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Characterization and tissue distribution of conjugated metabolites of pyrene in the rat

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ABSTRACT. Pyrene (PY) is a polycyclic aromatic hydrocarbon (PAH) that is often used as a biomarker for human and wildlife exposure to PAHs. As the metabolites of PAHs, similar to their parent compounds, pose public health risks, it is necessary to study their characteristics and tissue-specific distribution. The present study was performed to experimentally characterize PY metabolites and analyze the tissue-specific distribution of the conjugated metabolites after oral administration of PY to rats. PY metabolites, such as pyrenediol-disulfate (PYdiol-diS), pyrenediol-sulfate (PYdiol-S), pyrene-1-sufate (PYOS), pyrene-1-glucuronide (PYOG) and 1-hydroxypyrene (PYOH), were detected in rat urine. Although glucuronide conjugate was the predominant metabolite, the metabolite composition varied among tissues. Interestingly, the proportion of PYOH was high in the large intestine. Furthermore, PYOH was the only PY metabolite detected in feces.
KEY WORDS: conjugated metabolites, glucuronidation, rat, sulfation, tissue distribution

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Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants that are released into the environment due to incomplete combustion of organic matter. These compounds undergo several reactions, including metabolic bioactivation, in their metabolic pathways. Thus, a number of these PAHs gain promutagenic and procarcinogenic activities that contribute to the incidence of cancer in humans and animals. Pyrene (PY) is a PAH consisting of four fused benzene rings. PY is primarily metabolized to 1-hydroxypyrene (PYOH) by cytochrome P450 enzymes (CYP) [5, 12, 19, 33]. PY and its metabolite, PYOH, are commonly studied as models of PAH exposure in humans, rats, aquatic crustaceans, snails, crabs, fishes, amphibians and reptiles [1, 2, 7, 8, 16, 23, 25, 36, 37]. Measurement of the hydroxylation metabolites of PY, including PYOH, has been recommended for evaluation of daily PAH exposure due to occupational and environmental exposure. PYOH was detected in milk, urine and fecal samples collected from mammals [6, 17, 36, 37]. In addition, there have been a number of reports of PY and PYOH as good biomarkers of PAH exposure.

PYOH is further metabolized by phase II reactions to form conjugates, such as glucuronide and sulfate. Phase II conjugation reaction was reported to play an important role in the deactivation of metabolically activated PAHs by phase I oxidative reaction. Therefore, it is important to characterize the metabolic pathway and the tissue-specific distribution of the conjugated metabolites of PY, which may be useful for predicting the tissue-specific cancer risk due to PAH exposure. However, the metabolism of PY and tissue distribution of its metabolites have not been clarified in the rat.

The present study was performed to investigate the conjugated metabolism of PY and to determine the tissue-specific, urine, plasma and feces distributions of oxidized and conjugated PY metabolites in the rat.

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 limpet Type V; 34 U/mg), β-glucuronidase (from bovine liver, Type B-1; 1,240 U/mg), β-glucosidase (from almond; 3.4 U/mg) and bovine serum albumin were obtained from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). 6-Hydroxychrysene (6-OH chrysene) as an internal standard was purchased from AccuStandard Inc. (New Haven, CT, U.S.A.). 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. (Hama-

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matsu, Japan) and acclimated for one week in the laboratory. The rats had an average ± SD body weight of 248 ± 10 g and were kept under conditions of 40% humidity and 25°C in a temperature-controlled room with a 12-hr light/dark cycle. The animals were given laboratory food and clean water *ad libitum*. All of 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 hr before exposure. PY was dissolved in 100% propylene glycol and administered orally at a dose of 4 mg/kg body weight. The rats were kept in a metabolic cage for 24 hr for urine (9.50 ± 2.2 m*l*) and feces (3.04 ± 0.5 g) collection. Rats were sacrificed by asphyxia with CO_2 gas. Plasma, liver (6.78 ± 0.08 g), kidney (0.88 ± 0.01 g), spleen (0.47 ± 0.06 g), heart (0.75 ± 0.01 g), lung (0.75 ± 0.01 g), brain (1.51 ± 0.02 g), testes (1.05 ± 0.01 g), tongue (0.31 ± 0.04 g) and gastrointestinal tract organs, such as stomach (0.98 ± 0.05 g), duodenum (0.40 ± 0.04 g), jejunum (1.22 ± 0.01 g), ileum (0.35 ± 0.1 g), cecum (0.66 ± 0.13 g) and colon (0.88 ± 0.12 g), were collected immediately. After removing the content, stomach and intestines were cleaned with 0.9% normal saline.

Extraction of pyrene metabolites: Urine: A mixture of 1 m*l* of urine and 1 m*l* of 70% methanol was added to 10 μl of 200 ppm 6-OH chrysene (dissolved in methanol) as an internal standard. The mixtures were extracted by vortex mixing and centrifugation at 9,000 × *g* for 10 min at 20°C. Then, the supernatants were filtrated with a 0.2 μm syringe filter (SupraPure; Recenttec, Tokyo, Japan). Filtered samples were analyzed by HPLC with fluorescence detector (HPLC/FD).

Tissues: Approximately 1 g of tissue was homogenized using a mechanical homogenizer (Eurostar; IKA, Staufen, Germany) and extracted with 10 m*l* of 70% methanol. Aliquots of 75 μl of 200 ppm 6-OH chrysene were added to the extract. Intestinal samples were washed with 0.9% normal saline before extraction. The rinsed pieces of tissues were homogenized and extracted as described above. The homogenates were centrifuged at 9,000 × *g* for 10 min at 20°C. The supernatants were transferred to new tubes. The pellets were extracted once more with 5 m*l* of 100% methanol by shaking vigorously (10 min), and the samples were then centrifuged once again. The pooled supernatant was filtrated with a 0.2 μm syringe filter (SupraPure; Recenttec). Aliquots of 5 μl of the supernatant were injected into HPLC/FD.

Plasma: Aliquots of 250 μl of plasma were mixed with 1 μl of 200 ppm 6-OH chrysene and extracted with 500 μl of methanol. The samples were kept at −20°C for 15 min and centrifuged at 13,000 × *g* for 15 min at 4°C. The supernatant was reduced in volume under a stream of nitrogen gas and resuspended in 50% methanol up to 200 μl. After centrifugation, 5 μl of the supernatant was injected into HPLC/FD.

Feces: Samples of approximately 3–5 g were homogenized with 20 m*l* of 70% methanol, and 75 μl of 200 ppm 6-OH chrysene was added. Extraction was performed twice by vortex mixing, followed by sonication for 20 min and centrifugation at 9,000 × *g* for 10 min at 4°C. The supernatant was filtrated and analyzed by HPLC/FD.

Analysis of PY metabolites: The samples were analyzed by HPLC (20A series; Shimadzu, Kyoto, Japan) with FD (RF-10AXL; Shimadzu) equipped with an ODS column (ODS-120T 4.6 mm ×300 mm; Tosoh, Tokyo, Japan). HPLC was performed according to a modification of the method of Beach *et al.* [1] and Ueda *et al.* [36]. Mobile phase A consisted of 10 mM ammonium acetate buffer, and pH was adjusted to 5.0 with acetic acid, and mobile phase B consisted of a mixture of methanol:acetonitrile:water (38:57:5, v/v/v). The solvent gradient was 10% mobile phase B from 0 to 2 min, followed by a linear gradient to 100% mobile phases A and B from 2 to 35 min. The gradient was held at 100% mobile phases A and B for 5 min until 45 min. The solvent flow rate was set at 0.5 m*l*/min, and the column temperature was 45°C. The excitation (Ex) and emission (Em) wavelengths for fluorescence detection 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 >90%: 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). We confirmed that methanol solution of each metabolite was stable under −20°C for at least 2 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 electrospray ionization ion-trap mass spectrometry (ESI/ion-trap/MS, LTQ Orbitrap; Thermo Fisher Scientific, Waltham, MA, U.S.A.). The ESI conditions were fully scanned (*m/z* 80–750) negative mode, with an ion source voltage and temperature of −5.0 kV and 300°C, respectively.

Pyrenediol (*PYdiol*) *conjugation compound synthesis*: PYdiol was synthesized under a photoreduction and photoaddition reaction of pyrenedione [35]. 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 m*l* of 4 N sulfuric acid (H_2SO_4) [10]. Briefly, the mixture was heated at 90°C for 1 hr and then refluxed for 1 hr. The reaction mixture was diluted with cold water and filtrated, producing an insoluble material that was then washed and dried. Then, 4 g of the pyrenedione mixture was dissolved in 150 m*l* of hot acetic acid and filtrated through glass wool. The filtrate was separated by silica gel column chromatography (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 [27, 35]. A fast reaction occurred in isopropanol. The fluorescence spectrogram was obtained with excitation and emission wavelengths of 385 nm and 425 nm, respectively [26].

Deconjugation: Deconjugation was performed using the method described by Ikenaka *et al*. [18]. Briefly, sulfatase,

β-glucuronidase and β-glucosidase were dissolved in 0.1 M sodium acetate buffer. The pH was adjusted to 5.0 with acetic acid. The enzyme concentrations were 10, 4,000 and 17 U/m*l*, respectively. Aliquots of 30 *μl* of samples containing PY metabolites were mixed with 270 *μl* of buffer, and each deconjugation enzyme (200 *μl*) was added. As a control, the same quantity bovine serum albumin (1 mg/m*l*) was added, and reactions were performed under the same conditions as used the deconjugation enzymes. All samples were incubated at 37°C for 8 hr. The reaction was stopped by adding 500 *μl* of methanol. The deconjugated solutions were analyzed by HPLC/FD.

Quantification of PY metabolites, analytical limit of detection (LOD) and limit of quantitation (LOQ): The concentration of PY metabolites was estimated from the fluorescence peak areas of deconjugated metabolites. The standard solution of PYOH was quantified by calculation of the net amount of PYOG and PYOS after deconjugation. The correlation coefficient of the standard curve of PYOH was R^2=0.9998. The LOD and LOQ were defined as concentration by the linear calibration curve parameter of PYOH. LOD and LOQ of PYOH were 0.64 and 6.42 ppb, respectively. Moreover, LOD and LOQ of PY were 0.89 and 8.89 ppb, respectively.

Statistical analyses: The results were subjected to cluster analyses, principal component analysis (PCA) and Tukey–Kramer test (JMP 10.0; SAS, Cary, NC, U.S.A.). In all analyses, $P<0.05$ was taken to indicate statistical significance.

RESULTS

Identification of PY metabolites in urine: Figure 1 shows the HPLC/FD chromatograms of urine after PY exposure. Based 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, B, C, D, E and F) were observed in the urine of PY-exposed rats. These six peaks were considered to be PY-derived substances. The retention times (RT) and mass to charge ratio (*m/z*) of each peak are shown in Table 1. 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 of 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 present in the conjugated metabolites

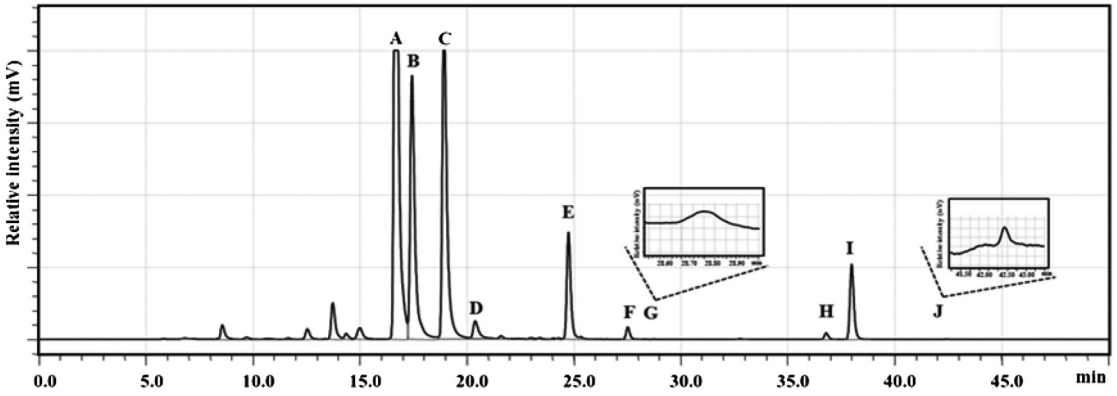

Fig. 1. HPLC/FD chromatogram of PY metabolites in rat urine. Glucuronide and sulfate conjugates were detected, A–D) and F) were sulfate-conjugated metabolites. E) Glucuronide-conjugated metabolite. G and H) Hydroxylation metabolites. Peaks I and J are internal standard and pyrene, respectively.

Table 1. PY and its metabolites under MS, MS/MS and deconjugation 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 decrease.

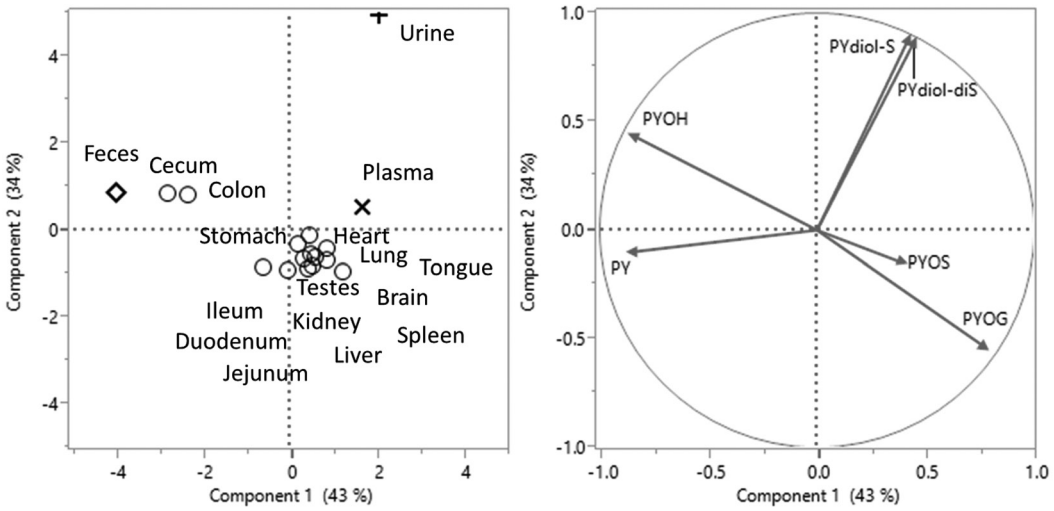

Fig. 2. Characterization of PY and PY metabolites in tissues, plasma, urine and feces based on Principal Component Analysis (PCA). Samples with the same symbol (e.g., circle, diamond, cross, plus sign) indicate the same cluster as determined by cluster analysis. Plot of tissue and letter codes are related to each metabolite, as PYOH and PY are mainly distributed in the large intestine and feces. PYOG is abundant in the small intestine and various tissues. PYdiol-S and PYdiol-diS are mainly distributed in urine.

of peak-A, B, C and D.

Deconjugation was also used to identify each peak of PY metabolites. Peaks-A, B, C, D, E and F were PY metabolites, which presented in rat urine. Each peak was collected using a fraction collector. Then, each collected metabolite was treated with the deconjugation enzymes, sulfatase, β-glucuronidase and β-glucosidase to identify each peak (Table 1). With sulfatase treatment, peaks-A, B, C, D and F disappeared, and PYdiol (peaks-A, B, C, and D) and PYOH (peak-F) were formed. Interestingly, peak-B was formed after treatment of peak-A with sulfatase. With β-glucuronidase treatment, peak-E disappeared, and PYOH was formed. However, none of the metabolite peaks were deconjugated after β-glucosidase treatment. Therefore, all PY metabolites in rat urine were suspected to be peak-A as pyrenediol-disulfate (PYdiol-diS); peaks-B, C and D as pyrenediol-sulfate (PYdiol-S) (the congener different for PYdiol); peak-E as pyrene-1-glucuronide (PYOG); and peak-F as pyrene-1-sulfase (PYOS). Moreover, peak-I was the internal standard, and peak-J was PY (Fig. 1).

Distribution of PY and PY metabolites in tissues, plasma, urine and feces by PCA: Figure 2 shows the results of PCA analysis using the peak area of each PY metabolite (peaks A, B, C, D, E, F and H). The tissue-specific distribution pattern of PY metabolites could be divided into three major clusters: 1) PYOH and PY group, 2) PYOG and PYOS group, and 3) PYdiol-S and PYdiol-diS group. The PYOH and PY group was predominant in the large intestine (colon and cecum) and feces. PYOG was present at high levels in the small intestine (duodenum, jejunum and ileum). On the other hand, all of these metabolites showed similar accumulation patterns in other tissues, including the liver, kidney, lung, spleen, heart, brain and testes.

Quantification of PY and PY metabolites in tissues, plasma, urine and feces: Table 2 shows the concentrations of PYOG, PYOS, PYOH and PY in various tissues, plasma, urine and feces.

The recorded PYOG concentrations were higher than those of PYOS in all tissues examined (Table 2). The concentration of PYOG followed the following pattern: ileum >duodenum >jejunum>kidney >liver >lung >stomach >cecum >colon >tongue >testes >heart >spleen and brain. The order of PYOS concentration was as follows: ileum >liver >cecum >kidney >colon >jejunum >lung >heart >stomach and testes. Interestingly, the highest PYOH concentrations were recorded in the cecum and colon ($59.5 \times 10^3 \pm 760$ and $13.1 \times 10^3 \pm 796$ ppb, respectively). PYOH distribution varied as follows: large intestine >small intestine >liver >stomach >kidney and testes. PY was higher distributed in feces than intestine and other tissues. Although PYdiol S and PYdiol-diS were mainly detected from the urine, they were not quantified in the present study due to the low fluorescence sensitivity of PYdiol (estimated LOQ is 9.19 ppm) or other factor during the de-conjugation process. Even we could not quantified these compounds, estimated amount was small compared to other metabolites, especially to the PYOG. In addition, we determined PY metabolite concentrations in plasma, urine and feces. PYOG and PYOS were detected only in the plasma and urine. However, the highest concentration of PYOH was recorded in feces.

DISCUSSION

Phase II metabolism of xenobiotics, such as PAHs, is a major determinant for the safety of animals and biota. PY and its metabolites are ideal models for studying phase II

Table 2. Concentrations of PY metabolites and PY (wet-g) in tissues, plasma, urine and feces of rats and total amount of PY metabolites in each tissues weight (*n*g) (mean ± SD)

	PYOG	PYOS	PYOH	PY
Liver	$10.2 \times 10^3 \pm 300^{c,d}$ $(70 \times 10^3 \pm 220)$	$96 \pm 38^{a,b}$ (650 ± 260)	$1.3 \times 10^3 \pm 200^{b}$ $(8.5 \times 10^3 \pm 100)$	$18.4 \pm 3.2^{c,d,e}$ (124.5 ± 20.4)
Lung	$2.4 \times 10^3 \pm 900^{c,d}$ $(1.7 \times 10^3 \pm 640)$	38 ± 13^{b} (29 ± 9)	nd	$14.2 \pm 0.2^{d,e}$ (10.7 ± 0.2)
Heart	$1.1 \times 10^3 \pm 200^{d}$ (860 ± 140)	12 ± 4^{b} (9 ± 3)	nd	$16.8 \pm 1.8^{c,d,e}$ (12.7 ± 1.5)
Spleen	790 ± 20^{d} (380 ± 120)	nd	nd	$26.7 \pm 5.2^{c,d,e}$ (12.4 ± 0.8)
Kidney	$20.6 \times 10^3 \pm 100^{c,d}$ $(18 \times 10^3 \pm 880)$	$76 \pm 38^{a,b}$ (67 ± 33)	154 ± 23^{b} (135 ± 19)	$13.8 \pm 2.1^{d,e}$ (12.1 ± 2.0)
Brain	210 ± 60^{d} (310 ± 100)	nd	nd	$11.6 \pm 0.9^{d,e}$ (17.5 ± 1.5)
Testes	$1.0 \times 10^3 \pm 360^{d}$ $(1.1 \times 10^3 \pm 390)$	11 ± 3^{b} (12 ± 3)	88 ± 36^{b} (92 ± 37)	$12.6 \pm 3.3^{d,e}$ (13.3 ± 3.7)
Tongue	$1.4 \times 10^3 \pm 500^{d}$ (420 ± 110)	nd	nd	$36.7 \pm 5.0^{b,c,d}$ (11.4 ± 0.4)
Stomach	$1.7 \times 10^3 \pm 10^{d}$ $(1.7 \times 10^3 \pm 100)$	11 ± 1^{b} (11 ± 1)	871 ± 64^{b} (871 ± 64)	$22.9 \pm 5.2^{c,d,e}$ (22.4 ± 4.1)
Duodenum	$65.9 \times 10^3 \pm 800^{b,c}$ $(2.6 \times 10^3 \pm 500)$	nd	$1.1 \times 10^3 \pm 80^{b}$ (441 ± 29)	$49.2 \pm 14.3^{b,c}$ (19.4 ± 5.3)
Jejunum	$87.1 \times 10^3 \pm 400^{b}$ $(11 \times 10^3 \pm 500)$	39 ± 17^{b} (48 ± 21)	$1.3 \times 10^3 \pm 200^{b}$ $(1.6 \times 10^3 \pm 200)$	$28.3 \pm 12.4^{c,d,e}$ $(34.5 \pm 14.8.)$
Ileum	$170.1 \times 10^3 \pm 700^{a}$ $(58 \times 10^3 \pm 300)$	261 ± 21^{a} (96 ± 10)	$10.7 \times 10^3 \pm 1.0^{b} \times 10^3$ $(4.1 \times 10^3 \pm 400)$	$67.0 \pm 17.9^{a,b}$ (22.0 ± 1.7)
Cecum	$1.4 \times 10^3 \pm 100^{d}$ (950 ± 70)	$89 \pm 58^{a,b}$ (57 ± 35)	$59.4 \times 10^3 \pm 800^{a,b}$ $(40.1 \times 10^3 \pm 100)$	$51.2 \pm 12.3^{b,c}$ (33.0 ± 4.8)
Colon	$1.2 \times 10^3 \pm 300^{d}$ $(1.0 \times 10^3 \pm 200)$	54 ± 20^{b} (47 ± 16)	$13 \times 10^3 \pm 800^{b}$ $(11.4 \times 10^3 \pm 700)$	$37.5 \pm 13.8^{b,c,d}$ (32.0 ± 7.7)
Plasma	446.0 ± 30^{d}	2 ± 1^{b}	4 ± 2^{b}	1.4 ± 0.2^{e}
Urine	$10.1 \times 10^3 \pm 400^{c,d}$ $(140 \times 10^3 \pm 100)$	13 ± 7^{b} $(1.9 \times 10^3 \pm 800)$	546 ± 78^{b} $(63.2 \times 10^3 \pm 800)$	$4.3 \pm 0.0^{d,e}$ (40.7 ± 9.6)
Feces	nd	nd	$226.8 \times 10^3 \pm 200^{a}$ $(755.8 \times 10^3 \pm 700)$	96.1 ± 25.8^{a} (380.9 ± 31.2)

a–e) Tukey-Kramer test ($P<0.05$) among the tissue. nd: not detected.

PAH metabolic pathways. A recent *in vitro* study of PY glucuronidation using human recombinant UGT showed that UGT1A6, 1A7 and 1A9 were mainly involved in metabolism. In addition, PYOH could also be metabolized by UGT1A1 and 1A6 in mice [24]. The glucuronide levels account for more than 80% of total PY metabolites in human urine [32, 34]. Based on our results, the rat also eliminates high levels of glucuronide conjugate in the urine, which are greater than those of sulfate conjugate and PYOH. Sulfation is also associated with conjugation of PY. It has been reported that SULT1A1 contributes to PY conjugation in mice [22, 24].

Characterization of PY metabolites formed in the rat: Glucuronide and sulfate conjugates of PY were identified and detected in various tissues of the rat. Boyland and Sims [4] reported that glucuronide and sulfate conjugates of PYOH, 1,6- and 1,8-PYdiol, and *trans*-4,5-PYdiol were formed in the urine of both rats and rabbits treated with PY. In previous studies, PYdiol-S and PYdiol-diS were identified in amphibian species and marine snails [1, 36]. To our knowledge, PYdiol-S and PYdiol-diS have not been clearly detected in mammalian species. We assumed that PYOH and PYdiol metabolized by phase I reaction (mainly by CYP) were rapidly conjugated by microsomal UGT and cytosolic SULT. These results indicated that attention should be paid not only to PYOG, which was reported as a biomarker for PAH exposure, but also to sulfate-conjugated metabolites (PYOS, PYdiol-S and PYdiol-diS).

Tissue distribution and elimination pattern of PY in the rat: It has been reported that the bioavailability of orally administered PY is between 50% and 60% [14, 15, 20, 29]. Bouchard *et al.* [3] and Withey *et al.* [38] have been studied about the kinetic parameters of PY in the rat, indicating that PY itself is considered to be rapidly distributed, metabolized and eliminated from the body. 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 hr [3, 38]. Therefore, PY can be found in various tissues as liver, kidney and intestine. From the present study, 99% of the injected PY were eliminated via urine and feces. The results also indicated that around 10% were excreted via urine as PYOG and 70% were excreted via feces as PYOH.

Tissue distribution and elimination pattern of PY metabolites in the rat: In the present study, the tissue distribution

and elimination of PY metabolites were observed in various tissues of the rat. The PY metabolites from phase I reaction (mainly PYOH) were produced during metabolism, and the conjugated compounds from phase II conjugation (mainly PYOG) were distributed in various tissues, such as liver, kidney and GI tract of the rat (Fig. 1 supplementary). Harper [14, 15] also reported that PY conjugated as PYOG was detected in the liver, kidney, bladder and small intestine, especially the duodenum, in rats.

Interestingly, PCA clearly showed different patterns of PY metabolites in the GI tract; e.g., PYOG was the dominant metabolite detected in the small intestine (duodenum, jejunum and ileum), and its concentrations were significantly higher than those in other tissues. In agreement with our findings, Shiratani *et al*. [30] reported higher UGT activities from various UGT substrates in the intestines (duodenum, jejunum, ileum and colon) of rat and mouse microsomes. The UGT activities in rats tended to decrease from the small intestine to large intestine, whereas this tendency in mice differed according to the substrate. This may explain the high levels of PYOG in the small intestine compared to other tissues examined. However, the levels of conjugated metabolites were significantly reduced in the large intestine, while those of PYOH increased. The biodegradation of PYOG could be explained by decreased levels of conjugated metabolites of PY. PY conjugated metabolites could be biodegraded by bacteria and fungi through initial dioxygenase (bacteria) and monooxygenase (bacteria and fungi) reactions (deconjugation reactions) [21, 28]. The normal flora in the large intestine may digest conjugated compounds, such as PYOG, to form dominant metabolites, such as PYOH.

Considerable cancer risk in the large intestine: Our finding revealed that the large intestine recorded the high levels of deconjugated form PY (PYOH). Although, PYOH is not the carcinogenic compounds, some species of hydroxylated PAHs have carcinogen as 3-hydroxybenzo[a]pyrene, 1-, 2-, 3-, 4- and 9-hydroxyphenanthrenes and 1- and 2- hydroxynaphthalenes [7, 11, 31]. They could be shown to be a sensitive biomarker for evaluating carcinogenic PAHs exposure. Epidemiological studies indicated PAH-associated risk of colorectal cancers in humans and animals [9]. In addition, Sinha *et al.* [33] reported that consumption of well-done red meat was associated with adenoma of the colon due to B[*a*]P. In parallel, Gunter *et al.* [13] reported an association between high intake of barbecued red meat and colorectal adenoma in a sigmoidoscopy-based study. Generally, the extent of production of PAH-activated or conjugated metabolites is the major determinant for cancer incidence. Interestingly, PYOH was mainly detected in the large intestine, suggesting that de-conjugated PAHs may contribute to cancer of the large intestine.

In conclusion, phase II conjugated metabolites of PY were identified in various tissues of the rat, as well as plasma, urine and feces. PY metabolites, such as PYOG, PYOS, PYdiol-diS and PYdiol-S, were identified in the present study, and they were eliminated in urine and feces (Fig. 1 supplementary). Furthermore, the tissue distribution patterns of PY metabolites were different among tissues.

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