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Accidental chlorophacinone exposure of lactating ewes: Clinical follow-up and human health dietary implications

Meg-Anne Moriceau^{1,2} , Sébastien Lefebvre¹ , Isabelle Fourel¹ , Etienne Benoit¹ , Barnett A. Rattner³ , Virginie Lattard¹

Abstract

Anticoagulant rodenticides are widely used for rodent control in agricultural and urban settings. Their intense use can sometimes result in accidental exposure and even poisoning of livestock. Can milk, eggs or meat derived from such accidentally exposed animals be consumed by humans? Data on the pharmacokinetics of chlorophacinone in milk of accidentally exposed ewes were used to estimate the risk associated with its consumption. Three days after accidental ingestion, chlorophacinone was detected in plasma of 18 ewes, with concentrations exceeding 100 ng/mL in 11 animals. Chlorophacinone was detected in milk on day 2 post-exposure and remained quantifiable for at least 7 days in milk of these 11 ewes. Concentrations in milk were much lower than in plasma and decreased quickly (mean half-life of 2 days). This study demonstrated dose-dependent mammary transfer of ingested chlorophacinone. Variation in prothrombin time (PT) on Day 3 suggested that some of the ewes that ingested chlorophacinone may have been adversely affected, but PT did not facilitate estimation of the quantity of chlorophacinone consumed. Using safety factors described in the literature, consumption of dairy products derived from these ewes after a one-week withdrawal period would pose low risk to consumers.

Keywords

Chlorophacinone residues ; Ewes ; Food safety ; Intoxication ; Mammary transfer ; Ovis aries ; Prothrombin time ; Sheep

¹ USC 1233 RS2GP, INRA, VetAgro Sup, University of Lyon, F-69280 Marcy l'Etoile, France ² CNITV, VetAgro Sup, 1 Avenue Bourgelat, 69 280, Marcy-l'Étoile, France ³ U.S. Geological Survey, Patuxent Wildlife Research Center, Beltsville, MD, 20705, USA

*Corresponding author: virginie.lattard@vetagro-sup.fr

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Introduction

Anticoagulant rodenticides (ARs) are widely used for pest control in urban and agricultural settings [1]. They disrupt the vitamin K cycle, antagonizing vitamin K epoxide reductase in liver and other tissues [2, 3], and thus interfere with the synthesis of active forms of blood clotting factors II, VII, IX and X. Their action results in coagulopathy that can lead to death of target rodents in several days[4].

As these products are widely used, they are all too frequently involved in domestic animal poisoning, especially in dogs (*Canis lupus familiaris*). For example, the National Centre for Veterinary Toxicological Information data from 2007 to 2017 (CNITV – Lyon, France) indicate that ARs are the main category of potential poisoning incident calls among all pesticides (40.4%), with dogs being 82.8% of incidents [5]. Ruminants are apparently less often exposed than other domestic species (only 2.2% of calls about rodenticide poisoning are for ruminants), and display clinical signs less frequently (only 10.5% of exposed animals displaying clinical signs according to CNITV data). This decreased incidence of clinical signs is possibly due to a lower sensitivity to ARs [6].

Clinical signs of rodenticide intoxication are similar for higher vertebrate species: hemorrhagic syndrome, with main localizations being respiratory and digestive tract, and less specific symptoms including lethargy, anorexia, or ataxia [4, 7–9]. Clinical onset is usually delayed from 2 to 10 days post-ingestion [5, 10]. No simple and definitive diagnostic procedures are available, and clinical suspicion is based on coagulation testing by measuring prothrombin time (PT) which is the clinical standard for diagnosis of AR intoxication [4, 11, 12]. Exposure can be definitively assessed by concentration of the ARs in liver and plasma [5]. Another issue with ARs lies in the dairy production field, as they undergo mammary transfer [13]. Residues can be found in the milk of an exposed and potentially intoxicated dairy animal, resulting in human exposure to ARs. The milk transfer rate of ARs in lactating animals has not been adequately documented in the literature, although it is mentioned in some older studies investigating the risks of vampire bat (*Desmodus rotundus*) mitigation campaigns in Latin America involving intraruminal injection of ARs in cattle (*Bos taurus*) [14, 15]. Many older publications report dose-dependent effects related to AR transfer through milk. For example, administration of massive doses of dicoumarol [16] or warfarin [17] to lactating rats (Carworth Farm albino rats - *Rattus norvegicus domestica*) produced hypoprothrombinemia in suckling young. Administration of diphenadione (i.e., diphacinone) to lactating cows resulted in increased PT in suckling calves [18]. Investigations have also been conducted in lactating women receiving anticoagulant drugs, allowing study of the effects of anticoagulant therapy on breastfed infants [19–21]; these children were usually asymptomatic [19, 20], with no residues of the drug in their mother's milk [21].

As there is no established Maximum Residue Limit (MRL) for these products in animal tissues, no withdrawal period has been defined. This brings into question the safety of dairy products for human consumption in suspect dairy livestock exposure incidents. Chlorophacinone (CAS number: 3691-35-8) is a first-generation AR, derived from indanedione. In Europe this product is no longer used as a plant protection agent but is still approved as a biocide (EU regulation n°528/2012) for control of pest rodents (e.g., brown rat, *Rattus norvegicus*; Black rat, *R. rattus*; house mouse, *Mus musculus*). It can

be hazardous for domestic animals (ACTA, 2018; Standing committee on Biocidal Products, 2009), and to a lesser degree a public health issue if accidentally ingested by dairy animals. Herein we report results of an investigation on the pharmacokinetics of chlorophacinone in milk of accidentally exposed dairy ewes to estimate the risk associated with its consumption by humans.

1. Material and methods

1.1 Animal population, case history

In May 2017, a flock of 23 healthy Lacaune adult lactating ewes (*Ovis aries*), of standard weight for the breed (70–80 kg), from a farm of the french department of Creuse (23), accidentally ingested an entire 20-kg bag of grain bait (ROZOL GRAIN'TECH - Liphatech SAS) containing 50 mg/kg of chlorophacinone (i.e. 1 g of chlorophacinone in total). Exposure distribution among the flock was obviously unknown. A veterinarian was contacted about 12 h post-ingestion, and all animals were asymptomatic at that time.

1.2 Samples

Samples collection was conducted under a veterinary license issued by France. For each animal, milk and blood samples were collected. Milk was obtained daily in dry tubes by the farmer from days 2 to day 7 post-ingestion. Whole blood was drawn by the attending veterinarian from the jugular vein into 10-mL tubes containing citrate on day 3 post-ingestion from all animals, and on day 11 from the subset of animals with plasma chlorophacinone concentration >100 ng/mL in the first blood sample.

1.3 Determination of AR concentrations in plasma and milk

Liquid-Liquid Extraction (LLE) was used to extract ARs from plasma and milk of the sheep. Briefly, 200 μ L of blood plasma was homogenized with 4 mL of acetone using a rotator for 10 min. The mixture was centrifuged for 5 min. The liquid fraction was separated and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residues were reconstituted and mixed in 0.2 mL of acetonitrile. For milk, 1 mL was homogenized with 4 mL of acetone using a rotator for 30 min. The mixture was centrifuged for 5 min. The liquid fraction was separated and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residues were resuspended in 4 mL of acetone using a rotator for 10 min, then centrifuged (2000g) for 5 min. The supernatant was resuspended in 200 μ L of acetonitrile, vortexed for 10 s, then placed in an ultrasonic tank for 5 min and again vortexed for 10 s. In order to freeze the lipidic portion, the sample was placed in the freezer for about 10 min. The supernatant was recovered and purified through a 0.2 μ m filter.

AR residue analysis was carried out by using the LC-MS/MS method previously described [22]. The ARs analysed with this method are three first-generation ARs: warfarin, coumatetralyl, chlorophacinone and 5 s-generation ARs

potentially used in European countries: bromadiolone, difenacoum, brodifacoum, flocoumafen and difethialone. The chromatographic separation was achieved with a semi-porous Poroshell 120 StableBond C18 column (2.1 × 100mm, 2.7 μm) and MS/MS detection was carried out by a 6410B Triple Quadrupole from Agilent Technologies (Palo Alto, CA, USA) equipped with ElectroSpray Ionization source in negative mode. Two fragment ions were recorded in dynamic Multiple Reaction Monitoring mode for each analyte. Limits of quantification (LOQ) were 0.1 ng/mL for milk and 2 ng/mL for plasma.

1.4 Prothrombin time determination

Blood samples were collected into citrated tubes, then centrifuged at 2000 g for 10 min. The PT (in seconds) was assessed in duplicate using a Biomerieux Option 2 Plus (Behnk Electronick, Norderstedt, Germany) with the Neoplastin CI, INR Determination kit (Diagnostica Stago, Asniere, France). The PT was determined in 100 μl samples of plasma with thromboplastin, according to the manufacturer’s instructions. Each value was the mean of 2 measurements.

1.5 Data analysis

Statistical analyses were also performed using GraphPad Prism 7.04 (GraphPad Software, La Jolla CA, USA) and SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Pharmacokinetic calculations were performed using the noncompartmental approach. Chlorophacinone concentrations in milk were used to estimate the elimination rate constant and half-life from daily samples collected between 48 and 168 h post-exposure, except for one animal having a concentration below the LOQ for the 168-h time point. The half-life of elimination ($t_{1/2}(el)$) was calculated using a linear regression.

For residues in milk (<50% of samples under the LOQ), the Kaplan-Meier method was used to estimate the extremes of the mean in the whole flock[23]. Lowest bound was calculated replacing values under the LOQ by 0.001 ng/mL, while highest bound was obtained by replacing those values by the value of the LOQ (i.e. 0.1 ng/mL).

2. Results

2.1 Clinical description of the flock

None of the ewes exhibited overt signs of intoxication at the time accidental chlorophacinone exposure was discovered. Vitamin K1 was not administered, and in the month following ingestion no clinical signs appeared in any of the animals.

2.2 Post-exposure measurements during week 1

On day 3 post-exposure, chlorophacinone was detected at quantifiable concentrations (LOQ = 2 ng/mL) in plasma of 18 of the 23 ewes (78.3%). Plasma concentrations (median = 47.6 ng/mL) are presented in Fig. 1A. Seven ewes had plasma chlorophacinone concentrations > LOQ to ≤50 ng/mL, and 8 were between >50 and 500 ng/mL. Three ewes had plasma

levels >500 ng/mL, with a maximum concentration of 1800 ng/mL found in 2 animals.

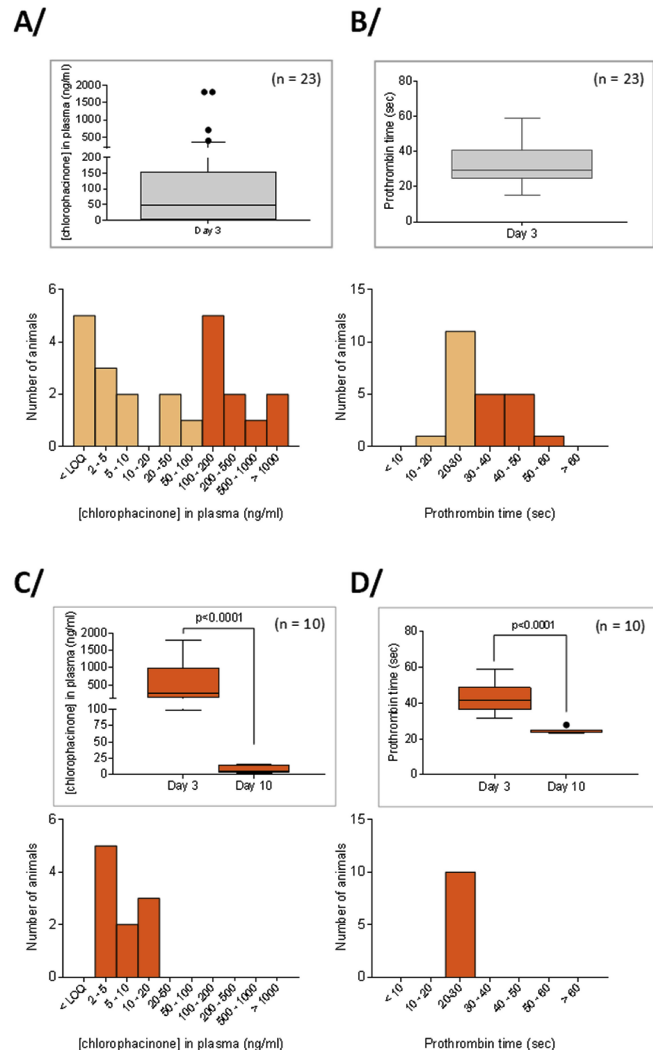


Figure 1. Distribution of chlorophacinone concentrations in plasma and prothrombin time on Days 3 and 11. A/Box plot and distribution of chlorophacinone in plasma on Day 3. B/Box plot and distribution of prothrombin time on Day 3. C/Box plot and comparison of chlorophacinone on Days 3 and 11, and distribution of chlorophacinone in plasma on Day 11. D/Box plot and comparison of prothrombin time on Days 3 and 11, and distribution of prothrombin time on Day 11.

On day 3 post-exposure, PT was determined for each animal (Fig. 1B). Median value in the flock was 29.3 s, and all values were within 15–60 s. Clotting time was compared among groups of ewes with plasma chlorophacinone concentrations assigned the LOQ or in the ranges of >LOQ to 50 ng/mL, >50–500 ng/mL, and >500 ng/mL, and differences were apparent (analysis of variance F3,19 = 23.08, $p < 0.0001$). On day 3, ewes with plasma chlorophacinone >50 ng/mL exhibited prolonged PT (Tukey’s honestly significant difference, $p = 0.025$) compared to those with plasma chlorophacinone assigned the LOQ (Table 1).

Table 1. Prothrombin time and plasma chlorophacinone concentration of accidentally exposure flock ewes on day 3.

Plasma chlorophacinone	N	Prothrombin time (mean ± SD in sec)
LOQ	5	27.45 ± 4.46 ^a
>LOQ to 50 ng/mL	7	23.68 ± 1.92 ^a
>50–500 ng/mL	8	28.02 ± 10.67 ^b
>500 ng/mL	3	50.83 ± 7.47 ^d

Means with different letter superscripts are different by Tukey’s HSD test ($p < 0.025$).

Chlorophacinone was detected at quantifiable concentrations in milk in 13 of 18 animals sampled on day 2 and remained quantifiable in 12 of these animals for 5 additional days (Table 2). For these 12 animals, the mean chlorophacinone value in milk on day 3 was 3.37 ng/mL, and all values were ≤ 8.9 ng/mL. Notably, for these 12 animals, plasma concentrations were near or >50 ng/mL (one individual at 47.59 ng/mL) on day 3. In milk of the other 11 animals, chlorophacinone was not detected or detected at concentrations ≤ 1 ng/mL on day 3. The frequency distribution of chlorophacinone concentration in milk of all ewes on day 3 is presented in Fig. 2. Seven days after accidental exposure, chlorophacinone concentration in milk of all animals was <1 ng/mL.

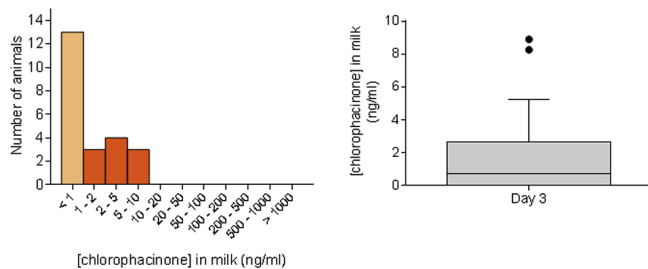


Figure 2. Distribution and box plot of chlorophacinone concentrations in milk on Day 3 ($n = 23$ animals).

2.3 Post-exposure measurements day 11

On day 11 (i.e., 8 days after the initial sample collection), chlorophacinone concentration was measured in plasma of 10 animals that exhibited concentrations ≥ 100 ng/mL on day 3 post-exposure. Values ranged from 2.01 to 16.37 ng/mL (Fig. 1 C) and were significantly lower (paired t-test, $p = 0.0256$) than those collected on day 3. For each individual, the remaining fraction of initial plasma concentration varied between 0.80 and 4.12%, with a mean value of 1.93% (SD = 0.99).

Prothrombin time values in the samples collected 11 days after chlorophacinone ingestion were distributed in a narrow range (23.2–27.85 s), with a mean value of 24.25 s (SD: 0.44) (Fig. 1D), and did not differ from PT values of the 10 of 23 animals with plasma chlorophacinone concentration below 10 ng/mL on day 3 (i.e., animals with low exposure to chlorophacinone on day 3)(unpaired t-test, $p = 0.58$).

2.4 Pharmacokinetics of chlorophacinone in milk

Chlorophacinone was quantifiable in milk of 12 ewes from days 2 through 7. Two ewes exhibited an unexpected increase in the concentration of chlorophacinone in milk on day 4 and those animals were excluded from pharmacokinetic calculations (i.e., R^2 values of 0.44 and 0.24 for excluded individuals compared to $R^2 \geq 0.85$ for other ewes; Fig. 3 and discussed in section 3.1). Using simple regression modelling of chlorophacinone elimination from milk, elimination half-life for each animal was estimated (mean $t_{1/2} = 48$ h) (Table 3). Extrapolated initial concentrations in milk varied between 1.60 and 30.12 ng/mL (Table 2). Kinetics were similar for all tested animals (Fig. 3 – right: mean $K_{el} = 0.01489$; $R^2 = 0.9676$) over a wide range of initial concentrations in plasma and milk.

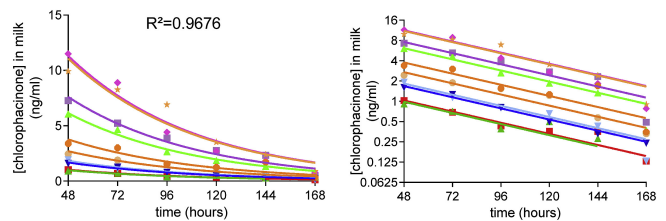


Figure 3. Changes in chlorophacinone concentrations in milk over time (mean $K_{el} = 0.01489$; $R^2 = 0.9676$, $n = 10$ animals)..

2.5 Relationship between chlorophacinone concentration in plasma and milk

A positive relation between the residues in the plasma and the milk was observed, as determined by linear regression ($R^2 = 0.9603$, $p = 0.002$; Fig. 4). Such a correlation suggests a dose-dependent mammary transfer.

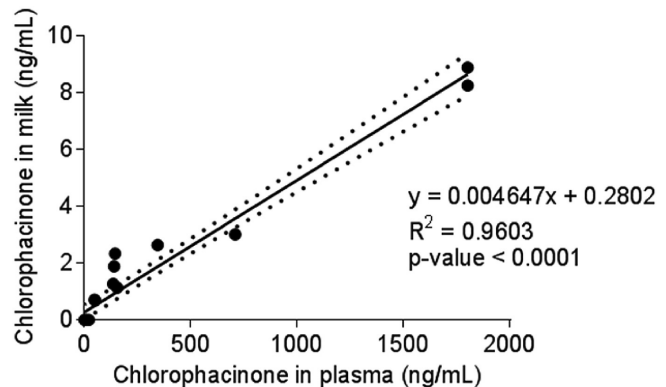


Figure 4. Relation between chlorophacinone residues in milk and plasma. Dotted lines represent the 95% confidence intervals for the linear regression model. Statistical analysis was done to test the hypothesis of a slope significantly non zero.

3. Discussion

Table 2. Concentration of chlorophacinone in milk (>LOQ) of ewes between days 2 and 7 post-accidental exposure for animals with detectable chlorophacinone and for the entire flock.

Day post-exposure	2	3	4	5	6	7
<i>Animals with detectable chlorophacinone in milk^a</i>						
Number of animals	13	12	12	12	12	12
[chlorophacinone] in milk (ng/ml)	3.84 [0.40–11.50]	3.37 [0.69–8.89]	2.57 [0.39–6.91]	1.53 [0.36–3.52]	1.08 [0.28–2.41]	0.37 [0.13–0.90]
<i>Entire flock (n = 23)^b</i>						
[chlorophacinone] in milk (ng/ml)	2.17–2.22 [LOQ–11.50]	1.76–1.80 [LOQ–8.89]	1.45–1.50 [LOQ–6.91]	0.80–0.85 [LOQ–3.52]	0.57–0.61 [LOQ–2.41]	0.19–0.24 [LOQ–0.90]

^a Values are mean and [range of values] for animals with detectable chlorophacinone in milk.

^b Values are Kaplan-Meier extremes of the mean and [range of values] for the entire flock.

Table 3. Changes in chlorophacinone concentrations in milk over time (mean $K_{el} = 0.01489$; $R^2 = 0.9676$, n = 10 animals).

	K_{el} (h ⁻¹) [95% CI]	$T_{1/2}$ (h) [95% CI]	C_0 (ng/ml) [95% CI]	R^2
Animal 1	0.01277 [0.007909–0.01762]	54.26 [39.33–87.62]	4.68 [2.89–6.48]	0.951
Animal 2	0.01461 [0.009647–0.01957]	47.33 [35.41–71.83]	2.03 [1.27–2.78]	0.9591
Animal 3	0.01179 [0.003483–0.02011]	58.77 [34.46–198.9]	1.60 [0.57–2.63]	0.8891
Animal 4	0.01729 [0.01272–0.02186]	40.08 [31.70–54.48]	4.32 [2.93–5.71]	0.978
Animal 5	0.01424 [0.009652–0.01882]	48.66 [36.82–71.79]	14.58 [9.51–19.64]	0.9653
Animal 6	0.01706 [0.01255–0.02157]	40.62 [32.13–55.22]	14.26 [9.71–18.81]	0.9789
Animal 7	0.01557 [0.01222–0.01892]	44.50 [36.63–56.71]	3.53 [2.66–4.39]	0.9848
Animal 8	0.01913 [0.01324–0.02502]	36.22 [27.69–52.34]	30.12 [18.13–42.11]	0.9737
Animal 9	0.01258 [0.004087–0.02106]	55.08 [32.90–169.56]	6.44 [2.11–10.77]	0.8527
Animal 10	0.01386 [0.007176–0.02055]	50.00 [33.72–96.5]	20.70 [10.11–31.29]	0.9284
All animals	0.01489 [0.01321–0.01657]	47.55 [42.35–52.76]	/	

Results are means with 95% confidence interval. K_{el} : calculated elimination constant; $T_{1/2}$: calculated elimination half-life; C_0 : extrapolated initial concentration in milk.

3.1 Chlorophacinone transfer in milk

As previously reported in the literature [13] our findings demonstrate mammary transfer of ingested chlorophacinone to milk. However, only a small fraction of the chlorophacinone in plasma was found in milk, with proportions varying between 0.8 and 4.1%. Three days after accidental chlorophacinone ingestion, concentrations in milk were very low (<15 ng/mL) while plasma concentrations remained high (ranging up to 1800 ng/mL). Seven days after chlorophacinone ingestion, concentrations in milk were <1 ng/mL.

It should be noted that 2 ewes exhibited an unexpected increase in chlorophacinone concentration in milk on day 4. Two hypotheses might explain such elevated values: (i) possible new exposure, or (ii) a delay in absorption of chlorophacinone from the digestive tract of those two animals. A larger-scale study would be necessary to more fully examine inter-individual variability.

This mammary transfer appears to be dose-dependent, as suggested by the positive relation between the residues in plasma and milk (Fig. 4). This same dose-dependent transfer has been observed in cows intra- ruminally treated with another AR, diphenadione (i.e., diphacinone) citebullard-DiphenadioneResiduesMilk1977. Proportions of mean quantifiable plasma to milk concentrations in cows, calculated from the data provided at each time point, varied between 0.5 and 1%, a rate similar to that estimated in the present study.

One of the limitations of the present accidental exposure event was that the quantity of chlorophacinone ingested by each animal was unknown. It would be interesting to explore the possible relation between ingested amounts of chlorophacinone and concentrations in milk. The total ingested quantity of chlorophacinone was 1 g, and based on plasma concentrations at day 3, only 10 animals had values above 100 ng/mL, suggesting a significant quantity of chlorophacinone was ingested by those animals. Assuming that all of the AR was ingested by those 10 ewes, and was distributed equally among them, the dose per animal would be 1.4 mg/kg BW (using a standard weight of 70 kg). For sheep treated intra- ruminally with 1 mg chlorophacinone/kg BW [6], mean plasma concentration after 72 h was about 1000 ng/mL. In the present accidental exposure event, if in fact 1 g was ingested by only 10 ewes, the observed plasma concentrations at 72 h post-exposure (range 100–1800 ng/mL, mean = 570 ng/mL) were within the range of values reported by Berny and coworkers (2006) [6]. Thus, the present hypothesis of a mean ingested amount of 1.4 mg/kg BW per animal corresponds to a consumption of 98 mg per animal for a standard 70-kg ewe. In the present study, mean extrapolated initial concentration (C_0) in milk is 10.53 ng/mL. Assuming that daily milk production for one ewe is about 1.5L, at day 1 each animal would have 0.016 mg ($C_0 \times$ milk production) of chlorophacinone in its milk. Assuming that all chlorophacinone was transferred into milk in 24 h, this amount represents 0.00016% of the ingested quantity. Thus, only a small fraction of ingested AR undergoes mammary transfer.

3.2 Milk residues of chlorophacinone and human health issues

There is no MRL defined for ARs in feed, and only limited data are available regarding the toxicity of chlorophacinone in humans (Standing Committee on Biocidal Products, 2009). Data from literature indicate that acute ingestion of between 100 mg and 250 mg of chlorophacinone causes prolonged clotting time in adults, but these dosages are only based on a few clinical cases [21, 24]. Using these data for prolonged clotting time, we attempted to estimate the human health risk associated with consumption of milk produced by the exposed ewes.

Notably, risk estimations have been conducted for diphenadione (i.e., diphacinone; [14, 24]) that was used both as an AR and as a therapeutic agent in man, with a dosage between 2.5 and 30 mg per individual. It was estimated that an adult human would have to drink about 115L of milk containing the maximal quantity found in milk of ewes orally administered with 2.75 mg/kg BW of diphenadione (i.e., diphacinone, 21 ng/mL), to reach the therapeutic dosage of 2.5 mg [14]. However, such data are not available for chlorophacinone, making extrapolation challenging. A safety factor of 1000 was used to extrapolate the human health risk associated with consumption of game animals containing diphacinone employing toxicity data in rats [24]. If such a safety factor is applied to the LD50 of chlorophacinone in rats (3.15 mg/kg BW; ACTA, 2018), a dose with unacceptable risk for humans would be 0.003 mg/kg BW (i.e., 0.18 mg of chlorophacinone for a standard adult of 60 kg). The maximal concentration of chlorophacinone found in milk in our study was 12 ng/mL on day 2 post-ingestion (Fig. 2). If this chlorophacinone concentration in milk is used to assess the human health risk associated with consumption, one standard adult would have to drink 14.3L of milk to reach this dosage, or eat 2.4 kg of ewe cheese [(as 6 L of milk are needed to make 1 kg of cheese; this assumes that the AR would not affect the coagulation properties of milk, which may not be the case [25] and that cheese formation would not affect AR concentration]. To provide even a more accurate estimate, one should use the concentration found in the entire milk tank (i.e., a mixture of milk from all 23 ewes) rather than the greatest value found in a single animal at the beginning of the accidental exposure period. While this approach uses a large safety factor, risk assessments based solely on LD50 are not robust.

Another study presented the use of a safety factor of 300 to calculate the human health risk from the NOAEL of chlorophacinone after repeated oral exposure in rats (i.e., 0.005 mg/kg BW/day for 90 days; Standing committee on biocidal products, 2009). Using this factor, a standard adult weighing 60 kg would have to ingest 0.0010 mg/day, which corresponds to a daily consumption of 0.09L of the most contaminated milk or 17 g of cheese. Taking the regular safety factor generally used to calculate the Acceptable Daily Intake (ADI) of chemicals in feed (i.e., 100), exceedance would require more than 0.27L of milk or 51g of cheese daily for a standard adult. Such

estimations are a “worst-case scenario” that do not take into account previously described considerations (i.e., dilution in the tank by the addition of milk from unexposed ewes and declining chlorophacinone concentrations related to its 2 day elimination half-life in exposed ewes). If applying this same worst-case scenario at day 7 post-ingestion (with maximal measured concentration of 0.9 ng/mL), a standard adult could drink up to 1.1 L of the most contaminated milk daily for 90 days without exceeding the estimated ADI.

To obtain a more conservative estimate, this approach could be applied to the mean chlorophacinone concentration in milk from the entire flock ($n = 23$) at day 2 post-ingestion (i.e., 2.2 ng/mL). In this scenario, using a safety factor of 300, a standard adult weighing 60 kg (CVMP, 2018) could consume 0.45 L of milk or 76 g of cheese daily, and three times those quantities if using a safety factor of 100.

In this more realistic scenario, it is possible to estimate the “withdrawal period” for the milk from the entire flock (exposed and unexposed individuals) that was mixed in the tank. According to WHO’s model food basket (FAO/WHO Expert Committee on Food Additives, 2006), an adult consumes 1.5L of milk daily. Taking the dose of 0.0010 mg/day as a reference for the ADI, the “maximal residue limit” would be 0.7 ng/mL. According to Table 2, using the Kaplan-Meier method, the mean chlorophacinone concentration in the tank would be under this value at day 6. It is to be noted that this estimation is liberally adapted from the reference method to establish MRLs for veterinary drugs, and provided results are only rough estimations.

Thus, even in the worst-case scenario, the human health concern for consumption of chlorophacinone contaminated dairy products seems low. Still, this study is only a case report based on a small sample size, and not a robust controlled blinded study. It should be viewed as a preliminary risk assessment, and may only be valid for chlorophacinone.

3.3 Using clinical parameters to estimate residues levels in milk?

3.3.1 Species sensitivity to chlorophacinone intoxication

During the study, and in the absence of vitamin K1 administration, none of the ewes exposed to the AR chlorophacinone displayed overt clinical signs, even those with highest plasma levels. Those observations are in line with a suspected lack of sensitivity of ruminants to AR intoxication, as described in literature. For example, cattle experimentally administered doses up to 5 mg/kg BW of diphenadione (i.e., diphacinone) did not display any clinical signs [14, 15], and Texel sheep orally administered 1 mg/kg BW of chlorophacinone did not display overt signs of intoxication, but did exhibit a slight increase of bleeding time at the venipuncture site [6].

These findings are corroborated by statistics from the French National Center for Veterinary Toxicological Information (CNITV). Between 2007 and 2017, this poison call-center received 398 calls about possible or probable intoxications with AR in ruminants (cattle, goats, sheep - i.e., 2.2% of

the calls regarding this category of biocide). Total number of exposed animals was 2043 individuals, and only 216 of them (i.e. 10.5%), displayed clinical signs, without further details on the severity of those signs. This presumed lack of sensitivity could be related to a dilution effect in the rumen, endogenous production of vitamin K1 in the rumen, or dietary vitamin K in their feed composed of leafy greens. Thus, based on these results, overt symptoms are not a good indicator of the quantity of AR ingested in ruminants.

3.3.2 Evaluation of the relationship between PT and residues in plasma and milk

An increase in PT was observed in some of the “exposed” ewes (plasma chlorophacinone concentration > LOQ: mean PT value = 34.1 s, $n = 18$ animals) when compared to “seemingly unexposed” individuals (assigned LOQ: mean PT value = 27.45 s, $n = 5$ animals). Mean clotting time on day 3 increased by about 137% in ewes with plasma chlorophacinone between >50 and 500 ng/mL, and by about 185% in animals with plasma chlorophacinone >500 ng/mL. This variation is similar to the one observed in cattle [14, 15], sheep [6] or goats receiving pindone [13]. In the study by Berny and coworkers [6], baseline PT was around 13.25 s (SD = 0.52), increasing up to 27–40.7 s after administration of the AR. No definitive reference baseline value could be found for PT in sheep, and values may differ among breeds and the analytical method used [26]. Median values found in the literature varied from 7.31 s (Leite Carlos et al., 2017[27] – $n = 20$ “mixed breed” ewes) to 40 s (Siller-Matula et al., 2008[28] – $n = 6$ Austrian Mountain ewes). In our study, a control group (known to be unexposed) would have permitted determination of a baseline value for the breed. However, the mean PT value of “unexposed” animals is a good proxy of such a reference value.

Despite the observed increase, no simple linear relation exists between PT and concentrations of chlorophacinone in plasma and milk (Fig. 5), but a “threshold-level effect” is seemingly apparent on day 3 at plasma concentrations >50 ng/mL and milk concentrations >2 ng/mL. Thus, while PT can serve as an indicator of exposure and adverse AR effects, it cannot be used to predict the residue levels in the milk of dairy animals intoxicated with chlorophacinone, highlighting the need for better indicators of exposure.

4. Conclusion

This study demonstrates minimal mammary transfer of ingested chlorophacinone in accidentally-exposed ewes, and seemingly low risk for consumers of their dairy products after a one-week withdrawal period. Based on plasma chlorophacinone levels, transfer to milk is dose-dependent and suggests that milk could serve as a non-invasive proxy for AR exposure. However, clinical markers used for routine monitoring in animals (overt signs of intoxication, changes in PT) did not permit estimation of the magnitude of the primary exposure of ewes, and therefore the potential risk related to consumption

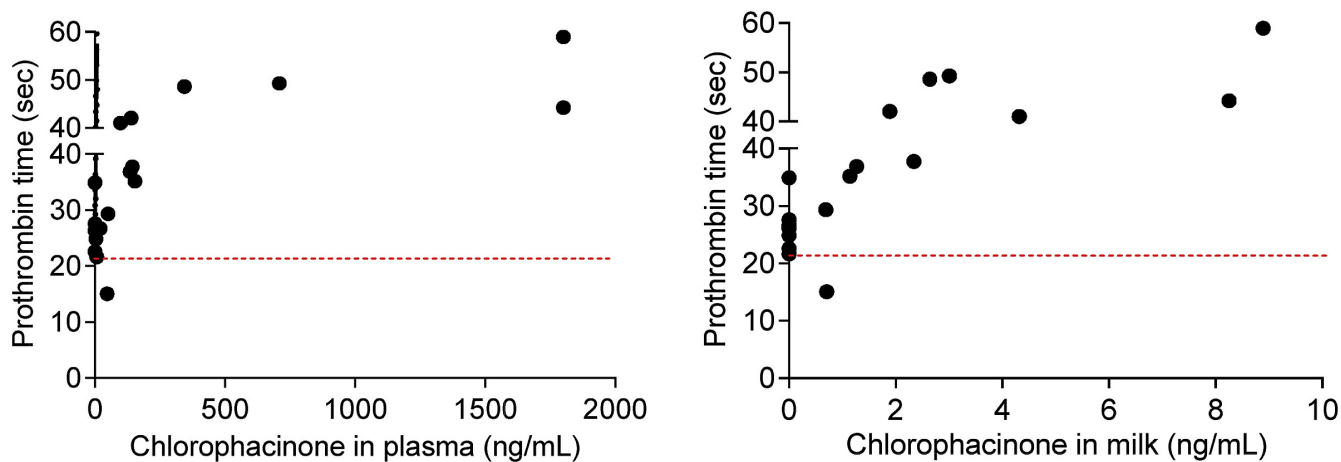


Figure 5. Prothrombin time versus chlorophacinone concentration in plasma (A) and milk (B).

of their dairy products.

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