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TWO NEW ORAL CHEMICAL BIOMARKERS FOR COYOTES

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ABSTRACT: Pentachlorobenzene (PeCB) and 1,2,3,4-tetrachlorobenzene (TeCB) were evaluated as oral chemical biomarkers when administered to coyotes (*Canis latrans*) during the period of 31 January to 10 August 1994. Three coyotes each received 100 mg of PeCB and three received 100 mg of TeCB, each in a mineral oil formulation. Three additional coyotes received only the mineral oil carrier. Muscle and adipose tissues, blood serum, and fecal samples were evaluated by capillary gas chromatography with electron capture detection for 120 days following administration. Residues of PeCB were detected in serum, feces, and adipose and muscle tissues for 120 days post-treatment; TeCB residues were detected in feces and serum at 1 and 8 days post-treatment and in adipose tissue at 30 days post-treatment. Residues of TeCB were not detected in muscle tissue at any point in the study.

Key words: Baits, *Canis latrans*, chemical biomarker, coyote, pentachlorobenzene, residues, tetrachlorobenzene.

INTRODUCTION

Biomarkers have been used to estimate population abundance (Davison, 1980), assess delivery of vaccines (Linhart et al., 1991), assess diets of individuals, and examine social and scent-marking behavior of mammals (Crabtree et al., 1989). Desirable characteristics of biomarkers include that they be readily ingested by target species, safe to target and nontarget species, sampled by nondestructive methods, accurately detectible by available means, inexpensive, present for appropriate lengths of time, and free from excessive restrictions for use.

Both physical and chemical markers have been employed in studying vertebrates (Savarie et al., 1992). Physical markers include particles such as metal flakes and coded particles (Fall and Johns, 1987), and can be detected either in feces or in digestive tracts. Chemical markers include dyes, drugs, and other chemicals such as iophenoxic acid (Larson et al., 1981) which binds with serum-bound proteins and tetracycline which binds with calcium in teeth or bones (Savarie et al., 1992). They may also include chemicals which provide persistent, easily detectable residues such as chlorinated pesticides. Selection of appropriate markers is difficult due to their inherent limitations. Physical markers would not provide the persistence required for many applications and may have adverse effects on bait acceptance (Fall and Johns, 1987). Chemical markers may not provide suitable persistence, may be toxic, not readily available, or otherwise perceived as unsuitable. For example, mirex has been employed as a chemical biomarker (Knowlton et al., 1988), but its use has been restricted by the U.S. Environmental Protection Agency. The use of radioisotopes (Knowlton et al., 1989) requires extensive administrative documentation and safety precautions, and may be negatively perceived by the public (Crabtree et al., 1989).

To study the characteristics of coyotes (*Canis latrans*) that attack livestock, we sought two relatively non-toxic, easily detectable, long-lived (≥4 mo) chemical biomarkers which could be delivered with the livestock protection collar (Connolly and Burns, 1990) and be detected using available analytical methodology. A primary requirement for our application, as well as for potential oral contraception, vaccination, and census applications, was that the marker be persistent. Pentachlorobenzene

(PeCB) and 1,2,3,4-tetrachlorobenzene (TeCB) were regarded as promising candidates because they are fairly non-toxic, as indicated by their oral lethal doses (LD_{50}), and residues probably would be persistent because of their lipophilic nature. The oral LD_{50} concentrations determined in rats were 1,125 mg/kg and 1,470 mg/kg for PeCB (Hazardous Substances Databank, 1992a) and TeCB (Hazardous Substances Databank, 1992b), respectively.

MATERIALS AND METHODS

Nine captive-reared coyotes were selected from the captive colony as test-animals and confined in outdoor chain-link kennels (1.2 × 3.6 × 1.8 m) at the U.S. Department of Agriculture, Predator Research Facility near Millville, Utah, USA, (41°40′N, 111°49′W). Total body mass and length (tip of nose to base of tail) of each animal were determined prior to the study. Animals were fed daily 650 g of a commercial ration consisting primarily of poultry, fish, and cereal (Furbreeders Agricultural Cooperative, Sandy, Utah), and provided water ad libitum. This study was conducted during the period 31 January to 10 August 1994 under conditions prescribed by animal-use standard operating procedures following approval by the Denver Wildlife Research Center Animal Care and Use Committee.

Analytical grade PeCB, TeCB, and mineral oil were obtained from Aldrich Chemical Company, Inc. (Milwaukee, Wisconsin, USA). The solvents used for chemical analysis, isooctane and isopropyl alcohol, were obtained from Baxter Diagnostics Inc. (McGaw Park, Illinois, USA). Reagent grade hexachlorobenzene (HeCB) was used as a surrogate standard during chemical analysis (Aldrich Chemical Company, Inc.). We obtained 2,3,4,5-Tetrachlorophenol from Supelco, Inc. (Bellefonte, Pennsylvania, USA).

The test animals were randomly assigned to three treatment groups of three animals each 45 days prior to treatment. Each test compound was formulated in solution with mineral oil at a concentration of 50 mg/ml. A 100-mg oral dose of either PeCB or TeCB was delivered via two 1-ml gelatin capsules. Three test-animals received PeCB, three test-animals received TeCB, and three test-animals received capsules containing mineral oil only (controls). All test-animals were force-fed capsules during recovery from anesthesia following Day 0 surgical collection of adipose and muscle tissues. Animals were anesthetized with intravenous injection of sodium thiamylal (Boehringer Ingelheim Animal Health Inc., St. Joseph, Missouri, USA) at an average dose of 4 mg/kg.

TABLE 1. Study protocol used in evaluation of oral chemical biomarkers for coyotes.

Sampling day	Samples taken				
	Blood profile	Adipose tissue	Muscle tissue	Serum	Feces
45 Days Pre-dose	X				
Day 0		X	X	X	X
Day 1	X			X	X
Day 8				X	X
Day 30		X	X	X	X
Day 45					X
Day 60		X	X	X	X
Day 90		X	X	X	X
Day 120	X	X	X	X	X

Biological samples were obtained periodically over a 120-day period post-treatment (Table 1). Samples were extracted and analyzed for PeCB and TeCB within 2 days of collection. Fecal samples (40 to 100 g) were collected from kennels of test-animals on each sample date in a manner to include representative portions of the deposits accumulated during the preceding 20 hr. The composite fecal material was manually homogenized with a spatula. To obtain serum, two 10-ml vacutainers of whole blood were drawn from the cephalic vein of test animals immobilized with 1 ml intramuscular injections of 100 mg ketamine hydrochloride (Avco Co. Inc., Fort Dodge, Iowa, USA) and 1 mg acepromazine maleate (Fermenta Animal Health Co., Kansas City, Missouri). Serum was collected by allowing the blood to clot at 25 C for 1 to 3 hr prior to centrifugation. Adipose and muscle tissue samples were surgically collected. Skeletal muscle (0.5 to 1.0 g) was excised from the biceps femoris muscle of a rear leg. Intraabdominal adipose tissue (0.5 to 1.0 g) was excised from the falciform-ligament deposit. Tissue samples were weighed and placed in 50-ml glass screw-cap culture tubes. All biological samples were maintained frozen at −24 C until analysis.

Each solid biological sample (0.5 to 1.0 g) or serum sample (1.0 ml) was placed into a 50-ml glass screw-cap culture tube for analysis. The samples were fortified with the surrogate standard solution, hexachlorobenzene in iso-octane, to yield a concentration of approximately 20 ng HeCB/g in the solid samples and 10 ng HeCB/ml in the serum samples. The samples were extracted with approximately 1.4 ml of a 25% isopropyl alcohol/75% isooctane solution by vigorous mixing with a vortex mixer. After de-

canting the extract into a graduated tube, the extraction was repeated two more times with approximately 0.9 ml of the extraction solution. The combined extracts were then brought to a final volume of 3.00 ml and mixed. Extracts were transferred to gas chromatographic autosampler vials for injection into the gas chromatograph.

Quality control samples were also prepared at each analysis for each matrix and analyzed using the same procedures. Adipose and muscle tissue, feces, and serum samples were obtained from euthanized coyotes (4 ml intravenous injection of 390 mg/ml sodium pentobarbital and 50 mg/ml sodium phenytoin, Steris Laboratories, Phoenix, Arizona, USA) prior to beginning the study and fortified with PeCB, TeCB, and HeCB, at concentrations of 20 ng/g each in the solid samples and 10 ng/ml each in the serum. The quality control results were used to normalize the quantitative data. Quantitative analysis of the samples was achieved versus PeCB, TeCB, and HeCB external standards.

A Hewlett-Packard Model 5890 gas chromatograph equipped with an electron capture detector was used for all residue analyses (Hewlett-Packard Co., Avondale, Pennsylvania). A vortex mixer was used to perform the solvent extractions (Glas-Col Apparatus Co., Terre Haute, Indiana, USA). A 30 m × 0.25 mm poly(dimethylsiloxane) gas capillary column with a 0.25 μm film thickness (DB-1, J&W Scientific, Folsom, California, USA) was used with the following oven program: 90 C initial temperature for 1 min; 15 C/min ramp to 160 C, hold for 8.5 min; 30 C/min ramp to 180 C, hold for 4.5 min; 50 C/min ramp, hold for 9 min. The injection port temperature was 250 C and the detector temperature was 325 C. One μl splitless injections (30 sec purge time) were made. The helium carrier flow rate was 1.5 ml/min and the make-up gas (10% methane in argon) flow rate was 55 ml/min.

An unknown compound present in the feces of TeCB-dosed test-animals was positively identified by combining several feces sample extracts and concentrating by evaporation. The concentrated extract was subjected to gas chromatography with mass selective detection. The chromatographic parameters were the same as described. The electron impact ionization was 70 ev.

Normalization of analyte concentration in each matrix was performed by dividing the observed analyte concentration by the normalization factor (F) described in the equation below. Analyte recovery values were expressed as decimal values.

$$F = \frac{\text{Quality Control Analyte Recovery}}{\text{Quality Control HeCB Recovery}} \times \text{Surrogate HeCB Recovery}$$

We evaluated two relationships in the normalization equation. First we compared analyte recovery between the quality control sample and the actual sample through the measured HeCB recovery in the quality control sample and the HeCB surrogate recovery from the actual sample. We also compared HeCB recovery from the quality control sample to PeCB or TeCB recovery from the quality control sample. Thus, the overall effect was determined by dividing the observed analyte concentration by the analyte recovery from a quality control sample with the added feature of controlling the varying matrix effects on recovery.

The method limits of detection were defined to be the lowest detectable quantity of the analyte present in the matrix. The lowest detectable quantity was calculated from the chromatographic peak-to-peak noise at the retention times of the analytes in control matrices. The concentration of the analyte corresponding to three times the peak-to-peak noise was then calculated versus a fortified sample. Where a chromatographic interference was present in a control matrix, the interfering peak was considered to be the analyte and quantified. All concentrations were normalized and the 99% confidence interval (Ott, 1993) was calculated for the mean normalized concentration for each analyte in each matrix. The value of mean +99% confidence interval was then defined as the method limit of detection.

Potential pathologic effects in test animals related to treatment with PeCB and TeCB were assessed by routine visual inspection of the animals, body mass monitoring, and evaluation of blood profiles. Body mass of each animal was determined at 45 days pre-treatment, day 0, and 30, 60, 90, and 120 days post-treatment. Blood samples were obtained 45 days pre-treatment, 1 day post-treatment and 120 days post-treatment (Table 1). Because TeCB residues were no longer observed in samples after 30 days, blood profiles were not taken for TeCB dosed animals at 120 days post-treatment.

Blood samples consisted of one 10-ml vacutainer of blood (to yield serum as previously described) for analysis of 20 serum biochemical variables, and one 3-ml vacutainer of whole blood containing ethylenediaminetetraacetic acid (EDTA) for analysis of 16 hematologic variables. Analyses were performed by the Logan Regional Hospital Laboratory, Logan, Utah, USA. The serum biochemical variables:

TABLE 2. Pentachlorobenzene (PeCB) residue concentrations determined in four sample matrices following oral delivery of 100 mg PeCB.

Matrix	Day 0	Day 1	Day 8	Day 30	Day 45	Day 60	Day 90	Day 120
Adipose (μg/g)	ND[a,b]	NS	NS	99 ± 105[c]	NS	8.9 ± 1	6.2 ± 1	3.5 ± 1
Muscle (ng/g)	ND	NS	NS	580 ± 330	NS	62 ± 16	31 ± 7	22 ± 11
Serum (ng/ml)	ND	200 ± 110	250 ± 74	140 ± 15	NS	65 ± 19	55 ± 17	20 ± 10
Feces (ng/g)	ND	5,500 ± 6,500	450 ± 250	360 ± 26	230 ± 61	92 ± 25	72 ± 9	40 ± 10

[a] ND, not detected; NS, not sampled.
[b] The concentration of one of the three samples was 0.2 μg/g.
[c] Mean ± SD; sample size is three coyotes for all reported mean values.

sodium (Na), potassium (K), chloride (Cl), carbon dioxide (CO_2), glucose, blood urea nitrogen (BUN), creatinine, calcium (Ca), magnesium (Mg), phosphorous (P), total protein, albumin, uric acid, cholesterol, triglycerides, total bilirubin, alkaline phosphatase (ALP), lactic dehydrogenase (LDH), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were obtained with a Hitachi 717 autoanalyzer and Boehringer reagent (Boehringer Mannheim Corporation, Indianapolis, Indiana). The 16 hematological variables: white blood cell count (WBC), red blood cell count (RBC), hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration, red blood cell distribution width, platelets, mean platelet volume, and the differential white blood counts: band neutrophils, segmented neutrophils, basophils, eosinophils, lymphocytes, and monocytes were obtained using a Coulter STKS autoanalyzer with Coulter reagent (Coulter Corporation, Miami, Florida, USA).

Blood profile and body mass results were each subjected to two factor analyses of variance to identify differences among treatments (control, PeCB, TeCB) and over the treatment period using an α level of 0.05 (SAS Institute, Inc., 1990). Furthermore, comparisons were also made with control animals and normal ranges for domestic dogs (*Canis lupus*).

RESULTS

Pentachlorobenzene was present at levels above the limit of detection in all matrices at each sampling interval following administration of PeCB (Table 2). Adipose tissue concentrations were the highest, with values near 100 μg/g (100,000 ng/g). While the variation was quite high in most samples in the first 30 days, variability decreased with time.

The method limits of detection calculated for PeCB in adipose tissue, muscle tissue, and feces were 17, 9.4, and 14 ng/g, respectively, and 7.2 ng/ml for serum. While PeCB was not detected at concentrations greater than the detection limit in any pre-dose muscle, serum, or feces samples, it was detected in one of the three adipose tissue sample extracts.

The other candidate marker, TeCB, was not observed in any pre-dose matrix sample. Residues of TeCB were detected in the feces and serum of TeCB-treated test-animals at days 1 and 8, and in the adipose tissue samples collected from two of the three test-animals at 30 days post-treatment, but not afterwards. Residues of TeCB were not observed at levels greater than the detection limit in muscle tissue at any sampling period. The calculated detection limits for TeCB in adipose tissue, muscle tissue, and feces were 12, 8.2, and 16 ng/g, respectively, and 6.8 ng/ml for serum.

Additionally, an unknown compound was observed in the serum and fecal samples of TeCB dosed animals on days 1 and 8. The unknown was identified as 2,3,4,5-tetrachlorophenol by its mass spectrum and its retention time versus a known standard.

No visible symptoms of pathologic effects in test-animals related to one-time treatment with PeCB or TeCB were observed during the study. Variations in body mass of test-animals were minor and unrelated to treatments ($P = 0.58$) or time ($P = 0.42$). Furthermore, there were no interactions ($P = 0.99$).

Based on analysis of variance, there were significant differences for the variables AST ($P = 0.0001$), albumin ($P = 0.004$), BUN ($P = 0.04$), basophils ($P = 0.04$), Cl ($P = 0.04$), creatine ($P = 0.01$), eosinophils ($P = 0.004$), glucose ($P = 0.001$), hematocrit ($P = 0.05$), LDH ($P = 0.03$), MCH ($P = 0.009$), MCH concentration ($P = 0.0001$), total protein ($P = 0.0003$), RBC ($P = 0.05$), segmented neutrophils ($P = 0.004$), and WBC ($P = 0.004$) over the period of the study but there were no treatment effects. Thus, the changes in these variables were observed in all animals regardless of administration of PeCB or TeCB.

DISCUSSION

Residues of PeCB were persistent in all samples from the PeCB-dosed test animals for 120 days. Since the PeCB concentration observed in adipose tissue at 120 days post-treatment was 3.5 ± 1 μg/g (Table 2), the lone observation of 0.2 μg/g PeCB in the Day 0 adipose tissue of one test animal was considered an artifact of contaminated glassware. Great care was taken after this observation to individually solvent-clean each sample tube.

Because no tissue samples were collected at days 1 and 8, the relationships between PeCB concentration in the four matrices can not be compared for the first 30 days. However, from days 30 to 120, the decline of PeCB residues in adipose tissue were reflected by corresponding declines of PeCB residues in feces, serum, and muscle tissue. Similar relationships between whole blood DDE levels and DDT residues in adipose tissue have been previously investigated in humans (Edmundson et al., 1972). Radomski et al. (1971) demonstrated that organochlorine pesticide residues in blood plasma are in equilibrium with residues in adipose and other body tissues.

Based on the residue data, TeCB would not be a suitable long-term chemical biomarker. The presence of TeCB residues in serum and feces of TeCB-dosed test-animals for 8 days is evidence that 1,2,3,4-TeCB may be useful as a short-term chemical biomarker. Chu et al. (1983) found that 1,2,3,4-TeCB residues accumulated in adipose tissues at concentrations far less than the other isomers (1,2,3,5- and 1,2,4,5-TeCB).

The limited persistence of TeCB was apparently due to its rapid metabolism and elimination. Mammalian metabolism of chlorinated benzenes primarily involves hydroxylation leading to the formation of phenols which are then typically subjected to enzymatic glucuronidation or sulfation (Sipes and Gandolfi, 1993). These conjugation reactions render the lipophilic chlorinated benzenes more hydrophilic and therefore easier for the mammal to excrete via urination and defecation (Klaassen and Rozman, 1993).

Kohli et al. (1976) found the principal metabolites of 1,2,3,4-TeCB to be 2,3,4,5-tetrachlorophenol and 2,3,4,6-tetrachlorophenol in yields of 20% and 2%, respectively, in rabbits. We also identified 2,3,4,5-tetrachlorophenol in the serum and feces samples from TeCB-dosed test animals.

The dosage rate of biomarkers may also affect persistence of residues. A 100-mg dose was selected based on data from previous studies and the toxicities of the compounds. The single dose delivered in this study of 100 mg resulted in an approximate dosage of 9 mg/kg for the test animals which had an average weight of approximately 11 kg. This is far less than the reported oral LD_{50} in rats for either compound. Increased or multiple doses may be employed to increase persistence while not approaching toxic levels.

No change in serum biochemical or he-

matologic values were attributed to administration of PeCB or TeCB. Values for ALP, LDH, ALT, AST, total bilirubin, albumin, cholesterol, and total protein were evaluated specifically as indicators of hepatic abnormalities resulting from toxicity. In addition to observing no differences in these variables as a result of treatment, all values (except AST) were within the normal range for domestic dogs. As recommended by Duncan and Prasse (1986), the values used for comparison were provided by the laboratory which conducted the analyses (Logan Regional Hospital Laboratory). Elevation of AST in all animals following surgery; including untreated controls, was attributed to muscle excision.

In this study, we found that PeCB and TeCB are promising long- and short-term oral chemical biomarkers for use in coyotes. These readily available chlorinated benzenes can be delivered orally via liquid carriers, such as mineral or corn oils. Besides the potential for adding an oil solution containing one of these markers to various prepared baits, the non-polar nature of these compounds also makes them suitable for direct inclusion in paraffin wax baits. Such baits have been demonstrated to be useful in the delivery of an oral rabies vaccine (Linhart et al., 1991). Furthermore, the equipment and expertise required for the gas chromatographic technique employed here is available in nearly all university and commercial laboratories.

Samples for residue analysis can be collected in the field from live animals by blood sampling and collection of fresh feces. Analysis of adipose tissue, which is readily sampled from carcasses of mammals collected during field studies, offers the most definitive measure of PeCB residues. Based on our study, further evaluation of chlorinated benzene compounds for various field applications as safe, persistent biomarkers in mammals is warranted.

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