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Simultaneous assay of pentachlorophenol and its metabolite, tetrachlorohydroquinone, by gas chromatography without derivatization

BRUNO G. REIGNER*, JEAN F. RIGOD and THOMAS N TOZER

Department of Pharmacy, School of Pharmacy, University of California San Francisco, San Francisco, CA 94143-0446 (U.S A.)

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ABSTRACT

A sensitive capillary gas chromatographic method was developed for the simultaneous determination of pentachlorophenol and its major metabolite, tetrachlorohydroquinone, in plasma, urine and feces The method involved a simple one-step liquid–liquid extraction with diethyl ether and electron-capture detection gas chromatography on a fused-silica capillary column coated with 50% methylsilicone–50% trifluoropropylsilicone The detection limit of both compounds was 50 ng/ml in plasma (from an initial volume of 0 1 ml), 100 ng/ml in urine and 100 ng/g in feces Optimal conditions for both chemical and enzymatic hydrolysis were defined to measure conjugates of both pentachlorophenol and tetrachlorohydroquinone in urine. Tetrachlorohydroquinone was found to be unstable in plasma and urine, means to prevent its degradation during sample collection and storage by addition of ascorbic acid and ethylenediaminetetraacetic acid are presented This chromatographic method was shown to be precise, accurate and specific It was successfully applied to toxicokinetic studies in rat.

INTRODUCTION

Pentachlorophenol (PCP) is a general biocide used extensively as a wood preservative [1]. Studies have shown that PCP uncouples oxidative phosphorylation [2], is fetotoxic in rats [3] and, from a recent investigation carried out by the National Toxicology Program, is carcinogenic in mice [4]. This toxicant is of concern as an occupational hazard for people exposed to it in the wood industry (3 to 6 million people) and as an environmental pollutant since widespread use and improper disposal have made PCP an occasional contaminant of ground water supplies [5].

Metabolism studies carried out in rodents have indicated that PCP is conjugated with glucuronic acid and undergoes oxidative dechlorination to form tetrachlorohydroquinone (TCHQ), which is further metabolized to a glucuronide [6–8] The formation of TCHQ in humans remains controversial since, in a few studies, this compound has not been detected in urine [6,9,10]. Although the contribution of TCHQ to the toxicity observed after administration of PCP to

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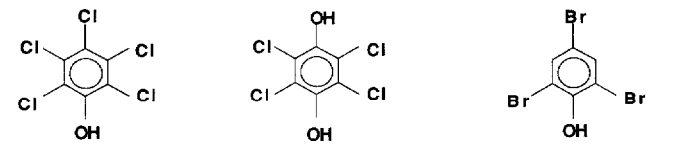
animals has not been investigated, TCHQ has a lower intraperitoneal median letal dose (LD_{50}) (28 ± 2 mg/kg) than PCP (59 ± 4 mg/kg) in mice [11]. Moreover, as suggested by *in vitro* studies [12], TCHQ is likely to be oxidized *in vivo* to tetrachloro-1,4-benzoquinone (TCBQ) which belongs to a chemical class containing several well known carcinogenic compounds [13]. Toxicokinetic studies of both PCP and TCHQ (Fig. 1) should allow a better understanding of PCP toxicology; a reliable assay method is therefore required.

Many papers have been published on the use of gas chromatography (GC) with electron-capture detection (ECD) for analysis of PCP in biologic fluids (see review [1]). However, most of the methods are assays for PCP alone. Only one method describes the simultaneous assay of PCP and the major metabolite, TCHQ, by GC but it involves derivatization and mass spectrometric (MS) detection [6]. The purpose of this study was to develop a sensitive and simple GC method with ECD to measure simultaneously PCP and TCHQ in plasma, urine and feces collected during toxicokinetic studies in rats. The conditions required to hydrolyze completely the conjugated metabolites of PCP and TCHQ in urine by chemical hydrolysis and treatment with β-glucuronidase and sulfatase were investigated. Data on TCHQ instability in plasma and urine, and means to prevent its degradation during sample collection and storage, are also presented.

EXPERIMENTAL

Chemicals

PCP (99% purity), 2,4,6-tribromophenol (TBP) (Fig. 1), TCBQ, hexachlorocyclohexane and diethylenetriaminepentaacetic acid (DTPA) were obtained from Aldrich (Milwaukee, WI, U.S.A.). TCHQ was purchased from Kodak (Rochester, NY, U.S.A.). Ethyl acetate was GC grade and supplied by EM Science (Cherry Hill, NJ, U.S.A.). Diethyl ether (organic residue analysis grade) and ethylenediaminetetraacetic acid (tetrasodium salt, dihydrate, EDTA) were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). Ascorbic acid, tris(hydroxymethyl)aminomethane (Trizma® base), β-glucuronidase and sulfatase were pur-



Pentachlorophenol (PCP)

Tetrachloro-1,4-hydroquinone (TCHQ)

2,4,6-Tribromophenol (TBP)

Fig 1. Structures of pentachlorophenol, its metabolite tetrachloro-1,4-hydroquinone and 2,4,6-tribromophenol (the internal standard)

chased from Sigma (St Louis, MO, U.S.A.) All other chemicals were of reagent grade (Fisher Scientific, Springfield, NJ, U.S.A.).

Standard stock solutions

The standard stock solutions of TCHQ and TBP were made by dissolving 50 mg of each in 50 ml of ethanol (with 10 m*M* ascorbic acid) and ethyl acetate, respectively These solutions were stored at −20°C and diluted with water just before use. An isotonic pH 7.4 phosphate buffer solution of 1.5 mg of PCP per milliliter, for intravenous injection to the rat, was used as PCP standard stock solution. This solution was stored at 4°C and diluted with water just before use.

Instrumentation

GC analyses were carried out using a Hewlett-Packard Model 5890 chromatograph (Palo Alto, CA, U.S.A.) with a ^{63}Ni electron-capture detector and a Hewlett-Packard Model 3396A integrator. Separation was performed on a silica capillary DB-210 column (30 m × 0.32 mm I.D. film thickness 0.5 μm) from J & W Scientific (Folsom, CA, U.S.A.).

An Ultra-2 (Hewlett-Packard, Palo Alto, CA, U.S.A.) column (0.2 m × 0.32 mm I.D., 0.52 μm film thickness) was used as a pre-column and post-column to protect the DB-210 column from the high temperatures set at the injection and detection ports, 250 and 300°C, respectively. The column was operated isothermally at 190°C for 7 min to separate and elute the compounds of interest. When analyzing urinary and fecal samples, the oven temperature was increased at 40°C/min to 230°C at the end of the 7-min run and maintained at this level for 5 min, to elute undesirable endogenous compounds. The carrier gas (helium) column head pressure was 83 kPa. The chromatograph was equipped with a split–splitless capillary injection port and a silanized splitless liner. Split sampling was performed with a ratio of 10:1. Split vent and septum purge flow-rates were 30 and 3 ml/min, respectively.

Separation of TCHQ and TCBQ by high-performance liquid chromatography (HPLC)

Separation of TCHQ and TCBQ was achieved using an HPLC method similar to that described previously by Van Omnen *et al.* [12]. An Alltech (Deerfield, IL, U.S.A.) 25 cm × 4.6 mm I.D. column packed with C_{18} (5 μm, Econosil) was used. The flow-rate of the mobile phase, 55% methanol in a 1% aqueous acetic acid solution, was 1 ml/min. The eluent was monitored with a UV detector set at 288 nm, the TCBQ maximum absorption wavelength.

Collection of samples

Because PCP glucuronide is unstable at pH values below 7.4 [14] and TCHQ is unstable in urine and plasma (see Results and discussion), the biologic samples from rat were collected using the following procedures. Blood, about 0.4 ml, was

collected in small tubes containing about 0.01 g of a mixture of EDTA and ascorbic acid (70:30 w/w) to obtain a final concentration in blood close to 42 m*M* of each compound and to keep the pH equal to 7.4. After stirring, the blood was centrifuged at 1000 *g* for 10 min and then the plasma was transferred to a culture tube. The rats were housed in metabolism cages to collect urine and feces separately. Under the container designed to receive the urine, a magnetic stirrer was used to mix continuously the urine with 1 ml of a solution containing 0.1 *M* ascorbic acid and 0.1 *M* EDTA (pH 7.4). Since the daily urine production in rat is about 20 ml, the final concentration was about 5 m*M* ascorbic acid. A solution containing 5 m*M* ascorbic acid and 5 m*M* EDTA (pH 7 4) was used to dilute feces so that 1 ml of fecal suspension corresponded to 0.1 g of feces. The biologic samples were stored frozen at −20°C until they were analyzed.

Preparation of plasma samples

Frozen plasma samples were thawed at room temperature. To 0.1 ml of plasma in a borosilicate culture tube were added 0.3 ml of citrate buffer (0.1 *M*, pH 3), 80 μl of an aqueous solution of the internal standard (1 μg/ml TBP) and 200 μl of water. After vortex-mixing, extraction was performed twice with 2 ml of diethyl ether using a rotator (Glas-Col Apparatus Company, Terre-Haute, IN, U.S.A.) at 20 rpm for 10 min. After centrifugation (1000 *g* for 5 min), the ether phases were pooled in a silanized culture tube. The combined ether phase was evaporated at room temperature under a nitrogen stream to a smaller volume (about 0.5 ml). After adding about 60 mg of anhydrous sodium sulfate (to remove traces of water from organic phase), a 2-μl volume was injected directly into the gas chromatograph.

Preparation of urine samples

Unconjugated PCP and TCHQ. Frozen urine samples were thawed at room temperature. To 0 1 ml of urine were added 0.2 ml of phosphate buffer (0.1 *M*, pH 7.4) and 80 μl of TBP (1 μg/ml). After vortex-mixing, extraction was performed twice with 2 ml of diethyl ether for 10 min on the rotator. The ether phases were pooled and evaporated under nitrogen to a smaller volume (about 0.5 ml). After addition of about 60 mg of anhydrous sodium sulfate, a 2-μl volume was injected into the gas chromatograph.

Conjugated PCP and TCHQ. To measure conjugated PCP and TCHQ concentrations, both chemical hydrolysis and enzymatic hydrolysis with β-glucuronidase and sulfatase were performed.

For chemical hydrolysis, 200 μl of water and 100 μl of 1.5 *M* sulfuric acid were added to 0.1 ml of urine in a pyrex screw-cap culture tube. The tubes were capped and placed in a 70°C heating block for selected times up to 8 h. After the tubes were cooled to room temperature, an aqueous solution (80 μl) of the internal standard (1 μg/ml) was added. Extraction with diethyl ether and subsequent steps of preparation of samples for chromatography were the same as those with plasma samples.

For enzymatic hydrolysis, 80 μl of a TBP solution (1 μg/ml) and 200 μl of a solution containing 0.1 *M* ascorbic acid, 5 m*M* DTPA, 250 U/ml β-glucuronidase (type VII, from *Escherichia coli*) or 0.57 U/ml sulfatase (type VI, from *Aerobacter aerogenes*) were added to 0.1 ml of urine, and the pH was adjusted to 7.4 with Trizma® base. The tubes were then capped and incubated at 37°C in a water bath with constant shaking, for selected times, up to 4 and 48 h for β-glucuronidase and sulfatase, respectively. After cooling to room temperature, subsequent steps were the same as those for unconjugated urine.

Preparation of feces samples

To 1 ml of fecal suspension (0.1 g of feces) in a pyrex screw-cap culture tube were added 200 μl of water and 500 μl of 1.5 *M* sulfuric acid. The tubes were capped and placed in a 70°C heating block for 4 h. After the tubes were cooled to room temperature, a 80-μl volume of TBP solution (1 μg/ml) was added. The extraction was performed twice with 2 ml of diethyl ether. Subsequent steps were similar to those used in the treatment of plasma samples.

Calibration curves

Calibration curves in plasma, urine and feces were obtained by spiking blank samples with PCP and TCHQ and using TBP as an internal standard. The peak-height ratios (PCP/TBP and TCHQ/TBP) were plotted as a function of concentration. Values of slopes and intercepts, obtained from linear regression analysis, were used to calculate the unknown concentrations in biologic samples.

RESULTS AND DISCUSSION

Gas chromatographic separations

Methods using derivatizations with diazomethane, diazoethane, acetic anhydride, acetyl chloride, triethylsulfonium hydroxide and trimethylchlorosilane have been used to determine PCP in biologic fluids [1]. Because these methods may require the use of hazardous reagents (*e.g.* diazomethane) and are time-consuming, we tried to analyze PCP and TCHQ without derivatization. Butte *et al.* [15] considered that "GLC separation and quantification of traces of PCP without derivatization is not recommended because this compound is rather a strong acid and may be adsorbed irreproducibly during the chromatographic separation". The chromatogram obtained after extraction of plasma spiked with TBP, PCP and TCHQ (Fig. 2) exhibited satisfactory peak shapes, without tailing and a good intra-day assay precision was obtained (see *Reproducibility and accuracy*). To date, two other authors [16,17] could measure PCP concentrations by capillary GC without derivatization, a worthwhile simplification of sample work-up.

Attempts to separate PCP and its major metabolite, TCHQ, were not succesful with non-polar columns. These two relatively polar compounds are well separat-

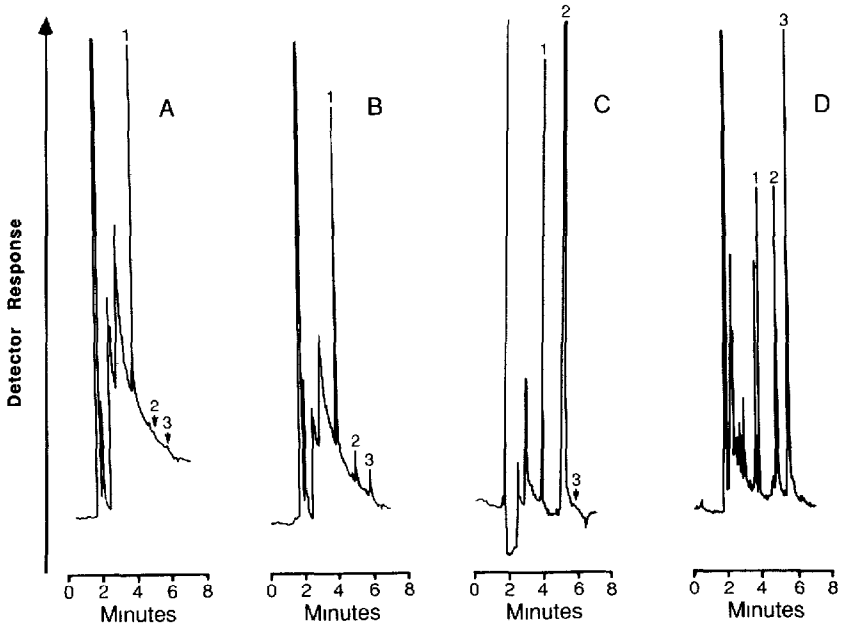


Fig 2 Chromatograms of extracts from (A) a blank plasma sample, (B) the same plasma spiked with 50 ng/ml PCP (2) and TCHQ (3), (C) a plasma sample taken 1 5 h after administration of 2.5 mg/kg PCP to a rat and (D) a chemically hydrolyzed urine sample from a rat administered 2 5 mg/kg PCP. All samples contain the internal standard TBP (1)

ed on a capillary column coated with 50% trifluoropropylsilicone –50% methylsilicone (DB-210) as shown in Fig. 2B and D.

The retention times of the putative metabolites of PCP which can potentially interfere in the assay were determined (Table I). TCBQ exhibits the same retention time as TCHQ Attempts to separate these compounds with a more polar

TABLE I

RETENTION TIMES OF PCP, TCHQ, TBP AND PUTATIVE METABOLITES

Compound	Retention time (min)
PCP	4 9
TCHQ	5.5
TBP	3.8
2,3,5,6-Tetrachlorophenol	3 4
2,4,6-Trichlorophenol	2 6
Tetrachlorocatechol	No peak[a]
Tetrachloro-1,2-benzoquinone	No peak[a]
TCBQ	5.5

[a] Peaks may have occurred in solvent front These compounds have melting points much lower than the corresponding paraisomers.

column (Carbowax 20M, Hewlett-Packard) were not successful. The occurrence of TCHQ and not TCBQ as a urinary metabolite of PCP was established by the following HPLC experiment To 1 ml of fresh rat urine spiked with TCBQ (concentration 10 μg/ml) was added 0 5 ml of pH 3 citrate buffer and the extraction was performed with 4 ml of diethyl ether. The extract was evaporated to dryness, a 500 μl-volume of mobile phase was added and a 100-μl volume was injected into the HPLC system. The resulting chromatogram showed no peak at the retention time of TCBQ (15.1 min) but a TCHQ peak (11 2 min) was obtained. Reduction of TCBQ to TCHQ occurs in fresh urine since, when water (instead of urine) was spiked with TCBQ and the extraction carried out as previously described with urine, a peak at only the TCBQ retention time was observed. The presence of endogenous ascorbic acid in rat urine (about 0.1 m*M*) [18] may explain the reduction of TCBQ to TCHQ. Due to its instability, TCBQ does not contribute to the TCHQ peak height. Therefore, the GC method, as described in this paper, specifically measures TCHQ.

Extraction

As expected from the pH partition hypothesis, the extraction recovery of PCP, an acid with a pK_a of 4.7 [1], is increased when the aqueous phase is buffered to a low pH. However, metabolites of PCP are conjugates, and undesirable hydrolysis at low pH may occur during sample work-up. Consequently, urine collected for 24 h and plasma withdrawn 5 h after administration of PCP (2.5 mg/kg) were extracted using different pH values (all buffers were 0.1 *M*). As shown in Fig. 3, hydrolysis, as measured by an increase in the concentration, is observed under more acidic conditions. For PCP in plasma and TCHQ in urine, it occurred at pH values lower than 2, whereas hydrolysis of PCP conjugates in urine started when the pH was lower than 6. These observations corroborate the results of Lilienblum [14] who showed that PCP glucuronide at 37°C is stable at pH 7.4 but

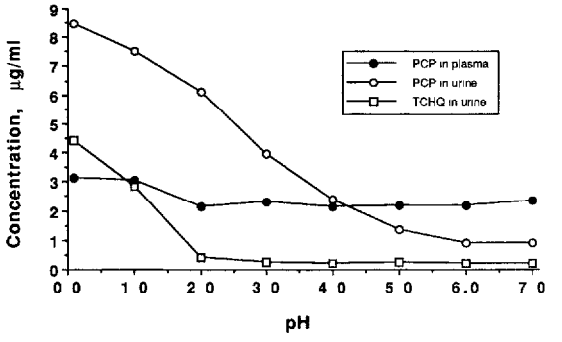


Fig 3 Concentrations measured in urine collected for 24 h and in plasma withdrawn 5 h after administration of PCP (2 5 mg/kg to a rat) as a function of pH of the buffer added to the biologic medium before extraction with diethyl ether

TABLE II

ABSOLUTE EXTRACTION RECOVERY OF PCP, TCHQ AND TBP FROM PLASMA

Concentration (μg/ml)	Absolute recovery[a] (%)		
	PCP	TCHQ	TBP
10	93±5	84±5	79±2
1 0	83±4	76±8	81±2
0 1	89±6	84±18	85±6

[a] Absolute recovery in percent was determined by comparing peak-height ratios with and without performing the extraction procedure The peak-height ratio was the ratio of the peak height of the compound and the external standard, hexachlorocyclohexane Each value represents the mean ± S D of four determinations

degrades when the pH is 6.2 or less. This finding is unexpected with PCP glucuronide, since ether glucuronides are usually considered to be stable compounds [19]. The effect of extraction pH on PCP hydrolysis yield is quantitatively different in urine and plasma. This difference can be explained by the concentration of the conjugate being low, relative to total PCP, in plasma and high in urine. Practically, this result led us to extract urine after addition of a pH 7.4 buffer and plasma after acidification to pH 3.

Extraction with diethyl ether gives high recovery from plasma as shown in Table II. Recoveries were independent of the concentration within the tested range (0 1–10 μg/ml). Diethyl ether was found to give better recovery than pen-

TABLE III

INTRA-DAY ASSAY PRECISION AND ACCURACY IN THE DETERMINATION OF PCP AND TCHQ IN SPIKED PLASMA

Compound	Concentration added (μg/ml)	Concentration measured[a] (μg/ml)	C.V (%)	Relative error[b] (%)
PCP	0 05[c]	0.052	8.0	+3.7
	1	0 96	9 7	−4 1
	10	9 39	2 4	−6 1
TCHQ	0 05[c]	0 054	7 8	+7 3
	1	1 09	6 0	+8 9
	10	9 98	1 3	−0 2

[a] Mean value of six determinations

[b] Relative error = $\frac{(\text{concentrations measured} - \text{concentration added})\ 100}{\text{concentration added}}$

[c] Limit of detection

tane, which was expected because molecules containing hydroxyl groups tend to require more polar solvents for extraction.

Reproducibility and accuracy

Intra-day assay precision and accuracy were evaluated from the analyses of six plasma samples at three concentrations over the range 0.05–10 μg/ml for both PCP and TCHQ. Accuracy was within 9% of theoretical and the coefficient of variation (C.V.) was lower than 10% for PCP and TCHQ. These results are presented in Table III. Inter-day assay variabilities for both PCP and TCHQ were also investigated (Table IV). The coefficient of variation ranged from 4.5 to 10.7% for PCP and from 4.0 to 6.0% for TCHQ.

Linearity and limit of detection

Standard curves were linear ($0.992 < r^2 < 0.999$) at both low (0.05–0.5 μg/ml) and high (0.5–20 μg/ml) ranges when PCP and TCHQ were added to biologic fluids.

The limit of detection, defined as the concentration at which the signal-to-noise ratio is about 3, was 50 ng/ml for both PCP and TCHQ when 0.1 ml of plasma was used; at this concentration, the reproducibility was about 8% (Table III). Typical chromatograms from a blank plasma and from the same plasma spiked with 50 ng/ml PCP and TCHQ are presented in Fig. 2A and B. Although lower concentrations could have been measured in urine and feces, particularly with a higher volume of the biologic medium, the limit of detection was 100 ng/ml using 0.1 ml of urine and 100 ng/g using 0.1 g of feces.

TABLE IV

INTER-DAY ASSAY PRECISION AND ACCURACY IN THE DETERMINATION OF PCP AND TCHQ IN SPIKED PLASMA

Compound	Concentration added (μg/ml)	Concentration measured[a] (μg/ml)	C.V. (%)	Relative error[b] (%)
PCP	0.1	0.102	10.7	+ 2.2
	1	1.02	8.9	+ 1.8
	10	10.6	4.5	+ 6.5
TCHQ	0.1	0.112	6.0	+11.8
	1	1.00	5.9	+ 0.2
	10	9.6	4.0	− 4.1

[a] Mean value of six determinations.

[b] Relative error = $\frac{(\text{concentrations measured} - \text{concentration added}) \ 100}{\text{concentration added}}$

Hydrolysis experiments

To assay both PCP and TCHQ conjugates in urine and feces, measurements of PCP and TCHQ concentrations were performed before and after both chemical and enzymatic (β-glucuronidase and sulfatase) hydrolyses. Chemical hydrolysis was carried out with sulfuric acid and the formation of PCP and TCHQ was measured as a function of time (Fig. 4). In urine, the hydrolysis of conjugates appeared to be virtually complete within an hour. To assure total hydrolysis, 4 h were used in the toxicokinetic studies. Due to the presence of β-glucuronidase and sulfatase in the gastrointestinal tract of the rat [20] and the consequent problem of interpreting the results, we did not measure the unconjugated PCP and TCHQ in feces Concentrations in this medium were measured only after chemical hydrolysis.

Hydrolysis of urine with β-glucuronidase enzymes from *E. coli* showed the presence of PCP glucuronide and TCHQ glucuronide as illustrated in Fig. 5. The hydrolysis was complete after about 1 h. These results were quite unexpected since Ahlborg *et al.* [6] reported that enzymatic hydrolysis of urine was unsuccessful because TCHQ strongly inhibits β-glucuronidase with a concentration causing 50% inhibition (I_{50}) of 0.5 μg/ml (with *p*-nitrophenol-β-D-glucuronide as a substrate). In our experiment, the concentration of TCHQ (in the incubation solution, not in urine) was below the I_{50}, which may explain why we could observe PCP and TCHQ formation with β-glucuronidase treatment. Later, the same author reported that bovine liver β-glucuronidase had been used successfully to hydrolyze the conjugates present in urine [7]. Using both enzymes (β-glucuronidase from *E. coli* and bovine liver) at their optimal pH values, we observed similar results: the differences between each pair of concentrations (enzyme minus control, for a given time) were about the same, for both sources of the enzyme. The higher concentrations obtained with the enzyme from bovine liver in both control and enzyme curves likely came from an increased non-specific hydrolysis due to the lower optimum pH used with the β-glucuronidase from bovine liver.

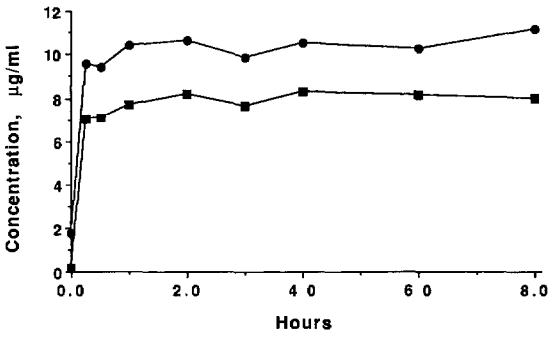


Fig 4 Formation of PCP (●) and TCHQ (■) with time after treatment of the urine with 1 5 *M* sulfuric acid and heating at 70°C. The urine was collected for 24 h after an intravenous dose of 2 5 mg/kg PCP to a rat.

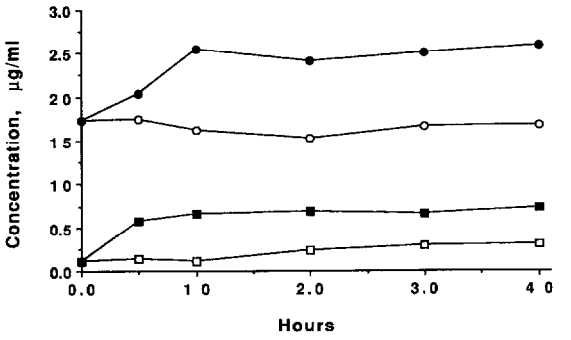


Fig. 5 Formation of PCP (●, ○) and TCHQ (■, □) with time after treatment of urine at 37°C with β-glucuronidase from *E. coli* (●, ■) and without enzyme (○, □) The urine, same sample as in Fig 4, was collected for 24 h after intravenous administration of 2 5 mg/kg PCP to a rat

Formation of PCP and TCHQ with time after treatment of the urine with sulfatase are presented in Fig 6. The hydrolysis of the sulfates of PCP and TCHQ was virtually complete in 12 h. The hydrolysis of PCP sulfate appeared to be the faster of the two compounds. Non-specific hydrolysis occurred in controls, particularly with PCP conjugates. Since the sulfatase used is considered specific (no β-glucuronidase activity at pH 7.0, according to the manufacturer), these results proved indirectly the presence of PCP sulfate and TCHQ sulfate as metabolites of PCP in rat after administration of a 2.5 mg/kg dose. In toxicokinetic studies, the experiments with sulfatase were performed over 24 h for complete hydrolysis of TCHQ sulfate.

These hydrolysis experiments indicated that PCP sulfate and TCHQ sulfate accounted for a large proportion of the conjugates hydrolyzed chemically. These observations are in contrast to previous metabolism studies in the rat, in which the conjugates were claimed to be only glucuronides [6,7].

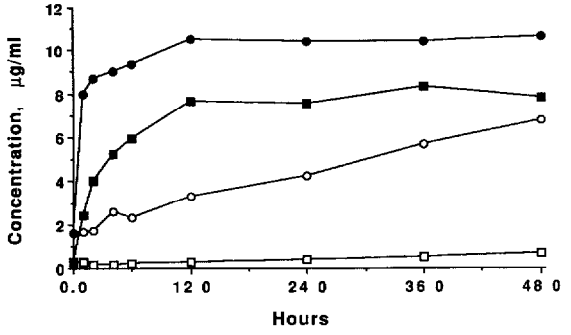


Fig 6 Formation of PCP (●, ○) and TCHQ (■, □) with time after treatment of urine at 37°C with sulfatase from *Aerobacter aerogenes* (●, ■) and without enzyme (○, □) The urine, same sample as in Figs 4 and 5, was collected for 24 h after intravenous administration of 2 5 mg/kg PCP to a rat

Stability

PCP was stable in aqueous solutions at 4°C for at least two weeks. No PCP stability problem was observed with samples of plasma, urine and feces frozen at −20°C for a month. At room temperature, the concentration of PCP was unchanged when plasma and urine samples were analyzed within 24 h. These results support the stability of PCP, as reported before by McConnell [4].

In contrast to PCP, TCHQ is very unstable in urine and plasma, as shown in Fig. 7. At room temperature (24°C), the concentration in blank plasma and urine spiked to 10 μg/ml with TCHQ declined very quickly, particularly in plasma. In our experimental conditions, the apparent half-lives of degradation were 92 and 11 min in urine and plasma, respectively. The addition of ascorbic acid (5 m*M*) and EDTA (5 m*M*) was shown to prevent the degradation of TCHQ for 24 h at room temperature and for a month when the samples were frozen at −20°C. The prevention of the TCHQ degradation by ascorbic acid, a well known antioxidant, suggested that TCHQ is oxidized spontaneously to TCBQ. The latter, if formed, may be further transformed, since the addition of ascorbic acid after disappearance of TCHQ does not give back the original compound. This observation is consistent with the covalent binding of TCBQ to bovine serum albumin previously reported by Van Omnen *et al.* [12].

Since ascorbic acid itself undergoes autooxidation in aqueous solution, chelating agents, which sequester metal ions catalyzing this reaction, were added to stabilize this antioxidant. As expected from previous studies [21,22], EDTA gave satisfactory results at room temperature, 4°C and −20°C. However, in the hydrolysis experiments with β-glucuronidase and sulfatase (incubation at 37°C), EDTA did not totally prevent the loss of TCHQ, presumably because of ascorbic acid degradation, and was replaced by DTPA as suggested by Nishikimi and Ozawa [23]. TCHQ instability might explain inconsistent results concerning the occurrence of TCHQ as a metabolite of PCP in humans [6,9,10] and the fact that, in

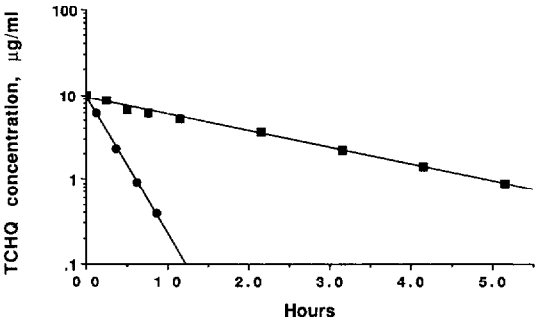


Fig 7 Stability of TCHQ with time in blank plasma (●) and blank urine (■) Experiments were carried out at room temperature (24°C) without addition of ascorbic acid and EDTA. Frozen plasma and urine samples were thawed at room temperature and left on the bench 2–4 h before they were spiked to 10 μg/ml

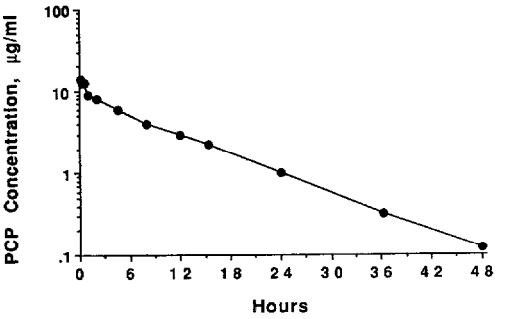


Fig 8 PCP concentrations in plasma *versus* time after intravenous administration of 2 5 mg/kg PCP to a rat

rat, the percentage of the administered dose excreted in urine as TCHQ has been underestimated in previous studies [6–8].

Application

This method was successfully applied to toxicokinetic studies in the rat. Concentrations of PCP in plasma and PCP, TCHQ and their conjugates in urine and feces were determined after intravenous administration of 2.5 mg/kg PCP. Plasma PCP half-life was found to be about 8 h and therefore this method allowed measurement of PCP concentrations for more than five half-lives in plasma (Fig. 8) and concentrations of PCP and its metabolites in urine after daily collection for three days. An example of concentrations of PCP and its metabolites measured in urine collected for 24 h after administration of PCP is shown in Fig. 9.

After more than a thousand samples had been analyzed, the column retained the same chromatographic resolution of peaks in spite of the injection at a non-washed ether phase obtained from a one-step extraction of the biologic samples. The low volume of biologic fluid used, which limits the amount of endogenous

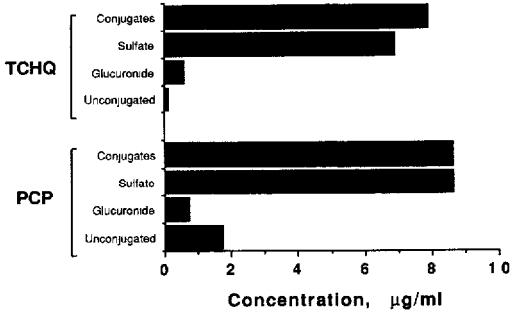


Fig 9 Concentrations of PCP, TCHQ and their conjugates in urine collected for 24 h (same sample as in Figs. 4, 5 and 6) after administration of 2 5 mg/kg PCP to a rat Concentrations were measured before hydrolysis and after treatment with sulfuric acid, β-glucuronidase and sulfatase

compounds extracted and then injected, may explain the longevity of the column. Tailing of peaks and irreproducibility of the peak-height ratio after injection of the same extract were observed during the development of the method. These problems were solved by using a silanized splitless liner and replacement of the liner every other week.

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