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Thomas M. Primus
USDA/APHIS/WS/National Wildlife Research Center

Dennis J. Kohler
USDA/APHIS/WS/National Wildlife Research Center, dennis.kohler@aphis.usda.gov

Carol A. Furcolow
USDA/APHIS/WS/National Wildlife Research Center

Margaret J. Goodall
USDA/APHIS/WS/National Wildlife Research Center

John J. Johnston
USDA/APHIS/WS/National Wildlife Research Center

See next page for additional authors

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Determination of Acetaminophen Residues in Whole Body Brown Treesnakes

Thomas M. Primus,[1,*] Dennis J. Kohler,[1] Carol A. Furcolow,[1] Margaret J. Goodall,[1] John J. Johnston,[1] and Peter J. Savarie[2]

[1]Analytical Chemistry Project and [2]Product Development Section, USDA/APHIS/WS/National Wildlife Research Center, Colorado, USA

ABSTRACT

Acetaminophen was extracted from brown treesnakes (*Boiga irregularis*) and analyzed by reversed-phase high-performance liquid chromatography (HPLC). Acetaminophen was quantified by UV absorbance at 250 nm. Recoveries were determined by analyzing acetaminophen-fortified blank homogenized tissue. The mean recovery of acetaminophen in whole body brown treesnakes was 87.9% ± 5.9% and 92.2% ± 5.8% for the

*Correspondence: Thomas M. Primus, Analytical Chemistry Project, USDA/APHIS/WS/National Wildlife Research Center, 4101 LaPorte Ave, Ft. Collins, CO 80521, USA; E-mail: thomas.m.primus@usda.gov.

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fortification levels of 20 and 2400 μg/g, respectively. The method's limit of detection (MLOD) with UV detection was 0.70 μg/g.

Key Words: Acetaminophen (CAS# 114-26-1); Brown treesnake; High-performance liquid chromatography; UV detection; Tissue.

INTRODUCTION

The brown treesnake (*Boiga irregularis*) is not indigenous to the island of Guam, but was probably introduced accidentally after the end of World War II. Since then, this snake has been responsible for the extinction of nine species of native forest birds[1,2] and the decline of several lizards species on Guam.[3] The brown treesnake is mildly venomous and poses a health risk to children,[4] causes electrical power outages by climbing on wires,[5] and preys on domesticated birds.[6] An integrated control strategy is being developed for these snakes with chemical control being investigated as one possibility. Dermal and oral toxicity experiments were conducted on brown treesnakes with several candidate compounds.[7] Currently, acetaminophen, in a carrion-based delivery system, is being investigated as an oral toxicant for control of brown treesnake populations on Guam[8] due to its availability, reasonable cost, and the potential for registration under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA).

Acetaminophen is an over-the-counter analgesic, antipyretic drug and has no prior history of use as a pesticide. Studies related to its use in clinical research tends to focus on the analysis of plasma and urine, therefore, analytical methods for the determination of acetaminophen in whole bodies, muscle, or liver tissue[9] are rarely published. A method was developed in-house for the determination of acetaminophen in white mice and was used for a study to evaluate acetaminophen stability in mouse baits.[10,11]

A rapid and reliable analytical method was needed to determine acetaminophen residues in brown treesnakes to help interpret efficacy experiments, and as a tool to potentially assess secondary hazards. This work reports the development of a validated method for the assay of acetaminophen residues in brown treesnake tissue at concentrations of 20–2400 μg/g. The wide range of acetaminophen concentrations was required because some snakes regurgitated the baits shortly after ingestion while other snakes did not.

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EXPERIMENTAL

Samples

The samples consisted of whole brown treesnakes (control and treated) from the island of Guam. Control snakes were analyzed to assure the absence of analyte or chromatographic interferences. Control snakes were fortified (see fortification of controls) with known amounts of acetaminophen for method development and quality control samples.

Apparatus

A Hewlett Packard (Palo Alto, CA) 1090M high-performance liquid chromatography (HPLC) system equipped with a Hewlett–Packard computer workstation was operated at 25°C with a Phenomenex Prodigy ODS (Torrance, CA) analytical column (250 mm × 4.6 mm i.d.) and a guard column (15 mm × 4.6 mm i.d.). The HPLC was equipped with an ultra-violet/visible diode array detector set at 250 nm to detect acetaminophen. Sample extracts and standards (25 μL) were chromatographed with a mobile phase consisting of a 15 : 85 mixture of methanol : 50 mM potassium phosphate, monobasic (pH = 3.25) aqueous solution with a flow rate of 1.0 mL/min and a runtime of 20 min. The solvents were filtered through a 0.45 μm nylon filter and degassed by sparging with helium.

Reagents

Liquid chromatography grade acetone (Fischer Scientific, Denver, CO) was used as the extraction solvent. Potassium phosphate, monobasic (99%) was obtained from Fischer (Denver, CO). Acetaminophen (99%) was obtained from Chem Service, Inc. (West Chester, PA). A solution consisting of a 20 : 80 mixture of methanol : 50 mM potassium phosphate, monobasic (pH = 3.25) aqueous solution was used to reconstitute the final extracts and dilute calibration standards.

Concentrated standard solutions were prepared by accurately weighing 10.0 mg of acetaminophen into a 10.00-mL volumetric flask. The acetaminophen was dissolved in methanol, followed by dilution to volume with the same solvent. The concentration of the standard was ~1 mg/mL (1000 μg/mL).

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Calibration Curve

Calibration standard solutions for ultra-violet detection ranged from 0.5 to 150 μg/mL. All calibration standard solutions were prepared in 10.00-mL volumetric flasks with the methanol : water buffer solution as the diluent.

Extraction of Acetaminophen in Brown Treesnake

Partially frozen whole brown treesnakes were cut into approximately 2 in. pieces. The pieces were frozen with liquid nitrogen in a stainless steel cylindrical container. The frozen snake was shattered into a powder with a steel piston. Once most of the tissue was powdered, the large unhomogenized pieces of tissue were removed. The powdered tissue was transferred to a separate container and the remaining pieces were refrozen with liquid nitrogen and shattered into powder, and combined with the rest of the powdered sample.[12]

For each sample, a 1.00–1.10 g portion of powdered tissue was weighed into a 50-mL glass tube. A 10.0 mL aliquot of acetone was added from a calibrated volumetric device. Each tube was sealed with a screw cap and vortex mixed. All of the tubes were shaken on a mechanical shaker for 10 min on the high setting. The samples were then placed in an ultrasonic bath for 30 min, followed by centrifuging for 5 min. A 1.00 mL aliquot of the resulting extract was transferred to a 15-mL graduated centrifuge tube and the solvent was evaporated by blowing a stream of nitrogen over the solution with the tube in a warm water bath at 60–65°C. The dry residue was reconstituted by adding 2.00 mL of the methanol : water buffer solution and vortex mixed. The reconstituted samples were placed in an ultrasonic bath for 10 min in a glass beaker. A portion of each sample was filtered through a 0.45 μm Teflon disk into an amber LC vial, and sealed with a crimp cap. Each standard and sample were analyzed by HPLC.

Operating conditions were adjusted to obtain optimum response and reproducibility. For 15 : 85 methanol : water with phosphate buffer mobile phase, the retention time of acetaminophen was approximately 12.8 min. At the end of each analysis sequence, a column wash was performed by pumping 80 : 20 water : methanol through the guard and analytical columns for approximately 30 min.

Fortification of Controls

Acetaminophen fortification standard solutions at 20,000 and 1000 μg/mL in methanol were used for fortifying the control tissue to evaluate analyte

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recovery. Each 1.00 g portion of control brown treesnake tissue was fortified at 2400 or 20 μg/g with 0.120 or 0.020 mL aliquots of the 20,000 and 1000 μg/mL fortification standard solutions, respectively. The methanol was allowed to evaporate for 20 min and the extraction and cleanup procedure was followed as previously described.

RESULTS AND DISCUSSION

Instrumental Data

Two sets of calibration standard solutions were prepared ranging in concentrations from 0.50 to 150 μg/mL for UV detection. Each standard solution was injected twice, and a plot was constructed of acetaminophen chromatographic peak area response (y-axis) vs. acetaminophen concentrations (x-axis). A linear regression was performed on the data set. Linearity of chromatographic response was assessed for the chromatography conditions required for this system. In each case, a range of acetaminophen standard solutions at concentrations including the expected acetaminophen concentrations in quality control and field samples were analyzed. In all cases, linear regression analysis of concentration vs. response yielded an r^2 greater than 0.99. Linear regression analysis of the log : log plot of these data yielded a slope that was not significantly different than 1 ($P > 0.05$). These data indicated that the chromatographic responses for acetaminophen were linear and proportional, which justified the use of single point external standard for quantification of acetaminophen in the sample extracts.

Instrumental and Method Limits of Detection

Instrument limit of detection (ILOD) was defined as the concentration of acetaminophen in a calibration standard required to generate a signal equal to 3× the baseline noise (measured peak-to-peak) observed in the blank diluent. The ILOD was estimated from the mean chromatographic peak height of a acetaminophen standard solution (1.0 μg/mL for UV) and the peak-to-peak noise observed from three reagent blank samples. The ILOD for UV detection was determined to be 0.034 μg/mL for acetaminophen.

Method limit of detection (MLOD) was defined as the concentration of acetaminophen in the sample required to generate a signal equal to 3× the baseline noise (measured peak-to-peak) observed in the control sample. The MLOD was estimated from the mean chromatographic peak

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height of acetaminophen in seven fortified samples (20 μg/g for UV) and the peak-to-peak noise observed from seven control samples. The MLOD for UV detection was determined to be 0.72 μg/g for acetaminophen.

Residue in Brown Treesnakes

The recoveries of acetaminophen from control brown treesnake samples at two fortified levels, assayed for four different sets of samples over a 2 week period, are listed in Table 1. The recoveries ranged from 72.2% to 102% with a mean of 90.1% and a CV of 6.2% for 42 replicates at fortification levels of 20 and 2400 μg/g. Chromatograms of a control sample extract (Fig. 1), and extracts from control samples fortified at approximately 2400 μg/g acetaminophen (Fig. 2) and 20 μg/g acetaminophen (Fig. 3) analyzed with field study samples, are shown. As shown from the attached chromatograms, no interferences were observed for UV detection at 250 nm.

The residue results of brown treesnakes from an efficacy study in which the snakes ingested 80 mg acetaminophen fortified baits are shown in Table 2. Samples were homogenized and assayed in duplicate. The method yielded very reproducible results over a wide range of incurred residues, as the mean coefficient of variation for all the samples listed in Table 1 was less than 10%. Incurred residues ranged from 708 μg/g to less than the MLOD. A chromatogram of an actual snake sample extract (Fig. 4) collected during a field study is shown. The snake weighed 88.9 g with a concentration of 204 μg/g observed in this extract.

Table 1. Fortification levels and percent recoveries for acetaminophen in brown treesnake tissue.

	Fortification levels (μg/g)	
	20	2,400
Replicates	21	21
Mean	87.9	92.2
SD	5.9	5.8
CV	6.7	6.3

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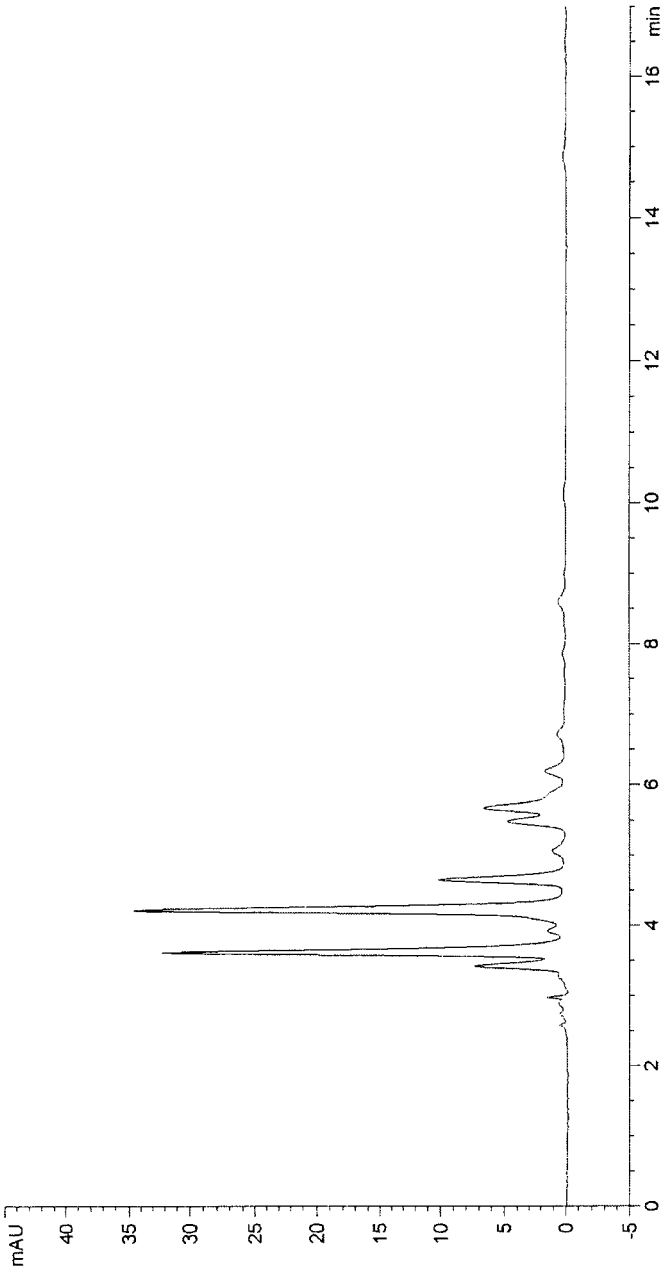

Figure 1. Chromatogram of a control brown treesnake sample extracted and analyzed with UV detection (acetaminophen typically has a retention time of 12.8 min).

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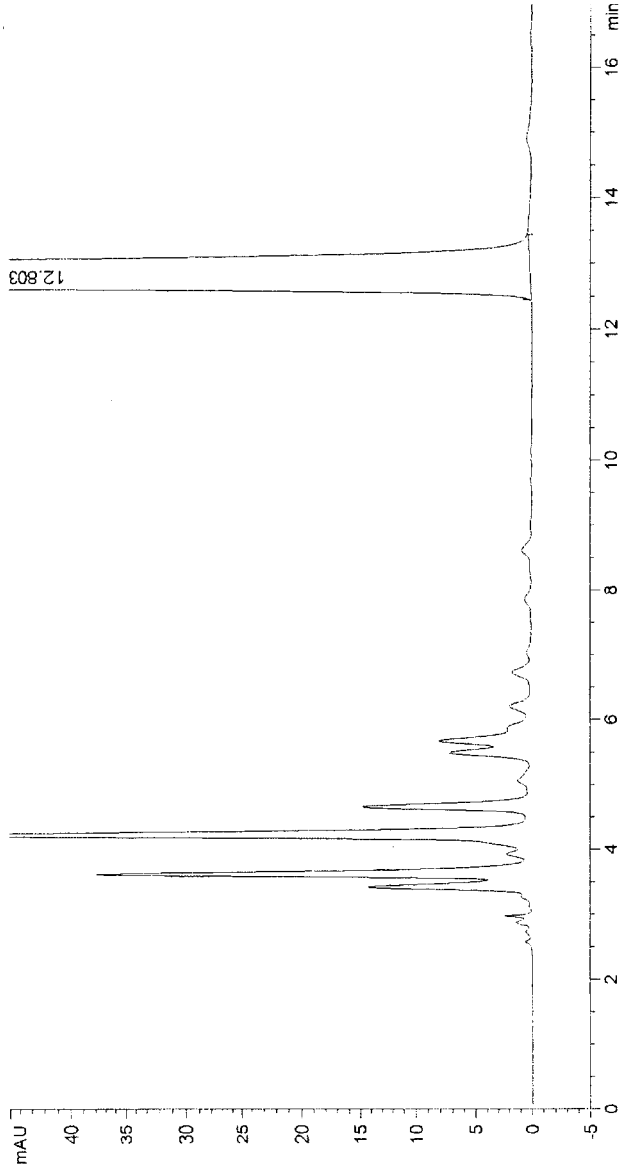

Figure 2. Chromatogram of a 2400 μg/g acetaminophen (12.8 min) fortified control sample extracted and analyzed with UV detection.

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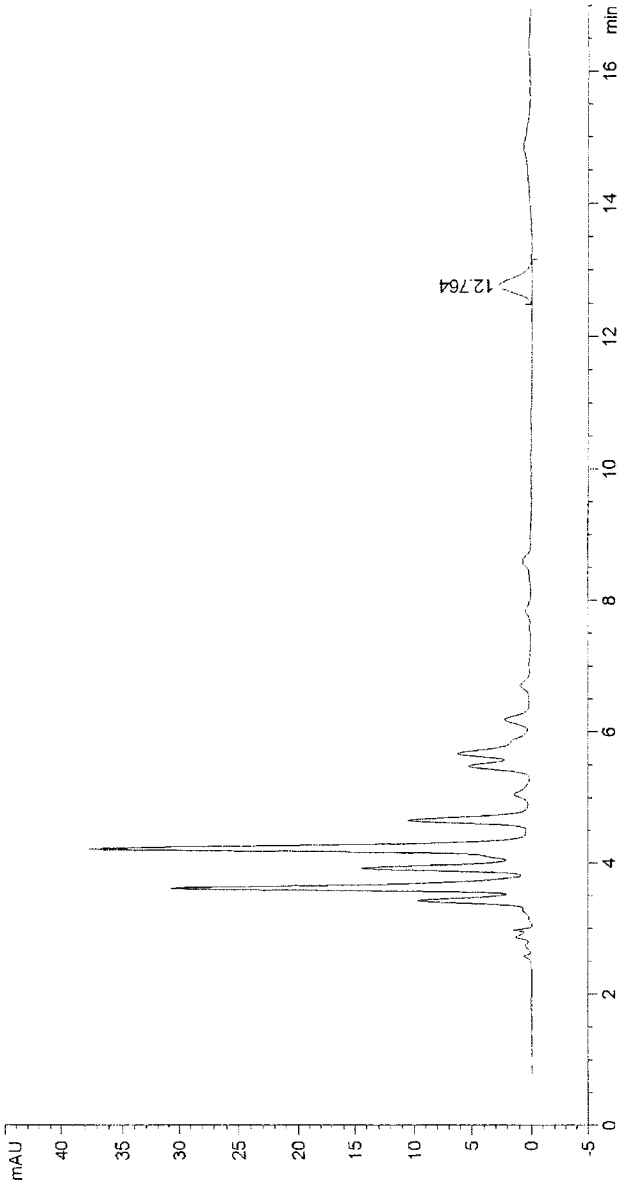

Figure 3. Chromatogram of a 20 μg/g acetaminophen (12.8 min) fortified control sample extracted and analyzed with UV detection.

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Table 2. Orally treated brown treesnakes.

Sample #	Description of treatment	Weight of snake (g)	Acetaminophen (μg/g)	Note
1	0 mg—day 0	29.9	<MLOD	
2	0 mg—day 0	90.6	<MLOD	
3	0 mg—day 0	108.4	<MLOD	
4	80 mg—day 0	52.5	7.0	Regurgitated bait
5	80 mg—day 0	56.1	3.3	Regurgitated bait
6	80 mg—day 0	79.3	505	
7	80 mg—day 0	88.4	398	
8	80 mg—day 0	102.7	358	
9	80 mg—day 0	139.8	151	
10	0 mg—day 1	53.0	<MLOD	
11	0 mg—day 1	90.5	<MLOD	
12	0 mg—day 1	128.8	<MLOD	
13	80 mg—day 1	59.9	388	
14	80 mg—day 1	58.1	19.3	Regurgitated bait
15	80 mg—day 1	88.9	291	
16	80 mg—day 1	154.4	126	
17	0 mg—day 2	55.3	<MLOD	
18	0 mg—day 2	85.9	<MLOD	
19	0 mg—day 2	127.0	<MLOD	
20	80 mg—day 2	48.4	735	
21	80 mg—day 2	25.4	648	
22	80 mg—day 2	73.6	391	
23	80 mg—day 2	73.5	461	
24	80 mg—day 2	78.9	16.0	Regurgitated bait
25	80 mg—day 2	110.8	246	
26	0 mg—day 3	40.2	<MLOD	
27	0 mg—day 3	27.7	<MLOD	
28	0 mg—day 3	75.9	<MLOD	
29	80 mg—day 3	20.3	16.4	Regurgitated bait
30	80 mg—day 3	21.2	5.4	Regurgitated bait
31	80 mg—day 3	39.8	164	
32	80 mg—day 3	30.7	213	
33	80 mg—day 3	35.0	24.4	

(*continued*)

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Table 2. Continued.

Sample #	Description of treatment	Weight of snake (g)	Acetaminophen (μg/g)	Note
34	80 mg—day 3	97.2	118	
35	0 mg—day 4	12.4	1.4	
36	0 mg—day 4	35.2	<MLOD	
37	0 mg—day 4	46.8	<MLOD	
38	80 mg—day 4	38.7	230	
39	80 mg—day 4	17.6	344	
40	80 mg—day 4	19.9	228	
41	80 mg—day 4	29.3	9.00	Regurgitated bait
42	80 mg—day 4	38.5	19.5	
43	80 mg—day 4	32.7	63.7	

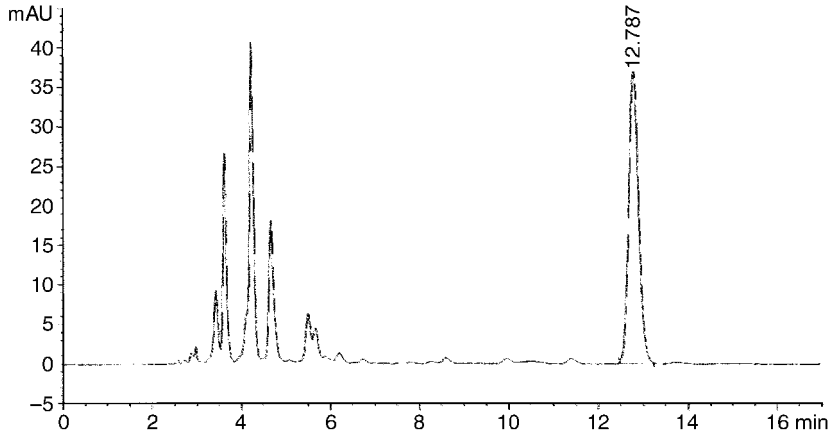

Figure 4. Chromatogram of a brown treesnake carcass collected from a baited trap extracted and analyzed with UV detection. The snake carcass weighed 88.9 g with an assayed concentration 204 μg/g acetaminophen (12.8 min).

This method was used to support multiple field and laboratory studies conducted on Guam. The resulting residue data are being used to determine nontarget hazards to scavengers and/or predators that might consume acetaminophen poisoned snake carcasses. These hazard assessments play a key role in the development of safe baiting strategies for the control of brown treesnakes on Guam.

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