analyzed, making the method better adapted to the number of samples encountered in a monitoring program.

LITERATURE CITED

Plapp, F. W., Casida, J. E., *Anal. Chem.* **30**, 1622 (1958).
St. John, L. E., Jr., Lisk, D. J., *J. Agric. Food Chem.* **16**, 408 (1968).
Shafik, M. T., Bradway, D. E., Biros, F. J., Enos, H. F., *J. Agric. Food Chem.* **18**, 1174 (1970).
Shafik, M. T., Bradway, D. E., Enos, H. F., *J. Agric. Food Chem.* **21**, 625 (1973).

Received for review July 19, 1976. Accepted September 24, 1976.

Diphenadione Residues in Milk of Cattle

Roger W. Bullard,* R. Daniel Thompson, and Stephen R. Kilburn

The mammillary transfer of diphenadione (2-(diphenylacetyl)-1,3-indandione) into the milk of treated cows is an important health aspect of the use of this systemic anticoagulant in vampire bat (*Desmodus rotundus*) control. Milk of cows dosed intraruminally with diphenadione at the recommended 1-mg/kg level did not contain detectable levels of residues, whereas cows treated with 2.75 mg/kg contained 21.3 ppb or less at 12, 24, and 48 h posttreatment. There were no residues in samples after 72 h. In nursling calves the prothrombin clotting time did not change and there were no detectable residues in their blood plasma. It appears that the mammillary transfer of diphenadione, like that of other anticoagulants, is dose dependent. Our data indicate that it is safe to consume milk from cows dosed at the recommended rate of 1 mg/kg. Even if cows are accidentally given 2.75 times that amount, and milk collected within 72 h after treatment is consumed, there are no apparent health hazards involved.

Diphenadione (2-(diphenylacetyl)-1,3-indandione) is a systemic anticoagulant that has been found to be effective in killing vampire bats (*Desmodus rotundus*) that consume blood from cattle treated intraruminally. One feeding is effective if it takes place within 3 days after cattle are given a 1 mg/kg dose (Thompson et al., 1972). This promising control method could save the Latin American cattle industry up to $250 million annually (Greenhall, 1970).

Currently, the major emphasis is to determine how the diphenadione treatment affects livestock and if there is a potential health hazard to humans who consume meat and milk from treated animals. Thompson et al. (1972) reported that a moderate increase in clotting time of plasma prothrombin is the only observable sign of intoxication in adult cattle. Later, residues were found in liver (about 0.15 ppm) and kidneys (about 0.08 ppm) of treated animals at 30 to 90 days posttreatment but not in heart, brain, muscle from the hindquarter, fat, or blood plasma (Bullard et al., 1976). Calculations and secondary hazard tests in albino rats indicated that these levels are of no danger to humans that consume liver and kidneys from treated animals.

Other anticoagulants have been found to undergo mammillary transfer. Their appearance in the milk of treated subjects appears to be dose dependent. The administration of massive doses of dicumarol (Field, 1945) or warfarin (Blumberg et al., 1960) to lactating rats produced hypoprothrobinemia in suckling young. However, dicumarol administered prophylactically to 125 nursing mothers did not affect prothrombin activity of the infant (Brambel and Hunter, 1950). Furthermore, in 4000 mothers given oral anticoagulants prophylactically, all of the breast-fed infants remained asymptomatic throughout the period of therapy (Fries et al., 1957). Warfarin could not be found by chemical analysis in the milk of two women that were on a therapeutic anticoagulant regimen (O'Reilly and Aggeler, 1970).

U.S. Fish and Wildlife Service, Wildlife Research Center, Federal Center, Denver, Colorado 80225.

However, since there are no reports concerning diphenadione in milk, it was imperative that we determine if mammillary transfer occurs in treated cows. Cows were tested at 2.75 mg/kg as well as 1 mg/kg so that dose-dependent responses could be observed in case of accidental overdosing.

EXPERIMENTAL SECTION

Treatment of Animals and Collection of Samples. Three lactating cows were given the recommended 1-mg/kg intraruminal doses of diphenadione and three others were given 2.75 mg/kg. Each cow had a nursling calf. A Carbopol 941 aqueous suspension of the compound was injected with a pistol grip automatic syringe (Vaco HL 013700) having a 14 gauge, 1.5-in. disposable needle. A control cow (also with nursling calf) received a "sham" injection of physiological saline.

Samples of milk and blood from each cow and blood from each calf were collected immediately pretreatment and at 12, 24, 48, 72, 96, 120, and 144 h posttreatment. Ten-milliliter blood samples were obtained by venipuncture from the jugular vein. Prothrombin clotting times (Quick, 1935) were determined immediately after collection. All samples were stored at −12 °C until analyses could be conducted.

Residue Analysis. A gas–liquid chromatographic (GLC) procedure reported earlier in this journal was used for analysis of all milk and blood samples (Bullard et al., 1975). The only difference in analysis of milk and blood is in the sample preparation. The plasma fraction of venous blood is extracted with acetone, and proteins are removed by centrifugation. In milk, most of the water is removed through evaporation and then the residue is mixed with anhydrous sodium sulfate and extracted with acetone. The acetone extract in both cases is processed the same way through the remainder of the procedure. Diphenadione cannot be analyzed by GLC directly but is oxidized to benzophenone which chromatographs readily and is sensitive to electron-capture detection.

An Aerograph 1520B gas chromatograph equipped with a 0.0625-in. i.d. injection port liner and a tritium foil

Table I. Recoveries of Diphenadione from Fortified Milk

Added ppb	ppb recovd[a,b] (mean ± SD)
0.00	0.00 ± 0.00
5.00	3.11 ± 0.16
10.00	6.48 ± 0.45
15.00	8.37 ± 0.46
20.00	10.32 ± 1.02
40.00	16.62 ± 0.37

[a] This recovery is affected by oxidation yield (Bullard et al., 1975). [b] Samples were run in quadruplicate, and values have been corrected for the molecular weight conversion of the derivative measurement.

Table II. Diphenadione Residues in Milk of Dosed Cows

h post-treatment	ppb found (mean ± SD) Control[a]	1 mg/kg[b]	2.75 mg/kg[b]
0	<3	<3	<3
12	<3	<3	19.33 ± 7.28
24	<3	<3	21.33 ± 5.16
48	<3	<3	20.00 ± 6.78
72-144	<3	<3	<3

[a] Means of two values from one animal. [b] Means of six values from three animals at each period run in duplicate; values have been corrected for the molecular weight of the derivative and for recoveries.

Table III. Diphenadione Residues in the Blood Plasma of Test Cows (ppm)[a]

h post-treatment	1.0 mg/kg (mean ± SD)	2.75 mg/kg (mean ± SD)
0	<0.01	<0.01
12	0.91 ± 0.29	3.61 ± 0.73
24	1.49 ± 0.28	4.09 ± 0.65
48	1.20 ± 0.20	1.91 ± 0.93
72	0.42 ± 0.41	1.42 ± 0.77
96	<0.01	0.76 ± 0.41
120	<0.01	0.33 ± 0.13
144	<0.01	<0.01

[a] Means of six values from three animals at each period run in duplicate; values have been corrected for the molecular weight of the derivative and for the recoveries.

electron-capture detector was used for all analyses. A 100 ft long × 0.03 in. i.d. stainless steel capillary column coated with OV-101 containing 5% Igepal CO-880 provided adequate resolution for all analyses. The operating parameters were: injection port, 225 °C; column, 175 °C for blood and 142 °C for milk; and 12-ml/min nitrogen flow. A 23-ml/min flow of nitrogen makeup gas was added between the column and detector.

Under these conditions benzophenone had retention times of 9.1 and 24.6 min, respectively, for blood and milk samples. The samples were quantitated by comparison of the peak height with that of an appropriate standard. Regression equations derived from the GLC analysis of fortified samples were used to predict the sample residue levels in both blood plasma and milk.

Mass Spectral Confirmation. The eluate from the GLC peak suspected to be diphenadione in milk samples was collected and analyzed by GLC–mass spectrometry. The procedure, instrumentation, and conditions were described earlier (Bullard et al., 1976).

RESULTS AND DISCUSSION

The difficulty of obtaining adequate resolution of benzophenone from extraneous interfering peaks in milk was discussed earlier (Bullard et al., 1975). Packed columns coated with a large variety of liquid phases were unsatisfactory, even when an additional cleanup step was tried. The 100-ft long large bore open tubular column solved this dilemma. Still, the column temperature required for resolution was critical and analyses were slow because of the long retention time. A chromatogram of typical analyses is given in Figure 1.

The recovery of diphenadione added to milk samples in the 5 to 40 ppb range is given in Table I. A similar table for plasma analysis is given elsewhere (Bullard et al., 1975). Small quantities of diphenadione were detected in 12-, 24-, and 48-h posttreatment milk samples of cows treated at 2.75 mg/kg but no residues were detected in the milk of cows dosed at 1 mg/kg (Table II).

The presence of diphenadione in 12-, 24-, and 48-h milk samples from cows that were dosed at 2.75 mg/kg was confirmed by mass spectrometry. The mass spectrum verified that the peak being measured was benzophenone. In an earlier experiment (Bullard et al., 1976) benzophenone could not be found in unoxidized kidney or liver samples which were known to contain diphenadione. This confirmed the fact that benzophenone was not present as a metabolite in detectable levels before the oxidation step in the analytical procedure. We assume that this is also true for milk.

The presence of diphenadione in the milk of cows dosed at 2.75 but not 1 mg/kg indicates that mammillary transfer of the anticoagulant depends upon the blood plasma level (Table III). This is similar to the dose dependency discussed earlier for other anticoagulants. It appears that the blood threshold level for mammillary transfer of detectable quantities into the milk (3 ppb) lies between 1.49 and 1.91 ppm.

The plasma prothrombin clotting times (Table IV) also appear to be dose dependent. The prothrombin response lags several hours behind the increase of diphenadione levels in the blood. The change in clotting time seems to climb at about the same rate for the two dose levels. Therefore, since the maximum elapsed clotting time is dose dependent, it is reached at an earlier time for the 1-mg/kg

Table IV. Prothrombin Clotting Time of Diphenadione Treated Cows and Suckling Calves (in Seconds)

Animal age and treatment group	h posttreatment 0	12	24	48	72	96	120	144
1. Control cow	19.0	17.0	16.5	19.0	15.8	16.1	16.8	15.0
Control calf	18.0	15.7	15.4	17.0	15.4	15.5	16.0	15.5
2. 1 mg/kg cows (mean ± SD)	15.3 ± 0.3	18.7 ± 2.5	19.0 ± 2.2	25.3 ± 4.2	24.6 ± 6.8	21.2 ± 4.4	20.3 ± 2.8	16.7 ± 2.5
Suckling calves (mean ± SD)	14.8 ± 0.7	16.7 ± 1.6	15.8 ± 0.3	18.5 ± 1.6	15.2 ± 0.2	15.7 ± 0.8	16.3 ± 0.2	14.4 ± 1.2
3. 2.75 mg/kg cows (mean ± SD)	16.7 ± 1.4	16.7 ± 0.4	22.0 ± 0.9	29.2 ± 2.6	39.8 ± 4.5	42.0 ± 8.0	30.5 ± 10.6	23.2 ± 8.7
Suckling calves (mean ± SD)	17.3 ± 0.6	15.5 ± 0.2	15.9 ± 0.1	16.0 ± 1.3	15.3 ± 0.6	15.7 ± 0.6	15.2 ± 0.2	14.3 ± 0.6

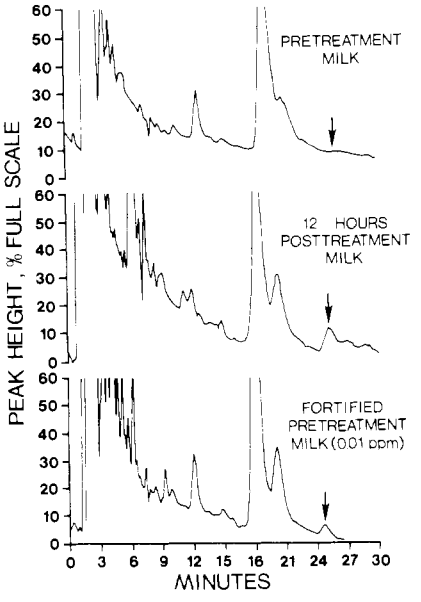

Figure 1. Typical gas-liquid chromatograms of milk samples that were analyzed for diphenadione residues.

rate than for 2.75 mg/kg; i.e., the response at 2.75 mg/kg is higher and does not reach a maximum until several hours after that of animals dosed at 1 mg/kg.

Remington's Pharmaceutical Sciences (1970) lists the range of daily doses for this prothrombinopenic anticoagulant as 2.5 to 30 mg for human therapy. Hence, even if milk contained the 0.021-ppm maximum resulting from accidentally administering 2.75 times the recommended dosage, a person would have to drink 31 gallons of milk to obtain the 2.5-mg minimum daily dosage of diphenadione used in human anticoagulant therapy. The fact that there was neither a detectable plasma level or observable prothrombin response in the nursling calves (Table IV) confirmed this proposition. Since the recommended 1 mg/kg dosage did not induce mammillary transfer of detectable quantities of diphenadione into the milk, the safety of the systemic method vampire bat control with respect to milk residues is assured.

ACKNOWLEDGMENT

The authors gratefully acknowledge the technical assistance provided by Stanley E. Gaddis, Kenneth A. Crane, and Thomas J. Trujillo.

LITERATURE CITED

Blumberg, H., Dayton, H. B., Gordon, S. M., *Proc. Soc. Exp. Biol. Med.* **105**, 35 (1960).
Brambel, C. E., Hunter, R. E., *Am. J. Obstet. Gynecol.* **59**, 1153 (1950).
Bullard, R. W., Holguin, G., Peterson, J. E., *J. Agric. Food Chem.* **23**, 72 (1975).
Bullard, R. W., Thompson, R. D., Holguin, G., *J. Agric. Food Chem.* **24**, 261 (1976).
Field, J. B., *Am. J. Physiol.* **143**, 238 (1945).
Fries, K., Konig, F. E., Reich, T., *Schweiz. Med. Wochenschr.* **87**, 615 (1957).
Greenhall, A. M., *Proc. Vertebrate Pest Conf.* **4**, 41 (1970).
O'Reilly, R. A., Aggeler, P. M., *Pharm. Rev.* **22**, 35 (1970).
Quick, A. J., *J. Biol. Chem.* **109**, 73 (1935).
"Remington's Pharmaceutical Sciences," 14th ed, Hoover, J. E., Ed., Mack Publishing Co., Easton, Pa., 1970, pp 832–833.
Thompson, R. D., Mitchell, G. C., Burns, R. J., *Science* **177**, 806 (1972).

Received for review July 23, 1976. Accepted September 28, 1976. This research was conducted with funds provided to the U.S. Fish and Wildlife Service by the U.S. Agency for International Development under the project "Control of Vertebrate Pests: Rats, Bats, and Noxious Birds", PASA (RA(ID) 1-67. Reference to trade names does not imply U.S. Government endorsement of commercial products.

Insect Chemosterilants. Analogues of 2,5-Dichloro-*N*-(2,4-dinitrophenyl)benzenesulfonamide

Albert B. DeMilo,* Alexej B. Borkovec, and Richard L. Fye

From the 51 analogues of 2,5-dichloro-*N*-(2,4-dinitrophenyl)benzenesulfonamide synthesized and tested as candidate insect chemosterilants, 4-bromo-*N*-(2,4-dinitrophenyl)benzenesulfonamide and 3,4-dichloro-*N*-(2,4-dinitrophenyl)benzenesulfonamide exhibited outstanding effectiveness when fed to adult male *Musca domestica* L.

Several substituted *N*-(1-naphthalenyl)benzenesulfonamides induced sexual sterility in male and female house flies, *Musca domestica* L.; structure–activity studies indicated that the activity in males increased when the naphthalenyl (naphthyl) group was replaced with a phenyl group (DeMilo et al., 1974). Because the male-sterilizing activity is from a practical standpoint more important than the activity against females (Borkovec, 1972) we attempted to identify the structural features that would optimize the sterilizing effectiveness of 2,5-dichloro-*N*-(2,4-dinitrophenyl)benzenesulfonamide (**13**) in male insects. Herein we describe the preparation, properties, and chemosterilant activity of 51 substituted *N*-phenylbenzenesulfonamides and related compounds.

U.S. Department of Agriculture, Agricultural Research Service, Insect Chemosterilants Laboratory, Beltsville, Maryland 20705 (A.B.D., A.B.B.), and Insects Affecting Man Research Laboratory, Gainesville, Florida 32604 (R.L.F.).

EXPERIMENTAL SECTION

Synthesis of Chemicals. The melting points are uncorrected. Microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. Compounds reported in this section gave acceptable analyses for C, H, and N. Tables of complete analytical data for the sulfonamides not mentioned in this section will appear in the microfilm edition; see paragraph at end of paper regarding supplementary material. The majority of compounds listed in Tables I and II are new.