Allosteric Kinetics of Human Carboxylesterase 1: Species Differences and Interindividual Variability

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ABSTRACT: Esterified drugs such as imidapril, derapril, and oxybutynin hydrolyzed by carboxylesterase 1 (CES1) are extensively used in clinical practice. The kinetics using the CES1 substrates have not fully clarified, especially concerning species and tissue differences. In the present study, we performed the kinetic analyses in humans and rats in order to clarify these differences. The imidaprilat formation from imidapril exhibited sigmoidal kinetics in human liver microsomes (HLM) and cytosol (HLC) but Michaelis-Menten kinetics in rat liver microsomes and cytosol. The 2-cyclohexyl-2-phenylglycolic acid (CPGA) formation from oxybutynin were not detected in enzyme sources from rats, although HLM showed high activity. The kinetics were clarified to be different among species, tissues, and preparations. In individual HLM and HLC, there was large interindividual variability in imidaprilat (31- and 24-fold) and CPGA formations (15- and 9-fold). Imidaprilat formations exhibited Michaelis-Menten kinetics in HLM and HLC with high activity but sigmoidal kinetics in those with low activity. CPGA formations showed sigmoidal kinetics in high activity HLM but Michaelis-Menten kinetics in HLM with low activity. We revealed that the kinetics were different between individuals. These results could be useful for understanding interindividual variability and for the development of oral prodrugs. © 2008 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 97:5434–5445, 2008

Keywords: phase I enzyme; prodrugs; enzyme kinetics; phase I metabolism

INTRODUCTION

Carboxylesterase (CES) is one of the phase I drug metabolizing enzymes and is responsible for the hydrolysis of many ester- and amido-type compounds. CES belongs to a serine hydrolase superfamily and is expressed in various mammalian tissues.[1] Mammalian CESs are mainly expressed in liver and small intestine.[2] Recently, human CES was clarified to be expressed in cytosol as well as microsomes in our laboratory.[3] In humans, there are two major isoforms, CES1 and CES2. The human CES1 gene spans approximately 30 kb on chromosome 16q13-q22.1 and has 14 exons.[4] CES1 is mainly expressed in the liver but also in the small intestine at low expression levels.[5] CES1 plays an important role in the metabolism and bioactivation of esterified prodrugs such as capecitabine,[3] imidapril,[6] methylphenidate,[7] and oseltamivir.[8] Esterification is one of the beneficial approaches to increase the oral bioavailability or reduce the adverse reactions of drugs. Therefore, CES1 can be a major

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Abbreviations: CES, carboxylesterase; CPGA, 2-cyclohexyl-2-phenylglycolic acid; HLM, human liver microsomes; HLC, human liver cytosol; HJM, human jejunum microsomes; HJC, human jejunum cytosol; RLM, rat liver microsomes; RLC, rat liver cytosol; RJM, rat jejunum microsomes; RJC, rat jejunum cytosol; LC-MS/MS, liquid chromatography-tandem mass spectrometry; BNPP, bis (*p*-nitrophenyl) phosphate; CYP, cytochrome P450; SNP, single nucleotide polymorphism.

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determinant of the pharmacological and toxicological effects of prodrugs. Understanding the CES function is necessary to predict the pharmacokinetics of prodrugs. Kinetic analyses are helpful to characterize the enzymatic properties of CES1. The typical CES1 substrates are imidapril, derapril, and oxybutynin, which are clinically used in Japan. Imidapril and derapril are angiotensin-converting enzyme inhibitors and oxybutynin is an anticholinergic drug. Imidapril, derapril, and oxybutynin are hydrolyzed to imidaprilat,[9] deraprilat,[10] and 2-cyclohexyl-2-phenylglycolic acid (CPGA),[11] respectively. In the present study, we selected these three drugs as typical CES1 substrates.

Mammalian CES activities are present in various tissues such as liver, small intestine, lung and blood, and the liver shows the highest hydrolase activity.[12] Some compounds are hydrolyzed by mammalian CESs in microsomes from liver and intestine.[12] In comparison with human CES, there are many isoforms in rats and mice.[12] In addition, as reported by Li et al.,[13] CES was not present in human plasma although it was highly expressed in that of rodents indicating differences in the localization of CES expression between mammalians. Recently, species differences in the pyrethroid hydrolysis reaction have been reported.[14,15] However, sufficient kinetic analyses of the hydrolysis reaction by CES concerning species and tissue differences have not been performed. It is still unclear whether cytosolic CES exhibits similar kinetics to microsomal CES. For drug development, it is important to elucidate the species and tissue differences in CES kinetics. The purpose of the present study was to clarify the kinetics of CES1 hydrolase activity in human liver, human jejunum, rat liver, and rat jejunum. In addition, the differences in microsomal and cytosolic CES1 kinetics were investigated.

MATERIALS AND METHODS

Materials

Imidapril and imidaprilat (a major metabolite of imidapril) were kindly supplied by Tanabe Seiyaku (Osaka, Japan). Derapril was kindly provided by Takeda Pharmaceutical (Osaka, Japan). Oxybutynin, CPGA (a metabolite of oxybutynin), phenytoin, and clonazepam were obtained from Wako Pure Chemicals (Osaka, Japan). Pooled human liver microsomes (pooled HLM), pooled human liver cytosol (pooled HLC), microsomes from 13 individual human livers, and cytosol from 13 individual human livers were purchased from BD Gentest (Woburn, MA). Pooled human jejunum microsomes (pooled HJM) and cytosol (pooled HJC) were obtained from KAC (Shiga, Japan). All other chemicals and solvents were of analytical or the highest grade commercially available.

Preparation of Microsomes and Cytosol from Rat Liver or Jejunum

Wister rats, 7 weeks old, were obtained from Japan SLC, Inc. (Shizuoka, Japan). Pooled microsomes and cytosol from 5 rat livers (RLM and RLC) and jejunums (RJM and RJC) were prepared according to the method of Emoto et al.[16]

Imidaprilat Formation

Imidaprilat formation from imidapril was determined according to the method of Mabuchi et al.[17] with slight modifications. A typical incubation mixture (200 μL of total volume) contained the enzyme source and 100 mM tris(hydroxymethyl)aminomethane-HCl buffer (pH 7.4). After a 2-min preincubation at 37°C, the reaction was initiated by the addition of imidapril and then the mixture was incubated at 37°C for 30 min except for RLM and RLC (10 min). The reaction was terminated by adding 100 μL of ice-cold acetonitrile. After centrifugation at 9000*g* for 5 min, 10 μL of the supernatant were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) system.

LC was performed using an HP 1100 system including a binary pump, an automatic sampler, and a column oven (Agilent Technologies, Waldbronn, Germany), which was equipped with an Inertsil ODS-3 analytical column (2.1 × 100 mm; GL Science, Tokyo, Japan). The column temperature was 40°C and the flow rate was 0.2 mL/min. The mobile phase consisted of acetonitrile/10 mM ammonium formate, 20:80 (v/v). The LC was connected to a PE Sciex API 2000 tandem mass spectrometer (Applied Biosystems, Langen, Germany) operated in the positive electrospray ionization mode. The turbo gas was maintained at 500°C. Nitrogen was used as the nebulizing gas, turbo gas, and curtain gas at 40, 80, and 30 psi, respectively. Parent and/or fragment ions were filtered in the first quadrupole and dissociated in the collision cell using nitrogen as the collision

gas. The collision energy was 27 V. The mass/charge (m/z) ion transitions were recorded in the multiple reaction monitoring mode: m/z 378.2 and 206.3 for imidaprilat. The retention time of imidaprilat was 2.2 min. The limit of detection for imidaprilat was 0.2 pmol. The limit of quantification in the reaction mixture was 20 nM with CV's $< 10\%$. In the preliminary study, a specific CES inhibitor, bis (p-nitrophenyl) phosphate (BNPP) (300 μM) inhibited the imidaprilat formation with all enzyme sources at 150 μM imidapril. A CES2 inhibitor, loperamide (20 and 200 μM) did not inhibit the imidaprilat formation in pooled HLM and HLC at 10 and 100 μM imidapril.

Deraprilat Formation

Deraprilat formation from derapril was determined according to the method of Ito et al.[18] with slight modifications. A typical incubation mixture (200 μL of total volume) contained the enzyme source and 100 mM tris(hydroxymethyl)aminomethane-HCl buffer (pH 7.4). Derapril was dissolved in methanol. The final concentration of methanol in the reaction mixture was $<1.0\%$. After a 2-min preincubation at 37°C, the reaction was initiated by the addition of derapril and then the mixture was incubated for 30 min at 37°C. The reaction was terminated by adding 200 μL of ice-cold acetonitrile. Clonazepam (1 nmol) was added as an internal standard. After centrifugation at 9000g for 5 min, 50 μL of the supernatant was subjected to high-performance liquid chromatography equipped with a CAPCELL PAK CN UG120 analytical column (4.6×150 mm; Shiseido, Tokyo, Japan). The eluent was monitored at 210 nm with a noise-base clean Uni-3 (Union, Gunma, Japan). Deraprilat was quantified using a standard curve of derapril because authentic deraprilat could not be obtained. The column temperature was 35°C and the flow rate was 1.0 mL/min. The mobile phase consisted of acetonitrile/100 mM KH_2PO_4 (pH 3.0), 25:75 (v/v). The retention times of deraprilat and clonazepam were 6.1 and 11.4 min, respectively. In the preliminary study, BNPP (20 μM) inhibited the deraprilat formation with all enzyme sources at 10 μM derapril.

CPGA Formation

CPGA formation from oxybutynin was determined according to the method of Malcolm et al.[19] with slight modifications. A typical incubation mixture (200 μL of total volume) contained the enzyme source and 100 mM potassium phosphate buffer (pH 7.4). After a 2-min preincubation at 37°C, the reaction was initiated by the addition of oxybutynin and then the mixture was incubated for 30 min at 37°C. The reaction was terminated by adding 200 μL of ice-cold acetonitrile. Phenytoin (1 nmol) was added as an internal standard. After centrifugation at 9000g for 5 min, 90 μL of the supernatant was subjected to high-performance liquid chromatography equipped with a Develosil C30-UG-5 analytical column (4.6×150 mm; Nomura chemical, Aichi, Japan). The eluent was monitored at 220 nm. The column temperature was 35°C and the flow rate was 1.0 mL/min. The mobile phase consisted of acetonitrile/20 mM potassium phosphate buffer (pH 5.0), 40:60 (v/v). The retention times of CPGA and phenytoin were 6.2 and 11.1 min, respectively. The limit of detection for CPGA was 20 pmol. In the preliminary study, BNPP (5 μM) inhibited the CPGA formation with all enzyme sources at 200 μM oxybutynin. Loperamide (20 and 200 μM) did not inhibit the CPGA formation in pooled HLM and HLC at 10 and 100 μM oxybutynin.

Kinetic Analyses of Imidaprilat, Deraprilat, and CPGA Formation

The kinetic analyses were performed using pooled HLM, pooled HLC, RLM, RLC, RJM, and RJC. When the kinetic parameters were determined, the substrate concentrations ranged from 2 to 500 μM for imidapril, from 5 to 1000 μM for derapril, and from 4 to 500 μM for oxybutynin. The imidaprilat formation in HJM and that in HJC were examined at 200 μM imidapril. The protein concentrations of microsomes and cytosol were 0.2 and 1.0 mg/mL, respectively. The protein concentrations of RLM and RLC in the imidaprilat formations were 0.01 and 0.05 mg/mL, respectively. In the preliminary study, the linearity of the protein concentrations and incubation times were confirmed. The kinetic parameters were estimated from the fitted curves using a KaleidaGraph computer program (Synergy, Reading, PA) designed for nonlinear regression analysis. The following equations were used: Michaelis-Menten equation: $V = V_{max} \times [S]/(K_m + [S])$ and Hill equation: $V = (V_{max} \times [S]^n)/(S_{50}^n + [S]^n)$, where V is the velocity of the reaction, S is the substrate

concentration, K_m is the Michaelis-Menten constant, V_{max} is the maximum velocity, S_{50} is the substrate concentration showing the half-V_{max}, and n is Hill coefficient. Intrinsic clearance was calculated as V_{max}/K_m for the Michaelis-Menten kinetics. For the sigmoidal kinetics, the maximum clearance (CL_{max}) was calculated as $V_{max} \times (n-1)/S_{50} \times n(n-1)^{1/n}$ to estimate the highest clearance.[20]

Species and Tissue Differences in CPGA Formation

The CPGA formation was examined at 200 μM oxybutynin in pooled HLM, pooled HLC, pooled HJM, pooled HJC, RLM, RLC, RJM, and RJC. The protein concentrations of microsomes and cytosol were 0.5 and 2.5 mg/mL, respectively.

Interindividual Variability in Imidaprilat and CPGA Formation

Imidaprilat formation and that of CPGA were examined at 20 μM imidapril and 20 μM oxybutynin in 13 HLM and 13 HLC. The protein concentrations of HLM and HLC were 0.2 and 1.0 mg/mL, respectively. For the kinetic analysis of the imidaprilat formation, the individual HLM and HLC that could yield sufficient volumes were used as follows: HLM with high activities (HG3 and HH31), HLM with low activities (HH47 and HG64), HLC with high activities (HH18 and HG89), and HLC with low activities (HH47 and HH31). For the kinetic analysis of the CPGA formation, the individual HLM and HLC were used as follows: HLM and HLC with high activities (HG3 and HH18) and HLM and HLC with low activities (HH47 and HG64).

Statistical Analysis

The correlations between imidaprilat and CPGA formation in individual HLM or HLC were determined by Pearson's product moment method. A p value of less than 0.05 was considered statistically significant.

RESULTS

Kinetic Analysis of Imidaprilat Formation in Human Liver, Rat Liver, and Rat Jejunum

To investigate the differences in the CES1 characteristics between humans and rats or liver and jejunum, kinetic analyses of the imidaprilat formation were carried out in pooled HLM, pooled HLC, RLM, RLC, RJM, and RJC. Kinetic parameters were different between human and rat livers or rat livers and jejunums (Tab. 1). As shown in Figure 1, Eadie-Hofstee plots in pooled HLM and HLC exhibited curved lines at low substrate concentrations although the Hill coefficients were close to 1.0. The apparent S_{50} value in pooled HLM was lower than that of pooled HLC (Tab. 1). The kinetics in RLM and RLC fitted to the Michaelis-Menten equation and the V_{max}/K_m value of RLM was approximately two-fold higher

Table 1. Kinetic Parameters of Imidaprilat Formation in HLM, HLC, RLM, RLC, RJM, and RJC

	High Affinity Isoform			Low Affinity Isoform			
Enzyme Source	K_m (μM)	V_{max} (nmol/min/mg)	V_{max}/K_m (μL/min/mg)	K_m (μM)	V_{max} (nmol/min/mg)	V_{max}/K_m (μL/min/mg)	n
HLM	—	—	—	245[a]	2.4	7.4[b]	1.1 (1.1[c])
HLC	—	—	—	397[a]	0.2	0.5[b]	1.0 (1.1[c])
HJM	—	ND	—	—	—	—	—
HJC	—	ND	—	—	—	—	—
RLM	52	76.6	1459	—	—	—	—
RLC	33	22.4	677	—	—	—	—
RJM	41	1.0	25	346	3.7	10.6	—
RJC	53	0.2	4	382	0.8	2.1	—

n, Hill coefficient calculated using all concentrations of imidapril.
ND; not detected.
[a] S_{50}.
[b] CL_{max}.
[c] Hill coefficient calculated below 100 μM imidapril.

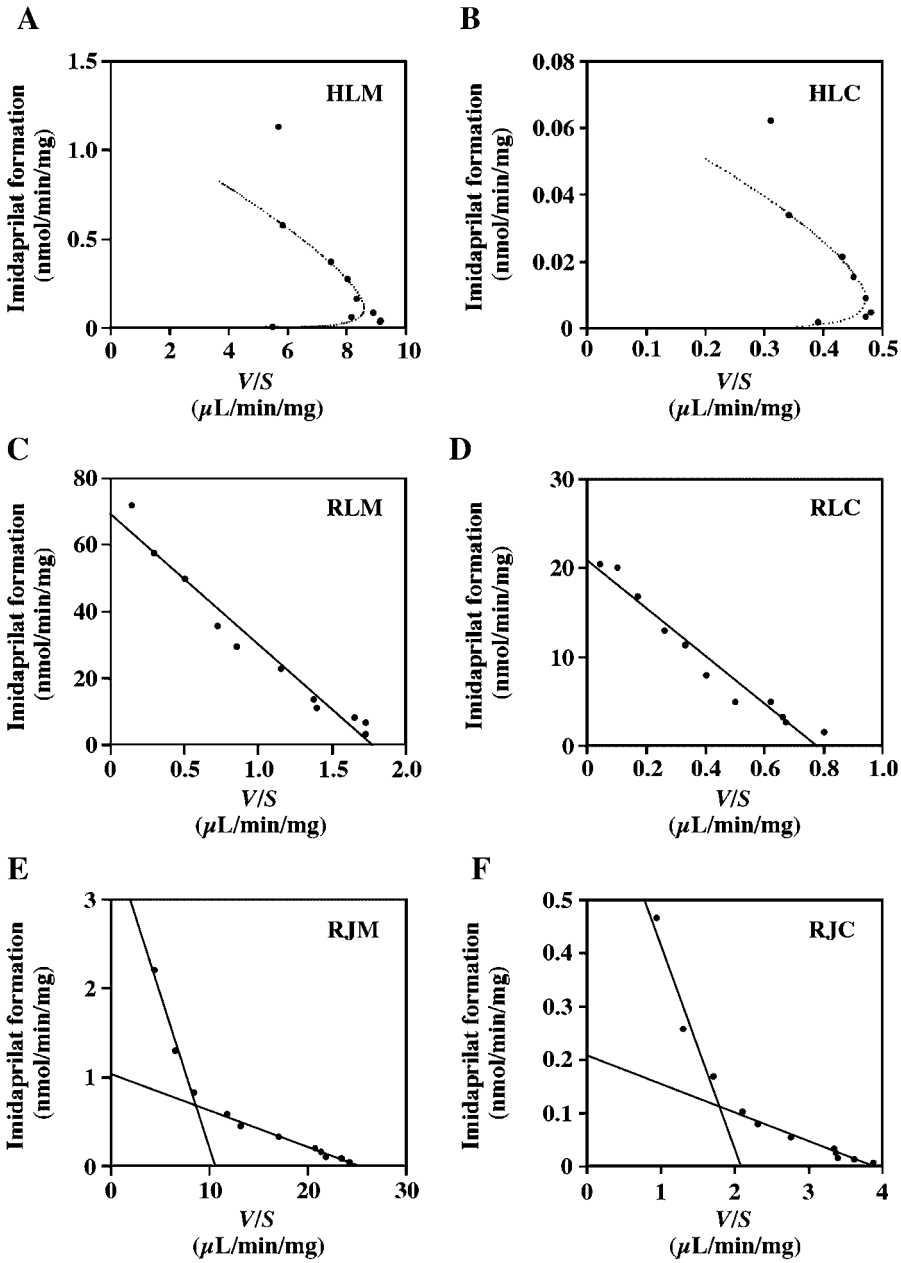

Figure 1. Kinetic analyses of imidaprilat formation catalyzed by pooled HLM (A), pooled HLC (B), RLM (C), RLC (D), RJM (E), and RJC (F). The concentration of imidapril was 2–500 μM. The curved lines (A and B) were estimated by the concentration below 100 μM imidapril. Each data point represents the mean of duplicate determinations.

than that of RLC. RLM and RLC hydrolyzed imidapril more efficiently than pooled HLM and HLC. RJM and RJC appeared bi-phasic in the Eadie-Hofstee plots. The apparent K_m values in RJM and RJC were similar in high and low affinity isoforms, but the V_{max} values of both isoforms in RJM were approximately five-fold higher than those in RJC. Imidaprilat formation in pooled HJM and HJC could not be detected.

Interindividual Variability of Imidaprilat Formation in HLM and HLC

Imidaprilat formation in microsomes from 13 human livers and in cytosol from 13 human livers was determined at 20 μM imidapril (Fig. 2). The interindividual variability of imidaprilat formation in HLM and HLC was 31- and 24-fold, respectively. The imidaprilat formation in HLM

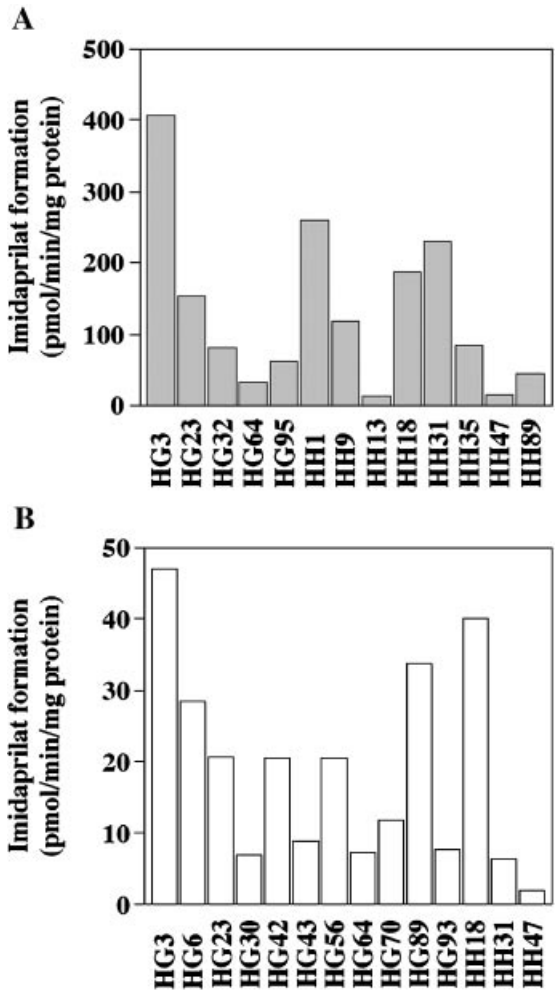

Figure 2. Interindividual variability of imidaprilat formation in 13 HLM (A) and in 13 HLC (B). The concentration of imidapril was 20 μM. Each column represents the mean of duplicate determinations.

ranged from 13.2 pmol/min/mg protein in HH13 to 406.9 pmol/min/mg protein in HG3. On the other hand, the imidaprilat formation in HLC ranged from 2.0 pmol/min/mg protein in HH47 to 47.1 pmol/min/mg protein in HG3. The imidaprilat formation between individual HLM and HLC from the same donors was well-correlated ($n = 6$, $r = 0.77$).

Individual Kinetic Analysis of Imidaprilat Formation in HLM and HLC

Kinetic analyses of the imidaprilat formation were performed in some individual HLM and HLC (Tab. 2). The imidaprilat formation in both HLM and HLC with high activities fitted to the Michaelis-Menten equation, whereas those with low activities fitted curved lines in the Eadie-Hofstee plots (Fig. 3). The K_m values in HLM with high activities were higher than the S_{50} values in HLM with low activities.

Kinetic Analysis of Deraprilat Formation in Human Liver, Rat Liver, and Rat Jejunum

To investigate the effects of structural diversity on the kinetic analysis, kinetic analyses of the deraprilat formation were performed in pooled HLM, pooled HLC, RLM, RLC, RJM, and RJC. The kinetic parameters of the deraprilat formation are shown in Table 3. The Eadie-Hofstee plot of the deraprilat formation was bi-phasic in pooled HLM whereas it was curved in pooled HLC. RLM also appeared bi-phasic in the Eadie-Hofstee plot. The V_{max}/K_m values of high and low affinity isoforms in RLM were higher than those in pooled

Table 2. Kinetic Parameters of Imidaprilat Formation in Individual HLM and HLC

Enzyme Source	Activity	Donor	K_m (μM)	V_{max} (pmol/min/mg)	V_{max}/K_m (μL/min/mg)	n	Model
HLM	High	HG3	119	2310	19.4	—	Michaelis-Menten
		HH31	175	1963	11.2	—	
	Low	HH47	15[a]	48	1.7[b]	1.5 (1.5[c])	Hill
		HG64	84[a]	349	2.9[b]	1.1 (1.1[c])	
HLC	High	HH18	444	905	2.0	—	Michaelis-Menten
		HG89	205	305	1.5	—	
	Low	HH47	420[a]	56	0.1[b]	1.0 (1.2[c])	Hill
		HH31	340[a]	124	0.4[b]	1.0 (1.2[c])	

n, Hill coefficient calculated using all concentrations of imidapril.
[a] S_{50}.
[b] CL_{max}.
[c] Hill coefficient calculated below 100 μM imidapril.

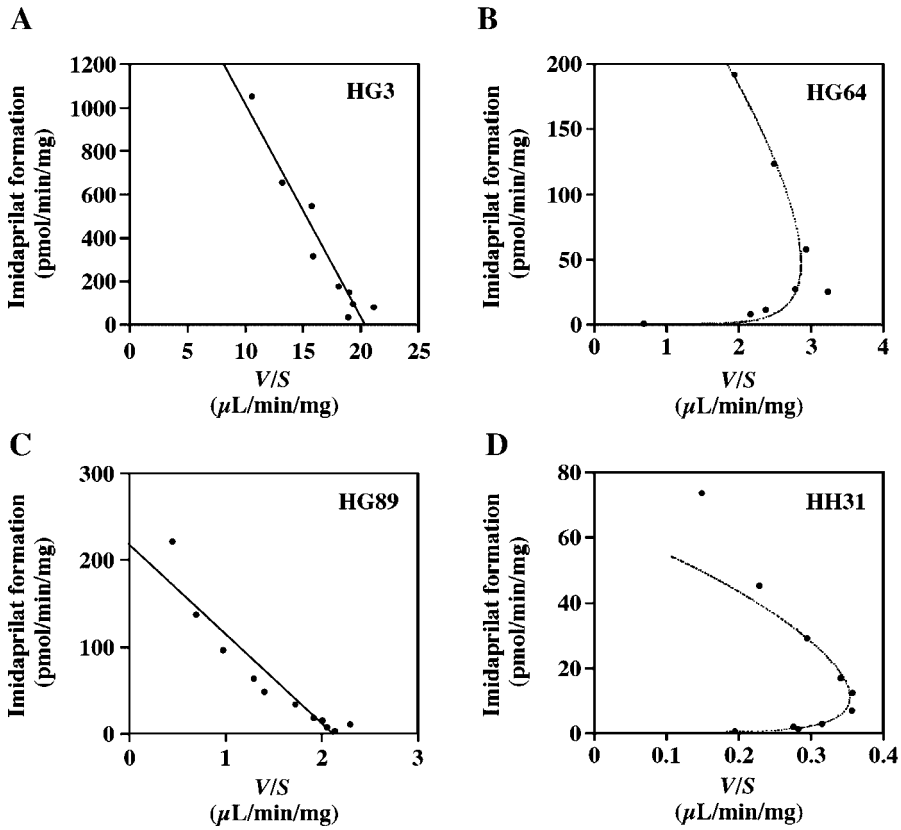

Figure 3. Kinetic analyses of imidaprilat formation catalyzed by individual HLM and HLC. (A) HLM with high activity (HG3); (B) HLM with low activity (HG64); (C) HLC with high activity (HG89); (D) HLC with low activity (HH31). The concentration of imidapril was 2–500 μM. The curved lines (B and D) were estimated by the concentration below 100 μM imidapril. Each point represents the mean of duplicate determinations.

HLM. Eadie-Hofstee plots in RJM and RJC exhibited curved lines at low substrate concentrations ($n = 1.2$). The apparent S_{50} value of RJM was similar to that of RJC, but the CL_{max} value in RJM was higher than that of RJC.

CPGA Formation in Humans and Rats

To clarify the differences of CES1 activity between humans and rats or between liver and jejunum, the CPGA formation was determined at 200 μM

Table 3. Kinetic Parameters of Deraprilat Formation in HLM, HLC, RLM, RLC, RJM, and RJC

	High Affinity Isoform			Low Affinity Isoform			
Enzyme Source	K_m (μM)	V_{max} (nmol/min/mg)	V_{max}/K_m (μL/min/mg)	K_m (μM)	V_{max} (nmol/min/mg)	V_{max}/K_m (μL/min/mg)	n^c
HLM	5.6	1.1	193	460	16.7	36.4	—
HLC	—	—	—	1118[a]	2.8	2.0[b]	1.1
RLM	0.2	0.1	700	33	1.6	49.5	—
RLC	1.7	0.1	29	101	0.8	7.5	—
RJM	—	—	—	28[a]	1.5	32.9[b]	1.2
RJC	—	—	—	41[a]	0.1	1.5[b]	1.2

[a] S_{50}.
[b] CL_{max}.
[c] n, Hill coefficient.

oxybutynin in pooled HLM, pooled HLC, pooled HJM, pooled HJC, RLM, RLC, RJM, and RJC. The pooled HLM showed higher activity (773.5 pmol/min/mg protein) than pooled HLC (38.2 pmol/min/mg protein), pooled HJM (36.8 pmol/min/mg protein), and pooled HJC (5.4 pmol/min/mg protein). No activities were detected in RLM, RLC, RJM, and RJC. Kinetic analyses of the CPGA formation in pooled HLM and HLC were performed and the Eadie-Hofstee plots showed curved lines when the Hill coefficients calculated below 100 μM were 1.5 and 1.1, respectively. As fitted to the Hill equation, the apparent S_{50} values in pooled HLM and HLC were 119.7 and 146.3 μM, respectively and the V_{max} values were 739.1 and 92.4 pmol/min/mg protein, respectively.

Interindividual Variability of CPGA Formation in HLM and HLC

As shown in Figure 2, 15- and 9-fold interindividual variability in the CPGA formation was observed in HLM and HLC, respectively. In HLM, the CPGA formation at 20 μM oxybutynin showed the highest in HG3 (375.3 pmol/min/mg protein) and the lowest in HH47 (25.1 pmol/min/mg protein). In HLC, the CPGA formation showed the highest in HG3 (72.8 pmol/min/mg protein) and the lowest in HG64 (7.6 pmol/min/mg protein) (Fig. 4). The CPGA formation was significantly correlated between individual HLM and HLC from the same donors ($n = 6$, $r = 0.89$, $p < 0.05$). Moreover, the CPGA formation was significantly correlated with the imidaprilat formation in both HLM ($r = 0.98, p < 0.001$) and HLC ($r = 0.97$, $p < 0.001$).

Kinetic Analysis of CPGA Formation in Individual HLM and HLC

Kinetic analyses of CPGA formation were performed in individual HLM and HLC (Tab. 4). The apparent K_m values of CPGA formation in HLM and HLC were similar. CPGA formation in HLM and HLC with high activities also exhibited curved lines in the Eadie-Hofstee plots (Fig. 5) and their Hill coefficients calculated below 100 μM showed 1.2 and 1.6, respectively. The Eadie-Hofstee plots in HLM with low activities were monophasic but those in HLC were curved.

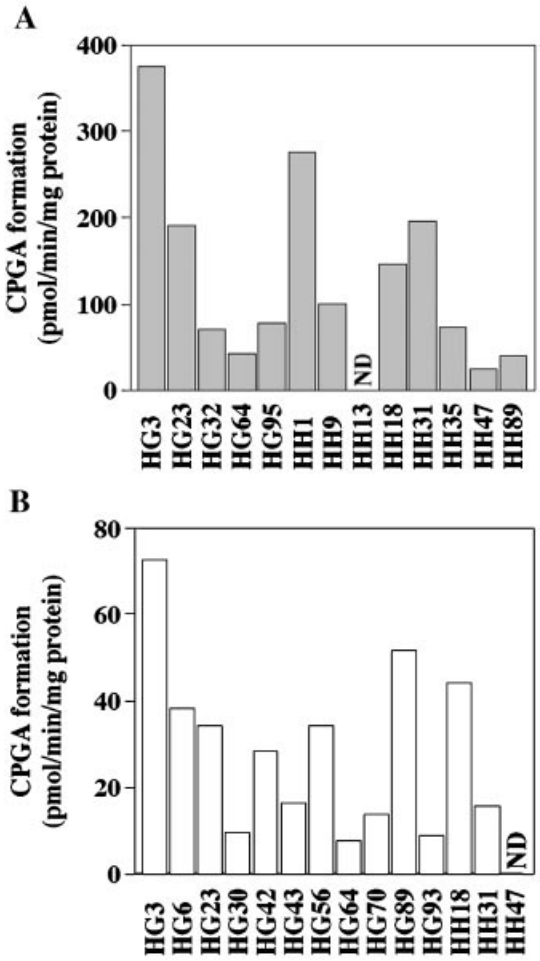

Figure 4. Interindividual variability of CPGA formation in 13 HLM (A) and in 13 HLC (B). The concentration of oxybutynin was 20 μM. Each column represents the mean of duplicate determinations. ND, not detected.

DISCUSSION

It is important to clarify the characteristics of drug metabolizing enzymes for understanding the *in vivo* pharmacokinetics. CES1 is involved in the metabolism of various prodrugs such as imidapril and capecitabine. To investigate the formation of an active metabolite from the prodrug would be important for drug development. However, sufficient information on the enzymatic properties of CES1 is not available. In the preliminary study, the imidaprilat and CPGA formation in HLM and HLC were inhibited by BNPP, but not by loperamide which was a potent CES2 inhibitor,[21] indicating that CES1 would be involved in these hydrolysis. In the present study, kinetic analyses of drugs hydrolyzed by CES1 were performed in humans and rats.

Table 4. Kinetic Parameters of CPGA Formation in Individual HLM and HLC

Enzyme Source	Activity	Donor	K_m (μM)	V_{max} (pmol/min/mg)	V_{max}/K_m (μL/min/mg)	n	Model
HLM	High	HG3	75[a]	1187	10.0[b]	1.2 (1.2[c])	Hill
	Low	HH47	123	107	0.9	—	Michaelis-Menten
HLC	High	HH18	93[a]	283	2.9[b]	1.0 (1.6[c])	Hill
	Low	HG64	94[a]	38	0.3[b]	1.1 (1.5[c])	Hill

n, Hill coefficient calculated using all concentrations of oxybutynin.
[a] S_{50}.
[b] CL_{max}.
[c] Hill coefficient calculated below 100 μM oxybutynin.

In this study, it was first clarified that imidaprilat and CPGA formation showed sigmoidal kinetics in pooled HLM and HLC. The Eadie-Hofstee plots in pooled HLM and HLC were curved sharply at low substrate concentrations (less than 20 μM). The Hill coefficients were estimated to be close to 1.0 when we used all points ranging from 2 to 500 μM imidapril and from 5 to 500 μM oxybutynin. However, the Hill coefficients appeared higher when plotting the activities at only low substrate concentrations. For example, in the CPGA formation in pooled HLM, the Hill coefficient was 1.5 at from 5 to 100 μM oxybutynin. CES1 may behave differently at high substrate concentrations compared with low concentrations. Based on the Eadie-Hofstee plots, it is suggested that CES1 would function allosterically.

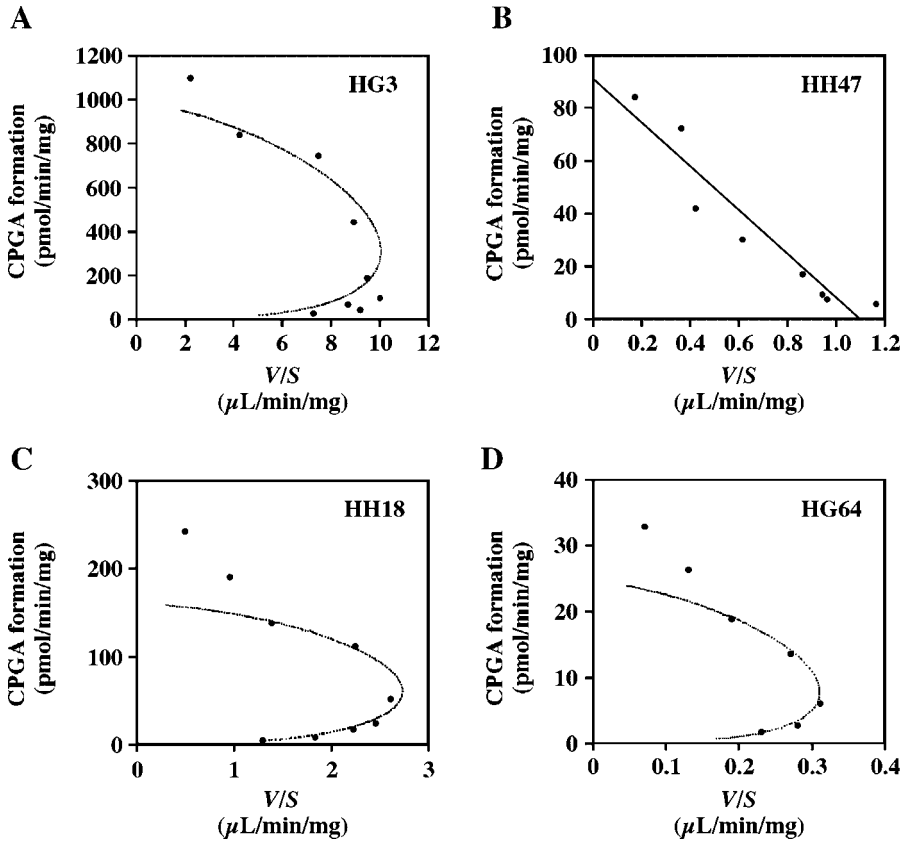

Figure 5. Kinetic analyses of CPGA formation catalyzed by individual HLM and HLC. (A) HLM with high activity (HG3); (B) HLM with low activity (HH47); (C) HLC with high activity (HH18); (D) HLC with low activity (HG64). The concentration of oxybutynin was 4–500 μM. The curved lines (A, C, and D) were estimated by the concentration below 100 μM oxybutynin. Each point represents the mean of duplicate determinations.

Imidaprilat formation in RLM and RLC exhibited Michaelis-Menten kinetics, suggesting that species difference exists. In rat CESs, many isoforms have been identified.[22] In the preliminary study, a specific CES inhibitor, BNPP, inhibited the imidaprilat formation in all enzyme sources from rats. Yamada et al.[23] reported that imidapril was hydrolyzed more efficiently by CES in rat liver than in other tissues. Thus, rat CES should be involved in imidapril metabolism. In the case of another angiotensin-converting enzyme inhibitor, derapril, the kinetics of deraprilat formation in all enzyme sources were different from those of imidaprilat formation. However, derapril has a similar chemical structure to imidapril. Derapril is more bulky than imidapril, thus such a difference might be responsible for the difference in kinetics. On the other hand, no CPGA formation in rats could be detected, suggesting that large species difference would also exist.

In both humans and rats, the V_{max}/K_m or CL_{max} values of the imidaprilat formation in liver were higher than those in jejunum. Human CES1 is mainly expressed in the liver but is found at low expression levels in the small intestine.[24] The differences in the enzymatic activities between liver and jejunum may be explained by the expression levels of CES1. In comparison with microsomes and cytosol in the present study, the V_{max}/K_m or CL_{max} values of imidaprilat formation in all microsomes were higher than those in cytosol. Our previous report indicated that the hydrolysis activity of cytosolic CES1 might be almost equal to that of microsomal CES when taking the protein concentrations into account.[3] Cytosolic proteins are approximately five-fold higher than microsomal proteins in human liver S9.[25] The contribution of cytosolic CES to hydrolysis may be as important as that of microsomal CES in both humans and rats. Recently, human CES1A1 (AB119997) and CES1A2 (AB119998) were registered in GenBank. Their coding regions showed high homology and they had four amino acid differences in their N-terminal regions.[26] Hosokawa et al.[27] speculated that CES1 genes had inverted duplication and were differently regulated at the transcriptional level. However, there are few reports on the differences in the structure, enzymatic activity, and substrate specificity between CES1A1 and CES1A2.

In the present study, there was large interindividual variability in the CES1 activities, which was consistent with previous reports.[3,28] In the imidaprilat formation, the Eadie-Hofstee plot in HLM with low activity was curved sharply around 20 μM, indicating that CES1 may show autoactivation kinetics. The kinetics of imidaprilat formation appeared different depending on the individuals. HLM and HLC with low activity exhibited sigmoidal kinetics in the imidaprilat formation. Since the serum concentration (79 nM) of imidapril in clinical doses (10 mg/day) is lower than the S_{50} value, sigmoidal kinetics may result in the low serum concentration in individuals in comparison with the Michaelis-Menten kinetics. The allosteric effects of CES1 could affect the interindividual variability, but further studies are needed to clarify this issue. There was large interindividual variability in the CPGA formation as in the imidaprilat formation. In individual HLM and HLC, the CPGA formation was significantly correlated with the imidaprilat formation (HLM, $r = 0.98$, $p < 0.001$; HLC, $r = 0.97$, $p < 0.001$), which has been reported to be specifically catalyzed by CES1.[6] Takai et al.[6] also demonstrated that oxybutynin was hydrolyzed by carboxylesterase with pI 4.5, which seems to be CES2, but loperamide did not inhibit the CPGA formation in pooled HLM and HLC in this study. Recombinant CESs are needed to identify the catalytic isoforms, but are not now available. In the present experimental condition, the CPGA formation would be catalyzed by human CES1 because of the significant correlation between the imidaprilat and CPGA formation. In individual HLM and HLC with high activities, the kinetics of the CPGA formation exhibited sigmoidal curves. However, imidaprilat formation in high activity individuals showed Michaelis-Menten kinetics, suggesting that CES1 may behave differently depending on the substrates.

Recently, crystal structures of human CES1 have been reported in complexes with several substrates.[29,30] In such reports, human CES1 contains three ligand binding sites, an active site, a side door, and a Z-site.[31] Bencharit et al.[32] reported that some compounds could bind to the Z-site and that the Z-site may function as an allosteric site, which may be related to the sigmoidal kinetics observed in the present study. Cytochrome P450 3A4 (CYP3A4) has a surface ligand binding site near its active site that may play a role in the recognition of substrates and in the allosteric behavior.[33] The surface ligand binding site of CYP3A4 may correspond to the Z-site of CES1.

Genetic polymorphisms of various drug metabolizing enzymes have been reported and many mutants can affect the catalytic activity.[34] In the case of CYP2B6, the G516T substitution in exon 4 altered the Michaelis-Menten kinetics to sigmoidal kinetics for 7-ethoxycoumarin *O*-deethylase activity.[35] Some single nucleotide polymorphisms (SNPs) can change the kinetic behavior. In the CES1 gene, several SNPs have been identified.[36] Geshi et al.[37] reported that a A(-816)C SNP on the CES1A2 promoter region might affect its transcriptional activity and the elevated responsiveness to imidapril. It is surmised that the genetic polymorphism of CES1 might be responsible for the allosteric effect, but further study is needed to clarify this issue.

In conclusion, species and tissue differences in the CES1 kinetics were elucidated. Moreover, we first clarified that CES1 exhibited sigmoidal kinetics depending on the substrates and the donors of the enzymes. Information on species and tissue differences in CES1 kinetics is important for drug development. For understanding the pharmacokinetics of a CES substrate, we should accumulate basic information concerning the enzyme characteristics. Because the allosteric effects of CES1 may be one of the determinants of the large interindividual variability in clinical doses, the allosteric mechanism should be clarified.

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