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USE OF PENTACHLOROPHENOL AS LONG-TERM INHIBITOR OF SULFATION OF PHENOLS AND HYDROXAMIC ACIDS IN THE RAT *IN VIVO*

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Abstract—Inhibition of sulfation of the phenolic compound harmol (7-hydroxy-1-methyl-9*H*-pyrido[3,4-*b*]indole) by pentachlorophenol (PCP) was studied in the Wistar rat; PCP was administered in various ways to find a convenient method for long-term inhibition of sulfation. High doses of PCP or sodium pentachlorophenolate (NaPCP) in the diet (350 ppm) or NaPCP in the drinking water (1.4 mM) of Wistar rats for one week inhibited the sulfation of harmol by 30–45%. The plasma concentration of PCP in rats with NaPCP (1.4 mM) in their drinking water was highest (270 μM) in the period that the animals were kept in the dark and consumed food and water. This is explained by a rapid elimination: the elimination of PCP from plasma, after intravenous administration, showed a biphasic disappearance curve with half-lives of 2.17 and 7.24 hrs, respectively. This is much faster than in Sprague–Dawley rats.

A log-linear correlation was found between the plasma concentration of pentachlorophenol and the inhibition of harmol sulfation.

Although administration of NaPCP to rats in their drinking water inhibited the sulfation of harmol only by 45%, it inhibited the sulfation of the carcinogenic arylhydroxamic acid *N*-hydroxy-2-acetylaminofluorene by 70–75%.

Several carcinogenic *N*-hydroxy-arylamines and *N*-hydroxy-arylamides, for instance *N*-hydroxy-2-acetylaminofluorene (N-OH-AAF) [1], *N*-hydroxy-*N*-methyl-4-aminoazobenzene [2], *N*-hydroxy-*N*,*N*′-diacetyl-benzidine [3] and *N*-hydroxy-2-acetylaminophenanthrene [4] can be metabolized to *N*-*O*-sulfates which are unstable and react with proteins, RNA and DNA. Therefore, these *N*-*O*-sulfates are believed to be involved in the carcinogenesis by these compounds [5].

Long-term inhibition of sulfation of these carcinogenic arylamine derivatives offers a tool to study the importance of *N*-*O*-sulfation for their carcinogenic action. PCP inhibits the sulfation of *N*-hydroxy-phenacetin [6], *N*-hydroxy-2-acetylaminofluorene [7] and *N*-hydroxy-4-acetylamino-4′-fluorobiphenyl (Meerman, unpublished results). Therefore, we have tried to find a convenient method to inhibit sulfation for a prolonged period of time by administration of PCP by several routes and modes.

The *N*-*O*-sulfates of the carcinogenic *N*-hydroxy-arylamines and *N*-hydroxy-arylamides are reactive and they decompose before they can be excreted in bile or urine. Thus, *N*-*O*-sulfation of these compounds can only be measured indirectly, for instance by covalent binding of the reactive break-down product to glutathione or cellular macromolecules. This gives no accurate data but only an estimate on the amount of these carcinogens that is sulfated *in vivo*. Therefore, we have used harmol to measure the inhibition of sulfation during the long-term administration of PCP because its sulfate conjugate can easily be measured and is rapidly excreted quantitatively in bile and urine [8].

Toxic effects have been reported of doses of PCP (40 μmol/kg body weight, i.p.) that inhibit sulfation [8]. In later studies [9] we did not observe such toxicity at this dose after 24 or 48 hr (unpublished results). The reason for this difference may be that in the earlier study [8] no highly purified PCP was used; impurities in that PCP may have been responsible for the observed toxicity. The published values for the oral LD_{50} of PCP for the rat range from 100 to 750 μmol/kg body weight. Uncoupling of oxidative phosphorylation by PCP might be responsible for the acute toxicity [10]. The chronic toxicity of PCP that was reported in several studies seems to be due to impurities in the PCP preparations that were used [10]: no toxic effects were noticed when very pure PCP was administered with the food at concentrations of 100–500 ppm for 8 months [11, 12]. Based on these toxicological data, we expected that pure PCP may be used for long-term inhibition of sulfation in rats without severe toxic effects in these animals.

MATERIALS AND METHODS

Materials. PCP (Gold label) was from Aldrich Chemical Co., Beerse, Belgium. Harmol-HCl was from Sigma Chemical Co., St. Louis, MO, USA. [9-^{14}C]-*N*-OH-AAF (1.8 mCi/mmol) was from New England Nuclear, Dreieich, F.R.G.; it was mixed with unlabeled compound to obtain a lower specific activity. Sodium pentachlorophenolate (NaPCP) was prepared from PCP by dissolving 8 g of PCP in a

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minimum amount of ethanol and adding this to 150 ml of water. Aqueous 1 M NaOH, equivalent to 95% of the PCP, was added, and the solution was stirred until the pH was 7. The solution was filtered to remove remaining PCP yielding a clear solution. The water was removed under reduced pressure at 50° and the remaining NaPCP was dried for 8 hours at 60°. All other used chemicals were of analytical reagent grade (Merck, Darmstadt, F.R.G.) unless stated otherwise.

Animals and diets. Male Wistar rats (100–120 g) of the inbred strain of the Department of Pharmacology, State University of Groningen, were used in most experiments. In the experiments in which the pharmacokinetic parameters of PCP were determined, the correlation between plasma concentration of PCP and inhibition of harmol sulfation was established, male Wistar rats of 140–160 g were used. In some experiments (indicated in the text) male Sprague–Dawley rats (100–120 g) were also used (these were obtained from TNO, Zeist, The Netherlands). All rats had free access to food and water; the lights were switched on at 7.00 a.m. and switched off at 7.00 p.m. The rats received either a control diet (MRH-B, Hope Farms, Woerden, The Netherlands) or a diet to which PCP or NaPCP was added. These diets were made by mixing the dry, powdered food with water (225 ml/kg of dry food) in which NaPCP was dissolved, and glycerol (100 ml/kg of dry food) in which PCP was dissolved. Pellets were made from this mixture and these were stored at 0–4°. The intake of PCP or NaPCP by the rats through their diet was calculated from the food and water consumption by weighing; corrections were made for the loss of weight from the pellets during the experiments. The rats were given tap water or a solution of NaPCP in tap water (1.4 mM). When PCP was administered intraperitoneally (40 μmol/kg) it was dissolved in propane-1,2-diol and was given 60 min before the administration of harmol.

Rats were anaesthetized with sodium pentobarbital at 9.30 a.m. and artificial respiration was applied through a trachea cannula. The bile duct and urine bladder were cannulated and body temperatures were kept at 38 ± 0.5°. A mannitol infusion (75 mg/ml in saline) was given via a cannula in the vena jugularis externa (13.6 ml/kg/hr) to induce an osmotic diuresis. Harmol (8.67 mM in saline) was given via a lateral tail vein (26 μmol/kg body weight) usually at 10.0–10.15 a.m. when a regular urine production was achieved. In the experiments whereby sulfation of *N*-OH-AAF was inhibited, rats were anaesthetized with sodium pentobarbital and the trachea was cannulated to ensure unhindered respiration. The bile duct was cannulated and the urethra was ligated. Body temperatures were kept at 38 ± 0.5°. [9-^{14}C]-*N*-OH-AAF (0.9 mCi/mmol) was administered intravenously via a lateral tail vein, at a dose of 60 μmol/kg. The dose was prepared freshly before each experiment by dissolving it in 0.5 M aqueous NaOH solution; then the pH was adjusted to approximately 10.5 and the volume was adjusted with water to 3.0 ml. The solution was injected within 5 min after its preparation; no breakdown of *N*-OH-AAF occurs under these conditions [7]. Bile was collected in tubes on ice during 4 hr after the administration of *N*-OH-AAF and urine was collected from the bladder with a syringe at the end of the experiments.

Blood was collected from the aorta in heparinized tubes. Plasma was obtained by centrifugation and stored at −20° for the determination of PCP.

In the experiments to determine the correlation between PCP plasma concentration and inhibition of sulfation of harmol, various priming doses of NaPCP were administered via a lateral tail vein in 2.15 ml saline/kg body weight. The rats were anaesthetized with sodium pentobarbital, cannulated and treated as described above. To keep the concentration of PCP in plasma constant, a combined infusion of NaPCP and mannitol in saline was given after the priming dose of NaPCP via a cannula in the vena jugularis externa at a rate of 13.6 ml/kg/hr. The concentration of mannitol in the infusion was 75 mg/ml and that of NaPCP was either 0.286, 0.143, 0.069 or 0.034 μmol/ml. The infusion rates of NaPCP were calculated to make up for the loss of PCP from plasma by elimination.

Harmol in saline was administered (26 μmol/kg body weight) within 1 min after the start of the combined infusion via a lateral tail vein, and bile and urine were collected for 4 hr and stored at −20° for the determination of harmol sulfate and harmol glucuronide as described elsewhere [13]. At the end of the experiments, blood was collected from the aorta for the determination of PCP.

Determination of PCP in plasma. PCP in plasma was determined according to a modification of the procedure of Haugh *et al.* [14]: 100 μl of a 1 M potassium dihydrogen phosphate solution of pH 4.4 was added to 1 ml of plasma and PCP was extracted 3 times with 2.5 ml of ethyl acetate. After each addition of ethyl acetate, the resulting two layers were mixed for 1 min on a Vortex-Mixer apparatus. The layers were separated by centrifugation, and ethyl acetate was removed from the combined ethyl acetate fractions with nitrogen at 25°. The residue was dissolved in 250 μl of ethanol and 25 μl was used for separation by HPLC on a μBondapak-C_{18} column (Waters Ass., Millford, MA, USA) that was eluted with a mixture of methanol/0.02 M aqueous acetic acid (78:22). The solvent was delivered with a Waters model 6000 A pump at a rate of 1.5 ml/min. The effluent was monitored at 254 nm with a Waters model 440 absorbance detector. PCP elutes from the column under these conditions at 7.5 min. The amount of PCP was determined by peak height; a linear correlation was observed in the range from 5 to 75 nmol. Samples were compared directly to standard peaks to avoid the slow decrease in response due to ageing of the column.

Recovery of PCP (added to blank plasma) in this procedure was 91.5 ± 0.5% (mean ± S.E.M., n = 5) and experimental values were corrected for this.

Determination of 1- and 3-(glutathion-S-yl)-2-acetylaminofluorene and the N-O-*glucuronide conjugate of* N-*OH-AAF in bile and urine of rats*. The glutathione conjugates of *N*-OH-AAF, 1- and 3-(glutathion-*S*-yl)-2-acetylaminofluorene (1- and 3-GS-AAF) were determined in bile and urine as described in reference 15. The *N*-*O*-glucuronide of *N*-OH-AAF was determined during the same HPLC

run: when [9-^{14}C]-*N*-OH-AAF was injected in rats, a large amount of radioactivity in bile eluted from the HPLC column at 9 min in this system, preceding the 1- and 3-GS-AAF peaks. Because the *N*-*O*-glucuronide conjugate of *N*-OH-AAF is a major biliary metabolite [7], the large amount of radioactivity at 9 min was expected to represent this metabolite. Therefore, bile and urine of rats that had received [9-^{14}C]-*N*-OH-AAF were incubated with β-glucuronidase as described in reference 7; after 1 hr an equal volume of methanol was added, and the mixture was centrifuged at 5000 rpm to remove protein. Aliquots were subsequently separated by HPLC; the compound that elutes at 9 min had disappeared while it was still present in control incubations in which β-glucuronidase was omitted. Treatment of bile with β-glucuronidase liberates *N*-OH-AAF [7]; therefore, it was concluded that the peak in the chromatogram at a retention time of 9 min represents the *N*-*O*-glucuronide conjugate of *N*-OH-AAF. The amount of this metabolite was determined by radioactivity: column fractions of 0.8 min were collected and radioactivity was counted after addition of 5 ml of Hydro-Luma (Lumac Systems Inc., Titusville, FL). In some experiments, the *N*-*O*-glucuronide of *N*-OH-AAF was determined as described before [7] and by the above method; both methods gave similar results.

RESULTS AND DISCUSSION

Inhibition of harmol sulfation by administration of PCP and NaPCP in the diet or drinking water

When harmol is given intravenously to rats, it is conjugated with inorganic sulfate or glucuronic acid, and the glucuronide and sulfate conjugates are excreted in bile and urine [8].

The inhibitor of sulfation, PCP, administered by intraperitoneal injection (40 μmol/kg, 60 min before the administration of harmol), inhibited the sulfation of harmol by 71% and, by consequence, more of the dose of harmol was conjugated with glucuronic acid (Table 1) as was shown earlier [8].

When PCP was administered with the food (350 ppm), it inhibited the sulfation of harmol much less efficiently (Table 1). This was also observed when PCP was administered with the food as the sodium salt (350 ppm NaPCP) or with the drinking water (1.4 mM NaPCP). Concentrations of PCP in blood plasma were measured at the end of the experiments (between 2.00 and 2.15 p.m., 4 hr after the administration of harmol); the highest concentrations were measured in rats that had been pretreated by intraperitoneal injection of PCP, while the lowest concentrations were measured in the rats that had received PCP with their food (Table 1). Intermediate concentrations of PCP in plasma were measured in the other groups. These data suggested that the concentration of PCP in plasma of rats that had received PCP or NaPCP with their food or in their drinking water, is too low to inhibit sulfation of harmol to a high extent.

Pharmacokinetics of PCP in Wistar rats

The concentration of PCP in the plasma of rats that had received PCP or NaPCP with their food or drinking water (Table 1) is much lower than expected from the published pharmacokinetic parameters of PCP ($t_{\frac{1}{2}}$ for its biphasic elimination from plasma of 17.4 and 40.2 hr) in Sprague–Dawley rats by Braun *et al.* [16]. We had assumed complete absorption of the PCP consumed (mainly at night because rats are nocturnal animals). $t_{\frac{1}{2}}$ of 17.4 and 40.2 hr should have resulted in much higher plasma levels (>480 μM). One of the possible explanations for the low plasma concentration of PCP that we found might be a faster elimination of PCP from plasma in our Wistar rats as compared to Sprague–Dawley rats. Therefore, we have studied elimination of PCP from plasma in our Wistar rats after intravenous administration of NaPCP.

At various times after the injection of NaPCP rats were anaesthetized with diethylether (2 for every point of time), and blood was collected from the aorta. PCP was determined in blood plasma and the results are shown in Fig. 1. The pharmacokinetic parameters were obtained by fitting the plasma concentrations of PCP to a multi-exponential model by means of a least-squares method, using the computer programme RUGFIT (1980), developed by Dr. A. H. J. Scaf, Department of Pharmacology, State University of Groningen. This programme was tested and compared to other programmes in a recent study [17]. The results are given in Table 2. The distribution and elimination of PCP was best described by a two-compartment, open system model with half-life values of 2.17 and 7.24 hr for the two phases of the plasma disappearance curve of PCP. The concentration of PCP in the central compartment $C_1(t)$ is described in this model by

$$C_1(t) = Ae^{-\alpha t} + Be^{-\beta t} \quad (1)$$

in which A and B are related to the dose and the parameters in Table 1 as described in ref. [18].

The value of 0.192 hr^{-1} for r_e in our Wistar rats (Table 2) is much greater than that of 0.0343 hr^{-1} in Sprague–Dawley rats reported by Braun *et al.* [16]. The volume of V_1 in Wistar rats is 58% greater than in Sprague–Dawley rats. Thus, the elimination of PCP from plasma in Wistar rats is faster than in Sprague–Dawley rats; this may in part explain the low plasma concentrations of PCP in rats when PCP or NaPCP was administered with the food or with the drinking water.

As rats are nocturnal animals and consume food and water mainly at night time, variations in the concentration of PCP in plasma are to be expected due to the rapid elimination of PCP from plasma. Therefore, the plasma concentration of PCP was determined at various times during a 24 hr day after administration of NaPCP with the drinking water of rats for 1 week. The results show that the highest concentrations were reached at night (260–280) μM, Fig. 2) while at daytime the plasma concentration was lower due to the rapid elimination of PCP from plasma. A similar situation probably exists when PCP or NaPCP were administered with the food.

The area under the curve (AUC) for PCP from Fig. 2 for the whole period or 24 hr yields a value of 3975 μmol l^{-1} hr. The AUC can also be calculated for the case that the oral dose is completely absorbed

Table 1. Inhibition of sulfation of harmol in the rat *in vivo* by dietary administration of PCP and NaPCP for 1 week

	Number of animals	Intake of PCP (μmols/kg/24 hr)	Body weight after 1 week§†	Plasma concentration of PCP‡ (μM)	% of the dose excreted in bile and urine		% inhibition of sulfation of harmol
					Harmol glucuronide	Harmol sulfate	
Controls	4	0	128 ± 10	0	34 ± 1	63 ± 3	0
PCP in food (350 ppm)	4	190 ± 11	114 ± 9	30 ± 3	50 ± 4*	45 ± 2*	29 ± 4
NaPCP in food (350 ppm)	5	200 ± 14	116 ± 10	36 ± 5	52 ± 3*	41 ± 4*	35 ± 7
NaPCP in drinking water (1.4 mM)	4	162 ± 16	108 ± 9**	40 ± 8	54 ± 3*	35 ± 2*	45 ± 3
Single dose of PCP (40 μmol/kg; intraperitoneally) 60 min before harmol	4	(40)	128 ± 2	55 ± 4	75 ± 1*	18 ± 1*	71 ± 1

† The body weight of the animals at the start of the experiments was 94 ± 1 g (mean ± S.E.M.; $n = 17$).

‡ Blood was taken from the rats at the end of the experiments, 4 hr after the administration of harmol. For further experimental conditions refer to the materials and methods section. All values are expressed as means ± S.E.M. * means significantly different from controls at $P < 0.05$; ** the same at $P < 0.1$ (Wilcoxon's test [21]).

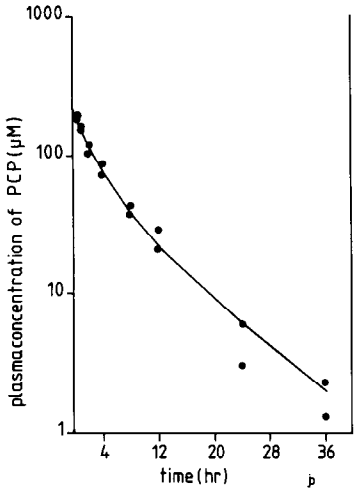

Fig. 1. Plasma concentration of PCP in the Wistar rat after a single intravenous dose of 40 μmol/kg of NaPCP.

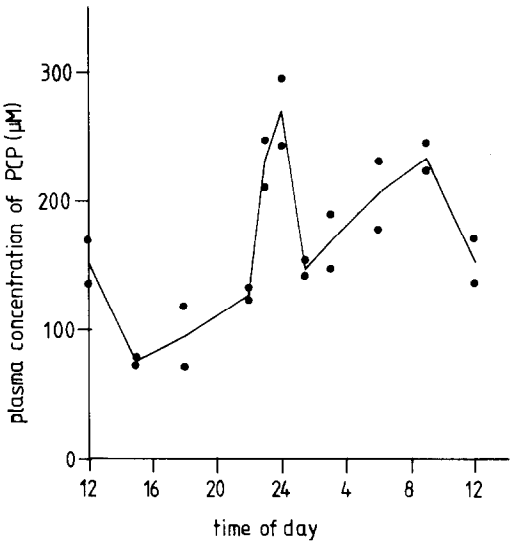

Fig. 2. Variation of the plasma concentration of PCP in rats during administration of NaPCP (1.4 mM) in their drinking water. The rats had received NaPCP in their drinking water for one week preceeding the experiment.

by using the equation (2) from reference [18].

$$\mathrm{AUC} = \frac{A}{\alpha} + \frac{B}{\beta} \qquad (2)$$

A, B, α and β are the same as used in equation [1]. This gives a value for the AUC of 4400 μmol l^{-1} hr. The experimentally determined value for the AUC is 90% of the calculated value. Thus, the 24-hr intake of NaPCP with the drinking water becomes almost totally available in plasma. This indicates that NaPCP is almost completely absorbed from the intestinal lumen; this is in agreement with the results of Braun *et al.* [16] who found that PCP, administered orally in corn oil, is completely absorbed.

Correlation between plasma concentration of PCP and inhibition of sulfation of harmol in the rat in vivo

The data in Table 1 suggested a correlation between the plasma concentration of PCP and the degree of inhibition of harmol sulfation. Therefore, experiments were performed in which the inhibition of harmol sulfation was measured at a constant concentration of PCP in plasma; this was achieved by giving to the rats an intravenous priming dose of NaPCP, followed by an intravenous infusion at a rate that compensated for the elimination of PCP from plasma. This rate, $Q(t)$, was calculated by using equation (3)

$$Q(t) = C_1(t) \times r_e \times V_1 \qquad (3)$$

$C_1(t)$ is given in equation (1) and r_e and V_1 are the same as in Table 2. The infusion was started 6.3 or 11 hr after the priming dose. These times are sufficiently long after the initial rapid distribution phase of PCP. Harmol was administered at the same time as the infusion with PCP was started, and bile and urine were collected during 4 hr. To determine the plasma concentration of PCP at the onset of the infusion, other groups of rats were injected with NaPCP intravenously (80, 40 or 20 μmol/kg) and the plasma concentrations of PCP were determined 6.3 and 11 hr thereafter. The results show that the plasma concentration of PCP remains more or less constant during the 4 hr period after the start of the infusions (Table 3). When the plasma concentration of PCP is high, less harmol sulfate is formed than at low PCP concentrations. An opposite effect was observed for the amount of harmol glucuronide that was formed. If the logarithm of the PCP plasma concentration at the beginning of the 4 hr period (most of the harmol is conjugated during the first minutes; Koster *et al.* [19]) is correlated with the inhibition of harmol sulfation (least-squares method) a correlation coefficient of 0.963 is obtained for linear correlation. If the data of Table 3 are extrapolated to a situation of complete inhibition of sulfation, the corresponding plasma concentration of PCP would be 500–600 μM. This indicates that sulfation of harmol (and maybe other phenolic compounds) can

Table 2. Pharmacokinetic parameters of PCP in male Wistar rats calculated from the plasma elimination of PCP after intravenous administration of NaPCP (40 μmol/kg body weight)

Parameter	Parameter value
r_e (hr^{-1})	0.192
r_{12} (hr^{-1})	0.064
r_{21} (hr^{-1})	0.160
α (hr^{-1})	0.319
β (hr^{-1})	0.096
V_1 (l/kg)	0.192
V_2 (l/kg)	0.077

The plasma elimination of PCP was described by a two-compartment, open system model:

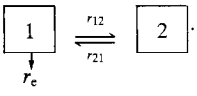

Table 3. Correlation between the plasma concentration of PCP and inhibition of harmol sulfation

Priming dose of NaPCP (μmol/kg)	Start of NaPCP infusion (hr after priming dose)	Infusion rate of NaPCP (μmol/hr/kg)	Plasma concentration of PCP at the start of the infusion (μM)	Plasma concentration of PCP 4 hr after the start of the infusion (μM)	Metabolites of harmol excreted in bile and urine during 4 hr (% of the dose)		% inhibition of sulfation of harmol
					Harmol sulfate	Harmol glucuronide	
0	0	0	0	0	63 ± 3†	34 ± 1†	0
20	11	0.47	13 ± 0.2	14–20	56–64	37–41	0–11
40	11	0.94	25 ± 0.3	26–29	56–57	44–46	10–11
40	6.3	1.94	48–48	52–52	41–43	52–58	32–35
80	6.3	3.89	81 ± 0.8	69–n.d.	28–33	63–63	48–56

The priming doses of NaPCP were given intravenously via a lateral tail vein. The infusions of NaPCP were combined with the administration of mannitol in saline at an infusion rate of 13.6 ml/kg/hr in the vena jugularis externa. Harmol was administered via a lateral tail vein when the combined NaPCP/mannitol was started. Bile and urine were collected during 4 hr after the administration of harmol. Harmol sulfate, harmol glucuronide and PCP in plasma were determined as described in the materials and methods section. Individual values are given for most experiments; some values, however, are means ± S.E.M. of 3 experiments.

† These data were taken from Table 1 and were used to calculate the percentage of inhibition of harmol sulfation. n.d. means not determined.

Table 4. Effect of administration of NaPCP with the drinking water on the excretion of metabolites of *N*-OH-AAF in bile and urine in Sprague–Dawley and Wistar rats

	% of the dose			
	Sprague–Dawley		Wistar	
	Controls	NaPCP	Controls	NaPCP
Total metabolites in bile and urine after 4 hr	75.0 ± 3.4	94.9 ± 1.1*	84.0 ± 2.0	89.5 ± 1.3
N-*O*-glucuronide	29.4 ± 2.7	60.9 ± 1.1*	36.4 ± 3.4	57.3 ± 2.8*
1-GS-AAF	6.6 ± 0.8	2.1 ± 0.3*	3.8 ± 0.7	1.0 ± 0.3*
3-GS-AAF	9.3 ± 0.8	3.1 ± 0.5*	6.3 ± 1.0	1.5 ± 0.5*

NaPCP (1.4 mM) was administered with the drinking water for 1 week. [9.^{14}C]-*N*-OH-AAF (0.9 mCi/mmol; 60 μmol/kg) was administered intravenously, and bile and urine were collected on ice subsequently during 4 hr. Metabolites of *N*-OH-AAF were analysed by HPLC as described in the materials and methods section. All values are the mean ± S.E.M. of four experiments.

* Means significantly different from control values at $P < 0.05$ (Wilcoxon's test [21]).

never be inhibited completely by PCP in rats *in vivo* because this high plasma concentration of PCP would kill the rats. The data of Table 3 suggest that below a concentration of 10 μM PCP no inhibition of sulfation occurs.

Inhibition of sulfation of N-*OH-AAF by administration of NaPCP with the drinking water*

We have investigated whether the administration of NaPCP with the drinking water inhibits the *N-O*-sulfation of N-OH-AAF in the rat *in vivo*, and whether it might be used for long-term inhibition of sulfation of this carcinogen. NaPCP (1.4 mM) was added to the drinking water of Sprague–Dawley rats for one week at the end of this period, *N*-OH-AAF was injected as described in Materials and Methods. As described above, the *N-O*-sulfate conjugate of *N*-OH-AAF cannot be measured directly because it is unstable in aqueous media [20] and decomposes before it can be excreted in bile or urine. However, we have shown previously that *N-O*-glucuronidation takes over when *N-O*-sulfation of N-OH-AAF is inhibited; therefore, under these conditions, the amount normally *N-O*-sulfated can be inferred from the increase in the amount of the stable *N-O*-glucuronide in bile and urine [7].

In both rat strains, a major part of the dose of *N*-OH-AAF was excreted in bile and urine as the *N-O*-glucuronide conjugate (predominantly in bile). When NaPCP was given with the drinking water, the *N-O*-glucuronide conjugate was increased by 32% in Sprague–Dawley rats and by 21% in Wistar rats. This indicates that *N-O*-sulfation of the *N*-OH-AAF most likely accounts for at least 32% of the dose in Sprague–Dawley rats and by 21% in Wistar rats. The latter value is the same as reported before [7]. This shows that administration of NaPCP with the drinking water effectively inhibits *N-O*-sulfation of *N*-OH-AAF in the rat. This is supported by the finding that NaPCP also inhibited the formation of the glutathione conjugates 1-GS-AAF and 2-GS-AAF in Sprague–Dawley rats by 67% and in Wistar rats by 75% (Table 4). Since these conjugates most likely are formed from the reactive *N-O*-sulfate ester of *N*-OH-AAF in rats *in vivo* [15], the inhibition of their formation indicates that *N-O*-sulfation of *N*-OH-AAF was inhibited by about 70%.

In conclusion, administration of NaPCP to rats with their drinking water is a convenient way to inhibit *N-O*-sulfation of *N*-OH-AAF (and, most likely, similar compounds) and may be used in long-term studies to investigate the role of sulfation in the carcinogenesis by *N*-OH-AAF.

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