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9. ABSTRACT

Blood and tissue of cattle were tested for residues of diphenadione (2-(diphenylacetyl)-1,3-indandione), an anticoagulant livestock systemic intended for use in vampire bat (Desmodus rotundus) control. Liver and kidney samples from heifers given single 1 mg/kg intraruminal injections, contained 0.15 ppm or less up to 90 days post-treatment. Detectable levels (>0.01 ppm) could not be found by gas-liquid chromatography in blood, brain, heart, fat, and muscle tissue samples taken at 30, 60, and 90 days post-treatment. Adult Sprague Dawley rats were fed the liver from test cattle in a 14-day secondary hazard feeding study. No rats died; treated and control rats did not differ in prothrombin clotting time, and diphenadione was not detected in the liver or blood of treated rats. Calculations based on residue levels in this study indicate that humans may safety eat the meat, including liver and kidney, of treated cattle.

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Diphenadione Residues in Tissues of Cattle

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Blood and tissue of cattle were tested for residues of diphenadione (2-(diphenylacetyl)-1,3-indandione), an anticoagulant livestock systemic intended for use in vampire bat (*Desmodus rotundus*) control. Liver and kidney samples from heifers given single 1 mg/kg intraruminal injections contained 0.15 ppm or less up to 90 days posttreatment. Detectable levels (>0.01 ppm) could not be found by gas–liquid chromatography in blood, brain, heart, fat, and muscle tissue samples taken at 30, 60, and 90 days posttreatment. Adult Sprague Dawley rats were fed the liver from test cattle in a 14-day secondary hazard feeding study. No rats died, treated and control rats did not differ in prothrombin clotting time, and diphenadione was not detected in the liver or blood of treated rats. Calculations based on residue levels in this study indicate that humans may safely eat the meat, including liver and kidney, of treated cattle.

Vampire bats (*Desmodus rotundus*) are reported to cost the Latin American cattle industry up to $250 million annually (Greenhall, 1970). They are important carriers of paralytic rabies, which claims an estimated 1 million head of cattle every year. In addition, there is widespread belief that severe debilitating effects are caused through malnutrition, myiasis, and loss of blood resulting from vampire bat attacks. In 1960, the Mexican Agriculture and Livestock Ministry's National Institute of Livestock Research and the U.S. Fish and Wildlife Service (sponsored by the U.S. Agency for International Development) cooperated in a program to develop methods of controlling vampire bat populations in areas where bat-borne rabies was a problem. The livestock systemic method of control was developed under this program.

In this method, cattle are injected with an anticoagulant, diphenadione (2-diphenylacetyl)-1,3-indandione). A single intraruminal dose of about 1 mg/kg is capable of killing vampire bats that feed on the blood of a treated animal within 3 days after dosing (Thompson et al., 1972). The cattle apparently do not suffer any ill effects. However, it was not known whether this control method results in significant residues in their tissues.

The residue levels of diphenadione in the tissues of treated animals, and the effects of these levels, must be considered before the method can be adopted. Therefore, we determined tissue residue levels at 30, 60, and 90 days following a 1 mg/kg injection and investigated the secondary hazard to rats that ate the liver of treated cattle.

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EXPERIMENTAL SECTION

Treatment of Animals and Collection of Samples. Six Hereford heifers weighing approximately 230 kg each were dosed with 1 mg/kg of diphenadione by injecting a Carbopol 941 suspension into the rumen. A pistol-grip automatic syringe (Vaco HL 013700) fitted with a 14 gauge, 1.5-in. disposable needle was used for the injection.

At 30, 60, and 90 days posttreatment, two animals were randomly selected and killed. Samples of blood plasma, liver, heart, kidney, brain, muscle from the hindquarter, and fat (1:1 mixture visceral and subcutaneous) were collected from each animal. Pretreatment blood samples and samples of other tissues from a local butcher shop served as untreated controls. All samples were stored at −12 °C until analyzed or used in feeding tests.

Residue Analysis. A gas–liquid chromatographic (GLC) procedure developed earlier in our laboratory (Bullard et al., 1975) was used for all analyses. In this procedure, diphenadione is oxidized to benzophenone, which chromatographs well on silicone columns and is sensitive to electron-capture detection. An oxidation step following sample cleanup provides constant yields of benzophenone under standardized conditions.

An Aerograph 1520B gas chromatograph equipped with a tritium foil electron-capture detector was used for all analyses. The 5 ft × 1/8 in. glass column was packed with 3% XE-60 on 100–120 mesh Gas-Chrom Q. The operating parameters were: injection port, 225 °C; column, 115 °C; and nitrogen flow rate, 35 ml/min. Under these conditions, benzophenone had a retention time of 14.2 min. The

Table I. Recovery of Diphenadione from Tissue Samples Fortified with 0.1 ppm of Diphenadione[a]

Tissue	ppm recovd (mean ± SD)[b]
Brain	0.087 ± 0.004
Liver	0.090 ± 0.004
Kidney	0.091 ± 0.003
Heart	0.095 ± 0.004
Hindquarter muscle	0.095 ± 0.008
Fat	0.100 ± 0.005

[a] Fortified just before sample cleanup by adding the diphenadione into the extraction vessel containing the tissue, ground with sodium sulfate, and the acetone mixture (Bullard et al., 1975). [b] Samples were run in triplicate, and values have been corrected for the molecular weight conversion of the derivative measurement.

samples were quantitated by comparing the peak height with a benzophenone standard. The sample residue levels were predicted by a regression equation which had been derived from the analysis of fortified samples.

Mass Spectral Confirmation. For one liver and one kidney sample from treated animals, the eluate corresponding to the GLC peak identified and measured as benzophenone was collected in 13 cm × 1.8 mm melting point capillary tubes. (The hole in the Teflon exit plug of the Aerograph electron-capture detector was enlarged to 1.8 mm in order to hold the tube in place during collection.) The collected material was dissolved by adding 4 μl of hexane to the tube, and the solution was then removed by microliter syringe and injected into an Aerograph 204 gas chromatograph coupled to a Nuclide 1290G mass spectrometer. The GLC column and operating parameters were the same as used for quantitative analyses. The operating parameters of the mass spectrometer were: 70 eV ionizing potential, 200 °C source temperature, 200 °C separater temperature, 1.2×10^{-5} Torr source pressure, and 5000 V accelerating voltage.

Metabolite Experiment. To determine whether any of the benzophenone found in the liver and kidney was present as a metabolite before the oxidation step, we conducted a separate experiment with pooled samples of each of these tissues from treated animals. Extracts of the two pooled samples were analyzed for benzophenone after the last cleanup step by spotting triplicate aliquots on Type K301R Eastman thin-layer chromatogram (TLC) sheets and eluting with benzene. To verify that this method detected benzophenone, samples of butcher shop liver and kidney were fortified with 0.15 ppm of benzophenone and similarly analyzed. The chromatograms were viewed under uv light (254 mμ). Under these conditions, the R_f for benzophenone is 0.35. For verification, the TLC spots representing benzophenone were removed and extracted, and the extracts were analyzed by GLC.

Secondary Hazard Determination. The liver from each treated heifer was fed to two groups of five adult Sprague Dawley rats weighing about 200 g each. Two control groups of five rats each were fed beef liver from a butcher shop. Each rat was fed a 70-kg human equivalent of 1.4 kg/day for 14 days. The liver was mixed 2:1 with Quaker regular cooking oats, and lab chow was offered ad libitum to meet any remaining nutritional needs.

After 14 days of feeding, one of the two test groups fed each liver sample was returned to a diet of lab chow and observed an additional 14 days for mortality or signs of chronic toxicity. The remaining test group was killed at 14 days, and blood and liver samples were taken from each rat. The prothrombin clotting time was determined for part of each blood sample by the Sera-Tek Reaction Chamber technique, which is based on the one-stage method developed by Quick (1935). The remaining 35 blood and liver samples were pooled by test group and analyzed in duplicate for diphenadione residues by GLC.

Table II. Detectable Diphenadione Residues (>0.01 ppm) in Tissues of Cattle Given a Single 1 mg/kg Injection

Days posttreatment	ppm found (mean ± SD)[a] Liver	Kidney
30	0.15 ± 0.01	0.08 ± 0.01
60	0.14 ± 0.01	0.10 ± 0.02
90	0.15 ± 0.00	0.08 ± 0.00

[a] Means of four values from two animals at each period run in duplicate; values have been corrected for the molecular weight of the derivative and for recoveries (Table I).

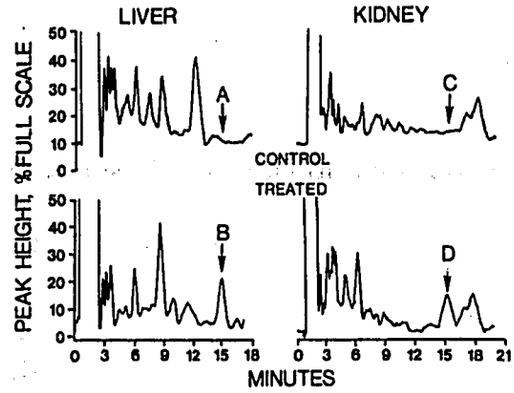

Figure 1. Typical gas-liquid chromatogram for determining diphenadione residues in liver and kidney samples of control (A and C) and treated (B and D) animals killed 60 days after being given a 1 mg/kg dose.

RESULTS AND DISCUSSION

Recovery values from tissue fortified with 0.1 ppm of diphenadione ranged from 87% for brain to 100% for fat (Table I). Values for bovine plasma were reported earlier (Bullard et al., 1975). There were no peaks that interfered with the measurement of benzophenone in any of the sample materials.

Small quantities of diphenadione were present in liver and kidney samples from treated cattle (Table II), but detectable levels (>0.01 ppm) could not be found in any of the other five tissues analyzed (plasma, brain, heart, muscle, and fat) or in any control samples. Since diphenadione is susceptible to protein binding (Bullard et al., 1975), small quantities may be tightly bound in the kidney and liver. This may explain the almost constant residues in those organs from 30 to 90 days posttreatment. Typical chromatograms are given in Figure 1 for samples of liver and kidney taken from test animals.

Representative liver and kidney samples were also chromatographed on QF-1, OV-17, and Carbowax 20M columns, in addition to the XE-60 column, in order to confirm that the observed peak was benzophenone. A peak representing the proper quantity and retention time was present in all cases. Final confirmation was achieved by mass spectral analysis. For both liver and kidney samples, mass spectra of the GLC peaks were identical with those of benzophenone.

In the metabolite experiment, benzophenone from fortified samples could be readily detected by both TLC and GLC, but none could be found in unoxidized kidney or liver samples from treated animals. This, plus the absence of benzophenone in tissues from untreated animals, convinced us that the benzophenone we measured in liver and kidney samples was the oxidation product of

diphenadione and did not represent a metabolite already present in the tissues.

Secondary hazards were evaluated by feeding the livers of treated cattle to laboratory rats because this species is highly susceptible to the anticoagulant effects of diphenadione (Saunders et al., 1955; Bentley and Larthe, 1959). The liver–oats formulation was highly palatable and all animals consumed their daily ration soon after it was offered. None of the test animals died or exhibited signs of chronic toxicity during the test or the following 14-day observation period; all appeared vigorous and showed weight gains comparable with those of the controls. After 14 days of feeding, prothrombin clotting times (means ± standard deviation) were similar in all groups: 17.7 ± 0.4 s for 10 rats fed liver from cattle killed at 30 days post-treatment, 18.6 ± 0.6 s for 10 fed liver from cattle killed at 60 days, 17.4 ± 0.5 s for 10 fed liver from cattle killed at 90 days, and 18.7 ± 0.5 s for 10 fed untreated liver. Blood and liver samples from these rats contained no detectable diphenadione. These results indicated that the minute diphenadione residues in the tissues of treated cattle caused no observable adverse effects in secondary consumers.

Diphenadione has been used as a prothrombinopenic anticoagulant in human therapy. "Remington's Pharmaceutical Sciences" (1970) lists the range of human daily dosages as 2.5 to 30 mg for anticoagulant therapy. Therefore, if the liver of treated cattle contained the highest residue level found, 0.15 ppm, a 70-kg human would have to eat about 17 kg of it per day to receive the minimum dosage used in therapy.

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