Levamisole Residues in Chicken Tissues and Eggs

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ABSTRACT Levamisole is currently being used to treat capillaria infection in chickens even though there is no published withdrawal information available for levamisole in chickens. Tissue residue withdrawal of levamisole in chickens was studied in 32 healthy broiler breeder chickens at the age of 32 wk (peak of egg production). Levamisole residues in chicken tissues, eggs, and plasma were determined by HPLC with ultraviolet (UV) detection at 225 nm. The highest level of residue and longest withdrawal after oral administration of 40 mg/kg levamisole to chickens was in the liver. On d 3 the level of levamisole were undetectable in the plasma. On d 9, levamisole residue in eggs was 0.096 μg/g and on d 18 it was 0.06 μg/g or less in all the analyzed chicken tissues. Those levels were lower than the recommended maximum residue limit (MRL). The withdrawal time for levamisole in chickens was longer than for other species tested, which is due in part to a larger dose of levamisole being recommended for chickens. In conclusion from this research, 9 d are needed for levamisole in eggs to be less than the MRL, and 18 d of withdrawal are needed before medicated birds are slaughtered if their tissues are to be safe for human consumption.

(*Key words*: broiler breeder, levamisole, residue, withdrawal)

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INTRODUCTION

Residue is any compound present in edible tissues of the target animal that results from the use of a xenobiotic, including the xenobiotic, its metabolites, or any other substances formed in or on food due to use of the xenobiotic. Residue monitoring is essentially a matter of risk management in which risks to human and trades, confidence in products, the extent of increase in product value, and compliance with regulations must be balanced against costs. In domestic and export markets, there is a growing focus on residues, and it is likely that requirements for testing will increase (O'Flynn, 1999). According to the Food and Marketing Institute Report (1988) food safety has become one of the most visible and emotional issues confronting affluent societies. In a national survey, they found that the first concern of consumers pertained to residues in meat. Health-related issues, such as cholesterol and saturated fat content, are perceived by the public as less threatening than chemical residues. There is a risk that residues of hypersensitivity-inducing drugs may elicit hypersensitivity in human consumers of food of animal origin. The residual levels present in food are unlikely to be sufficient to cause initial sensitization. Levels that would illicit sensitization in human are most likely to occur by therapeutic use of these substances. However, these levels may occasionally elicit hypersensitivity in previously sensitized patients. The available data suggest that incidences of such reactions are exceedingly low, and the risk can be minimized by the careful use and observance of sufficiently long withdrawal periods of substances fed to livestock (Woodward, 1991).

Levamisole is the levo isomer of ditetramisole, which is a racemic mixture. The parent compound tetramisole was first marketed as an anthelmintic in 1965, but it was soon noted that its anthelmintic activity resided almost entirely in the L-isomer, levamisole. Thus it was determined that by using the L-isomer alone the dosage could be reduced by half. Reduction of the dosage has an advantage of decreasing the risk of toxicity while benefiting from the same anthelmintic potency (Barragry, 1994). Levamisole is widely used as an anthelmintic in cattle, sheep, goats, swine, and poultry. It is effective against lungworms and gastrointestinal nematodes. It is also used as adjuvant therapy in the treatment of human cancer (Gilt, 2000).

Nematodes constitute the most important group of helminthes that infest poultry in number of species and extent of damage they cause. They far exceed trematodes and cestodes (Ruff and Norton, 1997). Adult worms are commonly diagnosed by necropsy in broiler flocks from 4 to 9 wk and in breeder pullets and males from 4 to 25

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Abbreviation Key: MRL = maximum residue limit; UV = ultraviolet.

wk of age (Dawe and Hofacre, 2002). One reason to study levamisole is that *Ascaridia* species appear to be developing drug resistance to piperazine, the widely used anthelmentic for poultry. Piperazine is only effective against adult forms, allowing recurrence of worm problems as immature larvae mature. Growers occasionally repeat treatment of pullets 4 to 8 times with 2 to 4 times higher than the recommended dose of piperazine and still have little success (Malone et al., 1986). Pankavich et al. (1973) reported that most of older anthelmintics recommended to be administered in one dose and are available for control of mixed infection are effective against only a part of the helminth spectra that comprise the species *Ascaridia galli*, *Heterakis gallinarum*, and *Capillaria obsignata*. To be effective against these species, these drugs are usually administered by continuous or extensive dosing regimens.

Levamisole is currently being used to treat capillaria infection in poultry (USDA, 1998). Recommendations for doses of levamisole to be used in poultry are available (Plumb, 1999), although these are not approved for use in the USA. Levamisole is approved in Australia and is administered at 28 mg/kg (Arundel, 1985). No information is available from Food Animal Residue Avoidance Databank (FARAD) or Food and Agriculture Organization (FAO) regarding the residue potential, metabolism, or withdrawal times of levamisole used in poultry (Sundlof et al., 1992). In the USA the maximum residue limit (MRL) in edible tissues of 0.1 μg/g only applies to cattle, sheep, goats, and swine (Craigmill et al., 1994; USDA, 1998). The withdrawal time is different in various animal species (Table 1), and it is hard to predict the withdrawal time in chickens after they are medicated with levamisole.

The dose of levamisole used in this study (40 mg/kg) was selected according to previously published results (Pankavich et al., 1973; Manger, 1991; Charles and Edward, 2001; Ruff and Norton, 1997). This dose effectively killed more than 95% of adult *Ascaridia galli*, *Heterakis gallinarum*, and *Capillaria obsignata* and eliminated a high percentage of the larval stages of these parasites. Advantages of using levamisole include its tendency to be self-regulating medicine when given in drinking water due to its bitter taste and its long-term protection that extends to 2 wk (Coles, 1997).

The purpose of this research was to determine the withdrawal time of levamisole after its administration as an oral dose to chickens at the peak of egg production to allow for acceptable residue levels in their tissues and eggs.

MATERIALS AND METHODS

Birds

Thirty-two healthy chickens, at 6 wk of age, were kept on standard ration in floor pens with diet ingredients for different age stages are shown in Tables 2, 3, and 4. At 29 wk of age, they were moved, placed in individual wire cages, and provided a drug-free ration at Auburn University Poultry Science farm. At 32 wk of age (peak of egg production) they were randomly segregated into 8 groups, with 4 replicate pens per group. One group served as a control group, which was euthanized on d 0, and the other 7 groups served as test groups. The 7 test groups of chickens were administered a single dose of levamisole (40 mg/kg) orally, and a group was chosen randomly and euthanized on d 3, 6, 9, 12, 15, 18, and 21.

TABLE 1. Dose and withdrawal time of levamisole in various animals

Species	Dose (mg/kg)	Withdrawal time (d)	Reference
Cattle	8	5	Berger et al., 1984
Goats	11.8	9	Babish et al., 1990
Sheep	8	3	Plumb, 1999
Swine	6	6	Berger et al., 1987
Poultry	28	0 (eggs) 7 (tissue)	Coopers Animal Health, 2004

Samples

From every euthanized chicken an egg and a 10-g tissue sample was taken from each of the following organs: liver, breast muscle, thigh muscle, fat and fat plus skin, and 2 mL of blood was collected into a heparinized tube. The blood samples were centrifuged at 848 × *g*, and plasma was separated and kept at –20°C until analyzed. Each egg was homogenized separately and analyzed each day. Tissue samples were kept at –20°C until analyzed.

Chemicals and Standards

Levamisole hydrochloride[2] was used for oral administration, and methyllevamisole hydrochloride[3] was used as an internal standard. The solvents used for extraction of levamisole and its internal standard and for chromatographic analysis were HPLC grade.[4] The chemicals used in extraction and for adjusting the pH were analytical reagent grade.[4] The ion-pairing compound (Pic B-7) used in the mobile phase was a low UV reagent.[5]

Chemical Extraction of Levamisole and Its Internal Standard from Plasma

Extraction from plasma was done according to Garcia et al. (1990). Polypropylene centrifuge tube (15 mL) containing 1 mL of plasma was spiked with 1 μg of the internal standard. To each tube, we added 0.9 mL water, mixed by vortex, added 0.5 mL of 10 *N* sodium hydroxide, mixed by vortex, added by 5 mL of ethyl ether:n-hexane (80:20, vol/vol), and shook vigorously. The mixture was centrifuged for 5 min at 848 × *g*, and the organic

[2]Sigma Chemical Co., St. Louis, MO.
[3]Jansen Pharmaceuticals, Beers, Belgium.
[4]Fisher Scientific, Fair Lawn, NJ.
[5]Waters Corp., Milford, MA.

TABLE 2. Diet ingredients for broiler breeder growers (from 6 to 12 wk of age)

Ingredient	Percentage
Corn, yellow #2	60.38
Soybean meal	30.5
Poultry Fat (54.7 U)	5.25
Dicalcium phosphate (18.5% P, 24% Ca)	1.65
Limestone (38%)	1.1
DL-Methionine	0.2
Salt	0.35
Coccidiostat	0.05
Vitamin A	0.013
Cholcalciferol	0.00002
Vitamin E	0.0029
Vitamin B_{12}	0.000007
Riboflavin	0.002
Niacin	0.013
Calcium D-pantothenate	0.005
Choline chloride	0.21
Menadione sodium bisulfide complex	0.0022
Folic acid	0.000175
Pyridoxine hydrochloride	0.000975
Thiamine mononitrate	0.0004
D-Biotin	0.00002
Manganese	0.0065
Zinc	0.0055
Iron	0.0055
Copper	0.0006
Iodine	0.0001
Selenium	0.00003
Calcium	0.23
Crude protein	20
Crude fat	7
Crude fiber	2.6
ME, kcal	455

TABLE 3. Diet ingredients for broiler breeders from 12 wk of age to 5% of egg production

Ingredient	Percentage
Corn, yellow #2	68.49
Soybean meal	15.30
Alfalfa meal (17%)	13.55
Dicalcium phosphate (18.5% P, 24% Ca)	1.1
Limestone (38%)	0.50
DL-Methionine	0.15
Salt	0.35
Coccidiostat	0.05
Vitamin A	0.013
Cholcalciferol	0.00002
Vitamin E	0.0029
Vitamin B_{12}	0.000007
Riboflavin	0.002
Niacin	0.013
Calcium D-pantothenate	0.005
Choline chloride	0.21
Menadione sodium bisulfide complex	0.0022
Folic acid	0.000175
Pyridoxine hydrochloride	0.000975
Thiamine mononitrate	0.0004
D-Biotin	0.00002
Manganese	0.0065
Zinc	0.0055
Iron	0.0055
Copper	0.0006
Iodine	0.0001
Selenium	0.00003
Calcium	0.23
Crude protein	20
Crude fat	3.6
Crude fiber	4.8
ME, kcal	1,325

layer was separated and dried at room temperature under a stream of nitrogen. The residue was redissolved in 100 μL of the mobile phase, and 20 μL was injected in the chromatographic system.

Chemical Extraction of Levamisole and Its Internal Standard from Tissue and Egg

Extraction from tissue and egg was done according to Heitzman (1994). Ten grams of tissue or egg homogenate was transferred into a capped 50-mL polypropylene centrifuge tube and spiked with 1 μg of the internal standard. After vortex mixing for 15 s, 5 g of anhydrous sodium sulfate was spread on the surface of the tissue, and 1 ml 50% potassium hydroxide was added and vortex mixed. To this mixture, 15 mL ethyl acetate was added and homogenized[6] at maximum speed. The homogenized mixture was shaken with a horizontal shaker for 10 min and left motionless for another 10 min. The tubes were centrifuged at 848 $\times$ g for 15 min and the upper organic layer was transferred to another polypropylene tube. The tissue sample was re-extracted with another 15 ml ethyl acetate and centrifuged for 5 min

[6]Tekmar tissue-mizer, Cincinnati, OH.

TABLE 4. Diet ingredients for broiler breeder chickens (at laying age)

Ingredient	Percentage
Corn, yellow #2	67.83
Soybean meal (48%)	21.90
Poultry fat	0.35
Dicalcium phosphate (18.5% P, 24% Ca)	1.30
Limestone (38%)	7.10
DL-Methionine	0.09
Salt	0.43
Vitamin A	0.026
Cholcalciferol	0.00004
Vitamin E	0.0058
Vitamin B_{12}	0.000014
Riboflavin	0.004
Niacin	0.026
Calcium D-pantothenate	0.010
Choline chloride	0.42
Menadione sodium bisulfide complex	0.0044
Folic acid	0.000350
Pyridoxine hydrochloride	0.001950
Thiamine mononitrate	0.0008
D-Biotin	0.00004
Manganese	0.013
Zinc	0.011
Iron	0.011
Copper	0.0012
Iodine	0.0002
Selenium	0.00006
Calcium	0.46
Crude protein	16
Crude fat	3.6
Crude fiber	2.5
ME, kcal	1,325

TABLE 5. Concentration of levamisole in chicken plasma, eggs and tissues (mean ± SD) after oral administration of 40 mg/kg levamisole to chickens (n = 4)

Sample	Day 0	Day 3[1]	Day 6[1]	Day 9[1]	Day 12[1]	Day 15[1]	Day 18[1]	Day 21[1]
Plasma	ND	ND[2]	ND	ND	ND	ND	ND	ND
Egg	ND	0.55 ± 0.04	0.19 ± 0.004	0.096 ± 0.002	0.03 ± 0.003	ND	ND	ND
Breast muscle	ND	0.31 ± 0.02	0.24 ± 0.03	0.21 ± 0.006	0.10 ± 0.003	0.08 ± 0.008	0.019 ± 0.001	ND
Thigh muscle	ND	0.47 ± 0.01	0.37 ± 0.02	0.23 ± 0.01	0.11 ± 0.002	0.07 ± 0.006	0.03 ± 0.004	ND
Liver	ND	0.6 ± 0.05	0.43 ± 0.02	0.21 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.06 ± 0.01	ND
Fat	ND	0.47 ± 0.02	0.35 ± 0.006	0.23 ± 0.011	0.099 ± 0.005	0.06 ± 0.003	0.02 ± 0.005	ND
Fat plus skin	ND	0.39 ± 0.005	0.22 ± 0.01	0.15 ± 0.003	0.9 ± 0.005	0.03 ± 0.003	ND	ND

[1]Number of days from when chickens were dosed with 40 mg/kg levamisole and euthanized.
[2]Not detected.

at 848 × *g*. The ethyl acetate extracts were pooled and 5 mL 0.5 M HCl was added and vigorously shaken. After centrifugation, the organic layer was discarded and the acidic layer was transferred to a 12 mL polypropylene tube. The solution was made alkaline with 1 mL 50% KOH. The analyte and the internal standard were extracted with 100 μl chloroform and transferred to polypropylene micro-vial. After evaporation, the chloroform residue was dissolved in 125 μl methanol and vortex mixed and 125 μl water added giving a total volume of 250 μl. Volume injected in the HPLC system was 50 μl.

Chromatographic Analysis of Levamisole and Methyllevamisole

The chromatographic analysis of levamisole and its internal standard was done according to El-Kholy and Kemppainen, 2003. The column was Luna[7] 5μ C18 150 mm × 4.6 mm and the mobile phase consisted of one liter 2% acetic acid in water:methanol (50:50, v/v) and one bottle of PIC B-7 low UV reagent. The pH of the mobile phase was adjusted to 7.31 with concentrated ammonium hydroxide solution. Flow rate was 1 ml/min. The UV detection was at wavelength of 225 nm.

Statistical Analysis

Statistics were computed with Minitab software.[8]

RESULTS AND DISCUSSION

Tissue concentrations after oral administration of levamaisole (40 mg/kg) are shown in Table 5. The results demonstrated that levamisole was not detected in chicken plasma on the third day after medication. The highest residue concentration was in the liver. Levamisole residues were less than 0.1 μg/g, the MRL, in eggs on d 9, in fat and fat plus skin on d 12, in breast muscle and thigh muscle on d 15, and in the liver on d 18.

It is difficult to compare levamisole withdrawal times in chickens to studies with other species because the dose used in other species is considerably smaller (Table 1). The results from this study are different from the very short withdrawal times reported for cattle and sheep. Berger et al. (1984) studied levamisole residues in cattle orally administered levamisole gel (8 mg/kg) and found that levamisole residues were under the MRL in fat, muscle, and kidney on d 2 and in liver on d 5. Berger et al. (1987) found that levamisole residues were less than 0.1 mg/kg 6 d after oral treatment of swine with an oral dose of levamisole (6 mg/kg). The residues in chickens may be closer to that in goats. Babish et al. (1990) reported that after goats are orally dosed with 11.8 mg/kg levamisole, 9 d is the recommended withdrawal period.

This long withdrawal time for broiler breeders may be because blood lipids level are high during egg laying (Christie and Moore, 1972), and levamisole is a lipid-soluble compound (Nielsen and Rasmussen, 1983). Although there is no information in open literature about withdrawal time of levamisole in chickens in the USA, it has been reported that the withdrawal times for chicken tissue and eggs are 7 and 0 d, respectively, in Australia (Coopers Animal Health, 2004); however, these reports are not consistent with those. This may be because the dose of levamisole for chicken in Australia (28 mg/kg) (Arundel, 1985) was lower than that used in this research. Additionally, the MRL of levamisole in Australia in chicken tissues and eggs are 0.1 and 1 μg/g, respectively, (W. Korth, 2001, National Residue Survey, Canberra, Australia, personal communication).

In conclusion, withdrawal of levamisole needs to occur for 9 d for eggs to be under the MRL and for 18 d before slaughter of medicated birds for their tissues to be safe for human consumption. The liver had the highest levamisole residues and needed the longest withdrawal time.

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