Toxicokinetics of Toxaphene in Japanese Quail (*Coturnix coturnix japonica*)

Henk-Jan Drenth, Chris J. C. van Oevelen, Martin van den Berg.
Research Institute Toxicology, Utrecht University, PO Box 80176, NL-3508 TD, Utrecht, the Netherlands.

Introduction

Toxaphene has been used as a pesticide on a huge scale, especially in cotton farming in the U.S. [1]. Toxaphene is a complex mixture consisting of chlorinated bornanes (CHBs) mainly. Presence of toxaphene has been reported in various environmental compartments, even in remote areas[1]. Relative high concentrations are detected in fish but also in birds[1].
Effects of toxaphene on liver mixed function oxidases in the rat have been reported[1,2]. To investigate effects of toxicants on cytochrome P450 enzyme activities hydroxylation of testosterone is often used. The change in formation rate of site-specific hydroxy testosterone metabolites by hepatic microsomes gives an indication of the cytochrome P450 enzymes induced or suppressed by a toxicant[3]. Haake *et al.*[2] reported induction of testosterone hydroxylase activities after exposure of rats to toxaphene. Formation of 6*β*-, 16*α*- and 16*β*-hydroxy testosterone was induced, while the formation of 6*α*-, 15*β*-, 7*α*-hydroxy testosterone and androstenedione were not affected. Effects of toxaphene on enzyme activity induction in quails are unknown, however, induction of cytochrome P450 activities after treatment with other toxicants has been reported [4,5]. Fetal Japanese quail hepatocytes exhibit testosterone hydroxylase activity. The formed metabolites include 16α-, 7α-, 15α-, 16β-, 6β-, 2α-hydroxy testosterone, and androstenedione. The formation rates of 6β-, 16α- and 16β-hydroxy testosterone appeared to be inducible by phenobarbital[6].

Four congeners of toxaphene have been identified which are present in relative high concentrations in environmental samples. These are CHB26 (also T2), CHB50 (also T12 or Toxicant Ac), CHB62, and CHB32 (also Toxicant B) [7]. De Boer et al. [8] estimated the presence of these congeners in a technical toxaphene mixture to be 0.2% for CHB26, 0.7-0.9% for CHB50, 0.6% for CHB62, and 0.5% for CHB32.

The present study was designed to investigate the kinetic behavior of these four CHBs in a bird species. As an indicator of phase I enzyme induction the effect on hepatic microsomal testosterone metabolism is investigated in the same animals. The aim of the study is to derive a toxico-kinetic model for toxaphene distribution and elimination in Japanese quails after intravenous injection.

Material and Methods

34 Female Japanese quails, 5 weeks of age, were obtained from 'De Kempische Kwartel', Lommel, Belgium. The animals were housed in two cages (2×2×2 m), with water and feed ad libitum. The light regime was 8 h. light, 16 h. darkness, to prevent the birds from laying eggs. After two weeks of acclimatization, 27 quails were exposed to 6 mg toxaphene (Campheclor, Sigma) per kg bodyweight, by intravenous injection. Toxaphene was dissolved in a 5% tween/water emulsion. Carrier control animals, injected with tween/water, were housed together with control animals, which received no treatment at all.
After 4 h., and after 1, 2, 3, 5 and 12 days four animals, and after 19 days 3 animals were sacrificed by cervical dislocation. From each animal blood, liver, brains, muscle- and adipose tissue were sampled. The liver was divided into two pieces. One piece was used for microsomal testosterone hydroxylase activity, the other, as all other samples, was analyzed for CHBs.

Microsomal enzyme activity

Liver microsomes were isolated as described by Bouwman et al.[9]. Protein content of the microsomal fraction was determined using the method of Bradford with BSA as a standard. To determine the testosterone hydroxylase activity, 20 µl microsomal suspension (300-500 µg protein) was added to 0.98 ml phosphate-buffer (50 mM phosphate, 1 mM EDTA, pH 7.4) containing 277 µM testosterone, 1 mM NADP, 5mM glucose-6-phosphate, 1 iU glucose-6-phosphate dehydrogenase. The incubation mixture was placed in a waterbath of 42°C. After 30 min the reaction was terminated by adding 5 ml dichloromethane. After adding 100 µl 11β-hydroxy testosterone (41µM) as an internal standard, vortexing and centrifugating, the aqueous phase was discarded and the organic phase was transferred to a clean tube. The solution was evaporated to dryness under a mild nitrogen stream, and the residue was dissolved in 130 µl 50% methanol/water. The hydroxy testosterone metabolites were analyzed using HPLC, as described by Funae and Imaoka[10].

Toxaphene analysis

The four Toxaphene congeners CHB26, CHB32, CHB50 and CHB62 were quantified using extraction, clean-up, and GC-NCIMS analysis as described by De Boer[11-13], with some modifications.
Adipose, muscle, brain and liver tissue were freeze-dried for 18 h. The samples were subjected to soxhlet extraction for 8 h. with 50% dichloromethane/hexane and 1,2,3,4-tetrachloronaphtalene as an internal standard. Fat content was determined by weighing the residue of the hexane/dichloromethane extractable fraction. The residue was dissolved in 5 ml hexane. A sample, containing not more than 250 mg fat, was transferred on top of a 5 g alumina column (6% water deactivated). Elution took place with 40 ml hexane, 1 ml iso-octane was added as a keeper. The solution was evaporated to approximately 1 ml. If necessary, samples were concentrated or diluted.
A sample of 2 µl was subjected to GC-NCIMS analysis, some samples were concentrated. Concentrations of the four Toxaphene congeners were determined by using an analytical standard (USL 421, Promochem, Wesel, Germany) calibration curve. GC-NCIMS conditions were as follows:

GC (Carlo Erba, MFC500)	Column:	J&W, DB-5, 625, 30 m, 0.25 µm film thickness, 0.32 mm internal diameter.
	Carrier gas	Helium (75 kPa).
	Temperatures	Injector: 270°C, oven: 80°C 2 min., 20°C/min to 200°C, 15°C/min to 230°C, 10 °C/min to 280°C, 10 min at 280°C.
	Injection:	2 µl splitless (1 min).
NCIMS (Carlo Erba QMD1000)	Temperatures	Transferline: 175°C, source: 150°C
	Ionisation gas	Methane (tuned using PFTA masses 312, 414, 595 m/z).
	Scanned ions	Internal standard: 264, 266 m/z. Toxaphene: 340, 341, 342, 343, 375, 377, 411, 413, 447, 449 m/z.

Results and Discussion

Testosterone hydroxylase activity

The hepatic microsomal fraction of female Japanese quails was able to hydroxylate testosterone. The formed hydroxy metabolites included 2α-, 6*β*-, 7*α*-, 15*α*-, 15*β*-, 16*α*-, 16*β*-hydroxy testosterone, and androstenedione. Highest enzyme activities were observed for formation of androstenedione and 6β-hydroxy testosterone (0.4 and 0.2 nmole/mg protein × min.). The other enzyme activities ranged from 1 to 60 pmole/mg protein × min).
In figure 1 the formation rates of 7α-, and 15α-hydroxy testosterone by the hepatic microsomal fractions are shown as a function of the time after dosing. Control values were obtained from untreated quails and tween-control quails that were injected with carrier (tween/water) only. The formation rates of 7α-, and 15α-hydroxy testosterone on day 1 and 2 after toxaphene injection were higher than of the control groups. All other hydroxy testosterone formation rates appeared to be unaffected after toxaphene injection of 6 mg/kg bodyweight.

The lowest 7α-, and 15α-hydroxy testosterone formation rates were observed 4 h. after injection (day 0). This is also true for formation rates of 16*α*-, and 16*β*-hydroxy testosterone, but not for androstenedione, 2*α*-, 6*β*-, and 15*α*-hydroxy testosterone. This observation might be explained by the fact, that these animals were kept in a separate cage for four hours without feed. These animals were sacrificed at 7 p.m., while all other animals were killed at 2 p.m.

Results of this experiment correspond well with the results described by Roelandt et al[6]. Both in fetal quail hepatocytes and female quail hepatic microsomes 16α-, 7α-, 15α-, 16β-, 6β-, 2α-hydroxy testosterone, and androstenedione were formed. The latter activity being the highest. The main difference between (control) fetal hepatocytes and (control) liver microsomes from female quails, seems to be the lower rate of formation of 2α-hydroxy testosterone by hepatic microsomes.

In rats 6*β*-, 16*α*-, and 16*β*-hydroxy testosterone were induced after toxaphene treatment[2]. This pattern has also been observed after phenobarbital treatment[14]. In fetal Japanese quail

hepatocytes phenobarbital also induced the formation of 6β-, 16α-, and 16β-hydroxy testosterone, while 7α-, 15α-, hydroxy testosterone formation rates were not affected. Results of the present experiment did not confirm a phenobarbital-like induction pattern by toxaphene in adult female Japanese quails, although inter-species comparisons are very complicated and should be considered with caution. Obviously, with this experimental design no dose-dependent effects could be detected, it is possible that the dose was too low to induce these enzyme activities.

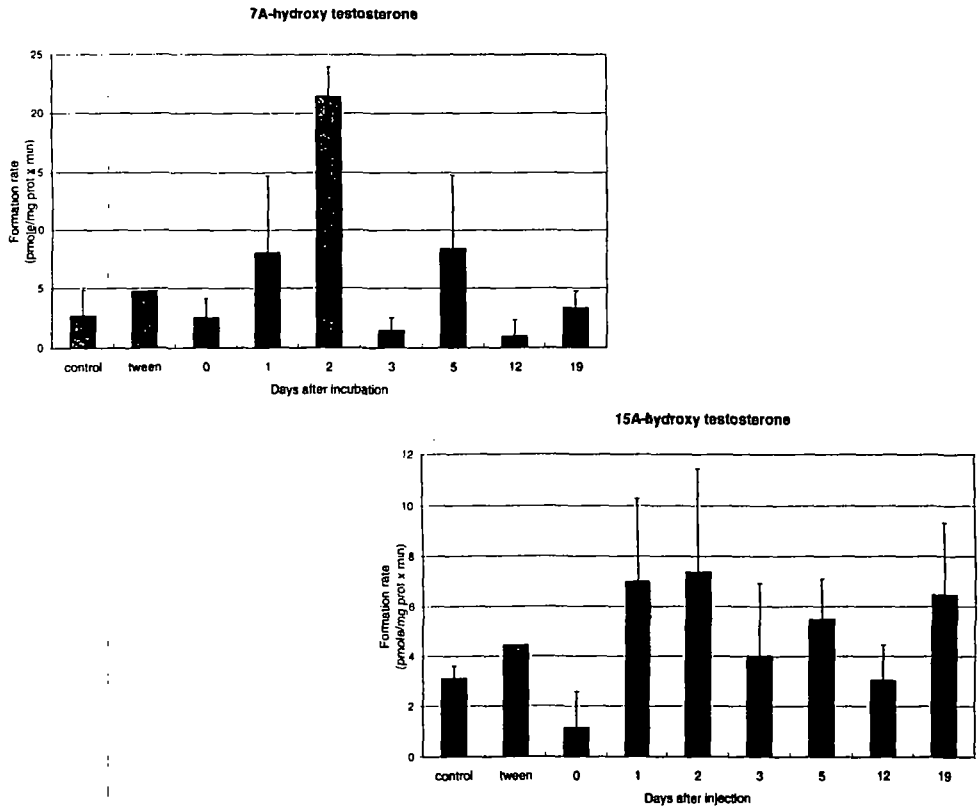

Figure 1. Formation rates of 7α-hydroxy testosterone (7A) and 15α-hydroxy testosterone of hepatic microsomes of adult female Japanese quails. Quails were injected (i.v.) with toxaphene (6 mg/kg bodyweight) at day 0, and sacrificed on the day as indicated (n=4 for each day). Carrier-control animals (n=2) were injected with tween/water, controls (n=3) were not injected at all. Given are mean formation rates and standard errors.

Kinetic models

With the described method for CHB analysis, it was not possible to completely separate the four congeners of interest. As described by De Boer [8,15], GC-ECD peaks corresponding to CHBs 26, 32, 50 and 62, consist of more than one congener if only one column is used (1-dimensional GC). In multi-dimensional GC, using a DB5 column in the first and a FFAP column in the second dimension with ECD detection, De Boer et al. [8,15] reported the following minimal number of congeners: CHB26, 4; CHB32, 6; CHB50, 4; and CHB62, 7. In some biological samples the number of congeners present in these peaks drop to: 3 for

CHB26, 1 to 7 for CHB32, 1 to 2 for CHB50, and 4 for CHB62. De Boer concluded that only CHB50 at higher trophic levels can be determined accurately by single-column GC. Therefore, in our experiment we can only derive models on peak-areas corresponding to the congeners CHB26, 32, 50, and 62. In order to analyze what fraction of the peaks really can be ascribed to the four congeners, a few samples from this experiment could be determined using multi-dimensional GC. Data analysis of the present study are currently under investigation.

Literature Cited

1)Saleh, M. A. *Rev. Environ. Contam. Toxicol.* **1991**, *118*, 1-85.
2)Haake, J.; Kelley, M.; Keys, B.; Safe, S. *Gen Pharmac* **1987**, *18*, 165-169.
3)Waxman, D. J. *Biochemical Pharmacology* **1988**, *37*, 71.
4)Stouvenakers, N.; Hugla, J. L.; Goffinet, G.; Kremers, P.; Thomĺe, J. P. *Bull Environ Contam Toxicol* **1996**, *5*, 839-46.
5)Miranda, C. L.; Wang, J. L.; Henderson, M. C.; Carpenter, H. M.; Nakaue, H. S.; Buhler, D. R. *Toxicology* **1983**, *1*, 75-82.
6)Roelandt, L.; Dubois, M.; Todaro, A.; Thome, J. P.; Kremers, P. *Ecotoxicol Environ Saf* **1995**, *2*, 158-63.
7)Muir, D. C. G. *Chemosphere* **1993**, *27*, 1827-1834.
8)De Boer, J.; De Geus, H.-J.; Brinkman, U. A. T. *18th International Symposium on Capillary Chromatography, May 20-24* **1996**.
9)Bouwman, C.; W. S.; JG, K.; M, V. d. B. *Toxicology* **1992**, *75*, 109-120.
10)Funae, Y.; Imaoka, S. *Biochimica Biophysica Acta* **1987**, *926*, 349-358.
11)De Boer, J. *Chemosphere* **1988**, *17*, 1803-1810.
12)De Boer, J. *Chemosphere* **1988**, *17*, 1811-1819.
13)De Boer, J.; Wester, P. G. *Chemosphere* **1993**, *27*, 1879-1890.
14)Waxman, D. J.; Azaroff, L. *Biochemical Journal* **1992**, *281*, 577.
15)De Boer, J.; De Geus, H.-J. *Organohalogen Compounds*. **1995**, *26*, 345-350.