Characterization and interspecies diversity of xenobiotic metabolism: A study of phase I oxidation and phase II conjugation reactions

（異物代謝酵素の酵素学的特徴と多様性: 第 I 相反応と第 II 相抱合反応）

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ABBREVIATIONS

AHR	Aryl hydrocarbon receptor
CPR	Cytochrome P450 reductase
CYP or P450	Cytochrome P450
EC_{50}	Half maximal effective concentration
Ex	Excitation
Em	Emission
ESI	Electrospray ionization
HPLC/UV	High-pressure liquid chromatography/ultraviolet detector
HPLC/FD	High-pressure liquid chromatography/fluorescence detector
Km	Michaelis constant
KPB	Potassium phosphate buffer
LD_{50}	Medium lethal dose
MS	Mass spectrometry
m/z	Mass-to-charge
OP	Organophosphate
PAHs	Polycyclic aromatic hydrocarbons
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PCBs	Polychlorinated Biphenyls
ppb	Part per billion
ppm	Part per million
PY	Pyrene
PYdiol	Pyrenediol
PYdiol-S	Pyrenediol-sulfate
PYdiol-diS	Pyrenediol-disulfate

PYOG	Pyrene-1-glucuroniude
PYOH	1-hydroxypyrene
PYOS	Pyrene-1-sulfate
RT	Retention time
SRS	Substrate recognition sites
SULT	Sulfotransferase
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
UGT	UDP-glucuronosyltransferase
VKOR	Vitamin K 2,3-epoxide reductase
Vmax	Maximum velocity
Vmax/Km	Enzymatic efficiency

INTRODUCTION

Xenobiotic metabolisms and species differences

Xenobiotic compounds refer to various chemicals that are found in an organism, but are not normally produced or expected to be present in the organism. Generally, natural compounds that have biological effects on organisms can become xenobiotics, including drugs, poisons, and environmental pollutants. However, the body removes xenobiotics by xenobiotic metabolism. Identifying the mechanistic basis for species differences in xenobiotic metabolism, an important area of toxicology, may be achieved by understanding and verifying the relevance of animal data to the human response. Intoxication influences differences in metabolic pathways and capacities of xenobiotics, and xenobiotic metabolites are important to cause high- or low-risk effects on human and animals. Moreover, it is typical for sensitivity to drugs or chemicals to have species specificity.

Pesticides are chemicals that are widely used in globally. They can contaminate the environment and have toxic effects on animals (Hayes et al., 2010). For example, organophosphate (OP) insecticides are more toxic to birds than to mammals because of differences in OP metabolism. Dimethoate is a widely used OP insecticide used to kill mites and insects. Dimethoate is 20 times more toxic to bird species, such as mallard ducks, pheasants, and sparrows, than to rats. This is indicated by the large difference in median lethal dose (LD_{50}) between bird species (17.8 to 63.5 mg/kg) and rats (310 mg/kg) (JMPR, 1996 and 1998). Dimethoate is metabolized in birds to a toxic metabolite, whereas the toxic metabolite is detoxified in rats (JMPR, 1996 and 1998). Aldicarb, an oxime carbamate insecticide, has been reported to have a different toxicity profile. A study has found the oral LD_{50} of aldicarb is higher in rabbits than in rats and mice (approximately 10 times) (Risher et al., 1987). In addition, atrazine is the most common pesticide contaminant in ground and

surface water (Hayes et al., 2010). It is a potent, active endocrine disruptor that has a high LD_{50} in rats (672 ppm), mice (850 ppm), rabbits (750 ppm), birds (500 to 2000 ppm) and humans (1000 ppm) (Ghosh and Philip 2006; Hayes et al., 2010). Interestingly, atrazine has a lower LD_{50} in fish, amphibians, and reptiles (30 to 600 ppb), especially in frog species (0.1 ppb) (Hayes et al., 2010). Rodenticides such as warfarin and second-generation rodenticides also have different susceptibilities to anticoagulants compared with bird species, rats, cats, and dogs (Watanabe et al., 2010). The LD_{50} was found to be highest in Northern Bobwhite birds, followed by chickens, mallards, dogs, cats, and rats (Erickson and Urban, 2004).

Furthermore, differences in LD_{50} via chemical poisoning have been reported. The LD_{50} for theobromine (i.e., resulting in chocolate poisoning) has been reported for humans, cats, dogs, rats, and mice (Gwaltney-Brant, 2001). The most common victims of theobromine poisoning are dogs, to which it can be fatal (LD_{50}: 300 mg/kg) (Gwaltney-Brant, 2001). In addition, mycotoxin poisoning, such as that involving aflatoxins, also presents higher toxicity in bird species (LD_{50} in ducks: 0.335 mg/kg) than in cats, pigs, guinea pigs, rats, mice, and hamsters (Newberbe and Butler, 1969). Moreover, the LD_{50} for the highly toxic dioxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) differs by more than 1000-fold between guinea pigs and hamsters. In addition to data for LD_{50}, the half-maximal effective concentration (EC_{50}) values for assessing biological activities of TCDD in a wide range of species are also available (Lipp et al., 1992; Eaton and Klaassen, 1996). The aryl hydrocarbon receptor (AHR) protein is a ligand-activated transcription factor through which TCDD and other halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons (PAHs) cause altered gene expression and toxicity (Hahn et al., 2004). AHR polymorphisms have been described as responsible for species diversity in TCDD toxicity profiles between mice and humans (Ema et al., 1994).

Phase I oxidation reactions and cytochrome P450

The ability to metabolize xenobiotics is one of the most important factors determining species sensitivity to xenobiotic toxicity, which is generally regarded as a two-stage process: phase I and phase II reactions. In various mammals, the basic patterns of xenobiotic metabolism are common in all species, involving phase I and II reactions. The final products are water-soluble compounds, which are easily excreted from the body. However, interspecies differences in the pathway of xenobiotic metabolism may arise because of the ability or inability to perform particular metabolic reactions. Generally, the first biotransformation occurs in the phase I reaction and involves cytochrome P450s (CYP or P450) as the main enzymes. CYP isoforms have been identified in the metabolism of xenobiotic compounds and activation of procarcinogens in various species (Lewis et al., 1998). However, the phase I reaction also involves other enzymes such as oxygenases, hydroxylases, flavin-containing monooxygenases, monoamine oxidases, cyclooxygenases, dihydrodiol dehydrogenases, DT-diaphorase, alcohol dehydrogenases (ADH), aldehyde dehydrogenases, oxidoreductase, esterases, amidases, and epoxide hydrolases (Filser, 2008). The activity of ADH has been reported to display species differences, being highest in rabbits, followed by mice, rats, guinea pigs, and dogs (Rachamin and Israel, 1985). Therefore, the levels and activities of ADH can be an important step in the metabolism of ethanol in humans and other animals.

The liver microsomal CYP enzymes involved in xenobiotic biotransformation belong to three main CYP gene families, namely CYP1, CYP2, and CYP3. The gene families generally correspond to a single subfamily of CYP enzymes, such as CYP3A. The CYP1 family is divided into four subfamilies: CYP1A, CYP1B, CYP1C, and CYP1D (Goldstone et al., 2007). Exposure to dioxins and polychlorinated biphenyls (PCBs) can lead to activation of the AHR and increased levels of CYP1A, CYP1B, and CYP1C mRNA. CYP1D genes

appear to have a different regulation than their sister forms and appear to be a nonfunctional pseudogene in human and bovine species (Kawai et al., 2010). The CYP1A family contains two major isoenzymes, CYP1A1 and CYP1A2, which appear to be highly conserved across most species. CYP1A shows moderately strong conservation among species (Mugford and Kedderis, 1998), with homology to the human gene higher than 80% in rat (83 and 80%, respectively for CYP1A1 and CYP1A2), mouse (83 and 80%, respectively for CYP1A1 and CYP1A2), dog (84% for CYP1A2), and monkey genes (95% for both CYP1A1 and CYP1A2). Furthermore, there are species differences in their function and regulation. For example, PAHs appear to induce CYP1A enzymes in all mammalian species. In contrast, omeprazole is an inducer of CYP1A enzymes in humans, but not in mice or rabbits (Diaz et al., 1990). CYP1B is constitutively expressed in normal tissues, such as heart, brain, placenta, lung, liver, kidney, and prostate (Sutter et al., 1994), but it is expressed at much higher levels in tumor cells compared with the surrounding normal tissue (Murray et al., 1997; McFadyen et al., 1999). Thus, CYP1B1 induction is an important factor in determining risks associated with hormone-mediated cancers. In human and rodent species, CYP1B1 can bioactivate carcinogenic PAHs, such as benzo(a)pyrene, to DNA-reactive forms associated with toxicity, mutagenesis, and carcinogenesis (Harrigan et al., 2006; Uno et al., 2006). Furthermore, benzo(a)pyrene can induce expression of CYP1B1 via the AHR (Harrigan et al., 2006). Enzymes belonging to the CYP2A gene family show marked species differences in catalytic function. However, the CYP2 family possesses five main subfamilies, CYP2A through to CYP2E. Differences in CYP2A function have important implications for the adverse effects of coumarin rodenticides (CYP2A6), which is hepatotoxic to rats but not to humans (Parkinson 1996). Human CYP2A6 shows different substrate specificity compared with CYP2A enzymes in animal species (Honkakoski and Negishi, 1997). CYP2C is the largest and most complicated subfamily in several species including humans, rats, and mice

(Matsunaga et al., 1994; Nedelcheva and Gut, 1994; Doherty et al., 2002). CYP2C is detected in the liver of rodent and nonrodent species, and its expression in extrahepatic tissue is isoform-specific. Remarkably, the expression of CYP2C is sex-dependent in adult in rats only (Riedl et al., 2000). Substrate specificities differ significantly between human and animal isoforms; in particular, CYP2C-mediated metabolism in dogs poorly represents that in humans. In addition, CYP2C is not expressed in all dogs, making general predictions risky (Graham et al., 2003). CYP3A is the most abundant CYP enzyme subfamily in human liver microsomes, which includes CYP3A4, CYP3A5, and CYP3A7. The various CYP3A isoforms expressed in different species show different substrate specificities, which also makes the extrapolation of observations from animals to humans also risky. The function and regulation of CYP3A vary the effect of some inducers, such as rifampicin and dexamethasone, which display differences in humans, rabbits, and rats (Pichard et al., 1990).

Phase II conjugation reactions

After phase I reactions, phase II enzymes play an important role in the biotransformation of xenobiotic compounds. Conjugation compounds are formed by various transferase enzymes, including UDP-glucuronosyltransferase (UGT), sulfotransferase (SULT), N-acetyltransferase, glutathione S-transferase, catechol O-methyltransferase and various methyltransferases (Bock, 2003). Phase II reactions also display individual and interspecies differences, which depend on both external and internal factors (Jancova et al., 2010). The study of conjugation compounds is useful for characterizing phase II reactions. For example, UGT is an enzyme that has a high detoxification pathway that catalyzes metabolism of drugs and xenobiotic compounds (deBethizy and Hayes, 1989). Aromatic amines are popular examples in studies on the role glucuronidation plays in metabolic activation of carcinogens. Large differences among mammalian species have been reported in the glucuronidation of xenobiotics (deBethizy and Hayes, 1989). The guinea pig generally

has higher UGT activity for co-planar PCBs than do most rats (deBethizy and Hayes, 1989; Oguri et al., 1993). Furthermore, slow glucuronidation of drugs such as acetaminophen and acetylsalicylic acid (aspirin) have been observed in cats; these animals display slow clearance and exquisite sensitivity compared with dogs and most other mammalian species (Davis and Westfall, 1972; Savides et al., 1984). Recently, UGT1A6 has been reported to be responsible for detoxification of phenolic drugs, but has not been observed to be expressed in cat liver (Court and Greenblan, 2000). Moreover, evaluations of phylogenetic timing showed that UGT1A6 is an inactivated gene (pseudogene) in the Felidae family (i.e., margay; *Felis weidii*), brown hyena (*Parahyaena brunnea*), and northern elephant seal (*Mirounga angustirostris*) (Shrestha et al., 2011). It is also possible that UGT1A6 is a pseudogene in other carnivores.

On the other hand, sulfation has a significant role in the biotransformation of numerous xenobiotic substrates such as drugs, steroids, and low-molecular-weight procarcinogenic compounds. SULT enzymes catalyze conjugation reactions of the co-substrate, 3'-phosphoadenosine-5'-phosphosulfate (PAPS). Xenobiotic conjugation with SULT is an important route for conversion of lipophilic xenobiotics to polar metabolites that are more readily excreted into urine. Sulfation of xenobiotics with an aliphatic or aromatic hydroxyl group readily occurs. Although sulfation can occur in most species, including mammals, birds, reptiles, amphibians, fish, and invertebrates, pigs have been shown to have low SULT activity (Williams, 1974; deBethizy and Hayes, 1989). Large differences in the relative ratios and kinetic parameters of SULT have been observed in various mammals (Sharer et al., 1995). Various isoforms of UGT and SULT also have different activities and specificities toward isoflavones. Although pigs have been reported to have a low sulfation capacity (Williams, 1974; deBethizy and Hayes, 1989), the UGT and SULT metabolic profiles of pigs have been found to be closer to that of humans than to those of rats or

monkeys (Gu et al., 2006). The formation reaction of 7-hydroxycoumarin sulfate shows similar *Km* in humans, monkeys, and rats, but is higher in dogs (*Km* 8.7 μM). In addition, its *Vmax* is slightly higher in dogs (Wang et al., 2006). The aryl-SULT (SULT1A) family plays a major role in xenobiotic metabolism. In humans, SULT1A1, 1A2, 1A3, and 1A4 have been identified and recognized as responsible for many differences in metabolism of phenolic and dopamine compounds such as 1-naphthol and *p*-nitrophenol (Bradley and Benner, 2005). In order to further understand xenobiotic metabolism, SULT1A1 has been identified in a large range of species, including rats, mice, cattle, dogs, rabbits, monkey, pigs, and platypus (Gamage et al., 2006). Additionally, SULT1A1 polymorphisms have gained considerable attention, as variations in its activity have been implicated in individual differences in the ability to metabolize xenobiotics such as dopamine and aromatic amines (Hung et al., 2004).

Model compounds for xenobiotic metabolism: warfarin and pyrene

There are various xenobiotic compounds that are conveniently used to characterize phase I oxidation and phase II conjugation reactions. Here, I introduce warfarin, as known to show higher toxicity in rodents than those in other species. The metabolite compounds of warfarin were catalyzed by various CYP isoforms. In addition, pyrene (PY) is also typical model compound for phase II conjugation reactions in mammals. PY and its metabolite are commonly detected from environment, and the conjugation metabolites seem to show species-specific difference patterns.

1. Warfarin

Warfarin (3-(α-acetonylbenzyl)-4-hydroxycoumarin), an anticoagulant drug, is a well-known xenobiotic used to characterize CYP forms in humans and various animals (Fig. 1). Metabolism of warfarin in humans is mainly catalyzed by a variety of CYPs to form a series of monohydroxylated metabolites (Kaminsky and Zhang, 1997). An investigation of *(R)*- and

(S)-warfarin metabolism by human liver CYPs revealed various hydroxywarfarin products. 4'-, 6-, 7-, 8-, and 10-hydroxywarfarin are products from stereoselective *(R)*- and *(S)*-warfarin (Wang et al., 1983; Kaminsky et al., 1984). The predominant human CYPs catalyzing *(R)*- and *(S)*-warfarin metabolism are CYP1A1, CYP1A2, CYP2C9, CYP2C19 and CYP3A4. Reductive metabolism of the acetonyl side chain of warfarin to yield diastereomeric alcohols is also an important step in the metabolic clearance of warfarin. This side chain ketone moiety is reduced by carbonyl reductases, and the phase I metabolites are catalyzed by conjugated enzymes (Hermans and Thijssen, 1989; Jansing et al., 1992). Meanwhile, a comparison of warfarin metabolites of phase I reactions in humans and animals has been studied (Martignoni et al., 2006). Moreover, the mechanism of anticoagulant rodenticides such as warfarin involves vitamin K 2,3-epoxide reductase (VKOR; subunit 1, VKORC1), which is responsible for variations in both warfarin sensitivity and warfarin resistance in rodenticide targets (i.e., rats) (Tanaka et al., 2012; Tanaka et al., 2013). Warfarin metabolic activities in resistant rats are higher than those in sensitive animals. In addition, NADPH cytochrome c reductase activity dependent on NADPH cytochrome P450 reductase (CPR) is markedly higher in resistant rats than in sensitive rats (Ishizuka et al., 2007 and 2008). Recent poisoning of non-targets such as birds, small mammals, and humans have been reported to be due to interspecies differences in the VKOR kinetic parameters of warfarin metabolism (Ishizuka et al., 2008; Watanabe et al., 2010).

2. Pyrene

PY, consisting of four fused benzene rings, is an abundant PAH, which is frequently measured as an indicator of external exposure to PAHs (Fig. 2). The PY metabolite PYOH serves as a biomarker for PAH exposure in humans, as the biotransformation of PY to PYOH is very rapid, and it is not the limiting factor in urinary elimination (Ramesh et. al., 2012). Additionally, detected PYOH is mainly a result of PAH hydroxylation, and has a strong

correlation with PAH concentrations (Jongeneelen et al., 1986; Kuljukka et al., 1997). At present, PY and PYOH are conveniently used as a phenolic model compound to study the characteristics of PY metabolites in various animals. In addition to PYOH, a hydroxylation product that is commonly detected as an indicator of PY exposure, conjugation compounds such as glucuronide, glucoside, and sulfate are also important in observations on various animals (Ikenaka et al., 2006; Ikenaka et al., 2007; Beach et al., 2009; Ueda et al., 2011). Furthermore, interspecies differences were observed by using PY metabolites as phenolic xenobiotics substrates (Ueda et al., 2011). Although PY metabolites in humans have been reported, those in various mammalian species remain unknown. This may be because PY is commonly found in the environment as PAH mixtures. PAHs are ubiquitously distributed carcinogens in the environment, and their carcinogenic potentials are formed from the products of incomplete combustion of carbon-containing fuels such as wood, coal, diesel, tobacco, and cooked foods. PAHs are absorbed onto particles in the air, soil, water, and sediments. These are released into the environment as mixtures, which are composed of carcinogenic and procarcinogenic compounds. Both food ingestion and inhalation represent the major routes of PAH exposure in humans and animals. Generally, humans and other animals are exposed to PAHs, including PY, either directly or indirectly. The direct pathway involves ingestion of PAH mixtures contaminated in the environment, including soil, water, and food. The secondary or indirect pathway involves exposure to PAHs via the consumption of animal products as food, such as cattle and pigs (Ciganek et al., 2002; Ramesh et. al., 2012).

Aim and overview of thesis

In this thesis, studies were performed to identify and clarify interspecies differences in the metabolism of xenobiotic compounds as described below.

Chapter I: Study of phase I reactions: comparative metabolism of warfarin in rats and chickens

Regarding the phase I reaction, I focused on the characterization of warfarin as a model compound to identify interspecies differences in CYP and non-CYP metabolites that have been reported in humans and various animals. Therefore, hydroxywarfarins and warfarin alcohols were measured in laboratory animals (rats) and bird species (chickens).

Chapter II: Phase II conjugation of pyrene (PY) as a model of xenobiotic metabolism

In this chapter, phase II conjugation reactions were performed using PY metabolites. PY and its metabolite 1-hydroxypyrene (PYOH) are commonly used as phenolic xenobiotic compounds to characterize conjugation in mammalian urine. In order to classify PY metabolites in urine and observe interspecies differences, the studies were divided into three sections as follows.

Section I: Characterization of phase II metabolites of pyrene in rats: identification and tissue distribution of metabolites

In this section, rats were exposed to PY, and then the metabolites in urine, feces, plasma, and tissues were identified. Both glucuronide- and sulfate-conjugated metabolites were observed, and the amount of each metabolite was measured. Finally, urine samples were studied to analyze the interspecies differences in mammalian species, since almost all PY metabolites are eliminated via urine.

Section II: Interspecies differences in phase II reactions in mammals: determination of PY metabolites in urine of various mammalian species

Urine samples were collected from various mammals to analyze PY metabolites. After processing and analysis, the levels of glucuronide- and sulfate-conjugated metabolites were

measured. Subsequently, interspecies differences in the ratio of each metabolite were assessed.

Section III: Study of xenobiotic metabolism phase II reactions in pigs: high sulfotransferase enzymatic efficiency observed by in vitro kinetic analyses.

Sulfate-conjugated metabolites were detected in pig urine. Therefore, sulfation activity in pig liver cytosol was measured in order to clarify interspecies differences. Remarkably, significant differences in sulfation activity and sequence analysis of SULT1A1 gene expression between pigs and rats were observed.

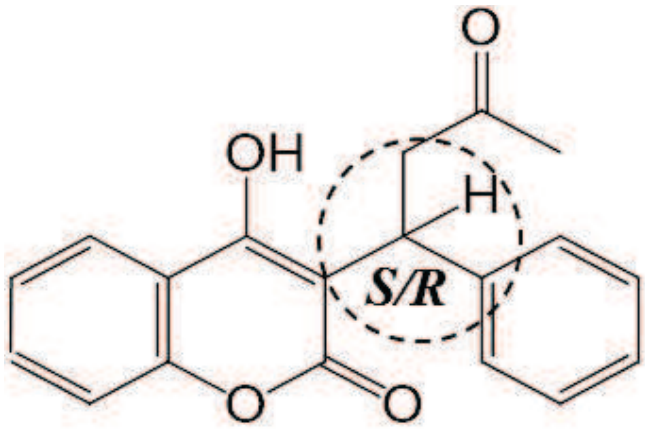


Fig. 1 The chemical structure of warfarin

The structure of *(R)*- and *(S)*-warfarin.

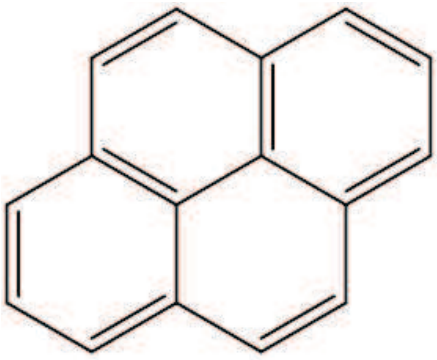


Fig. 2. The chemical structure of pyrene (PY)

PY is one of PAHs that consists of four fused benzene rings. The molecular formula and molecular mass for PY are $C_{16}H_{10}$ and 202.26, respectively.

CHAPTER I

The study of phase I reaction: comparative metabolism of warfarin in rats and chickens

Introduction

Warfarin and other coumarin derivative are used to eliminate vermin that circulate and garner in households, farms, and island areas. This results in a high risk of poisoning of nontarget animals, such as dogs, cats, swine, wild mammals, and birds (Stone et al., 1999; Eason et al., 2002; Valchev et al., 2008; Dowding et al., 2010). In particular, an increasing incidence of anticoagulant poisoning in birds has been reported (Eason et al., 2002; Erickson and Urban, 2004). The evident causes in birds are divided into 2 categories: primary exposure by direct consumption of bait, and secondary exposure by consumption of poisoned prey (Murray and Tseng, 2008; Redig and Arent, 2008; Albert et al., 2010).

In mammals, warfarin is extensively catalyzed to hydroxywarfarin forms in liver microsomes by hydroxylation reactions in phase I metabolic pathway using cytochrome P450 (CYP). The CYP oxidizes warfarin into 5 different hydroxywarfarins (4'-, 6-, 7-, 8-, and 10, hydroxywarfarin). Then, the conjugation enzymes, UDP-glucuronosyltransferases (UGT), catalyze the hydroxylwarfarins to hydroxyglucuronides in phase II of metabolism (Miller et al., 2009; Zielinska et al., 2009). In bird species, however, the detoxification pathway of warfarin remains unclear. In the previous study (Watanabe et al., 2010), they compared the activities of warfarin hydroxylation between bird species and rats. Authors reported that chicken showed a higher ability to hydroxylate warfarin compared with that of rats. Interestingly, they found a large amount of an unidentified metabolite in the reaction mixture of the chickens, but not in the rats. In a previous study by Hermans and Thijssen (1989) to determine which reduction of aldehydes and ketone reductases were responsible for the reaction in microsomal and cytosolic fractions, the metabolite was suggested to be non-hydroxywarfarin. Contrary to this hypothesis, I assumed that the metabolite might be from a non-CYP-dependent reaction, given that I could detect it in the cytosol fraction. Thus, in this

chapter, I aimed to identify the unknown metabolite of warfarin in the cytosolic fraction in order to compare and clarify the species differences between rats and chickens in warfarin metabolism.

Materials and Methods

Synthesis of warfarin alcohols

Warfarin alcohol was synthesized according to the method of Trager et al. (1970) and Chan et al. (1972). Briefly, 1 g of warfarin (Sigma-Aldrich Inc., St. Louis, MO) was dissolved into a solution of 0.86 g of $NaBH_4$ (Kanto Chemicals, Tokyo, Japan) in 5 mL of Mili-Q water (Millipore, Molsheim, France). Then, I kept the solution at room temperature (approximately 24ºC) for 1 h. The product precipitated upon acidification with 1 *N* HCl (Wako pure Chemical Industries, Osaka, Japan). The amorphous precipitate was filtered, washed with Milli-Q water, and vacuum dried. The resulting synthesized warfarin alcohol included 2 diastereomers: *RS/SR*- and *RR/SS*-alcohol warfarin. For the purification of the synthesized products, I used liquid-liquid extraction with water and ethyl acetate. The ethyl acetate layer contained the warfarin alcohols; they were collected and dried completely under a gentle nitrogen stream. Then, the mixture of warfarin alcohols was dissolved in a small amount of acetone and applied to activated silica-gel (60 *N*, spherical, neutral; 63-210 μm Kanto Chemicals) column chromatography (20 g; activated at 200ºC for 4 h; column was 30 × 2 cm). The synthesized products were eluted and fractionated with a mixture of hexane, benzene, ethyl acetate, and methanol in ratios of hexane:benzene (3:7), benzene:ethyl acetate (3:7), and ethyl acetate:methanol (9:1) (Wako Pure Chemical Industries).

Electrospray ionization (ESI) ion trap mass spectrometry (Thermo Scientific, Newington, NH) was used for identification of the mass spectrum of each warfarin alcohol isomer. The ESI conditions were full scan (range *m/z* of 50 – 650) by negative mode with an ion spray voltage of 1.5 kV, capillary voltage of –10 V, and an ion source temperature of 200ºC. In addition, the diastereomers of the warfarin alcohols were analyzed by high-pressure liquid chromatography with an ultraviolet detector (HPLC/UV).

Birds

The livers of 10-wk-old male chickens (*Gallus gallus*; n = 3) and 8-wk-old male Wistar rats (*Rattus norvegicus*; n = 3) were used in this experiment. The chickens were kept in a house with experiment. The chickens were kept in a house with a controlled 12L:12D cycle. The rats were housed with a 12L:12D cycle and provided commercial food and water ad libitum. Both chickens and rats were killed by CO_2 asphyxiation. The livers were rapidly moved to liquid nitrogen and kept at – 80ºC until use.

Treatments of all animals were performed according to the policies of the Institutional Animal Care and Use Committee of Hokkaido University (Sapporo, Japan).

Preparation of Liver Microsomes and Cytosol Fractions

Liver microsomes and cytosol fractions were prepared according to the method of Omura and Sato (1964). The liver samples were homogenized with potassium phosphate buffer (KPB; 0.1 *M*, pH 7.4) in ice conditions. The homogenates were transferred to a tube and centrifuged at 9,000 × *g* at 4ºC for 20 min. The supernatants were decanted to an ultracentrifugation tube and centrifuged at 105,000 × *g* at 4ºC for 60 min. Each homogenate consisted of 2 parts: the supernatant contained the cytosol fraction, and the pellet contained the microsome fraction. The pallets were homogenized in ice conditions with KPB and centrifuged for washing at 105,000 × *g* at 4ºC for 60 min. The microsomal pellets were homogenized with KPB again. The microsome and cytosol fractions were transferred to 1.5-mL tubes and stored at – 80ºC.

Protein and CYP Quantification

The protein concentrations in the microsomes and cytosol were measured using the Lowry method (Lowry et al., 1951). The CYP content was measured following the methods of Omura and Sato (1964) and Guengerich et al. (2009).

Warfarin Metabolic Activity

The warfarin metabolic activities in the microsome and cytosol fractions were analyzed following the method of Kaminsky and Zhang (1997). The final concentration of the microsomes and cytosol fractions was 1.0 mg of protein/mL of KPB, which contained magnesium chloride (3 mM, final concentration; Wako Pure Chemical Industries), glucose 6-phosphate (5 mM, final concentration; Oriental Yeast Co., Ltd. Tokyo, Japan), and warfarin sodium (400 μM, final concentration; Wako Pure Chemical Industries). In addition, KPB alone was used as a negative control. The samples were equilibrated for 5 min at the body temperature of each species (37ºC for rats and 41ºC for chickens) in a water bath (Richards, 1971; Watanabe et al., 2010). The mixtures for the start solution (glucose 6-phosphate dehydrogenase, 2 IU/mL, final concentration; and β-NADPH, 0.25 mM, final concentration; Oriental Yeast Co. Ltd., Tokyo, Japan) were added to each sample. The final volume of the reaction solution was 500 μL. The incubation time to measure microsomal and cytosolic fraction activities were 10 and 30 min, respectively (Zhang et al., 1993). The reactions were stopped with 10 μL of 60% perchloric acid (Wako Pure Chemical Industries). The samples were centrifuged at 3,000 × *g* at 4ºC for 10 min, and the 50 μL of each supernatant was injected into the HPLC/UV. The HPLC/UV system was composed of an LC-20AB pump (Shimadzu; Kyoto, Japan), SPD-20A detector (Shimadzu), and a TSKgel-120T column of 250 × 4.6 mm and 5 μm (Tosoh; Tokyo, Japan); the UV wavelength was 307 nm. The mobile

phase was 15 mM KH_2PO_4 (pH 3 adjusted with 1 *N* HCl; Wako Pure Chemical Industries):methanol:acetonitrile (55:30:15), and run at 0.3 mL/min (flow rate) in isocratic mode.

The quantification was calculated using the area of each metabolite of warfarin derived from the calibration curve, which was created from the standard mixture of *(R)*-4'-, 6-, 7-, 8-, and 10-hydroxywarfarin. All standard solutions were dissolved in KPB.

The *(S)*-, and *(R)*-warfarins were used as substrates in the warfarin metabolic reaction in the cytosol fractions of rats and chickens. These substrates were dissolved in dimethyl sulfoxide, and then 100 μM of the substrates were used to measure the reaction activity. The reaction volume contained a final concentration of 0.01% dimethyl sulfoxide. To stop the reaction and create acid conditions, 10 μL of 6 *N* HCl was added to the reaction mixture. Then, 50 μL of each sample was injected into the HPLC/UV. The conditions for the HPLC/UV analysis were the same as in the method described above. The concentrations of the *(S)*- and *(R)*-warfarin metabolites were derived from our calibration curve of warfarin alcohols.

Enzyme Inhibition Assay

The inhibitions used for the cytosol fractions were 1.0 m*M* menadione (an aldehyde oxidase inhibitor; Merck, Darmstadt, Germany), 3 mM pyrazole (an alcohol dehydrogenase inhibitor; Sigma-Aldrich Chemical, Steinheim, Germany), and 1.0 mM indomethacin (a prostaglandin reductase inhibitor; Wako Pure Chemical Industries). The dose of each inhibitor followed previous studies: menadione (Yoshihara and Tatsumi, 1985; Clarke et al., 1995), pyrene (Zhang et al., 1993), and indomethacin (Hermans and Thijssen, 1992), which

showed the inhibition effect on the production of warfarin metabolites in the cytosol fractions. Pyrazole was dissolved in KPB, whereas menadion and indomethacin were dissolved in acetone. The final concentration of acetone did not exceed 0.5%, and I confirmed that the activity was not influenced by this concentration of acetone.

Statistical Analysis

JMP 9.0 (SAS, North Carolina, USA) was used to analyze warfarin metabolic activities. The data were analyzed by the Student *t*-test for paired data and the Tukey-Karmer Honestly Significant Difference test for multiple comparisons, with a significance level of $P < 0.05$. The values are shown as mean ± SE.

Results

Warfarin Metabolism

Warfarin metabolites in rat and chicken microsome fractions consisted of 5 peaks for hydroxywarfarin: 4'-, 6-, 7-, 8-, and 10-hydroxylation. The amount of each metabolite is summarized in Table 1. The hydroxywarfarin values in chickens showed a large difference from those in rats.

In the cytosol, two warfarin metabolites were found by the HPLC/UV detector in both chickens and rats (Fig. 3). Although these two metabolites showed identical retention times in both species (peak A and B), the detection response to warfarin activities in chickens differed from that in rats. The warfarin metabolites found in rats presented with peak B being higher than peak A; in chickens, the converse was true. Moreover, the optimum temperatures for this reaction in rats and chickens were 37ºC and 41ºC, respectively.

Identification of Warfarin metabolites in Cytosol

The methods of Trager et al. (1970) and Chan et al. (1972) were used to identify the warfarin metabolites in both cytosol fractions. In this study, I identified the mass spectra of warfarin metabolites in the form of alcohol by negative mode ESI ion trap mass spectroscopy. These metabolites showed *m/z* 309 of MS^2 (*m/z* 291 and 250) and MS^3 (*m/z* 206) were identical to those reported by Trager et al. (1970) (Fig. 4).Their results showed that each warfarin metabolite peak was identified as an enantiomer of warfarin alcohol. The structures of warfarin alcohol have been reported as two pairs of each diastereomer and enantiomer. Therefore, I assumed that each peak contained two enantiomers of warfarin alcohol.

Peak A and B from the HPLC/UV data were suspected to be *(R)*-warfarin-*(R)*-alcohol/*(S)*-warfarin-*(S)*-alcohol (*RR*-alcohol and *SS*-alcohol, respectively) and *(R)*-warfarin-*(S)*-alcohol/*(S)*-warfarin-*(R)*-alcohol (*RS*-alcohol and *SR*-alcohol, respectively; Fig. 5).

To compare the production of the diastereomer products of the warfarin alcohols in the cytosol fractions of rats and chickens, the purified *(R)*- and *(S)*-warfarins were used as the substrates (Table 2). From these substrates, I detected the stereoisomers of the warfarin alcohol metabolites: *SS*-alcohol in *(S)*-warfarin metabolism. For *(S)*-warfarin, the level of *SS*-alcohol produced was 32-fold higher in chickens than in rats. For *(R)*-warfarin, the trend for *RS*-alcohol production in chickens presented almost at a similar level compared with that in rats. In the rat liver cytosol, *RS*-alcohol (3.6 ± 0.3 nmol/min per mg of protein) was dominant and 2-fold higher than *SS*-alcohol production (1.7 ± 0.5 nmol/min per mg of protein). In contrast, in the chicken liver cytosol, the production of SS-alcohol (54.3 ± 16.9 nmol/min per mg of protein) was markedly higher than *RS*-alcohol production (2.4 ± 0.2 nmol/min per mg of protein).

Enzyme Inhibition Assays

To clear the enzymes that may contribute to the production of warfarin alcohol in the cytosol fraction, several selected enzyme inhibitors were used. The effects of these selected inhibitors on the metabolism of warfarin to warfarin alcohol products in the cytosol are summarized in Table 3. Pyrazole showed no inhibiting effect on warfarin alcohol products in either chickens or rats when the inhibition concentration was 3 mM. On the other hand, formation of those metabolites was completely inhibited after treatment with 1 mM menadione, a vitamin K_2 precursor. In addition, indomethacin also inhibited the formation of warfarin also inhibited the formation of warfarin alcohols in both rats and chickens.

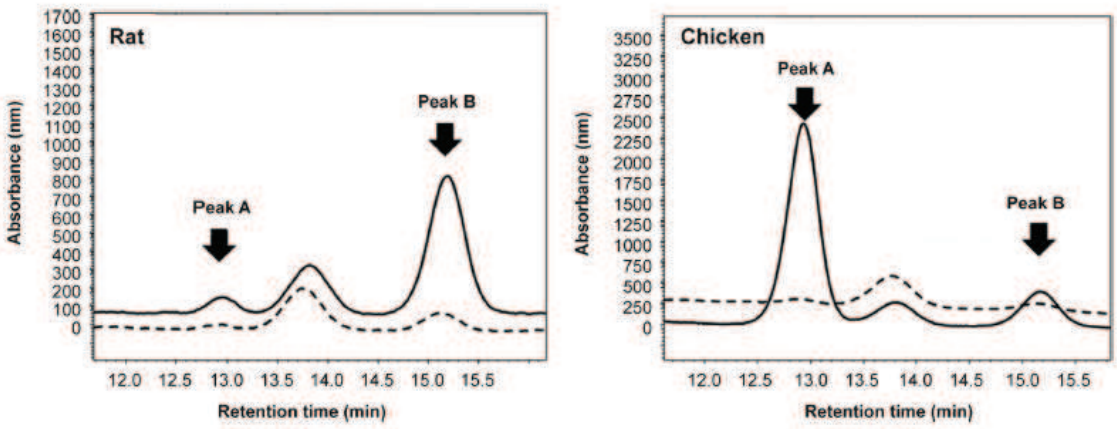


Fig. 3. The chromatograms of warfarin metabolites in chicken and rat cytosol fractions were detected as 2 peaks (A and B).

The retention time of the metabolites was identical in the 2 species (chicken and rat), though the peak pattern of warfarin metabolites in chickens differed from that in rats. The dashed line is the baseline (0.1 M potassium phosphate buffer, pH 7.4).

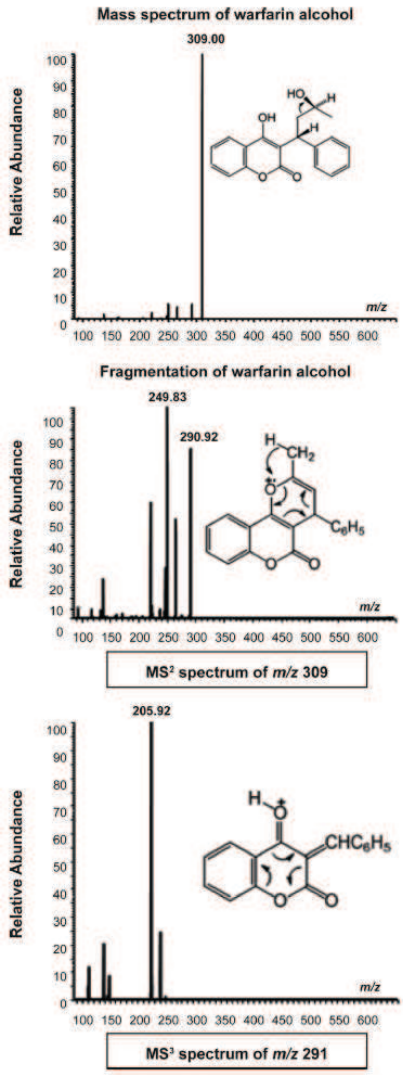


Fig. 4. The mass spectrums and structures of warfarin alcohols.

Mass spectra and structural development of the warfarin metabolite as warfarin alcohols and its fragmentation pattern in chicken and rat cytosol fractions detected by electrospray ionization ion trap mass spectroscopy (range *m/z* of 50 – 650). Each fragmentation pattern was composed of *m/z* 309 of MS^2 (*m/z* 291 and 250) and MS^3 (*m/z* 206).

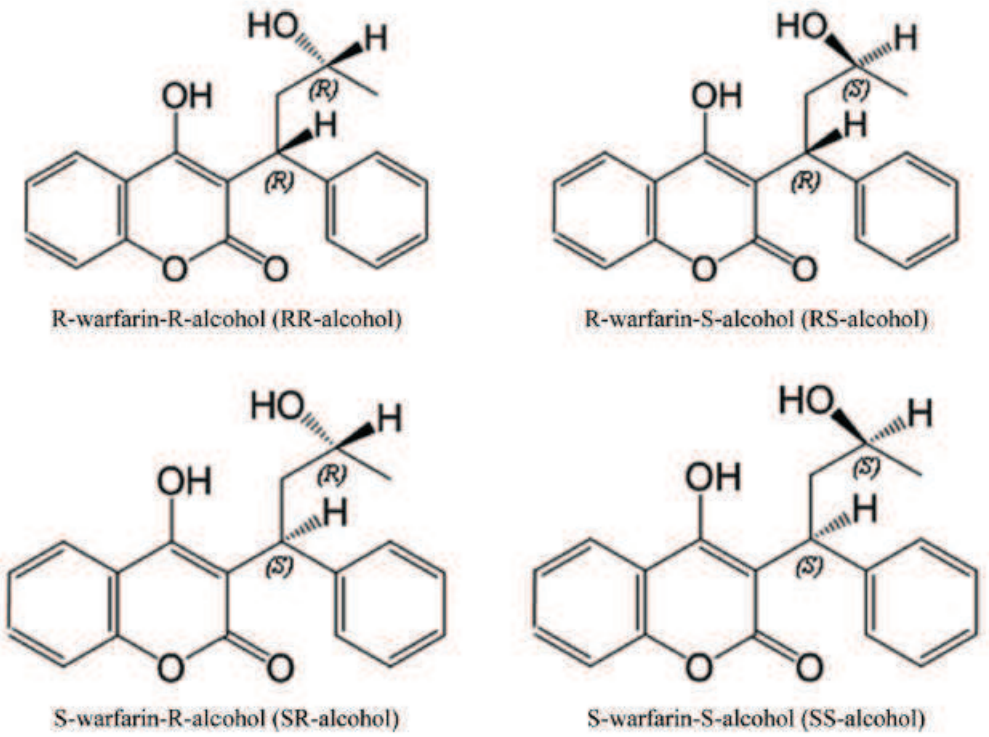


Fig. 5. The structure of warfarin alcohols.

Structure of warfarin alcohols and enantiomers: *(R)*-warfarin-*(S)*-alcohol (*RS*-alcohol), *(S)*-warfarin-*(R)*-alcohol (*SR*-alcohol), *(R)*-warfarin-*(R)*-alcohol (*RR*-alcohol), and *(S)*-warfarin-*(S)*-alcohol (*SS*-alcohol).

Table 1. Activities of warfarin hydroxylation in liver microsomes of chickens and rats

	4'-OH	6-OH	7-OH	8-OH	10-OH	Total
Rat	16.3 ± 1.97	8.00 ± 11.4	7.80 ± 0.690	4.60 ± 0.515	22.5 ± 0.297	59.2 ± 4.28
Chicken	668 ± 122	192 ± 475	31.2 ± 10.1	16.3 ± 6.13	28.9 ± 5.14	936 ± 161

Data show the means ± SE (n = 3) for metabolic activities of each hydroxylated form of warfarin (nmol/min per nmol of P450).

Table 2. Activities of warfarin alcohol production in liver cytosols of chickens and rats.

	SS-alcohol	*SR*-alcohol	*RR*-alcohol	*RS*-alcohol
Rat	1.70 ± 0.310	ND	ND	3.57 ± 0.495[B]
Chicken	54.3 ± 16.9[A,B]	ND	ND	2.37 ± 0.206

[A]Significantly different among the warfarin metabolic activity by Tukey-Kramer Honestly Significant Difference test ($P < 0.05$).

[B]Significantly different between SS- and RS-alcohols in each species by Student *t*-test ($P < 0.05$).

The value shows mean ± SE (nmol/min per mg of protein; n = 3).

ND = no activity was found; *SS*-alcohol = *(S)*-warfarin-*(S)*-alcohol; *SR*-alcohol = *(S)*-warfarin-*(R)*-alcohol; *RR*-alcohol = *(R)*-warfarin-*(R)*-alcohol; and *RS*-alcohol = *(R)*-warfarin-*(S)*-alcohol.

Table 3. The effect of selective enzyme inhibitors on warfarin alcohol production (%) in the cytosol of two species.

Inhibitors	% warfarin alcohol products			
	Rats		Chickens	
	SS-alcohol	*RS*-alcohol	*SS*-alcohol	*RS*-alcohol
Menadione	1.50 ± 0.410	ND	ND	ND
Pyrazole	97.1 ± 7.09	97.8 ± 5.24	90.3 ± 1.86	92.3 ± 2.34
Indomethacin	4.00± 0.820	ND	23.3 ± 3.26	14.1 ± 0.860

The values show mean ± SE (n = 3).

ND = no activity was found; *SS*-alcohol = *(S)*-warfarin-*(S)*-alcohol; *RS*-alcohol = *(R)*-warfarin-*(S)*-alcohol.

Menadione = aldehyde oxidase inhibitor (1 mM); pyrazole = alcohol dehydrogenase inhibitor (3 mM); indomethacin = prostaglandin reductase inhibitor (1 mM).

Discussion

In the current study, both chicken and rat cytosolic metabolisms produced the reduction reaction of warfarin. Because the CYP were mainly localized in the endoplasmic reticulum, the catalysis of warfarin in the cytosolic fraction did not occur by CYP activity. In this study, the diastereoisomers of warfarin alcohols were found both in chicken and rat cytosols. The total products form the warfarin alcohols were markedly high in chickens after I normalized the amount of cytosol protein and substrates in the reaction. Trager et al. (1970) reported two diasteroisomers of warfarin alcohol. Each warfarin alcohol isomer has two enantiomers: *RS*- and *SR*-alcohols (Chan et al., 1972; Wong and Davis, 1989; Hermans and Thijssen, 1989, 1992). Interestingly, the rate of formation of each warfarin alcohol diastereoisomer in the cytosol showed drastic interspecies differences.

In the present study, I detected warfarin alcohol as *SS*- and *RS*-alcohols in both rats and chickens (Table 2). The *SS*-alcohol enantiomer was especially dominant in chickens, and we could not detect the *SR*- or *RR*-alcohols in the cytosolic fractions of rats or chickens. However, Wong and Davis (1989) and Hermans and Thijssen (1989) reported a high level of *RS*-alcohol in microbial cell suspensions as models of mammalian metabolism and rat liver cytosolic fraction, respectively, using *(R)*-warfarin as a substrate. Therefore, it is possible that the reduction reaction of warfarin in the cytosol is different in rats than it is in chickens. Moreover, this reaction was related to the ketone reduction of the acetonyl side chain of warfarin (Moreland and Hewick, 1975). Based on the report of Hermans and Thijssen (1989), the liver cytosol fraction preference for the *(R)*-warfarin substrate led to the formation of *RS*-alcohols in mammals (humans, rats, horses, pigs and rabbits). In my study, the metabolism of *(R)*-warfarin to *RS*-alcohol was dominant compared with the conversion of *(S)*-warfarin to *SS*-alcohol in rat liver cytosols. However, chicken liver cytosols showed a higher ability to convert *(S)*-warfarin to *SS*-alcohol compared with the production of *RS*-alcohol from *(R)*-

warfarin, and this ability was drastically higher than that in rat cytosol. Although warfarin is mainly metabolized in microsomal fractions (Table 1), my results additionally show the interspecies differences between rats and chickens in cytosolic warfarin metabolism. It is possible that this reaction modestly contributes to the high median lethal dose (LD_{50}) of chickens against warfarin compared with that of rats.

Furthermore, I use of various enzymatic inhibitors helps to identify the role of specific classes of drug metabolizing enzymes. Pyrazole, which generally inhibits liver alcohol dehydrogenase, showed no inhibiting effect on the cytosol fraction in either the rats or chickens. Hermans and Thijssen (1989) reported that pyrazole disabled the inhibition of rabbit liver cytosolic warfarin reductases. For this reason, I suspected that warfarin metabolism in the cytosol fraction does not require an alcohol dehydrogenase enzyme. In contrast, indomethacin, the prostaglandin reductase inhibitor, showed strong inhibition of *(S)*-alcohol formation in both rats and chickens. The results were similar to Hermans and Thijssen (1992), which it was reported that indomethacin could inhibit warfarin alcohol formations of the rabbit liver cytosolic fraction (75 – 87% inhibition). Prostaglandins and other steroids may be physiological substrates for reductase enzymes in the cytosol fraction.

In particular, warfarin metabolites in both rat and chicken cytosol fractions were completely inhibited by menadione. Menadione is a typical inhibitor of aldehyde oxidase by inhibiting electron transport of flavin adenine dinucleotide (Yoshihara and Tatsumi, 1985, 1986; Robertson and Bland, 1993). Aldehyde oxidase is one of the cytosolic enzymes that has been involved in some pathological conditions, and plays an important role in the biotransformation of drugs and xenobiotics (Maia and Mira, 2002). In addition, ketone reductases and aldehyde reductases have been able to reduce the carbonyl group of alcohol forming from menadione activity (Herman and Thijssen, 1989). Finally, I suspected that

aldehyde oxidase may mainly contribute to the production of warfarin alcohol in the cytosol of chickens.

In conclusion, my study revealed large interspecies differences in warfarin metabolism between rats and chickens in both the microsomal and cytosolic fractions. The metabolic activity of warfarin is drastically higher in chicken microsomes and cytosol fractions compared with that of rats. This is the first report to show large species differences in the stereoselectivity of warfarin in rats and chickens.

CHAPTER II

Phase II conjugation in pyrene, the model of xenobiotic compounds

SECTION I

The characterization of phase II metabolites of pyrene in rats: the study of identification and tissue distribution of metabolites

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitously distributed promutagens and procarcinogens in environment. Pyrene (PY) is one of PAHs, consisting of four fused benzene rings, and is a major constituent in PAH mixtures (Zhao et al., 1990; Haddad et al., 1998). PY is primarily metabolized by CYP to 1-hydroxypyrene (PYOH) in phase I reaction (Grover et al., 1972; Cerneglia and Crow, 1981; Jacob et al., 1982; Singh et al., 1995). In a previous study on PY metabolites, oxidation metabolites were reported to present in urine and feces (Harper, 1957; Jongeneelen et al., 1987; Schooten et al., 1997; Bouchard et al., 1998; Ruzgyte et al., 2006). PY and its metabolite, PYOH, are commonly studied as a model compound of PAH exposure in humans, rats, aquatic crustaceans, snails, crabs, fishes, amphibians, and reptiles (Law et al., 1994; Viau et al., 1999; Hellou et al., 2004; Chetiyanukornkul et al., 2006; Dam et al., 2006; Beach et al., 2009, 2010; Ueda et al., 2011;). In addition, since PYOH was detected from milk, urine, and feces in mammalian and non-mammalian animals (Jongeneelen et al., 1990; Viau et al., 1999; Ikenaka et al., 2006; Chahin et al., 2008; Ueda et al., 2011), the measurement of the hydroxylation metabolites of PY, including PYOH, has been recommended for the evaluation of daily PAH exposure and of occupational and environmental exposure.

After reaction in phase I, metabolized PYOH undergoes phase II reactions to form conjugates with glucuronic acid and sulfate. However, information on phase II metabolites of

PY is still less known compare to the phase I reaction. Although most of the metabolites of PYOH are excreted as glucuronide and sulfate conjugates in phase II reaction, there is still unclear information about the metabolism pathway and distribution of PY conjugates in tissues. Since the metabolites of PAHs can induce carcinogenesis, the distribution of such metabolites should be elucidated.

Recently, a study of PY metabolites in human urine demonstrated that glucuronide forms were at higher levels than sulfate conjugates (Singh et al., 1995; Kakimoto et al., 2008). In this section, I aimed to investigate the metabolism of PY in rats, especially considering phase II metabolites in order to clarify PY metabolism in rats. Determination of PY metabolism in rats and the comparison with humans would be useful in understanding PY metabolic pathways and interspecies differences. This is the first report to show the detailed distribution and excretion of conjugated PY metabolites in various tissues, plasma, urine, and feces of rats. In addition, I identified novel metabolite of PY conjugate in mammals in the present study.

Materials and Methods

Chemicals and reagents

PY, methanol (HPLC grade), sulfuric acid, and acetonitrile (HPLC grade) were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). PYOH, sulfatase (from limpets Type V; 34 U/mg), β-glucuronidase (from bovine liver, Type B-1; 1240 U/mg), β-glucosidase (from almonds; 3.4 U/mg), and bovine serum albumin were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). 6-Hydroxychrysene (6-OH Chrysene) as internal standard was purchased from AccuStandard Inc. (New Haven, USA). Acetic acid, sulfuric acid, potassium dichromate, and ammonium acetate solution were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Animals

Eight-week-old, male Wistar rats (*Rattus norvegicus*) (n = 3) were obtained from Japan SLC Inc. (Hamamatsu, Japan) and acclimated for one week in the laboratory. The body weights of rats (average ± SD) were 248 ± 10 g. Rats were kept in 40% humidity at 25°C in a temperature-controlled room with a 12 h light/dark cycle. The animals were given laboratory food and clean water *ad libitum*. All the animal experiments were performed under the supervision and with the approval of the Institutional Animal Care and Use Committee of Hokkaido University.

Pyrene exposure and sample collection

Rats were fasted for 24 h before exposure. PY was dissolved in 100% propylene glycol and treated at a dose of 4 mg/kg body weight by oral administration. Then, rats were kept in a metabolic cage for 24 h for urine collection. Rats were sacrificed by asphyxia with CO_2 gas. Plasma, liver, kidney, spleen, heart, lung, brain, testes, tongue, gastrointestinal tract organs, and feces were collected immediately and kept at –20°C until analysis.

Extraction of pyrene metabolites

Urine: A mixture of 1 mL urine with 1 mL 70% methanol was added to 10 µL of 200 ppm 6-OH Chrysene (dissolved in methanol), as an internal standard. The mixture samples were cleaned by vortexing and centrifugation. Then, aliquots were analyzed by HPLC with fluorescence (HPLC/FD) to obtain the metabolite profile.

Tissues: Approximately 1 g of tissue was cut into small pieces and extracted with 10 ml of 70% methanol. Seventy-five microliters of 6-OH Chrysene were added in the mixture sample. For intestinal samples, they were washed with 0.9% normal saline before extraction. The rinsed pieces of tissues were homogenized by a mechanical homogenizer (Keurostar IKA®). The homogenates were centrifuged at 9000 × *g* for 10 min at 20°C. The supernatants were transferred to a new tube. The pellet was extracted once more with 5 mL of 100% methanol by shaking vigorously (10 min), and then the samples were centrifuged once again. The pooled supernatant was filtrated with a 0.2 *µm* syringe filter (SupraPure, Pecenttec®). Five microliters of an aliquot of the supernatant were injected into HPLC/FD.

Plasma: Two hundred and fifty microliters of plasma were mixed with one microliter of 6-OH Chrysene. Five hundred microliters of methanol were used for extraction. Then,

samples were kept at –20°C for 15 min and centrifuged at 13000 × *g* for 15 min at 4°C. The supernatant was reduced in volume under a nitrogen gas stream and resuscitated with 50% methanol up to 200 μL. After centrifugation, the 5 μL of supernatant was injected into HPLC/FD.

Feces: Approximately 3 to 5 g of sample was homogenized with 20 ml of 70% methanol. Seventy-five microliters of two hundred parts per million 6-OH Chrysene were added. The extraction was done two times by vortexing, followed by sonication for 20 min and centrifugation at 9000 × *g* for 10 min. The supernatant was filtrated and analyzed by HPLC/FD.

Analysis of PY metabolites

The samples were analyzed by using HPLC (20A series; Shimadzu, Kyoto, Japan) with FD (RF-1AXL; Shimadzu) equipped with an ODS column (ODS-120T 4.6 mm × 300 mm; Tosoh, Tokyo, Japan). The HPLC method was modified from Beach et al., 2009 and Ueda et al., 2011. Mobile phase A consisted of 10 mM ammonium acetate buffer, and pH was adjusted to 5.0 with acetic acid. The mixture of methanol:acetonitrile:water (38:57:5, v/v/v) was a mobile phase B. The solvent gradient was 10% mobile phase B from 0 to 2 min, and followed by a linear gradient to 100% mobile phase A and B from 2-35 min. Then, the gradient was held at 100% mobile phase A and B for 5 min until 45 min. The solvent flow rate was set at 0.5 ml/min, and the column temperature was 45°C. The excitation (Ex) and emission (Em) wavelengths for the fluorescence detector were 343 and 385 nm, respectively. Under these conditions, the retention time for the internal standard was 37.8 min. The recovery rate (percentage ± SD) of internal standard in each tissue was more than 90%, including liver (109 ± 8.5), kidney (105 ± 2.7), lung (103 ± 11.3), duodenum (106 ± 7.5),

colon (110 ± 6.9), urine (113 ± 0.1), feces (101 ± 9.1), and plasma (110 ± 1.2). I confirmed that methanol solution of each metabolite was stable under –20 ºC at least two years.

Identification of PY metabolites in rat urine

To identify the unknown metabolites in urine, the fraction collector (FRC-10A; Shimadzu) was connected to HPLC and used to separate each peak. Then, each separated peak was identified by an electrospray ionization ion-trap mass spectrometry detector (ESI/ion-trap/MS, LTQ Orbitrap; Thermo Fisher Scientific, MA, USA). The ESI conditions were fully scanned (*m/z* 80 to 750) negative mode, with an ion source voltage of –5.0 kV and an ion source temperature of 300°C.

Pyrenediol (PYdiol) conjugation compound synthesis

PYdiol was synthesized under a photoreduction and photoaddition reaction of pyrenedione (Tintel et al., 1987). The isomers of pyrenedione, including 1,6-pyrenedione and 1,8-pyrenedione, were activated from a mixture of PY (5 g) and potassium dichromate ($K_2Cr_2O_7$) (7.5 g) in 50 mL of 4 N sulfuric acid (H_2SO_4) (Fatiadi, 1965). Briefly, the mixture solution was heated at 90°C for 1 h and then refluxed for 1 h. The reaction mixture was diluted with cold water and filtrated, producing an insoluble material that was washed and dried. Then, 4 g of the pyrenedione mixture were dissolved in 150 mL of hot acetic acid and filtrated through glass wool. The filtrate was separated by silica gel columns (5 × 60 cm). This solution was eluted with acetic acid. The orange-red band was collected and re-chromatographed on the silica gel column. Finally, the orange-red color contained 1,6- and 1,8-pyrenedione. Then, the pyrenedione powder was oxygenated and deoxygenated by visible

light (wavelength > 400 nm) to give PYdiol (Ranganathan et al., 1984; Tintel et al., 1987). A fast reaction occurred in isopropanol. The spectrogram presented under fluorescent conditions with Ex385 and Em425 nm. The subsequent synthesis of glucuronic acid derivatives was performed via the Koenigs-Knorr reaction in order to produce PYdiol conjugation compounds (Fischer et al., 1984).

De-conjugation

De-conjugation was performed using the method described by Ikenaka et al., 2007. Briefly, sulfatase, β-glucuronidase, and β-glucosidase were dissolved in 0.1 M sodium acetate buffer. pH was adjusted with acetic acid to 5.0. Each enzyme concentration was 10, 4000, and 17 U/mL, respectively. Thirty microliters of samples, which contained PY metabolites, were mixed with two hundred and seventy microliters of buffer. Then, each de-conjugation enzyme (200 μL) was added. For control, bovine serum albumin (1 mg/mL) was used at the same quantity and reactions were performed under the same conditions as the de-conjugation enzymes. All samples were incubated at 37°C for 8 h. Five hundred microliters of methanol were used in order to stop the reaction. The de-conjugated solutions were analyzed by HPLC/FD.

Quantification of PY metabolites

The concentration of PY metabolites was estimated by using the fluorescence peak areas of de-conjugated metabolites, which were related to a calibration curve of PYOH standards. The standard solution of PYOH was quantified using the calculation of the net

amount of pyrene-1-glucuronide (PYOG) and pyrene-1-sulfate (PYOS) after de-conjugation. The correlation coefficient of the standard curve of PYOH was $R^2 = 0.9998$.

Statistical analyses

The results were analyzed by cluster analyses, principal component analysis (PCA) and Tukey-Kramer test (JMP 10.0, SAS, North Carolina, USA). The results were considered statistically significant if $P < 0.05$.

Results

Identification of PY metabolites in urine

Fig. 6 showed HPLC/FD chromatograms in urine seen with PY exposure. Base on the synthesized PYdiol and the standard solution of PYOH, peak-G (*m/z* 233) and peak-H (*m/z* 217) were identified as PYdiol and PYOH, respectively. Six characteristic peaks (peak-A, peak-B, peak-C, peak-D, peak-E, and peak-F) were observed from the urine in the PY exposed rats. Thus, these six peaks were considered to be PY-derived substances. The retention times (RT) and mass to charge ratio (*m/z*) of each peak were shown in Table 4. ESI negative mass spectra of peak-A (RT 16.8 min) had the parent ion of *m/z* 393 (MS) and the product ion of *m/z* 313 and 233 (MS^2). Peak-B, peak-C, and peak-D had RT at 17.4, 19.0, and 20.1 min that contained a major ion at *m/z* 313 (MS) with the product ion of *m/z* 233 (MS^2). The metabolite in peak-E (RT 24.7 min) contained a major ion at *m/z* 393 (MS) with the product ion of *m/z* 217 (MS^2). The metabolite in peak-F (RT 27.5 min) contained a major ion at *m/z* 297 (MS) with the product ion of *m/z* 217 (MS^2). From these results, peak-E and peak-F were considered to be the conjugation products of PYOH (*m/z* 217). On the other hand, PYdiol (*m/z* 233) was contained in conjugated metabolites of peak-A, peak-B, peak-C, and peak-D.

The de-conjugation was also used to identify each peak of PY metabolites. The compounds formed in the presence of rat urine (peak-A, peak-B, peak-C, peak-D, peak-E, and peak-F), each peak was collected by using fraction collector. Each collected metabolite was treated with the de-conjugation enzymes, sulfatase, β-glucuronidase, and β-glucosidase to identify each peak (Table 4). With sulfatase treatment, peak-A, peak-B, peak-C, peak-D, and peak-F disappeared and PYdiol (peak-A, peak-B, peak-C, and peak-D) and PYOH (peak-F) were formed. Interestingly, peak-B was formed after peak-A was treated with sulfatase.

With β-glucuronidase treatment, peak-E disappeared and PYOH was formed. However, all metabolite peaks were not-deconjugated after β-glucosidase treatment. Therefore, all PY metabolites in rat urine were suspected to be peak-A as pyrenediol-disulfate (PYdiol-diS); peak-B, peak-C, and peak-D as pyrenediol-sulfate (PYdiol-S) (the congener different for PYdiol); peak-E as PYOG; and peak-F as PYOS. Moreover, peak-I was the internal standard and peak-J was the PY (Fig. 6).

Distribution of PY metabolites in tissues, plasma, urine, and feces by PCA

Fig. 7 showed the results of PCA using each PY metabolite as a variable. PCA had three major categories of components, which presented the accumulation pattern of PY metabolites in each tissue and distribution tendency 1) PYOH group, 2) PYOG group and 3) PYOS, PYdiol-S and PYdiol-diS group. PYOH group was strongly correlated in the large intestine and feces. PYOG was highly conjugated in the small intestine (duodenum, jejunum, and ileum), while PYdiol-S and PYdiol-diS were highly correlated in urine. On the other hand, other tissues as liver, kidney, lung, spleen, heart, brain, and testes, all the metabolite had similar accumulation pattern.

Quantification of PY metabolites in tissues, plasma, urine, and feces

Table 5 showed the amount of PYOG, PYOS, and PYOH in various tissues, plasma, urine, and feces. PYdiol-S and PYdiol-diS were not quantify in the present study toward the product of de-conjugation (PYdiol) had low fluorescence sensitivity. Meanwhile, I focused on quantifying the levels of PYOG, PYOS, and PYOH in rat tissue. PYOG was detected in all tissues to be higher than PYOS (Table 5). The concentration of PYOG ranged from the

small intestine > kidney > liver > lung > stomach > large intestine > tongue > testes > heart > spleen, and brain. The concentration of PYOG in small intestine, especially jejunum and ileum (87185 ± 385 and 170123 ± 740 ppb, respectively) had significances higher than other tissues. Furthermore, the levels of PYOS ranged from the ileum > liver > cecum > kidney > colon > jejunum > lung > heart > stomach and testes. The concentration of PYOS in the ileum was slightly higher than other tissues, but I couldn't find the significant differences. On the other hand, PYOH concentrations in various tissues also observed, surprisingly PYOH was highly distributed in cecum and colon (59454 ± 760 and 13071 ± 796 ppb, respectively). The range of PYOH was ordered the large intestine > small intestine > liver > stomach > kidney and testes.

Furthermore, PY metabolites were also determined in plasma, urine, and feces. The amount of PYOG and PYOS were found in plasma and urine except in feces. Interestingly, the large amount of PYOH was concentrated in feces.

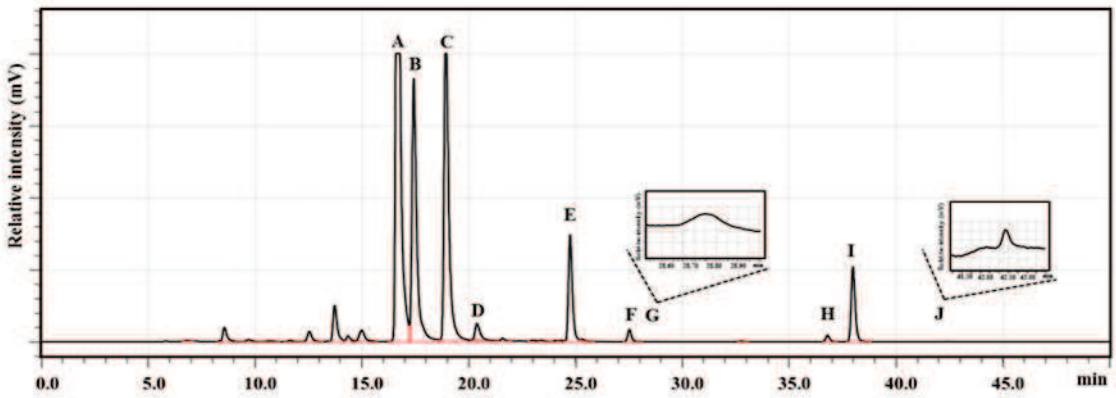


Fig. 6. The HPLC/FD chromatogram of PY metabolites in rat urine.

Glucuronide and sulfate conjugations were detected, peak-A, peak-B, peak-C, peak-D, peak-E, and peak-F were sulfate conjugated metabolites. Peak-E was glucuronide conjugate metabolite. Peak-G and peak-H were hydroxylation metabolites. Peak-I and peak-J were internal standard and PY, respectively.

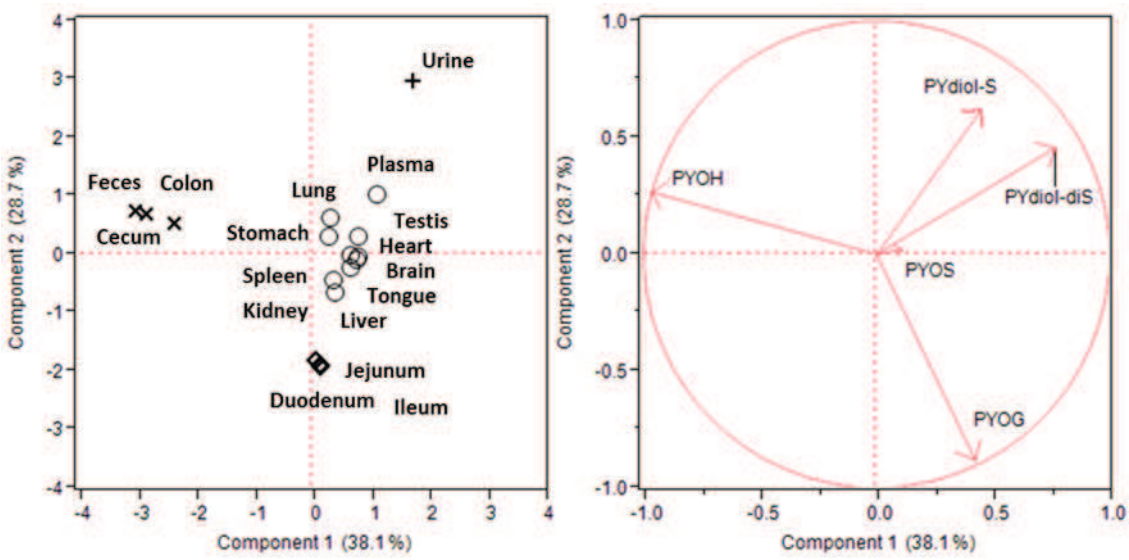


Fig. 7. Characterization of PY metabolites in tissues, plasma, urine, and feces base on Principal Component Analysis (PCA).

Samples with the same symbol (e.g., circle, diamond, cross, and plus) indicates the same cluster as determined by cluster analysis. Plot of tissues and letter codes were related to each metabolite, as PYOH is mainly distributed in the large intestine and feces. PYOG is highly distributed in the small intestine. PYdiol-S and PYdiol-diS were mainly distributed in urine.

Table 4. PY and its metabolites compounds under MS, MS/MS, and de-conjugation conditions.

Peak	Retention time (min)	MS *(m/z)*	MS^2 *(m/z)*	Sulfatase	β-glucuronidase	β-glucosidase	Metabolites
A	16.8	393	313, 233	++	-	-	Pyrenediol-disufate (PYdiol-diS)
B,C,D	17.4, 19.0, 20.1	313	233	++	-	-	Pyrenediol-sulfate (PYdiol-S)
E	24.7	393	217	-	++	-	Pyrene-1-glucuronide (PYOG)
F	27.5	297	217	++	-	-	Pyrene-1-sulfate (PYOS)
G	28.8	233	204	-	-	-	Pyrenediol (PYdiol)
H	36.8	217	-	-	-	-	PYOH
J	42.4	202	-	-	-	-	PY

++ decreased to disappeared, - no decreased

De-conjugation was performed to identify each peak of PY metabolite. Sulfatase treatment, peak-A, peak-B, peak-C, peak-D, and peak-F disappeared, then peak-B *m/z* 313 (peak-A), PYdiol *m/z* 233 (peak-B, peak-C, and peak-D), and PYOH *m/z* 217 (peak-F) were formed. β-glucuronidase treatment, peak-E disappeared, and PYOH *m/z* 217 was formed. However, the treatment with β-glucosidase, there were no de-conjugation peak.

Table 5. The concentrations of PY metabolites in tissues, plasma, urine, and feces of rats (ppb) (mean ± SD).

	PYOG	PYOS	PYOH
Liver	10262 ± 327^{C,D}	96 ± 38^{A,B}	1265 ± 168^{B}
Lung	2375 ± 859^{C,D}	38 ± 13^{B}	nd
Heart	1143 ± 186^{D}	12 ± 4^{B}	nd
Spleen	790 ± 18^{D}	nd	nd
Kidney	20592 ± 100^{C,D}	76 ± 38^{A,B}	154 ± 23^{B}
Brain	208 ± 60^{D}	nd	nd
Testis	1048 ± 356^{D}	11 ± 3^{B}	88 ± 36^{B}
Tongue	1366 ± 488^{D}	nd	nd
Stomach	1726 ± 11^{D}	11 ± 1^{B}	871 ± 64^{B}
Duodenum	65940 ± 795^{B,C}	nd	1140 ± 79^{B}
Jejunum	87185 ± 383^{B}	39 ± 17^{B}	1323 ± 164^{B}
Ileum	170123 ± 740^{A}	261 ± 21^{A}	10661 ± 972^{B}
Cecum	1423 ± 110^{D}	89 ± 58^{A,B}	59454 ± 760^{A,B}
Colon	1245 ± 312 ^{D}	54 ± 20^{B}	13071 ± 796^{B}
Plasma	446 ± 34^{D}	2 ± 1^{B}	4 ± 2^{B}
Urine	10123 ± 399^{C,D}	13 ± 7^{B}	546 ± 78^{B}
Feces	nd	nd	226870 ± 216^{A}

^{A,B,C,D}Tukey – Kramer test ($P < 0.05$). nd: not detected

Discussion

In this section, glucuronide and sulfate conjugations of PY were identified and detected from various tissues from rats. The study of pharmacokinetics and bioavailability of PY found that the bioavailability of orally administered doses was between 50 and 60% (Harper, 1957; Jacob et al., 1989; Ruzgyte et al., 2006). The elimination half-life ($t_{1/2}$) of PY in blood, liver, kidney, lung, muscle, and gastrointestinal tract averages between 6.2 and 8.7 h (Withey et al., 1991; Bouchard et al., 1998). Thus, PY itself was rapidly distributed, metabolized, and eliminated from the body (Bouchard et al., 1998). Possibly, phase II metabolites were produced, and then the conjugate compounds were distributed in various tissues of the rats. Harper (1958) reported that PYOG was detected in liver, kidneys, and small intestine. However in my study, I could also detect both glucuronide and sulfate conjugates in various tissues.

Interestingly, PCA clearly showed the different patterns of PY metabolites in GI tracts; for example, PYOG was a main metabolite detected in the small intestine (duodenum, jejunum and ileum) and had significant higher concentration than other tissues. The high UGT activities in the small intestine are considered to be the reason for this high PYOG concentration in the small intestine (Shiratani et al., 2008). Enzymatic activities of UGT and SULT to produce phase II metabolites represent the main pathways for many xenobiotic metabolisms. Such enzymes are expressed in various tissues, including intestines (Shiratani et al., 2008). Generally, the affinities of UGT and SULT enzymes become different under many circumstances and reflect metabolic capacity. On the other hand, the level of PYOG was significantly decreased in the large intestine, while PYOH increased. The biodegradation could be explained by decreasing PYOG. PY metabolites could be biodegraded by bacteria and fungi through initial dioxygenase (bacteria) and monooxygenase (bacteria and fungi) reactions (de-conjugation reaction) (Kanaly and Harayama 2000; Ravelet et al., 2000).

Possibly, normal flora that lives in the large intestine could digest conjugation compounds, such as PYOG, to form dominant metabolites, such as PYOH. Meanwhile, I could also detect PYOH in feces.

Surprisingly, I could detect the sulfate conjugate products of phase I metabolites as PYdiol-S and PYdiol-diS in urine, plasma and other tissues of rats. In previous report, PYdiol-S and PYdiol-diS were identified in amphibian species and marine snails (Beach et al., 2009; Ueda et al., 2011). To my knowledge, PYdiol-S and PYdiol-diS are novel metabolites in mammalian species. I presumed that PYOH and PYdiol produced by phase I reaction were rapidly released to cytosolic fractions after CYP reactions. Then PYOH and PYdiol in cytosolic fractions might be metabolized to PYOS, PYdiol-S, and PYdiol-diS by SULT. Further study is needed to clarify the detailed metabolism of PYdiol and sulfate conjugation. Based on the metabolites that were identified, a metabolic pathway might be proposed for PY and PYOH in Fig. 8.

Nowadays, studies of phase II metabolism in animals and biota have become important for xenobiotic metabolism. For PY, *in vitro* study of glucuronidation using human recombinant UGT showed that UGT1A6, 1A7, and 1A9 were mainly involved. In addition, in mice, PYOH also be catalyzed by UGT1A1 and 1A6 (Lee et al., 2007). The glucuronide levels account for more than 80% of total PY metabolites in human urine (Singh et al., 1995; Strickland et al., 1996). From my results, rats also eliminate high amount of glucuronide conjugates into urine more than that of sulfate conjugate and also PYOH.

In this section, I concluded that the phase II conjugated metabolites of PY in rats were identified in various tissues, including plasma, urine, and feces. In the present study, PY metabolites, such as PYOG, PYOS, PYdiol-diS, and PYdiol-S, could be identified, and they were eliminated in urine and feces. Although PYOH was not detected from all the tissues, it

remains possible to be a reliable indicator of systemic exposure to PAHs, especially in specific tissues with high incidence of this metabolite pattern such as the liver, kidney, small intestine and large intestine.

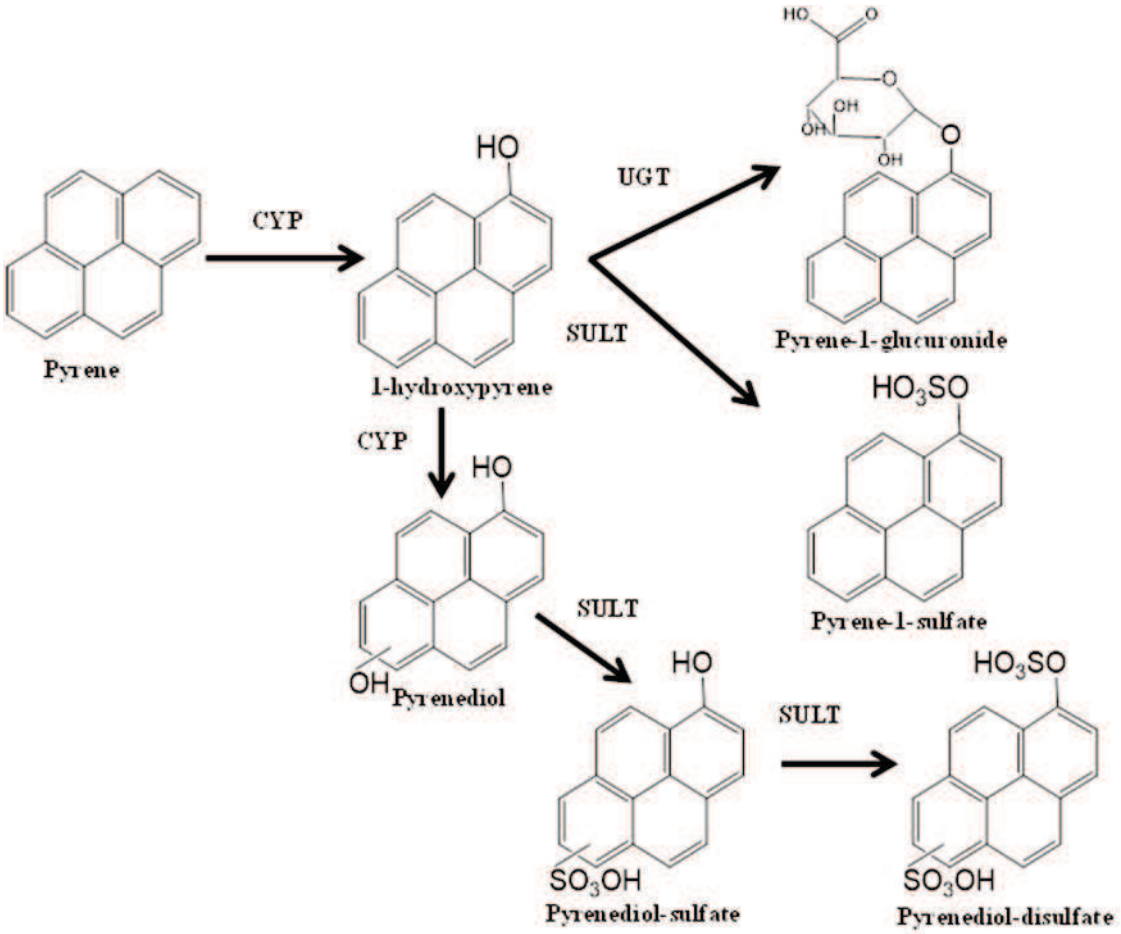


Fig. 8. Scheme pathway for the metabolism of pyrene and 1-hydroxypyrene in rats.

In rats, pyrene was metabolites base on CYP to be 1-hydroxypyrene (PYOH). Then the conjugated enzymes (UGT and SULT) catalyzed PYOH to pyrene-1-glucuronide (PYOG) and pyrene-1-sulfate (PYOS), respectively. However, PYOH also catalyzed belongs to CYP to pyrendiol form. Then pyrenediol-sulfate and pyrenediol-disulfate were formed to base on sulfation activity.

SECTION II

Interspecies differences in phase II reactions in mammals; determination of pyrene metabolites in urine of various mammalian species

Introduction

The biotransformation of xenobiotics, including drugs, is divided into phase I and II reactions. In phase I reaction, the main enzymes are the cytochromes P450 (CYP) (Anzenbacher et al., 2001). Phase II enzymes also play an important role in the biotransformation of xenobiotic metabolites to more water-soluble forms (Kretschmer and Baldwin, 2005). These include glucuronidation, sulfation, methylation, acetylation, and glutathione and amino acid conjugation (Jancova et al., 2010). In particularly mammals, glucuronidation and sulfation majorly contribute to metabolisms of various xenobiotics, such as drugs.

The species differences with regards to the capacity to metabolize and eliminate drugs and other xenobiotics from the body are typically substantial, complicating the effective use of drugs, as well as minimizing the ability to predict the adverse consequences of environmental pollutants (Caldwell, 1981). The best known species difference in drug metabolism is found in domestic cats that metabolize drugs and structurally related phenolic compounds by glucuronidation. Slow glucuronidation of acetaminophen and acetylsalicylic acid (aspirin) account for the slow clearance and exquisite sensitivity of cats to the adverse effects of these drugs compared with dogs and most other mammalian species (Davis and Westfall, 1972; Savides et al., 1984). This is because there are only a few UDP-glucuronosyltransferase (UGT) isoforms expressed in the feline liver that are comparable to those of other species (Court and Greenblatt, 2000; Court, 2001). The isoform, UGT1A6 was

found to be absent from feline liver because the gene that normally encodes this enzyme is a pseudogene, meaning it contains numerous deleterious mutations (Court and Greenblatt, 2000). The evolutionary basis for this species defect was proposed to result from the highly carnivorous nature of the natural feline diet conferring minimal selection pressure from dietary phytotoxins (Court and Greenblatt, 2000).

Sulfation also has a significant role in the biotransformation of a number of endogenous low-molecular weight compounds (Glatt and Meinl, 2004). However, there is limited information about interspecies differences in sulfotransferases (SULT). In some animal species, SULT forms are isolated, and they have been found to have no equivalent human form. The aryl-SULT (SULT1A) enzyme family plays a major role in xenobiotic metabolism. SULT1A1 is the most abundant SULT, exhibiting broad substrate specificity and wide tissue distribution (Wang et al., 2009). Nevertheless, SULT shows a preference for xenobiotic substrates. Therefore, SULT activity has been found to differ in various species, such as pigs, and it has been shown to have the lowest sulfation activity on 2-naphthol when compared with cattle, sheep, and rats (Smith et al., 1984).

Polycyclic aromatic hydrocarbons (PAHs) can be performed mutagenic and carcinogenic metabolites under metabolic activation by CYP in living organisms. Pyrene (PY) is known for a frequently encountered PAH. The metabolism of PY into a more soluble form is a necessary step for its excretion. Metabolism of PY involves the information of 1-hydroxypyrene (PYOH) as a phase I metabolite that undergoes phase II metabolism with conjugation to more water soluble forms. *In vitro* studies of PYOH with human recombinant UGT have shown that UGT 1A6, 1A7, and 1A9 are mainly involved in glucuronidation (Luukkanen et al., 2001). In a previous study, PY metabolites were found to have interspecies differences in various animals and biota. In aquatic animals, amphibians, and mammals, various conjugation forms have been discovered from those found in humans, rats, aquatic

crustacean, snails, crabs, fish species, amphibians, and reptiles (Law et al., 1994; Viau et al., 1999; Hellou and Leonard, 2004; Chetiyanukornkul et al., 2006; Dam et al., 2006; Ikenaka et al., 2006, 2007; Beach et al., 2009; Beach et al., 2010; Ueda et al., 2011). PY may be good model compounds to detect species difference in phase II xenobiotic metabolism.

In this section, urine samples from 16 mammalians species were used to predict the interspecies differences of phase-II (conjugation) metabolism of daily intake PY. This is the first study to provide comparative data on phase-II biotransformation in various mammalian species.

Materials and Methods

Chemicals

PY, PYOH, methanol (High-performance liquid chromatography, HLPC grade), ethyl acetate, sodium hydroxide, sulfuric acid, and acetonitrile (HPLC grade) were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). Sulfatase (from limpets Type V; 34 U/mg), β-glucuronidase (from bovine liver, Type B-1; 1240 U/mg), β-glucosidase (from almonds; 3.4 U/mg), and bovine serum albumin were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). 6-Hydroxychrysene used as an internal standard was purchased from AccuStandard Inc. (New Haven, USA). Acetic acid, formic acid, sodium phosphate, sulfuric acid, diethylamine, potassium dichromate, and ammonium acetate solution were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Animals

Urine samples from healthy mammals were collected in Japan and Thailand. They were taken from many species, including cattle (n = 9), deer (n = 14), horses (n = 13), elephants (n = 10), rabbits (n = 2), dogs (n = 2), guinea pigs (n = 2), hedgehogs (n = 2), cats (n = 2), ferrets (n = 2), pigs (n = 3), humans (n = 3), tapir (n = 1), chimpanzee (n = 1), and bear (n = 1). The sample data are presented in Table 6. Urine was collected in the mornings and kept at –20°C prior to analysis.

Nine-week-old male Wistar rat (*Rattus norvegicus*) (n = 3) (SLC, Hamatsu, Japan) were maintained according to the guideline of the Hokkaido University Institutional Animal Care and Use Committee. The body weights of rats (average ± SD) were 248 ± 10 g. Rats

were kept at 24 ± 1ºC and 12 h light and 12 h dark cycle. They were given feed and water ad libitum.

Sample preparation

Aliquots of urine (5 to 500 mL) were acidified (to approximately pH 6.8) with 10 mM formic acid before compound extraction. The acidified samples were then added to an internal standard (6-hydroxychrysene) and were loaded onto an Oasis WAX (Waters) solid phase extraction cartridge. The extraction process was a modified form of that used by Stewart et al. (2009). The cartridges were conditioned with methanol (10 mL) and Milli Q water (10 mL). Next, the samples were loaded onto the cartridges, which followed washing with sodium hydroxide (0.1 M; 5 mL), sodium phosphate buffer (0.1 M; pH 7.4; 5 mL), and Milli Q water (5 mL). The cartridges were dried briefly under vacuum, and then they were sequentially eluted with methanol:ethyl acetate (1:1, v/v; 10 mL; hydroxylation and internal standard fractions) and methanol:formic acid (9:1, v/v; 10 mL; β-glucuronide fraction). The last elution involved the sulfate fraction, employing methanol:Milli Q water:diethylamine (17:2:1, v/v; 10 mL) and methanol:ethyl acetate:diethylamine (50:50:1, v/v; 2 mL). All three eluents were reduced in volume by evaporation at 45°C under nitrogen. The final volume was 1 mL and 5 µL was used in HPLC employing a fluorescence detector (FD).

Rats were fasted for 24 h before exposure. PY was dissolved in 100% propylene glycol and treated at a dose of 4 mg/kg body weight by oral administration. Then, rats were kept in a metabolic cage for 24 h for urine collection. Extraction process was done following the previous explanation and analyzed using HPLC/FD.

PY metabolites analysis

The samples were analyzed using HPLC (20A series; Shimadzu, Kyoto, Japan) with FD (RF-1AXL; Shimadzu) equipped with an ODS column (ODS-120T 4.6 mm × 300 mm; Tosoh, Tokyo, Japan). The HPLC method was a modified form of that used by Beach et al. (2009) and Ueda et al. (2011). Mobile phase A consisted of 10 mM ammonium acetate buffer, and its pH was adjusted with acetic acid to pH 5.0. Mobile phase B was comprised of a mixture of methanol:acetonitrile:water (38:57:5, v/v/v). The solvent gradient was made up of 10% mobile phase B maintained from 0 to 2 min followed by a linear gradient to 100% mobile phase A and B occurring from minute 2 to 35. This was then maintained at 100% mobile phase A and B from minute 5 to 45. The solvent flow rate was set at 0.5 ml/min and the column temperature was 45°C. The excitation (Ex) and emission (Em) wavelengths for the fluorescence detector were 343 and 385 nm, respectively. Under these conditions, the retention time (RT) for the internal standard was 37.8 min, and the recovery rate (± SD) was 106.4 ± 10.7%. The recovery rate of each metabolite in each fraction was determined in the exposed rat urine. The recovery rate at RT 36.6 min was 105.1 ± 5.7% (Fraction 1). The recovery rate at 24.7 min was 97.6 ± 10.2% (Fraction 2). The recovery rates at RT 16.8, 17.4, 19.0, 20.1, and 27.5 min were 108.3 ± 2.0, 97.8 ± 8.0, 98.8 ± 3.3, 88.5 ± 1.5, and 89.8 ± 3.9%, respectively (Fraction 3).

PY metabolites identification

To identify the PY metabolites in the urine, the exposed rat urine was used for identification and quantification analysis, which followed from the previous study (Ueda et al., 2011). Each metabolite was collected from a fraction collector (FRC-10A; Shimadzu) that was connected to the HPLC system. Each separated peak was then identified by an

electrospray ionization ion-trap mass spectrometry detector (ESI/ion-trap/MS, LTQ Orbitrap; Thermo Fisher Scientific, MA, USA). The ESI conditions were fully scanned (m/z 80 to 750) in negative mode with an ion source voltage of –5.0 kV and an ion source temperature of 300°C, and the de-conjugation.

Chemical synthesis and analytical methods

Pyrenediol (PYdiol) was synthesized under a photoreduction and photoaddition reaction of pyrenedione (Tintel et al., 1987). The isomers of pyrenedione, as 1,6-pyrenedione and 1,8-pyrenedione, were activated from a mixture of PY (5 g) and potassium dichromate ($K_2Cr_2O_7$) (7.5 g) in 50 ml of 4 N sulfuric acid (H_2SO_4) (Fatiadi, 1965). Briefly, the solution was heated at 90°C for 1 h and then refluxed for 1 h. The reaction mixture was diluted with cold water and filtrated; an insoluble material was washed and dried. Then 4 g of the pyrenedione mixture was dissolved in 150 ml of hot acetic acid and filtrated through glass wool. The filtrate was separated on a silica gel column (5 × 60 cm) and was eluted with acetic acid. The orange-red band was collected and re-chromatographed on the silica gel column. The orange-red colored eluent contained 1,6- and 1,8-pyrenedione. Pyrenedione powder was then oxygenated and deoxygenated by visible light (wavelength > 400 nm) to give PYdiol (Ranganathan et al., 1984; Tintel et al., 1987). A fast reaction occurred in isopropanol. The spectrogram was visualized under fluorescent conditions using Ex385 and Em425 nm. The synthesis of the glucuronic acid derivative was then performed via the Koenigs-Knorr reaction in order to produce a PYdiol-conjugated compound (Fischer et al., 1984).

De-conjugation

De-conjugation was performed using the method described by Ikenaka et al. (2007). Briefly, the enzymes sulfatase, β-glucuronidase, and β-glucosidase were dissolved in 0.1 M sodium acetate buffer. The pH of these solutions was adjusted with acetic acid to pH 5.0. Each enzyme concentration was 10, 4000, and 17 U/mL, respectively. Thirty microliter aliquots of each sample containing PY metabolites were mixed with 270 μL of buffer. Each de-conjugation enzyme (200 μL) was then added. As the control, the same volume of bovine serum albumin (1 mg/mL) was used and subjected to the same conditions as the de-conjugation enzymes. All samples were incubated at 37°C for 8 h. A 500 μL volume of methanol was used to stop the reaction. After centrifugation the sample at 12000 × *g*, 10 min, the de-conjugated solutions were analyzed by HPLC/FD.

Quantification of PY metabolites

The concentration of the PY metabolite compounds was estimated using the fluorescence peak areas of de-conjugated metabolites and a calibration curve of PYOH standards. The transformation rates of pyrene-1-glucronide (PYOG) and pyrene-1-sulfate (PYOS) to PYOH were 0.21- and 0.24-fold, respectively. Then the PYOH calibration curve was constructed to calculate the level of PYOG and PYOS in urine. The standard curve for PYOH had an R^2 value of 0.9998.

Statistical analysis

The results were analyzed by Principle Component Analysis (PCA) and Spearman's ρ correlation (JMP 9.0). The results were considered statistically significant if the *P*-value was equal to or less than 0.05.

Table 6. The mammalian urine samples used in the study.

Animals (n)	Genus/species	Race/breed/strain	Age	Gender
Deer (14)	*Cervus nippon yesoensis*	Sika deer	1-6 years	Ten males, four females
Cattle (6)	*Bos tarurus*	Holstein Friesian	2-4 years	Females
Rabbit (2)	*Oryctolagus cuniculus*	Crossbreed	2-3 years	One male, one female
Horse (13)	*Equus ferus*	Thoroughbred	7-20 years	Males
Beef cattle (3)	*Bos primigenius*	Brahman bull crossbreed	2-4 years	Females
Dog (2)	*Canis familiaris*	Golden retriever	5-6 years	Males
Guinea pig (2)	*Cavia porcellus*	Cavies	2 years	Males
Hedgehog (2)	*Atelerix albiventris*	African pygmy	1 year	Males
Cat (2)	*Felis domesticus*	Domestic short hair	2-3 years	One male, one female
Ferret (2)	*Mustela putorus furo*	Domestic	5 years	Males
Pig (3)	*Sus domesticus*	Large white	4 years	Females
Human (3)	*Homo sapieus*	Asian	28-32 years	Two males, one female
Elephant (10)	*Elephas maximus*	Asian elephant	10-40 years	Two males, eight females
Tapir (1)	*Tapirus indicus*	Malayan tapir	20 years	Male
Chimpanzee (1)	*Pan troglodytes*	Common chimpanzee	16 years	Male
Bear (1)	*Ursus arctos yesoenis*	Hokkaido brown bear	12 years	Male

The mammalian urine samples as cattle (n=2), rabbit (n=2), horses (n=3), beef cattle (n=3), dogs (n=2), guinea pigs (n=2), hedgehogs (n=2), cats (n=2), ferrets (n=2), pigs (n=3), and elephants (n=10) were collected from Kasetsart University Veterinary Teaching Hospital (Kampangsan and Bangkhan campuses), Faculty of Veterinary Medicine, Kasetsart University, Thailand. The other mammalian urine samples such as deer, cattle, humans, tapir, chimpanzee, and bear were collected from Hokkaido, Japan. Samples were kept at –20°C until extraction.

Results

Identification of PY metabolites in rat urine (experimentally exposure study)

To identify the PY metabolites in mammalian urine, I analyzed rat urine samples as a mammalian model sample. Fig. 9 showed the typical chromatogram of PY metabolites observed from PY exposed rat urine. After solid phase extraction, I could observe two peaks (peak-a and peak of internal standard) from Fraction 1 (Fig. 9A) and one peak (peak-b) from Fraction 2 (Fig. 9B) and five peaks (peak-c, peak-d, peak-e, peak-f, and peak-g) from Fraction 3 (Fig. 9C). Base on the standard solution of PYOH, peak-a (*m/z* 217) was identified as PYOH. Moreover, the synthesized PYdiol (*m/z* 233) was detected with Ex385 and Em425 nm in low fluorescence intensity (therefore the peak was not in the Figure). Five peaks were considered to be PY-derived substances. The retention time (RT) and mass to charge ratio (*m/z*) of each peak were used to identify PY metabolites. ESI negative mass spectra of peak-b (RT 24.4 min) had the parent ion of *m/z* 393 (MS) and the product ion of *m/z* 217 (MS^2). Peak-c (RT 17.4) contained a major ion at *m/z* 393 (MS) with the product ion of *m/z* 313 and 233 (MS^2). Peak-d, peak-e, and peak-f had RT at 18.8, 20.5, and 22.3 min that contained a major ion at *m/z* 313 (MS) with product ion of *m/z* 233 (MS^2). The metabolite in peak-g (RT 26.8 min) contained a major ion at *m/z* 297 (MS) with the product ion of *m/z* 217 (MS^2). From these results, peak-a and peak-g were considered to be the conjugation products of PYOH (*m/z* 217). On the other hand, the ion at *m/z* 233 was identified as PYdiol (RT 28.5 min) that was contained very low fluorescence intensity in conjugated metabolites of peak-c, peak-d, peak-e, and peak-f.

De-conjugation

The de-conjugation was also used to identify each peak of PY metabolites. Each peak from rat urine (peak-a, peak-b, peak-c, peak-d, peak-e, peak-f, and peak-g) was collected by

using fraction collector connected to HPLC system. Each collected metabolite was treated with the de-conjugation enzymes, sulfatase, β-glucuronidase, and β-glycosidase to identify each peak. Sulfatase treatment, peak-c, peak-d, peak-e, peak-f, and peak-g disappeared and PYdiol (peak-c, peak-d, peak-e, and peak-f) and PYOH (peak-g) were formed. Surprisingly, peak-d was formed after peak-c was treated with sulfatase. With β-glucuronidase treatment, peak-b disappeared and PYOH was formed. However, all metabolite peaks were not de-conjugated after β-glucosidase treatment. From MS spectrum and de-conjugation study, I could identify PY metabolites in rat urine as following; peak-a as PYOH, peak-b as PYOG, peak-c as pyrenediol-disulfate (PYdiol-diS), peak-d, peak-e and peak-f as pyrenediol-sulfate (PYdiol-S), and peak-g as PYOS. By using these peaks information about RT and MS spectrum, PY metabolites from various mammalians urine were identified and quantified.

PY metabolites in various mammalians urine

PY metabolites were detected in mammalian urine which was collected from animals in both rural and urban areas. The concentration ranges of each metabolite (PYOG, PYOS, and PYOH) are summarized in Table 7. PYdiol-S and PYdiol-diS were not quantify in the present study toward the product of de-conjugation (PYdiol) had low fluorescence sensitivity. Higher concentrations of PY metabolites were detected in the urine of ungulates, such as deer, cattle, and horses. PYOG was highly eliminated in various mammals. The range of PYOG concentrations were deer > cattle > beef cattle > horses > rabbits > dogs > hedgehogs > bear > pigs > elephants > humans > guinea pigs > tapir and chimpanzee. PYOS was found in almost all urine samples except in hedgehogs, which ordered as horses > cattle > rabbits > deer > beef cattle > guinea pigs > dogs > cats > bear > elephants > pigs > ferrets > tapir > humans and chimpanzee. PYOH was also detected in the urine of various mammals. The

order of PYOH were as rabbits > guinea pigs > deer > horses > cats > ferrets > bear > cattle > hedgehogs > pigs > dogs > elephants > chimpanzee > tapir and beef cattle.

Species differences of PY metabolites

Fig. 10 shows the ratio of glucuronide and sulfate-conjugated metabolites detected from various mammalians urine. There were clear interspecies differences in PY metabolite composition. The ratio of each metabolite showed that portions of glucuronide conjugates were high in the urine of deer, humans, elephants, chimpanzee, beef cattle, hedgehogs, dogs, bear, cattle, pigs, tapir, and horses. From these species, sulfate conjugates were also detected, except hedgehog. The highest compositions of sulfate-conjugated metabolite were found in cats and ferrets. In addition, I found a correlation between certain PY metabolites (Fig. 11). A strong positive correlation was found between PYOG and total PY metabolites (r = 0.9968; Spearman's $\rho < 0.05$). Moreover, total PY metabolite levels were positively correlated to PYOS and PYOH (Spearman's $\rho < 0.05$).

The PY metabolites in mammalian urine were analyzed by PCA (Fig. 12). PCA had three major groups of components, which showed the pattern of PY metabolites detected from various mammalians urine. 1) PYOG group was strongly correlated in various mammalians urine such as cattle, deer, dogs, humans, hedgehogs, pigs, and elephants. 2) PYOH and PYdiol-S group was presented in the urine of ferrets, cats, and guinea pigs, while 3) PYOS and PYdiol-diS group was highly correlated in tapir, rabbits, horses, beef cattle, bear and chimpanzee.

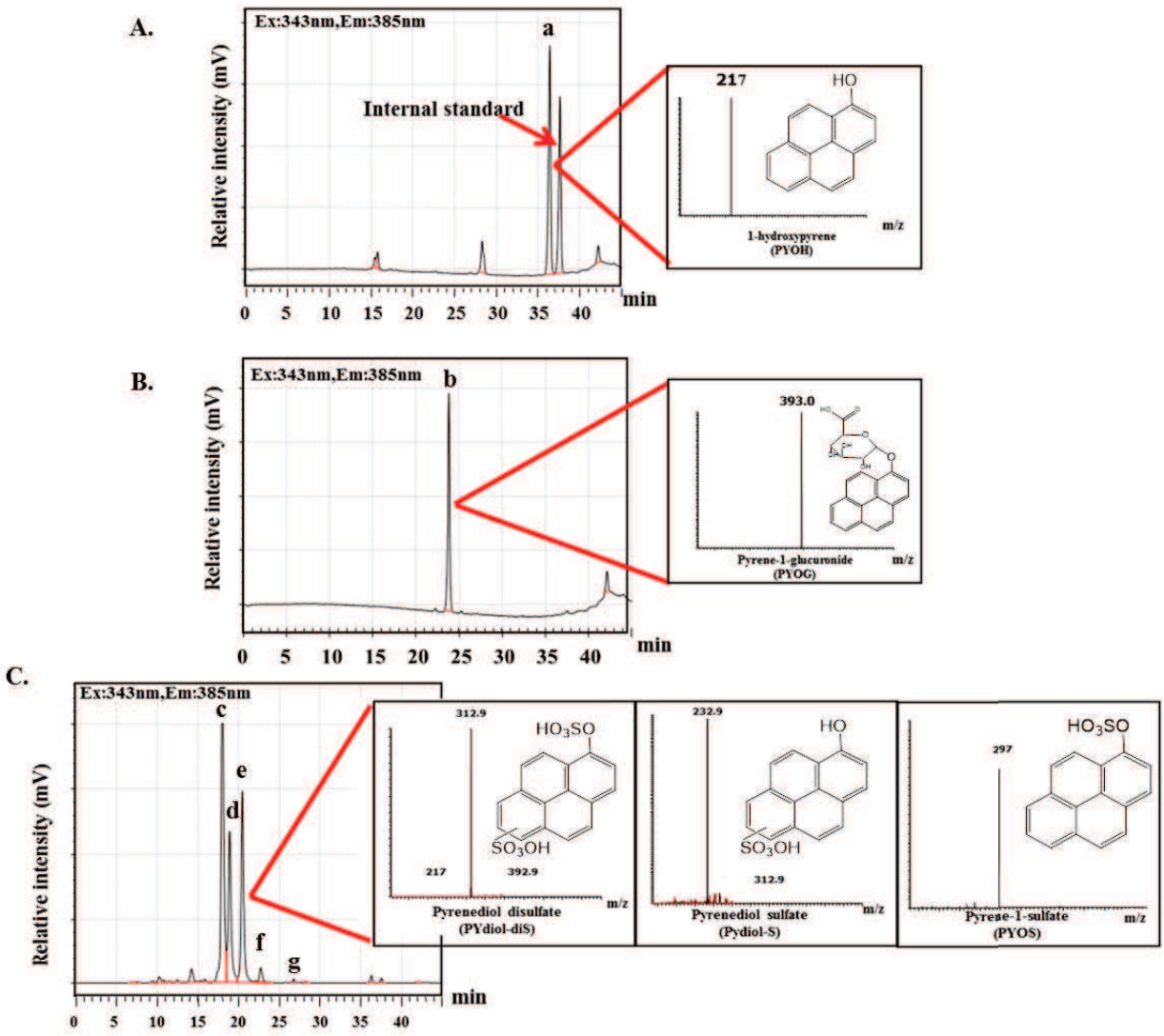


Fig. 9. The chromatograms and the ion trap results from the PY metabolites in urine after cleaning by Oasis WAX cartridges.

In the first fraction, 1-hydroxypyrene (peak-a) was detected with an *m/z* value of 217 and internal standard showed at RT 37.5 min (A). In the second fraction, the glucuronide conjugate (peak-b) was detected at *m/z* of 393 (B), and in the third fraction, sulfate conjugates (peak-c, peak-d, peak-e, peak-f, and peak-g) were detected at *m/z* of 393, 313, and 297(C).

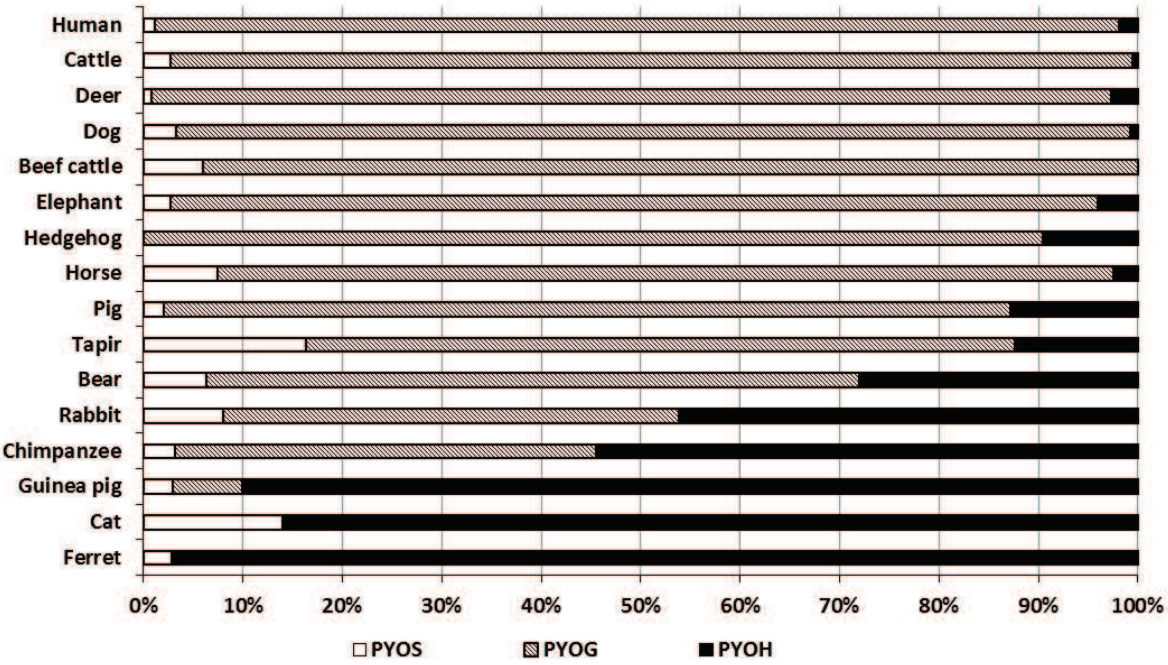


Fig. 10. The ratio of glucuronide and sulfate conjugates in mammalian urine.

The percentage of each PY metabolites (PYOS, PYOG, and PYOH) that contained in mammalian urine, PYOG had highly percentage in humans, cattle, deer, and dogs, etc. PYOS was mainly in ferrets and cats.

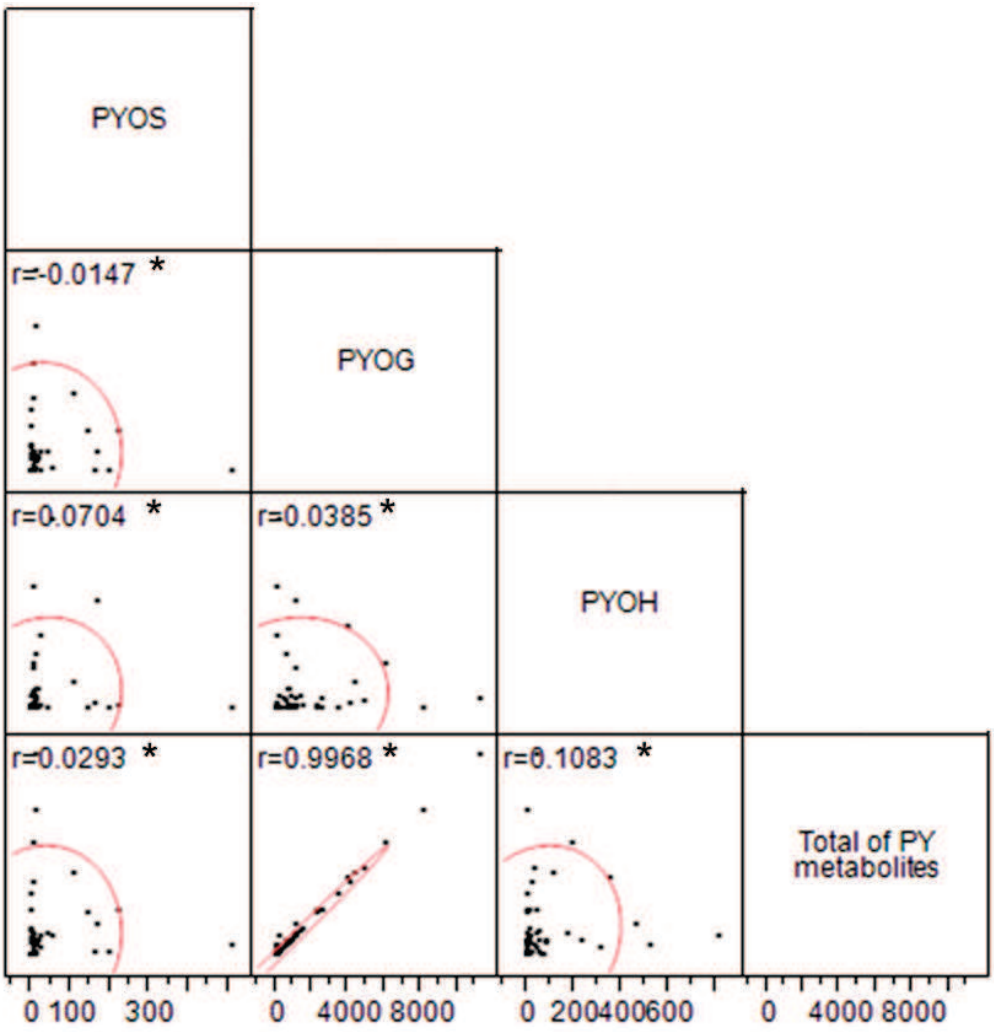


Fig. 11. The correlation of PY conjugates with the total amount of PY metabolites in urine.

PY metabolites were positive correlation with the total amount of PY metabolites (Spearman's ρ, $^{*}P < 0.05$).

The total amount of PYOG was strong correlation with the total amount of PY metabolites in urine. Additionally, I also found the positive correlation of the total amount of PYOG, PYOS, and PYOH with the total amount of PY metabolites.

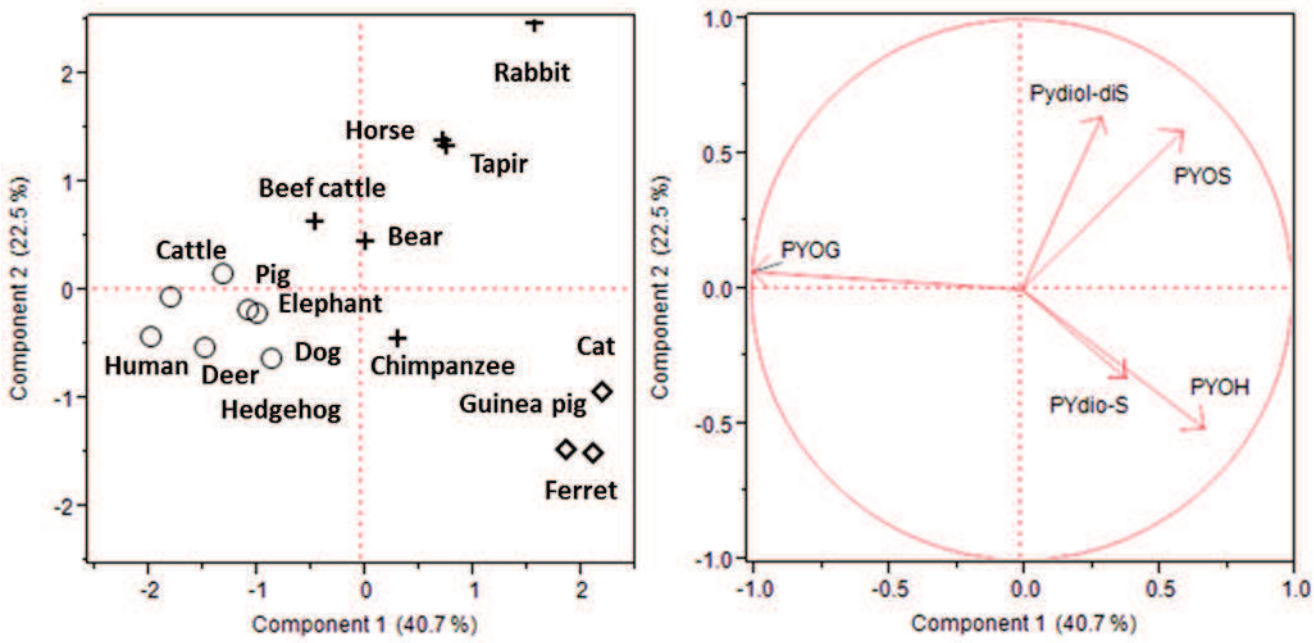


Fig. 12. The characterization of PY metabolites in mammalian urine base on principal component analysis (PCA).

Animals with the same symbol (e.g. circle, diamond, and plus) indicated the same cluster as determined by cluster analysis. PYOG was dominant of PY metabolites in cattle, pigs, elephants, dogs, humans, deer, and hedgehogs. PYOH and PYdiol-S were also presented in cats, ferrets, and guinea pigs. PYOS and PYdiol-diS were related to rabbits, horses, tapir, beef cattle, chimpanzee and bear.

Table 7. Species comparison of the concentration ranges and percentages (%) of the PY metabolites in urine (nM).

Animals (n)	PYOS	PYOG	PYOH
Deer (14)	0.02 – 12.70 (9.1)	4.20 – 64.85 (77.8)	0.13 – 10.97 (13.1)
Cattle (6)	0.22 – 15.98 (14.9)	3.63 – 46.65 (82.9)	0.03 – 1.53 (2.2)
Rabbit (2)	5.15 – 13.07 (25.7)	2.19 – 6.98 (12.9)	16.26 – 27.29 (61.4)
Horse (13)	0.11 – 2.74 (7.4)	1.59 – 61.44 (87.7)	0.03 – 2.64 (4.9)
Beef cattle (3)	0.04 – 10.21 (43.9)	0.06 – 12.87 (55.7)	0.02 – 0.03 (0.4)
Dog (2)	0.43 – 1.53 (21.0)	0.93 – 5.09 (64.5)	0.67 – 0.69 (14.6)
Guinea pig (2)	1.62 – 2.79 (12.1)	1.12 – 1.14 (6..2)	11.54 – 18.09 (81.6)
Hedgehog (2)	nd	1.08 – 3.36 (47.8)	1.80 – 3.05 (52.2)
Cat (2)	0.69 – 1.35 (34.3)	nd	0.89 – 3.01 (65.7)
Ferret (2)	0.63 – 0.72 (26.9)	nd	0.88 – 2.79 (73.1)
Pig (3)	0.03 – 0.15(17.6)	0.06 – 0.36 (42.6)	0.04 – 0.39 (39.8)
Human (3)	0.02 – 0.05 (13.0)	0.02 – 0.27 (76.6)	0.021 – 0.023 (10.4)
Elephant (10)	0.01 – 0.13 (24.0)	0.02 – 0.27 (57.4)	0.01 – 0.12 (18.6)
Tapir (1)	0.09 (51.7)	0.04 (21.9)	0.05 (26.4)
Chimpanzee (1)	0.019 (22.1)	0.016 (18.5)	0.05 (59.4)
Bear (1)	2.36 (29.6)	1.88 (23.5)	3.75 (46.9)

nd; Not detected

The PYOG was higher concentration in ungulates than other mammals, except in cats and ferrets urine. PYOS also detected in various mammals, unlike hedgehogs. Not only PY conjugated metabolites that found in urine, but the hydroxylation of PY as PYOH also presented in all mammalian urine.

Discussion

Daily exposure levels of hydroxylated and conjugated PY metabolites were detected in various mammalians urine. PY metabolites were reacted with CYP in phase I reactions, which produced PYOH and PYdiol. Then the hydroxylation compounds were conjugated to produce more water soluble forms. In present study, I did not mention the gender difference for PY metabolites. There have been reported that showed no gender differences of some PAH hydroxylation in human urine (Zhong et al., 2011). In case of rats, there is not also large sex difference in benzo(a)pyrene conjugation (van de Wiel et al., 1993).

In this section, I could find the different PY metabolite pattern, especially PYOG in various mammals. There also have been reported the interspecies differences in UGT activity with other phenol substrates such as *p*-nitrophenol, 4-methylumbelliferon, and silybin (Matal et al., 2008). Therefore glucuronidation of PY is possible to provide for prediction of metabolism of phenolic xenobiotics metabolism in mammals. From the results of my experiment, PYdiol-diS, PYdiol-S, PYOS, and PYOG were observed in mammalian urine such as cattle, deer, and horses. On the other hand, a large species variation in PY metabolites was observed. Glucuronide conjugates could be found in various species, except cats and ferrets. UGT1A6 preferentially conjugates complex phenols and primary amines (Jancova et al., 2010). Recent study of sequence and phyrogenetic analysis of UGT indicated that UGT1A6 has become a pseudogene in felidae, hyenas, and elephant seals (Shrestha et al., 2011). Court (2001) also reported about acetaminophen glucuronidation and cats and ferrets UGT1A6. I suggested that UGT1A6 might not have a significant role in PY metabolism in cats and ferrets and/or they received very low amount of PY. In humans, UGT1A6, 1A7, and 1A9 are mainly involved in glucuronidation of PYOH, which is similar in mouse. (Singh et al., 1995; Luukkanen et al., 2001; Lee et al., 2007; Kakimoto et al., 2008). The glucuronide

levels account for more than 80% of total PY metabolites in human urine, a result similar to that found in the present study.

Sulfation reactions were also found to exhibit species differences in terms of PY metabolism. In present study, PY sulfate conjugations were detected in various mammals such as rabbits, horses, cats, and ferrets. Although sulfation occurs in various mammals, the pig has been assumed to have little SULT activity (deBethizy and Hayes 1989). However, interestingly, I could detect PY sulfate conjugated such as PYOS and PYdiol-S in pig urine. Recently, SULT1A enzymes have been reported that are responsible for the sulfation of small phenolic substrates such as *p*-nitrophenol, 1-naphthol, acetaminophen, and dopamine (Tsoi et al., 2001). Additionally, PY was seen to induce SULT1A1 mRNA in mice (Lee et al., 2007). Thus, SULT1A1 is purported to be one important enzyme involved in the sulfation of PY. In contrast, there has been phylogenetic assessment of SULT1A genes in a large range of species (Bradley and Benner, 2005), and mice, rats, dogs, cattle, rabbits, and pigs all have genomes that contain a single SULT1A1 gene (Lin et al., 2004; Gamage et al., 2006). The simplest evolutionary model predicted that hominoid SULT1A loci were orthologous to the rodent SULT1A1. Although human SULT1A has four forms, human SULT1A1 is most like rodent SULT1A1 in sequence, and it functions as an orthologous (Bradley and Benner, 2005). In addition, I performed the phylogenetic tree especially for SULT1A genes, which based on my samples and previous reported (Fig. 13). There are found relationship of SUL1A1 between mammalian species such as humans and chimpanzee, dogs, pigs, and cattle. Therefore I assumed that PYOH was catalyzed by an enzyme encoded in the SULT1A1 gene, and sulfation of PY metabolite may occur at normal levels in mammals, including pigs. However, sulfation may be present as limited in some mammalian species as hedgehogs. Since in my study, I could not find PY sulfate metabolites in hedgehog urine. Nowadays, the xenobiotic metabolisms in hedgehog still have limited information, and my finding might be

the first report for phenolic xenobiotic metabolism in hedgehogs. Not only SULT1A1 gene expression that affect the sulfation of xenobiotics, but the bioavailability of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) also important substrate in sulfation (Falany, 1997). PAPS availability is dependent on sulfation that is conserved between different classes of SULT, within different tissues and between different species (Klaassen and Boles, 1997). It is possible that PAPS is a one factor to determine interspecies differences undergo the sulfation.

Since the majority of PYOH was found to be conjugated with glucuronide, a strong correlation was found with glucuronide conjugation. Possibly this was caused by high activities of UGT enzymes, which have been shown to be similar in studies using human urine (Strickland et al., 1994; Strickland and Kang, 1999). In particular, I found a good relationship between PYOG and total PY metabolites of ungulates in that they showed glucuronidation as mainly the conjugated form of PY. UGT activities in ungulates might be higher than that of SULT. Other several substrates were also reported to be highly conjugated by UGT in ungulate (Smith et al., 1983). In addition, the total amounts of PY metabolites of PYOG, PYOS, and PYOH in ungulates were highly eliminated into urine compared to other mammals. Moreover, in present study, PYOS and PYOH were also positively correlated with total PY metabolites. It was found that either metabolite could be used to determine PY and PY metabolite levels in urine. In addition, PCA characterization could demonstrate the xenobiotic metabolism of phenol compounds, and this was useful for observation of interspecies differences. Therefore, I would expect trends in ability to metabolize xenobiotic compounds.

This section, I characterized the phase II conjugation reaction of PY metabolites in mammalian urine. With regard to the profile of PY metabolism in mammals, interspecies differences were found clearly in that glucuronide was the dominant metabolite in certain

mammals, especially in ungulates, they had high elimination levels of PY metabolites. PYOG was detected very large amount in mammal urine, except in cats and ferrets. Although, unlike cats, ferrets had UGT1A6 as a non-pseudogene, the PY glucuronidation in ferrets was not performed. In my finding, I detected sulfate conjugates in pig urine, but not in hedgehogs. I assumed that hedgehogs might have low SULT activity against to PY. My study supports the idea that mammals have interspecies differences in their abilities to process phenol metabolites, especially in phase II reactions. However, there is little information on species difference in phase II conjugation *in vivo* study. I have strongly suggested that not only the genetic information can presume the xenobiotic metabolism, but also the characterization of phase II reactions by using *in vivo* sample should be considered as important way to clear interspecies differences.

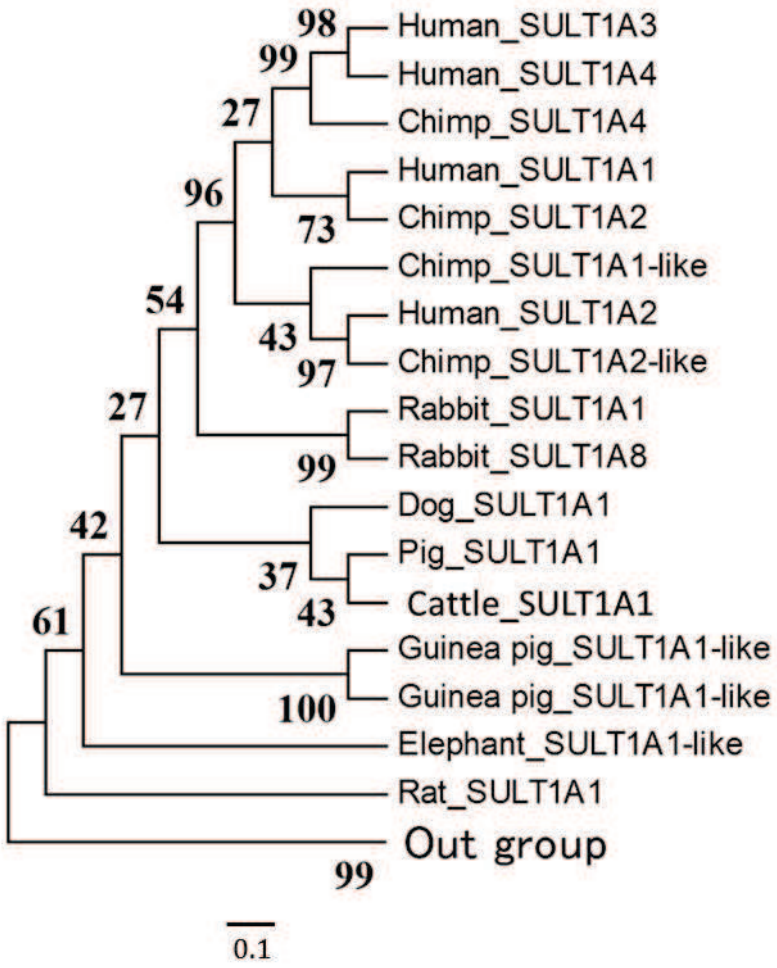


Fig. 13. Molecular Phylogenetic analysis of SULT1A by Maximum Likelihood method.

The SULT1A gene tree was modified from Bradley and Benner (2005). SULT1A1~4 were expressed in human. SULT1A1 was shown in various mammals including pigs. NCBI accession numbers of sequences used: chimpanzee 1A1 [Genbank: BK004887], chimpanzee 1A2 [Genbank: BK004888], chimpanzee 1A3 [Genbank: BK004889], cattle [Genbank: U34753], dog [Genbank: AY069922], human 1A1 [Genbank: L19999], human 1A2 [Genbank: U34804], human 1A3 [Genbank: L25275], human 1A4 [Genbank: BK004132], pig [Genbank: AY193893], rabbit [Genbank: AF360872], rat [Genbank: X52883], elephant [Genbank: LOC100654158], guinea pig [Genbank: LOC100728747].

The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). The bootstrap consensus tree inferred from 500 replicates Felsenstein (1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one-fourth of the total number of sites, the maximum parsimony method was used; otherwise, BIONJ method with MCL distance matrix was used. A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories (+G, parameter = 1.4537). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 17.5623% sites). The analysis involved 22 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 285 positions in the final data set. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2007).

SECTION III

The study of xenobiotic metabolism phase II reactions in pigs: high sulfotransferase enzymatic efficiency observed by *in vitro* kinetic analyses

Introduction

Interspecies differences in xenobiotic metabolism have a wide range of variations. These variations are due to differences in the pattern of xenobiotic bioaccumulation and biotransformation among organisms. Xenobiotic metabolism is often classified into two phases: phase I (oxidation) and phase II (conjugation) reactions. Phase I is mainly mediated by cytochrome P450. Phase II metabolic reactions are condensations of xenobiotics or phase I products with biomolecules. In particular, species differences in activities of phase II enzymes have been reported (deBethizy and Hayes 1989). The best-known example of a species-dependent variation in phase II metabolism is represented by the inability of felines, including domestic cats, to metabolize drugs and phenolic compounds by glucuronidation (Shrestha et al., 2011). Current knowledge of substrate specificity of UGT isoforms indicates that cats harbor UGT1A6 as a pseudogene (Court et al., 2000).

Sulfotransferase (SULT) has also been well known phase II enzyme which showed species difference. Sulfation is a major phase II biotransformation pathway for many xenobiotics, which utilize SULT to catalyze the sulfoconjugation of various groups. SULT produces highly water-soluble sulfuric acid esters using the ubiquitous donor 3'-phosphoadenosine 5'-phosphosulfate (PAPS) as a cofactor (Lin et al., 2004). Characterization of mammalian cytosolic SULT has led to their classification into two subfamilies, the aryl sulfotransferases (SULT1 family) and the hydroxysteroid sulfotransferases (SULT2 family) (Duanmu et al., 2000; Tsoi et al., 2001). SULT activities have showed tissue-, sex-, and

species-dependent differences (Kane et al., 1991; Nakamura et al., 1992). The sulfation of phenol compounds, steroid hormones (e.g., cortisol, dopamine, testosterone, pregnenolone, and estrogen), and drugs (e.g. acetaminophen) is catalyzed in the cytosolic fraction of many mammalian and aquatic animals (Gamage et al., 2006). In humans, four closely related SULT1A members that share > 90% sequence identity have been isolated (SULT1A1~ SULT1A4) (Bradley and Benner 2005). SULT1A subfamily enzymes are responsible for the sulfation of small phenolic compounds such as *p*-nitrophenol, 1-naphthol, acetaminophen, and dopamine (Duanmu et al., 2000). SULT1A1 is thought to be important for the sulfation of simple phenols (Bradley and Benner 2005). In both rats and humans, SULT1A1 enzymes are abundantly expressed in the liver, which becomes the site of drug metabolism in mammalian species (Tsoi et al., 2001). Moreover, members of SULT1A1 have been identified in a large range of species, including mice, cattle, dogs, guinea pigs, rabbits, monkeys, pigs, and platypuses (Lin et al., 2004; Bradley and Banner 2005).

Concerning species difference on SULT, until now, it has been widely assumed that pigs possess little ability of sulfoconjugation for phenolic compounds (deBethizy and Hayes 1989). However, contrary to previous belief, pig SULT1A1 has been recently fully characterized from pig liver cDNA, and identified as non-pseudogene (Lin et al., 2004). Furthermore, a previous study using [1-^{14}C] 1-naphthol and [8-^{14}C] 2-naphtol has found that pigs have the ability to transfer [1-^{14}C] 1-naphtol to both 1-naphthylglucuronide and 1-naphthylsulphate with a 2:1 ratio (Capel et al., 1974). Based on these results, I hypothesized that pig aryl-SULT has normal function and conjugates structurally related phenolic compounds into their sulfated forms. Additionally, in preliminary my work in the present study, sulfated derivatives of pyrene were detected in both pig and rat urine.

Thus, the aim of the current study was to characterize SULT kinetic parameters in order to predict the function of phase II conjugation reactions of phenolic compounds in pigs

as compared with rats. Pyrene (PY) was used as the model substrate in this study. Polycyclic aromatic hydrocarbons (PAHs), especially PY, may contaminate indoor and outdoor environments of pig farms through water, feed mixtures, and dust (Ciganek et al., 2002). 1-hydroxypyrene (PYOH) is a dominant metabolite of PY, which is detected and found in excretory products of animals and humans (Jacob et al., 1982; Keimig et al., 1983; Levin, 1995). In addition to the kinetic analyses, mRNA expression and sequences of SULT1A1 was compared between rats and pigs in order to explain interspecies differences. This is the first report to show that pig possesses an adequate level of physical activity of SULT *in vitro*.

Materials and Methods

Chemicals

Pyrene was obtained from Kanto Chemical Co. Inc. (Tokyo, Japan). PYOH, sulfatase, (from limpets, Type V; 34 U/mg), β-glucuronidase (from bovine liver, Type B-1; 1240 U/mg), bovine serum albumin, TRI reagent, GenElute Miniprep Kit, and GAPDH, pig and rat SULT1A1, and PAPS were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). UDP-glucuronic acid (UDP-GA) was purchased from Wako (Osaka, Japan). 5× RT-buffer, dNTP, SYBR® qPCR Mixand reverse transcriptase, and primers were purchased from Toyobo Co. Ltd (Osaka, Japan). All chemicals used for high-performance liquid chromatography (HPLC) were obtained from Kanto Chemical Co. Inc.

Animals

All experiments using animals were performed according to the guidelines of the Hokkaido University Institutional Animal Care and Use Committee.

Urine was collected from large white pigs (*Sus domesticus*) (females, 1.5 years old, n = 3) at the Kasetsart University farm, Thailand. Urine samples were kept at −20°C until extraction and clean up. Liver samples were collected from five male and five female large white pigs at the age of five months from a slaughterhouse in Hokkaido, Japan. Samples were immediately flash-frozen in liquid nitrogen. Afterward, they were kept in −80°C until analysis.

Nine-week-old male Wistar rats (*Rattus norvegicus*) (n = 3) (SLC, Hamamatsu, Japan) were maintained under conditions of 24 ± 1°C and 12 h light and 12 h dark cycle. They were given laboratory feed and water ad libitum. The rats were euthanized with carbon dioxide. Their livers were removed and perfused with cold 1.15% KCl to remove the blood, and samples were kept at –80°C.

Extraction of PY metabolites

According to the method of Stewart et al. (2009), aliquots of urine (250 mL) were acidified to pH 6.8 with 10 mM formic acid before extraction. Subsequently, the acidic samples were loaded onto Oasis WAX (Waters) solid-phase extraction cartridges. The cartridges were conditioned with methanol (10 mL) and Milli Q water (10 mL). The samples were loaded onto the cartridges, which were then washed with sodium hydroxide solution (0.1 M; 5 mL), sodium phosphate buffer (0.1 M; pH 7.4; 5 mL), and Milli Q water (5 mL). The cartridges were dried briefly under vacuum, and then they were sequentially eluted with methanol:ethyl acetate (1:1, v/v; 10 mL; hydroxylation and internal standard fractions) and methanol:formic acid (9:1, v/v; 10 mL; β-glucuronide fraction). The last elution occurred in the sulfate fraction, which had methanol:water:diethylamine (17:2:1, v/v; 10 mL) and methanol:ethyl acetate:diethylamine (50:50:1, v/v; 2 mL). All three eluates were reduced in volume by evaporation at 45°C under a stream of nitrogen. The final volume was 1 mL, and 5 μL were used for HPLC with fluorescence detection (FD).

Analyses of PY metabolites

The samples were analyzed by using an HPLC (LC-20 series; Shimadzu, Kyoto, Japan) unit equipped with a fluorescence detector (RF-1AXL; Shimadzu) and an ODS column (ODS-120T 4.6 mm × 300 mm; Tosoh, Tokyo, Japan). Mobile phase A consisted of 10 mM ammonium acetate buffer, and its pH was adjusted to 5.0 with acetic acid. The mixture of methanol:acetonitrile:water (38:57:5, v/v/v) constituted the mobile phase B. The solvent gradient was as follows: 10% of mobile phase B from 0 to 2 min, which was followed by a linear gradient to 100% mobile phase A and B from 2–35 min, and then the gradient was held at 100% mobile phase A and B for 5 min for 45 min. The solvent flow rate was set at 0.5

mL/min, and column the temperature was 45°C. The excitation and emission wavelengths for FD were 343 and 385 mm, respectively (Ueda et al., 2011). The recovery rate of each metabolite in each fraction was determined in the exposed rat urine. The recovery rates of PY metabolites were 88.5 ± 1.5% to 105.1 ± 5.7%.

Identification of PY metabolites formed by rats

To identify PY metabolites, rat urine was prepared through a procedure described in the previous study (Ueda et al., 2011). Each metabolite was collected by the fraction collector (FRC-10A; Shimadzu). Afterward, each separated peak was identified by an electrospray ionization ion-trap mass spectrometry (ESI/ion-trap/MS) detector (LTQ Orbitrap; Thermo Fisher Scientific, MA, USA). The ESI conditions were fully scanned (*m/z* 80 to 750). The negative mode used an ion source voltage of −5.0 kV and an ion source temperature of 300°C. Subsequently, PY metabolites in pig urine were estimated from the rat results.

De-conjugation

The levels of PY conjugation were estimated from the ratio of de-conjugation product. The transformation rates of pyrene-1-glucuronide (PYOG) and pyrene-1-sulfate (PYOS) to PYOH were 0.21- and 0.24-fold, respectively. The PYOH calibration curve was constructed to calculate the level of PYOG and PYOS in urine.

De-conjugation was performed using the method of Ikenaka et al. (2007). Sulfatase and β-glucuronidase were dissolved in 0.1 M sodium acetate buffer (pH 5.0) to concentrations of 10 and 4000 U/mL, respectively. The concentrated PY metabolites were dissolved in 300 μL of 0.1 M sodium acetate buffer (pH 5.0), and then 200 μL of each de-conjugation enzyme was added. Bovine serum albumin (1 mg/mL) was used in place of the de-conjugation enzyme in the control treatment. After 8 h of incubation at 37 °C, 500 μL of

methanol were added to stop the reaction. The de-conjugated compounds were analyzed by HPLC/FD.

Preparation of PAPS in pig and rat livers

Liver PAPS concentrations were measured by the method of Hazelton et al. (1985). Briefly, pig and rat livers were quickly excised and portions approximately 300 mg each was immediately boiled in two volumes of 50 mM glycine/NaOH buffer (pH 9.2) for 3 min. The time required removing the liver and then boil portions of it did not ever exceed 5 min. The boiled tissues were cooled on ice and homogenized in a glass homogenizer for 2 min. All subsequent procedures were performed at 4 ºC. Then the homogenate was centrifuged at 15,000 x *g*, 4ºC for 30 min. The resultant supernatants were decanted and extracted with an equal volume of chloroform by vortexing for 1 min. The aqueous and organic phases were separated by centrifugation at 15,000 x *g*, 4 ºC for 30 min and the aqueous phases (top layer) were used immediately for the analysis of PAPS.

The recovery experiments were performed. The known concentrations of PAPS standard were spiked directly to the 50 mM glycine/NaOH buffer, pH 9.2. Then they were done as same as tissue processes. Results were expressed as percentage of added PAPS recovered. The recovery of PAPS was 112.0 ± 8.2%. In addition, the PAPS concentration ranges (0.6 to 19.4 μM) were prepared for calibration curve.

Analysis of PAPS by LC-MS/MS

LC parameter

The LC-MS/MS has been modified in the previous study (Zhou et al., 2011). In briefly, the system was composed of LC-ESI-MS (LCMS 8030, Shimadzu, Japan). PAPS concentrations were separated on a KinetexTM 2.6 μm C18 100A analytical column with

guard column (50 mm length × 2.1 mm Phenomenex, Cheshire, England). The column was maintained at a temperature of 30 °C. The flow rate was 0.2 mL/min. The injection volume was 10 μL. Mobile phase A was 10 mM ammonium acetate buffer (pH 5) in distilled water, and mobile phase B was 95% methanol. A linear binary gradient was applied changing from 10 to 100% mobile phase B within 12 min.

MS parameter

A Shimadzu (LCMS 8030) triple quadrupole mass spectrometer equipped with an electrospray source analyzed the multiple reaction monitoring (MRM) negative ion mode with the following setting: nebulizing gas flow 3.0 L/min, drying gas flow 15.0 L/min, DL temperature 250°C, heat block temperature 400°C and CID gas 230 kPa.

Preparation of liver microsomal and cytosolic fractions

Livers microsomal and cytosolic fractions were prepared according to the method of Omura and Sato (1946). The liver samples were homogenized with potassium phosphate buffer (KPB; 0.1 M, pH 7.4) in ice condition. The homogenates were transferred to a tube and centrifuged at 9,000 × *g* at 4 °C for 20 min. The supernatants were decanted to an ultracentrifugation tube and centrifuged at 105,000 × *g* at 4°C for 70 min. Each homogenate consisted of 2 parts: the supernatant contained the cytosolic fraction. The pellets were homogenized in ice conditions with KPB and centrifuged for washing at 105,000 × *g* at 4 °C for 70 min. The microsomal pellets were homogenized with KPB again. The microsomal and cytosolic fractions were transferred to 1.5 mL tubes and stored at –80 °C. The cytosols were used to measure SULT activity; microsomes were used to determine UGT activity. The protein concentrations in the microsomes and cytosols were measured using the Lowry method (Lowry et al., 1951).

SULT activity

The SULT activity for PYOH was determined by using a modification of the method of Ueda et al. (2011). The concentration of the cytosolic protein fraction was set at 250 μg/mL. The fraction was mixed with 10 μL of 100 mM $MgCl_2$, 10 μL of 50 mM Na_2SO_3, and 1 μL of PYOH. Tris-HCl buffer (100 mM, pH 7.4) was added to make up to 97.5 μL. The mixtures were pre-incubated at 37 °C for 5 min. The reaction was initiated by adding 2.5 μL of 1 mM PAPS to a produce a final volume of 100 μL. The reaction was run by incubating at 37°C for 10 min, and then it was stopped by adding 400 μL of ice-cold methanol. Reaction samples were placed on ice for 15 min prior to centrifugation at 750 × *g* for 10 min. The supernatant was injected into the HPLC/FD system.

UGT activity

The UGT activity of PYOH was determined in microsomal fraction. 12.5 μL of the microsomal fraction (4 mg/mL) was mixed with 2.5 μL of 1% sodium cholate. Then, the microsomal mixture was added with 35 of 0.1M KPB and left on ice for 30 min. After the treatment, the microsomal solution (50 μL) was mixed with 41.5 μL of 0.1 M KPB, 5 μL of 100 mM $MgCl_2$, and 1 μL of PYOH. Next the mixture solution was pre-incubated at 37 °C for 5 min. The reaction was started with 2.5 μL of 100 mM UDP-GA in a final volume of 100 μL. After incubation for 10 min, the reaction was stopped by adding 400 μL of ice-cold methanol. The reaction samples were placed on ice for 15 min. After that they were centrifuged at 750 × *g* for 10 min. The supernatant was analyzed using the same HPLC/FD conditions as in the measurement of PYOS (Ueda et al., 2011).

RNA extraction and cDNA synthesis

Total RNA was extracted from pig and rat livers using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. Total RNA concentration and quality were checked by using a Nanodrop ND-1000 spectrophotometer (DYMO, Stamford, USA). The RNA quality was estimated by the 260 nm /280 nm and 260 nm /230 nm absorbance ratios, and confirmed by denaturing agarose gel electrophoresis. The cDNA was synthesized according to the previous report (Darwish et al., 2010). In brief, a mixture of 5 μg of total RNA and 0.5 ng of oligo dT primer in a total volume of 24 μL of sterilized ultrapure water was incubated at 70°C for 10 min and then removed from the thermal cycler. The volume was increased to 40 μL with a mixture of 4 μL (5×) RT-buffer (Toyobo Co. Ltd), 8 μL 10 mM dNTP, 2 μL water, and 2 μL reverse transcriptase (Toyobo Co. Ltd). The mixture was then re-incubated in the thermal cycler at 42°C for 45 min and at 90°C for 10 min to prepare the cDNA.

Quantitative real-time polymerase chain reaction of SULT1A1 mRNA expression

The cDNA generated were qPCR-amplified to determine the copy numbers of SULT1A1, using GAPDH as an endogenous control separately for both rats and pigs. The sequences of the rat SULT1A1 primers were 5′-GAGCACCCGGAGGCAAGGCTCAGAA-3′ (sense) and 5′-ACCCTTCACATGCACTAGCGGTGGA-3′ (antisense) (accession number NM031834). For GAPDH, the sense primer was 5′-CCAAGGCTGTAGGCAAAGTCATCCCAGA-3′ and the antisense primer was 5′-CAGGCCGCAGGTCAGGTCCACAAC-3′ (accession number BC161847). In the case of pigs, SULT1A1 primers were 5′-CATGAAGGAGAACCCCAAGA-3′ (sense) and 5′-TCATGAAGGCTGAGATGCTG-3′ (antisense) (accession number AY193893). For GAPDH, the sense primer was 5′-CCCATGTTTGTGATGGGCGTGAACCAT-3′ and the

antisense primer was 5′- TGAGTCCCTCCACGAAGTG-3′ (accession number DQ403065). PCR reactions were carried out in 10 μL volumes. The PCR reaction mixture was prepared with SYBR® qPCR Mix (Toyobo Co. Ltd), 10 μM of each primer, 600 ng cDNA, 50× ROX reference dye, and RNase-free water. The mixture was made up to a final volume of 10 μL. The reaction cycle consisted of an initial holding stage at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 1 min, and extension at 72°C for 30 s. Melting curve analysis and agarose gel electrophoresis confirmed the amplification of a single amplicon of the expected size as well as the absence of primer dimers and genomic DNA amplification. Each measurement was performed in duplicate and repeated three times. The number of copies of SULT1A1 and GAPDH for each rat and pig was calculated according to the standard curve method. The expression of SULT1A1 was normalized to the expression of GAPDH in each animal.

Sequencing of SULT1A1 in pigs and rats

The PCR products were cloned into the pCR 2.1 vector (Invitrogen). This was followed by transformation of *Escherichia coli* DH5-α with plasmid DNA. The bacterial cells were cultured for 14 h on average. Blue-white screening was employed to identify transformed cells, and the cDNA inserts of 300 bp were amplified by direct PCR using M13 primers (Invitrogen), specifically targeting the multiple cloning sites of the vector. The cells from the selected colonies were cultured for another 9 h in liquid Luria–Bertani broth. The plasmids were purified by a plasmid miniprep method using a GenElute Miniprep Kit (Sigma Chemical Co.). Purified plasmids were directly sequenced by using the dye terminator cycle sequencing method. The cycle sequencing was performed on a Program Temp Control System Thermal Cycler Personal (Takara) with vector-specific M13 Primers RV-N and M3 (Takara), using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit

(Applied Biosystems, Foster City, CA, USA). The nucleotide sequences were determined by an automated DNA sequencer (ABI Prism 310 genetic analyzer). A minimum of four clones were sequenced in both forward and reverse directions to avoid sequence ambiguities.

The translated amino acid sequences of pigs and rats SULT1A1s were aligned together using the Clustal W 1.83 program.

Statistical analyses

Kinetic parameters (maximum velocity, *Vmax*; Michaelis constant, *Km*; and enzymatic efficiency, *Vmax/Km*) were determined using the Michaelis–Menten equation and Graph Pad Prism 5 (GraphPad Software, CA, USA). The results were considered statistically significant by Student's *t* test (JMP 9.0, SAS, CN, USA) for *P*-values that were equal to or less than 0.05.

Results

Identification of pyrene metabolites in pig urine

PY metabolites were identified in pig urine according to retention time (RT, min), mass-to-charge ratio (*m/z*), and the two-stage MS (MS^2) (*m/z*) spectrum obtained by HPLC/FD, and MS system (Ueda et al., 2011). I could detect PYOH with MS peak at *m/z* 217 (M^-) (negative mode). The range of PYOH in pig urine was 0.09 to 11.32 nM. In addition, conjugated compounds were found both in sulfate and glucuronide forms. Sulfate conjugates of PYOS were detected, and had MS and MS^2 peaks at *m/z* 297 and 217, respectively. In pig urine, the range of PYOS was 0.04 to 1.75 nM. I could also detect PYdiol-S (MS and MS^2 at *m/z* 393 and 233, respectively). The peak for PYOG represented about 7.79 to 59.68 nM. The MS and MS^2 were *m/z* 393 and 217, respectively. The results are summarized in Table 8.

PAPS concentration in pig and rat livers

Figure 14A showed chromatogram of PAPS, which the molecular mass of product of PAPS was determined at *m/z* 506 by negative ion mode. The RT of PAPS was 1.1 min. The purified PAPS detected both in pig and rat livers. In pigs, PAPS concentration was significant lower than that of rats (16.3 ± 3.4 and 56.7 ± 3.4 nmol/g tissue in pigs and rats, respectively) (Fig. 14B).

Sulfation activity in pigs and rats

The sulfation activity in pigs and rats were performed by using PYOH as a substrate that was done in liver cytosol with co-substrate, PAPS. Kinetic analysis results of SULT-dependent activity toward PYOH were shown in Table 9. I could detect the PYOH sulfation activity as PYOS both in male and female pigs (Fig. 15A). The *Vmax* was 58.5 ± 10.6 and 53.7 ± 7.3 pmol/min/mg protein in male and female, respectively. *Km* of the both male and

female pigs were similarly as 9.4 ± 1.8 and 7.9 ± 4.7 nM, respectively. The enzymatic efficiency (*Vmax/Km*) of sulfation activity in pigs did not show significant differences between male and female. SULT-dependent activities toward PYOH were compared rat and pig liver cytosol samples (Fig. 15B). I found clear interspecies differences and were also significant differences ($P < 0.05$) in the enzyme kinetic parameters as *Vmax* and *Km*. In addition, the comparison with the ratio of *Vmax* to *Km* between pig and rat enzymes showed PYOH was a high efficient substrate for pig liver sulfation.

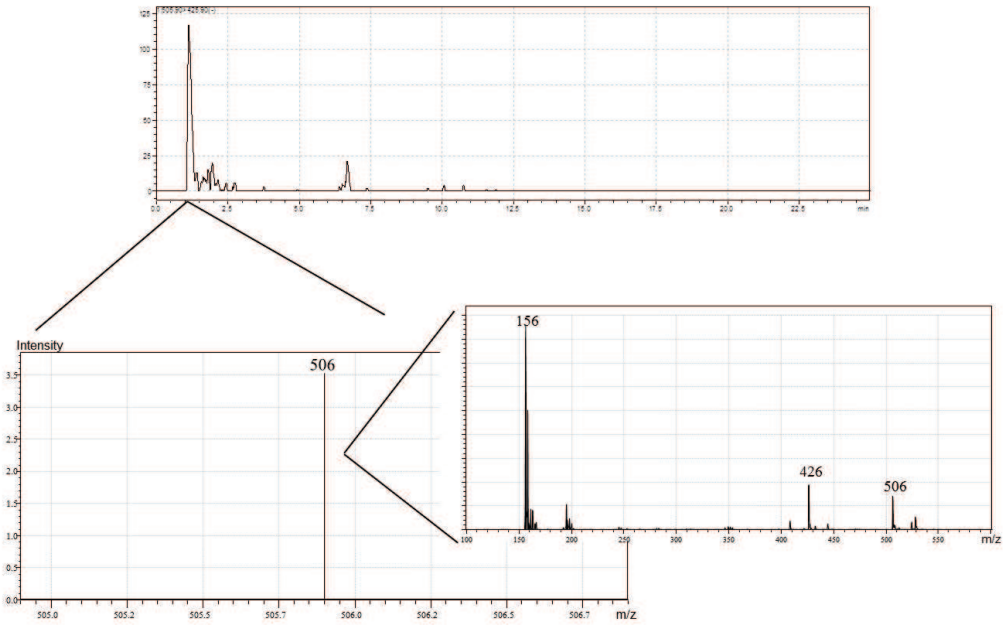
SULT1A1 mRNA expression and sequence analyses in pigs and rats

The mRNA expression in both pigs and rats were in line with the SULT activity, as it was significantly higher in rats than pigs (Fig. 16A). Furthermore, the partial amino acid sequence analysis of SULT1A1 in both pigs and rats also revealed critical differences in the substrate recognition sites (SRS) (Fig. 16B).

Glucuronidation activity in pigs and rats

UGT activities were performed in pig and rat microsomes. The PYOG was measured as a conjugate form. The kinetic parameters were summarized in Table 10. *Vmax* and *Km* of pig microsomes were similarly both in male and female pigs (Fig. 17A) as 942.3 ± 37.7 and 930.6 ± 85.3 pmol/min/mg protein and 144.3 ± 20.8 and 131.4 ± 36.4 μM, respectively. The kinetic parameters were no sex differences. When I compared kinetic parameter of UGT activity in pigs and rats, I found significantly lower *Vmax* and *Km* in rats than that of pigs ($P < 0.05$). Although, the glucuronidation of pigs showed much the maximum reaction velocity to catalyze PYOH, the apparent of reaction velocity for rats was significantly higher than that of pigs ($P < 0.05$). The Fig. 17B showed a plot of the enzyme activity of pigs and rats.

A.



B.

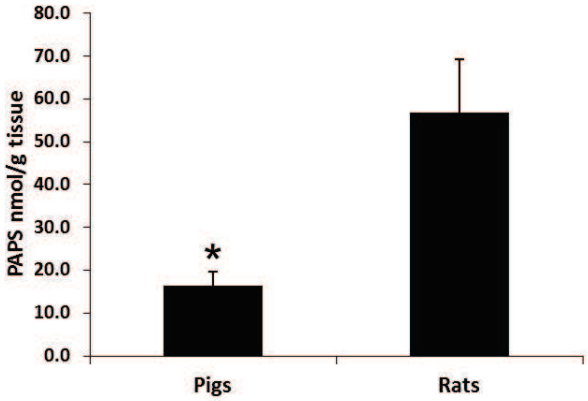
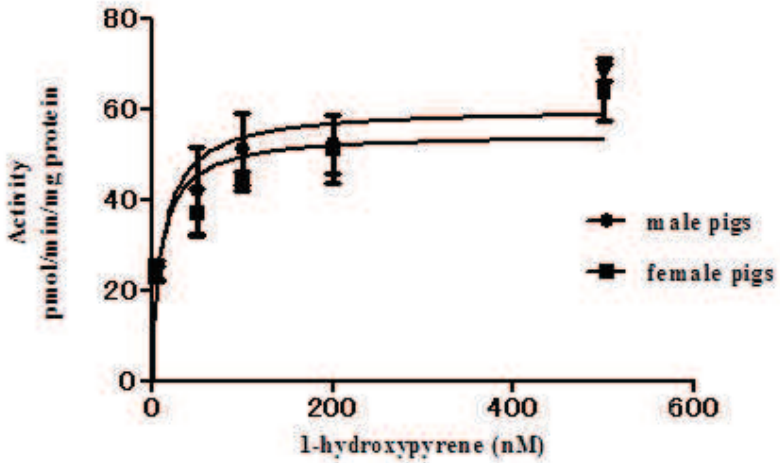


Fig. 14 Chromatogram of PAPS and PAPS concentrations in pig and rat livers.

The chromatogram and mass spectrum of PAPS, MS and MS^2 were 506 > 156 and 462 *m/z* in negative mode (A). PAPS concentrations between pig and rat livers, pig liver was lower level than that of rats, $*P < 0.05$ (B).

A.



B.

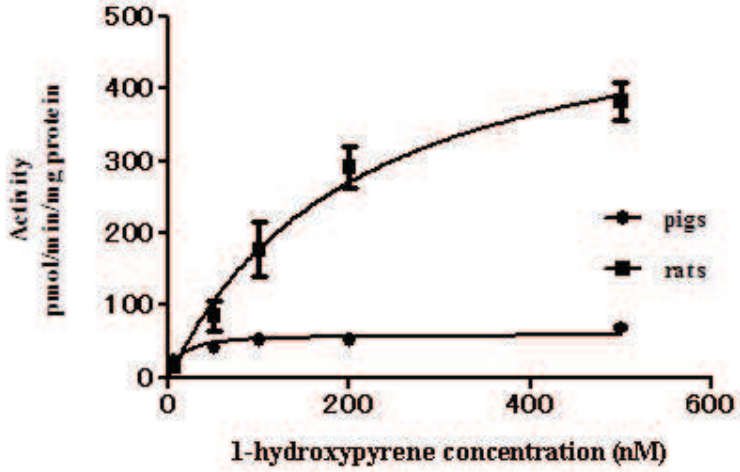
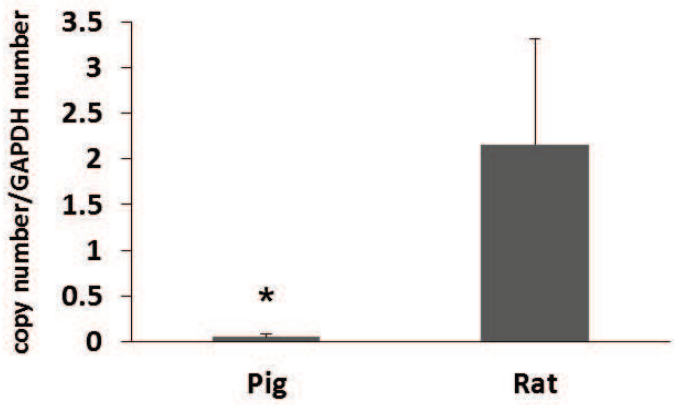


Fig.15. Hyperbolic regression kinetic analyses for SULT1A1-dependent PYOH activity in pigs and rats.

SULT1A1-dependent PYOH activity was measured over 5–500 nM substrate concentrations. Data represent means of three experiments performed at different times using liver cytosol from three animals. Male and female pigs had similar *Vmax* and showed no gender significant differences (A). However, SULT activity showed species differences between pigs and rats (B).

A.



B.

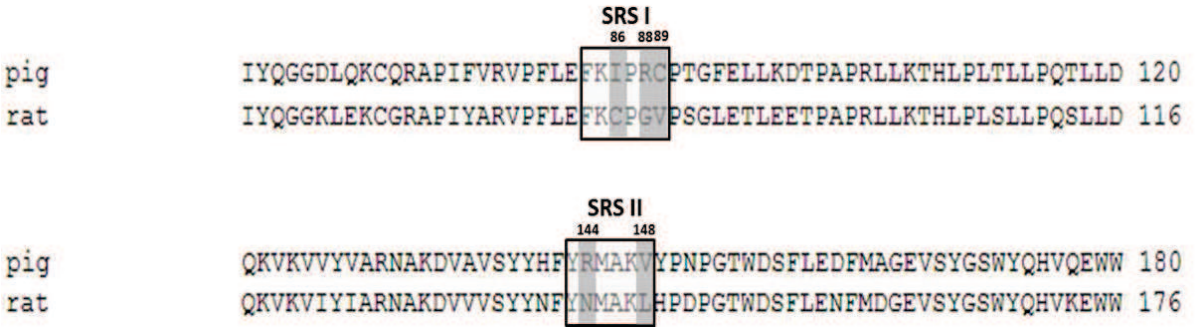
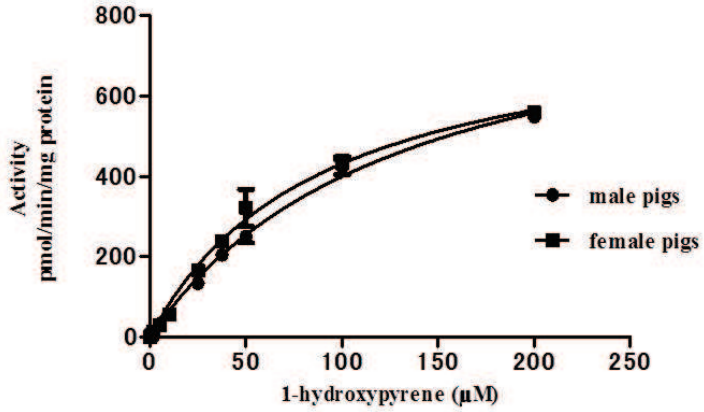


Fig. 16. SULT1A1 mRNA expression and deduced amino acid sequence alignment in pigs and rats.

(A) SULT1A1 mRNA expression in pig and rat livers using real-time RT-PCR. The cDNA samples were amplified as described in the Materials and Methods. The amount of each enzyme was normalized to the corresponding amount of GAPDH, and is presented as copy number/GAPDH copy number for each animal. Data are presented as the mean ± SD. $P < 0.05$.

(B) Alignments of deduced amino acid sequences of SULT1A1 in pigs and rats using the CLUSTAL W 1.83 program (substrate recognition site, SRS).

A.



B.

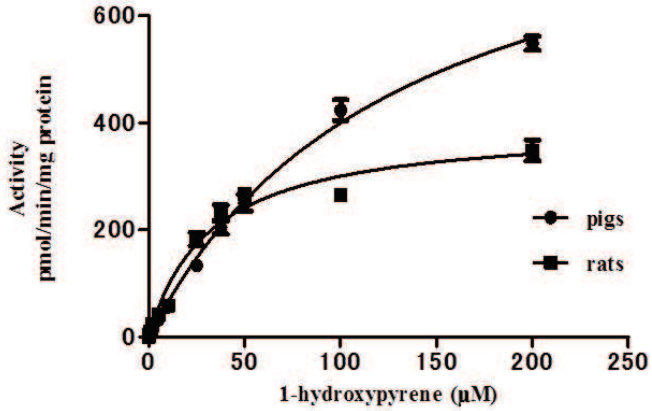


Fig. 17. Hyperbolic regression kinetic analyses for UGT1A-dependent PYOH activity in pigs and rats.

UGT1A-dependent PYOH activity was measured over 5–200 μM substrate concentrations. Data represent means of three experiments performed at different times using liver microsome from three animals. Male and female pigs had similar *Vmax* and showed no gender significant differences (A). However, UGT activity showed species differences between pigs and rats (B).

Table 8. Characteristics of PY metabolites identifies by HPLC/FD and MS

RT (min)	MS (*m/z*)	MS^2 (*m/z*)	Metabolites
17.1, 20.1	313	313 > 233	Pyrenediol-sulfate (PYdiol-S)
24.5	393	393 > 217	Pyrene-1-glucuronide (PYOG)
26.5	297	297 > 217	Pyrene-1-sulfate (PYOS)
36.2	217	217 > 189	1-hydroxypyrene (PYOH)

The pyrene metabolites were detected in pig urine. Each metabolite was detected by HPLC/FD and identified by MS.

Table 9. Summary of the kinetic analyses of SULT1A1-dependent activity toward PYOH in pigs.

	Vmax (pmol/min/mg protein)	*Km* (nM)	*Vmax/Km* (pmol/min/mg protein/nM)
Female pigs	53.7 ± 7.3	7.9 ± 4.7	8.1 ± 3.3
Male pigs	58.5 ± 10.6[b]	9.4 ± 1.8[b]	6.2 ± 0.7[a]
Male rats	563.0 ± 45.6[a]	258.7 ± 21.5[a]	2.2 ± 0.2[b]

Results were compared against similar data for rats. Kinetic parameters of SULT1A1-dependent PYOH activity were performed in the cytosol of murine and porcine livers. The data represent the means ± SD for three animals from each species (male pigs and male rats). Identical letters represent the absence of statistically significant difference between pigs and rats ([a,b]$P < 0.05$).

Table 10. Summary of the kinetic analyses of UGT1A-dependent activity toward PYOH in pigs.

	Vmax (pmol/min/mg protein)	*Km* (μM)	*Vmax/Km* (pmol/min/mg protein/μM)
Female pigs	930.6 ± 85.3	131.4 ± 36.4	7.4 ± 1.6
Male pigs	942.3 ± 37.7[a]	144.3 ± 20.8[a]	6.6 ± 0.8[b]
Male rats	398.7 ± 30.5[b]	33.4 ± 6.2[b]	12.2 ± 2.0[a]

Results were compared against similar data for rats. Kinetic parameters of UGT1A-dependent PYOH activity in the microsomal were done in murine and porcine livers. The data represent the means ± SD for three animals from each species (male pigs and male rats). Identical letters represent the absence of statistically significant difference between pigs and rats ([a,b]$P < 0.05$).

Discussion

Identification of PY metabolites in pig urine (environmental exposure)

In this section, PY metabolites were identified in pig urine according to the previous study (section II). The range of PYOH, PYOG, and PYOS in pig urine were observed, PYOG was mainly eliminated in urine. Interestingly, PYOS also perform according to phase II conjugation reaction. I assumed that sulfoconjugation could be active toward PY metabolite. The pig urine was suspected that the daily PAH exposure, since PAHs, including PY were contaminated in environment as pig farms (Ciganek et al., 2002).

PAPS concentration in pig and rat livers

In sulfation reactions, PAPS is a required universal sulfonate donor molecule. The availability of PAPS for sulfation has been reported that was dependent on its synthesis, transport, degradation, and utilization. PAPS tissue concentrations were low (4–80 nmol/g tissue) (Brzeznicka et al., 1987; Cappiello et al., 1989; Cappiello et al., 1990) compared with tissue concentrations of uridine 5'-diphosphoglucuronic acid (200 nmol/g liver) required for glucuronidation or with glutathione (5,000 mmol/g of liver) required for glutathione conjugation (Klaassen and Boles, 1997). In pig liver, PAPS was also determined, which had lower concentration than rat liver. Although PAPS was characterized in various tissues such as liver, kidney, lung, and intestines of human (Cappiello et al., 1989), liver was consistently the tissue with the highest PAPS concentration (Cappiello et al., 1989; Klaassen and Boles, 1997). In addition, the SULT activities of 2-naphthol were higher in the liver than non-hepatic tissues (Cappiello et al., 1989). Moreover, the concentrations of PAPS in liver from mammals have been observed as species–dependent differences (Wong et al., 1979; Hazelton et al., 1985). Klaassen and Boles (1997) and my present study found that PAPS in liver of human, mouse, dogs, rabbits, guinea pigs, and pigs were similar concentration and had lower

levels than that of rat liver. As known that sulfation is a high–affinity, low–capacity conjugation system in xenobiotic metabolism partly since the levels of sulfation activity may be limited by PAPS availability.

Sulfation activity in pigs and rats

Kinetic analysis results of SULT–dependent activity toward PYOH are observes. I could detect PYOH sulfation activity as PYOS in both male and female pigs. Although sex–dependent differences in the sulfation of phenol compounds have been reported in rats (as in the case of steroids and acetaminophen) (deBethizy and Hayes 1989; Smith and Griffiths 1976), *Km* values did not show significant differences in male and female pig. Similarly, the enzymatic efficiency (*Vmax/Km*) of sulfation activity in pigs did not show significant differences between males and females. Thus, sex had no effect on SULT activity in both male and female pigs.

However, a clear interspecies difference in SULT-dependent activity was observed. Pigs showed lower *Vmax* values than rats, indicating that SULT activity was higher in rats than that in pigs (deBethizy and Hayes 1989). Pigs had lower *Km* values compared with those of rats, indicating that pig SULT had higher substrate affinity than do rat SULT. Interestingly, the *Vmax/Km* values were significantly higher ($P < 0.05$) in pigs compared with rats, indicating that pigs had higher SULT1A1 enzyme efficiency as compared to rats.

SULT1A1 mRNA expression and sequence analyses in pigs and rats

In order to explain the causes of the observed interspecies differences in SULT–dependent activities among pigs and rats, SULT1A1 mRNA expression in both rats and pigs were compared in terms of copy number for each gene normalized separately with GAPDH as a house-keeping gene. These results suggest that pigs had higher SULT1A1 content than

rats, and this may be resulted in the low *Vmax* value in pig. Then the critical differences in the substrate recognition sites (SRS) was revealed in both pigs and rats revealed (Fig. 16B) The SRS I and II active site of SULT (Chapman et al., 2004) showed amino acid sequence different in two species. These differences may explain the low *Km* value in pigs compared with rats (Zhou et al., 2009).

Glucuronidation activity in pigs and rats

Furthermore, PYOH was characterized as a substrate for PYOG both in pig and rat microsomal fractions. Although pigs had high velocity both in male and female, *Vmax* and *Km* were no significant sex differences. When I compared kinetic parameters of pigs with rats, the interspecies differences were found. Pigs were estimated that higher efficient catalysis PYOH than that of rats. Moreover, rats had high the reaction rate of PYOH metabolism. Meanwhile, the glucuronidation in pigs showed high velocity to catalyze PYOH. However, the conjugated product in pigs was occurred slowly, when it was compared with rats. Nowadays, PYOH was also used to study glucuronidation kinetic constants of the catechol compounds (Elovaara et al., 2004). PYOH was the sensitive probe for detecting UGT induction by chemical inducers of the PAH types. The previous report, glucuronidation of PYOH was faster in rat liver microsomes than glucuronidation of 3-methylcholanthrene and polychlorinated biphenyls (Luukkanen et al., 1997). On the other hand, the previous study of UGT in pigs, they also showed interspecies differences in the fusarium toxin (zearalenone) biotransformation (Malekinejad et al., 2006). In zearalenone metabolism, there found a high capacity for glucuronidation in pigs. Its metabolite was lower ability in sheep, cattle, chicken, and rats than pigs (Malekinejad et al., 2006). It was similar as my study that found interspecies differences between pigs and rats.

In conclusion, the sulfate conjugation of PY metabolites was detected in pig urine. This is the first report to indicate that not only glucuronidation, but also sulfation, is important for the elimination of phenolic xenobiotics, such as PY, in pigs. Pig liver cytosol had lower sulfation activity *(Vmax)* compared with that of rats, and pigs had lower SULT1A1 mRNA expression compared to rats. This may be the reason why pigs have been assumed to possess little ability of sulfoconjugation to this date. Interestingly, pig enzyme had higher efficiency (*Vmax/Km*) compared to rat enzyme. Sequence analysis of SULT1A1 in pigs and rats revealed clear differences in substrate binding sites. Thus, the clear interspecies differences in sulfation activity between pigs and rats in this study may be attributed to both genetic and enzymatic differences.

SUMMARY

In this thesis, warfarin and PY were used as model compounds to determine species differences in xenobiotic metabolism. Although xenobiotic metabolites are produced by similar phase I and II reactions across species, understanding interspecies differences is important to aid prediction of xenobiotic metabolism. I observed the phase I reaction of warfarin and phase II conjugation of PY metabolites by using laboratory animals and various wild and domestic animal species. Interspecies differences based on their metabolites were established using *in vivo* and *in vitro* studies, and were used to demonstrate the process of xenobiotic metabolism in various animals in each chapter, described as follows.

In chapter I, species differences in the distributions of hydroxywarfarins and warfarin alcohols were observed between rats and chickens. The hydroxylation of warfarin metabolites such as 4'-, 6-, 7-, 8-, and 10-hydroxywarfarins were detected in both chickens and rats. Interestingly, the amounts of each hydroxywarfarin in chickens also showed large differences compared with those in rats. Moreover, the metabolic activity of warfarin was drastically higher in the cytosol fraction in chickens compared with that in rats. The cytosol fractions of both chicken and rat livers showed metabolic activity of two diastereomers and two enantiomers of warfarin alcohols. In the chicken cytosol, I found that the production level of *(S)*-warfarin-*(S)*-alcohol was markedly higher (32-fold) than that in rat cytosol. This is the first report to show large species differences in the stereoselectivity of warfarin (from the phase I reaction) in rats and chickens.

In chapter II, interspecies differences were observed in PY metabolism. This was because PY metabolites are commonly used as a biomarker and are easily detected in the environment and biota, including humans. In addition, glucuronide-conjugated metabolites of PY have been detected in human urine. There may be interspecies differences in mammalian

species. Therefore, phase II metabolic compounds of PY were studied separately in three sections, as follows In section I, rats (laboratory animals) were used to characterize the distribution of PY metabolites in tissues, plasma, urine, and feces. PYdiol-S and PYdiol-diS were novel phase II conjugates that could be identified in urine, plasma, and tissues. In addition, PYOG was the main PY conjugated metabolite in tissues such as liver, kidney, lung, brain, and GI tract, especially the small intestine (duodenum, jejunum, and ileum sections). PYOS was also distributed in tissues and was eliminated through the urine. Moreover, PYOH, the PY hydroxylation compound, was detected at a higher concentration in the large intestine (cecum and colon) and feces. In this study, all PY metabolites were primarily eliminated via urine. Therefore, urine sampling is a convenient method to study PY metabolites.

In section II, urine samples were collected from various mammals in urban and rural areas. Contaminant PAHs in the environment may contribute to daily intake of these compounds. I performed a protocol to process and identify the PY metabolites. The glucuronide-conjugated metabolites were the main metabolites detected in mammalian urine, including those from cattle, deer, horses, guinea pigs, hedgehogs, elephants, and pigs, except cats and ferrets. Unlike in cats, UGT1A6 is a non-pseudogene in ferrets. However, I propose that ferrets might have low UGT activity for PY metabolites. Surprisingly, sulfate-conjugated metabolites were detected in pig urine but not in hedgehogs. It is possible that sulfation of PY in pigs is a typical metabolic process.

In section III, sulfate-conjugated metabolites were detected in pig urine as a measure of daily intake of PAHs. Therefore, an *in vitro* study was performed to analyze interspecies differences in more detail. Differences in sulfation activities between pigs and rats were clearly observed. In liver cytosol, rats showed higher sulfation activity compared with that of pigs, and pigs had higher *Vmax/Km* compared with that of rats. Moreover, sequence analysis

of the SULT1A1 gene in rats and pigs revealed clear differences among the substrate recognition sites.

In conclusion, I found clear interspecies differences in both phase I and phase II enzyme reactions based on analysis of xenobiotic metabolites. I found that hepatic enzyme activities in phase I reactions by CYP or aldehyde oxidase enzymes displayed species-specific differences between rats and chickens. In phase II reactions, the glucuronide- and sulfate-conjugated metabolites differed in urine samples of each mammal. At present, genetic information on each species is drastically increasing because of development of sequencing technology. Genetic information is a powerful tool for understanding species specificity of xenobiotic chemicals. Remarkably, my research shows that chemical sensitivity is not only determined by the gene, but also by various factors. In particular, the screening of urinary metabolites is considered a powerful method for furthering the understanding of species differences in phase II conjugation reactions. The ability to determine high-risk species is important, but there is still limited knowledge of the toxicological effects of xenobiotics. The established screening technique in the present study successfully used urinary metabolites. This is one possible channel for advancing our understanding of species that are highly sensitive to or at high-risk of xenobiotic toxicity.

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