory effects compared with their parent drugs (Fig. 1). Recombinant CES1A2 was also constructed using a Bac-to-Bac Baculovirus Expression System, and the effects on the imidapril hydrolase activity by CES1A2 enzyme were investigated. However, the inhibitory profile of CES1A2 was quite similar to that of CES1A1 (data not shown). Therefore, the effects on only CES1A1 were evaluated in the present study.

Inhibition Constant and Inhibition Patterns of Imidapril Hydrolase Activities by Recombinant Human CES1A1 and HLM. The $K_i$ values and inhibition patterns of simvastatin and troglitazone showing strong inhibition for the imidapril hydrolase activities by recombinant CES1A1 and HLM were determined, and representative Lineweaver-Burk plots are shown in Fig. 2. The $K_i$ values of simvastatin and troglitazone for recombinant CES1A1 were 0.11 ± 0.01 and 0.62 ± 0.08 μM, respectively, with mixed-type inhibition. In contrast, the $K_i$ values of simvastatin and troglitazone for HLM were 0.76 ± 0.06 and 5.64 ± 0.23 μM, respectively, with competitive- and noncompetitive-type inhibition, respectively.

Inhibitory Effects of 23 Drugs on CPT-11 Hydrolase Activity by Recombinant Human CES2. To investigate the inhibitory effect on human CES2 enzyme activity, the CPT-11 hydrolase activity was evaluated (Fig. 3). The control activity by recombinant CES2 was 2.92 pmol/min/mg. It was confirmed that mock-transfected Sf21 cell
homogenates did not show the CPT-11 hydrolase activity. DMSO (1%) and ethanol inhibited the CPT-11 hydrolase activity by CES2 by 15.4 and 50.9%, respectively (data not shown), and 24 mM HCl also inhibited the CPT-11 hydrolase activity by CES2 by 33.2% (data not shown). If the drugs were dissolved in their solvents, the inhibition rate was calculated as percent inhibition of control activity in the presence of the respective solvents. The activity by recombinant CES2 was strongly inhibited by simvastatin (percentage of control: 0.0%), lovastatin (15.9%), and fenofibrate (0.1%), but the thiazolidinediones did not show strong inhibition (percentage of control: pioglitazone, 53.4%; rosiglitazone, 62.2%; and troglitazone, 50.7%). As with simvastatin and lovastatin, fenofibrate is also rapidly hydrolyzed to fenofibric acid, an active metabolite, in vivo in humans (Weil et al., 1990). Therefore, the inhibitory effects of fenofibric acid as well as those of simvastatin hydroxy acid and lovastatin hydroxy acid on the activity were examined, but they showed only slight inhibitory effects (Fig. 3).

Inhibition Constant and Inhibition Patterns of CPT-11 Hydrolase Activity by Recombinant Human CES2, HLM, and HJM. The $K_i$ values and inhibition patterns of simvastatin and fenofibrate showing strong inhibition of the CPT-11 hydrolase activity by recombinant CES2, HLM, and HJM were determined, and representative Lineeweaver-Burk plots are shown in Fig. 4. The $K_i$ values of simvastatin and fenofibrate for recombinant CES2 were $0.67 \pm 0.09$ and $0.04 \pm 0.01 \mu M$ with noncompetitive- and competitive-type inhibition, respectively. The $K_i$ values of simvastatin and fenofibrate for HLM were $1.85 \pm 0.28$ and $87.7 \pm 12.0 \mu M$, respectively, with non-competitive inhibition. The $K_i$ values of simvastatin and fenofibrate for HJM were $3.67 \pm 0.49$ and $0.50 \pm 0.06 \mu M$ with noncompetitive- and competitive-type inhibition, respectively. Thus, simvastatin showed relatively low $K_i$ values for the CPT-11 hydrolase activities by recombinant CES2, HJM, and HJM. In contrast, the $K_i$ value of fenofibrate for the activity in HLM was

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**Fig. 4.** Inhibitory effects of simvastatin (A, C, and E) and fenofibrate (B, D, and F) on the CPT-11 hydrolase activities by recombinant CES2 (A and B), HLM (C and D), and HJM (E and F). Each data point represents the mean of duplicate determinations. The $V_{max}$, $K_m$, and $K_i$ values represent the mean ± S.E.
approximately 2000- and 175-fold higher than those by recombinant CES2 and HJM, respectively.

**IC\(_{50}\) Value of p-Nitrophenyl Acetate Hydrolyase Activities by Recombinant Human CES1A1 and CES2.** Troglitazone and simvastatin strongly inhibited the imidapril hydrolyase activity by recombinant CES1A1 (Fig. 1), whereas simvastatin and fenofibrate strongly inhibited the CPT-11 hydrolyase activity by recombinant CES2 (Fig. 3). To compare the inhibitory effects of simvastatin, troglitazone, and fenofibrate on the CES1A1 and CES2 enzyme activities, the IC\(_{50}\) values of p-nitrophenyl acetate hydrolyase activity, which is catalyzed by both CES1A1 and CES2, were determined (Fig. 5). The p-nitrophenyl acetate hydrolyase activity was measured at a substrate concentration of 100 \(\mu M\), which was similar to the \(K_m\) values by recombinant CES1A1 and CES2 (150.8 \(\pm\) 17.8 and 123.7 \(\pm\) 13.3 \(\mu M\), respectively) (data not shown). In a preliminary study, it was confirmed that mock-transfected SF21 cell homogenates showed substantially lower p-nitrophenyl acetate hydrolyase activity [30 nmol/(min \cdot mg)] than CES1A1 and CES2 [542 and 300 nmol/(min \cdot mg), respectively]. Therefore, the content of p-nitrophenol, a hydrolyzed metabolite of p-nitrophenyl acetate, in the mixture incubated with mock-transfected SF21 cell homogenates was subtracted from those with recombinant CES1A1 and CES2 to correct the activity. The IC\(_{50}\) values of simvastatin and troglitazone for recombinant CES1A1 were 0.76 and 3.30 \(\mu M\), respectively, but that of fenofibrate was \(>2.0\) \(\mu M\). On the other hand, the IC\(_{50}\) values of simvastatin and fenofibrate for recombinant CES2 were \(<1.0\) \(\mu M\) (0.78 and 0.22 \(\mu M\), respectively), but that of troglitazone for recombinant CES2 (28.90 \(\mu M\)) was higher than that for recombinant CES1A1. Thus, the inhibitory effects of troglitazone and fenofibrate on CES1A1 and CES2 were different.

**Discussion**

Human CES plays important roles in the activation of a variety of prodrugs. In particular, most ACE inhibitors such as imidapril and temocapril are selectively biotransformed by CES1A into their pharmacologically active forms (Takai et al., 1997). Because patients with diabetes and hyperlipidemia frequently have hypertension and liver failure, it is possible that some patients are concurrently prescribed ACE inhibitors and antihyperlipidemic and/or antidiabetic drugs. In this study, we found that lactone-ring containing statins such as simvastatin and lovastatin strongly inhibited CES1A1 enzyme activity, whereas statins with an open acid form such as pravastatin and fluvastatin did not show strong inhibition of CES1A1 enzyme activity. In support of this result, simvastatin hydroxy acid and lovastatin hydroxy acid, which are the hydrolyzed metabolites of simvastatin and lovastatin, respectively, also did not show strong inhibition of CES1A1 enzyme activity. These results suggested that the lactone rings in simvastatin and lovastatin are important for inhibition of the CES1A enzyme activity. Fleming et al. (2005) reported that mevasstatin, which contains a lactone ring, inhibited \(\alpha\)-naphthyl acetate hydrolysis by CES1A (\(K_i\); 20.8 \(\mu M\)). Thus, it was considered that the concomitant use of simvastatin and lovastatin possibly attenuates the drug efficacy of ACE inhibitors via CES1A inhibition. However, in contrast to that in vitro study, the lack of interaction in vivo in human between enalapril and simvastatin (Shionoiri, 1993) and between ramipril and simvastatin was reported (Meyer et al., 1994). In vivo drug-drug interactions can be quantitatively predicted by comparing the maximum value of the unbound concentration at the inlet to the liver (\(I_{\text{inlet, u, max}}\)) estimated using pharmacokinetic data and the value of \(K_i\) obtained in vitro (Ito et al., 1998). \(I_{\text{inlet, u, max}}\) values were calculated using the equation as follows:

\[
I_{\text{inlet, u, max}} = f_u \times \{C_{\text{max}} + (k_d \times \text{dose} \times f_A/Q_h)\}
\]

where \(f_u\) is the unbound fraction in the blood, \(C_{\text{max}}\) is the maximum concentration in the blood, \(k_d\) is the first-order rate constant for gastrointestinal absorption, \(f_A\) is the fraction absorbed from the gastrointestinal tract into the portal vein, and \(Q_h\) is the hepatic blood flow.
rate. The $I_{\text{d}_{\text{inlet}}, \text{u}_{\text{max}}}$ value of simvastatin was estimated to be 0.11 μM after oral dosing at 40 mg. Because the $I_{\text{d}_{\text{inlet}}, \text{u}_{\text{max}}}$ value of simvastatin was lower than the $K_i$ value for the imidapril hydrolase activity in HLM (0.76 ± 0.06 μM), simvastatin may have a low inhibitory potential on the imidapril hydrolase activity in vivo in human. In addition to simvastatin and lovastatin, the thiazolidinediones also showed relatively strong inhibition on the imidapril hydrolase activity by CES1A1. Although troglitazone showed the strongest inhibition of activity among the drugs, it was withdrawn from commercial distribution after the U.S. Food and Drug Administration identified unacceptably high rates of acute liver failure. The $I_{\text{d}_{\text{inlet}}, \text{u}_{\text{max}}}$ value of troglitazone was estimated to be approximately 2.0 μM after oral dosing at 400 mg. Rosiglitazone and pioglitazone, which are commercially distributed, also inhibited the imidapril hydrolase activity, but their $I_{\text{d}_{\text{inlet}}, \text{u}_{\text{max}}}$ values were estimated to be 0.02 and 0.14 μM after oral dosing at 30 mg, respectively. These values are much lower than the $K_i$ value of troglitazone for imidapril hydrolase activity in HLM (5.64 ± 0.23 μM). Collectively, simvastatin, lovastatin, rosiglitazone, and pioglitazone may not affect imidapril hydrolase activity in vivo in humans, but we should take into consideration in drug development the fact that lactone ring-containing statins and thiazolidinediones preferentially inhibit the CES1A enzyme activity.

In this study, the inhibitory effect on CES2 enzyme activity was also evaluated. We found that the CPT-11 hydrolase activity by CES2 was strongly inhibited by fenofibrate as well as by simvastatin and lovastatin. However, the thiazolidinediones did not show strong inhibitory effects on the CES2 enzyme activity. Thus, the inhibitory effects of troglitazone and fenofibrate on CES1A1 and CES2 were different (Fig. 5). As in the case of imidapril hydrolysis, simvastatin may have a low inhibitory potential for the CPT-11 hydrolase activity in vivo because the $K_i$ values of simvastatin for the CPT-11 hydrolase activities in HLM and HJM (1.85 ± 0.28 and 3.67 ± 0.49 μM, respectively) were much higher than the $I_{\text{d}_{\text{inlet}}, \text{u}_{\text{max}}}$ value of simvastatin. The $K_i$ values of fenofibrate for the CPT-11 hydrolase activity were surprisingly quite different between HLM and HJM (87.7 ± 12.0 and 0.50 ± 0.06 μM, respectively). Fenofibrate is rapidly hydrolyzed to fenofibric acid after absorption from the gastrointestinal tract and is undetectable in plasma (Adkins and Faulds, 1997). We confirmed that fenofibrate was efficiently hydrolyzed in HLM [68.4 ± 4.9 nmol/(min · mg) at 10 μM fenofibrate], whereas it was not hydrolyzed in HJM (Supplemental Fig. 1A). This result was obvious from the finding that the recombinant CES1A1 used in this study could hydrolize fenofibrate [51.4 ± 3.0 nmol/(min · mg) at 10 μM fenofibrate], whereas CES2 could not (Supplemental Fig. 1B). Because fenofibrate acid did not show inhibitory effects on the CES2 enzyme activity (Fig. 3), the high $K_i$ value of fenofibrate for the CPT-11 hydrolase activity in HLM would be due to the efficient decrease of fenofibrate. Thus, CES2 may be inhibited by fenofibrate in the gastrointestinal tract because it is the first organ exposed to drugs after oral dosing.

In conclusion, we found that lactone ring-containing statins and thiazolidinediones showed strong inhibitory effects on CES1A1 enzyme activity, whereas CES2 enzyme activity was strongly inhibited by fenofibrate as well as by lactone ring-containing statins. In this study, antihyperlipidemic and antiangiogenic drugs were focused on in inhibition analyses of CES enzyme activity because CES1A is responsible for the biotransformation of a variety of ACE inhibitors. However, CES enzymes are involved in the biotransformation of not only ACE inhibitors but also many prodrugs. This study should provide useful information for the prediction of drug-drug interactions.

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References


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**Supplemental Fig. 1.** Fenofibrate hydrolase activities (A) in HLM and HJM, and (B) by CES1A1 and CES2. The activity was determined as follows: a typical incubation mixture (final volume of 0.2 ml) contained 100 mM potassium phosphate buffer (pH 7.4), and enzyme sources (0.2 mg/ml). Each data point represents the mean ± SD of the triplicate determinations. The reaction was initiated by the addition of 10 µM fenofibrate after 2-min preincubation at 37˚C. After the 15-sec incubation at 37˚C, the reaction was terminated by the addition of 200 µl of ice-cold acetonitrile. After removal of the protein by centrifugation at 9,500 g for 5 min, a 50 µl portion of the supernatant was subjected to HPLC. The HPLC analysis was performed using an L-7199 pump (Hitachi, Tokyo, Japan), an L-7200 autosampler (Hitachi), an L-7405 UV detector (Hitachi), and a D-2500 chromato-integrator (Hitachi) equipped with a CapcellPak C18 UG 120 coulmn (5-µm particle size, 4.6 mm i.d.x150 mm; Shideido, Tokyo, Japan). The eluent was monitored at 254 nm. The mobile phase was 60% methanol containing 20 mM citric acid (pH 3.7). the flow rate was 1.0 ml/min. The column temperature was 35˚C. The quantification of fenofibric acid was performed by comparing the HPLC peak height with that of an authentic standard. Each coulmn represents the mean ± SD of triplicate determinations.