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## Dicoumarol (moldy sweet clover) toxicosis in a group of Holstein calves

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During a 3-week period of mid-fall 1991, 6 of 25 6-month-old Holstein calves developed progressive rear limb stiffness and recumbency and died 4-12 hours after the onset of clinical signs. Affected animals were initially treated by the referring veterinarian with sulfadimethoxine<sup>a</sup> and vaccinated with a 7-way clostridial bacterin-toxoid.<sup>b</sup> Necropsy of 3 calves by the referring veterinarian revealed hemorrhage in the thorax and epicardial petechial hemorrhages. There was no previous history of vaccination or vitamin E/selenium or any other supplementation. The last calf to die was treated with thiamine and injectable vitamin E and selenium and developed swelling at the site of injection. The affected animals were born on the farm in a closed herd and fed grass hay alternating with alfalfa hay.

One animal was submitted for diagnostic investigation. At necropsy, the carcass appeared to be in good nutritional state and weighed 123 kg. There was a diffuse subcutaneous hemorrhage and edema throughout the body, including lateral aspects of the larynx and esophagus. There was approximately 1 liter of blood in the abdominal cavity. Diffuse hemorrhages were present on the neck, thigh, stifle, and hock muscle, lung, epicardium, endocardium, perirenal areas, and serosal surface of the colon, uterus, and urinary bladder.

Aerobic cultures of lung, liver, and small intestine yielded no significant growth after 48 hours. Bovine viral diarrhea and infectious bovine rhinotracheitis viruses were not detected in the lung, liver, and spleen using a fluorescent antibody test and virus isolation. *Clostridium* species (*C. chauvoei*, *C. sordelli*, *C. septicum*, *C. novyi*) were not identified in skeletal muscle using fluorescent antibody testing.

Microscopically, the liver contained diffuse, moderate, bridging, centrilobular degeneration and necrosis. Cardiac and skeletal muscles contained multifocal areas of myofiber degeneration and necrosis associated with severe hemor-

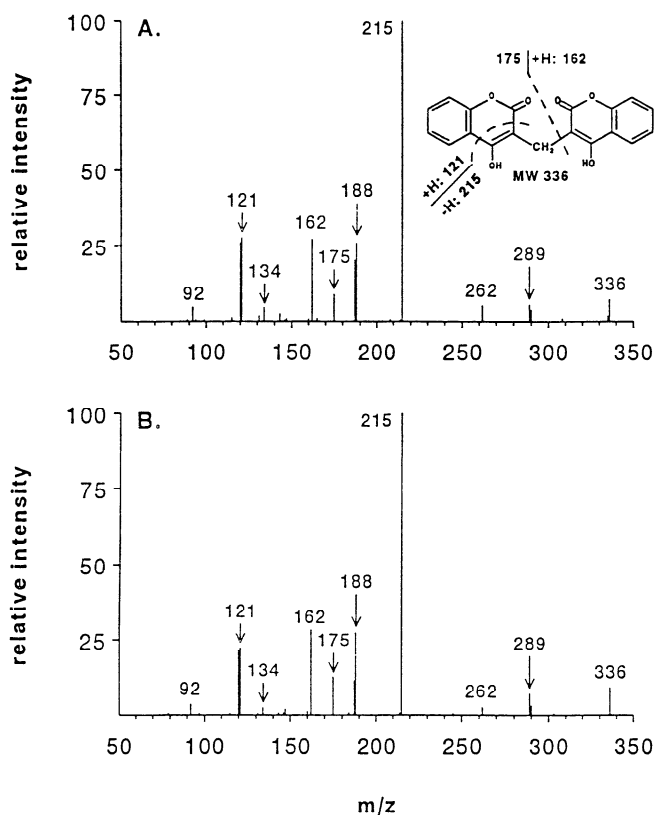
rhage. Multifocal to diffuse, severe hemorrhage was present in sections of lung, intestine, uterus, kidney, and urinary bladder. A diagnosis of dicoumarol (moldy sweet clover) toxicosis was made based on the clinical history, postmortem findings, and detection of dicoumarol in liver tissue by thin-layer chromatography (TLC).<sup>3</sup> Dicoumarol was confirmed and quantified by mass spectrometry/mass spectrometry (MS/MS).<sup>4</sup> Daughter ion spectra of dicoumarol were generated by collision-activated dissociation (CAD) at a collision offset of -10 V from the 70-eV electron impact (EI) parent ion,  $M^+ = 336$ , and also from the methane chemical ionization (CI) parent ion,  $MH^+ = 337$ . Characteristic EI daughter ions of  $M^+ = 336$  were  $m/z$  215, 175, 162, and 121 in both reference dicoumarol and liver extract (Fig. 1). Characteristic CI daughter ions of  $MH^+ = 337$  were  $m/z$  234 and 163, present in both reference dicoumarol and liver extract (data not shown). Results were confirmed by generation of parent ion scans of selected CAD daughter ions, using both EI and methane CI techniques. The direct insertion probe volatilization profiles of reference dicoumarol, the extract of liver from an affected calf, and extract from a bovine liver control were compared (Fig. 2). At 2:14-2:18 minutes, the reference dicoumarol and the extract from the affected calf both exhibited a peak in the reconstructed total daughter ion chromatogram and in the selected ion chromatogram of daughter ion  $m/z$  215. Only background noise at a signal strength 1-2 orders of magnitude less could be observed in the control liver chromatograms. Dicoumarol was quantified in the liver of the affected calf using the ratio of the peak heights of the  $m/z$  215 ion chromatograms (Fig. 2):

$$\text{liver conc. (ppm)} = (P_1/P_d) \cdot (100 \text{ ng}/50 \text{ mg}),$$

where  $P_1$  is the  $m/z$  215 ion current peak height of the liver extract equivalent to 50 mg liver and  $P_d$  is the  $m/z$  215 ion current peak height of 100 ng dicoumarol. The liver of the affected calf contained 2.6 ppm dicoumarol, which is within the range of 1-5 ppm found in liver in other reported cases of dicoumarol toxicity in cattle.<sup>2,7</sup> A sample of the suspect hay submitted at a later date contained 18 ppm dicoumarol on a dry weight basis (the sample contained < 10% moisture) by TLC and high-performance liquid chromatography. A

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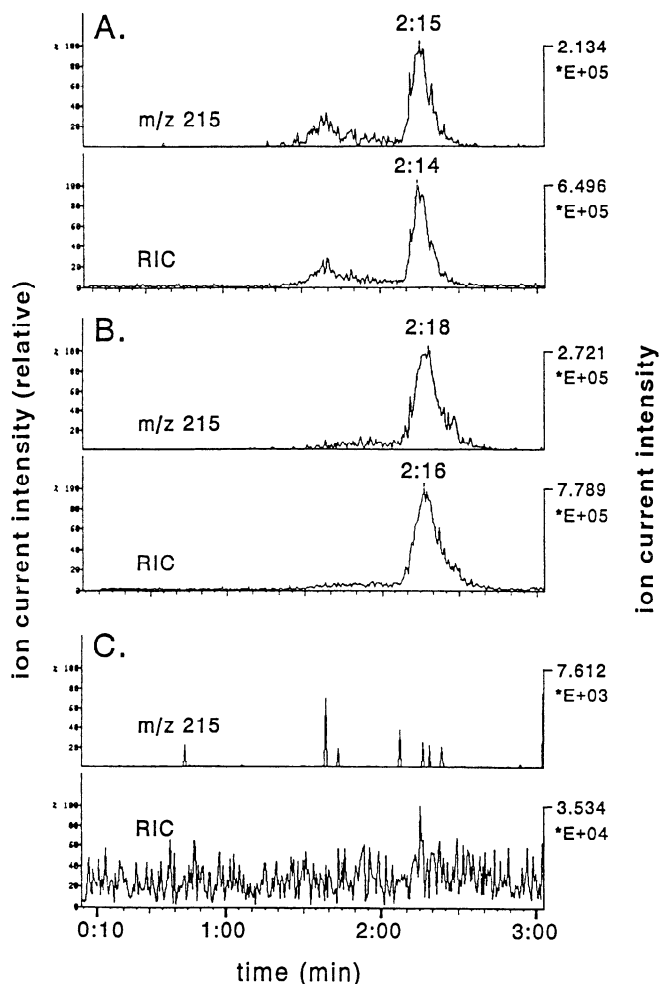


**Figure 1.** MS/MS positive EI (70 eV) daughter ion spectrum resulting from argon collision activated dissociation (Ar CAD) at a collision offset of  $-10$  V. **A.** 100-ng reference dicoumarol. **B.** 50-mg equivalent extract of liver from an affected calf.

sample of the alfalfa appeared to be visually free of clover. Therefore, a chemical analysis for dicoumarol was not performed.

Dicoumarol toxicosis may have to be differentiated from several other infectious and toxic etiologies marked by hemorrhage. These include clostridial disease (*C. chauvoei*, *C. septicum*, *C. novyi*, *C. hemolyticum*), leptospirosis, anaplasmosis, pasteurellosis, bovine viral diarrhea virus, and bracken fern (*Pteridium aquilinum*), anticoagulant rodenticide, nitrofurantoin, sulfonamide, aflatoxin, and trichlorethylene-extracted soybean meal toxicoses.<sup>9,11,12,14</sup> Leptospirosis and anaplasmosis were ruled out based on the gross and microscopic examinations. Bracken fern was not detected in the hay samples examined. Based on the information provided by the owner and referring veterinarian, anticoagulant rodenticide was not used on the farm and the liver sample was negative for anticoagulant rodenticides by chemical analysis. The animals were not previously treated with nitrofurantoin or sulfonamide and were not fed trichloroethylene-extracted soybean oil meal. Myocardial and skeletal muscle degeneration and necrosis were suggestive of vitamin E/selenium deficiency. However, liver vitamin E and selenium analyses were not performed.

In the present case, the animals were confined to dry lots and were fed large round bales of mixed grass hay. The hay was harvested the previous summer and was stored outside.



**Figure 2.** Direct insertion probe volatilization. The top tracing of each graph is the reconstructed ion current "chromatogram" of the daughter ion  $m/z$  215 resulting from Ar CAD of the dicoumarol-positive EI parent ion  $M^+ = 336$ , whereas the bottom tracing is the reconstructed total ion current "chromatogram" (RIC) of the daughter of  $M^+ = 336$ . The "chromatograms" have been normalized by the data system to the largest peak, with the absolute ion current intensities on the right y axis. **A.** 100-ng reference dicoumarol. **B.** 50-mg equivalent extract of liver from an affected calf. **C.** 50-mg equivalent extract of liver from a bovine control.

The owner noted the hay to be "musty" and was alternating the affected hay with good quality alfalfa hay. The animals had consumed 3 "musty" round bales over approximately 1 month. Visual examination of the submitted hay sample revealed the presence of sweet clover.

*Melilotus alba* (white sweet clover) and *M. officinalis* (yellow sweet clover) are found as weeds throughout the United States and are grown as forages in certain regions of the country. Moldy sweet clover toxicosis in cattle has been recognized for approximately 70 years.<sup>12</sup> Intoxication is due to the formation of the anticoagulant, dicoumarol, in improperly cured clover hay or silage. The plants contain coumarin, which is converted into 4-hydroxycoumarin; this subsequently condenses to form dicoumarol (3,3'-methylene-bis-(4' hydroxycoumarin). The conversion of coumarin to

4-hydroxycoumarin results from the action of several fungi, including *Penicillium*, *Aspergillus*, *Fusarium*, and *Mucor*.<sup>8</sup> Following ingestion, dicoumarol antagonizes vitamin K epoxide reductase, which is responsible for maintaining body stores of active vitamin K<sub>1</sub>. In the absence of sufficient active vitamin K<sub>1</sub>, vitamin K-dependent clotting factors (II, VII, IX, X) are consumed, which subsequently results in the occurrence of a hemorrhagic diathesis. Clinical signs include spontaneous hematomas, weakness, tachycardia, tachypnea, pale mucous membranes, and prolonged prothrombin times. Animals may be found dead without premonitory signs. Dicoumarol toxicosis has been reported primarily in cattle. Sheep can become intoxicated although they are more resistant to the anticoagulant effects of dicoumarol.<sup>13</sup> A feeding period of several weeks or longer is generally necessary for toxicosis to occur.<sup>6</sup>

The detected concentration of dicoumarol in the submitted hay sample is near a potentially toxic range of 20-30 ppm.<sup>6</sup> However, it is uncertain whether the detected concentration reflected the ingested concentration, because the forage sample was obtained several weeks after the toxicosis was diagnosed. Careful sampling of hay is critical to obtain a representative sample for analysis, because dicoumarol concentrations may vary within and between bales.<sup>5</sup> The sample of hay submitted had been obtained by the owner from 1 of the large bales. The bale was unrolled, and samples were collected from throughout the entire bale to obtain a representative sample.

This case is unique in 2 respects. First, sweet clover was a contaminant weed in the hay being offered to the calves. In other reported cases, sweet clover was the principle forage being fed at the time of intoxication. Thus, cases of moldy sweet clover toxicosis may occur when grass or legume pastures are heavily contaminated with this ubiquitous weed. Second, the use of MS/MS provides a powerful tool to confirm the presence of dicoumarol in biologic specimens. Because of its strong polarity and low pressure, dicoumarol cannot be readily analyzed by gas chromatographic techniques. MS/MS allows mass spectra of specific molecules in a mixture to be obtained without prior gas chromatographic separation. This is accomplished through selective choice of parent ions with 1 mass filter, generation of daughter ions by CAD of the selected parent ions with argon atoms, and separation of the daughter ions with a second mass filter. The first mass filter in effect replaces the chromatographic step by admitting a very select group of ions, those with the mass of the parent ion only, into the collision chamber to be fragmented for further mass spectral analysis.

Recommended treatment of dicoumarol toxicosis includes removing the animals from affected forage, administration of vitamin K<sub>1</sub> and, in severe cases, administration of whole blood or plasma. In most situations, the cost of treatment is prohibitive. Administration of less costly vitamin K<sub>3</sub>, in lieu of vitamin K<sub>1</sub>, has given inconsistent results,<sup>11</sup> possibly because of the inability of vitamin K<sub>3</sub> to activate clotting factors

prior to its own metabolism to active vitamin K<sub>1</sub>. Prolonged clotting times can return to normal within 4 days of withdrawal of affected feed without additional treatment.<sup>10</sup> These affected cattle were not treated with vitamin K but made uneventful recoveries over several days.

If forage is known to contain dicoumarol, toxicosis can be prevented if feeding of affected forage is alternated with feeding of good quality alfalfa hay every 10 days. However, in these cattle, this feeding strategy was not sufficient to prevent intoxication.

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