Determination of Diphacinone Residues in Hawaiian Invertebrates

Thomas M. Primus, Dennis J. Kohler, and **John J. Johnston**
National Wildlife Research Center, 4101 LaPorte Ave., Fort Collins, Colorado, 80521

Abstract

A reversed-phase ion-pair liquid chromatographic analysis combined with a solid-phase extraction clean-up method is used to assess the quantity of diphacinone residue found in invertebrates. Three invertebrate species are exposed to commercially available diphacinone-fortified bait used for rat control. The invertebrate samples are collected, frozen, and shipped to the laboratory. The samples are homogenized after cryogenic freezing. A portion of the homogenized samples are extracted with acidified chloroform–acetone, followed by cleanup with a silica solid-phase extraction column. Diphacinone is detected by UV absorption at 325 nm after separation by the chromatographic system. The method limit of detection (MLOD) for snail and slug samples averaged 0.055 and 0.066 mg/kg, respectively. Diphacinone residues in snail tissue ranges from 0.83 to 2.5 mg/kg for *Oxychilus spp.* The mean recoveries from snails at 0.20 and 2.0 are 97 ± 21% and 84 ± 6%. Diphacinone residues in slug tissue ranges from 1.3 to 4.0 mg/kg for *Deroceras laeve* and < MLOD to 1.8 mg/kg for *Limax maximus*, respectively. The mean recoveries from slugs at 0.20 and 2.0 mg/kg are 91% ± 15% and 86% ± 5%.

Introduction

Diphacinone [2-(Diphenylacetyl)-1H-indene-1,3(2H)-dione] is a registered late-first generation or early-second generation anticoagulant rodenticide commonly used to control populations of rats and mice in urban areas. This anticoagulant is also effective in the control of other rodents such as pocket gophers (*Thomomys bottae*), Beldings' ground squirrels (*Spermophilus beldingi*), and California ground squirrels (*Spermophilus beecheyi*) in rangeland rodents. The acute oral toxicity (LD_{50}) of diphacinone for rats is approximately 2 mg/kg, compared with the acute oral toxicity for other anticoagulants such as warfarin and pindone, which are approximately 59 mg/kg.

Rats (*Rattus spp.*) on the Hawaiian Islands are a nonindigenous species that have impacted the native ecosystems. On the Hawaiian Islands, as well as other islands, rats have contributed to the extinction of indigenous flora and fauna (1). Control methods being studied for rats in remote areas include the broadcast application of acute toxicants, including anticoagulants such as diphacinone (2). In remote areas, broadcast application of rodenticide baits has been shown to effectively control rat populations (3). In humid environments such as Hawaii, the use of all weather rodenticide baits usually consist of grain-fortified (at 0.005% w/w) diphacinone encapsulated in wax or pressed with oil.

Invertebrates (snails and slugs) have been observed to consume portions of rodenticide baits in bait stations and baits that were located on forest floors in treated areas (2). When considering the use of pesticides to control problem species, the risk of secondary toxicity to nontarget species such as birds must be evaluated. Therefore, the objectives of this study were to determine if snails and slugs consume enough bait to accumulate measurable quantities of diphacinone and, if so, to determine if the detected residues of diphacinone are significantly high enough to pose a risk to avian species that consume these invertebrates.

An analytical method to assess the levels of diphacinone residues in snails and slugs exposed to 0.005% diphacinone-fortified baits was developed. Typically, liver and serum are analyzed for residues of anticoagulants, as they accumulate and are metabolized in the liver. Obviously, this is not possible for snails and slugs because of the small body mass of individual animals. For all three species of invertebrates, whole body diphacinone residues were determined, and multiple numbers of each species were ground together into composite samples.

Several methods have been developed for analysis of indanediones (Figure 1) in baits, formulations, and tissues. A gas chromatographic method with derivatization (4) is sensitive and selective but suffers from low recoveries and is time consuming. Spectrophotometric methods (5,6) have been utilized for baits and formulations, but they are not selective when assaying multiresidue samples. Thin-layer chromatography (7–9) methods are not suited for determining low levels of residues in complex matrices such as plant and animal tissues. Reversed-phase (RP) high-performance liquid chromatography (HPLC) methods (10–14) provide sufficient sensitivity but often produce poor chromatographic resolution for the indandiones. Ion-pair RP-HPLC (15–20) for diphacinone (Figure 1) using tetrabutylammonium ion pairing reagent is sensitive and selective, but column lifetime is often short because of adsorption of the ion-pairing reagent onto the stationary phase of the column packing material. For this study, ion-pair RP-HPLC was used because good chromatographic

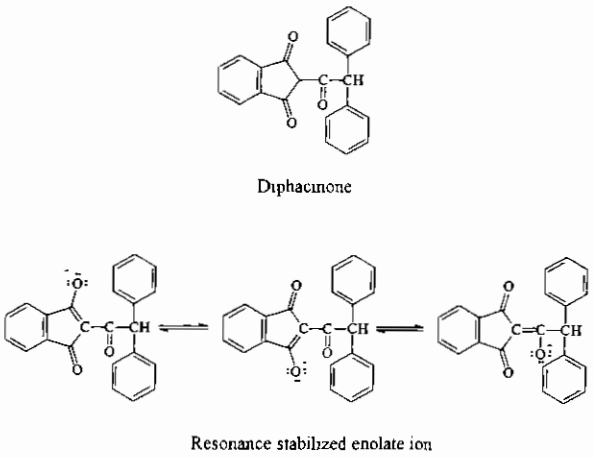

Figure 1. Sturcture of diphacinone and anion in basic solution that forms the ion pair.

resolution can be achieved, and column lifetime can be extended with regular column washing. Sample extraction utilized a normal-phase solid-phase extraction (SPE) sample cleanup with a silica column with an automated SPE workstation.

Experimental

Sample collection

Whole snails (*Oxychilus spp*) and slugs (*Deroceras laeve* and *Limax maximus*) were collected and placed in individual plastic bags, sealed, labeled, and frozen in a freezer at –12°C. The samples were stored in a freezer until shipped to our laboratory where they were stored in freezers at –12°C until homogenized and assayed. Method validation and analyses were completed under U.S. Environmental Protection Agency Good Laboratory Practice guidelines (40 CFR 160) (21).

Reagents

Chloroform, hexane, ethyl acetate, and methanol were liquid chromatography grade reagents (Fischer Scientific, Denver, CO). Deionized water was purified using a reversed osmosis water purification system (U.S. Filter Corp., Schaumburg, IL). Concentrated phosphoric acid (Fischer Scientific) was used to prepare the aqueous 1.33M phosphoric acid solution. The extraction solution of 1.7% (v/v) formic acid in acetone–chloroform (1:1) was prepared by mixing 20 mL of concentrated (88%) formic acid (Fischer Scientific) with 500 mL of acetone and 500 mL of chloroform. Anhydrous sodium sulfate (Fischer Scientific) was mixed with tissue samples to remove water.

Diphacinone (98.9%) was obtained from Hacco (Madison, WI). Concentrated stock standards of diphacinone were prepared by first drying the technical-grade compound for 4 h at 110°C, then dissolving 10.000 mg in 10.0 mL of ethyl acetate. Working standards, ranging in concentration from 0.050 to 2.3 mg/L, were prepared by dilution of stock solutions with mobile phase. All standard solutions were stored in a refrigerator at 5°C.

Tetrabutylammonium dihydrogen phosphate (97%) was purchased from Aldrich (Milwaukee, WI) and was used to prepare a 5mM solution in methanol. An aqueous solution of 5mM tetrabutylammonium dihydrogen phosphate with 50mM potassium dihydrogen phosphate buffer (Alltech, Inc., Deerfield, IL) was prepared.

Sample preparation

Sample homogenization and extraction

Approximately 2 to 10 g were frozen and homogenized with a Spex Centiprep 6850 freezer mill (Metuchen, NJ) (22). It required approximately 30 to 50 snails and 2 to 4 slugs to attain a 10-g sample. The samples were transferred to a polycarbonate cylindrical vessel that holds a metal rod. Each end of the cylindrical container was capped with a metal lid. The sample and container were placed in the freezer mill sample holder with liquid nitrogen and frozen over a 5-min period. The sample was homogenized by forcing the metal rod back and forth magnetically at 10 cycles/s between the metal end caps. This is typically done for approximately 1 to 2 min for 2 to 3 periods. The powdered, frozen snail or slug sample was transferred to a 20-mL glass amber sample jar. For snail samples, the soft tissue was not removed from the shell of the animal. Samples were stored at –12°C until assayed.

Homogenized tissue samples were weighed (0.500 to 0.550 g) into a 25-mL glass tube, and 5 g of anhydrous sodium sulfate was added. All samples were analyzed in duplicate if sufficient sample mass was available. The tissue and sodium sulfate were vortex mixed together for 10 s. A 15-mL aliquot of the extraction solution was added to each sample and vortex mixed for 5 s. The samples were shaken horizontally on a mechanical shaker (Eberbach, Ann Arbor, MI) at high speed (56 displacements/min) for 10 min. The samples were placed in an ultrasonic bath (power level of 150 W) for 10 min (a beaker partially filled with water was used to hold the tubes). Sample tubes were centrifuged at approximately $1000 \times g$ for 5 min.

The extract was transferred to a second 25-mL glass tube. The extraction was repeated twice with two subsequent 10-mL additions of extraction solution. The solvent in the extract was removed by placing the tubes in a warm water bath (60°C) and allowing nitrogen gas to flow over the surface of the extract until no solvent remained. The residue was reconstituted with 2.0 mL of chloroform, gently vortex mixed, and placed in an ultrasonic bath for 10 min. This was followed by the addition of 3.0 mL of hexane. This solution was vortex mixed and placed in an ultrasonic bath for 10 min. The reconstituted samples were filtered through a 0.45-µm Teflon syringe filter (30 mm, National Scientific Co., Part #F2500-3) into a 10-mL glass tube. A 1-mL portion of chloroform and a 1-mL portion of hexane was used to rinse the sample tube, filtered through the syringe filter, and added to the extract in the 10-mL glass tube.

Analyte concentration

The SPE procedure was completed using a Zymark RapidTrace automated workstation (Hopkinton, MA). Each aminopropyl SPE (500 mg sorbent in a 3-mL column) cartridge was conditioned with approximately 3 mL of hexane–chloroform (2:1). The packing material was not allowed to dry. The reconstituted sample extract (6.5 mL) was passed through the column at 2 mL/min. The eluate was discarded to waste. Each SPE column was rinsed by adding a 3-mL aliquot of hexane–chloroform (2:1),

followed by 3 mL of chloroform. This eluate was discarded to waste.

Analyte elution and sample reconstitution

The analyte was eluted from each SPE column by adding 10 mL (2- × 5-mL) of 4mM tetrabutyammonium phosphate in methanol and collected in a clean 10-mL screw-top glass tube. The volume of eluate was reduced by placing tubes in a warm water bath (60°C) and blowing a stream of nitrogen over the solution until the solvent was removed. The residue was redissolved with 1.0 mL of methanol–water (60:40) (with 5mM tetrabutyammonium phosphate), vortex mixed, and placed in an ultrasonic bath for 5 min. The reconstituted samples were filtered through a 0.45-µm Teflon syringe filter into a vial and capped before HPLC analysis.

HPLC

The HPLC system consisted of a Hewlett-Packard 1090 LC (Palo Alto, CA) equipped with a diode-array multiple wavelength detector (Table I). The initial mobile phase was prepared by mixing methanolic and aqueous solutions of 5mM tetrabutylammonium with 50mM dihydrogen phosphate (60:40, v/v) and adjusting the pH to 8.5 with 4N phosphoric acid. The mobile phase was degassed by sparging with helium. At the end of each set of analyses, the column was washed with a mixture of methanol–water (1:1, v/v) for 40 min. Each tissue sample was analyzed in duplicate.

Quality control samples and fortification of controls

Snails and slugs were collected by National Wildlife Research Center staff members at the Hilo Field Station in Hawaii prior to the beginning of any experiments. These control invertebrates were processed and screened for diphacinone prior to compositing of control samples. Control samples were fortified at 0.20 and 2.0 mg/kg diphacinone with aliquots of fortification standards of diphacinone in ethyl acetate. The quality control samples were then assayed with the method described previously.

Table I. HPLC Parameters for the Analysis of Snail and Slug Extracts

Parameter	Conditions		
	Combine the aqueous IPC solution and methanolic IPC solution in the ratio 60:40 (methanol–water)		
Mobile phase	Time	(60:40) MeOH –5mM TBA	MeOH–water –5mM TBA
Gradient	0.0 min	100%	0%
	12.0 min	100%	0%
	20.0 min	70%	30%
	26.0 min	70%	30%
	28.0 min	100%	0%
	34.0 min	100%	0%
Column cleaner	Methanol–water (1:1)		
Flow rate	1.0 mL/min		
Injection volume	100 µL		
Column	Keystone ODS/H (C18), 5 µm, 250- × 4.6-mm i.d. or equivalent (use guard column containing identical HPLC packing)		
Column temperature	35°C		
Detector	UV at 285 nm and 325 nm		
Run time	34 min		

Results and Discussion

Response linearity

Two sets of 6 diphacinone standard solutions were prepared, ranging from 0.050 to 2.3 mg/L. Data were collected from duplicate injections of each solution and a plot was constructed of analyte peak area (y-axis) versus diphacinone concentration (x-axis). A linear regression was performed on the data set and produced an $r^2 = 0.9988$. The plot of log (peak area) versus log (diphacinone concentration) produced a slope of 1.00608 and an $r^2 = 0.9981$. The average response factor over the range of the calibration curve produced a coefficient of variation of 4.1%. A linear and proportional relationship exists between chromatographic peak area and diphacinone concentration. Single-point calibration to calculate the concentration of diphacinone in the sample extracts was considered valid.

Method limit of detection

The method limit of detection (MLOD) was calculated as the concentration of diphacinone required in the sample to generate a signal equal to three times the baseline noise (peak-to-peak) observed in the chromatogram of the control extract. The MLOD was estimated from the chromatographic response in height of a control tissue extract and an extract from a control tissue sample fortified at 0.20 mg/kg. The MLOD for snail and slug tissue samples averaged 0.055 and 0.066 mg/kg, respectively. For the chromatographic parameters chosen, the retention time of diphacinone was approximately 23.5 min, as shown in Figure 2B. No significant chromatographic response was noted at the reten-

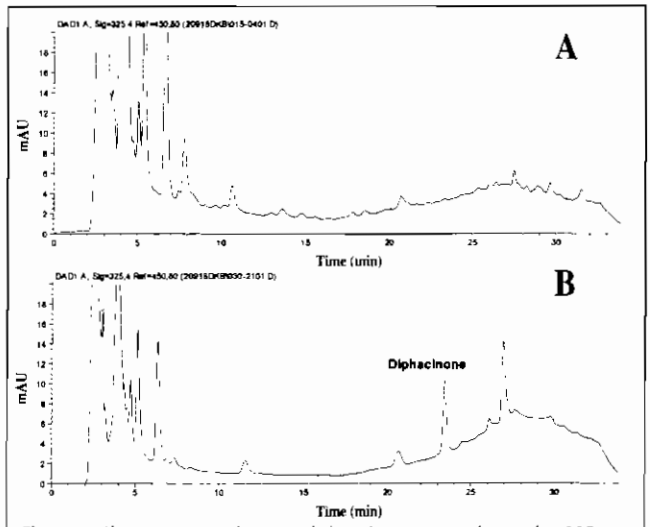

Figure 2. Chromatogram of a control slug, tissue extract detected at 325 nm (A) and chromatogram of a slug, tissue extract detected at 325 nm, which contains 1.1 mg/kg diphacinone (B).

tion time of diphacinone in the chromatogram of the control tissue extract (Figure 2A). Chromatograms of slug and snail extracts were virtually identical.

SPE clean-up

Based on the polar nature of the analyte and the solubility of the analyte in intermediate polar solvents, an SPE clean-up was attempted by the adsorption of the analyte on aminopropyl (NH_2), 2,3-dihydroxypropyl, strong anion exchanger (SAX), florisil, and silica SPE columns. All the SPE columns were conditioned and loaded with combinations of chloroform–hexane solutions. The analyte was only partially retained on the SAX, silica, and florisil SPE sorbents during the loading steps. The only SPE sorbents to retain greater than 85% to 90% of the analyte in the presence of matrix during the loading and washing procedure were the NH_2 and silica sorbents. During method development, the NH_2 sorbent proved more reproducible and typically yielded 10% to 15% higher recoveries for diphacinone in this matrix. The analyte was only partially eluted with methanol but was completely eluted from the NH_2 sorbent with the methanolic ion-pairing reagent. The NH_2 sorbent was adopted as the SPE column of choice for the remainder of the method.

Diphacinone residues in snails and slugs

Mean recoveries of snail (n = 15) and slug (n = 16) quality control samples were 90 ± 16% and 89 ± 11% (Table II). Diphacinone residues were calculated as the average of duplicate analyses of samples, when available. When duplicate analyses differed by more than 25%, a third replicate was analyzed. The reported residue concentration was then calculated as the mean of the three replicates when this occurred. Diphacinone residues in snail and slug tissue of the three species analyzed ranged from < MLOD to 4.00 mg/kg (Table III) with a mean value of 1.68 mg/kg. In comparison, diphacinone residues (all values reported are means) determined from carcasses of species exposed to diphacinone baits have ranged from 0.52 and 1.1 mg/kg for California ground squirrels (22,23), 4.4 mg/kg for black rats (24), 2.25 mg/kg for house mice (24), and 0.40 mg/kg for pocket gophers (25).

The primary wavelength for quantitative analysis was 325 nm, though absorption at 285 nm was also recorded. The ratio of absorbance at 285 and 325 nm was used to qualitatively confirm the presence of the analyte. The molar absorptivity of diphacinone at 285 nm was typically between 1.9 and 2.1 greater than the molar absorptivity at 325 nm. Observation of the UV–vis spectra of diphacinone was also a useful tool to qualitatively confirm the presence of any indandione. As shown in Figure 3, the spectrum is unique enough to confirm the presence of diphacinone. This is the spectra of the diphacinone peak from the slug extract chromatogram in Figure 2B.

Table II. Analytical Recoveries of Diphacinone in Snail and Slug Tissues

Fortification levels (mg/kg)	Species	Range (%)	Mean (%)	Standard deviation (%)	CV (%)
0.20 (n = 7)	Snails	78–140	97	21	22
2.00 (n = 8)	Snails	74–92	84	5.6	6.7
0.20 (n = 8)	Slugs	72–120	91	15	16
2.00 (n = 8)	Slugs	79–95	86	4.9	5.7

Table III. Residues of Diphacinone in Snail and Slug Tissues

Species	Range of residue concentration (mg/kg)	Mean residue* concentration (mg/kg)
Snails		
Oxychilus spp. (n = 28)	0.832–2.47	1.77
Slugs		
Limax maximus (n = 19)	< MLOD–1.80	0.806
Deroceras laeve (n = 15)	1.30–4.00	2.64

* To calculate the mean residue for samples reported as < MLOD, the MLOD was used as the value for these samples. Residue values have not been corrected for recovery.

Conclusion

The methodology developed for snail and slug tissue analysis proved to be reliable, efficient, and simple. The same method was used to determine the diphacinone residues from three different invertebrate species. In estimating potential secondary hazards for proposed use of indanedione rodenticides, it is critical to have analytical methods available to collect the necessary data to evaluate the risk to nontarget species.

Acknowledgments

The authors are grateful to Hacco, Inc. for supplying diphacinone. We would like to acknowledge Earl W. Campbell, Peter A. Dunlevy, Robert Sugihara, and William C. Pitt of the National Wildlife Research Center Hilo, Hawaii Field Station for assistance and providing control snails and slugs for method development. Mention of commercial products is for identification only and does not constitute endorsement by the U.S. Department of Agriculture.

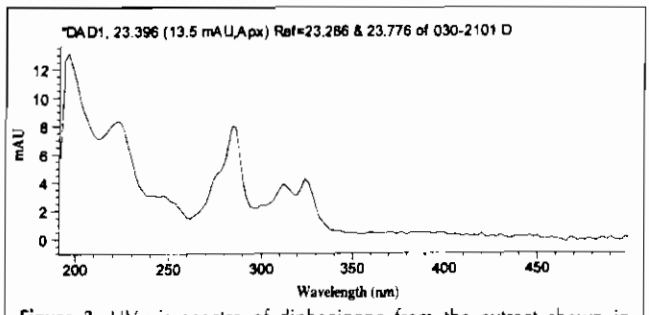

Figure 3. UV–vis spectra of diphacinone from the extract shown in Figure 2.

References

1. I.A.E. Atkinson. A reassessment of factors, particularly *Rattus L.*, that influenced the endemic forest birds in Hawaiian Islands. *Pacific Sci.* **31:** 109–33 (1977).
2. P.A. Dunlevy, E.W. Campbell, and G.D. Lindsey. Broadcast application of a placebo rodenticide bait in a native Hawaiian forest. *Inter. J. Biodeter. Biodegrade.* **45:** 199–208 (2000).
3. C.J. Miller and S. Anderson. Impacts of aerial 1080 poisoning on the birds of Rangitoto Island, Hauriaki Gulf, New Zealand. *New Zealand J. Ecol.* **16:** 103–107 (1992).
4. R.W. Bullard, R.D. Thompson, and G. Holguin. Diphendione residues in tissues of cattle. *J. Agric. Food Chem.* **24:** 261–63 (1976).
5. Y. Kawano and W. Chang. Spectrophotometric determination of Rozol in paraffinized formulations. *J. Assoc. Off. Anal. Chem.* **63:** 996–98 (1980).
6. R.L. Caswell. Report of rodenticides. *J. Assoc. Off. Anal. Chem.* **42:** 104–106 (1959).
7. V. Mallet, D. Surette, and G.L. Brun. Detection of naturally fluorescent pesticides on silica gel layers. *J. Chromatogr.* **79:** 217–22 (1973).
8. P. Owen, A. Pendlebury, and A.C. Moffat. Choice of thin-layer chromatographic systems for the routine screening for neutral drugs during toxicological analyses. *J. Chromatogr.* **161:** 187–93 (1978).
9. K. Opong-Mensah and W.R. Porter. Separation of some rodenticides and related compounds by thin-layer chromatography. *J. Chromatogr.* **455:** 439–43 (1988).
10. J.B. Addison. Improved method for high pressure liquid chromatographic determination of chlorophacinone in mouse tissue. *J. Assoc. Off. Anal. Chem.* **65:** 1299–1301 (1982).
11. J.E. Houglum, R.D. Larson, and R.M. Neal. High-performance liquid chromatographic separation of indanedione rodenticides. *J. Chromatogr.* **481:** 458–60 (1989).
12. J.D. Reynolds. Extraction and identification of ten anticoagulant rodenticides from baits and stomach contents by high pressure liquid chromatography. *Proc. Am. Assoc. Vet. Lab. Diagn.* **23:** 187–94 (1980).
13. B.R. Bennett and G.S. Grimes. Reverse phase liquid chromatographic determination of chlorophacinone and diphacinone in bait formulations. *J. Assoc. Off. Anal. Chem.* **65:** 927–29 (1982).
14. P. Mura, A. Piriou, Y. Papet, D. Lochon, and D. Reiss. Rapid high-performance liquid chromatographic assay of chlorophacinone and diphacinone in human serum. *J. Anal. Toxicol.* **16:** 179–81 (1992).
15. G. Vigh, E. Varga-Puchony, E. Papp-Hites, J. Hlavay, and S. Balogh. Determination of chlorophacinone in formulations by reversed-phase ion-pair chromatography. *J. Chromatogr.* **214:** 335–41 (1981).
16. K. Hunter. Reversed-phase ion-pair liquid chromatographic determination of chlorophacinone residues in animal tissues. *J. Chromatogr.* **299:** 405–14 (1984).
17. K. Hunter. High-performance liquid chromatographic strategies for the determination and conformation of anticoagulant rodenticide residues in animal tissues. *J. Chromatogr.* **321:** 255–72 (1985).
18. K. Hunter and E.A. Sharp. Modification to procedures for the determination of chlorophacinone and for multi-residue analysis of rodenticides in animal tissues. *J. Chromatogr.* **437:** 301–305 (1988).
19. T. Chalermchaikit, L.J. Felice, and M.J. Murphy. Simultaneous determination of eight anticoagulant rodenticides in blood serum and liver. *J. Anal. Toxicol.* **17:** 56–61 (1993).
20. T.M. Primus, D.A. Goldade, E.E. Petty, and J.J. Johnston. Solid-phase extraction and reversed-phase ion-pair high-performance liquid chromatographic determination of chlorophacinone and diphacinone residues in range grass. *J. Chromatogr. Sci.* **34:** 389–93 (1996).
21. Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA): Good Laboratory Practice Standards. *Code of Federal Regulations*, 40 CFR Part 160. Environmental Protection Agency, Washington, D.C., August 17, 1989.
22. T.P. Salmon, D.A. Whisson, and W.P. Gorenzal. Field efficacy studies comparing 0.005% and 0.01% diphacinone and chlorophacinone baits for controlling California ground squirrels (*Spermophilus beechyi*). Department of Wildlife, Fish and Conservation Biology, University of California, Davis, CA. Unpublished study submitted to the California Department of Food and Agriculture. MRID 45855101, 2002, p. 131.
23. J. Baroch. Field efficacy of diphacinone grain baits used to control the California ground squirrel. *Proc. 17th Vertebr. Pest Conf.* **17:** 127–32 (1996).
24. E.B. Spurr, G.D. Lindsey, C. Forbes Perry, and D. Foote. Efficacy of aerial broadcast application of pelletised baits containing 0.005% diphacinone in reducing rat populations in Hawai'ian forests. Pacific Islands Ecosystems Research Center, U.S. Geological Survey, Unpublished report #QA-02, 2004.
25. T.M. Primus. Determination of chlorophacinone and diphacinone in pocket gopher liver and whole body tissues. U.S. Department of Agriculture, National Wildlife Research Center, Analtyical Chemistry Project, Fort Collins, CO. Unpublished study submitted to the California Department of Food and Agriculture. 2004, p. 9, Invoice #04-018.

Manuscript received August 2, 2004;
revision received September 29, 2005.