ORIGINAL RESEARCH

PEER REVIEWED

Depletion of bromadiolone in tissues of hogs following oral exposure

Saad Enouri, MSc, PhD; Kristina Dekroon, BSc; Robert Friendship, DVM, MSc, Diplomate ABVP; Nick Schrier, MSc; Patricia M. Dowling, MSc, Diplomate ACVIM (LAIM), Diplomate ACVCP; Ron Johnson, PhD, Diplomate ACVCP

Summary

Objectives: To assess bromadiolone depletion in the edible tissues of swine and propose post-exposure withdrawal periods.

Materials and methods: Two groups of barrows and two groups of gilts were given a single oral dose of bromadiolone: low dosage (LD, 0.05 mg/kg; n = 20; 10 males, 10 females) and high dose (HD, 0.5 mg/kg; n = 20; 10 males, 10 females). Coagulation parameters were assessed before and after administration. Animals were sacrificed at 1, 2, 3, 5, and 6 weeks (LD) and 1, 2, 3, 6, and 9 weeks (HD) post dosing. Loin muscle, skin-adherent fat, liver, feces, and blood were analyzed for bromadiolone using liquid chromatography-tandem mass spectrometry.

Results: Partial thromboplastin times exceeded control values in the LD and HD groups 6 and 9 weeks post dosing, respectively. In the HD group, bromadiolone concentrations exceeded the limit of detection (LOD) at all time points in liver and skin-adherent fat and for up to 6 weeks in feces, muscle, and plasma. In the LD group, bromadiolone concentrations exceeded the LOD at all time points in liver and up to 3 weeks in fat, feces, and plasma. Estimated withdrawal periods for bromadiolone in liver were 83 and 176 weeks in the LD and HD groups, respectively, and 62 weeks in muscle in the HD group.

Implication: Bromadiolone residues persist in tissues such that it is impractical to wait for the hog to eliminate the rodenticide to a concentration that is safe for entry into the human food chain.

Keywords: swine, rodenticides, bromadiolone detection, withdrawal period, liquid chromatography-tandem mass spectrometry.

Received: January 8, 2015
Accepted: March 3, 2015

Resumen - Disminución de bromadiolona en tejidos de cerdos después de la exposición oral

Objetivos: Evaluar la reducción de bromadiolona en tejidos comestibles de cerdos y proponer periodos de retiro después de la exposición.

Materiales y métodos: Se administró una dosis única de bromadiolona a dos grupos de machos castrados y dos grupos de hembras: dosis baja (LD [dosis baja por sus siglas en inglés), 0.05 mg/kg; n = 20; 10 machos, 10 hembras) y dosis alta (HD [dosis alta por sus siglas en inglés], 0.5 mg/kg; n = 20; 10 machos, 10 hembras). Se evaluaron los parámetros de coagulación antes y después de la administración. Los animales fueron sacrificados a las semanas 1, 2, 3, 5, y 6 (LD) y a las semanas 1, 2, 3, 6, y 9 (HD) después de la administración de la dosis. Se analizaron el músculo del lomo, la grasa adherida a la piel, el hígado, las heces, y la sangre en busca de bromadiolona utilizando cromatografía de líquidos con espectrometría de masas en tándem.

Resultados: Los tiempos parciales de tromboplastina excedieron los valores control en los grupos de LD y HD 6 y 9 semanas después de la administración de la dosis, respectivamente. En el grupo de HD, las concentraciones de bromadiolona excedieron el límite de detección (LOD por sus siglas en inglés) en todos los muestreos en hígado y grasa adherida a la piel, y hasta por 6 semanas en heces, músculos y plasma. Los periodos de retiro estimados para la bromadiolona en hígado fueron 83 y 176 semanas en los grupos de LD y HD, respectivamente, y 62 semanas en músculo en el grupo HD.

Implicación: Los residuos de bromadiolona persisten en tejidos de tal manera que es impráctico esperar a que el cerdo elimine el raticida hasta una concentración que sea segura para entrar en la cadena alimenticia humana.

Résumé - Déplétion du bromadiolone dans les tissus de porcs suite à une exposition orale

Objectifs: Évaluer la déplétion du bromadiolone dans les tissus comestibles de porc et proposer une période de retrait post exposition.

Matériels et méthodes: Deux groupes de castrats et deux groupe de cochettes reçurent par voie orale une dose unique de bromadiolone: faible dosage (FD, 0,05 mg/kg; n = 20; 10 mâles, 10 femelles)

SE, RJ: Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada.

KD, RF: Department of Population Medicine, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada.

NS: Agriculture and Food Laboratory, Guelph, Ontario, Canada.

PMD: Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

Corresponding author: Dr Ron Johnson, Room 1683, Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, ON N1G 2W1, Canada; Tel: 519-824-4120, Ext 54041; Fax: 519-767-1450; E-mail: rjohns03@uoguelph.ca.

This article is available online at http://www.aasv.org/shap.html.

Enouri S, Dekroon K, Friendship R, et al. Depletion of bromadiolone in tissues of hogs following oral exposure. *J Swine Health Prod.* 2015;23(6):298–305.

et dosage élevé (DE, 0,5 mg/kg; n = 20; 10 mâles, 10 femelles). Les paramètres de coagulation furent évalués avant et après l'administration. Les animaux furent sacrifiés à 1, 2, 3, 5, et 6 semaines (FD) et à 1, 2, 3, 6, et 9 semaines (DE) suivant l'administration du produit. Le muscle de la longe, du gras adhérent à la peau, du foie, des fèces, et du sang furent analysés pour la présence de bromadiolone par chromatographie liquide couplée à la spectrométrie de masse en tandem.

Résultats: Les temps de thromboplastine partielle excédaient les valeurs de contrôle pour les groupes FD et DE à 6 et 9 semaines post administration, respectivement. Dans le groupe DE, les concentrations de bromadiolone excédaient la limite de détection (LD) à tous les moments testés dans le foie et le gras adhérent à la peau et jusqu'à 6 semaines dans les fèces, le muscle, et le plasma. Dans le groupe FD, les concentrations de bromadiolone excédaient la LD à tous les temps testés dans le foie et jusqu'à 3 semaines dans le gras, les fèces, et le plasma. Les périodes de retrait estimées pour le bromadiolone dans le foie sont de 83 et 176 semaines dans les groupes FD et DE, respectivement, et de 62 semaines dans le muscle du groupe DE.

Implication: Les résidus de bromadiolone persistent trop longtemps dans les tissus pour qu'il soit pratique d'attendre que le porc élimine le rodenticide à une concentration sécuritaire pour permettre l'entrée de la viande dans la chaîne alimentaire humaine.

Accidental ingestion of rodenticides in hogs is a significant food-safety concern, impacts animal welfare, and can result in substantial economic losses to producers. The hydroxycoumarins are a group of anticoagulant compounds that include bromadiolone, brodifacoum, coumafuryl, coumatetralyl, difenacoum, and warfarin.[1-3] These compounds are commonly used as rodenticides worldwide for the control of rats and mice. The increased commercial availability of these compounds has resulted in accidental ingestion by animals.[4] Further, the emergence of rodent strains resistant to older or first-generation anticoagulant rodenticides has spawned the development of more potent, second-generation compounds such as bromadiolone, with increased potential for toxicity following accidental ingestion by livestock and adulteration of food intended for human consumption.[5]

The true incidence of bromadiolone exposures in food-producing animals, including hogs, is not currently known. The Canadian Global Food Animal Residue Avoidance Databank (Canadian g-FARAD) has previously received queries regarding accidental ingestion of rodenticides in swine. Bromadiolone is one of the most commonly cited rodenticides in suspected accidental ingestion in swine, according to the Canadian gFARAD (`https://cgfarad.usask.ca/home.html`), making it an ideal candidate for study. The median oral lethal dose (LD_{50}) for bromadiolone in research hogs is estimated to be 3 mg per kg body weight following single dosing, but no data on toxicokinetics of bromadiolone in hogs are currently available.[1] Oral bioavailability of rodenticides is generally high in mammals (79% to 92%),[1] and residue depletion may be prolonged[6] due to liver accumulation and enterohepatic recycling,[1,7,8] with some studies in rodents suggesting that bromadiolone toxicokinetics are dose-dependent.[7] Clinical signs associated with anticoagulant rodenticide toxicosis may vary, including spontaneous bleeding or bruising or both, with elevations in prothrombin time (PT) and activated partial thromboplastin time (PTT) confirming blood-clotting abnormalities.[9,10] However, with lesser exposures, swine may not show clinical signs, yet still have violative residues in edible tissues. Information pertaining to exposure, tissue depletion, and possible withdrawal periods of rodenticides in suspected swine toxicosis would provide substantial guidance to veterinarians and producers regarding animal disposition. Therefore, the objectives of this study were to assess the depletion of bromadiolone in the edible tissues of swine and evaluate bromadiolone withdrawal periods following oral exposure. The study also sought to evaluate changes to coagulation profiles associated with bromadiolone exposure.

Materials and methods

The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Guelph and conformed to standards set forth by the Canadian Council on Animal Care.

Animals

Male (20 barrows) and female (20 gilts) Yorkshire hogs weighing 40.4 ± 1.4 kg and 38.4 ± 1.5 kg (mean ± standard error [SE]), respectively, were used in this study. A pilot depletion study (12 Yorkshire hogs) was carried out followed by a main depletion study (28 Yorkshire hogs). On the basis of physical examination, all animals were deemed healthy prior to study. Animals were born in the established herd at Arkell Research Station (Guelph, Ontario, Canada) and housed at the same facility according to standards of care for swine for the duration of the study, with free access to feed and water. Animals were randomly assigned to pens with stocking density as low as possible (four hogs per pen) in order to provide greater observation of each animal and reduce injuries caused by fighting. Additionally, animals were monitored daily for general health and adverse events throughout the study.

Study design

Our experimental design, including animal numbers per sacrifice group, was based on current recommendations of the Veterinary International Conference on Harmonisation (VICH) guideline (no. 48)[11] outlining marker residue depletion studies to establish compound withdrawal times. The marker residue, being the parent molecule or metabolite, is determined from total residue and metabolism studies in the target species. The marker residue depletes in known relation to depletion of total residues. This guideline is currently used by the Food and Drug Administration-Center for Veterinary Medicine (FDA-CVM; United States) and the Veterinary Drug Directorate of Health Canada for regulatory approval of drugs requiring withdrawal times in food-producing animals. Although the metabolism and safety profile of bromadiolone in hogs is unknown, it was not an objective of the study to determine the marker residue for bromadiolone in hogs. Rather, the depletion of the parent bromadiolone molecule was chosen for assay with the understanding that metabolites of bromadiolone may persist in edible tissues for longer periods than the parent bromadiolone molecule. A pilot study was first conducted to assist with determination of sampling time points (sacrifice times) for the main depletion study. Furthermore, because of the anticipated long residue depletion for bromadiolone in hogs, we included additional sacrifice time points in the main depletion study, on the basis of the pilot study results. Data generated in the pilot study were added to the main depletion study, giving a final number of four hogs (two male, two female) per sacrifice time point.

Because bromadiolone is not approved for use as a therapeutic, and there is no available dosage regimen, and because hogs may accidentally ingest varying quantities of bromadiolone, we chose to study both a low (0.05 mg per kg) and a high (0.5 mg per kg) bromadiolone dosage. All hogs were weighed 1 day before starting either the pilot or main depletion study. For the pilot study, 12 normal, healthy male (six barrows) and female (six gilts) Yorkshire hogs were used. The pilot study was conducted in two parallel arms of six hogs each, with a low dose (LD) single oral bromadiolone (Sigma Aldrich, Oakville, Ontario, Canada) dosage group (0.05 mg per kg body weight) and a high dose (HD) single oral bromadiolone dosage group (0.5 mg per kg body weight), with each arm of the study balanced across sexes. In the LD group, two hogs (one male and one female) were randomly chosen, by drawing ID numbers from a container, to be sacrificed at 7 days, 14 days, and 21 days post dosing, while in the HD group, two hogs (one male and one female) were sacrificed at 7 days, 14 days, and 42 days post dosing. A similar approach was followed in the main depletion study (28 Yorkshire hogs), with hogs in the low and high treatment dosage arms being sacrificed at 1, 2, 3, 5, and 6 weeks and 1, 2, 3, 6, and 9 weeks, respectively, taking into consideration the total number of animals to be sacrificed at each time point, based on the VICH guideline[11] and on the number of animals used per sacrifice time point in the pilot study.

Tissue sampling

Samples were collected from all animals at pre-dosing (plasma from anti-coagulated blood [sodium heparin] and fresh feces) and post dosing (plasma from anti-coagulated blood [sodium heparin], fresh feces, liver, loin muscle, and skin-adherent fat) at the time of sacrifice for each treatment group (LD group at 1, 2, 3, 5, and 6 weeks; HD group at 1, 2, 3, 6, and 9 weeks) for determination of bromadiolone concentrations by mass spectroscopy. In the pilot study, plasma from anti-coagulated blood (sodium heparin) was also collected from all animals at 12, 24, 48, and 72 hours post dosing, and fresh fecal samples were collected at 24 and 72 hours post dosing, in order to assist the Animal Health Laboratory (AHL) at the University of Guelph with the validation of a non-invasive detection assay to confirm bromadiolone exposure. Prothrombin time and PTT evaluation were performed on blood samples collected from all hogs at predosing, and 24, 48, and 72 hours post dosing during the first week, as well as on subsequent samples collected twice a week until sacrifice time. All animals were sacrificed by exsanguination following electrical stunning before collection of tissues samples, including liver (minimum of 250 g), loin muscle (approximately 500 g core sample), and skin-adherent fat (minimum of 50 g of natural tissue proportion). All hogs were sacrificed at the university abattoir according to standard handling protocols. All samples were identified and stored at -80°C until analysis. Blood samples for PT and PTT assessment were analyzed on the same collection day by the AHL in order to evaluate effect on coagulation. Using baseline (control) PT and PTT values from all hogs in the study, the AHL generated reference intervals for both PT and PTT using Rhoads EP Evaluator software (Release 8; David G. Rhoads Associates Inc, Kennett Square, Pennsylvania). The reference range is being defined as the so-called normal range (in seconds) for these respective coagulation tests, ie, the range of the central 95% of results obtained from a group of ostensibly healthy individuals.

Sample preparation for mass spectroscopy

Each plasma sample (0.5 mL) was transferred to a screw-cap centrifuge tube (13 mm × 100 mm), vortex-mixed for 30 seconds, and extracted using 4.5 mL of acetonitrile. After centrifugation for 10 minutes at 1932*g*, the acetonitrile layer was removed using a 1-mL syringe, and the extract was filtered through a syringe filter (13 mm; polytetrafluoroethylene [PTFE], 0.2 μm) into an autosampler vial. Liver, muscle, skin-adherent fat, and fecal samples were prepared by weighing 1.0 ± 0.2 g of each tissue into MediFASTH2 vials (Syntec International, Dublin, Ireland), then adding 10 mL of acetonitrile and 10 mL of acetonitrile:water (85:15, volume:volume) for liver and muscle, and skin-adherent fat and fecal samples, respectively, and processing using a MediFASTH2 homogenizer. The acetonitrile layer was transferred to a centrifuge tube (16 mm × 125 mm) and centrifuged for 10 minutes at 1932*g*. After centrifugation, the acetonitrile layer was removed using a 1-mL syringe, and the extract was filtered through a syringe filter (13 mm; PTFE, 0.2 μm) into an autosampler vial. In the case of skin-adherent fat and feces, the acetonitrile extract was washed with 2 × 5 mL of hexane. Finally, the obtained aliquots of tissue extracts were injected into the liquid chromatography-tandem mass spectrometry (LC-MS-MS) system for analysis.

LC-MS-MS analysis

The bromadiolone analytical method development and validation were carried out by one of the authors (NS) at the University of Guelph, Laboratory Services, AHL (Guelph, Ontario, Canada) utilizing a standard operating procedure based on the CITAC/Eurachem guide to quality in analytical chemistry.[12] Tissue extracts were assayed for bromadiolone concentrations in each matrix type using an LC-MS-MS system consisting of an Agilent 1100 series vacuum degasser, binary pump (Agilent Technologies Canada Inc, Mississauga, Ontario, Canada), and a Shimadzu autosampler (Mandel Scientific, Ontario, Canada) coupled to a triple quadrupole mass spectrometer (QTRAP 4000; AB SCIEX, Concord, Ontario, Canada). The following instrument variables were used: flow rate, 1.0 mL per minute; total run time, 10 minutes; ion spray voltage, 2000 V; source temperature, 650°C; polarity, electrospray ionization (ESI), negative; ESI probe setting: X-axis = 5 mm, Y-axis = 2 mm; curtain gas, 10 pounds per square inch; collision gas, medium. The injection volume was 1 μL, and chromatographic separation was achieved by use of Atlantis dC18 mini column (3.9 × 20 mm; internal diameter 3 μm) (Waters Corporation, Milford, Massachusetts). The column oven temperature was maintained at 10°C, and the autosampler temperature was 5°C. Bromadiolone concentrations for plasma, muscle, liver, skin-adherent fat, and feces were estimated from bromadiolone-spiked calibration curves. The validated limit of quantification (LOQ) and limit of detection (LOD) for calibration curves for skin-adherent fat (μg per kg), plasma (ng per mL), feces, muscle, and liver (μg per kg) were 1.0, 1.7, 3.3, 6.6, 10.0, and 0.3, 0.5, 1.0, 2.0, 3.0, respectively. Each matrix was validated at 3 × LOQ and run in triplicate. The calibration curve was evenly distributed, with a coefficient of determination (r^2) higher than 0.99 for each curve. The percent accuracy (mean ± SD) for bromadiolone determination in plasma, muscle, liver, skin-adherent fat, and feces was 88.0 ± 2.4, 66.7 ± 8.1, 78.7 ± 6.6, 106.7 ± 4.6, and 116.0 ± 8.2, respectively. The coefficient of variation for reference curve bromadiolone concentrations in all matrices including the LOQ was less than 15%.

Statistical analysis

All data analyses were conducted with the assistance of a biostatistician. Prior to analysis of data for withdrawal-period estimation, bromadiolone concentrations were transformed using the natural logarithm (Ln). Least squares means and 95% confidence levels were reported along with back-transformed values. The statistical significance level was set at $P < .05$. Statistical analyses were begun with a full factorial model that included all interactions (ie, dose, sex, tissue, and time), with terms being removed if P values were > .05. All terms involving sex had P values > .05. Tissue residue data for withdrawal-period determination were analyzed by a statistical method that determined the statistical tolerance limit for the central 99% of the population with 95% confidence using SAS (version 9.1.3; SAS Institute Inc, Cary, North Carolina).[13,14] Briefly, regression analyses were performed on tissue bromadiolone concentrations to determine the time required for bromadiolone in the LD and HD treatment groups to reach a predetermined tissue concentration, ie, level of detection for the analyte in each matrix. In order to evaluate whether elimination of bromadiolone in hog tissues was dose dependent, depletion of bromadiolone from the liver in HD and LD groups, and from liver HD and muscle HD groups, was assessed using a general linear model to test differences in slopes over time. Rodenticides are classified as pesticides in most countries and are not approved for use in food-producing animals. Therefore, no acceptable daily intake of bromadiolone has been set by most regulatory authorities. However some regulatory agencies may allow certain concentrations of pesticides, such as rodenticides, in edible foodstuffs.[15] Zero levels of an analyte, as determined by assay, are set at the limit of detection for that assay of the analyte in question and were used in the current study for the withdrawal period. In order to increase statistical power, results of pilot-study hogs were combined with results of main-study hogs. Slopes of the regression analysis for hogs in the pilot study did not differ significantly from the slopes in the main study, suggesting that all hogs eliminated bromadiolone similarly, and therefore could be combined as a single data set for statistical analysis. Prothrombin time and PTT values and bromadiolone concentrations obtained in both treatment groups up to 1 week post dosing, as well as subsequent data obtained twice a week after the first week and at sacrifice time points, were analyzed by ANOVA for repeated measures, respectively. To meet the assumptions of the ANOVA, log transformation was applied when appropriate. Prothrombin time values were presented as arithmetic means, and PTT values as geometric means.

Results

No injuries or adverse events occurred throughout the study period. All data reported include pilot-study data. Plasma and fecal samples collected prior to dosing in all hogs contained undetectable concentrations of bromadiolone. The normal range of swine PT values was 11.7 to 16.0 seconds (mean ± SD = 13.9 seconds ± 1.0 second), while for PTT, the normal range was 22.3 to 51.0 seconds (geometric mean ± SD = 37.4 seconds ± 6.9 seconds). In both treatment groups, mean PT values obtained in bromadiolone-treated animals did not differ statistically from control (baseline) values at any study sacrifice time points (data not shown; $P = .18$). In the LD and HD groups, geometric mean values of PTT obtained at 6 weeks (55.7 seconds; lower limit [LL] 42.9 seconds; upper limit [UL] 72.3 seconds) and 9 weeks (163.5 seconds; LL 82.0 seconds; UL 326.1 seconds) post dosing were significantly greater ($P < .001$) than the control value (37.7 seconds; LL 33.7 seconds; UL 42.3 seconds), respectively. Some individual hogs had PT and PTT values above the normal ranges at various times, returning to normal values on subsequent PT and PTT tests (data not shown). None of these hogs showed clinical signs of bleeding or bruising.

In the pilot study, all hogs in the LD and HD groups were positive (both above the LOD) for bromadiolone in feces collected at 24 and 72 hours post dosing (data not shown). However, in the LD group, one of six hogs was negative (below the LOD) for bromadiolone in plasma collected at 24 hours post dosing, and two of six hogs were negative at both 48 and 72 hours post dosing (data not shown). Bromadiolone concentrations in both plasma (Figure 1) and feces (Figure 2) were greater in the HD group than those obtained in the LD group ($P < .05$). The gradual decline in bromadiolone concentrations in feces at 72 hours, compared to 24 hours, was followed by a sharp elevation at 7 days post dosing, particularly in the HD group (Figure 2), then a gradual decrease at subsequent study time points (tables 1 and 2).

Bromadiolone residues in pig tissues at post-dosing sacrifice time points

Results of data analysis showed that sex had no effect on residue depletion of bromadiolone in the present study. At the 1, 2, 3, and 6 weeks sacrifice time points, for all tissues and feces collected, bromadiolone concentrations were higher in samples obtained in the HD treatment group (Table 1) than in those in the LD group ($P < .01$) (Table 2). In both treatment groups, bromadiolone concentrations decreased gradually in all tissues and feces across all sacrifice time points, but remained above the LOD at some time points. In the HD group, concentrations of bromadiolone remained higher than the corresponding LOD at all sacrifice time points in liver (Figure 3A, Table 2) and skin-adherent fat (Table 2), and up to 6 weeks post dosing in feces, plasma (Table 2), and muscle (Table 2, Figure 4). In the LD group, concentrations of bromadiolone were higher in liver (Figure 3B, Table 1), while lower in muscle (Table 1), than the corresponding LOD at all sacrifice time points. In the LD group, feces, plasma, and skin-adherent fat bromadiolone concentrations continued to be higher than the LOD up to 3 weeks post dosing (Table 1). It should be noted that in the LD group, bromadiolone was detected in muscle of only one of four hogs at week 2 post dosing. In the LD and HD groups, liver bromadiolone concentrations were higher than those detected in other tissues and feces at all post-dosing sacrifice time points ($P < .001$). At the 1-week sacrifice time point, all hogs were positive for bromadiolone in liver samples; however, one of the four hogs in the LD group was negative on fecal assay for bromadiolone, with an additional different hog being negative on plasma assay (data not shown).

Bromadiolone withdrawal period estimation

The tissue concentrations for bromadiolone were used to calculate a withdrawal period by applying the statistical tolerance limit method.[16] The withdrawal period provides a time interval within which the concentrations of bromadiolone are at the maximum predetermined concentration (eg, muscle, 2 μg per kg; liver, 3 μg per kg) or below for 99% of treated pigs with a 95% confidence level. The maximum predetermined concentration for this study was denoted by the current LOD for the assay used to quantify bromadiolone concentrations in tissues. The withdrawal period calculation was achievable on liver (LH and HD groups) and muscle (HD group) samples. In LD and HD skin samples, estimation of the withdrawal period was not possible because the ANOVA test (F test) was not significant and slopes were not obtained (ie, slopes = 0). Additionally, in the LD group, only one muscle sample

showed detectable bromadiolone concentration. The withdrawal period was calculated from the regression lines according to FDA-CVM guidelines[16] and was estimated as 83 weeks for LD liver, 176 weeks for HD liver, and 62 weeks for HD muscle. In order to evaluate the presence of dose-dependent elimination kinetics for bromadiolone in hog tissues, the slopes of the plots of HD and LD liver and HD muscle and HD liver over time were compared. Results showed no difference between liver HD and LD slopes ($P = .29$) or liver HD and muscle HD slopes ($P = .36$), suggesting dose-independent elimination kinetics.

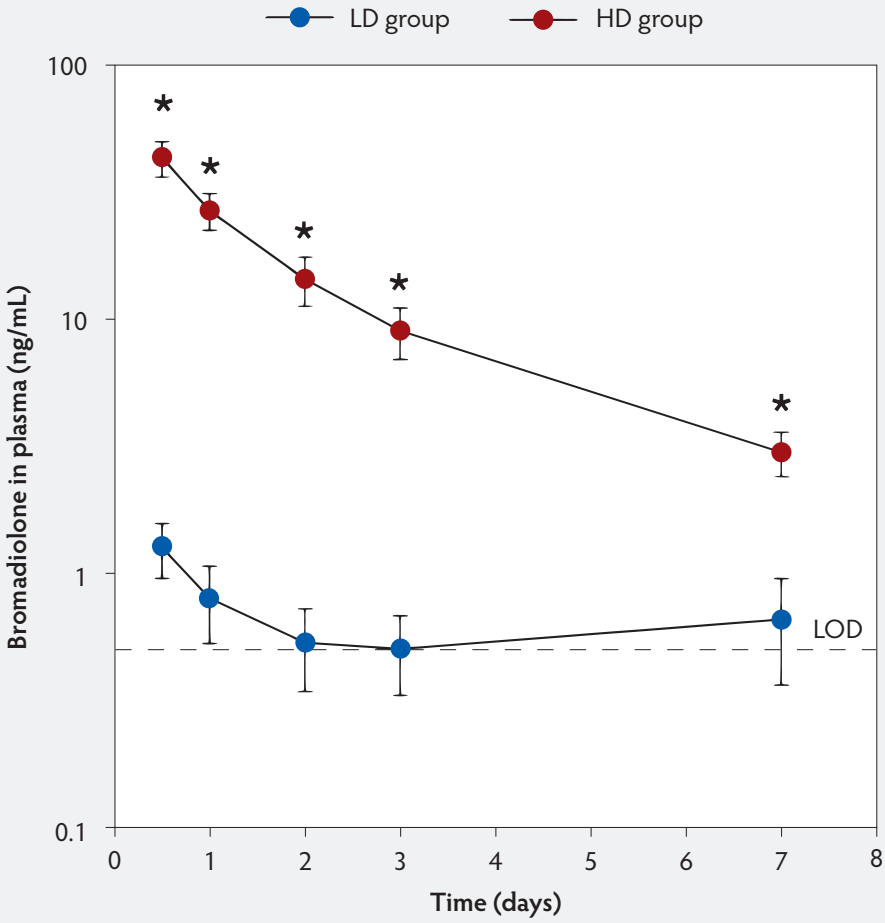
Discussion

In Canada, rodenticide toxicosis reported in hogs most likely results from direct exposure and ingestion of rodenticide baits such as bromadiolone or difethialone. Employing bait stations in the barn is crucial in order to reduce accessibility to rodenticides by non-target animals.

Exposure to second-generation rodenticides results in prolonged tissue residue depletion, particularly in the liver as reported in the present study. In the present study, bromadiolone was administered as a single oral dose of either 0.05 or 0.5 mg per kg body weight, on the basis of the following reasoning. Firstly, an oral LD_{50} (dose producing death in half of the animals tested after single oral dosing) of 3 mg per kg body weight has been reported for bromadiolone in swine.[1] Secondly, recommendations for application of bromadiolone bait in barns may vary; however, common approaches using 100 to 450 grams of bait per site interval will provide approximately 0.05 to 0.5 mg per kg of bromadiolone (0.005% weight by weight) with a single ingestion. Lastly, the selection of a low and high dosage group enables the evaluation of dose-dependent residue elimination.

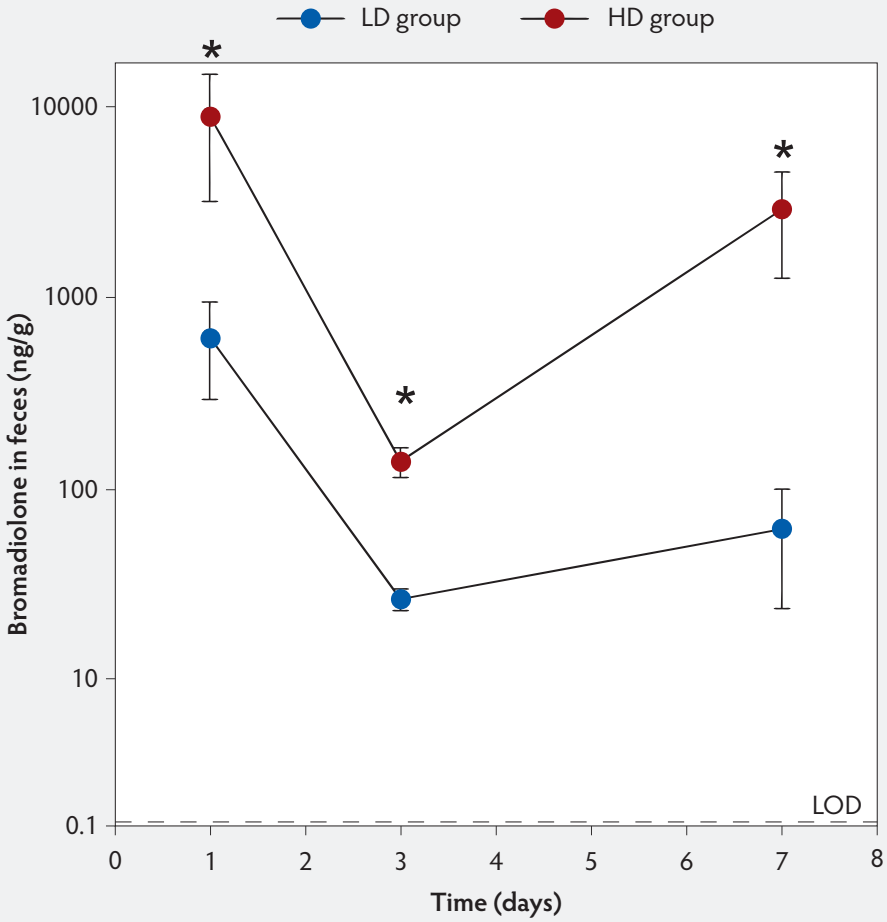
Anticoagulant rodenticides, including bromadiolone, interfere with the normal synthesis of vitamin K-dependent clotting factors in the liver, which results in extended bleeding time and possibly death.[1] However, in the present study, no clinical signs of hemorrhage or significant bruising were observed in any animal. In the current study, PT values were similar to control values in both groups; however, in the LD and HD groups, PTT values obtained at 6 and 9 weeks post dosing were significantly greater than control values, respectively. Therefore, these measures of blood clotting activity cannot be used as an indicator of the absence of anticoagulant rodenticide residues in swine. Alternatively, plasma and fecal rodenticide analyses could be used to assess the ingestion of bromadiolone. Any detectable plasma or fecal concentration of bromadiolone would confirm ingestion, with bromadiolone concentrations in edible tissues possibly being higher than those in either plasma or feces. Our results showed that all hogs receiving bromadiolone tested positive on fecal assay in the first 72 hours following exposure, but some animals in the LD group did record negative plasma assay results during the same time period post dosing. However, findings in the current study also suggest that starting at 1 week post dosing, negative results on either plasma or fecal bromadiolone tests could occur, with other edible tissues, such as liver, being positive for bromadiolone.

Figure 1: Mean plasma concentrations of bromadiolone versus time after single oral administration of low dosage (0.05 mg/kg; LD, n = 6) and high dosage (0.5 mg/kg; HD, n = 6) obtained during the first week post dosing in healthy Yorkshire pigs weighing (mean ± standard error [SE]) 40.4 ± 1.4 kg (males) and 38.4 ± 1.5 kg (females). Pigs were housed in groups according to standard of care for swine and had free access to feed and water. Data reported as mean ± SE. Asterisks (*) indicate a statistical difference (ANOVA; $P < .05$) between the LD group and the HD group at the corresponding time point. LOD = limit of detection.



Our results showed that bromadiolone had a biphasic depletion pattern in feces, with the highest bromadiolone concentrations being detected for all tissues and feces during the first week of the single oral administration. Plasma concentrations of bromadiolone increased rapidly following dosing, with maximum concentrations of 1.3 ± 0.3 ng per mL and 43.2 ± 6.9 ng per mL being detected at 12 hours post dosing in the LD group and HD group, respectively. The use of other anticoagulant rodenticides in other species have led to similar trends.[17] In the present study, bromadiolone concentrations gradually declined following dosing, but remained relatively high over the study period in the liver of the LD and HD groups and in the muscle of the HD group. This kinetic behavior of bromadiolone confirms previous results obtained with this compound, as well

Figure 2: Fecal concentrations of bromadiolone versus time after single oral administration of low dosage (0.05 mg/kg; LD, n = 6) and high dosage (0.5 mg/kg; HD, n = 6) obtained during the first week post dosing in healthy Yorkshire pigs (pigs and study described in Figure 1). Data reported as mean ± standard error. Asterisks (*) indicate a statistical difference (ANOVA; $P < .05$) between the LD group and the HD group at the corresponding time point. LOD = limit of detection.



as other anticoagulant rodenticides in various species.[7,9,18-20] The prolonged elimination of bromadiolone is due to liver accumulation and enterohepatic recycling.[1,7,8] Furthermore, the results of the present study showed that concentrations of bromadiolone were higher in the liver and feces than in other tissues; this finding is consistent with the toxicokinetics of this pesticide.[6] It should be mentioned that in the HD group, bromadiolone concentrations in skin-adherent fat remained higher than the corresponding LOD at all sacrifice time points, which suggests that bromadiolone also accumulates in fat. Finally, comparison of the slopes of the depletion over time plots for bromadiolone showed no differences between liver HD and LD groups, or between liver HD and muscle HD groups, suggesting that the elimination of bromadiolone from hog tissues is not dose dependent.

Withdrawal period estimations of bromadiolone following administration of a single low or high dosage were performed in the present study. Withdrawal periods were determined by defining the 99th percentile of the population with 95% confidence. The estimated withdrawal periods for liver were 83 weeks and 176 weeks in the LD group and HD group, respectively. In the muscle of the HD group, withdrawal period was estimated as 62 weeks. While these findings provide new insights in terms of anticoagulant rodenticide tissue-residue depletion and withdrawal period estimations in swine, our statistical power would have improved if we had had both a larger sample size and a longer study period. Interestingly, our findings in the liver of the LD group are similar to those obtained in dogs showing that residues of flocoumafen, a first-generation anticoagulant rodenticide, administered at 0.4 mg per kg body weight, were found in the liver for 43 weeks.[18]

In summary, second-generation anticoagulant rodenticides, including bromadiolone, are highly toxic, with the ability to kill rats and mice after a single oral dose. Our findings show that fecal samples, and possibly plasma samples, could be used to confirm bromadiolone exposure in hogs if carried out quickly after the suspected exposure. However, it is not known if ingestion of bromadiolone at dosages less than or greater than those tested in the current study would provide similar results. In the present study, administration of a single oral low or high dosage of bromadiolone to hogs showed considerable accumulation in the liver, with concentrations persisting for a prolonged period despite the absence of clinical signs. Similar results were reported in the muscle of the HD group. It should be mentioned that the metabolite profile of bromadiolone in hogs is unknown, but in humans bromadiolone is excreted as metabolites (glucuronides and sulfates) in the urine and feces.[21] The elimination of bromadiolone from tissues in hogs may involve metabolism, with the parent bromadiolone molecule being converted to metabolites that may also be pharmacologically active and present residue concerns. In this study, samples were assayed for the parent bromadiolone molecule; however, its metabolites may also persist, adding to total residue levels of the pesticide. Thus, persistent metabolites of bromadiolone may increase the withdrawal periods estimated in the current study. Nevertheless, the estimated withdrawal periods for the parent bromadiolone molecule in hog tissues are still very long.

Implications

- Fecal and plasma detection assays for bromadiolone may reveal exposure of a pig to bromadiolone.
- Bromadiolone residues persist in muscle, and particularly in liver, such that it is impractical to wait for the hog to eliminate the rodenticide to a concentration that allows safe entry of the hog into the human food chain.

Conflict of interest

None reported.

Acknowledgements

The authors would like to thank Ontario Pork and the Ontario Ministry of Agriculture and Food (OMAF) for providing the financial support of this study, and William Sears for his assistance with statistical analysis.

Table 1: Bromadiolone concentrations after single oral administration of low dosage (0.05 mg/kg) in 20 healthy Yorkshire pigs*

	Liver (µg/kg)			Skin & adherent fat (µg/kg)			Feces (µg/kg)			Plasma (ng/mL)		
LOD	**3.0**			**0.3**			**1.0**			**0.5**		
Week	**LL**	**Median**	**UL**	**LL**	**Median**	**UL**	**LL**	**Median**	**UL**	**LL**	**Median**	**UL**
1	96.9	175.1†	316.5	0.9	3.1	10.2	2.9	19.9	73.5	0.4	0.8	1.3
2	87.2	137.3†	216.1	0.6	1.6	4.1	3.3	8.9	24.4	0.4	0.8	1.3
3	74.3	107.6†	155.8	0.4	0.9	1.8	1.8	4.0	9.1	0.3	0.5	0.7
5	40.9	66.1†	106.6		< LOD			< LOD			< LOD	
6	27.8	51.8	96.8		< LOD			< LOD			< LOD	

* Study described in Figure 1. For each week, n = 4 pigs, which were euthanized for collection of tissues. Plasma and fecal samples were obtained at the time of euthanasia. For muscle tissue, all median values were < LOD. Values are expressed as median (geometric mean) with lower limit (LL) and upper limit (UL).

† Versus other tissues within a time point ($P < .05$; ANOVA and multiple *t* tests).

LOD = limit of detection; ANOVA = analysis of variance.

Table 2: Bromadiolone concentrations after single oral administration of high dosage (0.5 mg/kg) in 20 healthy Yorkshire pigs*

	Muscle (µg/kg)			Liver (µg/kg)			Skin & adherent fat (µg/kg)			Feces (µg/kg)			Plasma (ng/mL)		
LOD	**2.0**			**3.0**			**0.3**			**1.0**			**0.5**		
Week	**LL**	**Median**	**UL**	**LL**	**Median**	**UL**	**LL**	**Median**	**UL**	**LL**	**Median**	**UL**	**LL**	**Median**	**UL**
1	6.1	9.6	15.2	348.8	596.4†	1019.9	2.7	8.2	24.5	20.6	67.3	220.2	1.4	2.3	3.8
2	5.3	7.8	11.5	333.6	524.7†	825.3	2.8	7.0	17.7	13.8	37.5	101.9	1.2	1.8	2.7
3	4.5	6.4	8.9	312.1	461.6†	682.6	1.8	6.1	13.4	8.8	20.9	49.6	0.9	1.3	1.9
6	2.4	3.4	4.9	205.4	314.2†	480.6	1.6	3.8	9.1	1.4	3.6	9.2	0.4	0.6	0.9
9		< LOD		106.7	213.9	428.8	0.6	2.4	10.1		< LOD			< LOD	

* Study and pigs described in Figure 1. For each week, n = 4 pigs, which were euthanized for collection of tissues. Plasma and fecal samples were obtained at the time of euthanasia.

† Versus other tissues within a time point ($P < .05$; ANOVA and multiple *t* tests).

LOD = limit of detection; LL = lower limit; UL = upper limit.

Disclaimer

Scientific manuscripts published in the *Journal of Swine Health and Production* are peer reviewed. However, information on medications, feed, and management techniques may be specific to the research or commercial situation presented in the manuscript. It is the responsibility of the reader to use information responsibly and in accordance with the rules and regulations governing research or the practice of veterinary medicine in their country or region.

References

1. Murphy MJ. Anticoagulant rodenticides. In: Gupta RC, ed. *Veterinary Toxicology: Basic and Clinical Principles.* 1st ed. New York: Academic Press Elsevier; 2007:525–547.

*2. Murphy MJ. Rodenticides. *Vet Clin North Am Small Anim Pract*. 2002;32:469–484.

3. Watt BE, Proudfoot AT, Bradberry SM, Vale JA. Anticoagulant rodenticides. *Toxicol Rev.* 2005;24:259–269.

4. Caloni F, Cortinovis C, Rivolta M, Davanzo F. Animal poisoning in Italy: 10 years of epidemiological data from the Poison Control Centre of Milan. *Vet Rec.* 2012;170:415. doi:10.1136/vr.100210.

5. Jackson WB, Brooks JE, Bowerman AM. Anticoagulant resistance in Norway rats. *Pest Control.*1975;43:14–23.

6. Vandenbroucke V, Bousquet-Melou A, De Backer P, Croubels S. Pharmacokinetics of eight anticoagulant rodenticides in mice after single oral administration. *J Vet Pharmacol Therap.* 2008;31:437–445.

7. Kamil N. Kinetics of bromadiolone, anticoagulant rodenticide, in the Norway rat (*Rattus norvegicus*). *Pharmacol Res Commun*. 1987;19:767–777.

8. Huckle KR, Hutson DH, Warburton PA. Elimination and accumulation of the rodenticide flocoumafen in rats following repeated oral administration. *Xenobiotica.* 1988;18:1465–1479.

9. Woody BJ, Murphy MJ, Ray AC, Green RA. Coagulopathic effects and therapy of brodifacoum toxicosis in dogs. *J Vet Intern Med.* 1992;6:23–28.

10. Breckenridge AM, Cholerton S, Hart JA, Park BK, Scott AK. A study of the relationship between the pharmacokinetics and the pharmacodynamics of the 4-hydroxycoumarin anticoagulants warfarin, difenacoum and brodifacoum in the rabbit. *Br J Pharmacol.* 1985;84:81–91.

11. VICH GL48 (MRK). Studies to evaluate the metabolism and residue kinetics of veterinary drugs in food-producing animals: Marker residue depletion studies to establish product withdrawal periods. 2015. Available at: **http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM207941.pdf**. Accessed 04 August 2015.

12. CITAC/EURACHEM guide to quality in analytical chemistry: an aid to accreditation (2002 edition). Available at: **http://www.eurachem.org/images/stories/Guides/pdf/CITAC_EURACHEM_GUIDE.pdf**. Accessed 14 August 2015.

13. Riviere J. Tissue residues and withdrawal times. In: J Riviere, ed. *Comparative Pharmacokinetics: Principles and Applications*. Ames, Iowa: Iowa State Press; 1999:308–319.

14. Concordet D, Toutain PL. The withdrawal time estimation of veterinary drugs: a non-parametric approach. *J Vet Pharmacol Ther*. 1997;20:380–386.

Figure 3: Liver concentration-time profile of bromadiolone after single oral administration of high dosage, HD (Panel A; 0.5 mg/kg; n = 4 hogs per sacrifice time point) and low dosage, LD (Panel B; 0.05 mg/kg; n = 4 hogs per sacrifice time point) obtained at sacrifice time points of 1, 2, 3, 6, and 9 weeks and 1, 2, 3, 5, and 6 weeks, respectively, in healthy Yorkshire pigs (pigs and study described in Figure 1). LOD = limit of detection.

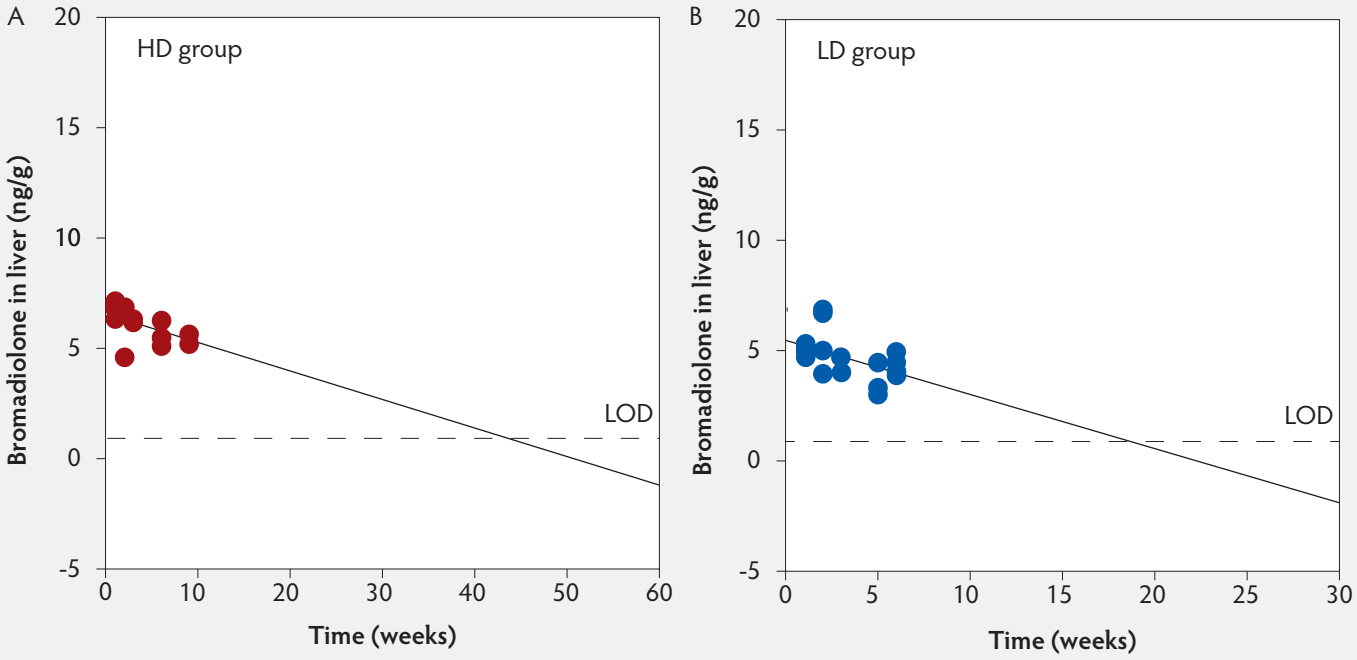
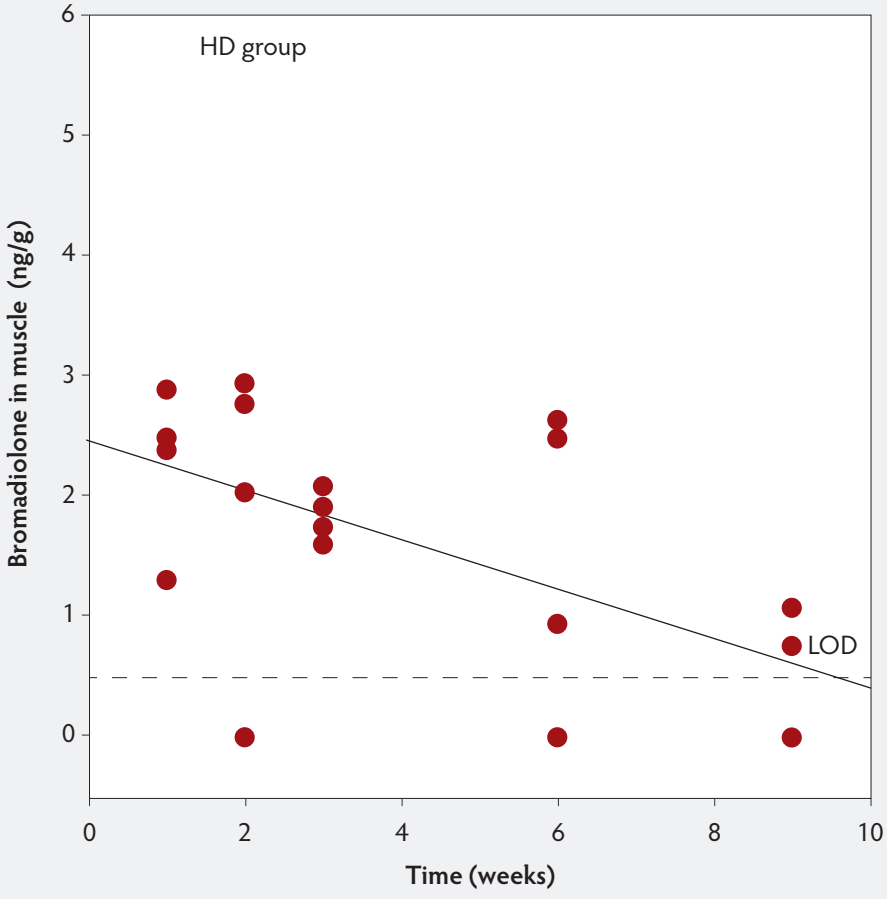


Figure 4: Muscle concentration-time profile of bromadiolone rodenticide after single oral administration of high dosage, HD (0.5 mg/kg; n = 20) obtained at sacrifice time points of 1, 2, 3, 6, and 9 weeks in healthy Yorkshire pigs (described in Figure 1). LOD = limit of detection.



15. Health Canada Food and Drug Regulations (C.R.C., c.870). Available at: http://laws-lois.justice.gc.ca/eng/regulations/c.r.c.,_c._870/page-158.html#h-109. Accessed July 12, 2015.

16. Food and Drug Administration. Guidance for Industry: General principles for evaluating the safety of compounds used in food-producing animals. US Department of Health and Human Services. Food and Drug Administration Center for Veterinary Medicine, Rockville, Maryland, USA. July 25, 2006. Available at: www.fda.gov/downloads/AnimalVeterinaryGuidanceComplianceEnforcement/GuidanceforIndustry/ucm052180.pdf. Accessed 31 July 2015.

17. Huckle KR, Hutson DH, Logan CJ, Morrison BJ, Warburton PA. The fate of the rodenticide flocoumafen in the rat: Retention and elimination of a single oral dose. *Pest Sci*. 1989,25:297–312.

18. Veenstra GE, Owen DE, Huckle KR. Metabolic and toxicological studies on the anticoagulant rodenticide, flocoumafen. *Arch Toxicol Suppl*. 1991;14:160–165.

19. Mosterd JJ, Thijssen HH. The long-term effects of the rodenticide, brodifacoum, on blood coagulation and vitamin K metabolism in rats. *Br J Pharmacol*. 1991;104:531–535.

20. Huckle KR, Warburton PA, Forbes S, Logan CJ. Studies on the fate of flocoumafen in the Japanese quail (*Coturnix coturnix japonica*). *Xenobiotica*. 1989;19:51–62.

21. Grobosch T, Angelow B, Schönberg L, Lampe D. Acute bromadiolone intoxication. *J Anal Toxicol*. 2006;30:281–286.

* Non-refereed reference.