Qureshi, A. A.; Abuirmeileh, N.; Burger, W. C.; Din, Z. Z.; Elson, C. E. "Effect of AMO 1618 on Cholesterol and Fatty Acid Metabolism in Chickens and Rats". *Atherosclerosis* **1983**, *46*, 203–216.

Qureshi, A. A.; Burger, W. C.; Peterson, D. M.; Elson, C. "Suppression of Cholesterogenesis by Plant Constitutents. Review of Wisconsin Contributions to NC-167". *Lipids* **1985**, *20*, 817–824.

Revis, N. W.; Bull, R.; Laurie, D.; Schiller, C. A. "The Effectiveness of Chemcial Carcinogen To Induce Atherosclerosis in the White Carneau Pigeon". *Toxicology* **1984**, *32*, 215–227.

Roe, F. J.; Pierce, W. E. "Tumor Promotion by Citrus Oils: Tumors of the Skin and Urethral Orifice in Mice". *J. Natl. Cancer Inst. (U.S.)* **1960**, *24*, 1389–1404.

Ross, R.; Glomsett, J. A. "Atherosclerosis and the Arterial Smooth Muscle Cell". *Science (Washington D.C.)* **1973**, *180*, 1332–1339.

Shapiro, D. J.; Nordstrom, J. L.; Mitschelen, J. J.; Rodwell, V. W.; Schimke, R. T. "Micro Assay for 3-Hydroxy-3-methylglutaryl-CoA Reductase in Rat Liver and in L-Cell Fibroblasts". *Biochim. Biophys. Acta* **1974**, *370*, 369–377.

Shaw, P. E. *Citrus Science and Technology*; AVI: Westport, CT, 1977.

Silberkange, M.; Havel, C. M.; Friend, D. S.; McCarthy, B. J.; Watson, J. A. "Isoprene Synthesis in Isolated Embryonic *Drosophila* Cells. I. Sterol-Deficient Eukaryotic Cells". *J. Biol. Chem.* **1983**, *258*, 8503–8511.

Sinensky, M.; Logel, J. "Defective Macromolecule Biosynthesis and Cell Cycle Progression in a Mammalian Cell Starved for Mevalonate". *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 3257–3261.

Siperstein, M. D.; Fagan, V. W. "Feedback Control of Mevalonate Synthesis by Dietary Cholesterol". *Cancer Res.* **1964**, *24*, 1108–1115.

Wattenberg, L. W. "Inhibition of Neoplasia by Minor Dietary Constituents". *Cancer Res. (Suppl.)* **1983**, *43*, 24485–24535.

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Determination of Furazolidone in Eggs by High-Performance Liquid Chromatography

Nickos A. Botsoglou

A high-performance liquid chromatographic (HPLC) method for the determination of furazolidone in eggs at levels as low as 1 ppb has been developed. Egg homogenate was acidified at pH 4 and extracted with dichloromethane. The extract, after solvent evaporation, was subjected to clarification by treatment with dry ice cooled acetone, partitioning in hexane–water, and back-partitioning in dichloromethane. HPLC determination was performed on a reversed-phase C_8 10-μm column. Peak characterization was based on simple sequential procedures, such as comparison of retention values, on-line UV–vis scanning and absorbance ratio technique, off-line thin-layer chromatography (TLC), and conversion of furazolidone to a fluorescent species. Precision data suggested an overall relative standard deviation of 8.3%. The accuracy was found to be 92.9 ± 2%, while the linearity was excellent ($\bar{R}^2$ = 0.999 81) in the range (1–104 ppb) examined.

Furazolidone [*N*-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone] is a widely used antimicrobial agent added in feed to control diseases in pig, poultry, and cattle (Leidahl, 1984). However, controversy regarding the carcinogenicity of furazolidone has arisen since 1976 (FDA, 1976). The compound has been found to be mutagenic in *Escherichia coli* WP2 (McCalla and Voutsinos, 1974), *Drosophila* (Blijleven et al., 1977), and *Salmonella typhimurium* TA100 (Tatsumi et al., 1978).

Furazolidone is extensively metabolized in animals after its absorption from the gastrointestinal tract (Tennent and Ray, 1971; Tatsumi et al., 1984); immediately after the last administration to chickens and pigs, residual concentrations in muscle, kidney, and liver tissues were found to be less than 0.5 ppb (Winterlin et al., 1984). In eggs, however, no further metabolism occurs during the long egg development time (Card, 1952). This fact has raised the possibility that considerable furazolidone levels might be present in eggs.

Two HPLC methods for the determination of furazolidone in eggs have been published recently (Petz, 1984; Beek and Aerts, 1985). However, both of them lack the sensitivity required to monitor the ultralow levels (<10 ppb) of furazolidone expected in eggs during the final stage of drug elimination.

In a study conducted in our laboratory on furazolidone concentrations in hen's eggs following feeding trials, a method capable of detecting in eggs as low as 1 ppb of the drug has been developed.

MATERIALS AND METHODS

Instrumentation. High-performance liquid chromatography was carried out on a Perkin-Elmer system consisting of a Series 3 modular chromatograph equipped with two reciprocating pumps controlled by microcomputer, a Model LC-100 column oven set at 35 °C, a Model LC-55-B single-beam variable-wavelength UV–vis spectrophotometer set at 365 nm, and a Model 023 variable-span recorder. A Perkin-Elmer digital scanner (Model LC-55-S) permitted monitoring corrected spectra of the eluted compounds on stop-flow conditions; flow could be stopped by simultaneously shutting off pump power and closing a valve located at the injector inlet. Injections were made on a Perkin-Elmer C_8 10-μm, 25 × 0.46 cm prepacked column, through a Rheodyne Model 7105 injector. The mobile phase used was a mixture of 0.01 M sodium acetate

Laboratory of Nutrition, School of Veterinary Medicine, Aristotle University, 54006 Thessaloniki, Greece.

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solution, acetonitrile, and methanol (45.5:35:19.5, v/v/v) adjusted at pH 5 with diluted (10%) acetic acid. The flow rate was set at 1 mL/min.

Thin-layer chromatography was carried out on 20 × 20 cm glass plates, coated (250 μm) with silica gel G by a Camag automatic TLC coater. They were left in position for 15 min to allow layer setting and, then, were activated by heating for 30 min at 110 °C. A Mineralight Model Chromato-view C5 UV–vis cabinet was used for visual examination of TLC plates.

Chemicals. Crystalline furazolidone was obtained from Sigma (Dorcet, U.K.) and silica gel G from Merck (Darmstadt, FRG). All other reagents used were of analytical grade. Furazolidone stock solution was prepared by dissolving 25 mg of the drug in dimethyl sulfoxide and diluting to 50 mL with distilled water. Aliquots of this solution were further diluted to give working solutions in the range 1–160 μg/50 mL. Working solutions were prepared daily and protected from direct sun and artificial light throughout the analysis.

Sample Extraction. An 8-g sample of homogenized egg was transferred to a 50-mL glass-stoppered centrifuge tube and acidified at pH 4 with 2.5 N HCl. A volume (30 mL) of dichloromethane was added, and the tube was shaken for 3 min. The suspension formed was centrifuged for 3 min at 2000g. A 25-mL aliquot of the bottom organic layer, containing the compound of interest, was filtered through anhydrous sodium sulfate on fritted glass disk funnel into a 50-mL evaporating flask. Following funnel washing with two portions of 3 mL of dichloromethane each, the combined filtrates were rotary-evaporated under reduced pressure at 30 °C. Traces of solvent in the viscous liquid that remained in the flask were removed by gentle nitrogen stream.

Cleanup Procedure. A volume (10 mL) of acetone was added under stirring in the flask, and the resulting mixture, after being cooled for 3 min in a dry ice/acetone bath, was filtered through fritted disk funnel. Following flask washing with 2 mL of acetone, the combined filtrates were submitted to rotary evaporation and to further drying with nitrogen. The remaining viscous liquid was partitioned in hexane–water (15 + 11 mL) and centrifuged. The upper layer was discarded, and the remaining aqueous layer was reextracted with 10 mL of hexane. A 10-mL quantity from the aqueous layer separated was pipetted to another centrifugal tube and extracted with 11 mL of dichloromethane. A 10-mL aliquot from the bottom organic layer that separated was filtered through anhydrous sodium sulfate on a fritted disk funnel into a 25-mL flask. Funnel washings with 5 mL of dichloromethane were collected in the same flask. The filtrates were evaporated to a volume of 2 mL and transferred to a 4-mL conical-bottom vial, with two rinses of dichloromethane, 1 mL each. The content of the vial was evaporated to dryness with nitrogen and redissolved, under vortexing, in 200 μL of distilled water or mobile-phase solution. A 50-μL volume, equivalent to 1.377 g of sample, was chromatographed under the aforementioned conditions.

Calibration curves were constructed by plotting peak height versus concentration from 50-μL injections of each of the prepared working solutions. The concentration of furazolidone in samples was calculated by reference to standard curve and multiplication by appropriate dilution factor.

Peak Characterization. On-line spectroscopic characterization of the recorded peak was performed by obtaining, in stopped-flow conditions, corrected spectra of the leading edge, top, and tail of the peak and comparing the absorbance ratios (Table I) calculated at five wavelengths close to the absorption maximum of the compound. Off-line characterization was made possible by collecting column effluents corresponding to the peak and extracting them with 2 mL of dichloromethane. The organic layer separated was transferred to a 2-mL conical-bottom vial, evaporated to small volume (20–30 μL) with nitrogen, and spotted on TLC plate. After development with chloroform–acetone (70:30, v/v), the plate was sprayed heavily with pyridine and illuminated for 5 min under long-wave length UV light. Visual examination of the plate could confirm the presence of furazolidone (R_f 0.40), since this compound should had been converted to a fluorescent species (Heotis et al., 1980).

Table I. Absorbance Ratios of Furazolidone Peak

	absorbance ratio			
point	330/350	340/350	360/350	370/350
leading edge	0.57	0.79	1.17	1.22
top	0.56	0.77	1.15	1.21
tail	0.57	0.75	1.13	1.22

RESULTS AND DISCUSSION

Sample extraction was more efficient when dichloromethane instead of ethyl acetate (Vroomen et al., 1986) was used. However, incomplete separation of the dichloromethane layer occurred, unless samples had been acidified at values lower than pH 4.5. On the other hand, acidification at values lower than pH 3.9 resulted in the appearance of an unknown peak, with varying intensity, interfering with the analysis.

Appearance of such a peak was observed even at pH 4.0, when cellulose filter papers, instead of fritted glass disk funnels, were used. Prewashing the cellulose filters with dichloromethane proved to be ineffective in removing the interfering substance, since the peak could be observed even after successive washings. Appearance of an interfering peak has also been reported in HPLC analysis of furazolidone in tissues (Winterlin et al., 1981) and eggs (Beek and Aerts, 1985).

Interferences due to the use of cellulose filter papers in spectrophotometric analyses have been attributed (Rekker, 1957) not to a simple solution process but to partial hydrolysis of cellulose molecules. This possibility could explain the appearance of the unknown peak, since carbohydrates, similarly structured to those derived from hydrolysis of polysaccharides, are present in eggs.

The partition process followed in the cleanup procedure was based on that used (Winterlin et al., 1981) for analysis of furazolidone in tissues. However, injections of egg samples, clarified solely by this process, caused an increase in column back-pressure and eventually column clogging. Since this was apparently due to precipitate formation in the mobile phase, the egg extracts were submitted to additional treatment with acetone cooled in a dry ice/acetone bath. By this treatment much soluble material was easily removed after filtration.

The effectiveness of this cleanup procedure permitted chromatographic analysis under isocratic conditions. The chromatograms recorded were free of extraneous peaks. Typical chromatograms of a spiked egg sample and an egg sample from hens fed furazolidone are shown in Figure 1.

Characterization of the recorded peaks was based on the retention behavior of furazolidone (retention time 4.7 min). Peak identity was also tested by the absorbance ratio technique, unless furazolidone concentration in egg samples was lower than 15 ppb. Absorbance measurements were made at five wavelengths to allow setting up four ratios for more positive corroboration. Close resemblance

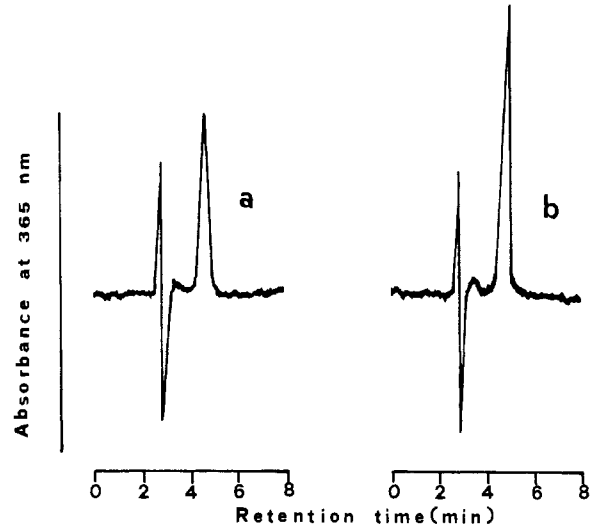

Figure 1. Typical chromatograms of an egg sample (a) spiked with furazolidone at 5.0 ppb and an egg sample (b) from a treated hen. Conditions: mobile phase, aqueous 0.01 M sodium acetate solution–acetonitrile–methanol (45.5:35:19.5, v/v/v), adjusted to pH 5 with 10% acetic acid; column, 25 × 0.46 cm, C_8 (10 μm); temperature, 35 °C; flow rate, 1 mL/min; wavelength, 365 nm; recorder sensitivity, 0.010 AUFS; chart speed, 0.5 cm/min; injection volume, 50 μL.

Table II. Recovery Data for the Determination of Furazolidone in Eggs

concn furazolidone added, ppb	mean concn found,[a] ppb	mean rec, %
1.0	1.1 ± 0.2	106.7
5.0	4.6 ± 0.2	91.7
10.0	9.1 ± 0.6	91.5
45.0	42.8 ± 1.0	95.1
104.0	96.5 ± 1.7	92.8

[a] Mean of six replicates ± SD.

of the absorbance ratios calculated for each pair of wavelengths indicated peak integrity (Table I). In cases where the recorder's sensitivity setting did not allow application of the absorbance ratio technique, because of excessive noise (samples containing less than 15 ppb), the presence of furazolidone in HPLC effluents was supplementary tested by off-line TLC.

Regression analysis of the data obtained by running a series of working solutions showed the response to be linear within the range studied (0.010 AUFS, $y = 0.33 + (4.76 \pm 0.09)x$, $\bar{R}^2 = 0.999\,54$; 0.040 AUFS, $y = 0.04 + (1.15 \pm 0.01)x$, $\bar{R}^2 = 0.999\,85$, y represents peak height and x the quantity of furazolidone injected (ng)).

The accuracy of the method was studied by adding varying amounts of working solutions to egg samples and analyzing six replicates. The concentrations examined ranged from 1.0 to 104.0 ppb. Least-squares and regression analyses of the data, presented in Table II, showed that linearity was excellent ($\bar{R}^2 = 0.999\,81$). The slope (0.929 ± 0.020) of this regression line was used as an estimate of furazolidone overall recovery (92.9 ± 2%) from spiked egg samples. Concentrations as low as 1 ppb could be readily determined, since the relative standard deviation found at this level was not greater than 20%.

The precision of the method was studied by assaying, on three different days, 18 egg samples spiked with furazolidone at the 5.0 ppb level. To estimate the overall precision, the raw data (Table III) were subjected to "analysis of variance and expected mean squares for the one way classification–random effects model" (Beyer, 1976). It was found that within-day precision was 7.0%, between-day precision 4.3%, and overall precision 8.3%.

Table III. Precision Data for the Determination of Furazolidone in Egg Samples Spiked with 5.0 ppb

day	concn furazolidone found,[a] ppb	mean value, ppb	std dev	rel std dev, %
1	5.3, 4.3, 4.4, 4.7			
	4.4, 4.9, 4.9, 5.3	4.8	0.4	8.3
2	4.4, 4.7, 4.9, 4.7			
	4.4, 4.4	4.6	0.2	4.7
3	3.9, 4.3, 4.6, 4.3	4.3	0.3	6.7

Variance Estimates

source	rel std dev, %
within day	7.0
between day	4.3
total	8.3

Table IV. Concentration of Furazolidone in Eggs of Hens Fed 330 ppm of the Drug for 14 Successive Days

	concn furazolidone in eggs of two hens,[a] ppb			
	during treatment		after withdrawal of treatment	
day				
0	<1	<1		
1	61	89	79	78.4
2	192.3	–	37.6	30
3	204	211	–	22.5
4	309	290.8	17	10.1
5	–	317	6.5	–
6	334.6	–	–	5.8
7	–	303.3	3	2.9
8	326.7	–	3.5	–
9	–	300.5	1.8	2.4
10	263	–	–	<1
11	–	347	<1	–
12	–	272	<1	<1
13	348.5	–		
14	–	293.7		

[a] Not corrected for recovery. –, no egg laid.

To test the method with real samples, a trial was undertaken to quantitate residues in eggs of two Warren SSL laying hens treated with furazolidone for 14 days by feed medication at a level of 330 ppm. Eggs were collected daily and analyzed according to the procedure described. For egg samples containing more than 100 ppb, HPLC analysis was repeated by injecting 10- or 20-μL volumes instead of 50 μL. The results, given in Table IV, showed that furazolidone levels of eggs laid reached a plateau the fourth day after the administration of medicated feed. It is still evident that the parent compound can be detected in eggs laid up to the ninth day after withdrawal of medicated feed.

Although the method presented involves a multistep extraction scheme, one analyst can easily process eight samples in an 8-h working day, the equivalent of 1 man-hour/sample. Therefore, this method could be applied in routine monitoring of furazolidone in field samples.

Registry No. Furazolidone, 67-45-8.

LITERATURE CITED

Beek, W. M. J.; Aerts, M. M. L. "Determination of Furazolidone Residues in Eggs by HPLC followed by Confirmation with a Diode Array UV/VIS detector". *Z. Lebensm. Unters. Forsch.* **1985**, *180*, 211–214.

Beyer, W. H. *Handbook of Tables for Probability and Statistics*, 2nd ed.; The Chemical Rubber Co.: Cleveland, OH, 1976.

Blijleven, W. G. H.; Kortselius, M. J. H.; Kramers, P. G. N. "Mutagenicity Testing of H-193, AF-2 and Furazolidone in *Drosophila melanogaster*". *Mutat. Res.* **1977**, *56*, 95–100.

Card, L. E. *Poultry Production*; Lea and Febiger, Eds.; Philadelphia, 1952.
FDA "Furazolidone, Nihydrazone, Furaltadone, Nitrofurazone". *Fed. Regist.* **1976**, *41*, 19906–19921.
Heotis, J. P.; Mertz, J. L.; Herret, R. J.; Diaz, J. R.; Van Hart, D. C.; Olivard, J. "Specific Programmed Multiple Development Thin Layer Chromatography of Furazolidone in Chicken, Turkey, Swine and Bovine Tissues: Collaborative Study". *J. Assoc. Off. Anal. Chem.* **1980**, *63*, 720–726.
Leidahl, R. *Feed Additive Compendium*; Miller Publishing: Minneapolis, MN, 1984.
McCalla, D. R.; Voutsinos, D. "On the Mutagenicity of Nitrofurans". *Mutat. Res.* **1974**, *26*, 3–16.
Petz, M. "Ruckstande im Ei Nach Behandlung von Legehennen mit Chloramphenicol und Furazolidone". *Arch. Lebensm. Hyg.* **1984**, *35*, 51–54.
Rekker, R. F. "The Influence of Filtration on Quantitative UV Measurements". *Spectrochim. Acta* **1957**, 542–547.
Tatsumi, K.; Ou, T.; Yamada, H.; Yoshimura, H.; Koga, H.; Horiuchi, T. "Isolation and Identification of the Metabolite of 5-Nitro-2-Furfurylidene-3-Amino-Oxazolidone (Furazolidone)". *J. Pharmacobio-Dyn.* **1978**, *1*, 256–261.
Tatsumi, K.; Nakabeppu, H.; Takahashi, Y.; Kitamura, S. "Metabolism In Vivo of Furazolidone: Evidence for the Formation of an Open-Chain Carboxylic Acid and α-Ketoglutaric Acid from the Nitrofuran in Rats". *Arch. Biochem. Biophys.* **1984**, *234*, 1, 112–116.
Tennent, D. M.; Ray, W. H. "Metabolism of Furazolidone in Swine". *Proc. Soc. Biol. Med.* **1971**, *138*, 808–810.
Vroomen, L. H. M.; Berghmans, M. C. J.; Van der Struijs, T. D. B. "Determination of Furazolidone in Swine Plasma, Muscle, Liver, Kidney, Fat and Urine based on High Performance Liquid Chromatographic Separation after Solid Phase Extraction on Extrelut 1". *J. Chromatogr.* **1986**, *362*, 141–145.
Winterlin, W.; Hall, G.; Mourer, C. "Ultra Trace Determination of Furazolidone in Turkey Tissues by Liquid Partitioning and High Performance Liquid Chromatography". *J. Assoc. Off. Anal. Chem.* **1981**, *64*, 1055–1059.
Winterlin, W.; Mourer, C.; Hall, G.; Kratzer, F.; Weaver, G. L. H.; Tribble, L. F.; Kim, S. M. "Furazolidone Residues in Chicken and Swine Tissues after Feeding Trials". *J. Environ. Sci. Health, Part B* **1984**, *B19*, 209–224.

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Metabolism of T-2 Toxin in Rats: Effects of Dose, Route, and Time

Richard L. Pfeiffer,*[1] Steven P. Swanson, and William B. Buck

Metabolic profiles of the excreta from rats following iv, oral, and dermal administration of tritium-labeled T-2 toxin at 0.15 and 0.60 mg/kg were determined. The major metabolites in urine were 3′-OH HT-2, T-2 tetraol, and unknown metabolite M5, whereas the major metabolites in feces were deepoxy T-2 tetraol, 3′-OH HT-2, and unknown metabolites M5, M7, and M9. The metabolite labeled M9 (major metabolite) was tentatively identified as deepoxy 3′-OH HT-2. There was no significant effect on metabolic profiles due to dose, but there was a variable effect associated with the route of administration. The increase over time of appreciable levels of deepoxy metabolites as a percentage of extracted radioactivity was both consistent and statistically significant.

The trichothecene mycotoxins are a chemical group of fungal metabolites characterized by a 12,13-epoxytrichothec-9-ene skeleton. T-2 toxin, one of over 40 naturally occurring trichothecenes, is a toxic metabolite produced primarily by species of *Fusarium* (Bamburg and Strong, 1971). Serious mycotoxicoses, including moldy corn toxicosis in the United States and fusariotoxicosis in Canada, have been attributed to T-2 toxin (Hsu et al., 1972; Puls and Greenway, 1976). This toxin was also possibly involved in the bean hull toxicosis of farm animals in Japan (Ueno et al., 1972). In addition, alimentary toxic aleukia, which has been a human health problem in Russia, was found to be associated primarily with the ingestion of moldy cereals infected with T-2 toxin producing strains of *Fusarium* (Joffe, 1971). The signs of trichothecene intoxication included emesis, decreased weight gain, lethargy, diarrhea, feed refusal, necrosis, lowered immunity, hemorrhage, and death (Hsu et al., 1972; Beasley et al., 1986; Kosuri et al., 1970; Osweiller et al., 1981; Boonchuvit et al., 1975; Wyatt et al., 1973; Glavits et al., 1983). Trichothecene mycotoxins, including T-2 toxin, and their effects on humans have received considerable international attention because of their alleged use in chemical warfare as the agent Yellow Rain in Southeast Asia (Watson et al., 1984).

The toxin, when administered to rodents, chickens, cattle, and swine, is rapidly metabolized into various products. T-2 toxin is rapidly metabolized and eliminated in feces to urine ratios of 3:1 in mice, 5:1 in rats, and 1:4 in guinea pigs (Matsumoto et al., 1978; Pace et al., 1985).

The major metabolites isolated from urine and feces of a lactating cow after daily oral administration of T-2 toxin were 3′-OH HT-2, 3′-OH T-2 toxin, and 3′-OH 7-OH HT-2 (Pawlosky and Mirocha, 1984; Yoshizawa et al., 1981, 1982). In a previous study using rats given an oral dose of T-2 toxin, the major metabolites isolated from feces were HT-2 and T-2 toxin at 2.7 and 7.5% of the administrated dose, respectively, whereas two unknown metabolites representing 25.8 and 9.1% of the dose were also present (Matsumoto et al., 1978). In urine from rats administered T-2 toxin, neosolaniol, HT-2, and three unknown metabolites accounting for less than 8% of the administered dose were identified (Matsumoto et al., 1978). The metabolites of T-2 toxin in the excreta of chickens, following oral administration, were T-2 toxin, HT-2, neosolaniol, T-2 tetraol, and several unknown metabolites. These were later identified as 3′-OH T-2 toxin, 3′-OH HT-2, 8-acetoxy and 15-acetoxy T-2 tetraol (Visconti and Mirocha, 1985). In guinea pigs administered T-2 toxin im, the major urinary

Department of Veterinary Biosciences, College of Veterinary Medicine, University of Illinois, 2001 South Lincoln Avenue, Urbana, Illinois 61801.
[1] Present address: Veterinary Diagnostic Laboratory, Iowa State University, Ames, IA 50010.

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