Reports the results of an experiment to determine if mammillary transfer of diphenadione occurs in treated cows. Diphenadione is a systemic anticoagulant effective in killing vampire bats that consume blood from cattle treated intraruminally. The experiments indicate that mammillary transfer of diphenadione, like that of other anticoagulants, is dose dependent. This data indicates that it is safe to consume milk from cows dosed at the recommended rate of 1mg/kg.
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 nursing 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.

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 nursing 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 nursing 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
A 23-ml/min flow of nitrogen makeup gas was added to blood and 142 parameters were: injection port, 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 mammary 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 mammary 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>
<thead>
<tr>
<th>Animal age and treatment group</th>
<th>h posttreatment</th>
<th>0</th>
<th>12</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>96</th>
<th>120</th>
<th>144</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control cow</td>
<td></td>
<td>19</td>
<td>17</td>
<td>16</td>
<td>15</td>
<td>15</td>
<td>18</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>Control calf</td>
<td></td>
<td>18</td>
<td>17</td>
<td>15</td>
<td>17</td>
<td>16</td>
<td>15</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>2. 1 mg/kg cows (mean ± SD)</td>
<td></td>
<td>15.3 ± 0.3</td>
<td>18.7 ± 2.5</td>
<td>19.0 ± 2.2</td>
<td>25.3 ± 4.2</td>
<td>24.6 ± 6.8</td>
<td>21.2 ± 4.4</td>
<td>20.3 ± 2.8</td>
<td>16.7 ± 2.5</td>
</tr>
<tr>
<td>(mean ± SD)</td>
<td></td>
<td>14.8 ± 0.7</td>
<td>16.7 ± 1.6</td>
<td>15.8 ± 0.3</td>
<td>18.5 ± 1.6</td>
<td>16.2 ± 0.2</td>
<td>15.7 ± 0.8</td>
<td>16.3 ± 0.2</td>
<td>14.4 ± 1.2</td>
</tr>
<tr>
<td>Suckling calves (mean ± SD)</td>
<td></td>
<td>16.7 ± 1.4</td>
<td>16.7 ± 0.4</td>
<td>22.0 ± 0.9</td>
<td>29.2 ± 2.6</td>
<td>39.8 ± 4.5</td>
<td>42.0 ± 8.0</td>
<td>30.5 ± 10.6</td>
<td>23.2 ± 8.7</td>
</tr>
<tr>
<td>(mean ± SD)</td>
<td></td>
<td>17.3 ± 0.6</td>
<td>16.5 ± 0.2</td>
<td>15.9 ± 0.1</td>
<td>16.0 ± 1.3</td>
<td>15.3 ± 0.6</td>
<td>15.7 ± 0.6</td>
<td>15.2 ± 0.2</td>
<td>14.3 ± 0.6</td>
</tr>
</tbody>
</table>

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

observable prothrombin response in the nursing calves (Table IV) confirmed this proposition. Since the recommended 1 mg/kg dose did not induce mammary 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.

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LITERATURE CITED


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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...