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PHARMACOKINETIC EVIDENCE FOR THE OCCURRENCE OF EXTRAHEPATIC CONJUGATIVE METABOLISM OF *p*-NITROPHENOL IN RATS

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Abstract—*p*-Nitrophenol (PNP), as a model compound for the study of conjugative metabolism, was administered intravenously to rats. PNP and its conjugated metabolites, i.e. PNP-glucuronide (PNP-Glu) and PNP-sulfate (PNP-Sul), were determined in body fluids by reversed-phase high-performance liquid chromatography using ion-pair systems. Linear pharmacokinetics was applicable in the dose range of 1.6 to 8 mg/kg. The metabolic clearance which was obtained from the area under the PNP blood concentration curve (AUC_{iv}) and from the excretion ratio of the total conjugates as PNP-Glu and PNP-Sul was so close to the hepatic blood flow that the PNP conjugation reactions seemed to be limited by the hepatic blood flow; that is, the hepatic extraction ratio (E_H) was expected to be 1. However, AUC_{pv}, following portal vein administration of PNP (4 mg/kg), was not zero but was significantly different from AUC_{iv} after the same dosing ($P < 0.05$). Consequently, comparison between the AUC values from both dosing routes and the excretion ratio of PNP-Glu and PNP-Sul gave an E_H of 0.43. Such a difference in E_H obtained by the two methods suggested a contribution by extrahepatic conjugative metabolism. It was shown that the intrinsic hepatic clearance obtained, assuming exclusively hepatic conjugative metabolism, was certainly overestimated. Furthermore, the results of the conjugation reaction in tissue homogenates suggested a contribution by extrahepatic glucuronidation.

Drug conjugative metabolism, especially glucuronidation and sulfation, is one of the most effective detoxication reactions in the body. Recently there have been studies which report that there is a good correlation between *in vivo* and *in vitro* drug oxidative metabolism rates [1–3], but there are few such studies for conjugation reaction rates.

p-Nitrophenol (PNP) is well-known to be conjugated to glucuronide (PNP-Glu) and sulfate (PNP-Sul), and it has often been used as a model compound in *in vitro* conjugation studies using hepatic microsomes [4–6], perfused liver [7, 8], and isolated liver cells [9]. Accordingly, PNP is a suitable compound to examine the correlation between *in vivo* and *in vitro* conjugation reaction rates. However, its pharmacokinetic study in the whole body has previously only been presented without urinary excretion data following the intraperitoneal administration of PNP [10].

The purpose of the present study is to report the pharmacokinetic behavior of PNP based on clearance concepts and to discuss the contribution of the extrahepatic metabolism that has been reported for phenols [11], as a first step in the study of a correlation between *in vivo* and *in vitro* conjugation rates.

MATERIALS AND METHODS

Chemicals and reagents. *p*-Nitrophenol (PNP), PNP-glucuronide (PNP-Glu), the monopotassium salt of PNP-sulfate (PNP-Sul), the ammonium salt of uridine-5′-diphosphoglucuronic acid (UDPGA), and the disodium salt of adenosine-5′-triphosphate (ATP) were obtained from the Sigma Chemical Co., St. Louis, MO. *p*-Fluorophenol and tetrabutylammonium bromide were obtained from Tokyo Kasei, Tokyo. All other chemicals and reagents were of analytical grade or better.

Animals and sample collection. Male Wistar rats weighing 200–350 g (Matsumoto Animals Laboratory, Chiba) were cannulated under ether anesthesia as described below depending on the administration routes. For intravenous and intra-arterial administration, cannulae of polyethelene tubing (PE-50) in the left femoral vein and the left carotid artery were used respectively. Administratoin via the hepatic portal vein was through a PE-50 cannula with the needle (24G) inserted and fixed with surgical glue (Aron Alpha, Sankyo Co. Ltd., Tokyo) in the portal vein. The rats were then fixed in a restraining cage and, after awakening, the experiments were started. Blood samples in all cases were taken from the cannula in the right femoral artery at 0, 1, 2, 4, 6, 8, 10, 20, 30, 60 and 90 min following the PNP administration. Urine and bile samples were collected from a cannula of polyethylene tubing in the urinary bladder (1 mm i.d., 2 mm o.d.) and a bile-duct cannula (PE-10) respectively. The sampling intervals were 0–4, 4–8 and 8–24 hr following the PNP administration. During all experiments, water and food were provided *ad lib.*

Dosing procedure. PNP was administered intravenously at three doses (1.6, 4 and 8 mg/kg) in physiological saline solution. The intra-arterial doses were 4 and 8 mg/kg, and the hepatic portal doses were 1.6 and 4 mg/kg.

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Distribution of PNP in red blood cells. The distribution of PNP between the plasma and red blood cells was calculated from the plasma and the whole blood concentrations of PNP, using the hematocrit. The PNP concentrations in plasma and whole blood samples at 2, 4 and 8 min following intravenous administration (8 mg/kg) were determined. The whole blood samples were diluted 3-fold with distilled water. The hematocrits of each blood sample were determined simultaneously.

The uptake of PNP into red blood cells *in vitro* was examined using the whole blood freshly prepared. The whole blood was preincubated for 5 min at 37°, and then the uptake reaction was started by adding PNP to the blood. The samples were taken at 0.5, 1, 3 and 5 min and divided into two parts. One part was used to determine the plasma concentration and the other to determine the whole blood concentration. The concentration of PNP in red blood cells was calculated from the means of the hematocrits and of the plasma and whole blood concentrations.

Serum protein binding of PNP. The ratio of unbound PNP to the total PNP in serum was determined after incubation for 24 hr at 4° in a dialysis cell (Kokugo-Gum Co., Tokyo) to which was attached Visking membrane (type 20/32) that separated the PNP in 0.05 M Tris–HCl buffer and the serum (predialyzed against 0.05 M Tris–HCl buffer for 24 hr at 4°).

Assay methods for PNP, PNP-Glu and PNP-Sul. After centrifuging blood samples at 3000 rpm for 10 min, the plasma was collected. The plasma (0.1 ml) was mixed with 10% perchloric acid (0.1 ml) containing *p*-fluorophenol (3.56 mM) as the internal standard. After centrifuging the mixture, the supernatant fractions (10–20 μl) were injected into the high-performance liquid chromatograph. Urine and bile samples were diluted adequately with saline solution. The diluent was treated similarly to the plasma sample. In this assay, a model JASCO Uniflow-211 (Japan Spectroscopic Co., Tokyo) equipped with a model JASCO Uvidec-100-II ultraviolet detector set at 300 nm and a reversed-phase column (250 × 4.6 mm i.d.) packed with octadecyl silane-bonded silica, particle size 10 μm (JASCO SS-10-ODS-B), with a variable loop injector (JASCO VL-611) were used. The assay was carried out at ambient temperature. The mobile phase is water–methanol–acetic acid (68.5:30:1.5 by vol.) containing 0.5 g/l KNO_3 and 30 mg/l tetrabutylammonium bromide. The flow rate was 1.5 ml/min. The capacity factors (k') of PNP, PNP-Glu and PNP-Sul were 5.1, 1.4 and 2.5 respectively. Linear relationships were observed up to concentrations of 5 μM for these three compounds in plasma, urine and bile. The correlation coefficient for each group was greater than 0.99.

Conjugation reactions in tissue homogenates. Freshly excised livers, lungs or kidneys from male Wistar rats weighing 235–265 g were homogenized with 4 vol. of 0.154 M KCl in Teflon–glass homogenizers. When preparing small intestine homogenates, the scraped mucosa was homogenized with a glass homogenizer. Each tissue homogenate, equivalent to 0.1 g/ml in incubation medium (pH 7.4), was incubated with 50 μM PNP, 2 mM UDPGA or 80 μM 3′-phosphoadenosine-5′-phosphosulfate (PAPS). PAPS was generated according to the method of Van Kempen and Jansen [12] and an 80 μM concentration of PAPS was used with 41.7 mM EDTA in the incubation medium. The incubation medium contained 131 mM NaCl, 5.2 mM KCl, 0.9 mM $MgSO_4 \cdot 7H_2O$, 0.12 mM $CaCl_2 \cdot 2H_2O$, 3.0 mM NaH_2PO_4, and 10.0 mM Tris–ethanolamine. The final pH was adjusted to 7.4 using HCl. Incubation was carried out for 10 min at 37° and was stopped immediately by adding 10% perchloric acid into the incubation mixture. The samples were analyzed as described above.

RESULTS

Femoral vein administration of PNP. The PNP concentration ratio of red blood cells to plasma was constant at 0.85 among the samples at 2, 4 and 8 min after administration. The same concentration ratio was obtained within 0.5 min from experiments of *in vitro* uptake into red blood cells using whole blood. These results illustrated the rapid equilibrium of PNP between plasma and red blood cells. Therefore, when necessary, the PNP blood concentration was given by $y = 0.937x$ with a corelation coefficient of 0.984, where y and x stand for blood and plasma concentrations respectively.

Figure 1 shows the plasma concentrations of PNP, PNP-Glu and PNP-Sul following the PNP femoral vein administrations of 1.6, 4 and 8 mg/kg. The elimination of PNP from plasma was so fast that the plasma appearance of PNP-Glu and PNP-Sul could be observed even at 1 min after dosing, in all cases. The saturation of sulfate formation of salicylamide [13], and of acetaminophen [14, 15], has been reported to cause a dose dependency of the drug elimination rate. Although, in the dose range shown

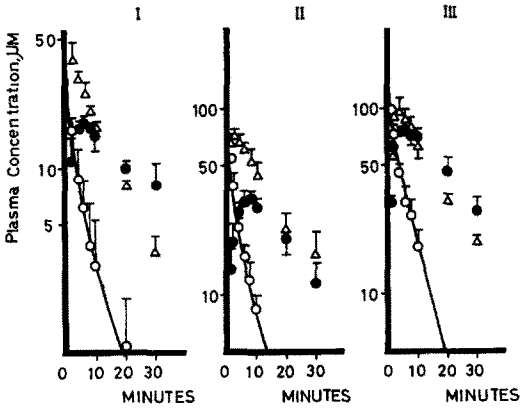

Fig. 1. Semi-logarithmic plots of plasma concentrations of *p*-nitrophenol (○), its glucuronide (●) and sulfate (△) versus time following intravenous (femoral) administration of *p*-nitrophenol to rats. Doses in (I), (II) and (III) were 1.6, 4 and 8 mg/kg respectively. The values are means ± S.E. of three (I), four (II) and six (III) rats. The curves of *p*-nitrophenol were obtained by the best fit of the data to the two-compartment model by the computer program PHART [16], using the HITACHI 3700/3800 digitial computer in the University of Tokyo Computer Center.

Table 1. Cumulative percentages of dose excreted in urine and bile as *p*-nitrophenol, and as its glucuronide and sulfate, in the 24 hr following femoral vein administration of *p*-nitrophenol to rats*

Intravenous dose (mg/kg)	*p*-Nitrophenol in urine	Glucuronide in urine	Sulfate in urine	Glucuronide in bile
1.6	1.8 ± 3.1	37.4 ± 0.8	61.6 ± 4.4	6.2 ± 3.7
4	2.2 ± 0.6	41.0 ± 3.8	40.0 ± 3.7	11.1 ± 4.0
8	7.4 ± 5.8	41.0 ± 8.8	30.7 ± 8.6	7.9 ± 3.9

* Each value is the mean ± S.D. of four (8 mg/kg) and three (1.6 and 4 mg/kg) rats.

in Fig. 1, the plasma elimination of PNP did not precisely indicate such a dose dependency, a high dose of PNP decreased the excretion ratio of PNP-Sul in urine (Table 1). But no significant dose dependencies were found of the total excretion ratios of PNP-Glu and PNP-Sul or of the total body clearances (Tables 1 and 2). And, as stated later, the index of hepatic extraction, i.e. the ratio of the area under the blood concentration curve after portal vein administration to that after intravenous administration, was also dose independent. These results show that the total metabolic elimination of PNP from blood and the total excretion of the conjugates in urine can be treated by linear pharmacokinetics. Accordingly, neglecting the small effects of sulfation dose dependency, the blood concentration data were analyzed by clearance concepts from linear pharmacokinetic theory. Only the results that could be derived from the total clearance values and from the total excretion ratios of the conjugates are discussed below.

Metabolic clearance and hepatic extraction ratio. Metabolic clearance (CL_M) for PNP glucuronidation plus sulfation is given by equation 1:

$$CL_M = \frac{f_{m,iv} \cdot \text{Dose}}{AUC_{iv}} \quad (1)$$

where $f_{m,iv}$ is the ratio of the cumulative excretion of PNP-Glu plus PNP-Sul to the intravenous (femoral) dose of PNP, and AUC_{iv} is the area under the PNP blood concentration curve calculated from the plasma concentration versus time curve following PNP femoral vein administration. CL_M, calculated from equation 1, was 17–21 $ml \cdot min^{-1} \cdot (250\,g\ rat)^{-1}$ (Table 2) and corresponded to the hepatic blood flow, i.e. 10–20 $ml \cdot min^{-1} \cdot (250\,g\ rat)^{-1}$ [2, 17–19]. This result suggests that PNP glucuronidation plus sulfation was limited by the hepatic blood flow, and that the hepatic extraction ratio for glucuronidation plus sulfation (E_H) was 1.

PNP injection into the hepatic portal vein. AUC_{pv} values following PNP administration of 1.6 and 4 mg/kg were 77.7 ± 8.4 (N = 3) and 168 ± 24 (N = 4) $\mu M \cdot min \cdot (250\,g\ rat)^{-1}$ respectively. A comparison between the PNP plasma concentrations following PNP femoral vein and portal vein administrations of 4 mg/kg is shown in Fig. 2 as an example. The differences between AUC_{iv} and AUC_{pv} at 1.6 and 4 mg/kg were significant ($P < 0.05$). The ratios of AUC_{pv} to AUC_{iv} at 1.6 and 4 mg/kg were

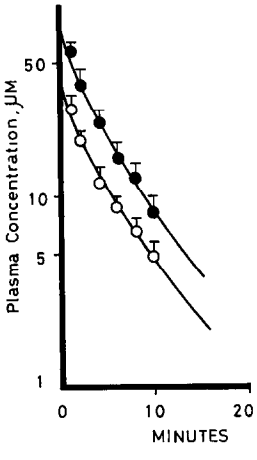

Fig. 2. Comparison of *p*-nitrophenol plasma concentration versus time following *p*-nitrophenol administration (4 mg/kg) into the femoral vein (●) and the hepatic portal vein (○) in rats. Each plot represents the mean ± S.E. of four rats.

Table 2. Area under the blood concentration curve (AUC_{iv}), total clearance (CL_{total}) and metabolic clearance (CL_M) following femoral vein administration of *p*-nitrophenol to rats*

Intravenous dose (mg/kg)	AUC_{iv}† ($\mu M \cdot min \cdot 250\,g^{-1}$)	CL_{total}‡ ($ml \cdot min^{-1} \cdot 250\,g^{-1}$)	CL_M§ ($ml \cdot min^{-1} \cdot 250\,g^{-1}$)
1.6	149 ± 73	19.3 ± 9.5	19.3 ± 9.5
4	312 ± 63	22.9 ± 4.6	21.0 ± 4.2
8	674 ± 179	21.3 ± 5.7	17.0 ± 4.5

* The values are means (± S.D.) of six (8 mg/kg), four (4 mg/kg), and three (1.6 mg/kg) rats.
† AUC is the total AUC calculated by the trapezoidal rule for the 10 min after dosing and $C_{t=10\,min}/\beta$ after 10 min.
‡ CL_{total} was calculated by $Dose/AUC_{iv}$.
§ CL_M was calculated by equation 1.

Table 3. Rates of *p*-nitrophenol glucuronidation in rat tissue homogenates*

Tissue	Rate of glucuronidation	
	(nmoles·min^{-1}·g^{-1})	(nmoles·min^{-1}·tissue^{-1})
Liver	23.7 ± 4.2	308 ± 18
Kidney	43.3 ± 1.4	79.8 ± 2.7
Lung	31.8 ± 4.9	40.8 ± 5.5
Small intestine	27.2 ± 2.5	21.0 ± 6.3

* Each value is the mean ± S.D. of three rats. The concentration of *p*-nitrophenol added to the reaction medium was 50 μM. Other experimental conditions are described in Materials and Methods.

0.52 ± 0.26 and 0.54 ± 0.13, respectively, showing no dose dependency in this dose range. The hepatic extraction ratio for total metabolism (E_{total}) is the sum of the E_H only for glucuronidation plus sulfation and of the hepatic extraction ratio for the unknown metabolism [7, 8], indicating that E_M can be expressed by equations 2 and 3:

$$E_H = f_m \cdot E_{total} \quad (2)$$

$$= f_m \cdot (1 - AUC_{pv}/AUC_{iv}) \quad (3)$$

where f_m is the ratio of the cumulative excretion of PNP-Glu and PNP-Sul to the total metabolites, i.e. 0.94 in the intravenous dose of 4 mg/kg (Table 1). Using f_m and the respective AUC values following the PNP femoral and portal vein administrations of 4 mg/kg, E_H was 0.43 ± 0.11 and thus inconsistent with the value expected to be 1 from CL_M described in the previous section.

PNP hepatic intrinsic clearance. The relation between the hepatic intrinsic clearance ($CL_{int,h}$) and E_H is expressed as equation 4:

$$E_H = \frac{CL_{int,h} \cdot f_B}{F_H + CL_{int,h} \cdot f_B} \quad (4)$$

where F_H is the hepatic blood flow, 10–20 ml·min^{-1}·(250 g rat)$^{-1}$ as described before [2, 17–19], and f_B is the ratio of unbound PNP to the total PNP in serum. PNP had a high and constant binding ratio in the blood concentration range discussed and its f_B value was 0.09. Therefore, putting 0.43, 10–20 ml·min^{-1} (250 g rat)$^{-1}$ and 0.09 into the E_H, F_H and f_B in equation 4, respectively, $CL_{int,h}$ was calculated to be 84–168 ml·min^{-1}·liver^{-1}·(250 g rat)$^{-1}$. This value represents the total ability of one rat liver to conjugate PNP as PNP-Glu and PNP-Sul.

Intra-arterial administration of PNP. PNP doses of 4 and 8 mg/kg were intra-arterially administrated to investigate the contribution of lung to the PNP conjugative metabolism that has been reported for phenol [11]. The AUC_{ia} values obtained were 278 ± 73 (N = 4) and 650 ± 261 (N = 4) μM·min·(250 g rat)$^{-1}$ values respectively. Since these values were not significantly different from those after intravenous dosing, a pulmonary contribution was not detected.

PNP conjugation reactions in tissue homogenates. The rates of PNP-Glu synthesis in tissue homogenates with UDPGA are listed in Table 3. The glucuronidation rate per gram of liver was not as high as in kidney and lung, but the order of the rate per whole tissue was liver > kidney > lung > small intestine, indicating that the glucuronidation ability in all other tissues combined was not negligibly small, i.e. it was approximately 46% of that in the liver.

PNP-Sul synthesis in the tissue homogenates containing PAPS is shown in Table 4. Unlike glucuronidation, there was a uniquely high sulfation activity in liver, and the slight activity per tissue found in kidney was only about 3% of that in liver.

DISCUSSION

Although CL_M of PNP conjugative metabolism obtained after administration through the femoral vein was so close to the hepatic blood flow that E_H could be considered to be 1, a comparison between AUC_{iv} and AUC_{pv} gave E_H 0.43. The most probable factor causing the difference in E_H was probably extrahepatic conjugative metabolism.

Assuming hepatic conjugative metabolism exclusively, CL_M in equation 1 can be replaced by the

Table 4. Rates of *p*-nitrophenol sulfation in rat tissue homogenates*

Tissue	Rate of sulfation	
	(nmoles·min^{-1}·g^{-1})	(nmoles·min^{-1}·tissue^{-1})
Liver	27.1 ± 2.7	324 ± 66
Kidney	5.3 ± 1.0	10.0 ± 2.2
Lung	ND†	ND
Small intestine	ND	ND

* Each value is the mean ± S.D. of three rats. The concentration of *p*-nitrophenol added to the reaction medium was 50 μM. Other experimental conditions are described in Materials and Methods.

† ND means that the sulfation was negligibly small or not detected.

hepatic clearance, CL_H, as shown in equation 5:

$$f_{m,iv} \cdot \text{Dose} = CL_H \cdot AUC_{iv} \quad (5)$$

Since CL_H is the product of F_H and E_H, equation 6 can be derived from equations 4 and 5:

$$f_{m,iv} \cdot \text{Dose} = (1 - E_H) \cdot CL_{int,h} \cdot f_B \cdot AUC_{iv} \quad (6)$$

Thus, substituting 0.92, 7.19 μmoles/250 g rat (4 mg/kg), 0.43, 0.09* and 312 μM·min·(250 g rat)$^{-1}$ for $f_{m,iv}$, Dose, E_H, f_B* and AUC_{iv}, respectively, $CL_{int,h}$ is 414 ml·min^{-1}·liver^{-1}·(250 g rat^{-1}). On the other hand, equation 4, in which no exclusively hepatic metabolism was assumed, gave a $CL_{int,h}$ of 84–168 ml·min^{-1}·(250 g rat)$^{-1}$, using E_H, F_H and f_B* values which were free from the assumption of an exclusively hepatic metabolism. These differences clearly indicate the extrahepatic contribution to PNP conjugative metabolism. An E_H of 0.43 suggests that the contribution of the extrahepatic metabolism reached approximately 50% of the total metabolic clearance.

The order of glucuronidation activity per tissue in tissue homogenates was liver > kidney > lung > small intestine (Table 3) whereas sulfation activity was detected almost exclusively in liver (Table 4), indicating that PNP glucuronidation occurred in tissues other than liver but that PNP sulfation proceeded almost exclusively in liver. The finding that pulmonary extraction, already reported for phenol [11], was not detectable by comparing AUC_{ia} and AUC_{iv} suggests that there was no pulmonary metabolism of PNP. But, since less than 0.1 of the pulmonary extraction ratio was considered as barely significant, due to the very rapid PNP elmination that produced a large AUC variance (Table 2), a PNP clearance about equal to or less than, the hepatic clearance of PNP cannot be ruled out for lungs, considering that pulmonary blood flow was three to four times as large as the hepatic flow [20]. The facts that a difference between AUC_{po} and AUC_{pv} was not detected and that small intestine had the lowest glucuronidation activity suggest that small intestine scarcely contributed to extrahepatic glucuronidation of PNP.

From these results it can be concluded that PNP glucuronidation proceeded in kidney and lung as well as in liver but that PNP sulfation proceeded almost exclusively in liver. And although PNP is widely used as a model compound to study the conjugation reaction *in vitro*, such as in hepatic microsomes or cells, it can be concluded that, when PNP is used to study the correlation between *in vivo* and *in vitro* glucuronidation rates, its extrahepatic glucuronidation as pointed out in this paper has to be considered in calculations.

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* f_B Value at 37° was 0.17. Using this value, the $CL_{int,h}$ values calculated using equations 4 and 6 were 44–89 and 219 ml·min^{-1}·liver^{-1}·(250 g rat^{-1}) respectively. Thus, the difference in the ratio of unbound PNP to the total PNP did not affect the above conclusion.

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