An HPLC Method for the Determination of Bromadiolone Plasma Kinetics and its Residues in Hen Eggs

Mario Giorgi* and **Grazia Mengozzi**

Department of Veterinary Clinics, Veterinary Teaching Hospital, Section of Pharmacology and Toxicology, University of Pisa, Via Livornese (lato monte) 1, 56010, San Piero a Grado, Pisa, Italy

Abstract

Cereal-based bromadiolone anticoagulant is often used for rodent control, and because these baits are attractive for poultry they may be accidentally ingested. Thus, the aim of this study was to develop a new high-performance liquid chromatography (HPLC) method for the determination of bromadiolone residues in hens' eggs and its plasma kinetics. Laying hens ($n = 48$) were divided into four groups of 12 animals each. Groups I and II received orally a single dose of bromadiolone 10 mg/kg, group III received a single dose of bromadiolone 60 mg/kg, and group IV was the control. Eggs were collected from groups I, III, and IV, whereas plasma was collected from groups II and IV. The HPLC method developed was reproducible, sensitive, accurate, and linear within the range 0.1–20 μg/g. The final HPLC conditions were as follows: mobile phase MeOH–ammonium acetate (0.5 M) triethylamine buffer (pH 5, 51:49, v/v); analytical column Luna C_{18} ODS2; wavelength 260 nm; flow rate of 1.5 mL/min; and warfarin as internal standard (5 μg/mL). Recoveries for bromadiolone were in the range of 72–80% with RSD lower than 10%. Pharmacokinetic behavior of bromadiolone in hens results faster than that reported in other animals and humans. Following 10 and 60 mg/kg treatment bromadiolone was not detected in albumen but was present in yolk from day 4 to 5 and from day 2 to 9. In conclusion, the bromadiolone amount found in eggs was well below the toxic dose of this anticoagulant for humans, and no anticoagulant effect should be observed.

Introduction

Bromadiolone (B) {3-[3-(4'-bromobiphenyl-4-yl)-3-hydroxy-1-phenyl propyl]-4-hydroxycoumarin} is a hydroxycoumarin-derived (Figure 1) and is called "second generation" or "superwarfarin" rodenticide. Like warfarin, it is based on the natural compound coumarin, acts as an anticoagulant, and at the same time is more effective than warfarin against pest species. Also, it is effective against warfarin-resistant rats and mice, including Norway rats (*R. norvegicus*) (1–3). B residues have been detected in tissues of *Arvicola terrestris* (4–6) and coypu (*Myocastor coypus*) (7) after field use. Its main activity is the

vitamin K epoxide reductase inhibition, an enzyme that causes blood clotting alteration and hemorrhages leading to death. B in cereal-based baits (0.005%) is often used to control rodent populations both outdoors and indoors. The recommended period of baiting ranges from few weeks up to 20 weeks (8). These baits are attractive for poultry and may be accidentally ingested. Although fatal intoxications in poultry have been previously described (9,10), ingestion of anticoagulant baits rarely lead to clinical signs of intoxication because poultry are much less susceptible to these compounds than mammals (11,12), and they eat just small quantities. The eggs laid by birds exposed to anticoagulants may contain residues of this compound, which is dangerous for human health. Previous studies on warfarin showed that the risks caused by first-generation rodenticides are negligible. B is more toxic than warfarin (13) and therefore has a higher octanol/water coefficient (4.27 at pH 7 and 20°C) residues at high concentrations that could possibly be present in eggs (14). In literature, several chromatographic methods for single (15,16) and multiple (17–19) anticoagulant agents determination in biologic matrices have been described but only a few in eggs (20,21). None of these last two methods seem to be completely suitable for the analysis of B in eggs and plasma at the same time. Many techniques request expensive extraction methods, long time of analysis (multi-residual detection), and expensive devices. Moreover, because of the quenching due to B (Figure 1), B is only weakly fluorescent (22,23), and fluorimetric detection seems not appropriate. The aim of this work was to develop a simple, rapid, and sensitive high-performance liquid chromatography (HPLC) method for B residues determination in albumen and yolk of laying hens eggs as well as for B pharmacokinetics in plasma.
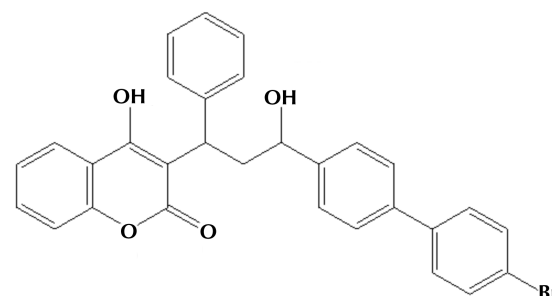

Figure 1. Molecular structure of bromadiolone.

*Author to whom correspondence should be addressed.

Experimental

Reagents and materials

Tap water was first purified in-house by a distiller device and then by a Milli-Q Plus water system (Millipore, Milan, Italy). Acetonitrile (ACN), methanol (MeOH), and ethylacetate (EtOAc) were of HPLC grade. B and warfarin (W) pure analytical standards were purchased from Lab Service Analytica (Bologna, Italy). B used for oral animal treatment (93% pure) was kindly donated from INDIA Chemical Industries (Padova, Italy). All the other reagents and materials were of analytical grade and supplied from commercial source. The aqueous and organic components of the mobile phase, degassed with helium under pressure, were mixed by the HPLC. The LC mobile phases were filtered through 0.22-μm nylon-66 (N-66) filters with a solvent filtration apparatus. Prior to injection onto the HPLC, sample extracts were filtered through 13-mm 0.2-μm Acrodisc nylon (Gelman, Ann Arbor, MI) or 22-mm 0.2-μm Millex-GP (Millipore, Bedford, MA) filters.

Standard solutions

Stock solutions (1000 μg/mL)

On the basis of the listed potency or purity of the standard, the amount of B and W needed to prepare 100 mL of the individual 1000 μg/mL standard solutions was calculated. The standards were weighed to the nearest 0.1 mg into a 100-mL volumetric flask and brought to the mark with MeOH. These solutions should be used immediately for the preparation of the fortification solution below.

Fortification solution (5 μg/mL)

Twenty mL of each stock standard solution (1000 μg/mL) were pipetted into a 100-mL volumetric flask and brought to the mark with MeOH. This operation was repeated once more to obtain final concentrations of 40 μg/mL. These solutions have been successively diluted to reach final concentration of 5 μg/mL. These solutions were kept in plastic 50-mL vials at –80°C, and they were stable for at least 12 months.

Calibration standard solutions

The first dilution of the fortification solution (200 μg/mL) was diluted with MeOH for the preparation of five-points of standard dilution for both analytes of interest at the following concentrations: 25, 50, 75, 100, and 150 μg/mL. For concentrations at or below 20 μg/mL, a second standard calibration curve was prepared at 0.1, 1, 5, 10, and 20 μg/mL. These solutions, used for the preparation of matrix-matched curves, should be freshly prepared every day before use.

Apparatus

The LC system consisted of a Thermo Finnigan Spectra system P2000 pump, a Thermo Finnigan Spectra system UV 2000 UV-vis detector (Waltham, MA) interfaced to a Thermo Finnigan ChromQuest data system and a Thermo Finnigan Spectra system autosampler/sampleprocessor equipped with a variable 1-100 μL loop. The LC columns tested were a LichroCART (4.6 × 250 mm, 5 μm) with guard column of the same packing (Merck, Darmstadt, Germany) and a Luna C_{18} ODS2 (4.6 × 150 mm, 3 μm) with guard column of the same packing (Phenomenex Synergy, Milan, Italy).

Animal treatment and sampling

Animal experiments were conducted at the poultry experimental facility of the Faculty of Veterinary Medicine at the University of Pisa. Animal care and handling were performing according to the provision of the EC Council Directive 86/609 EEC, recognized and adopted by the Italian Government (DL 27/1/1992, n°116). The study protocol was approved by the ethics committee of the University of Pisa (CASA) authorization n°4155 and transmitted to Italian Ministry of Health. A flock ($n = 48$) of laying hens (ISA-brown), weighing 1.7–2.2 kg and aged 31–39 weeks old, was used for the trial during the first half of their production cycle. The hens were foot ringed for a rapid identification and randomly divided into four groups of 12 animals each. These laying hens were housed in 24-tier battery pens of two laying hens each under conventional conditions of ventilation, temperature (18–22°C), and lighting (16 h light/day).

Food and water were available ad libitum during the whole trial. Each group was previously controlled for their laying persistency in order to evaluate the homogeneity of the entire flock. During the entire experiment, the hens were monitored daily for general health by qualified personnel supervised by a veterinarian. Eggs were collected daily during the complete course of the study. After the animals were placed in their pens, they were allowed to adjust to their environment for four weeks. During this adjustment period, all animals were fed with anticoccidial-free feed, and the only additives added were vitamins A and D (Galline Ovaiole C.A.P., Pisa, Italy).

The eggs collected during this period were used as controls. After the adaptation period, groups I and II orally received B at a single dose of 10 mg/kg body weight (BW) diluted in sunflower oil. Group III was received B at a single dose of 60 mg/kg BW diluted in sunflower oil, while group IV was received just sunflower oil. Eggs collection (groups I and III) was stopped 15 days after the administration of the compound of interest. About 8–12 eggs were daily collected from each experimental group, and from each egg, yolk and albumen were separated and stored at –18°C until analysis. Also, egg collection was carried on during the weekend. Group II was used for blood sample collection (1 mL) by a brachial vein puncture and pooled at 0, 1, 2, 3, 4, 5, 6, 8, 10, 24, 30 h. Group IV was used as control for eggs and blood samples.

Because it was not possible to collect much blood from each animal, we decided to combine blood from two animals and process it as a single sample. Then, blood was transferred into blood collection tubes containing lithium heparin, centrifuged within 30 min, and the plasma achieved was frozen at –20°C until analysis.

Extraction procedure

Egg yolk or egg albumen (2.0 ± 0.1 g) was weighed into a disposable 15 mL polypropylene centrifuge tube. For recovery measurement, the fortification solution of W (5 μg/mL) was added and gently vortex-mixed and left to react with the matrix for 15 min. Anhydrous Na_2SO_4 (0.5 g) was also added to the sample followed by 5 mL of organic solvent (MeOH, EtOAc, or ACN), and the sample was stirred using a spatula to ensure adequate mixing. The tube was capped and vortex-mixed at high speed for

30 s, followed by centrifugation for 5 min at 3800 rpm ($3000 \times g$) at 4°C for phases separation. The clear supernatant was transferred into another 15-mL polypropylene centrifuge tube using a disposable glass pipette. Then, 5 mL of organic solvent was added, and the extraction procedure was repeated one or two more times to evaluate the influence of the number of extractions. The supernatants were combined and centrifuged for 5 min at 3800 rpm ($3000 \times g$) at 4°C. The supernatant was carefully transferred into a glass test tube and evaporated to dryness at 40°C by a gentle stream of N_2. The sample was vortex-mixed again with 2 mL ACN, and the extracts were transferred into an insulin syringe fitted with a 13-mm Acrodisc nylon or a 22-mm Millex-GP filter to be filtered into a glass autosampler vial. Twenty microliter were injected onto the top of the LC column within 24 h after preparation. The plasma samples were extracted using the same procedure with that for the eggs, starting from 1 mL of plasma with 1 mL ACN final added.

Chromatographic conditions

Egg and plasma extracts were analyzed for B testing the following isocratic LC conditions: mobile phases consisted of MeOH–KH2PO4 0.05 M, ACN–H2O (9:1)–KH2PO4 0.05 M, MeOH–ammonium acetate (0.5 M) triethylamine buffer, blended at different % and different pH (Table I); flow rate from 0.8 to 1.6 mL/min; wavelength 260 and 280 nm; run-time from 5 to 15 min; column temperature 25 ± 0.5°C. One hundred microliters of pure MeOH was injected to equilibrate the LC system. Twenty micro-

liters of B standard series were injected prior to injecting a sample set. At the end of each day's analyses, the analytical column and guard column should be flushed with MeOH–ACN (50:50, v/v).

Quantification

A calibration curve of peak area versus concentration (µg/mL) of B was plotted. Least squares regression parameters for the calibration curve were calculated, and the concentrations of the test samples were interpolated from the regression parameters. Sample concentrations were determined by linear regression using the formula $Y = mX + b$, where Y = peak area and X = concentration of the standard in µg/mL. Correlation coefficients for each of the calibration curves were > 0.99. To estimate the analyte concentration, the areas under the curve specific for B were calculated using the internal standard (IS) method, according to equation:

B area = (plotted B area/plotted W recovery) × 100

When unknown samples were assayed, a control and a fortified blank sample should be processed along with each set for quality control. Limit of detection (LOD) and quantitation (LOQ) were determined as analyte concentrations giving signal-to-noise ratios of 3 and 10, respectively.

Pharmacokinetics and statistical analysis

Plots of plasma B concentration versus time were constructed for each pool of animals ($n = 6$). Pharmacokinetic analysis was performed for each data set using a computer program WinNonlin version 5.2.1 (Pharsight Corporation, Mountain View, CA). Akaike's information criterion (24) was used to select the best-fitting model (mono-exponential versus bi-exponential). Parameters computed included AUC (h µg/mL) area under the plasma concentration-time curve; K_{01} (h) and K_{10} (h), microconstants estimating the absorption of the drug in blood (K01) and its elimination from plasma (K_{10}); K_{01} $t_{1/2}$ (h), half-life of the absorption phase, K_{10} $t_{1/2}$ (h), half-life of the elimination phase; CL (mL/h/kg), total clearance; Vd (L/kg), volume of distribution; Tmax (h), time of peak plasma concentration; Cmax (µg/mL), peak plasma concentration. One-way analysis of variance (ANOVA) was applied to the principal pharmacokinetic parameters obtained from the different treatments and pools. $P < 0.05$ was taken as being significant.

Table I. Different Blends (v/v) Tested to Optimize Mobile Phase

Mobile phase (pH 4.0–9.0)		
I*	II‡	III‡
80:20	80:20	60:40
85:15	75:25	55:45
87:13	70:30	51:49
90:10		46:54
		36:64

* CH_3OH–KH_2PO_4 0.05 M.
† ACN–H_2O (9:1)–KH_2PO_4 0.05 M.
‡ CH_3OH–ammonium acetate (0.5 M) triethylamine buffer.

Table II. Influence of the Solvent Extraction on the Recoveries (%) of Bromadiolone (B) and Warfarin (W) from Egg (yolk and albumen) and Plasma Samples* (n = 3)

Substances	MeOH + NaCl		EtOAc		MeOH		ACN	
	R%	RSD%	R%	RSD%	R%	RSD%	R%	RSD%
Matrix: Yolk								
B	63	9	64	11	55	17	75	8
W	59	11	68	8	60	19	69	9
Matrix: Albumen								
B	66	9	61	10	58	21	72	9
W	60	11	64	9	55	18	74	7
Matrix: Plasma								
B	68	10	69	6	54	15	80	10
W	60	9	70	7	69	11	72	8

* Spiking level 5 µg/g, 1 cycle of 5 min.

Result and Discussion

Optimization of the extraction process

Initial studies were performed to evaluate the influence of solvent and the number of extractions on the extraction efficiency in order to find the best conditions to be used for B extraction. At first, various solvent mixtures were tested, and as the optimum solvent was found, the impact of the number of extractions was evaluated. Also several extraction solvents were tested, and B extraction efficiency from eggs and plasma matrices are shown in Table II. Organic

solvents, such as ACN, EtOAc, or MeOH, were able to extract B from the egg and plasma in a certain degree (Table II); however, ACN was shown to be the most effective solvent for B extraction. Using MeOH an emulsion was formed, which was difficult to eliminate, even though prolonged centrifugation or increase of the ionic strength with NaCl was applied. Thus, ACN was selected as the most suitable extraction solvent yielding cleaner extracts and better recoveries. No significant differences in recoveries were found among albumen, yolk, and plasma samples.

Number of extractions

To investigate the effectiveness of B extraction from egg and plasma matrices, a 5 min extraction time and a range of 1 to 3 repeated extractions were tested as well as an optional extraction
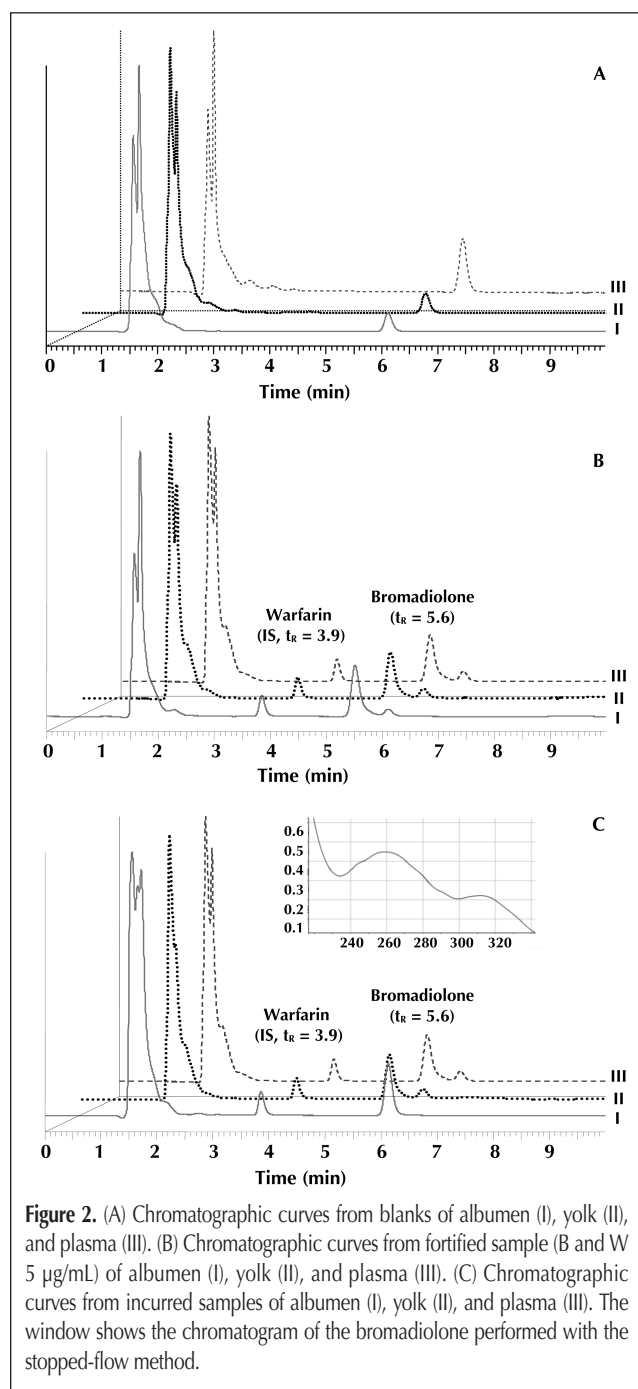

Figure 2. (A) Chromatographic curves from blanks of albumen (I), yolk (II), and plasma (III). (B) Chromatographic curves from fortified sample (B and W 5 µg/mL) of albumen (I), yolk (II), and plasma (III). (C) Chromatographic curves from incurred samples of albumen (I), yolk (II), and plasma (III). The window shows the chromatogram of the bromadiolone performed with the stopped-flow method.

time from 5 to 15 min. No significant differences were found between the three different matrices (data not shown). Generally, an increment of the number of extractions allows better matrix/solvent equilibration, which improved partitioning and increased analyte recoveries. It was found that the number of extractions did not play an important role in extraction efficiency because negligible differences were found between one, two or three extractions with recoveries ranging between 75 and 87% (RSD = 5–11%, n = 3). Finally, it was decided to adopt a 5 min extraction time, ensuring an efficient B and W extraction from incurred eggs and plasma in less time.

Method validation

Validation of the HPLC–UV method has been performed according to the requirements defined by the guidelines of the Commission Decision 2002/657/EC, which establishes the performance criteria for analytical residue methods (Official Journal of the European Communities, 2002). The validation procedure includes the determination of the system suitability test (SST), specificity, recoveries, precision in terms of repeatability and within-laboratory reproducibility, decision limit (CC_α), detection capability (CC_β), LOD, LOQ, and robustness. The primary SST parameters assessed were resolution, repeatability (relative standard deviations, RSD, of peak response and retention time), column efficiency, and tailing factor. These parameters were most important as they indicated system specificity, precision, and column stability. Other parameters included capacity factor (k) and signal-to-noise ratio (S/N) for impurity peaks. The acceptances of criteria were in accordance with those proposed by Vander Heyden et al. (25). The specificity of the method was assessed by testing a number of 20 control eggs (yolk and albumen) and 10 plasma samples to verify the absence of potential interfering compounds. Different mobile phases were tested in different % and pH (Table I), according to previous studies (20,26); the best was mobile phase III [MeOH–ammonium acetate (0.5 M) triethylamine buffer (pH 5, 51:49, v/v)], which allowed the best resolution, tailing factor, and rapidity of the analysis (retention time). The other best conditions were: analytical column Luna C_{18} ODS2, wave length 260 nm, flow rate of 1.5 mL/min, and W as internal standard (5 µg/mL). Typical chromatograms of control egg and plasma extracts and fortified egg and plasma samples are shown in Figures 2A–2B, respectively. Identification of the components by comparison of their retention factors (retention times) alone could be not sufficient owing to similar retention of various accompanying compounds in the sample. Hence, although a diode array or photo diode array detector was not available, a full spectrum of B by the stopped-flow method was obtained. The comparison this spectrum (Figure 2C) with that in library showed the absence of impurities.

The obtained chromatograms did not show any interference, as no detectable matrix peaks were eluted in the retention time window of the target W and B (3.7–4.1 and 5.4–5.8 min, respectively), despite a peak with retention time of 6.3 min, was detected after B elution in all the matrices. The LOD and LOQ of the method resulted in 0.1 and 0.03 µg/g, respectively. The limit of decision (CC_α) is defined as the analyte concentration at or above which it can be concluded with an error probability (α = 1%) that a sample is not compliant (CC_α is, in the case of banned

substances, the lowest concentration level at which the method can discriminate with a statistical certainty of $1-\alpha$ whether the identified analyte is present); whereas the detection capability (CC_β), for substances for which not permitted limit has been established, is the lowest concentration at which a method is able to detect truly contaminated samples with an error probability (β = 5%) (Official Journal of the European Communities, 2002). Following these criteria, specified in the Directive 96/23 Commission Decision 2002/657, CC_α and CC_β were calculated from three calibration curves, through analysis performed on different days by spiking control egg or plasma samples with B in the range 0.1–20 µg/g and 5 of µg/g the IS. These values were calculated on the basis of multiple-spiked samples on each day. In the three matrices the same values for CC_α and CC_β of 0.12 and 0.23 µg/g, respectively, were obtained. To quantify the absolute recoveries during method optimization and validation, five-point matrix-matched calibration curves were prepared by fortification of control egg and plasma extracts with B within the range 0.1–20 µg/mL, thus assuring a perfect match between analyzed samples and standard curves. Under the instrumental conditions reported in the Experimental Section, the calibration curves presented good linearity (R^2 for all curves were > 0.999). To evaluate the accuracy and intraday repeatability of the method, control egg samples, spiked at three concentration levels (1, 5, and 50 µg/g, n = 9) were analyzed using the optimized analytical method. Recoveries in the range of 72–80% were obtained for B at all fortification levels with RSD lower than 10%. Mean recoveries of the IS at the spiked level (5 µg/g) were 72 ± 9%. To determine the linearity and the reproducibility of the whole analytical method, control egg and plasma samples were fortified with B in the range 0.1–20 µg/g, and 5 µg/g of W was added as IS. Sample analysis, according to the procedure described in Experimental Sections, was performed in triplicate on three different days. The method's validation parameters are shown in Table III. No significant differences (95% confidence limit) were observed between the calibration slopes obtained for B in different assay days, confirming robustness of the analytical method. Thus, the reproducibility of the complete analytical procedure was evaluated from the percentage recovery at each spiked level, using the regression curves by interpolation of the peak area values in the matrix calibration curves. Excellent reproducibility was achieved for B with mean recovery values of 70–80% and RSD lower than 11% in all cases.
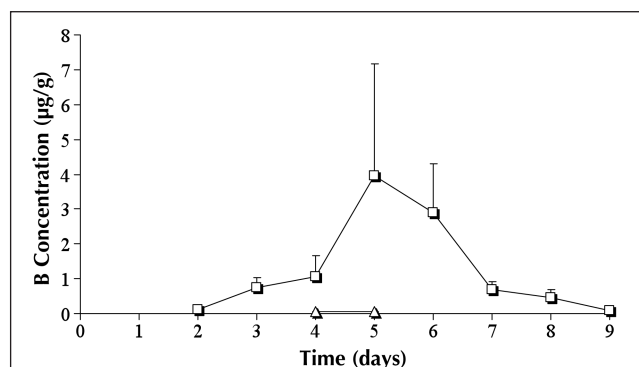

Figure 3. B concentrations (mg/gr) in yolk following single oral dose of 10 mg/kg (Δ) and 60 mg/kg (❑) of B in laying hens (six pools of two hens). Day zero is the day of starting treatment. Bars represent the standard deviation.

Analysis of egg (yolk and albumen) and plasma samples

To further validate the applicability of the method for the analysis of real samples, eggs were collected over a 15 days period from hens dosed with a single oral dose of B (60 and 10 mg/kg BW). These samples have been processed with the final validated method. The chromatograms of incurred egg and plasma sample are shown in Figure 2C. Quantification of B in the incurred samples was performed by using five-point matrix calibration curves made by spiking control eggs. The results obtained were used to construct the depletion curves of B in yolk (Figure 3). After 10 mg/kg dose treatment, no B residues were found in albumen collected for 10 days and low B concentrations were identified in yolk on the fourth and fifth day. Some of the albumen samples were processed starting from 10 g sample and final reconstitution with 200 µL of MeOH (concentration factor 50×). After such procedure, no residues were found in the sample. After 60 mg/kg dose, B concentrations found in yolk were above the LOQ almost 2 days after treatment and with highest values observed on the fifth day (Figure 3). Thereafter, B concentrations were dropped rapidly and were above the LOD 9 days after dosing. Concerning albumen, no B residues were observed during this period, which means that this finding does not agree with the depletion profile of W in eggs (21) because of its detection in both yolk and albumen. It is possibly due to the partition coefficient differences between B (Log P 4.27) and W (Log P 2.52). The higher lipophylicity might not allow the repartition of B in albumen. Moreover, these results indicate that B residues are present for several days in yolk and not in albumen, which could be justified by the fact that albumen is produced and excreted in 24 h, while the turnover time for yolk is 6–8 days (27).

Table III. Inter-day Mean Recovery (n = 5) and Precision (RDS%) Data for the Determination of Bromadiolone (B) in Fortified Egg (Yolk and Albumen) and Plasma Samples*

Spiked amount (µg/g)	R%	RSD%	Matrix
Substance: B AB/AIS = (115 ± 31) 10^{-4} C + (0.03 ± 0.02)†			
0.1	70	10	
1	74	9	
5	75	8	Yolk
10	78	10	
20	78	7	
Substance: B AB/AIS = (188 ± 49) 10^{-4} C + (0.04 ± 0.01)†			
0.1	70	11	
1	79	10	
5	72	9	Albumen
10	74	11	
20	70	6	
Substance: B AB/AIS = (123 ± 45) 10^{-4} C + (0.05 ± 0.02)†			
0.1	71	11	
1	75	8	
5	80	10	Plasma
10	78	8	
20	75	9	

* Performed on three different days.
† Average calibration equation. Uncertainly values calculated at a 95% confidence limit; five data points (n = 3). C=bromadiolone concentration (µg/mL); IS: warfarin (5 mg/mL).

The main objectives of plasma kinetics studies in general are to define the drug/toxic bioavailability, dose proportionality, gender differences, and species differences in pharmacokinetics and metabolism from which the target organ toxicity can be predicted and the safety doses in animals and humans can be established. These studies may also serve as a tool for the toxicologic pathologist in understanding models used for predicting and assessing drug/toxic-related toxic response. In order to evaluate the pharmacokinetic behavior of B in laying hens, plasma concentrations of the drug were detected after 10 mg/kg oral single-dose treatment. An equation, at one compartment of 1st order input and output (28), best describes curves Figure 4 ($R^2 = 0.94$). The main pharmacokinetic parameters are reported in Table IV. The constant of the absorption phase (K_{01}) was higher than the constant of the elimination phase (K_{10}), suggesting that the absorption rate is higher than the elimination rate. These results are in contrast with the similar half-life (1.78 h versus 1.77 h; Table IV), and this might be due to the value of the last point (24 h) deriving from two on six tested pools. The elimination half-life resulted of 1.77 h suggests a predictable washout period of about 13 h, despite residues very close to the LOQ were found after 24 h in few hens. The low value of clearance parameter (CL) is substantial enough to ascribe to the high lipophylicity of B. Pharmacokinetic behavior of B in hens results faster than that reported in mice (29), rats (13), and humans (30). In rats, B is detectable in plasma up to 100 h after a single oral dose of 3

mg/kg, while it is detectable up to 5 days following 0.17 mg/kg ingestion in humans. A longer persistence of B has been also reported in mice (31). Moreover, B shows AUC, half-life, and Vd are lower in hens than in rats, suggesting that in birds, B might be poorly absorbed. Further studies are needed to confirm such speculation. Half of the hens administered with 60 mg/kg died on sixth day because of hepatic hemorrhage. Despite the fact that LD_{50} of B is not well-known in poultry (138 mg/kg in *Colins virginianus*), this manuscript suggests that it is higher than in rodents (1.125 mg/kg) (13), as early reported for other anticoagulant coumarin derivatives (31). Such difference could be related to a faster metabolism of B or to the difference in the coagulation pattern between birds and mammals (31). The Factor-VII is present in the hens in low quantities and seems not to play a primary role during coagulation (33,34). Hence, this difference in B toxicity between poultry and rats is adequate to produce "selective cereal-based baits" for practical purposes. Besides, B is one of the most popular rodenticides to combat rodent pests, and poultry is the major non-target species. Hens of 1.7–2.1 kg could be intoxicated only if they consume at least 2 kg of 0.005% B bait (recommended concentration) at a time, which is unlikely.

In conclusion, when laying hens accidentally ingest anticoagulant rodenticide baits, their eggs would be of very low risk to human health. The lowest dose used in this study (10 mg/kg) corresponds to two-fold the maximum risk (maximum dose consumed) under conditions of normal use of these baits. In case someone eats four eggs coming from contaminated with this dose laying hens, he would receive about 4 mg of total B. This amount is also well below the human therapeutic dose of W (2–10 mg/kg). An anticoagulant effect consequently seems very improbable.

Acknowledgments

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5. M. Sage, M. Coeurdassier, R. Defaut, E. Lucot, B. Barbier, D. Rieffel, P. Berny, and P. Giraudoux. How environment and vole behaviour may impact rodenticide bromadiolone persistence in wheat baits after field controls of Arvicola terrestris? *Environ. Pollut.* **148:** 372–379 (2007).
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Table IV. Pharmacokinetic Parameters (mean ± SD) of Bromadiolone Following Single-Dose Administration (10 mg/kg BW)

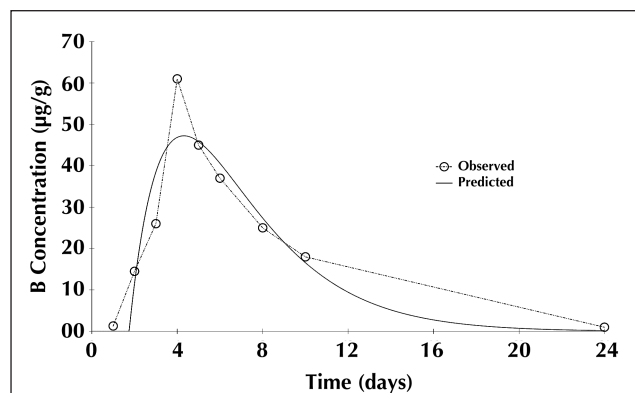| Parameters	Single dose
R^2	0.94 ± 0.02
AUC (h µg/mL)	329.7 ± 98.0
$K_{01}\ t_{1/2}$ (h)	1.78 ± 0.23
$K_{10}\ t_{1/2}$ (h)	1.77 ± 0.98
K_{10} (1/h)	0.50 ± 0.10
K_{01} (1/h)	0.21 ± 0.06
CL (mL/h/kg)	0.09 ± 0.01
Vd (L/kg)	0.23 ± 0.05
T_{max} (h)	4.31 ± 1.89
C_{max} (µg/mL)	47.2 ± 12.5

Figure 4. Mean plasma concentrations of B following a single oral dose (10 mg/kg BW) in 6 pools of laying hens (*n* = 2). Solid line represents predicted concentrations, dotted line represents observed concentrations.

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