Characterization of Plasma Hydrolases and Their Contribution to Hydrolase Activities in the Plasma of Different Species

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In the drug discovery stages, esterification is frequently used to develop prodrugs and/or soft drugs. Inside the body, these drugs can be enzymatically converted to their preferred forms by esterases in liver, intestine and plasma. In some cases, this hydrolysis process is more rapidly occurs in plasma rather than liver and intestine. But plasma esterase activity is markedly different in between humans and other animals. To understand interspecies variation, it is important to know the identities and properties of the plasma esterases responsible for drug hydrolysis. Hence, current study was focused on plasma esterase activities and aimed to provide useful information regarding the unknown features of species differences and to propose a suitable animal model for predicting the pharmacokinetics of xenobiotics in human.

(1) Expression patterns of plasma esterases were revealed in between human plasma and the plasma of several other preclinical species such as monkey, dog, minipig, rabbit, mouse and rat. It was shown that PON and BChE are ubiquitous in all species, but are highly expressed in primates and dogs while CES was abundant in only rabbits, mice and rats. In addition to these esterases, albumin of all species showed weak hydrolase activity. Except these well-known four hydrolases, the presence of several unknown proteins which has hydrolyzing abilities was also observed in minipigs and mice.

(2) Interspecies variation was evaluated by the plasma hydrolase activities. Five types of compounds (PNPA, irinotecan, oseltamivir, temocapril and propranolol derivatives) were selected for activity analysis based on their structure differences. All substrates except irinotecan were rapidly hydrolyzed by CES of mouse and rat plasma. Although CES is highly expressed in rabbit plasma, only propranolol derivatives were rapidly hydrolyzed by its CES. In contrast, human, monkey, dog and minipig plasmas were barely hydrolyzed these substrates with their abundantly expressed BChE enzymes. But, only one exception was observed in monkey plasma in which *O*-valeryl-(R)-propranolol was rapidly

hydrolyzed by monkey plasma BChE even though the fact that human and monkey plasma BChEs shares 96% homology.

(3) Interspecies variations were further evaluated with an appropriate model substrate, aspirin. Human, monkey, and dog plasma hydrolyzed this substrate by their major hydrolases, PON, BChE and albumin. The slow hydrolysis in minipig plasma was confirmed to be due to its BChE. CES of rabbit, mouse and rat plasmas was determined to be the responsible enzyme for aspirin hydrolysis. Furthermore, the studies in order to confirm the responsible esterases in the aspirin hydrolysis activity were revealed the enhancing effect of divalent cations such as calcium and magnesium on BChE activities especially in human plasma. The increasing concentrations of divalent cations resulted in two phase of stimulating effects on the human plasma activity for aspirin hydrolysis.

(4) Detailed studies were performed to understand the divalent cation effect on human plasma purified BChE. An allosteric effect was observed during aspirin hydrolysis by binding of calcium. In addition to EF-hand binding site, a second binding site with lower binding affinity, which probably creates an extra hydrolyzing environment for aspirin and therefore makes more active form of BChE, was found. In contrast to its enhancing effect on aspirin hydrolysis, calcium was inhibited the hydrolase activity of BChE for BTCh, the well-known BChE substrate. Presence of different concentration-dependent hydrolyzing patterms with low and high affinities was observed on BChE during BTCh hydrolysis. It is found that at low concentrations of BTCh calcium causes an allosteric effect and changes the conformation of BChE to more inappropriate position but at high BTCh concentrations calcium addition did not cause that much conformational changes in BChE.

In this study, differences in substrate-specific hydrolase activities are found in animal's plasma. Human plasma was found to be closer to both monkey and dog plasma when esterase expression, hydrolyzing pattern and effect of divalent cations are all considered. Furthermore, human plasma BChE showed allosteric properties which caused by divalent cations and BTCh. Although the endogenous function of plasma BChE is unclear, its changeable conformation may lead us to discover its function. Therefore, the correlations between species differences and allosteric properties is complicated but yet should be considered when selecting animal models in the discovery stage of drugs.

Abbreviations

AChE	Acetylcholinesterase
ACN	Acetonitrile
BChE	Butyrylcholinesterase
BNPP	Bis-*p*-nitrophenyl phosphate
BTCh	Butyrylthiocholine iodide
CES	Carboxylesterases
DMSO	Dimethyl sulfoxide
DTNB	5', 5'-Dithio-bis (2-nitrobenzoic acid)
EDTA	Di-sodium dihydrogen ethylenediamine tetraacetate dihydrate
Em	Excitation wavelength
Ex	Emission wavelength
HEPES	2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethane sulfonic acid
HPLC	High-performance liquid chromatography
HSA	Human serum albumin
HuBChE	Butyrylcholinesterase from human serum
k_{cat}	Catalytic constant for the conversion of substrate to product
K_M	Michaelis constant
N.D.	Not detected
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate-buffered saline
PNP	*p*-Nitrophenol
PNPA	*p*-Nitrophenylacetate
PON	Paraoxonase
RSA	Rabbit serum albumin
S.D.	Standard deviation
U (unit)	1 U HuBChE hydrolyzes 1.0 μmole of butyrylcholine to choline and butyrate per min at pH 8.0 at 37 °C.
V_{max}	Maximum velocity

Contents

General Introduction

Esterified compounds are found everywhere in the human environment, as xenobiotics such as medicines and pesticides or as environmental chemicals and foodstuffs.[1,2] Their characteristics has been utilized in pharmaceutical development in the form of esterified prodrugs and/or soft drugs. Prodrugs are simple chemical derivatives that require only one or two transformation steps to yield their active drug[3-5] whereas the soft drugs are the opposites. Soft drugs are the active drugs that are designed to undergo a predictable metabolism to inactivate metabolites after exerting their therapeutic effect.[6,7] Inside the body, these compounds are metabolized into non-toxic or active compounds by esterases.[8,9] After years of extensive researches, liver and the small intestine are determined to be the main organs in the metabolism of these drugs.[10-16] However, activity in the plasma and red blood cells, especially during the systemic circulation, are also important. Red blood cells comprise various enzymes but most of the drugs cannot be metabolized by these enzymes. Although we will also need to characterized the activity of red blood cells, in the current study we have focused only on esterase activities in plasma.

The main esterases that present in the plasma are butyrylcholinesterase (BChE; EC 3.1.1.8), paraoxonase (PON; EC 3.1.8.1) and carboxylesterases (CES; EC 3.1.1.1). Acetylcholinesterase (AChE; EC 3.1.1.7) is also presented in the plasma, but only in negligible amounts.[17] As shown in Table 1, in addition to these esterases, albumin is abundantly present in the plasma.[17-19] Although albumin is a carrier protein rather than an esterase, human serum albumin (HSA) has been reported to have esterase-like activity with some substrates such as α- or β-naphthylacetate[20] and this esterase-like activity of HSA is reported to be due to its irreversible acylation in the one or more of 82 acetylated residues in total.[20,21]

Table 1. Major plasma esterases and their protein properties.

Plasma Esterases	Protein Properties	
Carboxylesterase (CES)	B-esterase Catalytic triad (S:E:H)	Monomeric glycoprotein (65-75kDa)
Butyrylcholinesterase (BChE)	B-esterase Catalytic triad (S:E:H)	Tetrameric glycoprotein (monomer; 85kDa)
Paraoxonase (PON)	A-esterase Catalytic diad (H:H)	Associated with high-density lipoprotein (43kDa)
Albumin	Carrier protein Esterase-like activity	Monomeric protein (66kDa)

Generally, esterases can be classified into three groups based on their substrate specificities, A-, B- and C-esterases. A-Esterases, e.g., PON can hydrolyze organophosphate esters while B-esterases, e.g., CES, BChE and AChE, are strongly inhibited by these compounds.[22] C-Esterases, e.g., acetylesterases, do not interact with organophosphates.[23] The A-esterase, PON, and the B-esterase, BChE, are ubiquitously expressed in the plasma of all animal species. Interestingly, although CES is present in the plasma of most rodents, it is not found in primates. It is known that CES isozymes have been classified into five subfamilies.[24] Among these families, CES1 is the main esterase that expressed in liver and other several organs whereas CES2 mainly expressed in small intestine and kidney. Unlike these two isozymes, plasma CES is grouped under CES1 family (CES1G) and is secreted into the blood from the liver.[24,25] CES1G family is lack of a consensus sequence for the retention in the endoplasmic reticulum and has higher molecular weight due to higher number of sugar chains.[24,25]

Both CES and BChE are members of the α/β hydrolase fold family.[26,27] They are also referred to as serine esterases, since serine (Ser) is essential to their catalytic activity.[27] Their mechanism of action is analogous to serine proteases.[27] In other words, although these enzymes show some selectivity for substrates, they all work by the same basic mechanism. They contain a catalytic triad consisting of three hydrogen bond connected amino acid residues. These amino acids are Ser, glutamate (Glu) and histidine (His). During the hydrolysis of substrates, Ser, Glu and His they act as a nucleophile, an acid, and a base,

respectively. Mechanism for ester hydrolysis by these serine esterases is shown in Figure 1.[1)]

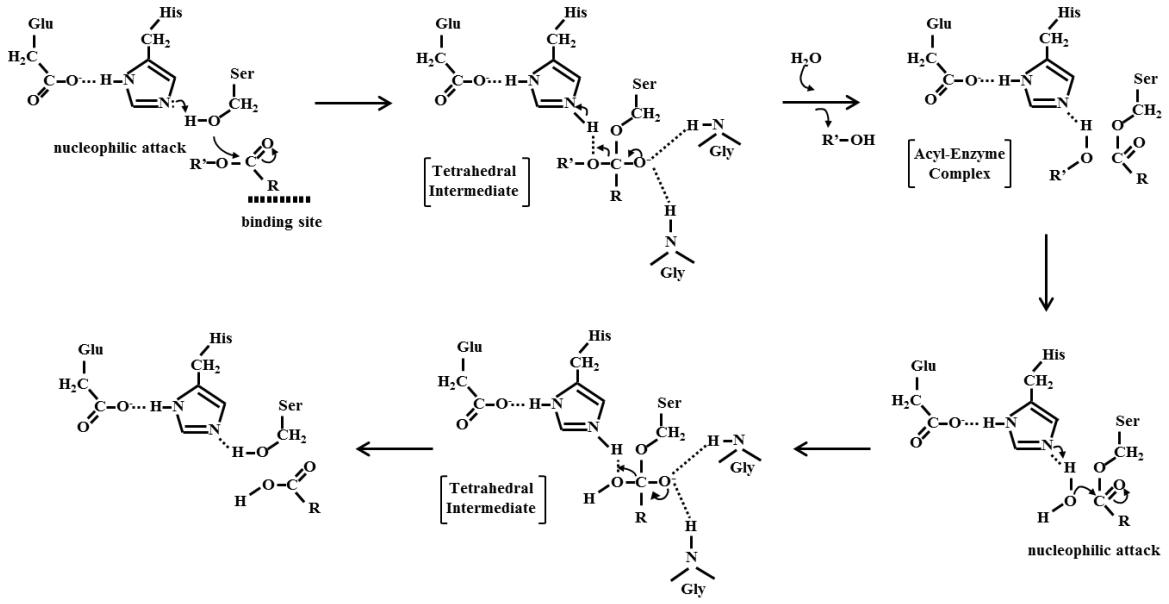


Figure 1. Mechanism for the hydrolysis of ester compounds by B-esterases.[1)]

This mechanism includes a two-step reaction.[1,8,24,28)] At neutral pH, Glu residue of active site exists as the charged form, which facilitates removal of a proton from His. This loss subsequently results in transfer of a proton from the adjacent Ser to the opposing nitrogen (N) of His, generating an oxygen nucleophile that can attack the carbonyl carbon of the substrate. After formation of the tetrahedral intermediate, the reverse reaction occurs to release the alcohol product and reform the carbonyl group, which is then attacked in an identical fashion with water acting as the nucleophile, leading to release of the carboxylic acid and return of the catalytic amino acids to their original forms.

The mechanism for hydrolyzing the substrates is same in between CES and BChE but the substrate specificities are different in each enzyme. CES has been found to be responsible for the metabolism of various pharmaceutical compounds such as angiotensin-converting enzyme inhibitors (e.g., temocapril)[29)] and antitumor drugs (e.g., irinotecan)[30)] while BChE can metabolize a more limited number of compounds, including cocaine[31)] and

heroin.[32] BChE also has a scavenging effect against poisons such as succinylcholine, pyrethroids and organophosphate pesticides.[33-35]

The A-esterase PON, otherwise known as arylesterase, is a six-bladed β-propeller with the two calcium ions located in the central tunnel. One calcium ion is essential for maintenance of structure (Ca2) whereas the other calcium ion which assigned as catalytic calcium (Ca1) is involved in hydrolysis.[36] The catalytic site of plasma PON is composed of a calcium atom, a phosphate ion and a His115-His134 dyad.[36] Mechanism for lactone and ester hydrolysis by PON is shown in Figure 2.[36] In this mechanism, His115 acts as a general base, deprotonates a water molecule, and generates a hydroxide ion that attacks the carbonyl group of the substrate. His134 shuttles a proton to increase the basicity of His115. The catalytic calcium ion serves as an oxyanion hole and stabilizes the negative intermediate produced by the attacking hydroxide ion. Unlike the mechanism of B-esterases, there is no evidence for the existence of an acyl-enzyme intermediate for the mechanism of PON.[37] It is the main plasma esterase responsible for the hydrolysis of pesticides that possess an aryl group or organophosphorus esters, such as paraoxon, sarin and soman.[38] PON also hydrolyzes lactones such as lovastatin, a drug used to control cholesterol levels.[39]

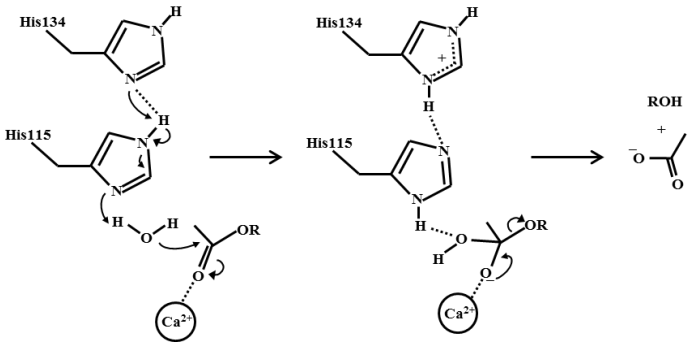


Figure 2. Mechanism for lactone and ester hydrolysis by PON.[36]

The relative abundance and substrate specificities of these plasma esterases vary in different species. The species differences in esterase expression and activities are clearly a complex topic. However, it is very important information when dealing with the

pharmacokinetics and pharmacological activities of drug subjects to ester hydrolysis during drug discovery studies. Generally, the process of generating new drugs consists of discovery and development stages. The discovery stage includes target selection, lead identification and preclinical studies, while the development stage includes clinical trials, manufacturing and product lifecycle management.[40] In the third step of discovery stage, the drug candidate will be tested for safety and efficacy in animal studies. As highlighted in Figure 3, Rosetta Stone approach is proposed by some researchers.[41] A crucial aspect of this approach is the dynamic feedback from animal models and clinical data which can be used to identify the treatments that are likely to succeed in clinical trials and to facilitate further development of animal and human models.[41] These studies with model animals are one of the most time-consuming steps in the drug discovery stage and sometimes a good drug candidate can be eliminated due to the inappropriate results obtained from animal screening studies. Therefore, after starting with thousands of compounds, only a few drug candidates continue into the development stage for clinical trials and manufacturing.

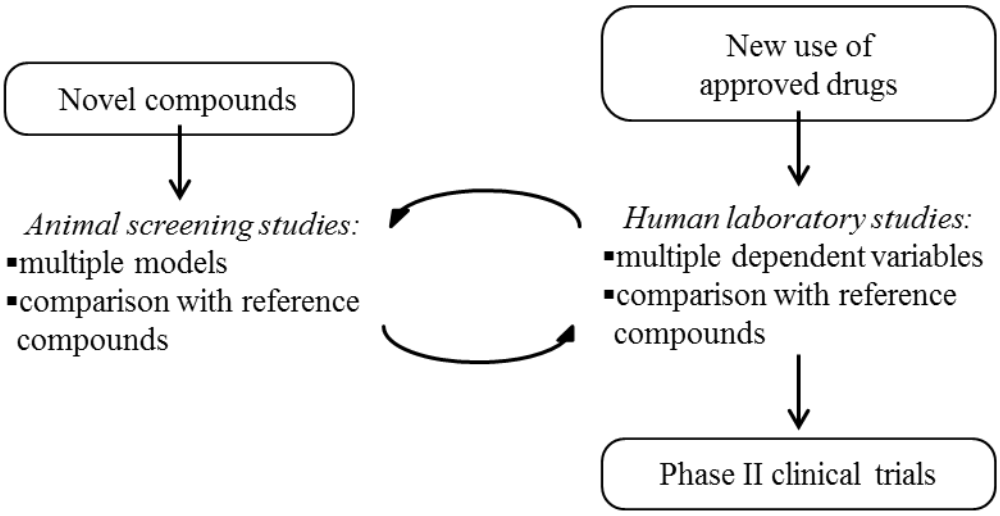


Figure 3. The 'Rosetta Stone approach' to drug development.[41]

It is possible to outline many such differences of esterase activities in human plasma and several other species with metabolism studies and native polyacrylamide gel electrophoresis (PAGE) staining techniques. Hence, this thesis was initiated to reveal the

above mentioned unknown features of differences in between human plasma and the plasma of several other preclinical species such as monkey, dog, minipig, rabbit, mouse and rat; with the ultimate aim to propose a suitable animal model for predicting the pharmacokinetics of xenobiotics in human.

This thesis is organized into 4 chapters. Chapter 1 deals with the expression pattern of plasma esterases. Native-PAGE was performed and gels were stained to visualize the plasma esterase activities in the human and several other animals to reveal the types of expressed esterases. Chapter 2 involves the hydrolase activities of human and several other animal plasmas. The species differences depending on their plasma esterases were discussed using five different compounds (*p*-nitrophenylacetate (PNPA), irinotecan, oseltamivir, temocapril, and propranolol derivatives). In Chapter 3, these differences in the hydrolase activities of human and other animal plasma has been evaluated in more detail with an appropriate model substrate (aspirin) that can be hydrolyzed by all plasma hydrolases. These studies were suggested the enhancing effect of divalent cations such as calcium and magnesium on aspirin hydrolysis in plasmas especially in human plasma. And therefore Chapter 4 deals with the divalent cation effect on human plasma. The hydrolysis kinetics of aspirin in the presence and absence of divalent cations has been studied in the purified human plasma BChE since it has been reported as the responsible plasma esterase for the changing activities in human plasma. An interesting finding about the allosteric effects of these divalent cations on human plasma purified BChE was also reported in this chapter. Finally a general summary including conclusion noting the future potentials of this study have been made from the overall investigation.

Chapter 1

Expression Pattern of Plasma Esterases

1.1. Introduction

Plasma esterases play an important role for the metabolic detoxification and/or activation of ester-linked compounds and xenobiotics including organic phosphates.[1,8,28,42] As mentioned in general introduction, esterases can be classified into three groups based on their substrate specificities, A-, B- and C-esterases. The A-esterase PON and the B-esterase BChE are the major plasma esterases, and are ubiquitously expressed in the plasma of animal species. Interestingly, although CES is present in the plasma of most rodents, it is not found in primates.

The relative abundance and substrate specificities of these plasma esterases vary in different species. The purpose of this chapter was to elucidate these species differences by comparing the expression of plasma esterases in human plasma and the plasma of several other animals (monkeys, dog, minipig, rabbit, mouse and rat) by performing native-PAGE.

1.2. Results

1.2.1. Detection of Esterase Activity on Native PAGE Gels

Figure 4 shows the native PAGE gel stained for esterase activity by β-naphthylacetate in the presence of calcium ($CaCl_2$). Bands corresponding to PON and BChE were observed in the upper part of the gel, whereas albumin and CES bands were seen in the lower part. In the middle part of the gel, undefined bands corresponding to the activity of unknown esterases were observed especially in minipig and mouse plasma.

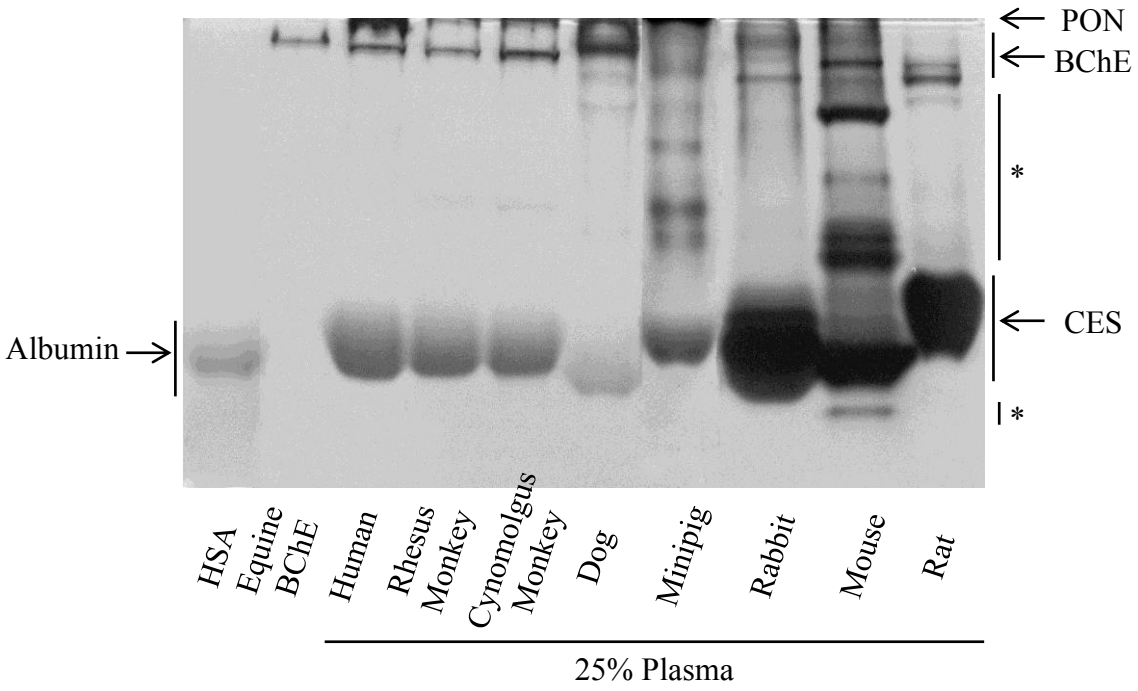


Figure 4. PAGE of animal plasma (25%) stained for esterase activity using β-naphthylacetate with $CaCl_2$ (10 mM); 1% HSA and 200 μg/mL equine BChE were used as controls. *Unknown bands.

When the native PAGE gels were stained by β-naphthylacetate in the absence of calcium, the same pattern of bands was observed in all species; however, the uppermost bands were indistinct. Because of its association with the high-density lipoprotein complex,[17] plasma PON (PON1) migrates slowly on the native PAGE gels. PON1 requires two calcium ions for the maintenance of its structure and for the activation of its catalytic center.[37] Therefore, the uppermost bands were confirmed as PON. Human, monkey, dog and minipig plasma showed prominent PON bands while rabbit, mouse and rat plasma showed weaker PON bands, corresponding to their hydrolytic activities for β-naphthylacetate.

The second bands from the top were confirmed as BChE using equine BChE as a control marker. Strongly stained BChE bands were observed in human, monkey and dog plasma. The weaker bands in other animals indicated their lower expression levels and/or the low activity of BChE against β-naphthylacetate.

In rats, mice and rabbits, the most intense bands were found in the lower part. These bands were shown to be CES by their disappearance on preincubation with BNPP, a specific CES inhibitor.[14,43,44] The CES band was more intense in rabbit and rat plasma than in mouse plasma. The migration of this band was different in rabbits, mice and rats, due to their enzymes having different numbers of glycosylation sites, which affects their molecular weights.[24]

Nearly in line with CES bands, lighter and more disperse bands, corresponding to a different source of lower hydrolase activity, were observed in all species tested. Purified HSA showed a similar light, disperse band, confirming these bands as albumin. It was not possible to distinguish between albumin and CES bands in rabbit, mouse and rat, because of their similar molecular weights (CES 65–75 kDa, albumin 66 kDa).

1.2.2. Detection of BChE Activity on Native PAGE Gels

Figure 5 shows a native PAGE gel stained to determine expression levels of BChE by butyrylthiocholine iodide, a specific substrate. Human, monkey, dog and minipig plasma showed intense bands corresponding to high expression levels of BChE. In rabbit, mouse and rat, BChE bands were more visible than those stained with β-naphthylacetate, but they were still only weak. In rabbit and rat, visible BChE bands were only observed in non-diluted plasma. Interestingly, another enzyme showed slight activity for butyrylthiocholine iodide in rabbit plasma. RSA was used to determine this band, which migrated in the center of the gel. As shown in the rightmost lane in Figure 5, RSA showed no such band after staining with butyrylthiocholine iodide. This band was also inhibited by preincubation with BNPP, confirming that it was because of the CES enzyme.

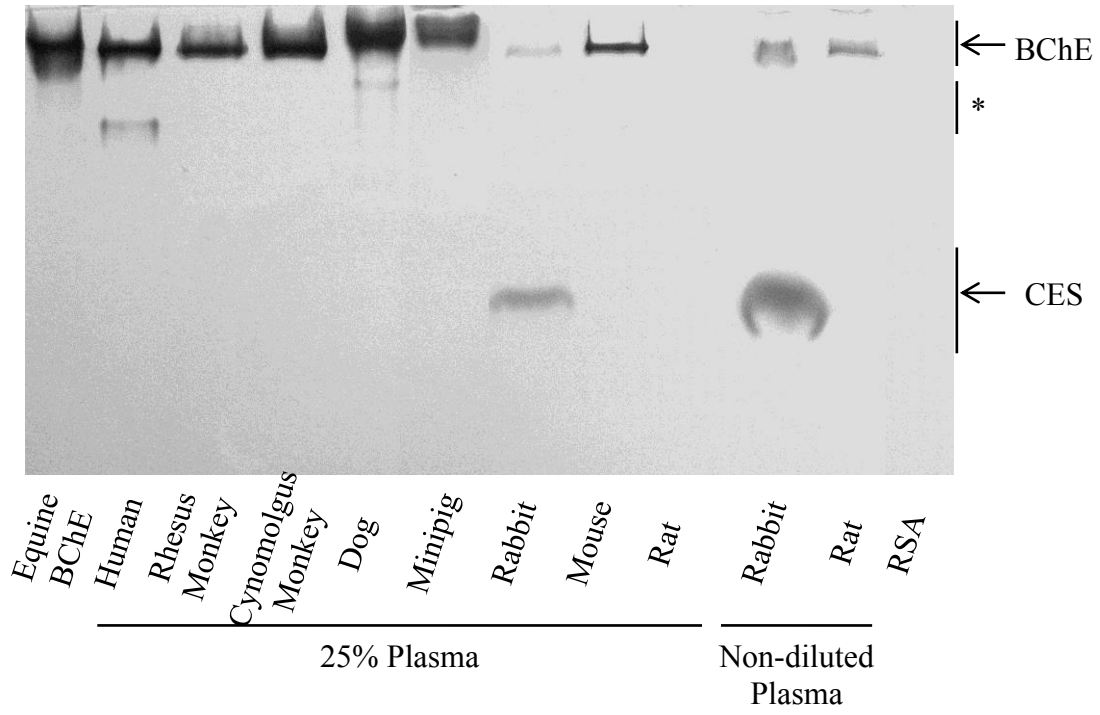


Figure 5. PAGE of human, rhesus monkey, cynomolgus monkey, minipig, dog, rabbit, mouse and rat plasmas (25% plasma) and of rabbit and rat plasmas (non-diluted plasma) stained for butyrylcholinesterase activity using butyrylthiocholine iodide. 4% RSA and 200 μg/mL equine BChE were used as controls. *Unknown bands.

1.3. Discussion

Several esterases, mainly CES, BChE, PON and AChE, are found in the blood. These four enzymes can be determined using α- or β-naphthylacetate. Although AChE, is abundant in red blood cells, it is present in only negligible amounts in plasma.[17] All tested animals showed two strong bands corresponding to PON and BChE (Figure 4). This study confirms that human plasma does not contain CES, a finding reported earlier.[17,18] Further, neither monkey, dog nor minipig plasma contained CES, which is the major component of rabbit, mouse and rat plasma. A weak band representing albumin was observed in all tested animals. Albumin is the most abundant soluble protein in the plasma (approximately 4%) and makes a significant contribution to drug binding. HSA has esterase activity with α- or β-naphthylacetate as well as with PNPA, aspirin and other substrates.[17] Lockridge *et al.*[20] reported that HSA possesses 82 acetylated residues in total, including high affinity

acetylation residue Tyr411. The high esterase activity of albumin is the result of the irreversible acetylation of these 82 residues. The hydrolysis activity of albumin is significantly lower than that of the serine esterases BChE and CES. Therefore, the presence of a weak and dispersed albumin band might indicate a high concentration, given its low activity. We also found PON to be a less active hydrolase. The hydrolysis mechanism of PON is substantially based on its catalytic dyad, which consists of His115 and His134.[36] His115 acts as a general base to deprotonate water molecules and generates the hydroxide ions that attack the carbonyl group of an ester substrate. In this mechanism, the catalytic calcium of PON serves as an oxyanion hole and stabilizes the negatively charged intermediate produced by the attacking hydroxide ion.[37] It has been reported elsewhere that PNPA is hydrolyzed by PON.[45] In the present study, it has been shown that PON also hydrolyzes β-naphthylacetate.

Species differences were also observed in the BChE content of the different plasmas (Figure 5). Human, monkey, minipig and dog plasma showed intense BChE bands but rabbit, mouse and rat plasma, particularly the latter, appeared to show only weak expression. It is conceivable that the low BChE content of rabbit, mouse and rat plasma might compensate for their high expression of CES. Additionally, only rabbit plasma CES was stained by butyrylthiocholine iodide, indicating the substrate specificity of rabbit CES.

After all, among the tested animals, esterase expression pattern of monkey plasma appeared to be closest to that of human plasma. However, hydrolase activities of these esterases should also be evaluated in order to characterize the hydrolytic properties of plasma esterases in human and several other animals.

Chapter 2

Characterization of Hydrolytic Properties of Animal Plasma Using Several Model Compounds

2.1. Introduction

In pharmaceutical development, ester compounds are frequently used in the development of prodrugs, either to improve bioavailability, to target a specific organ, or to prevent the adverse effects associated with their parent drugs. Esterase activity in the blood therefore plays an important role in the pharmacokinetics of these prodrugs. Since animal models are used to predict the pharmacokinetics of a prodrug during the preclinical phase, it is important to compare the substrate specificities of humans with commonly used animal models.

Study with many prodrugs might give some information about the relationship between plasma hydrolases and their hydrolytic properties against prodrugs, which are very important because on the basis of these relations it is possible to produce guidelines concerning systemic circulation of animals. Therefore, in this chapter, the species differences were tried to elucidate by comparing the hydrolase activities of plasma esterases in human plasma and the plasma of several other animals using different substrates: *p*-nitrophenylacetate (PNPA), irinotecan, oseltamivir, temocapril, and propranolol derivatives.

2.2. Results

2.2.1. Model Compounds and Their Structures

The structures of the compounds selected for characterization of hydrolysis in the different species are shown in Figure 6. PNPA is a substrate of several hydrolase enzymes, which allows it to be used for estimation of total esterase activity in animal plasma. Irinotecan is a substrate with a carbamate group while oseltamivir and temocapril have larger acyl moieties than alcohol moieties. Two propranolol derivatives, *O*-butyryl-propranolol and *O*-valeryl-propranolol, are substrates with relatively small acyl moieties.

These five types of prodrug, PNPA, irinotecan, oseltamivir, temocapril and propranolol derivatives were used to evaluate interspecies variation in plasma hydrolysis activity.

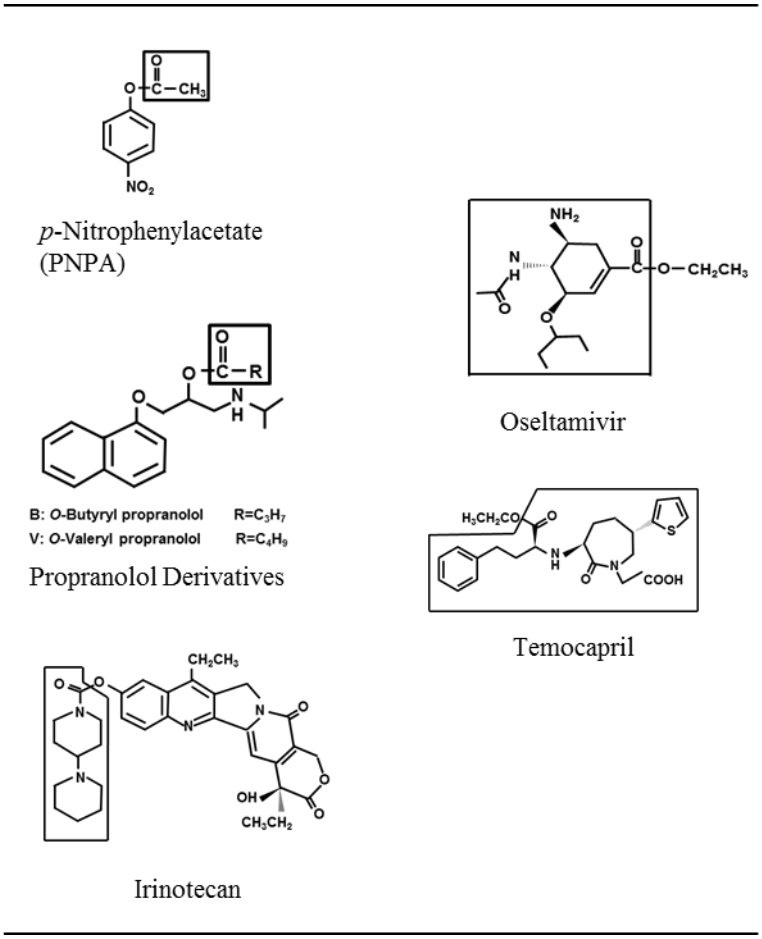


Figure 6. Structures of the substrates.

2.2.2. Characterization of the Hydrolytic Properties of Animal Plasma

As shown in Table 2, PNPA was rapidly hydrolyzed by the plasma of all species, the greatest activity being found in minipig plasma, which was 10-fold higher than that of human plasma. Rabbit and mouse plasma showed relatively higher activities than human plasma while monkey, dog and rat plasma activities were similar to human.

In all species, hydrolase activities for irinotecan, oseltamivir and temocapril were noticeably lower than their activities for PNPA (Table 2). There was no detectable hydrolase activity for irinotecan in human, monkey, dog, minipig and rabbit plasma, while

it was barely hydrolyzed in mouse and rat plasma. Oseltamivir and temocapril were only significantly hydrolyzed by mouse and rat plasma. The relatively high hydrolase activity of mouse and rat plasma is compatible with previous reports.[18,35] Rabbit plasma showed low hydrolysis activity for these three substrates, in contrast to its high activity for PNPA. It has been reported that irinotecan can be rapidly hydrolyzed by CES1 isozyme in rabbit liver[30] however, in our experiments, rabbit plasma CES showed no activity against this substrate, even though the plasma CES belongs to the CES1 family.

Table 2. Hydrolysis of PNPA, irinotecan, oseltamivir and temocapril by animal plasma. Values represents the mean ± S.D. (*n=3*). N.D. = not detected.

	Hydrolase Activity (μM/min)			
Species	PNPA	Irinotecan	Oseltamivir	Temocapril
Human	3000 ± 400	N.D.	0.305±0.011	0.064±0.026
Rhesus Monkey	2530 ± 280	N.D.	0.121±0.012	0.848±0.102
Cynomolgus Monkey	2710 ± 310	N.D.	0.103±0.011	0.176±0.029
Dog	2860 ± 450	N.D.	0.026±0.013	0.164±0.022
Minipig	31500 ± 10900	N.D.	1.030±0.255	N.D.
Rabbit	12730 ± 3700	N.D.	0.080±0.036	0.339±0.043
Mouse	6840 ± 3400	0.940±0.005	88.1±10.2	131±18
Rat	2540 ± 240	0.458±0.063	53.4±12.0	162±15

Figure 7 shows plasma hydrolase activities for the two types of propranolol derivatives. The R isoform of *O*-acyl-propranolol is preferentially hydrolyzed in all species. Further, derivatives with longer acyl chains are also more rapidly hydrolyzed (valeryl > butyryl). The hydrolysis of *O*-acyl-(R)-propranolol showed distinct species differences compared to that of irinotecan, oseltamivir and temocapril. Among the animals tested, the highest activity was obtained in rabbit plasma, followed by mouse plasma. However, rat plasma showed low activity, even lower than that of monkey plasma. The hydrolase activity of monkey plasma was approximately 10-fold faster than that of human plasma. It is interesting that monkey and rabbit plasma showed rapid hydrolysis activities only for

propranolol derivatives.

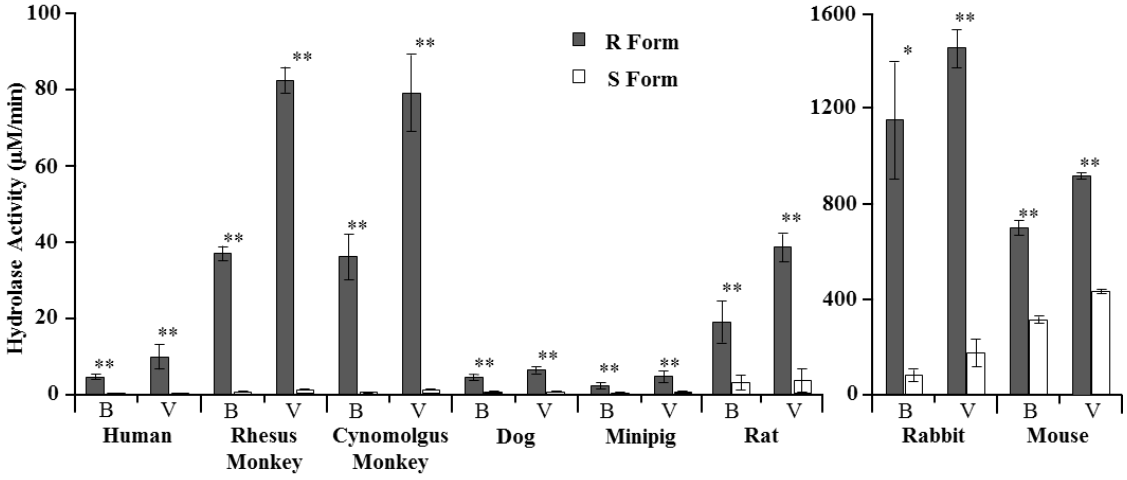


Figure 7. Hydrolysis of racemic propranolol derivatives. B and V represent *O*-butyryl-propranolol and *O*-valeryl-propranolol, respectively. Open and filled columns show hydrolase activity for S- and R-propranolol derivatives, respectively. Values represents the mean ± S.D. (*n=3*). R-isomers were preferentially hydrolyzed in the plasma of all species (*, $p<0.05$; **, $p<0.01$).

2.2.3. Esterases Responsible for Hydrolysis in Animal Plasma

In order to examine the esterases responsible for the highly hydrolyzed substrates, inhibition experiments were performed using ethopropazine and BNPP as specific inhibitors for BChE and CES, respectively.

Figure 8 shows the effect of the inhibitors on the hydrolysis of irinotecan, oseltamivir and temocapril in mouse and rat plasma. In mouse plasma, the hydrolytic activities of irinotecan and temocapril were significantly decreased in the presence of high concentrations of BNPP while similar concentrations of ethopropazine caused 50% and 80% decreases in activity, respectively. In the case of oseltamivir, the hydrolase activity of mouse plasma was completely inhibited by high concentrations of both inhibitors. These data indicate that CES was substantially and BChE slightly involved in the hydrolysis of irinotecan and temocapril in mouse plasma, while BChE and CES were both responsible for

hydrolyzing oseltamivir. In rat plasma, the inhibition patterns for irinotecan and oseltamivir were similar to that of mouse plasma however the pattern for temocapril was different (Figure 8). The hydrolysis of temocapril in rat plasma was almost completely inhibited by high concentrations of BNPP, suggesting that CES is the major enzyme responsible for this activity.

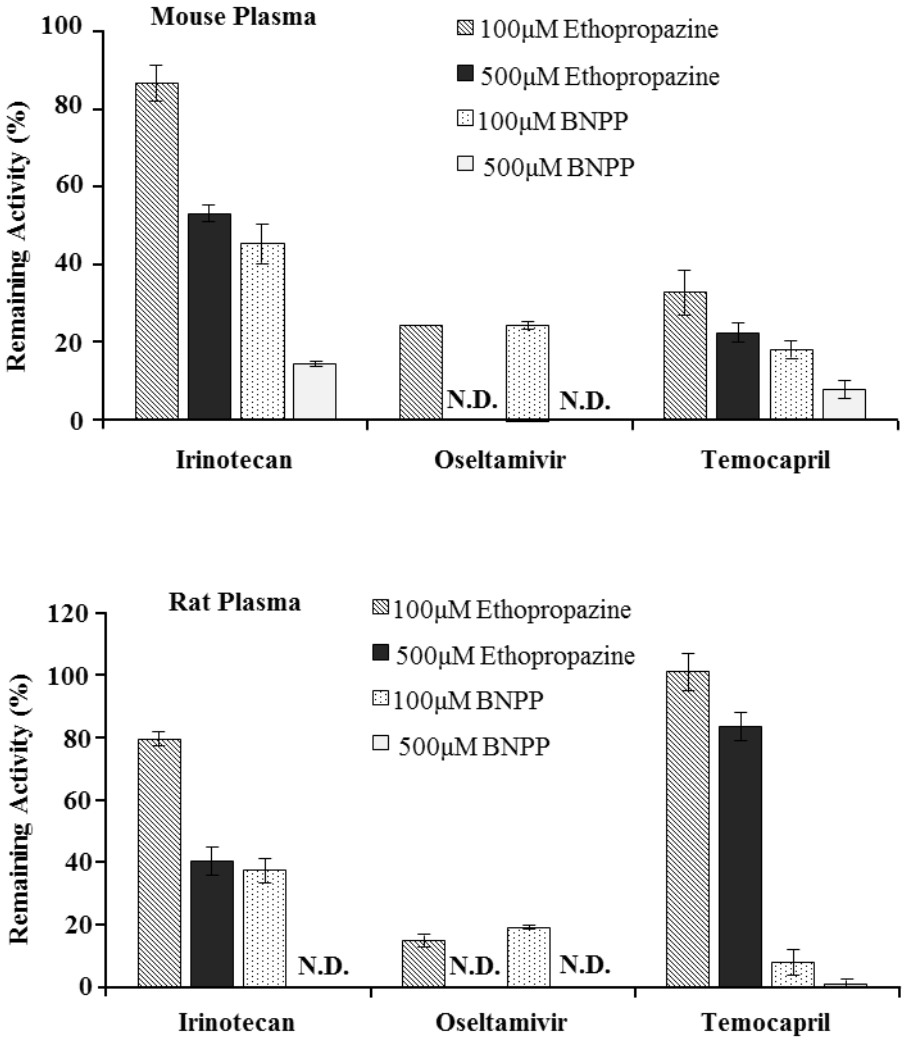


Figure 8. The effect of ethopropazine and BNPP on the hydrolysis of irinotecan, oseltamivir and temocapril in mouse and rat plasma. Values represents the mean ± S.D. (*n=3*). N.D. = not detected.

O-Valeryl-(R)-propranolol was highly hydrolyzed in plasma from monkey, rabbit, rat and mouse. The esterases responsible for this activity are shown in Figure 9. The extremely high activity of rabbit plasma was completely inhibited by even low concentrations of BNPP but not by ethopropazine. Similar results were obtained with rat plasma. This indicates that the hydrolysis activity is based substantially on CES enzymes in rabbit and rat plasma. In mouse plasma, the hydrolysis of *O*-valeryl-(R)-propranolol was inhibited in a pattern similar to that of temocapril inhibition, indicating that both BChE and CES are responsible for this activity. In rhesus and cynomolgus monkey plasma, the hydrolysis activity of *O*-valeryl-(R)-propranolol was significantly inhibited by both low and high ethopropazine concentrations, but not by BNPP. These inhibition data are supported by the fact that monkey plasma has no CES enzyme (Figure 4 in Chapter 1). Therefore, BChE is the enzyme responsible for the hydrolysis of *O*-valeryl-(R)-propranolol in the plasma of rhesus and cynomolgus monkeys.

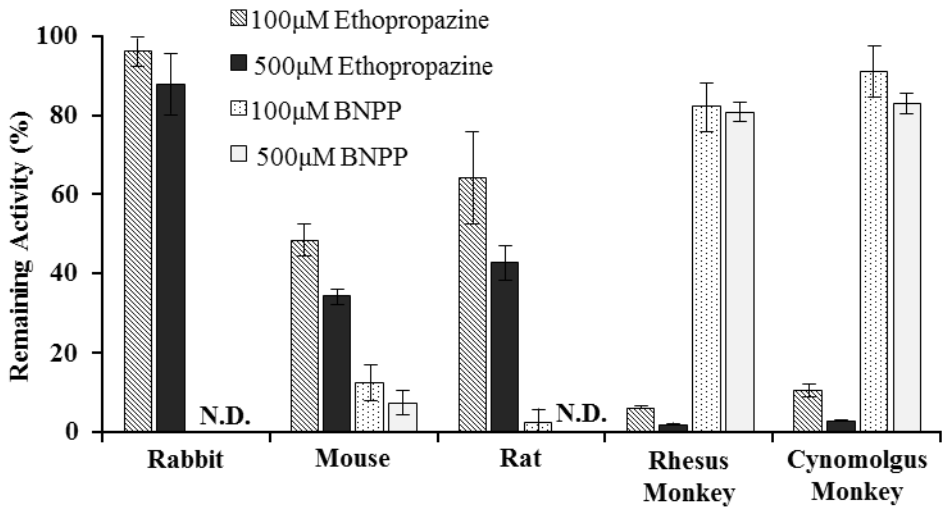


Figure 9. The effect of ethopropazine and BNPP on the hydrolysis of *O*-valeryl-(R)-propranolol in monkey, rabbit, mouse and rat plasma. Values represents the mean ± S.D. (*n=3*). N.D. = not detected.

2.3. Discussion

Five types of prodrugs, PNPA, irinotecan, oseltamivir, temocapril and propranolol derivatives, were used to evaluate interspecies variation in plasma hydrolysis activity. The hydrolysis activity of irinotecan was low, even in rat and mouse plasma, indicating the resistance of plasma BChE and CES to the carbamate group.[46] PNPA was used as control substrate, to indicate the maximum esterase activity in all species (Figure 6). However, interspecies variation in the hydrolysis of the other substrates was different from that found for PNPA. For example, minipig plasma showed several bands representing unknown esterases (Figure 4 in Chapter 1) and rapidly hydrolyzed PNPA (Table 2), but none of these esterases could hydrolyze the other substrates tested in this study. Also, the hydrolysis of PNPA in rat plasma was relatively low, despite the strongly staining CES enzyme seen on the native PAGE gel (Figure 4 in Chapter 1).

Rabbit, mouse and rat plasmas showed high expression of CES. Recently, CES isozymes have been classified into five subfamilies.[24] Among these families, CES1 (expressed in several organs) and CES2 (mainly expressed in small intestine and kidney) play important roles in the activation of prodrugs. The CES1 family consists of eight subfamilies (CES1A–CES1H). Plasma CES is grouped in the CES1G subfamily and is secreted into the blood from the liver, due to its lack of a consensus sequence for retention in the endoplasmic reticulum.[24,25] Sequence homology, calculated by CLUSTALW alignment, between the CES1G isozymes in rat and mouse plasma is 83% (rat CES1, NP_058700; mouse CES1, NP_0311980). There have been no reports on rabbit plasma CES1G family isozyme, so its sequence homology cannot be calculated.

Oseltamivir, temocapril and the propranolol derivatives, are known as good substrates, especially for CES.[9,28] High levels of hydrolase activity for oseltamivir, temocapril and propranolol derivatives were obtained in rat and mouse plasma. However, in rabbit plasma, although CES is highly expressed, substrate hydrolysis was slow in comparison with mouse and rat plasma, except for that of the propranolol derivatives which was rapid. Interestingly, only rabbit plasma CES1 isozyme hydrolyzed butyrylthiocholine (Figure 5 in Chapter 1). On the other hand, mouse plasma CES showed higher activity than rat plasma CES against all substrates except temocapril (Table 2), despite their high

sequence similarity. But, mouse hydrolase activity against oseltamivir, temocapril and the propranolol derivatives was inhibited by BNPP and ethopropazine. Mouse plasma expressed more bands than rat plasma, including the unknown esterases (Figure 4 in Chapter 1). Although BNPP and ethopropazine are known to inhibit CES and BChE, respectively, there is also a possibility to inhibit the unknown esterases. Taken together, these results suggest that the relatively high activity of mouse plasma may be slightly dependent on these unknown esterases.

In contrast to rabbits, mice and rats, the hydrolysis activities of human, monkey, dog and minipig plasmas were mainly due to their abundant expression of BChE enzymes. Interestingly, in monkey plasma, *O*-valeryl-(R)-propranolol was rapidly hydrolyzed by BChE while in human plasma hydrolysis by BChE was slow (Figure 6 and 8), in spite of the fact that human and monkey plasma BChEs show nearly 96% homology, differing in only 23 amino acids.[47] BChEs have three important regions determining their substrate specificity: a catalytic triad consisting of Ser198, Glu325 and His438;[48] the initial binding site of positively charged substrates, known as the peripheral anionic site; and the large acyl-binding pocket.[18,42] The peripheral anionic site is positioned at the entrance of the active center, and in human BChE an aromatic amino acid Tyr332 plays an important role in opening the active site gorge through hydrogen-bonded Asp70.[49] Flexible Leu286 and Val288 residues of the acyl pocket also affect the substrate specificity of BChE.[48]

In sequence analysis of human and monkey plasma BChE, the peripheral anionic site is conserved; however the acyl-binding pocket only differs in one amino acid. As shown in Figure 10, the Pro285 residue in human BChE is replaced by the more flexible Leu residue in monkey BChE. This increased flexibility of the acyl-binding pocket in monkey BChE may facilitate the binding of a greater variety of substrates. This would explain the difference in hydrolase activity indicated by different substrate specificities of human and monkey plasma BChEs. However, more detailed analysis will be required to verify this theory.

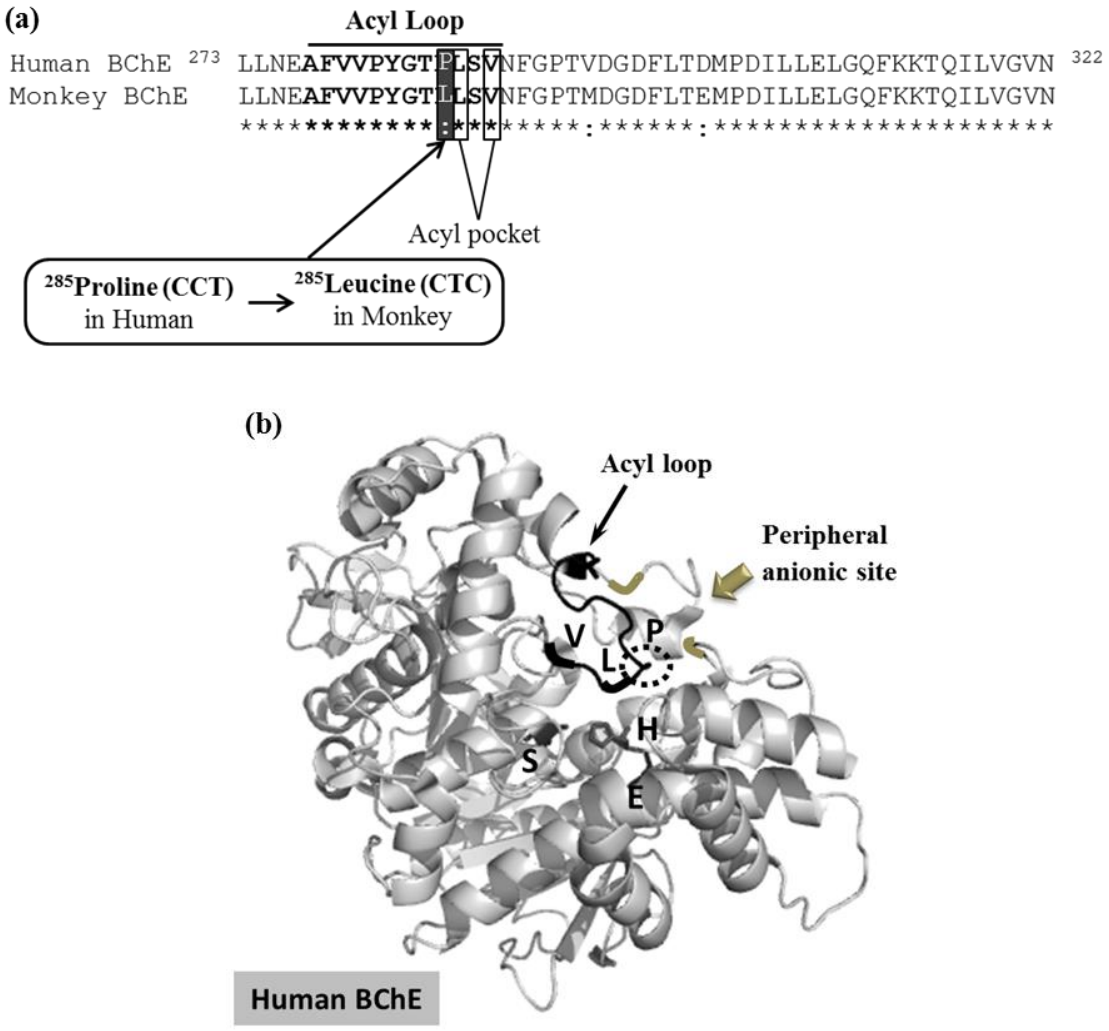


Figure 10. (a) Comparison of amino acid sequences in between human BChE and monkey BChE from the 273th residue to 322th residue. *(asterisk) means that the residues in that column are identical in all sequences in the alignment, :(colon) means that conserved substitutions have been observed. **(b)** The ribbon model for showing the crystal structure of human plasma butyrylcholinesterase (Protein Data Bank code 2XMB). S, E and H represent the catalytic amino acids (Ser, Glu and His) of BChE. The acyl loop was shown in black color and acyl pocket which consists of Leu286 and Val288 residues were pointed in bold as L and V, respectively. The Pro285 residue in human BChE was indicated by dashed-line circle as P. The structure was drawn with PyMOL software.[50)]

Chapter 3

Characterization of Hydrolytic Properties of Animal Plasma Using Aspirin

3.1. Introduction

In chapter 1 and chapter 2, the expression pattern and substrate specificities of plasma esterases of humans and several other animals were confirmed. However, the differences of expressing esterases and their substrate specificities in each plasma is urged to study in more detail especially by using an appropriate model substrate that can be hydrolyzed by all plasma hydrolases.

Aspirin is the most widely used drug in the world and it is hydrolyzed to salicylic acid by most of the esterases, even by albumin. Following its oral administration in man, aspirin undergoes hydrolysis in the intestine, liver and plasma. The specific hydrolases that plays active role for this hydrolysis in human tissues are carboxylesterase 1 (hCE1) in liver, carboxylesterase 2 (hCE2) in intestine[51-54] and in plasma are cholinesterases[55-59] paraoxonase[60,61] and albumin.[62-64]

Hence, in this chapter, the hydrolyzing properties of plasma esterases for aspirin were investigated in several animal and interesting findings were observed about the enhancing effects of divalent cations such as calcium and magnesium on the aspirin hydrolysis activities of plasma. BChE of human, monkey, dog together with mouse and rat plasma is reported as the responsible esterase enzyme for this increased hydrolysis activities.

3.2. Results

3.2.1. Hydrolysis of Aspirin in Animal Plasma

Aspirin hydrolysis was analyzed in the plasma of human and other animals. In addition to plasma samples, 4% HSA was also used to evaluate the effect of albumin on aspirin hydrolysis in the human plasma. Hydrolase activities in the plasma of all tested

Table 3. Aspirin hydrolysis by HSA and plasma of human and several animals and effect of esterase inhibitors- EDTA, BNPP and ethopropazine- on their hydrolase activities. Data showed statistically significant differences from control activity in the absence of inhibitors (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Values represents the mean ± S.D. (*n=3*). Values in parenthesis represent the remaining hydrolase activities (%) after inhibition by related inhibitor.

Species	**Hydrolase activity (µmol/min/mL plasma)**				
		In the presence of esterase inhibitors			
	Control	1 mM EDTA	0.5 mM BNPP	0.5 mM Ethopropazine	0.5 mM Ethopropazine & 0.5 mM BNPP
HSA (4%)	4.82				
Human	21.6 ± 0.81	10.7 ± 0.63 (49.5 ± 5.50)***		15.1 ± 2.43 (70.3 ± 19.8)*	
Monkey	19.5 ± 2.14	12.3 ± 1.60 (63.2 ± 5.54)*		15.3 ± 5.04 (76.6 ± 37.6)	
Dog	7.51 ± 1.09	5.13 ± 1.46 (68.3 ± 8.67)*		7.06 ± 1.59 (93.7 ± 12.6)	
Minipig	4.79 ± 1.66	4.83 ± 0.20 (101 ± 3.12)		3.74 ± 1.67 (78.2 ± 9.21)	
Rabbit	37.0 ± 2.58	25.4 ± 4.60 (68.6 ± 11.6)*	3.78 ± 0.26 (10.2 ± 0.24)**	32.7 ± 13.5 (88.3 ± 7.31)	
Mouse	12.3 ± 4.68	12.0 ± 0.37 (98.6 ± 15.6)	1.18 ± 0.35 (10.0 ± 3.50)*	20.5 ± 1.74 (173 ± 54.6)*	0.76 ± 0.61 (6.75 ± 5.47)*
Rat	15.5 ± 2.81	16.0 ± 2.03 (103 ± 4.68)	2.27 ± 0.97 (14.7 ± 4.72)*	71.8 ± 14.3 (468 ± 70.9)**	3.65 ± 0.45 (26.4 ± 5.77)*

species were verified by the hydrolysis of PNPA, the control substrate. Its rapid hydrolysis was obtained in the order of minipig > rabbit > mouse > human > dog > monkey > rat (Table 2, Chapter 2).

Results for aspirin hydrolysis are shown in Table 3. Hydrolysis activities were presented as their rate in one mL of plasma. The highest hydrolase activity for aspirin was observed in rabbit plasma, which was approximately 2-fold higher than that in human plasma. Aspirin hydrolysis rate in human plasma was nearly the same with monkey plasma, 21.6 ± 0.81 and 19.5 ± 2.14 μmol/min/mL, respectively. Similar hydrolase activities were observed in mouse and rat plasma but these activities were lower than that observed in human and monkey plasmas. Dog and minipig plasma show the lowest hydrolase activities. Furthermore, hydrolase activity of minipig plasma was almost the same as the activity of 4% HSA for hydrolyzing aspirin.

3.2.2. Effect of Enzyme Specific Inhibitors on Aspirin Hydrolysis

To identify the plasma esterases responsible for aspirin hydrolysis, inhibition experiments were performed. BNPP and ethopropazine were used as specific inhibitors for CES[14,43,44] and BChE,[65,66] respectively. Moreover, EDTA, a chelating agent, was used as inhibitor of PON, because plasma PON requires calcium ions for the maintenance of its structure and for its esterase activity.[37,67,68]

Table 3, also, shows the effect of these inhibitors on the hydrolysis of aspirin in human and animal plasma. Since human, monkey, dog and minipig plasma does not contain CES,[17,19] inhibition experiment with BNPP was not performed in these species. In human plasma, hydrolytic activity of aspirin decreased 50% by EDTA and 30% by ethopropazine, suggesting a substantial affect of PON and less contribution of BChE. Plasma hydrolase activities of monkey and dog were inhibited by EDTA suggesting a considerable effect of PON on these activities. On the other hand, hydrolase activity of minipig plasma was inhibited only by ethopropazine, but this inhibition was not statistically significant.

In the species that express CES in their plasma, i.e. rabbit, mouse and rat; only 10–15% hydrolase activities remained after inhibition by BNPP, suggesting the CES as the major responsible enzyme. As previously shown in Chapter 2, in most cases, plasma CES

activity is much higher than any other plasma esterases. Even, in case of aspirin which is a poor substrate for plasma CES, hydrolysis of this compound was primarily due to CES in mouse and rat plasmas.

Interestingly, hydrolase activities of mouse and rat plasma were significantly increased by the addition of ethopropazine as inhibitor, whereas rabbit plasma activity was decreased (Table 3). From this result, we postulate that ethopropazine could possibly have a positive effect on CES of rodent animals and so, hydrolysis of aspirin is activated by addition of ethopropazine. In order to clarify this hypothesis, we added firstly ethopropazine and then BNPP to inhibit the activated activity of CES. As shown in Table 3, the increased hydrolysis activity of mouse and rat plasma due to ethopropazine addition was inhibited after addition of BNPP.

3.2.3. Calcium Effect on Aspirin Hydrolysis

As mentioned earlier, aspirin is a well-known substrate that is rapidly hydrolyzed by human plasma BChE and albumin. However, inhibition experiments with EDTA also confirmed the contribution of plasma PON on aspirin hydrolysis in human, monkey, dog and rabbit. In order to determine the role of PON, aspirin hydrolysis experiments were conducted by adding calcium into reaction mixture.

The effect of calcium on aspirin hydrolysis was tested using two different concentrations (low, 2 mM and high, 25 mM). The presence of low calcium concentration is assumable as the mean free calcium concentration in the range of physiologically normal conditions (1.1 – 1.4 mM) and conditions of disorders such as hyperthyroidism and/or cancer with bone metastases and/or more severe cases (< 2.5 mM). As seen in Figure 11, simultaneous addition of aspirin and calcium positively affected the hydrolase activities in the plasma of human and other species. Change of the order in the addition of aspirin and calcium into plasma caused the same effect on the hydrolase activities. In human, monkey and dog plasma the addition of calcium even in low concentration (2 mM) increased the hydrolysis activity by 2.7-, 1.9- and 2.3-fold, respectively, and hydrolase activities were increased more than 5-fold at high concentration of calcium (25 mM). On the other hand, minipig, rabbit, mouse and rat plasma hydrolase activities were only slightly increased with

the addition of 2 mM calcium, while their hydrolyze activity for aspirin were noticeably increased in case of 25 mM calcium addition.

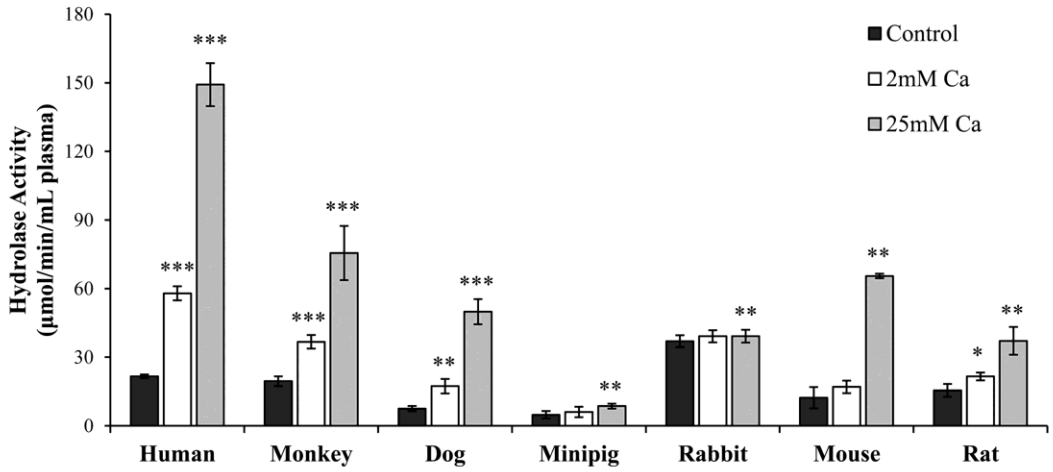


Figure 11. Hydrolysis of aspirin in the human and other animal species in the presence of two different calcium concentrations. Control refers to the hydrolysis condition in the absence of calcium at constant concentration of aspirin (2.5 mM). Values represent the mean ± S.D. (n = 3). Statistically significant differences were observed vs. control activities (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

In order to confirm PON involvement in the increased plasma activity observed by calcium addition, inhibition experiments using BNPP and ethopropazine were conducted in the presence of 25 mM calcium. Results are shown in Table 4. Highly increased activities of human, monkey and dog plasma were effectively inhibited by ethopropazine in the presence of calcium, which resulted in similar hydrolase activities after inhibition by ethopropazine in the absence of calcium (Table 3 and 4). In the plasma of these species, BChE contributed to 70-85% of aspirin hydrolysis in the presence of calcium in comparison with only 7-30% contribution in the absence of calcium. These data indicate that BChE is the responsible esterase for the calcium-activated aspirin hydrolysis in the plasma of these species. In the case of mouse and rat plasma, their CES activities were inhibited by BNPP but 48% and 38% of their hydrolase activities were remained in the

Table 4. The effect of BNPP and ethopropazine on aspirin hydrolysis in the presence of 25 mM calcium. Data showed statistically significant differences from the control activity that observed in the absence of inhibitors (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Values represents the mean ± S.D. (*n=3*). Values in parenthesis represent the remaining hydrolase activities (%) after inhibition by related inhibitor.

Species	**Hydrolase activity (µmol/min/mL plasma)**			
		In the presence of esterase inhibitors		
	Control	0.5 mM BNPP	0.5 mM Ethopropazine	0.5 mM Ethopropazine & 0.5 mM BNPP
Human	149 ± 9.42		20.6 ± 8.83 (13.9 ± 6.23)***	
Monkey	75.6 ± 11.7		20.9 ± 7.07 (27.6 ± 6.42)***	
Dog	49.9 ± 5.49		14.1 ± 5.19 (28.0 ± 4.27)***	
Mouse	65.5 ± 0.99	31.2 ± 0.30 (47.7 ± 3.29)**	50.8 ± 1.24 (77.7 ± 7.96)*	1.89 ± 1.36 (2.90 ± 2.73)***
Rat	37.2 ± 6.09	12.1 ± 0.92 (37.9 ± 23.7)*	78.9 ± 12.3 (214 ± 27.0)*	3.14 ± 0.26 (8.45 ± 0.84)***

presence of 25 mM calcium, respectively. When BNPP and ethopropazine were simultaneously used, almost complete inhibition was observed in both plasmas. Furthermore, even if the addition of ethopropazine is activated the plasma CES, the effect of ethopropazine becomes more evident in the presence of 25 mM calcium than in the absence of it (Table 3 and 4). Taken together, these results suggest that BChE contributes the aspirin hydrolysis in mouse and rat, in the presence of calcium.

3.2.4. Effect of Other Divalent Cations on Aspirin Hydrolysis in Human, Monkey and Dog Plasma

Addition of calcium perceivably increased the aspirin hydrolysis in the plasma of all tested animals, except in minipig and rabbit; mostly by affecting their BChE activities (Figure 11 and Table 4). In order to determine the role of divalent cations on plasma hydrolase activities, further experiments were conducted using human, monkey and dog plasmas in the presence of magnesium and zinc. Results are shown in Figure 12. Addition of magnesium at the same concentration of calcium (2 mM) increased the plasma hydrolase activities of these species by 2.1-, 1.6- and 1.5-fold respectively. In contrast, the hydrolysis of aspirin in the human, monkey and dog plasma was decreased after zinc addition (2 mM). In order to confirm the esterase responsible for the increased activity of aspirin hydrolysis, inhibition experiments were again performed using ethopropazine in the presence of 2 mM magnesium, and the increased hydrolase activities of human, monkey and dog plasma were inhibited by 80%, 50% and 63%, respectively. Based on these data, BChE of the human, monkey and dog plasma is considered as the responsible esterase enzyme involved in the increased activity of aspirin hydrolysis in the presence of calcium and magnesium.

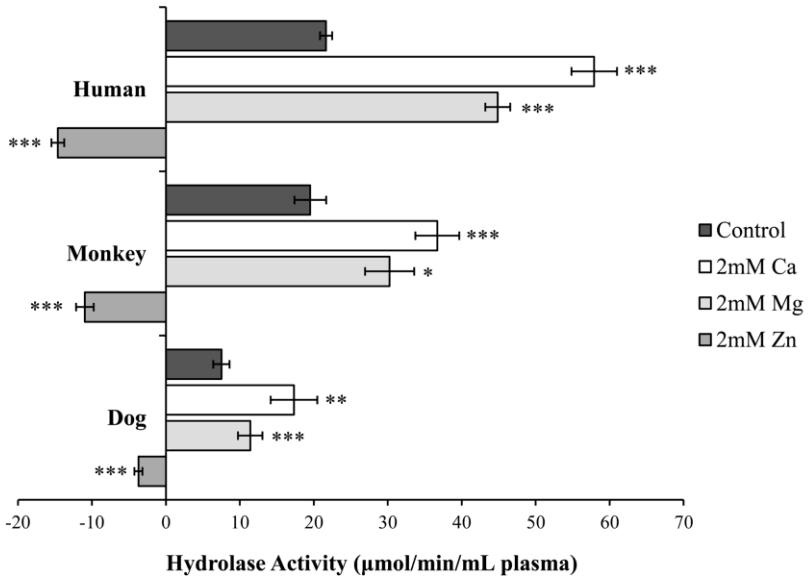


Figure 12. Effect of divalent cations on aspirin hydrolysis in human, monkey and dog plasma. Control refers to the hydrolysis condition in the absence of divalent cation at constant concentration of aspirin (2.5 mM). Values represent the mean ± S.D. (n = 3). All data showed statistically significant differences when compared to their control activities (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

3.2.5. Kinetic Behavior of Divalent Cation Effect during Aspirin Hydrolysis in Human Plasma

A further experiment was conducted to examine the effect of divalent cations on hydrolysis of aspirin in human plasma via changing concentrations of calcium and magnesium. Figure 13 (a, b) shows the effects of increasing divalent cation concentrations (1–10 mM) on the hydrolase activity of human plasma. As shown in Figure 13a, increasing concentrations of both calcium and magnesium activated the aspirin hydrolysis in human plasma. When the data (Figure 13a) is replotted as hydrolase activity vs. hydrolase activity over divalent cation concentration (v vs v/[M]) in Figure 13b, two crossing linear lines were obtained with the effect of calcium and magnesium on the hydrolase activity of human plasma. The slopes of these plots were related to the binding affinity (K_M) of divalent cations for the esterase enzyme that is responsible for aspirin hydrolysis in human plasma

(eq 2, see the section Material and Methods). We supposed that one of the linear relations occurred at less than 4 mM calcium concentrations with a binding affinity of 1.46 mM. The second linear relation occurred in between higher calcium concentrations (4–10 mM) with lower binding affinity (K_M is 5.65 mM). Similar results were also obtained for magnesium, showing a high binding affinity with a K_M value of 1.00 mM and lower binding affinity with the K_M value of 3.02 mM.

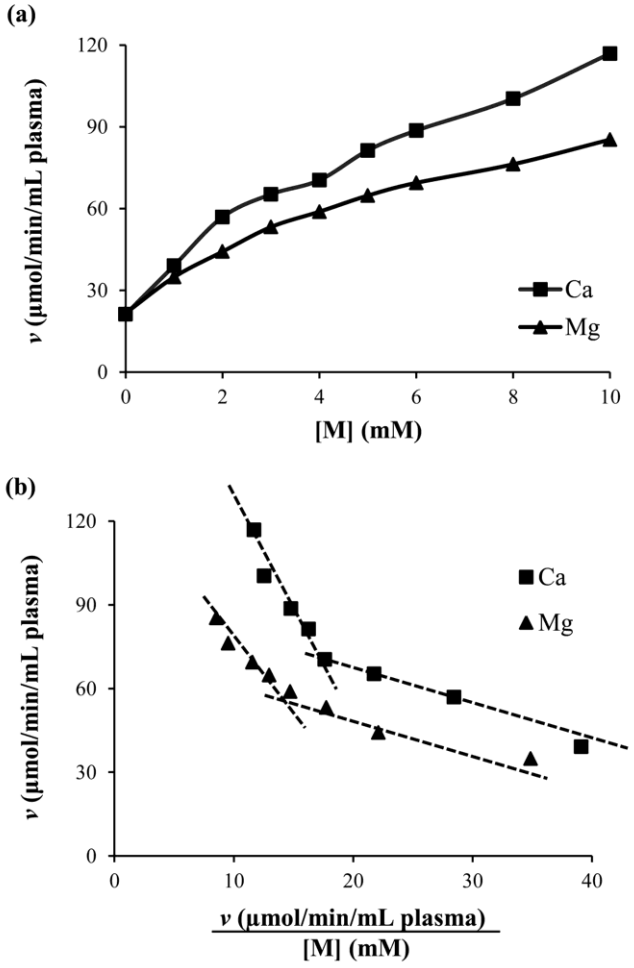


Figure 13. (a) Comparison of the hydrolase activity (v) that is activated by calcium and magnesium ions at different concentrations of the divalent cation (metal [M]) and constant concentration of aspirin (2.5 mM) in the human plasma. **(b)** v vs. v/[M] plot that resembles Eadie-Hofstee plot. The slopes are related to affinity of divalent cations for the enzyme that is responsible for aspirin hydrolysis in human plasma.

3.3. Discussion

As previously reported, various proteins with esterase activity are present in the plasma of all animals that tested in this study (Chapter 1). PON and BChE are expressed in the plasma of all animals together with albumin, while CES is only expressed in rabbit, mouse and rat plasma. BChE contents were rich in human, monkey and dog plasma; moderate in minipig plasma and were low in rabbit, mouse and rat plasmas. It was thought that low BChE contents might compensate for the high expression of CES. Furthermore, in the plasma where CES is present, except the rabbit plasma, various substrates hydrolyzed rapidly by their CES. CES of rabbit plasma showed distinct substrate specificity and it can hydrolyze limited substrates like butyrylthiocholine and propranolol derivatives (Figure 5 in Chapter 1 and Figure 9 in Chapter 2). In this chapter, our results suggested aspirin as another example for the substrates rapidly hydrolyzed by rabbit plasma CES. Although aspirin is also hydrolyzed by CES of mouse and rat plasma, this activity was very low when compared to other substrates like oseltamivir, temocapril and propranolol derivatives (Table 2 in Chapter 2).

In the inhibition experiments, we observed an interesting phenomenon that rat and mouse plasma CES activity for aspirin hydrolysis was enhanced by ethopropazine, especially in rat plasma by 4.6-fold (Table 3). But, enhancing effect of ethopropazine was not observed for rabbit plasma CES. Although the enhancing mechanism is unclear, there are two possible mechanisms; the activation of active center of CES and the appearance of non-specific catalytic sites via conformational change by ethopropazine. Since aspirin can be also hydrolyzed by non-specifically binding like the case of albumin, the binding of ethopropazine to CES may cause an exposure of non-specific binding sites. The substrate specificities and enhancing activity by ethopropazine were pointed the similar characteristics of mouse and rat plasma CES and the distinct property of rabbit plasma CES. Similarity between mouse and rat plasma CES is supported by their 83% sequence homology (calculated by CLUSTALW alignment, rat CES1, NP_058700; mouse CES1, NP_0311980). Nevertheless, sequence homology of rabbit plasma CES with these species cannot be calculated due to lack of information.

In the plasma where CES is absent, aspirin is hydrolyzed by other hydrolases in human, monkey and dog plasma. On the other hand, minipig plasma showed the lowest aspirin activity which is nearly same as the activity of 4% HSA. There are some reports that acetyl group of aspirin binds with the lysine residue of HSA and thus, the hydrolyzing activity from human plasma includes acylation of HSA.[62-64] Besides albumin activity, as a matter of fact, aspirin is long been known to be a substrate of human plasma BChE.[55,56,58,59,62] In addition to these two proteins, it is recently reported that aspirin can hydrolyzed by plasma PON.[60,61] Findings of our present study also indicated three proteins, PON, BChE and albumin, as the responsible plasma hydrolases for the aspirin hydrolysis in human, monkey and dog plasma (Table 3). During the hydrolysis experiments, addition of calcium noticeably increased the hydrolase activities in the plasma of human and some other animals (Figure 11). This significant increase considered to be not due to PON but due to BChE (Table 4) although the fact that PON requires calcium for substrate hydrolysis. Calcium was not affected the activities of rabbit and minipig, since aspirin is primarily hydrolyzed by plasma CES in rabbit or not hydrolyzed by any hydrolases in minipig plasma.

It is conceivable that highly activated hydrolase activities of human, monkey and dog plasma in the presence of calcium can be related to their intense BChE contents. Interestingly, even though the dog plasma BChE was slightly involved in the activity, aspirin hydrolysis was noticeably increased in the presence of calcium. In contrast to these species, the lower BChE contents of rat and mouse plasma (Figure 5 in Chapter 1) can be the reason for their slightly altered activities for aspirin hydrolysis. Additionally, even in the species with low contents of BChE, calcium stimulated the hydrolase activity of this enzyme, since the effect of ethopropazine inhibition on mouse and rat plasma is significantly greater in the presence of calcium than its absence (Table 3 and 4). Also as in Figure 14, the sequence analysis revealed that human and monkey plasma BChEs show nearly 96% homology whereas human and dog plasma BChEs shows 87% (calculated by CLUSTALW alignment; human BChE, NP_000046; monkey BChE, ADD64703; dog BChE, XP_545267). Surprisingly, CLUSTALW alignment analysis for BChE enzymes in human, mouse, rat plasma showed high sequence similarity (80%) in between human and

these two species (mouse BChE, NP_033868; rat BChE, NP_075231). Similarities between the BChE of these species may be the reason for the similar effects that observed on aspirin hydrolysis in the presence of calcium. Hereby, we are reporting for the first time that calcium has also stimulating effect on the plasma BChE of not only human, monkey and dog but also mouse and rat.

In human, monkey and dog, addition of magnesium also showed positive effect on the hydrolase activities by affecting plasma BChE (Figure 12). For these positive effects of calcium and magnesium on BChE, Valentino *et al.*[58] and Masson *et al.*[59] have suggested the facilitating approach in which the negatively charged substrates (i.e. aspirin) forms a complex with divalent cations and enters more easily to the active center of the BChE that possesses a negative charge. However, another divalent cation, zinc, was negatively affected the hydrolase activities (Figure 12). Therefore, the positive effect of calcium and magnesium on BChE cannot be explained by the complex formation of aspirin and divalent cations.

In an attempt for confirming the positive effect of calcium and magnesium on plasma BChE activity in the physiologically normal and disease conditions, their concentration dependency was studied in human (Figure 13). We found that BChE possesses high and low binding sites of calcium and/or magnesium. At higher concentrations of calcium and magnesium, these divalent cations bind to weaker binding affinity sites which resulted in more increased BChE activities. It is known that in the physiologically normal conditions, the free calcium and magnesium concentrations are 1.1 – 1.4 mM and 0.5 – 0.8 mM in the plasma, respectively. However, in some diseases, free calcium concentration in the plasma increases to higher levels (hypercalcemia) whereas more than 1.8 mM concentrations suggest cancer, particularly carcinoma and leukemia. In contrast, magnesium concentrations are less changeable than plasma calcium levels during diseases but in case of renal failure it can increase up to 1.6 mM. The high binding affinities of calcium and magnesium (1.46 mM, 1.00 mM respectively) are similar to their free concentrations in the physiologically normal conditions.

Some researchers performed activity analysis experiments with purified human plasma BChE using propionylthiocholine and/or butyrylthiocholine and indicated a

possible allosteric increase in the activity which is produced by the addition of 1mM calcium[69] whereas other researchers reported that 1 mM calcium and 1 mM magnesium do not effect activity of BChE, but they help to recover its activity after inhibited by the other divalent cations like zinc, nickel, cobalt and etc.[70,71] Since BChE is an allosteric protein, we have considered that calcium and magnesium binds to BChE and changes its conformation to more appropriate position for hydrolysis and therefore makes more active form of BChE. Furthermore it is also a possibility that the non-specific hydrolyzing sites to appear on the surface of BChE by divalent cations just like the case of albumin and caused further increases in the hydrolysis.

There are several reports that define different plasma proteins as the responsible enzymes for aspirin hydrolysis. This can be due to the dissimilar experimental conditions. Hence, our results confirmed BChE as the major responsible plasma protein for aspirin hydrolysis in physiologically normal conditions of free calcium concentrations. However, we have no accurate explanation about the number of bounded divalent cations and whether it is significant for increasing the hydrolase activity of BChE. In addition, human BChE is known to be soluble sugar-coated protein (23.9% by weight) from nine N-linked carbohydrate chains.[72,73] We have no information whether any of these sugar chains are related with the binding of divalent cations. We are planning to conduct further investigations in order to clarify these undetermined parts and to reveal exact interaction in between BChE and divalent cations.

Figure 14. Diagrammed comparison of amino acid sequences of human, monkey, dog, mouse, and rat plasma BChE. The residues that written in bold refer to the residues of catalytic triad. *(asterisk) means that the residues in that column are identical in all sequences in the alignment, :(colon) and .(period) means that conserved and semi-conserved substitutions have been observed, respectively.

Chapter 4
Divalent Cation Effect on Purified Human Plasma BChE

4.1. Introduction

As shown in Chapter 3, divalent cations are either positively or negatively affected the human plasma hydrolase activity for aspirin. For increased or decreased activities caused by divalent cations, we have proposed BChE as the responsible enzyme and considered the possibility of allosteric effects. In order to confirm these allosteric effects and the interaction between BChE and divalent cations, we have used purified human plasma BChE (HuBChE). In the previous chapters, we observed these noteworthy allosteric effects only for aspirin, the non-specific substrate. However, in this chapter, we studied the divalent cation effect not only in aspirin but also in butyrylthiocholine (BTCh), a well-known specific BChE substrate.[70,74,75)]

4.2. Results

4.2.1. Effect of Divalent Cations on Aspirin Hydrolysis

Hydrolysis of aspirin was firstly tested in HuBChE in the absence of divalent cations and it was shown that HuBChE rapidly hydrolyzed aspirin to salicylic acid with a rate of 2.36 nmol/U/min. Hydrolysis of aspirin by HuBChE was later tested in the presence of divalent cations. As in chapter 3, the role of divalent cations was tried to determine using calcium, magnesium, and zinc. Aspirin hydrolysis activities of HuBChE were shown in Figure 15. The control value refers to the above mentioned HuBChE activity for aspirin hydrolysis in the absence of divalent cations (2.36 nmol/U/min).

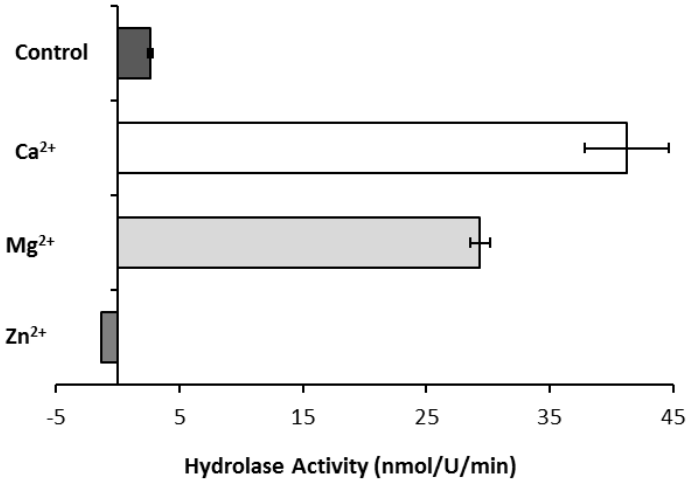


Figure 15. Effect of divalent cations on aspirin hydrolysis in HuBChE. Control refers to the hydrolysis condition in the absence of divalent cations at constant concentration of aspirin (1.0 mM). Final divalent cation concentration in the reaction mixture is 2 mM. Values represent the mean ± S.D. (*n*=2).

It can be seen clearly that HuBChE activity for aspirin hydrolysis is positively affected by the addition of calcium and magnesium (Figure 15). Calcium is found to be more effective than magnesium, since an 18-fold increase in the activity is observed in the presence of calcium whereas magnesium caused a 9-fold increase. In contrast to calcium and magnesium effect, addition of zinc is decreased the aspirin hydrolysis in HuBChE. These results are also compatible with the results of human plasma in Chapter 3.

4.2.2. Increased Aspirin Hydrolysis by Calcium

Addition of calcium is perceivably increased aspirin hydrolysis in HuBChE. To examine the effect of calcium on HuBChE, the rate of hydrolysis is plotted as a function of aspirin concentration in the absence (as a control) and the presence of two different calcium concentrations (low, 2 mM and high, 20 mM) (Figure 16). The concentrations of aspirin used to construct these plots were between 0.89 mM to 6.24 mM, since initial rate constants of aspirin hydrolysis were found to decrease at nearly 8 mM aspirin concentrations.[59)]

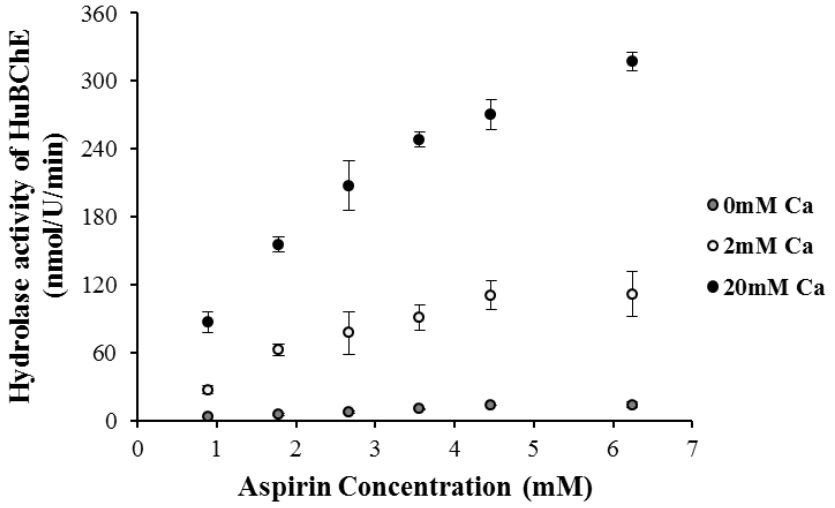


Figure 16. Effect of calcium on aspirin hydrolysis in HuBChE. "0 mM Ca" data refers to control as the hydrolysis condition in the absence of divalent cations.

As seen in Figure 16, both calcium concentrations is caused noticeable increases on the activity of HuBChE when compare to no metal conditions. It is known fact that addition of calcium ions may dissociate the aspirin molecules and may cause an increase in the free substrate concentration. Then, decreasing aspirin concentration together with the increased calcium concentration may help us to examine the calcium effect on HuBChE. Therefore, a further experiment was conducted under the low aspirin concentrations and the ratio of increased HuBChE activity vs. calcium concentration plots were drawn (Figure 17).

As it can be clearly seen from Figure 17, even in the lower than 1 mM aspirin concentrations, addition of 5 mM calcium is increased the activity about 30-fold. In case of higher calcium concentrations, such as 10 and 25 mM, hydrolase activity is kept increasing and suggested that calcium affects the BChE activity independent from the free concentration of aspirin.

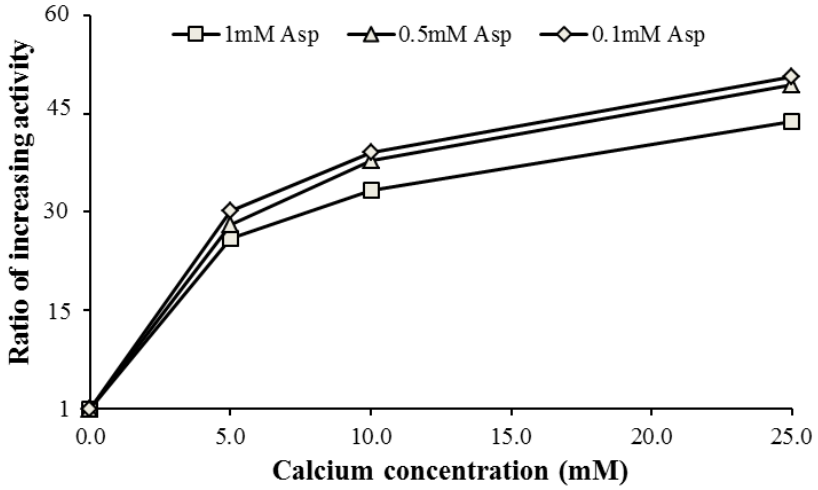


Figure 17. Calcium effect on hydrolysis of aspirin by HuBChE when the lower aspirin concentrations are used. Ratio of increasing activity was calculated by comparing the activities with the control data as no calcium addition conditions.

4.2.3. Kinetic Analysis of BChE-Catalyzed Aspirin Hydrolysis in the Presence of Calcium

In order to examine characteristics of the calcium activated hydrolysis, enzyme kinetic parameters were determined using aspirin in the absence and presence of calcium ions. K_M and V_{max} values are estimated by fitting the Michaelis-Menten equation to the data using none-linear regression analysis (MULTI)[76] and are listed in Table 5.

Table 5. Kinetic parameters for hydrolysis of aspirin by HuBChE as a function of calcium concentration. Values represent the mean ± S.D. (n=2).

Calcium	Aspirin hydrolysis		
Conc.	K_M (mM)	k_{cat} (sec^{-1})	k_{cat}/K_M ($sec^{-1}\ M^{-1}10^{-3}$)
0 mM	13.4 ± 4.20	65.4 ± 21.6	4.86 ± 0.09
2 mM	5.07 ± 0.33	308 ± 58.5	60.6 ± 7.55
20 mM	4.64 ± 0.45	789 ± 53.7	170 ± 5.02

K_M values are approximately 5-fold decreased when compared to that of no metal conditions in the presence of both low (2 mM) and high (20 mM) calcium concentrations. On the other hand, addition of 2 mM calcium is caused 4-fold increase in the k_{cat} of HuBChE for aspirin hydrolysis whereas 9-fold increase was observed in the presence of 20 mM calcium. These findings suggested that calcium has an enhancing effect on the HuBChE.

4.2.4. Kinetic Analysis of Calcium Effect on HuBChE

Further experiments were conducted to understand the effect of calcium on the increased HuBChE activity via changing concentrations of calcium in a constant aspirin concentration (1 mM). Figure 18 shows the effects of increasing divalent cation concentrations (0.5–25 mM) on the hydrolase activity of HuBChE. Data is plotted as hydrolase activity vs. hydrolase activity over divalent cation concentration (v vs. v/[M]) and the slope obtained from this plot will be related to the binding affinity (K_M) of calcium for HuBChE.

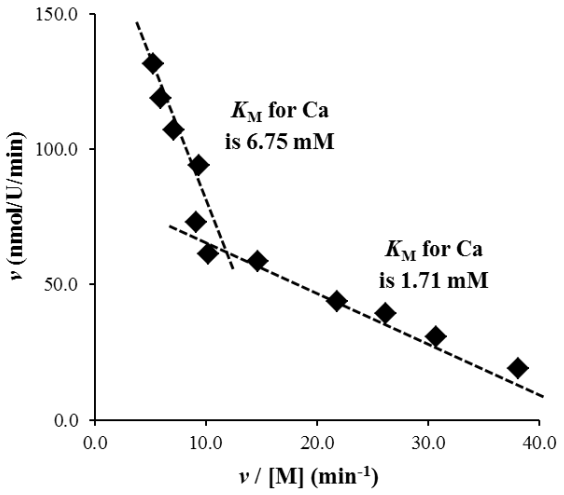


Figure 18. Comparison of the hydrolase activity (v) that is activated by calcium at different concentrations of the divalent cation (metal [M]) and constant concentration of aspirin (1 mM) in HuBChE. The plot resembles Eadie-Hofstee plot. The slopes are related to affinity of divalent cations for the enzyme.

When the activity plotted as a function of calcium concentration, two linear crossing lines were observed that suggests different binding sites of calcium. We supposed that one of the linear relations was occurred in between 0.5 mM and 8 mM calcium concentrations with a binding affinity of 1.71 mM. The second linear relation is occurred in between higher calcium concentrations (10–25 mM) with lower binding affinity (K_M is 6.75 mM) in contrast to that of low calcium concentrations. As shown in Chapter 3 (Figure 12), similar results were also obtained when hydrolase activity of human plasma is plotted in the same type of graph. Taken all together, these data suggested that addition of calcium affected the activity of BChE by causing two types of allosteric effect on the enzyme.

4.2.5. Hydrolysis of BTCh by HuBChE

Calcium effect on the hydrolase activity of HuBChE was further evaluated using another substrate, BTCh. As already mentioned in the introduction of this chapter, BChE rapidly hydrolyzes BTCh. For examining the hydrolyzing properties of HuBChE, enzyme kinetic parameters were determined. For this purpose, hydrolase activity of HuBChE is plotted against BTCh concentration (Figure 19). Then, K_M and V_{max} values are estimated by fitting the Michaelis-Menten equation to the data using none-linear regression analysis (MULTI).[76)]

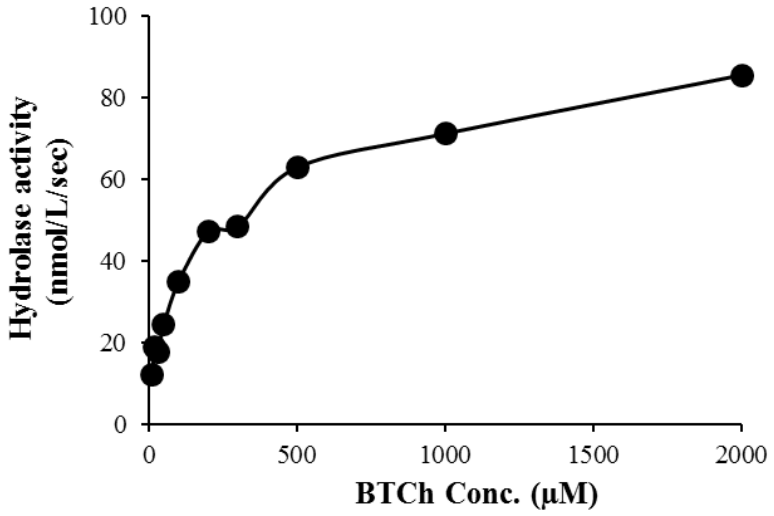


Figure 19. Hydrolysis of BTCh by HuBChE.

Hydrolase activity of HuBChE is continuously increased with the increasing concentrations of BTCh. However, in between 200 and 300 μM BTCh concentrations, activity of HuBChE was not significantly increased. These data makes us to consider about the presence of two different hydrolyzing patterns for this substrate. In order to visualize the presence of different hydrolyzing patterns, hydrolase activity of HuBChE *v* is plotted against hydrolase activity over substrate concentration *v*/[S] corresponding to Eadie-Hoftsee plot (Figure 20).

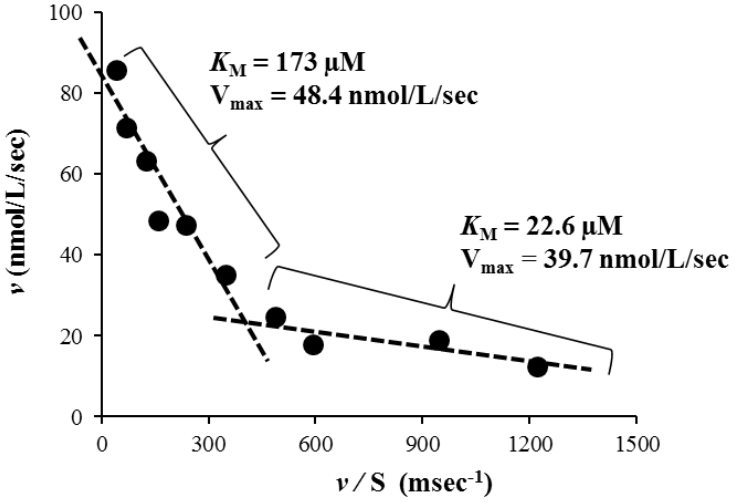


Figure 20. Enzyme kinetics of the hydrolysis of BTCh by HuBChE corresponding to Eadie-Hoftsee plot.

As seen in Figure 20, two crossing linear lines were observed as suggesting the two different hydrolyzing patterns. Kinetic evaluations of these plots were shown in Table 6. Higher binding affinity of HuBChE for BTCh with a 22.6 μM K_M value was shown if the substrate concentration is between 10 – 100 μM. However, if the substrate concentration is higher (200 – 2000 μM), K_M value is decreased by approximately 8-fold. Even though k_{cat} values were similar, about 6-fold slower catalytic efficiency (k_{cat}/K_M) was observed in the presence of high BTCh concentrations.

Table 6. Kinetic parameters for hydrolysis of BTCh by HuBChE.

BTCh	BTCh Hydrolysis		
Conc.	K_M (μM)	k_{cat} (sec^{-1})	k_{cat}/K_M ($sec^{-1}\,M^{-1}10^{-6}$)
10 – 100 μM	22.6	5398	239
200 – 2000 μM	172.8	6585	38

Effect of calcium on the hydrolysis of BTCh

Since two different hydrolyzing patterns were observed, two representative substrate concentrations (50 μM and 2000 μM) were selected to evaluate the calcium effect on the hydrolyzing activity of HuBChE. Four different calcium concentrations (low; 0.5 mM, 1 mM, 2 mM and high; 10 mM) were tested and the results are presented in Table 7. These results were evaluated as relative activity (%) by comparing the hydrolase activities in the presence of calcium with that of no calcium addition condition (Table 7).

Table 7. Effect of calcium on the hydrolysis of BTCh by HuBChE.

Relative Activity of HuBChE (%)		
Calcium	BTCh Concentrations	
Conc.	50 μM	2000 μM
0 μM	100	100
0.5 μM	71.1	93.9
1 μM	69.8	92.9
2 μM	61.6	89.8
10 μM	56.1	72.0

Calcium is inhibited the hydrolysis of BTCh in both concentrations. As shown in Table 7, only 62% of the hydrolysis activity was remained after addition of 2 mM calcium when a concentration from high binding affinity site (50 μM) is used. In contrast, only 10%

of the HuBChE activity was inhibited in the presence of 2 mM calcium when a concentration from low binding affinity site (2000 μM) is used. These data suggested that the hydrolyzing site with higher affinity is more sensitively inhibited by the addition of calcium.

4.3. Discussion

In this chapter, the divalent cation effect that observed on the aspirin hydrolysis of human plasma (Figure 12 in Chapter 3) is confirmed to be due to BChE by using HuBChE. Our results indicated calcium and magnesium as the activators and zinc as the inhibitor of BChE during the aspirin hydrolysis (Figure 15). Besides the positive effect of calcium and magnesium, we are showing for the first time that zinc has an inhibitory effect on HuBChE activity during the aspirin hydrolysis.

As for our knowledge, there are no reports of the inhibitory effect of zinc on HuBChE during the hydrolysis of aspirin. However, some researchers reported a strong inhibition of HuBChE by zinc when the activity was tested using BTCh.[70,71)] It was shown that BChE have Zinc-binding site that involves a His residue and constitutes the HXXE—H sequence on HuBChE.[77)] The divalent cations like nickel, cobalt, manganese, cadmium as well as aluminum and lithium are also binds to this Zinc-binding site of BChE and transform BChE to nonproductive or less productive conformational forms.[70,71,78)]

Another allosteric effect was observed during aspirin hydrolysis after the binding of calcium. In order to investigate the calcium binding to BChE enzyme, the concentration dependency of calcium vs. a constant aspirin concentration was studied (Figure 18). It is found that BChE is likely to have two calcium binding sites with high (K_M is 1.71 mM) and low binding affinities (K_M is 6.75 mM). There is some information in the literature that reports a single calcium binding site on BChE.

It was reported that calcium does not participate directly in catalysis of BChE and the calcium binding sites are at least 10 Å far from the catalytic residues.[79)] BChE belongs to the class of α/β hydrolase fold family and consists of central β-sheets surrounded by α-helices.[80)] Calcium binding sites found in proteins arise from E and F helices and its intervening loop. EF-hand motifs are also distributed in the α/β protease family.[81)]

Kretsinger[82] established a canonical consensus sequence and according to this motif sequence Schallreuter *et al*.[83] reported the presence of a single EF-hand binding site per each subunit of human plasma BChE. As shown in Figure 21, the potential ligands for EF-hand site in the enzyme are revealed to be Asp375, Asp378, Gln380, Pro382 and Glu387.[83]

(a)

Canonical	**α-helix E ~** X * Y * Z G # I –X * * –Z **~ α-helix F**
BChE	**α-helix E ~** D * D * Q R # E N * * E **~ α-helix F**

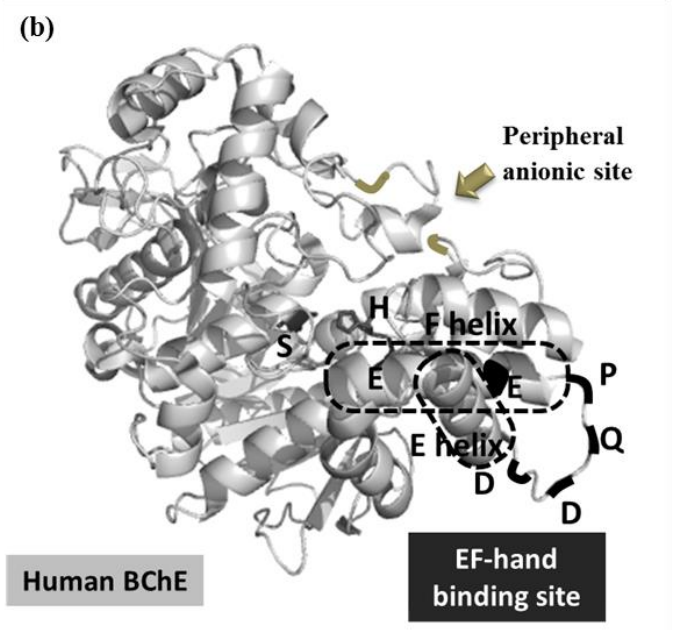


Figure 21. (a) EF-hand binding consensus sequences of a classical canonical calcium binding loop that is established by Kretsinger.[82] In this motif, X,Y,Z, -X and -Z refer to calcium coordinating amino acids (Asp, Asn, Ser, Thr, Glu, Gln). The potential ligands in human plasma BChE which is suggested by Schallreuter *et al*.[83] **(b)** The ribbon model for showing the crystal structure of human plasma butyrylcholinesterase (Protein Data Bank code 2XMB). S, E and H represent the catalytic amino acids (Ser, Glu and His) of BChE. α-helix E and α-helix F were indicated by dashed-line circles. The EF-hand binding site which consists of Asp375, Asp378, Gln380, Pro382 and Glu387 residues were shown in bold as D, D, Q, P and E, respectively. The structure was drawn with PyMOL software.[50]

Although only one EF-hand binding site was reported for BChE, we have found two binding sites including a different binding site rather than EF-hand site. The high binding affinity of calcium to BChE under the concentrations of 8 mM may be represents the EF-hand binding site. It is shown by the aspirin hydrolysis experiments (Figure 16, Table 5) that addition of low calcium concentrations caused a 5-fold increase in the binding affinity of BChE for aspirin and a 3-fold increase in the maximum reaction rate (Table 5). After this binding happened, calcium may cause a change in the conformation of BChE to a more appropriate position for aspirin hydrolysis and therefore makes more active form of BChE. On the other hand, in the presence of higher calcium concentrations, binding affinity of BChE for aspirin is similar to that of low calcium concentrations. Therefore we have considered that the lower binding affinity of calcium to BChE in the presence of higher than 8 mM calcium concentrations may be creating an extra hydrolyzing environment for aspirin. Our idea is also confirmed by the increased catalytic efficiency of the enzyme most likely to due to the created extra hydrolyzing environments.

In contrast to its enhancing effect on aspirin hydrolysis, even addition of low calcium concentrations is slightly decreased the hydrolase activity of HuBChE for BTCh. It has been reported elsewhere that (1mM) calcium and magnesium had no effect on the HuBChE activity[70] whereas another study reported that addition of (1mM) calcium produced possibly an allosteric increase in activity.[69,84] Our data also represented the report of Sarkarati *et al.*[70] when the lower concentrations of calcium and higher concentrations of BTCh were used.

Since BTCh is well-known substrate for activity analysis of BChE, we also have tested the characteristics of BChE enzyme using BTCh in the absence of divalent cations. Kinetic property analyses were suggested the presence of different hydrolyzing patterns on BChE with high and low affinities (Figure 19, Table 6). We have found the first hydrolyzing pattern with high affinity (K_M is 22.6 μM) when low concentrations of BTCh (10 – 100 μM) are used. However if higher concentrations of BTCh (200 – 2000 μM) were used, BChE showed lower hydrolyzing affinity (K_M is 172.8 μM) to this substrate. Since BChE has allosteric properties, this enzyme is very flexible to change its conformation. Therefore, we have speculated that in the presence of high BTCh concentrations, BTCh

binds non-specifically and creates some conformational changes on BChE and thus altering the hydrolyzing properties of BChE.

As already mentioned above, in the presence of low calcium concentrations (0.5 – 2 mM) BTCh hydrolysis experiments are showed the inhibitory effect on the hydrolysis in both BTCh hydrolyzing patterns. Furthermore, addition of higher calcium concentration (10 mM) caused a little more inhibition than that caused by low calcium concentrations again in both patterns. It can be considerable that just like calcium effect on BChE conformation for aspirin hydrolysis; in this instance, high concentrations of BTCh may cause conformational changes on BChE by non-specific binding. Thereon addition of calcium causes an allosteric effect at low concentrations of BTCh and change the conformation of BChE to more inappropriate position but at high BTCh concentrations this addition did not cause that much conformational change in BChE.

Taken all together, we have shown that calcium caused an allosteric effect on human plasma BChE enzyme by affecting its conformation and change it to more active or passive forms.

Summary

Esterified compounds can be found in the human environment as medicines and nutrients. In the drug discovery stages, the esterification is frequently used to develop prodrugs and/or soft drugs. Inside the body, these drugs can be enzymatically converted to their preferred forms by esterases mainly in the liver, intestine and plasma. However, in some cases the hydrolysis process is more rapidly occurs in the plasma rather than liver and intestine. But activity of plasma esterases is markedly different in between humans and other animals resulting in differences in the pharmacokinetics of these prodrugs and creates an added difficulty during development. To understand interspecies variation in response to drugs it is important to know the identities and properties of the plasma esterases responsible for drug hydrolysis. Therefore, in the current study we have focused on plasma esterase activities and aimed to provide useful information regarding the unknown features of species differences and to propose a suitable animal model for predicting the pharmacokinetics of xenobiotics in human. For this purpose, different types of studies were undertaken and the overall findings are summarized below.

In Chapter 1, expression patterns of plasma esterases were visualized on native-PAGE gels and species differences were revealed in between human plasma and the plasma of several other preclinical species such as monkey, dog, minipig, rabbit, mouse and rat. Two of the main plasma esterases, PON and BChE together with albumin, most abundant plasma protein, were observed in all tested animals. However, different contents of expressed esterases were indicated the species differences in these tested animals. Especially, these species differences were confirmed in BChE contents and it was shown that plasma of human, monkey, minipig and dog has intense BChE bands whereas rabbit, mouse and rat plasma appeared to show only weak expression. Furthermore, neither human and monkey nor dog and minipig plasma contained the other main plasma esterase, CES, which is the major component of rabbit, mouse and rat plasma. Since CES is abundantly expressed in rabbit, mouse and rat plasma, it was considered to be a possible reason for their lower BChE contents. Additionally, beside these main plasma esterases and albumin, various unknown esterases were observed in minipig and mouse plasmas.

In Chapter 2, interspecies variation was evaluated by the plasma hydrolase activities. Five types of compounds (PNPA, irinotecan, oseltamivir, temocapril and propranolol derivatives) were selected for activity analysis based on their structure differences. PNPA used for the estimation of total esterase activity and minipig plasma have shown the highest activity for PNPA in spite of the fact that other substrates cannot be hydrolyzed by minipig plasma. Irinotecan is a substrate with a carbamate group and both CES and BChE showed resistance to carbamate group of this substrate. Oseltamivir, temocapril and the propranolol derivatives, are known as good substrates, especially for CES with different size of acyl moieties. High levels of hydrolase activity for oseltamivir, temocapril and propranolol derivatives were obtained in rat and mouse plasma depending on their highly expressed CES enzyme. However, only propranolol derivatives were rapidly hydrolyzed by rabbit plasma CES. In contrast, the lower hydrolysis activities that observed in human, monkey, dog and minipig plasmas were mainly due to their abundant expression of BChE enzymes. Herein, one exception was observed. It is revealed that only monkey plasma BChE was rapidly hydrolyzed *O*-valeryl-(R)-propranolol in spite of the fact that human and monkey plasma BChEs shows 96% homology. Sequence analysis were revealed that the acyl-binding pocket only differs in one amino acid, the Pro285 residue in human BChE is replaced by the more flexible Leu in monkey BChE. Since acyl pocket is also affect the substrate specificity of BChE, the increased flexibility of the acyl-binding pocket in monkey BChE is suggested as a possible reason for the different substrate specificities of human and monkey plasma BChEs.

In Chapter 3, substrate specificities of plasma esterases were studied in more detail using aspirin as the model compound. All tested species were rapidly hydrolyzed aspirin to salicylic acid. Rabbit plasma showed faster aspirin hydrolysis than human and monkey, while mouse and rat plasma hydrolyzes aspirin slowly enough to be comparable to human. Although CES of rabbit plasma was rapidly hydrolyzed aspirin, CES activity of mouse and rat plasma was found to be low for hydrolyzing this compound. On the other hand, rapid hydrolysis in human plasma was found to be depending on its main hydrolases, PON, BChE and albumin which share the activity by 50%, 30% and 20%, respectively. Aspirin hydrolysis in monkey and dog plasma has found to be similar to that of human plasma and

due to their major plasma hydrolases. The studies in order to confirm the responsible esterases in the aspirin hydrolysis activity were revealed the enhancing effect of divalent cations such as calcium and magnesium on BChE activities especially in human plasma. The increasing concentrations of divalent cations resulted in two phase of stimulating effects. It is predicted that calcium and magnesium binds to BChE and causes a change in the conformation of enzyme to more appropriate position for aspirin hydrolysis and therefore makes more active form of BChE.

In Chapter 4, detailed studies were performed to understand the divalent cation effect on human plasma BChE. An allosteric effect was observed during aspirin hydrolysis by binding of calcium and the presence of two calcium binding sites with high and low binding affinities were found as dependent to its concentration. We have thought the first binding site with the high binding affinity as the EF-hand binding site which already reported in the literature. It is considered that, in the presence of low calcium concentrations (under 8 mM), calcium binds to EF-hand binding site of BChE and may cause a change in the conformation of BChE to a more appropriate position for aspirin hydrolysis. On the other hand, in the presence of higher calcium concentrations (above 8 mM), the second binding site of BChE for calcium with lower binding affinity may be creates an extra hydrolyzing environment for aspirin and therefore makes more active form of BChE. In contrast to this enhancing effect on aspirin hydrolysis, even addition of low calcium concentrations is inhibited the hydrolase activity of BChE for BTCh, the well-known BChE substrate. This inhibition effect by calcium was larger at low concentration of BTCh than that of high BTCh concentrations due to the fact that BTCh was hydrolyzed by two hydrolyzing patterns with low and high affinities. At low BTCh concentrations hydrolysis is occurred in the active site of BChE. However, if higher concentrations of BTCh are present, this substrate non-specifically bounded to BChE and creates some conformational changes and thus altering the hydrolyzing properties of BChE. In fact, at low concentrations of BTCh calcium causes an allosteric effect and change the conformation of BChE to more inappropriate position but at high BTCh concentrations calcium addition did not cause that much conformational changes in BChE.

As a conclusion to overall study; the expression pattern and substrate specificities of plasma esterases of humans and several other animals were confirmed. The esterase activity observed in human plasma could not be dependably represented by rabbit, mouse, rat, monkey, dog or minipig plasma. In rabbit, mouse and rat plasma, substrates are mainly hydrolyzed by their highly active CES enzyme, but CES is not expressed in human plasma. Among the animals tested, monkey plasma appeared to be the closest to human plasma, due to its high sequence homology with the abundantly expressed esterase enzyme. The hydrolyzing pattern of human plasma was also similar to that of dog and minipig plasma. However, if both esterase expression and hydrolyzing pattern are considered, human plasma was found to be closer to dog plasma. Furthermore, if the effect of divalent cations were also considered both monkey and dog plasma was found to be closer. Nevertheless, efficient effect of divalent cations on human plasma BChE could not be dependably represented by monkey and dog plasma. It is revealed that different divalent cations have different effects on human plasma BChE activity in concentration-dependent manner. Since BChE has allosteric properties, it is very flexible to change its conformation. Therefore, divalent cations like calcium cause an allosteric effect on human plasma BChE enzyme by affecting its conformation and change it to more active or passive forms. However more detailed structural analysis will be needed.

In general, these findings indicate that differences in substrate-specific hydrolase activities are found in many animals; these differences must be considered when selecting animal models in the discovery stage of the process of generating new drugs, especially ester-type prodrugs intended for human use.

Material and Methods

1. Plasma Samples

Blood was taken from humans (male, 22–26 years old), mice (ddy, male, 10 weeks old) and rats (Wistar, male, 8 weeks old, 0.20–0.25 kg) into heparinized syringes and centrifuged at 1500*g* for 10 min. All studies involving human samples were approved by the Institutional Review Board of Kumamoto University, Faculty of Life Sciences. Healthy volunteers provided written informed consent prior to their participation in the study. Plasma from Rhesus monkeys (male, 3–5 years old), Cynomolgus monkeys (male, 3–4 years old), dogs (Beagle, male, 1 year old) and rabbits (Japanese white strain, male, 10 weeks old, 1.5–2 kg) was kindly provided by Sekisui Medical Co., Ltd (Tokyo, Japan). Minipig plasma (Clown miniature, 13 months old, 28–32 kg) was a kind gift from Ono Pharmaceutical Co., Ltd. (Osaka, Japan). Plasma samples were kept in −80°C until used. All experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Kumamoto University.

2. Materials

Propranolol derivatives were synthesized as described in Shameem *et al.*[85] from propranolol hydrochloride (Wako Pure Chemicals, Osaka, Japan). Temocapril and temocaprilat were provided by Daiichi Sankyo Co., Ltd. (Tokyo, Japan). Irinotecan and SN-38 were donated by Yakult Honsha Co., Ltd. (Tokyo, Japan). Oseltamivir and Ro64-0802 were kind gifts from Prof. Ogihara (Takasaki University of Health and Welfare, Gunma, Japan). Aspirin (acetylsalicylic acid), salicylic acid (2-hydroxybenzoic acid), *p*-nitrophenylacetate (PNPA), butyrylthiocholine iodide (BTCh), α-naphthylacetate, bis-*p*-nitrophenyl phosphate (BNPP), di-sodium dihydrogen ethylenediamine tetraacetate dihydrate (EDTA 2Na dihydrate), calcium chloride dihydrate ($CaCl_2.2H_2O$), magnesium chloride hexahydrate ($MgCl_2.6H_2O$) and zinc chloride ($ZnCl_2$) were purchased from Nacalai Tesque (Kyoto, Japan). Butyrylcholinesterase from human serum (HuBChE), Butyrylcholinesterase from equine serum (Equine BChE), purified human serum albumin (HSA), rabbit serum albumin (RSA), 5',5'-dithio-bis (2-nitrobenzoic acid) (DTNB),

ethopropazine, Fast Red TR and Fast Blue TR were from Sigma-Aldrich (St. Louis, MO). *p*-Nitrophenol (PNP) and β-naphthylacetate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals and reagents were of analytical grade.

3. Methods

3.1. Chapter 1

3.1.1 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed as described by Mentlein *et al.*[86] For the separation of native enzymes, polyacrylamide gels (6% w/w) containing 1% (w/v) Nonidet P-40 were used. Plasma samples were diluted to 25% in HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl] ethane sulfonic acid) buffer (50 mM, pH 7.5), final volume 30 μL, and loaded into each well. After electrophoresis, gels were stained by β-naphthylacetate, in the presence or absence of $CaCl_2$ (10 mM), through coupling to liberated β-naphthol with Fast Blue TR salt to visualize esterase activities. The confirmation of BChE bands was performed by formation of copper ferrocyanide, based on the reduction of ferricyanide by thiocholine converted from butyrylthiocholine.[17] The staining solution contained 32.5 mL of 0.1 M maleate-tris buffer (pH 6.0), 2.5 mL 0.1 M sodium citrate, 5 mL 0.03 M copper (II) sulfate, 5 mL water, 5 mL 5 mM potassium ferricyanide, and 25 mg BTCh. Gels were incubated for 2–3 h with gentle shaking. Activity was visualized as dark brown bands on the gels.

3.2. Chapter 2

3.2.1. Hydrolysis experiments

Reaction conditions

Hydrolysis experiments with PNPA, irinotecan, oseltamivir, temocapril and propranolol derivatives were performed in plasma of human and other species. Plasma samples were diluted to appropriate concentrations with HEPES buffer (50 mM, pH 7.5) or, in the case of irinotecan, with phosphate-buffered saline (PBS) (pH 7.4). Hydrolysis reactions were initiated by the addition of substrates dissolved in dimethyl sulfoxide

(DMSO) after pre-incubation of the plasma samples (200 μL) at 37°C for 5 min. The final DMSO concentration was less than 1%, which has no effect on hydrolytic activity. The initial hydrolytic activity was measured under reaction conditions in which less than 25% of substrate was hydrolyzed. The rate of hydrolysis was determined by subtracting the hydrolysis rate in buffer from that in plasma samples. The hydrolytic activity was presented as the concentration of the hydrolysis product. In the PNPA hydrolysis reactions, *p*-nitrophenol (PNP) was periodically detected at 405 nm in the spectrophotometer (V-530; Jasco, Tokyo, Japan). For the other substrates, the formation of hydrolysate was determined after appropriate incubation periods by analyzing samples on high-performance liquid chromatography (HPLC). In the inhibition experiments, both inhibitors (BNPP and ethopropazine) were dissolved in DMSO to obtain two different final concentrations (low, 100 μM or high, 500 μM) and plasma samples were pre-incubated with these solutions for 5 min at 37°C before the addition of substrate. Control reactions were carried out in parallel by adding only DMSO to plasma samples. Inhibition percentage (%) was represented as the relative activity in the presence of the inhibitor compared with control activity.

Termination of reactions

Hydrolysis of the substrates (irinotecan, oseltamivir, temocapril and propranolol derivatives) was terminated by the addition of different solvents after appropriate incubation periods. The reaction mixtures obtained were prepared for HPLC analysis, as described below.

The hydrolysis reaction of irinotecan was terminated by addition of 400 μL methanol (MeOH). After centrifugation of the reaction mixture at 1600*g* for 10 min, the supernatant (300 μL) was added to 5% H_3PO_4 (10 μL). The hydrolysis of oseltamivir was terminated by addition of 600 μL MeOH. After centrifugation of the reaction mixture at 1600*g* for 10 min, the supernatant (600 μL) was added to 12% H_3PO_4 (7.5 μL) and 1.2 M 1-pentanesulfonic acid sodium salt (15 μL). The hydrolysis of temocapril was terminated by adding 6 mL ethyl acetate and 0.5 mL saturated NaCl adjusted to pH 2.7 by 1 M H_3PO_4. After the samples had been shaken for 10 min, the ethyl acetate phase was removed to a separate tube and evaporated off. The residue was dissolved in a mixture of 180 μL

acetonitrile (ACN) : water (1:2, v/v) and 20 μL 3.4 M H_3PO_4. The hydrolysis reaction of propranolol derivatives was terminated by addition of 6 mL ethyl acetate and 1 mL saturated NaCl adjusted to pH 1 by H_3PO_4. After the samples had been shaken for 10 min, the ethyl acetate was evaporated off. The residue was dissolved in a mixture of 160 μL hexane and 40 μL 2-propanol.

3.2.2. HPLC conditions

The concentrations of each hydrolysate were determined by HPLC. The HPLC system comprised a JASCO PU-980 pump, a JASCO 980-UV detector, a JASCO AS950 autosampler, a JASCO CO-965 column oven and a JASCO 1520S fluorescence detector (JASCO Co., Tokyo, Japan), and a Shimadzu chromatopac C-R7A plus (Shimadzu Co., Ltd., Kyoto, Japan). The column and composition of the mobile phase used for each assay are listed in Table 8. All substrates and hydrolysates were clearly separated. Each hydrolysate was measured in a quantitatively linear range.

3.2.3. Statistical analysis

Results are expressed as the mean ± S.D. (standard deviation). Data were analyzed using Student's t-test with $p<0.05$ as the minimal level of significance.

Table 8. Conditions for HPLC analysis of Chapter 2.

Compound	Column	Mobile Phase	Detection	Retention Time	Detection Limit[a]
Propranolol	Chiralcel OD[b]	Hexane/2-propranolol/Diethylamine (90:10:1.0) Isocratic Flow rate: 1 mL/min	Fluorescence Ex 285 nm Em 340 nm	R; 11.1 min S; 18.5 min	20 pmol
Temocaprilat	Inertsil ODS-2[c]	A: ACN/10 mM Phosphoric acid (70:30) B: 10 mM Phosphoric acid Gradient: 45% A (0−10 min), 45→100% A (10−20 min), 100% A (20−25 min), 100→45% A (25−26 min), 45% A (26−38 min) Flow rate: 0.8 mL/min	UV 258 nm	6.8 min	30 pmol
SN-38	Lichrospher 100 RP-8[d]	75 mM Ammonium acetate (pH 6.0)/ACN (70:30) Isocratic Flow rate: 1 mL/min	Fluorescence Ex 375 nm Em 568 nm	4.2 min	15 pmol
Ro64-0802	Inertsil ODS[e]	20 mM Phosphoric acid/MeOH (50:50) including 10 mM 1-Pentane sulphonic acid sodium salt Isocratic Flow rate: 1 mL/min	UV 220 nm	6.5 min	10 pmol

[a] Detection limit represented as injection amount on column.
[b] 4.6 mm i.d., 250 mm (Daicel Chemical Industries Ltd., Tokyo, Japan).
[c] 4.6 mm i.d., 250 mm (GL Science Inc., Tokyo, Japan).
[d] 4.0 mm i.d., 125 mm (Merck, Darmstadt, Germany).
[e] 4.6 mm i.d., 250 mm (Gasukuro Kogyo, Inc., Tokyo, Japan).

3.3. Chapter 3

3.3.1. Hydrolysis experiments

Reaction Conditions

Aspirin solutions were prepared freshly just before starting the experiments by dissolving aspirin in HEPES buffer (50 mM, pH 7.4). Plasma samples of human and other species were diluted with HEPES buffer (50 mM, pH 7.4) and their aliquots (100 µL) were preincubated at 37°C for 5 min, and the reactions were started by the addition of aspirin (100µL; final concentration is 2.5 mM). After 30 min incubation, reactions were terminated by addition of 400 µL ice-cold acetonitrile. Then, reaction mixture was centrifuged at 1600 g for 10 min, aliquot (400 µL) of the supernatant was added to 10% (v/v) H_3PO_4 (10 µL) and were analyzed by HPLC. The initial hydrolytic activity was measured under reaction conditions in which less than 20% of substrate was hydrolyzed. The rate of hydrolysis was determined by subtracting the hydrolysis rate in HEPES buffer from that in plasma samples. The hydrolytic activity was presented as the concentration of the hydrolysis product.

Effect of Enzyme Specific Inhibitors

Hydrolysis experiments were repeated in the presence of the following inhibitors: BNPP, ethopropazine and EDTA at the concentration of 0.5 mM, 0.5 mM and 1 mM, respectively. BNPP and ethopropazine were dissolved in DMSO whereas the latter was dissolved in HEPES buffer (50 mM, pH 7.4). Diluted plasma samples were incubated with these solutions for 5 min, at 37°C before the addition of the substrate and were then processed as above. The final DMSO concentration was less than 1%, which has no effect on hydrolytic activity. Control reactions were carried out in parallel by adding only DMSO or in case of EDTA inhibition, HEPES buffer (50 mM, pH 7.4) to plasma samples. Inhibition percentage (%) was represented as the relative activity in the presence of the inhibitor compared with control activity.

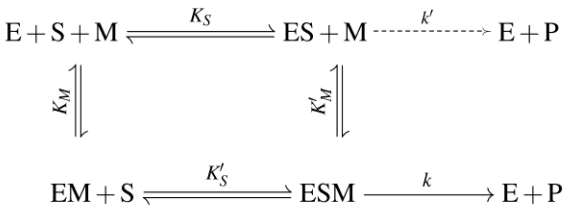
Effect of Divalent Cations

The effect of divalent cations, calcium (Ca), magnesium (Mg) and zinc (Zn) on the hydrolysis of aspirin were investigated. $CaCl_2$, $MgCl_2$ and $ZnCl_2$ were dissolved in HEPES buffer (50 mM, pH 7.4) to obtain needed concentrations (2–25 mM) when mixed with aspirin solutions. The reaction was started by simultaneous addition of aspirin and divalent cation to preincubated plasma samples. Reaction mixtures were then processed as above.

Evaluation of the Kinetic Behavior of Divalent Cation Effect

The Michaelian behavior of the activation by metal ions in the constant aspirin concentration was tried to describe by deriving an equation based on the model in Scheme 1,

Scheme 1.



where E is the enzyme, S is substrate (aspirin), M is the metal ion (i.e. divalent cation), ES is the enzyme-substrate complex, EM is the enzyme-metal complex, ESM is the enzyme-substrate-metal complex and P is the product. Additionally, K_{M} and K'_{M} are the Michaelis constants for EM and ESM, respectively while K_{S} and K'_{S} are the dissociation constants for ES and ESM, respectively. *k* and *k'* are the rate constants for the breakdown of ESM to E and P, and ES+M to E and P, respectively.

It is assumed that EM and ES is occurs independently and the activity of ES is much more low than that of ESM. Additionally, breakdown of ES+M, *k'*, is assumed to be much more low than that of ESM, *k*. Also, $K_{\mathrm{S}} \cdot K'_{\mathrm{M}}$ is assumed as equal to $K'_{\mathrm{S}} \cdot K_{\mathrm{M}}$. Under these assumptions, initial velocity (v) of the activation can be written as $v = k \cdot [ESM]$ and limiting maximal velocity (V_{max}) can be taken as $V_{max} = k \cdot [E]_o$ whereas $[E]_o$ resembles

the total enzyme concentration ($[E]_o = [\mathrm{E}] + [\mathrm{EM}] + [\mathrm{ESM}] + [\mathrm{ES}]$). In this way, a model that resembles Michaelis–Menten equation can be derived, as shown in equation 1 (eq 1).

$$v = \frac{V_{\max}[\mathrm{M}][\mathrm{S}]}{K_{\mathrm{S}}\,(K_{\mathrm{M}} + [\mathrm{M}]) + [\mathrm{S}](K_{\mathrm{M}} + [\mathrm{M}])} \qquad \text{(eq 1)}$$

This equation describes the rate of enzymatic reactions, by relating the reaction rate, *v* (hydrolase activity) to the [M], the concentration of divalent cation.

In order to represent enzyme kinetics graphically, a model equation (eq 2) that resembles the Eadie-Hofstee diagram is derived from the eq 1 in which reaction velocity (*v*) is plotted as a function of the velocity – divalent cation concentration ratio (*v*/[M]).

$$v = \frac{V_{\max}[\mathrm{S}]}{K_{\mathrm{S}} + [\mathrm{S}]} - K_{\mathrm{M}}\frac{v}{[\mathrm{M}]} \qquad \text{(eq 2)}$$

The *v* vs. *v*/[M] plot will yield $V_{\max}[\mathrm{S}]/K_{\mathrm{S}} + [\mathrm{S}]$ as the y-intercept, and K_{M} as the slope.

3.3.2. HPLC conditions

The concentration of the hydrolysate, salicylic acid, was determined by HPLC. The HPLC system comprised a JASCO PU-980 pump, a JASCO AS-950 autosampler, a JASCO UV-2075 Plus UV detector, a JASCO FP-1520 fluorescence detector, a JASCO LC Net II/ADC System (JASCO Co., Tokyo, Japan), and a HITACHI 655A-52 column oven (HITACHI Ltd., Tokyo, Japan). For determination, a TSK-GEL ODS-80Ts column (5 µm, 4.6 mm, 150 mm inner diameter (i.d.); TOSOH Bioscience, Tokyo, Japan) was used. The temperature of the column was maintained at 40°C. Aspirin was detected by UV at 277 nm whereas salicylic acid was detected by fluorescence at Ex and Em of 296 and 405 nm, respectively. During hydrolysis activity experiments, a mobile phase of 0.1% H_3PO_4 / ACN (60:40, v/v) was used at the flow rate of 1.0 mL/min. Retention time of aspirin and salicylic acid was 3.2 min and 4.4 min, respectively. A mobile phase of 0.1% H_3PO_4 / ACN (70:30, v/v) at the same flow rate was used while conducting the inhibition experiments. Retention time of aspirin and salicylic acid was 5.0 min and 7.2 min, respectively. Detection limit, which represents the injection amount on column, was 3 pmol and was measured in a quantitatively linear range.

3.2.3. Statistical analysis

All results are expressed as the mean ± S.D. for triplicate (n = 3). Data were analyzed using Student's t-test with $p < 0.05$ as the minimal level of significance.

3.4. Chapter 4

3.4.1. Hydrolysis experiments with Aspirin

Effect of Divalent Cations on Aspirin Hydrolysis

The effect of divalent cations, Ca, Mg and Zn, on the hydrolysis of aspirin were investigated by HuBChE. 0.5 Unit (U)/mL stock solution of HuBChE was prepared as described in the Sigma's product fact sheet and was kept in −80°C until used. 1 U HuBChE hydrolyzes 1.0 μmole of butyrylcholine to choline and butyrate per min at pH 8.0 at 37 °C. Before starting the experiments, HuBChE was diluted with HEPES buffer (50 mM, pH 7.4) to the required concentration (0.1 U/mL) and kept on ice. $CaCl_2$, $MgCl_2$ and $ZnCl_2$ were also dissolved in HEPES buffer (50 mM, pH 7.4) to obtain 2 mM final concentration when mixed with aspirin solution (final concentration is 1mM). Diluted HuBChE samples (100 μL) were preincubated at 37°C for 5 min, and reactions were started by the addition of aspirin and divalent cation mixture. After 30 min incubation, reactions were terminated by addition of 400 μL ice-cold ACN. Then, reaction mixture was centrifuged at 1600 *g* for 10 min, aliquot (400 μL) of the supernatant was added to 10% (v/v) H_3PO_4 (10 μL) and were analyzed by HPLC. The initial hydrolytic activity was measured under reaction conditions in which less than 20% of substrate was hydrolyzed. The rate of hydrolysis was determined by subtracting the hydrolysis rate in HEPES buffer from that in HuBChE samples. The hydrolytic activity was presented as the concentration of the hydrolysis product.

Kinetic Analysis of BChE Catalyzed Hydrolysis of Aspirin in the presence of Calcium

Kinetic analyses were carried out over the final aspirin concentration range 0.89 – 6.24 mM in the presence of needed calcium concentrations. Aspirin only or aspirin and divalent cation mixtures (180 μL) were preincubated at 37°C for 5 min, and the reactions were started by the addition of HuBChE (20μL; final concentration is 0.05 U/mL)

dissolved in HEPES buffer. After 60 min incubation, reactions were terminated and processed as above and the rate of hydrolysis was determined by subtracting the hydrolysis rate in HEPES buffer from that in HuBChE.

HPLC Conditions

The concentration of the hydrolysate, salicylic acid, was determined by HPLC as explained above in the Chapter 3. Briefly, a TSK-GEL ODS-80Ts column (5 μm, 4.6 mm, 150 mm inner diameter (i.d.); TOSOH Bioscience, Tokyo, Japan) was used with a mobile phase of 0.1% H_3PO_4 / ACN (60:40, v/v) at the flow rate of 1.0 mL/min. The temperature of the column was maintained at 40°C. Aspirin was detected by UV at 277 nm whereas salicylic acid was detected by fluorescence at Ex and Em of 296 and 405 nm, respectively. The substrate, aspirin, and hydrolysate, salicylic acid, were clearly separated and were measured in a quantitatively linear range.

3.4.2. Hydrolysis experiments with BTCh

HuBChE activity was measured spectrophotometrically (V-530; Jasco, Tokyo, Japan) according to the method of Ellman *et al.*[74] In this method, BTCh (used as substrate) is hydrolyzed by BChE and the product (thiocholine) together with DTNB gives a colored compound at 412 nm. In the kinetic studies, initial velocities were measured at 37°C by using 0.01 mM DTNB, dissolved in HEPES buffer (50mM, pH 7.4) as a function of BTCh concentrations from 0.1 to 2.0 mM in 0.8 ml final volume of reaction mixture. For the effect of calcium on BChE activity, appropriate amounts of calcium were mixed with BTCh concentrations and then added to reaction mixture as substrate. Final concentration of HuBChE in the reaction mixtures was 6.25×10^{-4} U/mL. All activities were measured up to 100 s.

3.4.3. Data analysis

K_M and V_{max} values are estimated by fitting the Michaelis-Menten equation to the data using none-linear regression analysis (MULTI).[76] K_M values for the calcium effect on HuBChE are calculated using eq.2, as mentioned above in the Chapter 3.

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