Poster Presentation

Anticoagulant Residues in Non-Target Wildlife – Assessing Sublethal Exposure to Brodifacoum without Lethal Sampling

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ABSTRACT: Residues of brodifacoum and other second-generation anticoagulant rodenticides are reported worldwide in a range of non-target wildlife, especially predatory or scavenging birds and mammals. Determination of exposure to brodifacoum in such cases relies on analysis of liver tissue. This limits current monitoring efforts to either destructive sampling of live birds or mammals to obtain liver, but more commonly opportunistic post-mortem liver sampling from carcasses in suitable condition. Also, detection of brodifacoum in liver often cannot be confirmed as a contributor to mortality, and within a potential 'sublethal' concentration range the toxicological significance of its presence is uncertain. We sought to determine whether dried blood spot (DBS) sampling could form the basis of a non-lethal, minimally invasive method for determining sublethal exposure to brodifacoum in live birds. Validation of a method for detecting brodifacoum in DBS samples, and preliminary comparison of brodifacoum concentrations in DBS and plasma samples, are described.

KEY WORDS: anticoagulant, birds, blood sampling, brodifacoum, dried blood spot, laboratory testing, nontarget species, residues, rodenticides

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INTRODUCTION

Anticoagulant rodenticides inhibit the synthesis of vitamin K-dependent blood clotting factors in the liver (e.g., Thijssen 1995) by binding to the active site of vitamin K epoxide reductase (VKER) (Gebauer 2007). When this inhibition occurs over a sufficient period, blood will fail to coagulate in response to injury. Typical signs of anticoagulant toxicity in mammals and birds are increased blood coagulation times, haemorrhage, and anemia, with death through haemorrhage generally occurring several days after a lethal exposure (Pelfrene 2001). Among a range of 'second-generation' anticoagulant rodenticides (SGAR), brodifacoum is used in many countries to manage commensal rodents that share habitat and food sources with humans, particularly Norway rats (*Rattus norvegicus*), black rats (*R. rattus*), and house mice (*Mus musculus*). Brodifacoum is also registered for field use in bait stations against rodents and brushtail possums (*Trichosurus vulpecula*) in New Zealand (Hoare and Hare 2006). Broad-scale aerial application of brodifacoum bait is also an important conservation tool, instrumental in successful eradication of invasive rodents from an increasing number offshore islands (Howald et al. 2007).

Monitoring reports residual brodifacoum and other SGAR in an increasing range of wildlife species, including predatory birds and mammals (Albert et al. 2009, Mineau et al. 2003, Shore et al. 2003, Stone and Okoniewski 2003) and omnivorous mammals (Dowding et al. 2010). Brodifacoum is one of the most toxic of the anticoagulants, and residual concentrations have been shown to persist for prolonged periods in the livers of live animals (Eason et al. 1996, Laas et al. 1985, Fisher et al. 2003), providing an obvious focus on liver tissue to determine exposure. However, this creates limitations as it is generally not acceptable to kill non-target wildlife considered at risk of exposure in order to sample liver. In turn, this means that current residue data from non-target wildlife are most often based on opportunistic sampling from carcasses (e.g., road kills), where degradation may preclude necropsy or liver sampling. Where field exposure to brodifacoum is confirmed by measurable liver concentrations, it is then uncertain how long ago or how often the exposure had occurred, and where liver concentrations are relatively low (less than 1 ppm), whether it was sufficient to have caused mortality. Thus, the current indication of the range of non-target wildlife exposed to brodifacoum is generally retrospective, in looking to confirm exposure and its possible contribution to mortality after the fact.

Sublethal exposure of wildlife to anticoagulants may be more widespread in the environment than currently thought, but this is not practicable to investigate using liver tissue. A reliable, non-lethal and minimally invasive sampling procedure to determine exposure in live animals would greatly expand the species and numbers of wildlife that could be monitored for residual brodifacoum and other SGAR. The plasma elimination half-life of brodifacoum is shorter (days) than hepatic half-life (months) in mammals (e.g., Breckenridge et al. 1985), thus detection of residues in blood samples could provide more precise estimates of when exposure occurred. Trudeau et al. (2007) describe application of a small volume dried blood spot (DBS) sampling for monitoring pesticide exposure in birds. To further investigate develop this as a minimally

invasive sampling procedure detecting sublethal brodifacoum exposure in live birds, a method for detecting brodifacoum in DBS samples from chickens (*Gallus gallus*) was validated using HPLC. In a second trial, chickens were orally administered a single sublethal dose of brodifacoum and residual brodifacoum concentrations were measured in DBS and plasma samples taken over the following 14 days.

METHODS

Validation of Dried Blood Spot Method for Detecting Brodifacoum

Seven 21-day-old Ross male broiler chickens were kept on sawdust litter on a concrete floor in a pen. A 0.5% stock solution of brodifacoum in ethanol was diluted 1:9 with water, and orally administered to chickens to deliver a dose of 1 mg/kg bodyweight. Blood was sampled from the right jugular vein at Days 1 (n = 3), 3 (n = 3), and 7 (n = 1) after dosing, with approximately 3 mL taken via a 21-gauge needle into a Vacutainer tube containing lithium heparin. The protocol described by Mei et al. (2001) was used for preparation of DBS samples. Aliquots (50 µL) of fresh blood were placed onto the marked 'wells' on Schleicher and Schuell No. 903 filter paper cards (Whatman International Ltd., Maidstone, Kent, UK). These were dried in a slide rack at room temperature for approximately 2 h, wrapped in weighing paper, and placed in water impermeable plastic bags with 1-g silica gel dessicant packages and humidity-indicating paper. The sealed bags were stored at -20°C for approximately 5 months before analysis. At analysis, the 50-µL spots were cut from each card, folded, and placed in 20-mL screw-cap tubes. Three milliliters of 0.05M tetra butyl ammonium dihydrogen phosphate (TBAP) in methanol was added and the blood spot extracted by vortexing and sonicating. The extract was transferred to a clean 10-mL test tube and gently evaporated at 50°C under air. The paper spot was also dried at 50°C under air, then re-extracted with 3 mL of 0.05M TBAP in methanol, and this second extract added to the first. The solvent was again gently evaporated at 50°C under air, the sample dissolved in a mobile phase of methanol/water/acetic acid, and passed through a 0.45-µm syringe filter for HPLC analysis. Aliquots were chromatographed on an Alltech® Alltima™ C18 column (Alltech Associates, Deerfield, IL) using methanol/water/acetic acid as the mobile phase. The post-column reagent was ammonia/methanol/water (10/10/80), with a flow rate adjusted until the effluent had a pH of approximately 10.1. The HPLC was run at a flow rate of 1.5 mL/min with degassing, with the gradient program: initial 65%A: 35%B; 8 minutes 84%A: 16%B; 16 minutes 95%A: 5% B; 20 minutes 95%A: 5% B; 23 minutes 65%A: 35%B. The fluorescence detector was at 310-nm excitation and 390-nm emission. Samples spiked with 1, 5, or 10 µL of brodifacoum in methanol were also analysed.

Comparison of Brodifacoum in DBS and Plasma Samples

A dose of 0.5 mg/kg brodifacoum was selected to represent a low sublethal exposure, based on known brodifacoum toxicity in chickens and other birds: Lund (1981) reported that brodifacoum killed 4 leghorn hens in 6-12 days after an average intake of 10.5 mg/kg, an LD_{50} estimate of 3.3 (95% CI 2.2-5.2) mg/kg in quail (*Callipepla californica*) (Godfrey 1985), and a report by Bailey et al. (2005) of 100% mortality in 10 chickens gavage-dosed with 3 mg/kg brodifacoum. Twenty 'Isabrown' pullets were given a numbered leg band and acclimatised for 3 weeks to group-housing in large outdoor grassed pens with sheltered roosts with fresh water and commercial pellet food freely available. After weighing, the chickens were randomly allocated to either 'sublethal brodifacoum' (n = 15) or 'control' (n = 5) treatments. A dosing solution of 0.4 mg/mL brodifacoum in monopropylene glycol (MPG) was administered by stomach tube to hens in the brodifacoum treatment at 1.25 mL solution per kg of bodyweight. Control hens were administered 1.25 mL/kg of MPG alone, with a 2.5 mL maximum dose volume in either treatment. After dosing, the hens were placed back in group housing outdoors.

Hens were sampled in groups of 5 (n = 4 brodifacoum-dosed and 1 control hen) on Days 1, 4, 7, and 14 after dosing. One hen was mistakenly dosed with control treatment, so the Day 14 sample included only 3 brodifacoum-dosed hens. At each sample day, DBS samples were taken first, where hens were restrained on their side, with the legs and upper wing held and the lower wing extended outwards to expose the brachial vein. The feathers underneath the wing were swabbed with disinfectant and held to one side, while a second person drew blood (maximum 0.2 mL) from the brachial vein with a 25 G needle. Single drops of blood were immediately placed directly from the syringe onto the 3 marked 'wells' on Schleicher and Schuell No. 903 filter paper cards. The DBS cards were dried in a slide rack at room temperature for approximately 2 h, then placed in ziplock plastic bags. Immediately after DBS sampling, the hens were euthanased by decapitation, and whole blood was collected directly into two 4.5-mL tubes (Vacutainer® blood collection tubes, 3.8% sodium citrate). Within an hour of sampling, whole blood was centrifuged at 2500×g for 15 min at 4°C, with plasma divided into 2 Eppendorf tubes and frozen at -80°C. Analysis of plasma samples for brodifacoum was based on methods described by Primus et al. (2001) and Jones (1996). Plasma was thawed in a hot water bath at 37°C and 1 mL extracted with acetonitrile to remove protein. Ethyl ether was added to remove water and the sample evaporated on a vacuum evaporator. The residue taken up in methanol was analysed by HPLC, where fluoresecence detection and a post-column pH switching technique was used to exploit the natural fluorescence of brodifacoum, with difenacoum as an internal standard.

RESULTS

Validation of Dried Blood Spot Method for Detecting Brodifacoum

The MDL for brodifacoum in DBS was 0.04 ppm, spike recovery ranged from 87 to 122%, and the method uncertainty (95% CI) was ±4%. At Day 1 after dosing, residual brodifacoum in DBS samples were 0.375, 0.411, and 0.411 ppm; at Day 3 after dosing, 0.045, 0.051, and

0.059 ppm, and at Day 7 after dosing 0.021 ppm.

Comparison of Brodifacoum in DBS and Plasma Samples

Spiked DBS samples had an 89-99% recovery and method uncertainty (95% CI) was ±10%, and spike recovery in plasma ranged from 88 to 100% with method uncertainty ±8%. Method detection limits were 0.04 ppm and 0.005 ppm for DBS and plasma samples, respectively. No brodifacoum was detected in any plasma or DBS samples from control hens. DBS samples taken at Day 1 after dosing with brodifacoum had a mean concentration of 0.144 ppm (± SE 0.036), and in plasma samples from the same day mean brodifacoum concentrations was 0.215 µg/mL (± SE 0.064). Plasma concentrations were below MDL by Day 7, and in DBS were nominally below MDL from Day 4 onwards (Table 1). The Spearman Rank Correlation Coefficient between detectable concentrations in DBS and corresponding plasma samples was 0.981.

Table 1. Concentrations of residual brodifacoum detected in corresponding chicken plasma and DBS samples after oral administration of 0.5 mg/kg brodifacoum. The analyzing laboratory noted that Day 4 DBS samples had repeatably detectable concentrations of brodifacoum which were below the calculated MDL that are shown in brackets.

Day	Chicken ID	Plasma [MDL 0.005]	DBS [MDL 0.04]
1	#247	0.40	0.25
	#686	0.19	0.11
	#666	0.16	0.12
	#683	0.11	0.097
	#229 (control)	<MDL	<MDL
4	#695	0.012	(0.010, 0.011)
	#690	0.011	(0.012, 0.011)
	#681	0.014	(0.016, 0.015)
	#652	0.012	(0.029, 0.020)
	#693 (control)	<MDL	(0.003, 0.002)*
7	#669	<MDL	<MDL
	#685	<MDL	<MDL
	#238	<MDL	<MDL
	#668	<MDL	<MDL
	#677 (control)	<MDL	<MDL
14	#218	<MDL	<MDL
	#653	<MDL	<MDL
	#700	<MDL	<MDL
	#205 (control)	<MDL	<MDL

*certainty on these below-MDL results could not be calculated

DISCUSSION

The correlation between brodifacoum concentrations measured in DBS and plasma samples from hens at Day 1 indicates that DBS provided an accurate index of circulating brodifacoum concentrations within this timeframe. Coumarin anticoagulants bind to plasma albumin proteins (Sutcliffe et al. 1987), so dilution by non-binding fractions such as erythrocytes may have accounted for the generally lower brodifacoum concentrations measured in DBS (whole blood) in comparison to plasma samples, suggesting that a correction factor accounting for erythrocyte packed cell volume could be applied to DBS samples. Considering a normal packed cell volume of 32 for broiler chickens, the DBS values should be about one-third higher for a 'plasma equivalent'. Plasma brodifacoum concentrations had fallen below MDL by Day 7, but in DBS were below MDL from Day 4 onwards (Table 1).

At 24 h after dosing with 0.5 mg/kg brodifacoum, chicken plasma concentrations in this trial were approximately 10 times higher than those measured by Howald (1997) in Japanese quail (*Coturnix japonica*) following sublethal exposure to brodifacoum. In his study, groups of 6 quail were dosed with 0.7, 1.4, or 0.35 mg/kg brodifacoum and plasma concentrations were not significantly different between the 3 dose groups 24 h later, with a mean of 0.028 ppm. In quail, plasma concentrations declined to a mean of 0.005 ppm at 5 days and then to 0.002 ppm by 10 days, showing a consistent rate of decline with the concentrations found here in chickens at 4 days (0.012-0.014 ppm) and 10 days (below 0.005 ppm MDL) after dosing. This suggests that, after sublethal exposure to brodifacoum, elimination of circulating (plasma bound) brodifacoum in birds occurs within approximately 7 days, representative of a "window of detectability" during which brodifacoum is at measurable concentrations in blood (plasma).

Although the plasma analysis was the more sensitive, it is probable that brodifacoum was also present in DBS samples at Day 4, as per the low but repeatable measurements noted by the reporting laboratory. It is also likely that improvements to the sensitivity of brodifacoum detection in DBS samples could be made. The utility of DBS as a minimally invasive monitoring technique could be improved by increasing the sensitivity of analyses for residual brodifacoum, potentially through more rigorous extraction or LC-MS detection. With currently established methods, using DBS over plasma samples is likely to slightly reduce the duration of detectability; however, we suggest that DBS has utility as a field monitoring tool for determining recent exposure to brodifacoum, especially where it is not desirable or possible to take sufficient blood to harvest a plasma sample, or to kill the animal to obtain liver tissue. Extending DBS sampling to also detect a range of second-generation anticoagulant rodenticides also fits well into a larger project to test birds for exposure to 6 groups of environmental contaminants (Shlosberg et al. 2009). A further reduction in invasiveness of monitoring for anticoagulant exposure may be possible using saliva samples. Sakai et al. (1983) found warfarin was excreted in the saliva of rabbits after oral administration; the salivary concentration corresponded with that in plasma and was correlated with the effect of warfarin on prothrombin complex activity. It would be useful to investigate whether coumarin anticoagulants occur in the saliva of sublethally-exposed birds in detectable concentrations comparable to those present in plasma and DBS.

In many countries, brodifacoum use is restricted to commensal rodent control (indoor) applications, yet predatory or scavenging wildlife species are being exposed, presumably through secondary environmental pathways of live rodent prey that have fed on bait, the carcasses of animals killed by brodifacoum, or invertebrates that have fed on bait and contain residual concentrations. Spurr et

al. (2005) provide recent evidence of commensal applications of anticoagulant bait resulting in transport of residues to a range of non-target wildlife in surrounding areas. Brakes and Smith (2005) demonstrate that routine commensal rat control using an anticoagulant reduced local populations of some non-target small mammals, demonstrating a significant route of secondary anticoagulant exposure for predators and scavengers. While the toxicological significance of apparently sublethal brodifacoum residues in wildlife remains uncertain (Kaukeinen et al. 2000), the potential for cumulative or chronic toxicosis needs further investigation. It may be that single or infrequent sublethal exposures do not significantly compromise long-term survival or reproductive fitness, but because the effects of cumulative or long-term exposure are not known, the ability to characterize field exposure profiles would be of great value in a proactive approach to identifying and minimizing risks to non-target wildlife. This is especially so, as the high toxicity and bioaccumulative potential of brodifacoum presents an additional concern where secondary exposure is likely to be repeated or sustained.

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