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# SHORT COMMUNICATIONS

## Formation of pentachlorophenol glucuronide in rat and human liver microsomes

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Pentachlorophenol (PCP)* is a widely used fungicide which has been increasingly found as an environmental contaminant. Toxicological properties and pharmacokinetics of this substance have recently been reviewed [1]. The major part of an oral dose was found in urine as free PCP [2–5]. In rats, 6–16% of the dose were recovered from urine as PCP glucuronide [3, 5]. In pilot experiments, PCP glucuronide was found to be unstable at pH 5, the pH normally used when glucuronides are identified by hydrolysis with β-glucuronidase. Therefore we investigated PCP glucuronide synthesis in rat and human liver microsomes and its stability at urinary pH. In the rat liver model of differential induction [6–11] it was found that glucuronidation of PCP is neither inducible by 3-methylcholanthrene nor by phenobarbital. Based on the appreciable rate of PCP conjugation in liver and the instability of the conjugate at the urinary pH it is concluded that the determination of PCP glucuronide in urine leads to an underestimation of the amount of this conjugate originally excreted via the kidney.

*Materials and methods*

Pentachlorophenol (purity 99%) was from EGA (Steinheim, F.R.G.). $^{14}$C-Pentachlorophenol (specific activity 6.8 mCi/mmole), originally supplied from Hoechst AG (Frankfurt), was a kind gift from Dr. G. Koss (Marburg, F.R.G.). UDP-[U-$^{14}$C]glucuronic acid, ammonium salt (specific activity 307 mCi/mmole) was from Amersham Buchler (Braunschweig, F.R.G.). β-Glucuronidase from rat preputial glands was purified as described [12].

Pretreatment of male Wistar rats with 3-methylcholanthrene or phenobarbital was carried out as described [9]. Microsomes were prepared as described [6]. Samples from human liver were kindly provided by Dr. G. Brunner (Hannover, F.R.G.). Microsomal protein was determined as described [13].

*UDP-glucuronosyltransferase assay with PCP as substrate*

The assay was carried out under conditions similar to those described for other substrates [10]. $^{14}$C-PCP (0.68 mCi/mmole, final concentration 0.5 mM) was added in a small volume (20 μl) of dimethylsulfoxide to the assay mixture [10] which contained microsomal protein (0.5 mg/ml) and the nonionic detergent Brij 58 (0.5 mg/mg protein) in a final volume of 1.0 ml. In blank samples, the cofactor UDP-glucuronic acid was omitted. Reactions were stopped by addition of chloroform (2 ml) and vigorous shaking. Precipitation of protein by perchloric acid [10] was avoided because of the lability of the glucuronide. After two extractions with chloroform (2 ml) radioactivity of PCP glucuronide was determined in the aqueous phase.

Glucuronidation rates were found linear for up to 20 min under these conditions. This rapid method is sufficient when using liver microsomes as enzyme source. However, radioactivity of the blank samples (without UDP-glucuronic acid) was found rather high (corresponding to about 3 nmoles PCP per assay), probably due to the incomplete extraction of PCP under these conditions. This may be explained by the low $pK_a$ (4.9) of PCP.

* Abbreviations used: PCP, pentachlorophenol; HPLC, high performance liquid chromatography.

*Isolation and determination of PCP glucuronide by HPLC*

PCP glucuronide was synthesized in microsomal suspensions as described above and the reaction was stopped by addition of an equal volume of methanol containing 5 mM tetrabutylammonium hydroxide. After centrifugation aliquots of 0.2 ml of the supernatant were injected onto a μ-Bondapak C-18 HPLC column (30 × 0.4 cm). Ion-paired, reversed phase HPLC was carried out using 50% (v/v) aqueous methanol containing 2.5 mM tetrabutylammonium as counterion and 25 mM phosphate, adjusted to pH 7.0. The flow rate was 1 ml/min.

Chromatography was followed either radiometrically by collecting fractions of 0.5 or 1 ml or by u.v. detection at 254 nm. 200 pmoles PCP glucuronide per assay could be determined by means of the peak area of absorption of the glucuronide.

*Hydrolysis of PCP glucuronide*

PCP glucuronide was synthesized enzymatically by incubating PCP (0.5 mM) in presence of 1 mg microsomal protein/ml for 1 hr. It was isolated by HPLC as described above. pH-dependent hydrolysis of PCP glucuronide was studied by incubating the glucuronide in presence of acetate buffer, pH 4.5–6.2, or phosphate buffer, pH 7.4, at 37°. At the time points indicated in Fig. 2, aliquots of 0.2 ml were mixed with tetrabutylammonium in buffered methanol and analysed by HPLC. Hydrolysis of the glucuronide was followed by determining the peak area of absorption of the conjugate.

Complete enzymatic hydrolysis of PCP glucuronide was achieved by incubation at pH 7.4 for 1 hr in presence of 25,000 Fishman units of β-glucuronidase. In control experiments carried out in presence of 10 mM saccharo-1,4-lactone, loss of PCP glucuronide was less than 10%.

*Results and discussion*

$^{14}$C-PCP glucuronide was synthesized in liver microsomes either with $^{14}$C-PCP or UDP-[U-$^{14}$C]-glucuronic acid and isolated by HPLC (Fig. 1). No radioactivity was found at the position of the glucuronide when microsomes, PCP or UDP-glucuronic acid were omitted (not shown). Complete hydrolysis of PCP glucuronide was achieved by incubation with β-glucuronidase at neutral pH (see Methods).

In earlier attempts to isolate PCP glucuronide the conjugate was found to be unstable during thin layer chromatography with silica as solid phase, particularly when acidic eluents were used. pH-dependent hydrolysis of PCP glucuronide is shown in Fig. 2. Whereas PCP glucuronide is stable at neutral pH for several hours, considerable hydrolysis occurs at pH 4–6. It is therefore conceivable that unconjugated PCP in urine [2–5] partially originates from hydrolysis of PCP glucuronide.

UDP-glucuronosyltransferase activities towards PCP in rat and human liver microsomes are shown in Table 1.

Glucuronidation rates of PCP are lower than those found with other planar phenols, such as 1-naphthol, 4-nitrophenol or phenol [6–10, 14, 15]. In rat liver microsomes, glucuronidation rates were not appreciably altered by pretreatment of rats with phenobarbital or 3-methylcholanthrene. Hence, in the rat liver model of differential induction, PCP would be classified as a group 3 substrate [11, 16]. From its planar structure, PCP would have been

expected to be a group 1 substrate [7, 8, 11, 14]. These findings suggest that planarity of the substrate cannot be the only requirement for classification as a group 1 substrate.

In a human liver sample, the rate of PCP glucuronidation was one third of the rate found in rat liver (Table 1). This sample appears to be representative since enzyme activities towards 1-naphthol, 4-nitrophenol and morphine were in the range found in other liver samples from a liver bank [15].

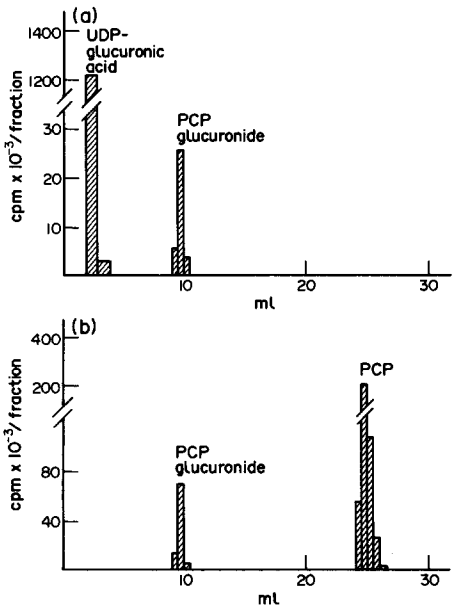


Fig. 1. Isolation and identification of labelled PCP glucuronide. Labelled PCP glucuronide was enzymatically synthesized either by incubation with UDP-$^{14}$C-glucuronic acid (2.7 mCi/mmole) (a) or with $^{14}$C-PCP (6.8 mCi/mmole) (b) and subsequently isolated by HPLC as described in Methods.

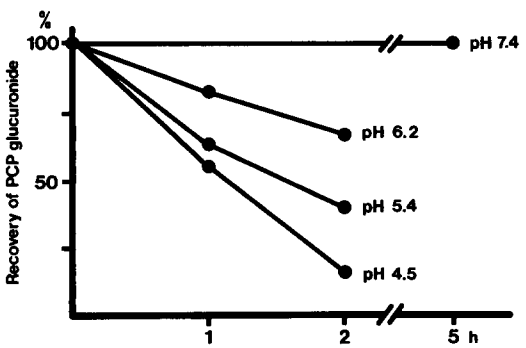


Fig. 2. pH-dependent hydrolysis of PCP glucuronide. Experiments were carried out as described in Methods. Data points are the means of 2 separate experiments.

In summary, a sensitive method for the isolation and determination of PCP glucuronide by HPLC is described. PCP glucuronide is formed at appreciable rates in rat and human liver microsomes. Since PCP glucuronide is partly hydrolyzed under the weakly acidic conditions normally found in urine, determination of PCP glucuronide in urine leads to an underestimation of the amount of this conjugate originally excreted via the kidney.

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Table 1. UDP-Glucuronosyltransferase activities in rat and human liver microsomes using pentachlorophenol as substrate

| Species | Enzyme activity (nmole/min/mg protein)* | | |
|---|---|---|---|
| | Untreated controls | 3-Methylcholanthrene-treatment | Phenobarbital-treatment |
| Rat | 5.7 ± 1.3 | 5.6 ± 1.4 (1.0) | 7.0 ± 1.0 (1.2) |
| Man | 2.0 ± 0.3 | — | — |

* Mean value ± S.D. of 4 experiments. The induction factor, i.e. the ratio of enzyme activities obtained from treated versus untreated animals, is given in parentheses.