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Pharmacokinetics and metabolism of a single subneurotoxic oral dose of tri-*o*-cresyl phosphate in hens

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Abstract. Hens were given a single oral dose of 50 mg (4.6 μCi)/kg [^{14}C] tri-*o*-cresyl phosphate (TOCP). Four groups of three hens each were killed after 0.5, 1, 2, and 5 days. The half-life of ^{14}C in plasma was 2 days. TOCP and its metabolites in the plasma, liver, kidneys, and lungs were analyzed by high-performance liquid chromatography and liquid scintillation counting. TOCP reached its highest concentration in plasma between 0.5 and 1 day after administration. Under these experimental conditions, the disappearance of TOCP from the plasma followed monoexponential kinetics with a half-life of 2.2 days. Appreciable concentrations of saligenin cyclic-*o*-tolyl phosphate, the active neurotoxic metabolite, were detected in the plasma as well as in the liver, kidneys, and lungs at all time points and had half-lives of 2.06, 1.36, 1.11 and 4.44 days, respectively. The presence of this active metabolite of TOCP might contribute to the sensitivity of the hen to TOCP-induced delayed neurotoxicity. Other hydrolytic and oxidative products of TOCP were also identified in tissues.

Key words: TOCP – OPIDN

Introduction

Tri-*o*-cresyl phosphate (TOCP), an industrial chemical used as a plasticizer, is among organophosphorus compounds capable of producing delayed and prolonged neurotoxicity (OPIDN, organophosphorus compounds-induced delayed neurotoxicity) (Smith et al. 1930; Abou-Donia 1981). Certain animal species (cats, dogs, cows and chickens) are susceptible to OPIDN, while others (rodents and some primates) are less sensitive. TOCP is used as positive control in screening organophosphorus chemicals for OPIDN in hens (EPA 1985).

Previous studies showed that TOCP was metabolized in several species to a more potent esterase inhibitor (Casida et al. 1961). This active metabolite, identified as saligenin cyclic-*o*-tolyl phosphate or 2-(*o*-cresyl)4H-1:3:2 benzodioxaphoran-2-one, has been found in rats (Eto et al. 1962; Nomeir et al. 1984), chickens (Sharma and Watanabe 1974), and cats (Taylor and Buttar 1967; Nomeir and Abou-Donia 1984, 1986a) treated with TOCP. Saligenin cyclic-*o*-tolyl phosphate has been demonstrated to be at least 5 times more neurotoxic to chickens as TOCP (Bleiberg and Johnson 1965; Buttar et al. 1968).

The present investigation reports the disposition, metabolism, and pharmacokinetics of TOCP in plasma and some tissues of the hen. These results may, in part, elucidate the mechanism of species sensitivity to OPIDN, since they contrast those reported in the rat (Nomeir et al. 1984) a less sensitive species.

Materials and methods

Chemicals. Radioactive tri-*o*-cresyl-(phenyl-U-^{14}C) phosphate (TOCP), specific activity 4.83 mCi/mmol, was obtained from Midwest Research Institute, Kansas City, MO. The radiochemical purity of [^{14}C]TOCP was 98% according to thin-layer chromatography on silica gel which was determined with the following solvents: methylene chloride (Rf = 0.30), 1:2 ethyl acetate:hexane (Rf = 0.34), and 1:4 ethyl acetate:hexane (Rf = 0.29). TOCP (99%) was obtained from Eastman Kodak Co., Rochester, NY, and purified as described earlier (Nomeir and Abou-Donia 1983). *o*-Cresyl dihydrogen phosphate (98%), di-*o*-cresyl hydrogen phosphate (99%), saligenin cyclic-*o*-tolyl phosphate (99%), hydroxymethyl TOCP (94%) and dihydroxymethyl TOCP (92%) were synthesized as described by Nomeir and Abou-Donia (1986b). Salicylic acid (99%), *o*-cresol (99%), *o*-hydroxybenzaldehyde (salicylaldehyde) (98%), and *o*-hydroxybenzylalcohol (97%) were purchased from Aldrich Chemical Co., Inc., Milwaukee, WI. Solvents for high-performance liquid chromatography (HPLC) were obtained from Fisher Scientific, Raleigh, NC. Nylon filters, 0.45 μm, were purchased from Rainin Instrument Co., Inc., Woburn, MA. All other chemicals used in this study were of the highest purity available.

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Treatment of animals. Laying hens (*Gallus gallus* domesticus), 18 months old, weighing approximately 1.88 ± 0.06 kg, (mean ± SE) were obtained from Featherdown Farm, Raleigh, NC. Twelve hens were each given a single oral dose of 50 mg (4.6 μCi)/kg [^{14}C]TOCP in a gelatin capsule (Abou-Donia et al. 1990). The birds were housed in individual metabolism cages in a temperature-controlled room (21–23° C) with a 12-h light cycle and free access to food (Layena chicken feed, Ralston Purina Co., St Louis, Mo.) and water. Groups of three hens per sampling time were killed at 0.5, 1, 2, and 5 days following treatment. The animals were anesthetized with a 30 mg/kg intraperitoneal injection of sodium pentobarbital (Letalis solution, Barber Veterinary Supply Co., Inc., Fayetteville, NC), and the blood was collected in a heparinized syringe via heart puncture and centrifuged to separate plasma from red blood cells. Liver, kidney, and lung were removed and weighed. Plasma and red blood cell volumes were calculated as 5% and 2% of body weight, respectively (Sturkie 1976).

Analysis of radioactivity. Radioactivity was determined by liquid scintillation counting (Packard Tri-Carb Model 3255, Packard Instrument Co., Inc., Downers Grove, Ill.). All data were corrected for background interferences, dilution effects, quenching, and counting efficiency. Samples from fresh tissues and body fluids were oxidized by combustion using a Packard tissue oxidizer (Model 306B) as described earlier (Abou-Donia 1980).

Extraction procedure. Extraction of TOCP and its metabolites from plasma and selected tissues was carried out as described (Nomeir and Abou-Donia 1984): ethyl ether was used for plasma extraction, ethyl acetate for liver, kidney and lung extraction.

Kinetic analysis. The kinetic analysis of TOCP and its metabolites was carried out by plotting their concentration versus time on semilogarithmic paper. The terminal half-life of TOCP was calculated from the elimination rate constant, β. The β value was obtained by linear regression of the terminal exponential decline in their concentration, using the expression

$$t_{1/2} = 0.693/\beta. \quad (1)$$

The total area under the TOCP and metabolites concentration versus time curves for plasma AUC_{plasma} and the various tissues AUC_{tissue} was calculated by the trapezoidal rule and extrapolated to infinity by using the last data point and the respective terminal linear exponential decline.

The relative residence, R_R (Abou-Donia et al. 1983), of TOCP and its metabolites in selected tissues with respect to plasma was calculated acccording to equation 2:

$$R_R = \frac{AUC_{tissue}}{AUC_{plasma}} \quad (2)$$

where the AUCs are as defined above. The R_R values reflect the exposure or accumulation of TOCP and its metabolites in specific tissue relative to plasma. The use of this parameter avoids the time-dependent changes in concentration ratios, which has recently been illustrated for TOCP and EPN (Abou-Donia et al. 1983; Nomeir and Abou-Donia 1984). R_R is a time-independent parameter that, in cases of linear kinetics, predicts the ratio of average concentrations of the xenobiotics in specific tissues C_{tissue}, to those in plasma, C_{plasma}, or equation 3:

$$R_R = \frac{C_{tissue}}{C_{plasma}} \quad (3)$$

The relative abundance, R_A, (Nomeir and Abou-Donia 1984) of each metabolite with respect to TOCP in specific tissues was calculated according to equation 4:

$$R_A = \frac{AUC_{metabolite}}{AUC_{TOCP}} \quad (4)$$

in the same tissue. The R_A values represent the concentration of each metabolite in each tissue relative to TOCP for all time points. The use of R_A and R_R avoids the time-dependent change in concentration ratios.

Chromatographic analysis. High-performance liquid chromatography (HPLC) (Waters Associates, Milford, MA) was used to analyze TOCP and its metabolites as described previously [Nomeir and Abou-Donia 1983). These compounds were eluted on a microparticulate C_{18} cartridge fitted into a radial compression separation system with a linear gradient of 22–80% acetonitrile in 2% aqueous acetic acid at a flow rate of 1.3 ml/min for 22 min at 25° C. The compounds were detected and quantified by monitoring the ultraviolet absorbance of the column eluates at 254 nm and measuring the peak area. A mixture of TOCP and authentic standards of nine of its metabolites was injected into HPLC with various tissue extracts. Radioactive material eluted from the HPLC was collected in scintillation vials. To each vial, was added 15 ml scintillation fluid consisting of ethylene glycol monomethyl ether/toluene (2:1 v/v) containing 1.67 g PRO and 67 mg POPOP/l. All samples were filtered through a 0.45 μm nylon filter prior to HPLC analysis. Duplicate analyses were carried out for each sample.

Results

Analysis of TOCP and its metabolites

TOCP and its nine metabolites were separated by reversed-phase HPLC. The relationship between peak area and the amount injected was linear over a 100-fold range for all compounds analyzed. The minimum detectable level was 3 μg for *o*-hydroxybenzyl aldehyde, 25 ng for *o*-hydroxybenzyl alcohol and *o*-hydroxybenzoic acid, and 50 ng for other compounds. Retention time for TOCP was 22.95 min.

Clinical and necropsy observations

Hens treated with a single oral dose of 50 mg/kg [^{14}C]TOCP did not exhibit signs of acute cholinergic or delayed neurotoxic effects. Also, tissues of treated hens were not different from controls; weight, color, and shape were comparable after dosing schedules.

Tissue deposit of radioactivity

Total content and percentage of TOCP-derived radioactivity measured in specific tissues are listed in Table 1. The highest amount of ^{14}C was present in plasma followed by liver, kidney, and lung. Radioactivity in plasma and other tissues reached a peak 1 day after dosing then declined thereafter.

Recovery of TOCP and metabolites from plasma and tissues

Plasma and selected tissues were extracted with organic solvents and analyzed by HPLC and liquid scintillation counting to identify and quantify TOCP and its metabolites (Table 2). The average percentages of recovery of radioactivity extracted at various time intervals were plasma, 35%; liver, 40%; kidney, 40%; and lung, 73%. Percentage recovery values represent the ratios of radioactivity extracted relative to total radioactivity determined for each tissue.

Plasma. TOCP and its nine metabolites were identified and quantified in the plasma and tissues of hens at all time points following the oral administration of a single oral

Table 1. Total[a] and percentage[b] radioactivity in some tissues of hens after a single oral dose of 50 mg/kg [^{14}C]TOCP

Specimen	Days after dosing							
	0.5		1		2		5	
	Total ^{14}C	%	Total ^{14}C	%	Total ^{14}C	%	Total ^{14}C	%
Plasma	394 ± 41	0.42	546 ± 55	0.58	246 ± 23	0.26	160 ± 18	0.17
Liver	326 ± 35	0.35	336 ± 40	0.36	192 ± 20	0.20	79 ± 7	0.08
Kidney	160 ± 16	0.17	198 ± 20	0.21	80 ± 9	0.09	28 ± 2	0.03
Lung	64 ± 6	0.07	84 ± 8	0.09	31 ± 3	0.03	16 ± 2	0.02

[a] Total radioactivity is expressed as µg TOCP equivalent per wet tissue or plasma. Each value is the mean ± SE for six samples from three hens
[b] Percentage of radioactivity were calculated from the total radioactivity determined in each tissue relative to the applied dose

Table 2. Concentration[a] of TOCP and metabolites in tissues of hens after a single oral 50 mg/kg [^{14}C]TOCP

Specimen	Day	Neurotoxic compounds		Non-neurotoxic metabolites							
		TOCP	Saligenin cyclic-*o*-tolyl phosphate	Di-*o*-Cresyl hydrogen phosphate	*o*-Cresyl dihydrogen phosphate	*o*-Cresol	*o*-Hydroxy-benzyl alcohol	*o*-Hydroxy-benzal-dehyde	*o*-Hydroxy-benzoic acid	Hydroxy-methyl TOCP	Dihydroxy-methyl TOCP
Plasma	1/2	0.18	0.17	0.23	0.33	0.18	0.14	0.16	0.15	0.17	0.15
	1	0.18	0.23	0.26	0.23	0.18	0.20	0.21	0.37	0.20	0.17
	2	0.16	0.13	0.13	0.13	0.13	0.14	0.04	0.12	0.13	0.13
	5	0.05	0.06	0.05	0.48	0.05	0.05	0.04	0.04	0.05	0.04
Liver	1/2	0.22	0.20	0.20	0.24	0.22	0.22	0.21	0.22	0.19	0.23
	1	0.35	0.32	0.38	0.49	0.35	0.31	0.39	0.46	0.29	0.44
	2	0.26	0.23	0.20	0.22	0.29	0.25	0.21	0.22	0.22	0.31
	5	0.05	0.04	0.43	0.04	0.06	0.04	0.06	0.06	0.05	0.05
Kidney	1/2	0.39	0.34	0.53	0.47	0.40	0.36	0.39	0.46	0.32	0.33
	1	0.60	0.60	0.78	0.73	0.61	0.80	0.66	0.70	0.53	0.54
	2	0.24	0.24	0.23	0.24	0.25	0.26	0.24	0.24	0.24	0.30
	5	0.15	0.05	0.05	0.05	0.24	0.05	0.05	0.05	0.04	0.06
Lung	1/2	0.25	0.20	0.25	0.18	0.19	0.21	0.27	0.20	0.31	0.18
	1	0.27	0.23	0.24	0.25	0.21	0.20	0.22	0.28	0.21	0.21
	2	0.18	0.12	0.12	0.12	0.12	0.12	0.13	0.12	0.12	0.12
	5	0.19	0.11	0.10	0.10	0.11	0.09	0.12	0.10	0.10	0.11

[a] Concentrations were calculated from the recovered radioactivity from HPLC and are expressed as ng TOCP or metabolite per g fresh tissue or ml plasma. Each value represents the mean of six determinations from three hens. SE value ($n = 3$) was not more than 15%

dose of 50 mg/kg TOCP (Table 2). TOCP reached its peak concentration at 12 h, while its metabolites exhibited their maximum levels between 12 and 24 h after dosing. TOCP disappeared from the plasma with a terminal half-life of 2.2 days (Table 3). Half-lives for TOCP metabolites in plasma ranged from 1.42 days for salicylic acid to 2.18 days for *o*-cresol. TOCP was the most abundant compound in plasma, followed by di-*o*-cresyl hydrogen phosphate and saligenin cyclic-*o*-tolyl phosphate, the active metabolite of TOCP. *o*-Hydroxybenzoic acid was the least abundant metabolite (R_A, 0.87) followed by dihydroxymethyl TOCP and hydroxymethyl TOCP with R_A values of 0.88 and 90, respectively.

Tissues. TOCP and its metabolites reached their highest concentration levels in the analyzed tissues between 12 and 24 h after dosing; these concentrations dropped significantly by day 5. In liver the most abundant compound was dihydroxymethyl TOCP (R_A, 1.16) followed by *o*-cresol (R_A, 1.11), with di-*o*-cresyl hydrogen phosphate being the least abundant (R_A, 0.88). The half-lives were longest (1.46 days) for *o*-cresol, *o*-hydroxybenzyl alcohol, and hydroxymethyl TOCP, while *o*-cresyl dihydrogen phosphate had the shortest half-life.

In the kidney, *o*-cresol was the only metabolite that was more abundant than TOCP (R_A, 1.5), and it also had the longest half-life ($T_{1/2}$, 3.80 days), followed by TOCP ($t_{1/2}$, 2.29 days). The least abundant metabolite was hydroxymethyl phosphate.

The kidney contained more TOCP than any of its metabolites, which had R_A values ranging from 0.27 to 0.51. TOCP also had the longest half-life of 11.71 days. Other metabolites had relatively longer half-lives than those in the plasma, liver, or kidney, and they ranged from 5.6 to 3.23 days for dihydroxymethyl TOCP and *o*-hydroxy benzoic acid.

TOCP and all of its metabolites had longer relative residence (R_R) values, which reflect their accumulation in

Table 3. Area under the concentration versus time curve (AUC) relative abundance (R_A)[a], and relative residence (R_R)[b] ratios of TOCP and its metabolites in hen tissues after a single oral dose of 50 mg/kg [^{14}C]TOCP

Specimen		Neurotoxic compounds		Non-neurotoxic metabolites							
		TOCP	Saligenin cyclic-*o*-tolyl phosphate	Di-*o*-cresyl hydrogen phosphate	*o*-Cresyl dihydrogen phosphate	*o*-Cresol	*o*-Hydroxy-benzyl alcohol	*o*-Hydroxy-benzalde-hyde	*o*-Hydroxy-benzoic acid	Hydroxy-methyl TOCP	Dihydroxy-methyl TOCP
Plasma	$t_{1/2}$	2.20	2.06	1.77	1.70	2.18	1.97	1.67	1.42	1.94	2.13
	AUC	775	762	768	773	702	713	674	741	696	679
	R_A	1.00	0.98	0.99	1.00	0.91	0.92	0.87	0.96	0.90	0.88
	R_R	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Liver	$t_{1/2}$	1.32	1.36	1.29	1.17	1.46	1.33	1.46	1.38	1.46	1.20
	AUC	1052	995	928	1062	1163	990	1017	1094	921	1220
	R_A	1.00	0.91	0.88	1.09	1.11	0.94	0.97	1.04	0.88	1.16
	R_R	1.36	1.25	1.21	1.37	1.66	1.39	1.51	1.48	1.32	1.80
Kidney	$t_{1/2}$	2.29	1.11	1.05	1.09	3.80	1.03	1.07	1.08	1.12	1.23
	AUC	1804	2229	1446	1411	2706	1378	1304	1378	1166	1353
	R_{AA}	1.00	1.23	0.80	0.78	1.50	0.76	0.72	0.76	0.65	0.75
	R_R	2.33	2.93	1.89	1.83	3.85	1.93	1.93	1.86	1.68	1.99
Lung	$t_{1/2}$	11.71	4.44	3.50	3.49	5.19	3.66	4.10	3.23	4.42	5.60
	AUC	4059	1312	1172	1116	2080	1076	1370	1120	1240	1520
	R_A	1.00	0.32	0.29	0.27	0.51	0.27	0.34	0.28	0.32	0.37
	R_R	5.24	1.72	1.53	1.44	2.96	1.51	2.03	1.50	1.78	2.24

[a] R_A ratio is calculated according to the equation $R_A = AUC_{metabolite}/AUC_{TOCP}$

[b] R_R ratio is calculated according to the equation $2R_R = AUC_{compound\ tissue}/AUC_{compound\ plasma}$

all analyzed tissues than the plasma. In general the largest R_R values were present in lung, followed by kidney, and liver had the smallest R_R values. TOCP had the highest R_R of 5.24 in lung, while the active metabolite, saligenin cyclic-*o*-tolyl phosphate, and R_R values of 1.25, 1.61, and 1.72 in the liver, kidney, and lung, respectively.

Discussion

In this study with a non-neurotoxic 50 mg/kg dose of TOCP, only 4.3% of the total radioactivity in the plasma, at 12 h following administration, was identified as TOCP, indicating that TOCP was rapidly metabolized to other products. This finding is in contrast to that found with a neurotoxic 770 mg/kg oral dose of TOCP in hens (Sharma and Watanabe 1974), where 86% of the radioactivity was identified as unmetabolized TOCP 12 h after dosing. The present and previous studies suggest that ingesting large doses of TOCP, unlike that of a small non-neurotoxic dose, may saturate or inhibit metabolic and elimination processes. This in turn would result in higher concentrations of TOCP and its active metabolite in circulation leading to OPIDN.

When the pharmacokinetic profile of TOCP in hen plasma is compared with that in the plasma of rats given the same single oral dose (Nomeir et al. 1984), it is evident that TOCP concentration in rats was far less (about 6- to 13-fold less) than that of the chicken. Also, the $t_{1/2}$ of TOCP was 53 h and 46 h in chicken and rat plasma, respectively. More significantly, the active metabolite of TOCP, saligenin cyclic-*o*-tolyl phosphate, was present in a much higher concentration in the plasma of chickens compared to the rat; differences ranged between 13- and 65-fold during the first 2 days. At day 5 it was not detected in the plasma of rats, while 56 µg/ml was found in the plasma of the chicken. Also, the plasma half-lives of this metabolite were 49 h and 18 h in the hen and rat plasma, respectively. These differences in the rate of deposition and disappearance of TOCP and its active metabolite in the plasma of the chicken and the rat may contribute to the selective sensitivity to OPIDN observed in these two species. The chicken seems to be less capable of clearing TOCP and its active metabolite from the plasma compared to the rat. Other factors that may be involved in the differential sensitivity of these animal species to OPIDN may be differences in the delayed neurotoxicity target proteins and/or rapid repair of the nervous tissue in the rat compared to the chicken (Abou-Donia 1983; Abou-Donia and Nomeir 1986).

The high level and long $t_{1/2}$ of TOCP in the lung may be explained by the highly perfused nature of the lung, which has a high density capillary bed. TOCP metabolites were less abundant than the parent compound, as indicated by smaller R_A values, suggesting that the lung does not play a prominent role in the metabolism of this compound.

Although the liver contained more radioactivity than the kidney, larger concentrations of TOCP and its active metabolite saligenin cyclic-*o*-tolyl phosphate were present in the kidney than in the liver (Suwita et al. 1986). These results may be explained by the nonionic absorption and passive reabsorption of TOCP and its non-polar metabolite in the renal tubule (Milne et al. 1958), which are increased

by their high lipid solubility. On the other hand, metabolites were more abundant than TOCP in the liver, as indicated by their high R_A values. This suggests extensive biotransformation of TOCP in liver and the important role of the liver in the metabolism of this compound.

The relative residence (R_R) values of TOCP in tissues relative to plasma is indicative of its accumulation and persistence, as well as its pool size, in these tissues. All three tissues studied had higher R_R values than the plasma, suggesting that TOCP is preferentially transferable from the plasma to these tissues.

Species sensitivity to OPIDN may be influenced by disposition, metabolism, and pharmacokinetics of delayed neurotoxic organophosphorus compounds (Nomeir and Abou-Donia 1983, 1986). In vivo TOCP undergoes metabolic activation and deactivation. Only saligenin cyclic-*o*-cresyl phosphate, of the nine metabolites identified, has more acute cholinergic and delayed neurotoxicity than TOCP. Its formation is considered an activation of TOCP. All other metabolites are more polar and less toxic than the parent compound. Their production is considered a detoxification process. The balance between activation and detoxification may influence the production of OPIDN. More importantly, the extent of exposure of tissues, in general, and the neurotoxicity target in particular, to the active metabolite and its persistence in tissues seem to be determining factors in species sensitivity to OPIDN. This study reveals that TOCP and its active metabolites were present in the plasma and other tissues of treated hens to a larger extent than that found in the rat, a less sensitive species. The larger extent of metabolic activation of TOCP to saligenin cyclic-*o*-tolyl phosphate in the chicken compared to the rat, coupled with faster clearance of TOCP and its active metabolite from plasma and various tissues of rats, may, in part, contribute to differences in species sensitivity.

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