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Development of a Cell Culture/ELISA Assay To Detect Anticoagulant Rodenticides and Its Application to Analysis of Rodenticide Treated Grain

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This study describes a generic biological screening assay designed to detect anticoagulant rodenticides based on their inhibitory action on the vitamin K epoxide reductase protein complex, resulting in an accumulation of under-carboxylated prothrombin or proteins induced by vitamin K antagonism (PIVKA-II). A combined cell culture/ELISA assay was optimized to measure PIVKA-II production by the human hepatoma HepG2 cell line cultured in the presence of anticoagulant rodenticides. The specificity and sensitivity of the assay was validated using 41 grain extracts containing representative concentrations of rodenticide or appropriate nonrodenticide control compounds. In all cases, PIVKA-II produced by HepG2 cells in response to grain extracts spiked with rodenticides was detected by ELISA, while PIVKA-II was not detected in supernatants collected from cells exposed to nonrodenticide controls. This represents a novel, class-specific biological assay for the detection of anticoagulant rodenticides present in contaminated grain.

KEYWORDS: Anticoagulant; rodenticide; detection; cell culture; ELISA; grain bait; WIIS; bioassay; PIVKA II

INTRODUCTION

Since the introduction of the 4-hydroxycoumarin compound warfarin in the 1950s, anticoagulant rodenticides have become the most commonly used chemical method for the control of rodents. Nowadays, warfarin has largely been superseded by more potent anticoagulant rodenticides. These are of two main types: 4-hydroxycoumarin superwarfarins such as coumatetralyl, bromadiolone, and difenacoum or acetyl-indandione derivatives such as chlorophacinone and diphacinone.

Anticoagulant rodenticides have a relatively high toxicity to vertebrate animals (*1*), and primary poisoning from consumption of rodent baits presents a direct hazard to wild mammals and birds, as well as to domestic animals. A number of studies has shown that secondary poisoning from consumption of poisoned animals is also a potential risk (*2*−*4*). Monitoring wildlife for the effects of anticoagulant rodenticide poisoning has been undertaken in France (*5*, *6*), New Zealand (*7*), and the U.S. (*8*). In the UK, the Wildlife Incident Investigation Scheme (WIIS) has investigated cases of suspected pesticide poisoning since the 1960s. Incidents involving anticoagulant rodenticides were first confirmed by the WIIS in 1973. To conduct such investigations, suitable analytical methods are required to detect anticoagulant rodenticides in tissues from potentially poisoned animals and in bait materials that may have been consumed. Methods capable of simultaneous detection of a number of anticoagulant rodenticides are preferred to minimize the number of analyses performed on each sample. A number of such multianalyte methods based on high-performance liquid chromatography (HPLC), with detection by ultraviolet (UV) absorption, fluorescence, or mass spectrometry (MS) has been reported for blood (*9*), serum (*10*−*13*), tissues (*10*, *14*, *15*), and soil (*16*). HPLC methods for simultaneous analysis of the two indandione compounds chlorophacinone and diphacinone in bait materials have also been reported (*17*, *18*). Maurer and Artl (*19*) reported a gas chromatography method for the analysis of urine after extractive methylation of 4-hydroxycoumarin anticoagulant residues. Although effective, methods such as these require the availability of sophisticated and expensive analytical equipment, housed in a well-equipped laboratory. Because of these limitations, there is a need for alternative approaches to the detection of anticoagulant rodenticides to reduce reliance on expensive equipment and complement existing techniques. Furthermore, a generic rodenticide screening assay, based on a common property of all anticoagulant rodenticides, would permit the detection of both existing and novel compounds without modifications to existing methodology. In this study, the potential for a generic screening assay to detect anticoagulant rodenticides by their biological actions was investigated.

Vitamin K is an essential cofactor for γ-carboxylation of key glutamic acid residues in blood clotting proteins by vitamin K-dependent carboxylase. During catalysis, vitamin K is converted from the active form to vitamin K 2,3-epoxide, which must be recycled to the active form by vitamin K epoxide

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reductase (VKOR) to maintain the coagulation cycle (*20*, *21*). Anticoagulants antagonize VKOR, preventing vitamin K recycling and resulting in an accumulation of des-γ-carboxy prothrombin (DCP) or proteins induced by vitamin K antagonism (PIVKA-II) (*22*).

Secretion of undercarboxylated prothrombin is a species-dependent event. PIVKA-II production is not detected in warfarin-treated rabbits, hamsters, or guinea pigs but has been reported for rat, cow, mouse, and chick species (*23*, *24*). PIVKA-II measurement in plasma samples routinely involves plasma coagulation assays (*24*, *25*), barium citrate absorption to separate soluble and insoluble prothrombin forms (*26*, *25*), chromogenic assays (*27*), or radiolabeling experiments (*28*).

PIVKA-II has been identified as a marker of hepatocellular carcinoma in humans. An enzyme-linked immunosorbant assay (ELISA) containing a monoclonal antibody that specifically detects undercarboxylated prothrombin (*29*) was developed to improve the diagnosis of hepatocellular carcinoma and is also used to clinically monitor the effectiveness of anticoagulant therapy (*30*). The PIVKA-II ELISA has also been successfully used to detect undercarboxylated prothrombin in culture supernatants of primary human liver cells (*31*). Several reports have provided evidence that the human hepatoma cell line, HepG2, secretes PIVKA-II in response to warfarin exposure (*32*, *33*), although this has not been demonstrated previously by ELISA. In this study, we report the development of a detection system for anticoagulant rodenticides using ELISA to detect PIVKA-II produced by HepG2 cells exposed to rodenticides. Such a system would be applicable to rodenticide detection in both animal tissues and bait materials; however, for the purposes of this study, it was decided to limit validation to grain-based bait materials. Detection in fluid or tissue samples was not considered at this stage since it is likely to be much more challenging as typical residues are much lower than in grain baits. As well as being useful for investigating wildlife poisoning cases, a generic screening assay for rodenticides would be applicable to other situations, such as the detection of rodenticide contamination of food samples (*18*).

MATERIALS AND METHODS

Materials. Cell culture reagents were purchased from Invitrogen, Paisley, UK, with the exception of dimethyl sulfoxide (DMSO; Sigma-Aldrich, Dorset, UK). The human hepatoma cell line HepG2 was purchased from the American Type Culture Collection (LGC Promochem, Middlesex, UK); PIVKA-II ELISA kits were from Axis-Shield Diagnostics, Cambridgeshire, UK. Analytical or HPLC-grade solvents and anhydrous sodium sulfate were purchased from Fisher Chemicals, Loughborough, UK. Brodifacoum (98.5% purity), coumatetralyl (98.5% purity), diphacinone (99% purity), warfarin (98% purity), and imidacloprid (99% purity) were purchased from QMx Laboratories Ltd., Thaxted, UK. Chlorophacinone (98% purity), coumaphos (99.8% purity), bromadiolone (98.8% purity), dimethoate (99.9% purity), fonofos (98% purity), and tefluthrin (98.6% purity) were purchased from Riedel-de Haen, Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany. Difethialone (98.5% purity) was kindly supplied by Liphatech SAS. Difenacoum (99% purity) was purchased from Greyhound Chromatography and Allied Chemicals, Birkenhead, UK, and flocoumafen (99.4% purity) was from BASF Aktiengesellschaft-67114 Limburgerhof, Germany.

Preparation of Solvent Standards. Warfarin, coumatetralyl, difenacoum, bromodialone, chlorophacinone, coumaphos, and dimethoate were individually weighed out into sterile 1.5 mL microtubes and resuspended at 30 mM in DMSO. Stocks were aliquotted and stored at −20 °C until required. For use in assays, 30 mM stocks were diluted in DMSO to 0.003−3 mM. Stocks were finally diluted 1000× in serum-free tissue culture medium prior to use, to give working concentrations in the range of 0.003−30 μM. Chemical structures for analytes used in this investigation are shown in **Figure 1**.

Extraction of Grain Samples and Preparation of Positive Control Extracts. Spiked or blank organic wheat grain (∼1 g) was weighed into a test tube (25 mL), and water (8 drops, ∼0.2 mL) was added. The tube was left to stand for at least 15 min. Extraction solvent (8 mL of dichloromethane:acetone, 1:1) was added, and the contents of the tube were mixed on a vortex mixer. A small quantity of anhydrous sodium sulfate (∼1 g) was added to the tube, and the contents were mixed again. Each extract was filtered through a glass wool plug in the neck of a glass funnel into another tube, rinsing with acetone (1−2 mL). The filtrate was evaporated to dryness and redissolved in DMSO (2 mL) with the help of an ultrasonic bath and vortex mixer. All extracts were finally diluted 1:100 in serum-free medium for addition to cells.

Portions of blank extract were also spiked post-extraction with 3 mM warfarin or coumatetralyl in DMSO and included as positive controls during initial assay optimizations. A final dilution of 1:100 in serum-free cell culture medium gave a working concentration of 30 μM.

Preparation of Spiked Grain Samples for Validation. Organic wheat grain (∼1 g) was weighed into a screw-capped vial and spiked with the test compound in methanol solution. Individual portions of grain were spiked with one of nine anticoagulant rodenticides (brodifacoum, bromodialone, chlorophacinone, coumatetralyl, difenacoum, diphacinone, flocoumafen, warfarin, and difethialone), each at four final concentrations (1.5−25 μg/mL), or with one of four other pesticides, each at one final concentration. Concentrations of all compounds present in grain samples are shown in **Table 1**. One portion of grain was spiked with methanol alone to serve as a blank. The methanol was carefully evaporated by directing a stream of nitrogen gas over the sample with a Pasteur pipet, before proceeding to the extraction as described.

Cell Maintenance. HepG2 cells (passage 80−90) were maintained in mimimal essential medium containing Earle's salts, 25 mM HEPES, and Glutamax I (MEM). For routine cell maintenance, the culture medium was supplemented with 10% foetal calf serum (FCS) and penicillin/streptomycin. Cells were maintained at 37 °C in a 5% CO_2 humidified environment and were passaged weekly. Cell culture assays were performed in a reduced-serum assay medium comprising MEM supplemented with 0.5% FCS and penicillin/streptomycin.

Cell Treatments with Solvent Standards and Grain Extracts. HepG2 cells were seeded at 5×10^5 per well of a six-well plate and cultured for 48 h prior to addition of treatments. Each experimental condition was tested in a single well. Wells were washed with Hank's balanced saline solution before the addition of 1.5 mL of reduced-serum assay medium supplemented with rodenticide solvent standards or grain extracts diluted as described. A no-treatment control was included in each assay. At the end of the treatment period, 1 mL of supernatant was collected from each culture well and stored at −70 °C until tested using PIVKA-II ELISA.

PIVKA-II ELISA. The PIVKA-II ELISA kit was used according to the manufacturer's instructions, except that supernatants were tested neat, diluted 1:1, or diluted 1:3 depending on the sensitivity required. On each ELISA plate, four reference standards (2, 50, 117, and 197 ng/mL) provided with the test kit were tested at the same dilution used for the experimental samples. Representative absorbance values obtained for neat PIVKA-II reference standards and those diluted 1:1 or 1:3 are shown in **Figure 2**.

RESULTS AND DISCUSSION

HepG2 Cell Supernatants Contain PIVKA-II After Treatment with Warfarin. Supernatants from HepG2 cells exposed to 0, 3, or 30 μM warfarin were assayed neat or diluted 1:3 to detect the presence of PIVKA-II. PIVKA-II was detected in both neat and diluted supernatants from cells that had been exposed to warfarin but not in supernatants from no-treatment controls (**Figure 3**). Similar absorbance values were recorded for supernatants from cells treated with 3 and 30 μM warfarin, suggesting that PIVKA-II production is not dose-responsive but may be continually produced above a threshold concentration

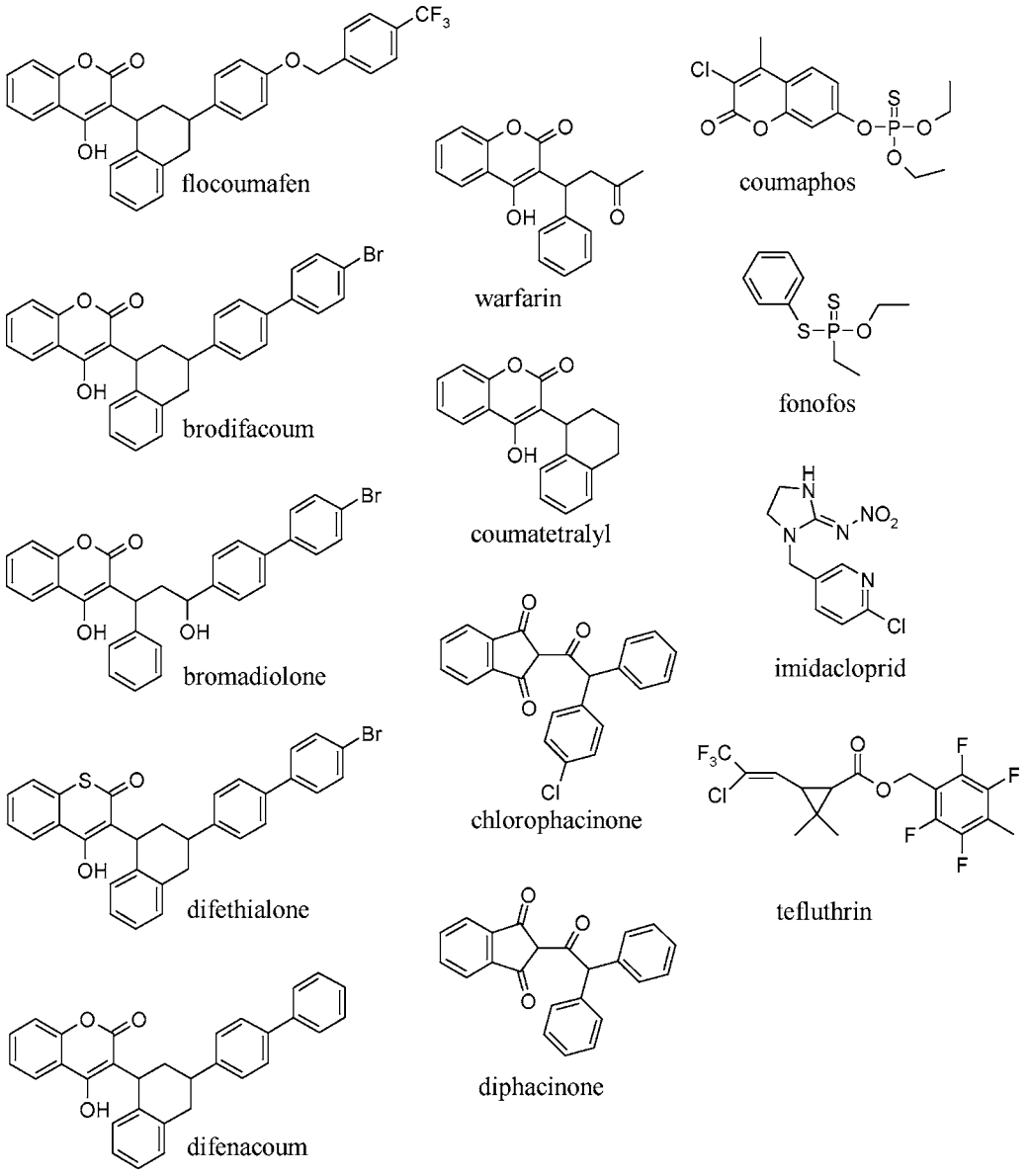

Figure 1. Structures of analytes investigated.

of warfarin. Although PIVKA-II was detected in both diluted and neat supernatants, diluted supernatants were selected for subsequent experiments, to reduce absorbance values from experimental samples to within the range of the standard curve.

HepG2 Cell Supernatants Contain PIVKA-II After Treatment with Coumarins and Indandiones But Not Two Organophosphate Compounds. Rodenticide grain baits are formulated with a 0.005% (w/w) active ingredient for most compounds, the exception being warfarin, which is usually in the range of 0.025−0.05 (w/w). This translates to concentrations on the grain of 250−500 μg/g for warfarin and 50 μg/g for the other rodenticides. Old or partly decomposed bait samples submitted to the WIIS may have much lower residues. Dose−response experiments were performed using standards prepared in DMSO, at final concentrations of 0.003−3 μM. This corresponds to theoretical grain extract concentrations of 0.3−300 μM, which is equivalent to 0.09−90 μg/mL and 0.18−180 μg/g for coumatetralyl (smallest molecule, 292.3 g/mol) and 0.16−160 μg/mL and 0.32−320 μg/g for bromadiolone (527.4 g/mol).

Following the 24 h treatment period, cell supernatants were diluted 1:3 and evaluated for PIVKA-II content. Pooled duplicate data from two experiments are shown in **Figure 4**. Absorbance readings for the two reference standards are included on each graph: 2 ng/mL, which is considered negative for PIVKA-II production, and 200 ng/mL, which represents a high positive result. Exposure of HepG2 cells to 0.03−3 μM warfarin (**Figure 4A**) resulted in clearly detectable PIVKA-II in cell supernatants. In contrast, less than 2 ng/mL PIVKA-II was detected following exposure to 0.003 μM warfarin The detection limit for warfarin, based upon the measurement of PIVKA-II in cell supernatants, was therefore considered to be 0.03 μM, although this only relates to cell supernatants that have been diluted 1:3 prior to analysis.

HepG2 cells were also exposed to three coumarin anticoagulants, one indandione anticoagulant, and two organophosphates, one of which (coumaphos) is a coumarin derivative. Each compound was tested at the three doses shown to induce PIVKA-II production by warfarin (0.03−3 μM). Supernatants were diluted 1:3 and evaluated for PIVKA-II content. Pooled duplicate data from two experiments are shown in **Figure 4B**. PIVKA-II production was clearly detected in supernatants collected from HepG2 cells treated with all three doses of difenacoum, bromodialone, chlorophacinone, and coumatetralyl.

Table 1. Results of a Validation Experiment Testing 41 Blind Samples for the Presence of Anticoagulant Rodenticide, Based on Production of PIVKA-II by HepG2 Cells

sample	analyte present in extract	Abs 1[a]	Abs 2[a]	PIVKA-II detected[b,c]
1	24.97 μg/mL brodifacoum	1.416	1.470	yes
2	9.99 μg/mL brodifacoum	1.518	1.524	yes
3	4.00 μg/mL brodifacoum	1.459	1.592	yes
4	1.60 μg/mL brodifacoum	1.589	1.670	yes
5	24.61 μg/mL bromadiolone	1.583	1.695	yes
6	9.85 μg/mL bromadiolone	1.609	1.674	yes
7	3.94 μg/mL bromadiolone	1.545	1.580	yes
8	1.58 μg/mL bromadiolone	1.600	1.624	yes
9	25.21 μg/mL chlorophacinone	1.583	1.600	yes
10	10.08 μg/mL chlorophacinone	1.542	1.565	yes
11	4.03 μg/mL chlorophacinone	1.578	1.612	yes
12	1.61 μg/mL chlorophacinone	1.643	1.674	yes
13	25.27 μg/mL coumatetralyl	1.544	1.575	yes
14	10.11 μg/mL coumatetralyl	1.645	1.675	yes
15	4.04 μg/mL coumatetralyl	1.510	1.509	yes
16	1.62 μg/mL coumatetralyl	1.411	1.401	yes
17	24.9 μg/mL difenacoum	1.610	1.592	yes
18	9.96 μg/mL difenacoum	1.548	1.525	yes
19	3.98 μg/mL difenacoum	1.567	1.571	yes
20	1.59 μg/mL difenacoum	1.509	1.554	yes
21	24.64 μg/mL diphacinone	1.637	1.703	yes
22	9.86 μg/mL diphacinone	1.612	1.664	yes
23	3.94 μg/mL diphacinone	1.556	1.611	yes
24	1.58 μg/mL diphacinone	1.575	1.596	yes
25	25.00 μg/mL flocoumafen	1.605	1.649	yes
26	10.00 μg/mL flocoumafen	1.586	1.622	yes
27	4.00 μg/mL flocoumafen	1.651	1.683	yes
28	1.60 μg/mL flocoumafen	1.549	1.648	yes
29	25.14 μg/mL warfarin	1.681	1.691	yes
30	10.05 μg/mL warfarin	1.575	1.584	yes
31	4.02 μg/mL warfarin	1.439	1.478	yes
32	1.61 μg/mL warfarin	1.179	1.198	yes
33	24.70 μg/mL difethialone	1.595	1.668	yes
34	9.88 μg/mL difethialone	1.570	1.571	yes
35	3.95 μg/mL difethialone	1.606	1.627	yes
36	1.58 μg/mL difethialone	1.566	1.601	yes
37	175.19 μg/mL imidacloprid	0.106	0.091	no
38	100.10 μg/mL tefluthrin	0.125	0.116	no
39	25.03 μg/mL coumaphos	0.117	0.103	no
40	402.89 μg/mL fonofos	0.110	0.097	no
41	Methanol-only blank	0.111	0.114	no
42	no-treatment control	0.052	0.450	no
43	negative control (30 μM coumaphos)	0.078	0.082	no
44	positive control (30 μM coumatetralyl)	1.542	1.544	yes

[a] Absorbance (Abs) measurements taken at 492 nm; each sample was tested in duplicate. [b] Less than 2 ng/mL PIVKA-II is considered to be negative.[29] [c] PIVKA reference standard duplicate absorbance values: 2 ng/mL standard: 0.169, 0.175; 50 ng/mL reference standard: 1.286, 1.284; 117 ng/mL reference standard: 1.937, 1.981; and 197 ng/mL reference standard: 2.353, 2.372.

No PIVKA-II production was detected at any of the three doses tested for coumaphos or dimethoate. The results confirm that the assay detects the coumarin and indandione anticoagulants that were tested but does not detect two organophosphates, one of which is a coumarin derivative.

PIVKA-II Production by HepG2 Cells in Response to Treatment with Extracts from Rodenticide-Spiked Grain Is Detectable by ELISA. It was necessary to determine (i) that the blank grain extract did not induce background PIVKA-II production from HepG2 cells and (ii) that PIVKA-II production was still detected when rodenticide was presented to cells within a solvent grain extract. Extracts of rodenticide-free grain were tested unspiked and spiked post-extraction with a final concentration of 30 μM warfarin. Extracts were diluted 1:100 in serum-free medium prior to the addition to HepG2 cells, which represents a final concentration of 0.3 μM warfarin present in culture medium. Data are presented in **Figure 5**. PIVKA-II was clearly detected in ELISA samples, which were diluted both 1:1 and 1:3 prior to analysis; furthermore, no background PIVKA-II production was detected following HepG2 treatment with blank grain extracts. Given the complete lack of background PIVKA-II production in response to blank grain extracts, it was decided to test all extract samples at 1:1 dilution, to maximize the likelihood of rodenticide detection in blind samples.

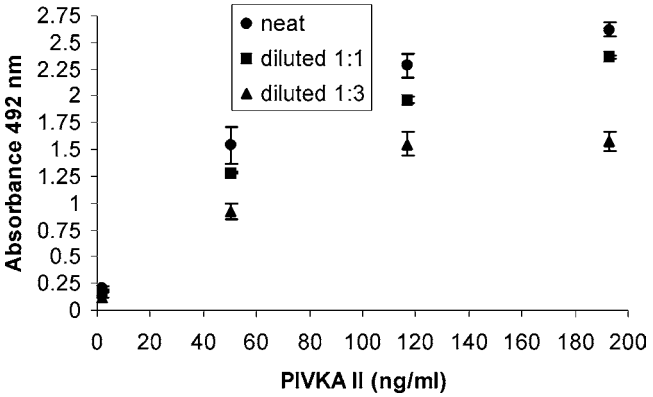

Figure 2. PIVKA-II standard curves. Standard curves were prepared using reference standards known to contain 2, 50, 117, and 193 ng/mL PIVKA-II. Reference standards were analyzed neat or diluted in dilution buffer provided with the ELISA kit. Standard curves were used to evaluate PIVKA-II concentrations in HepG2 cell supernatants. Data represent mean values from at least two replicate wells.

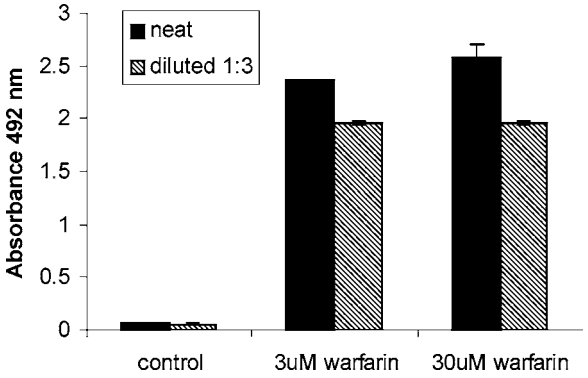

Figure 3. Detection of PIVKA-II in HepG2 supernatants following treatment with serum-free medium supplemented with warfarin. Supernatants were tested neat or diluted 1:3 in dilution buffer before testing using the PIVKA-II ELISA. Data represent mean values from at least two replicate wells.

PIVKA-II Production by HepG2 Cells Is a Reliable, Specific, and Sensitive Technique To Detect the Presence of Rodenticide Compounds in Grain Extracts. Forty-one samples containing various concentrations of nine rodenticides or single concentrations of four nonrodenticide compounds were extracted. Extracts contained rodenticides in the range of 1.6−25 μg/mL and were diluted 1:100 in reduced-serum assay medium, which represents final concentrations of 16−250 ng/mL present in cell culture medium. Four pesticide treatments were included as controls: imidacloprid and tefluthrin, because of their current usage as seed treatments that might be present on a grain sample submitted for analysis; fonofos, which was approved as a seed treatment in the 1990s and can occasionally be present in samples presented for Wildlife Incident analysis; and coumaphos, because of the structural resemblance to coumarin rodenticides.

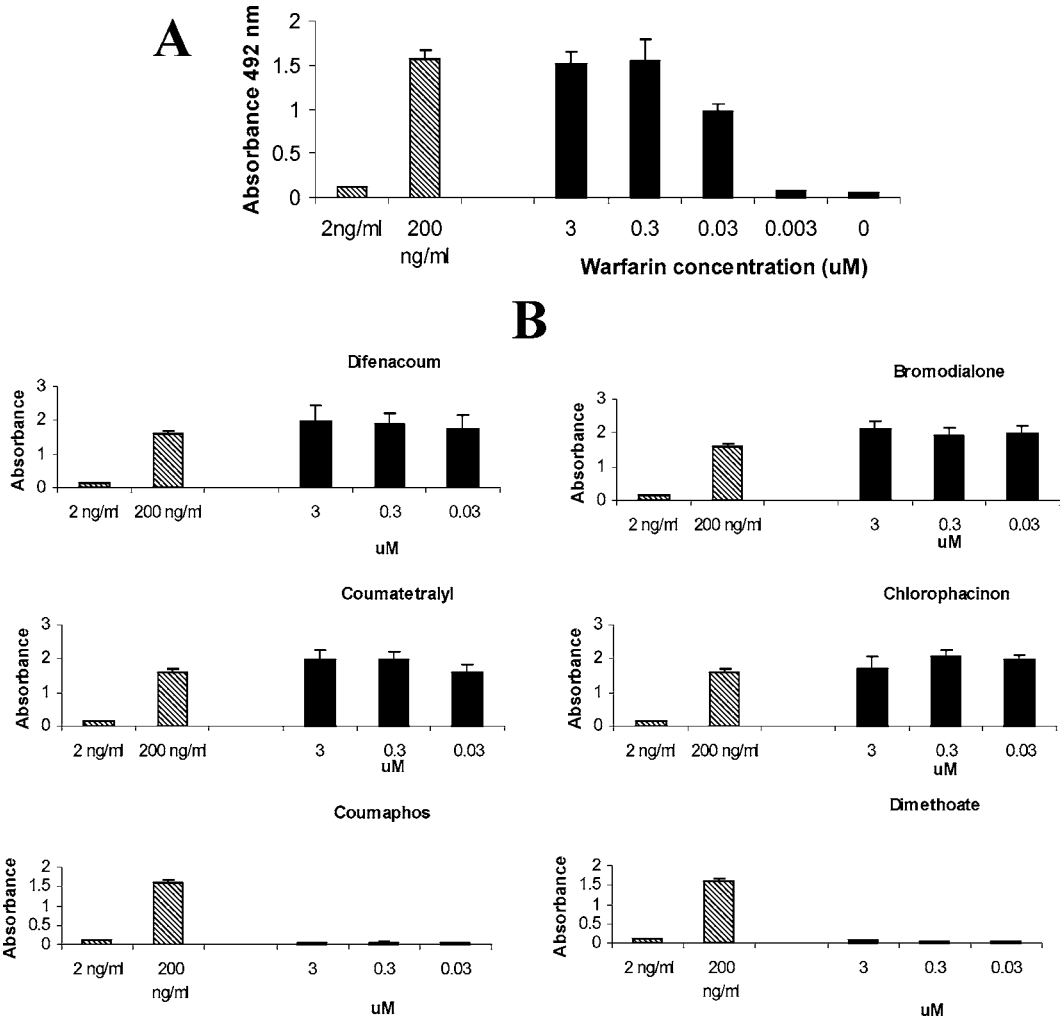

Figure 4. PIVKA-II production by HepG2 cells in response to treatment with reduced-serum assay medium supplemented with anticoagulant rodenticides or organophosphates. Data represent mean absorbance values from two experiments. Absorbance values for two reference standards are included for comparison (hatched bars). All samples and standards were diluted 1:3 before ELISA analysis. A five-point dose response curve is shown for warfarin (**A**) and a three-point dose response curve for a further six compounds (**B**).

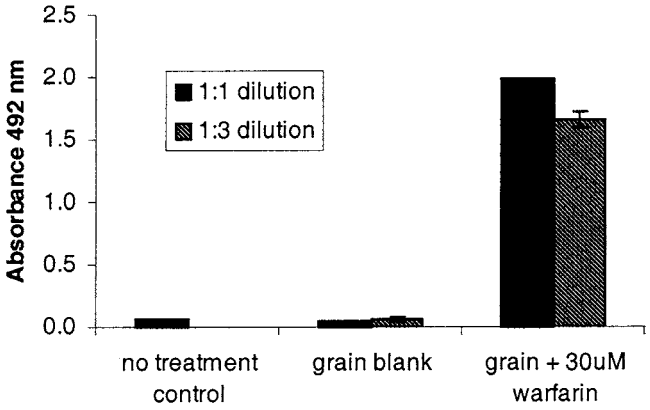

Figure 5. PIVKA-II production by HepG2 cells in response to treatment with blank grain extracts and grain extracts spiked post-extraction with warfarin (positive control). Grain extracts were diluted 1:100 in serum-free medium prior to addition to cells. Cell supernatants collected following 24 h treatment were diluted 1:1 or 1:3 prior to ELISA analysis.

Extracts diluted 1:100 in serum-free medium were added to HepG2 cells for a period of 24 h, after which supernatants were collected and diluted 1:1 for ELISA analysis. Each sample was analyzed in duplicate. Results for 41 samples and three controls are presented in **Table 1**, with raw absorbance values shown for each duplicate well. For samples where absorbance values were greater than those obtained from the 2 ng/mL reference standard, PIVKA-II was considered to be present. For samples where absorbance values were lower than those obtained from the 2 ng/mL reference standard, PIVKA-II was considered to be absent. For samples 1−36 and the positive control sample 44, absorbance values were greater than those obtained for the 50 ng/mL PIVKA-II reference standard, indicating a clear positive result. For samples 37−43, absorbance values were below those obtained for the 2 ng/mL PIVKA-II reference standard, and PIVKA-II was therefore considered to be absent. In all cases, there was clear detection of those extracts containing rodenticides with no false positives or negatives detected.

These data confirm that the presented method is sensitive and highly specific. Up to 44 samples can be tested in duplicate on a single ELISA plate and compared directly with a four-point standard curve, thereby permitting high-throughput analysis. This investigation confirms, using a commercial ELISA technique, that HepG2 cells produce PIVKA-II but only in response to anticoagulant treatment. In conclusion, a new cell culture/ELISA method is presented, which provides rapid detection of the presence of rodenticides over a wide dose range within grain samples.

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LITERATURE CITED

(1) Osweiler, G. D., Carson, T. L., Buck, W. B., Vangelder, G. A., Eds. *Clinical and diagnostic veterinary toxicology*, 3rd ed.; Kendall/Hunt: Dubuque, IA, 1985.

(2) Mendenhall, V. M.; Pank, L. F. Secondary poisoning of owls by anticoagulant rodenticides. *Wildlife Soc. Bull.* **1980**, *8*, 311−315.

(3) Townsend, M. G.; Fletcher, M. R.; Odam, E. M.; Stanley, P. I. An assessment of the secondary poisoning hazard of warfarin to tawny owls. *J. Wildlife Manage.* **1981**, *45*, 224−248.

(4) Townsend, M. G.; Bunyan, P. J.; Odam, E. M.; Stanley, P. I.; Wardall, H. P. An assessment of the secondary poisoning hazard of warfarin to least weasels. *J. Wildlife Manage.* **1984**, *45*, 628−632.

(5) Berny, P. J.; Buronfosse, T.; Buronfosse, F.; Lamarque, F.; Lorgue, G. Field evidence of secondary poisoning of foxes (*Vulpes vulpes*) and buzzards (*Buteo buteo*) by bromadiolone, a 4-year survey. *Chemosphere* **1997**, *35*, 1817−1829.

(6) Lorgue, G.; Nahas, K.; Keck, G.; Rampaud, M. *Intoxication of domestic and wild animals by anticoagulant rodenticides—a synthesis of data from the French national Antipoison Centre*; Proceedings of the 12th Vertebrate Pest Conference: University of California, Davis, 1986; pp 82−87.

(7) Eason, C. T.; Spurr, E. B. Review of the toxicity and impacts of brodifacoum on nontarget wildlife in New Zealand. *New Zealand J. Zool.* **1995**, *22*, 371−379.

(8) Stone, W. B.; Okoniewski, J. C.; Stedelin, J. R. Poisoning of wildlife with anticoagulant rodenticides in New York. *J. Wildife. Dis.* **1999**, *35*, 187−193.

(9) Guan, F. Y.; Ishii, A.; Seno, H.; Watanabe, K.; Kumazawa, T.; Suzuki, O. A method for simultaneous determination of five anticoagulant rodenticides in whole blood by high-performance liquid chromatography. *J. Pharm. Biomed. Anal.* **1999**, *21*, 179−185.

(10) Chalermchaikit, T.; Felice, L. J.; Murphy, M. J. Simultaneous determination of eight anticoagulant rodenticides in blood serum and liver. *J. Anal. Toxicol.* **1993**, *17*, 56−61.

(11) Kuijpers, E. A. P.; den Hartigh, J.; Savelkoul, T. J. F.; de Wolff, F. A. A method for the simultaneous identification and quantitation of five superwarfarin rodenticides in human serum. *J. Anal. Toxicol.* **1995**, *19*, 557−562.

(12) Lotfi, H.; Dreyfuss, M.-F.; Marquet, P.; Debord, J.; Merle, L.; Lachatre, G. A screening procedure for the determination of 13 oral anticoagulants and rodenticides. *J. Anal. Toxicol.* **1996**, *20*, 93−100.

(13) Palazoglu, M. G.; Tor, E. R.; Holstege, D. M.; Galey, F. D. Multiresidue analysis of nine anticoagulant rodenticides in serum. *J. Agric. Food Chem.* **1998**, *46*, 4260−4266.

(14) Jones, A. HPLC determination of anticoagulant rodenticide residues in animal livers. *Bull. Environ. Contam. Toxicol.* **1996**, *56*, 8−15.

(15) Fauconnet, V.; Pouliquen, H.; Pinault, L. Reversed-phase HPLC determination of eight anticoagulant rodenticides in animal liver. *J. Anal. Toxicol.* **1997**, *21*, 548−553.

(16) Medvedovici, A.; David, F.; Sandra, P. Determination of the rodenticides warfarin, diphenadione, and chlorophacinone in soil samples by HPLC-DAD. *Talanta* **1997**, *44*, 1633−1640.

(17) Primus, T. M.; Griffin, D. L.; Volz, S. A.; Johnston, J. J. Reversed-phase ion-pair liquid-chromatographic determination of chlorophacinone and diphacinone in steam-rolled oat baits and steam rolled oat/wax baits. *J. AOAC Int.* **1998**, *81*, 349−357.

(18) Mesmer, M. Z.; Flurer, R. A. Determination of chlorophacinone and diphacinone in commercial rodenticides by liquid chromatography-ultraviolet detection and liquid chromatography-electrospray ionization mass spectrometry. *J. Chromatogr. A* **2000**, *891*, 249−255.

(19) Maurer, H. H.; Arlt, J. W. Detection of 4-hydroxycoumarin anticoagulants and their metabolites in urine as part of a systematic toxicological analysis procedure for acidic drugs and poisons by gas chromatography−mass spectrometry after extractive methylation. *J. Chromatogr. B* **1998**, *714*, 181−195.

(20) Cain, D.; Hutson, S. M.; Wallin, R. Assembly of the warfarin-sensitive vitamin K 2,3-epoxide reductase enzyme complex in the endoplasmic reticulum. *J. Biol. Chem.* **1997**, *272*, 29068−29075.

(21) Goodstadt, L.; Ponting, C. P. Vitamin K epoxide reductase: homology, active site, and catalytic mechanism. *Trends Biochem. Sci.* **2004**, *29*, 289−292.

(22) Fasco, M. J.; Principe, L. M. R- and S-warfarin inhibition of vitamin K and vitamin K 2,3-epoxide reductase activities in the rat. *J. Biol. Chem.* **1982**, *10*, 4894−4901.

(23) Carlisle, T. L.; Shah, D. V.; Schlegel, R.; Suttie, J. W. Plasma abnormal prothrombin and microsomal prothrombin precursor in various species. *Proc. Soc. Biol. Exp. Med.* **1975**, *148*, 140−144.

(24) Zivelin, A.; Mohan Rao, L. V.; Rapaport, S. I. Plasma proteins in rabbits given warfarin. *Thromb. Res.* **1996**, *82*, 429−438.

(25) Shah, D. V.; Swanson, J. C.; Suttie, J. W. Abnormal prothrombin in the vitamin-K deficient rat. *Thromb. Res.* **1984**, *35*, 451−458.

(26) Suttie, J. W. Mechanism of action of vitamin K: synthesis of gama-carboxyglutamic acid. *CRC Crit. Rev. Biochem.* **1980**, *8*, 191−223.

(27) Harauchi, T.; Takano, K.; Matsuura, M.; Yoshizaki, T. Liver and plasma levels of descarboxyprothrombin (PIVKA II) in vitamin K deficiency in rats. *Jpn. J. Pharmacol.* **1986**, *40*, 491−499.

(28) Oka, T.; Touchi, A.; Harauchi, T.; Takano, K.; Yoshizaki, T.; Matsubara, T. In vivo effects of â-lactam antibiotics and heterocyclic thiol compounds on vitamin K dependent carboxylation activity and blood coagulation factors in vitamin K-deficient rats. *Biochem. Pharmacol.* **1988**, *37*, 2091−2095.

(29) Grosley, B. M.; Hirschauer, C.; Chambrette, B.; Bezaud, A.; Amiral, J. Specific measurement of hypocarboxylated prothrombin in plasma or serum and application to the diagnosis of hepatocellular carcinoma. *J. Lab. Clin. Med.* **1996**, *127*, 553−564.

(30) Bach, A. U.; Anderson, S. A.; Foley, A. L.; Williams, E. C.; Suttie, J. W. Assessment of vitamin K status in human subjects administered "minidose" warfarin. *Am. J. Clin. Nutr.* **1996**, *64*, 894−902.

(31) Biron-Andreani, C.; Bezat-Bouchahda, C.; Raulet, E.; Richard-Garcia, L.; Fabre, J.-M.; Saric, J.; Baulieux, J.; Schev, J.-F.; Maurel, P. Secretion of functional plasma haemostasis proteins in long-term primary cultures of human hepatocytes. *Br. J. Haem.* **2004**, *125*, 638−646.

(32) Wang, Z.; Wang, M.; Finn, F.; Carr, B. I. The growth inhibitory effects of vitamin K and their actions on gene expression. *Hepatology* **1995**, *22*, 876−882.

(33) Wu, W.; Bancroft, J. D.; Suttie, J. W. Differential effects of warfarin on the intracellular processing of vitamin K dependent proteins. *Thromb. Haem.* **1996**, *76*, 46−52.

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