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Glucuronide Conjugates of T-2 Toxin and Metabolites in Swine Bile and Urine

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Metabolite profiles in the bile and urine of two swine were determined following the intravascular administration of tritium-labeled T-2 toxin. A total of 13.1 and 1.3% of the dose was found in the gallbladders in addition to 17.9 and 42.5% in the urine of the two swine 4 h after dosing. Free metabolites represented less than 20 and 30% of the total metabolite residues in bile and urine, respectively, with the parent compound, T-2 toxin, never exceeding 0.25%. The major free metabolites were 3'-OH HT-2 and T-2 triol. Glucuronide conjugates represented 63 and 77% of the metabolite residues in urine and bile, respectively. The major conjugated metabolites were glucuronides of HT-2, 3'-OH T-2, 3'-OH HT-2, and T-2 toxin. Neosolaniol, 4-deacetylneosolaniol, and T-2 tetraol were also identified in addition to three unknown metabolites.

INTRODUCTION

T-2 toxin, $4\beta,15$-diacetoxy-8α-[(3-methylbutyryl)oxy]-3α-hydroxy-12,13-epoxytrichothec-9-ene, is a toxic fungal metabolite produced by several species of *Fusaria* (Bamburg and Strong, 1971; Pathre and Mirocha, 1977). T-2 toxin has been found in naturally contaminated corn, barley, and mixed feeds in the U.S. and Canada at concentrations of 0.076–25 ppm (Vesonder, 1983). When present in the diets of livestock and poultry, T-2 toxin has been associated with feed refusal, infertility, diarrhea, intestinal irritation, and possibly hemorrhage, perioral and pharyngeal irritation, and lowered immunity (Hsu et al., 1972; Palyusik and Koplik-Kovacs, 1975; Speers et al., 1977; Weaver et al., 1977; Weaver et al., 1978a; Weaver et al., 1978b; Rafai and Tuboly, 1982; Hoerr et al., 1982). Trichothecene mycotoxins, including T-2 toxin, and their effects on humans have attracted considerable international attention because of their possible use in chemical warfare as the agent "Yellow Rain" (Rosen and Rosen, 1982; Mirocha et al., 1983; Watson et al., 1984).

Many procedures have been reported for the analysis of T-2 toxin in grains and mixed feeds (Scott, 1982). Analytical procedures designed to detect T-2 toxin alone in body fluids, excrement, or tissues will probably fail to confirm exposure since several studies on the fate of T-2 toxin in laboratory animals, poultry, and livestock have demonstrated that the parent compound, T-2 toxin, is rapidly cleared from body fluids and tissues (Chi et al., 1978; Matsumoto et al., 1978; Robison et al., 1979; Yoshizawa et al., 1981). Toxicokinetic studies of T-2 toxin in growing gilts and heifers (Beasley, 1984) demonstrated that the disappearance of intravascularly administered T-2 toxin follows a two-compartment open model with mean plasma elimination phase half-lives of 13.8 min for swine and 17.4 min for cattle. In spite of administration of a lethal oral dose in swine (2.4 mg/kg) and a toxic oral dose in calves (3.6 mg/kg), no parent T-2 toxin was detected in plasma or urine at a detection limit of 25 ng/mL. These results indicate that the parent compound, T-2 toxin, is very rapidly eliminated in all species examined.

Studies of the in vivo metabolism of tritium-labeled T-2 toxin in laboratory animals, livestock, and poultry have demonstrated that hydroxylation at the 3'-carbon position and hydrolysis of ester linkages are important biochemical

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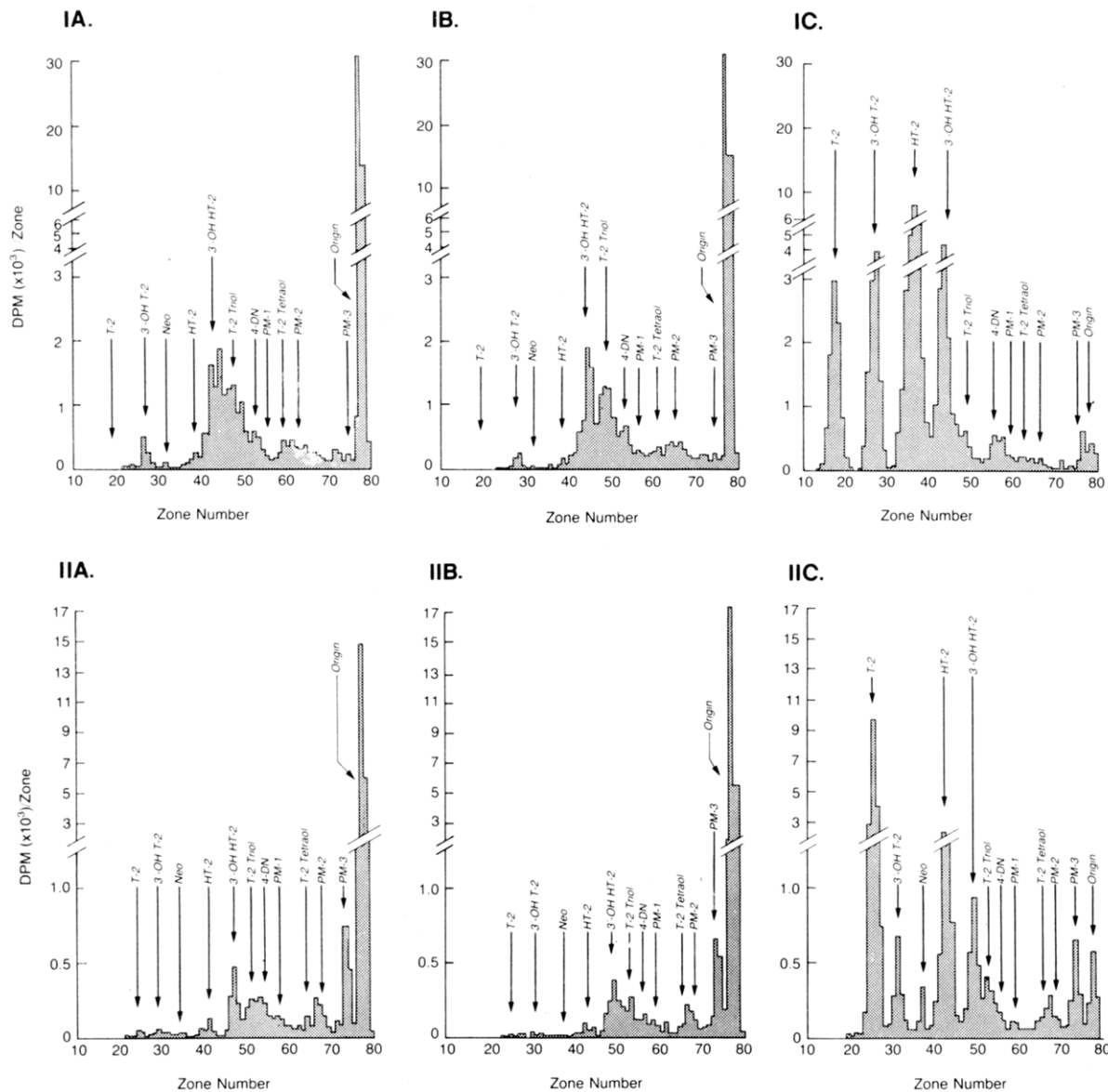
Table I. Chemical Structures of T-2 Toxin and Its Metabolites

generic name	designation	R^1	R^2	R^3	R^4
T-2 toxin		Ac	Ac	H	$COCH_2CH(CH_3)_2$
3'-OH T-2 toxin[b-e]	TC-1, TB-1, TB-2	Ac	Ac	H	$COCH_2C(OH)(CH_3)_2$
Neosolaniol (NEO)[a-c]		Ac	Ac	H	H
HT-2 toxin[a-c,e]	TC-2	H	Ac	H	$COCH_2CH(CH_3)_2$
3'-OH HT-2 toxin[b-e]	TC-3, TB-3	H	Ac	H	$COCH_2C(OH)(CH_3)_2$
T-2 triol[b,e]		H	H	H	$COCH_2CH(CH_3)_2$
4-deacetylneosolaniol (4-DN)[b,c,e]	TMR-1, TC-4, TB-6	H	Ac	H	H
3'-OH,7-OH HT-2 toxin[c,f]	TC-6	H	Ac	OH	$COCH_2C(OH)(CH_3)_2$
T-2 tetraol[b,e]		H	H	H	H

[a] Matsumoto et al., 1978. [b] Yoshizawa et al., 1980. [c] Yoshizawa et al., 1981. [d] Yoshizawa et al., 1982. [e] Visconti and Mirocha, 1985. [f] Pawlosky and Mirocha, 1984.

reactions for the metabolism of T-2 toxin (Yoshizawa et al., 1980; Yoshizawa et al., 1981; Yoshizawa et al., 1982). Although several metabolites were identified in these studies (Table I) many were not structurally characterized. In a study of the metabolic fate of tritium-labeled T-2 toxin in a lactating cow, a significant fraction of the original radioactivity in urine (24–54%) was present as very polar metabolites, possibly conjugates (Yoshizawa et al., 1981). Kosuri et al. (1971) reported that the excretion of glucuronides in rats was increased following intraperitoneal administration of T-2 toxin at 2 mg/kg and increased further when rats were pretreated with phenobarbital, suggesting that glucuronide conjugation is a possible route of metabolism for T-2 toxin. Since swine are physiologically similar to the human (Pond and Houpt, 1978) and are an important agricultural commodity, this species was used to investigate the metabolism of tritium-labeled T-2 toxin. This paper reports the extent of glucuronide conjugation of T-2 toxin and its metabolites excreted in swine bile and urine.

EXPERIMENTAL SECTION

Reference Standards. Tritium-labeled T-2 toxin (labeled in the C-3 position, radiopurity >99%, specific activity 1.287 mCi/mg) was synthesized by the method of Wallace et al. (1977). Unlabeled standards of T-2 toxin, neosolaniol, HT-2, T-2 triol, 4-deacetylneosolaniol, and T-2 tetraol were produced from cultures of *Fusarium tricinctum* in our laboratory. Additional standards of 3'-OH T-2 and 3'-OH HT-2 were kindly provided by T. Yoshizawa (Yoshizawa et al., 1982).

Animal Treatment. Two 20-kg female crossbred swine (Yorkshire × Hampshire; Thrushwood Farms, Fairbury, IL) were injected with erysipelas bacterin (Rhusigen; Pitman-Moore, Inc., Washington Crossing, NJ) and acclimated to the large-animal holding facility at the College of Veterinary Medicine, University of Illinois. All feeds offered to experimental swine were free from detectable concentrations of trichothecene mycotoxins and aflatoxins. Immediately prior to dosing, catheters were inserted into the urinary bladders of each swine. Tritium-labeled T-2 toxin was diluted with nonradioactive T-2 toxin in 1.5 mL of 50% ethanol such that each swine received 1 mCi of total radioactivity at a dosage of 0.15 mg/kg of body weight intravascularly as a single bolus injection.

During the course of the experiment, the animals were restrained in a plastic-lined sling. Urine was collected hourly. The animals were euthanized 4 h after dosing by administration of an anesthetic dose of pentobarbital followed by exsanguination. Bile was immediately collected from the gallbladders.

Determination of Total Radioactivity. The total radioactivity in urine was determined by adding 0.2 mL of urine directly to 5 mL of Aquasol-2 liquid scintillation cocktail (New England Nuclear Corp., Boston, MA). The total radioactivity in bile was determined by adding 0.02 mL of bile to 0.1 mL of 30% H_2O_2 followed by heating at 60 °C for 1 h. Aquasol-2 (5 mL) was added for scintillation counting. The counting of radioactivity was performed with a Packard Tri-Carb 300 M liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL). All data were corrected for background, dilution, quenching, and counting efficiency.

Extraction of Bile and Urine. A 0.2-mL volume of urine or 0.05 mL of bile was added in duplicate to 2 mL of 0.1 M acetate buffer (pH 3.8; Sigma Chemical Co., St. Louis, MO) and heated at 90 °C for 30 min to inactivate enzyme inhibitors. After cooling, 1 mL of either 0.1 M acetate buffer or β-glucuronidase (Sigma Chemical Co., type L-II from limpets; 4500 units/mL in 0.1 M acetate buffer) was added to duplicate samples and incubated with gentle mixing in a 38 °C water bath for 18 h. A positive enzyme control utilizing phenolphthalein glucuronide (Sigma Chemical Co.; 0.01 M, pH 7.0) and β-glucuronidase in bile or urine was included with each set of samples. The β-glucuronidase preparation used in this procedure also contained aryl sulfatase activity. Therefore, to confirm that metabolites liberated in this assay originated from conjugates of glucuronic acid, a specific inhibitor of β-glucuronidase, saccharic acid 1,4-lactone (10 mM final concentration; Sigma Chemical Co.), was added to replicate samples. Following incubation, samples were added to a 500-mg C18 cartridge (Baker-10 SPE; J. T. Baker Chemical Co., Phillipsburg, NJ; preconditioned with 2 column volumes of methanol followed by 2 column volumes of deionized water). The sample tubes were rinsed 2 × 1 mL with deionized water and added to the column. The aqueous eluates were combined and assayed for radioactivity to determine losses. Metabolites were eluted with 2 × 0.8 mL of methanol and concentrated to 0.5 mL for TLC radiochromatography. The extraction efficiencies for all metabolites (total radioactivity) were (mean ± SE) 93.5 ± 1.6% ($n = 40$).

TLC Radiochromatography. Aliquots of each sample were spotted onto the outer channels of a precoated silica gel TLC plate (5 × 20 cm, 0.25-mm gel thickness; J. T.

Figure 1. Thin-layer radiochromatograms of urine (Ia–c) and bile (IIa–c) following incubation with buffer alone (Ia,IIa), β-glucuronidase plus saccharic acid 1,4-lactone (Ib,IIb), and β-glucuronidase (Ic,IIc).

Baker; scored into three equal channels). Standard compounds were spotted in the middle channel. The plates were developed in chloroform–methanol (9:1) and allowed to air-dry. To obtain radiochromatographic profiles of each sample, 1–2-mm-wide bands were scraped from the TLC plates directly into scintillation vials. A 0.1-mL volume of water was added to each vial followed by 0.25 mL of methanol. The samples were then counted in 5 mL of Aquasol-2. After scraping sample zones, the remainder of the plates was sprayed with 30% H_2SO_4 in methanol and heated at 120 °C for 3–5 min. The standards were visualized under a long-wave (365-nm) UV lamp.

Two-dimensional high-performance thin-layer chromatography was used to aid in the identification of metabolites. After cospotting each sample with standards in the corner of a precoated silica gel HPTLC plate (10 × 10 cm, 0.2-mm gel thickness; Whatman HP-K), the plates were developed first in chloroform–methanol (9:1). After air-drying, the plates were developed the second direction in ethyl acetate–isooctane (3:1). The compounds were visualized with (p-nitrobenzyl)pyridine (Takitani et al., 1979). Individual spots corresponding to known standards were scraped and analyzed for radioactivity as above to confirm the presence of metabolites.

Table II. Total Concentration and Percent of Administered Dose of T-2 Toxin and Its Metabolites in Bile Collected from the Gallbladders of Two Swine (S1 and S2) at Time of Euthanasia (4 h)

animal	total vol, mL	concn, µg/mL	% dose
S1	13.3	24.87 ± 0.51[a]	13.1
S2	3.9	10.02 ☉ 0.13	1.3

[a] $\bar{x}$ ± SE (n = 15), based upon specific radioactivity of administered tritium-labeled T-2 toxin.

RESULTS

Bile. A total of 13.1% and 1.3% of the administered dose was found in the gallbladders of swine S1 and S2, respectively, at necropsy. Since the bile was not sampled continuously over the course of the experiment, the total fraction of the dose eliminated by the liver into the bile could not be determined. Results are summarized in Table II.

Several free metabolites, representing 9.9% (S1) and 19.4% (S2) of the total metabolite residues in bile, were identified by thin-layer radiochromatography (Table III) with 3′-OH HT-2 and T-2 triol as the major metabolites. A significant fraction of the total radioactivity in bile (82%) was present at the origin of the TLC plates (see

Table III. Concentration of T-2 Toxin and Its Metabolites in Bile Collected from the Gallbladders of Two Swine (S1 and S2) at Time of Euthanasia (4 h)

	S1				S2			
	concn, ng/mL			% dose (total)	concn, ng/mL			% dose (total)
metabolite	free	conjugated	total		free	conjugated	total	
T-2 toxin	63 ± 22[a]	11768[b]	11831 ± 271	6.2	12 ± 5	3622	3634 ± 29	0.5
3′-OH T-2	106 ± 30	1096	1202 ± 32	0.6	33 ± 2	399	432 ± 11	0.1
NEO	56 ± 7	177	232 ± 10	0.1	19 ± 6	74	93 ± 8	<0.1
HT-2	236 ± 35	4462	4698 ± 24	2.5	87 ± 8	2249	2336 ± 55	0.3
3′-OH HT-2	924 ± 98	1173	2097 ± 40	1.1	696 ± 50	1016	1712 ± 32	0.2
T-2 triol	497 ± 52	304	801 ± 23	0.4	770 ± 68	238	1008 ± 82	0.1
4-DN	289 ± 20	34	324 ± 10	0.2	119 ± 12	41	160 ± 12	<0.1
T-2 tetraol	298 ± 63	24	322 ± 4	0.2	209 ± 14	157	366 ± 11	0.1
total[c]	2469	19038	21507	11.3	1945	7796	9741	1.3

[a] $\bar{x}$ ± SE ($n = 3$), based upon the specific radioactivity of administered tritium-labeled T-2 toxin. [b] Concentration of conjugated metabolites determined by subtracting free from total concentration. [c] Total concentration of identified metabolites.

Table IV. Total Concentration and Percent of Administered Dose of T-2 Toxin and Its Metabolites in Urine from Two Swine (S1 and S2)

animal	time after dosing, min	total vol, mL	concn, μg/mL	% dose	cum % dose
S1	60	6.8	ND[a]	0	0
	120	9.4	26.89 ± 0.84[b]	10.0	10.0
	180	11.2	17.42 ± 0.76	7.7	17.7
	240	0.8	5.61 ± 0.13	0.2	17.9
S2	60	30.0	6.44 ± 0.05	6.4	6.4
	120	21.0	27.21 ± 0.41	18.9	25.3
	180	20.0	13.82 ± 0.30	9.1	34.4
	240	35.0	6.99 ± 0.12	8.1	42.5

[a] ND indicates none detected (detection limit of 0.1 ng/mL). [b] $\bar{x}$ ± SE ($n = 6$), based upon specific radioactivity of administered tritium-labeled T-2 toxin.

Table V. Free and Conjugated Metabolites of T-2 Toxin in Total Urine Collected over 4 h from Two Swine (S1 and S2) Administered Tritium-Labeled T-2 Toxin

metabolite	% dose (S1)[a]			% dose (S2)		
	free	conjugated	total	free	conjugated	total
T-2 toxin	ND[b]	2.3	2.3	<0.1	4.2	4.2
3′-OH T-2	0.1	3.0	3.1	0.3	7.1	7.4
NEO	<0.1	0.2	0.2	0.1	0.3	0.4
HT-2	0.1	3.4	3.5	0.6	8.9	9.5
3′-OH HT-2	3.3	1.5	4.8	5.5	7.5	13.0
T-2 triol	0.9	0.1	1.0	1.9	0.3	2.2
4-DN	0.3	0.1	0.4	1.0	<0.1	1.0
T-2 tetraol	0.6	<0.1	0.6	0.7	0.4	1.1
sum[c]	5.3	10.6	15.9	10.1	28.7	38.8
percent[d]	29.6	59.2	88.8	23.8	67.5	91.3

[a] The urine collected at 240 min was not included due to small sample size (0.8 mL). [b] ND indicates nondetected. [c] Total percent of administered dose represented by above metabolites. [d] Percent of total metabolite residues in urine represented by above metabolites.

Figure 1). When aliquots of bile from both swine were incubated with β-glucuronidase, the percentage of radioactivity at the origin decreased from 82% to an average of 8% of the total radioactivity. This decrease in radioactivity at the origin was matched by an increase in the concentration of several free metabolites with HT-2, 3′-OH HT-2, and the parent, T-2 toxin, as the major compounds identified. No significant differences ($p < 0.05$; t-test) were detected in metabolite profiles between samples incubated with β-glucuronidase in the presence of 10 mM saccharic acid 1,4-lactone and those incubated in buffer alone (see Figure 1). These results confirm that T-2 toxin and its metabolites are conjugated with glucuronic acid. Glucuronide conjugates of known metabolites (listed in Table I) represented 76.6% (S1) and 77.8% (S2) of the total radioactivity present in bile. Total known metabolites identified (free and glucuronide conjugates) accounted for 86.5% (S1) and 97.2% (S2) of the radioactivity in bile from both swine. An additional 2–8% of the total radioactivity was associated with metabolites whose structures are currently unknown (designated PM-1 to PM-3; see Figure 1).

Urine. A total of 17.9% and 42.5% of the administered dose was eliminated in the urine of swine S1 and S2, respectively, by 4 h after dosing (Table IV). Concentrations of total metabolite residues peaked between 60 and 120 min in the urine from both swine and decreased rapidly by 4 h.

Several free metabolites, representing 29.6% (S1) and 23.8% (S2) of the radioactivity in urine, were identified by thin-layer radiochromatography (Table V) with 3′-OH HT-2 and T-2 triol as the major metabolites. As with bile, a significant fraction of the radioactivity in urine (72%) remained at the origin of the TLC plate (see Figure 1). Following incubation with β-glucuronidase, the percentage

of radioactivity at the origin decreased from 72% to 2% while the concentration of several metabolites increased. No significant differences ($p < 0.05$; t-test) were detected in metabolite concentrations between urine incubated with β-glucuronidase in the presence of 10 mM saccharic acid 1,4-lactone and urine incubated in buffer alone. Glucuronide conjugates of T-2 and identified metabolites (see Table I) represented 59.2% (S1) and 67.5% (S2) of the radioactivity in urine. Total known metabolites identified (free and glucuronide conjugates) accounted for 88.8% (S1) and 91.3% (S2) of the radioactivity eliminated via the urine within 4 h. As with bile, several unknown metabolites (PM-1 to PM-3) were detected; however, these metabolites accounted for less than 4% of the total radioactivity present in urine.

It should be noted that storage of urine longer than 1 year at −20 °C resulted in a significant decrease in the ability of the β-glucuronidase enzyme system to liberate metabolites of T-2 toxin. This was evidenced by an increase in the radioactivity located at the origin of the TLC plates following enzyme hydrolysis from an average of 2% in fresh urine to 36% in urine stored longer than 1 year. This phenomenon did not occur in bile stored under identical conditions.

DISCUSSION

Glucuronidation plays an important role in the metabolism of T-2 toxin in intravascularly dosed swine. Glucuronide conjugates of T-2 toxin and its metabolites listed in Table I represented approximately 77% of the metabolite residues in the bile and 63% in urine of these swine.

The formation of glucuronide conjugates generally results in the elimination of pharmacological or toxicological activity of xenobiotics. If the glucuronides undergo hydrolysis by gut microflora, however, reabsorption of active compounds may occur.

The parent compound never exceeded one-fourth of 1% of the total metabolite residues in bile or urine. Analytical procedures designed to detect only the parent compound in bile or urine will be very difficult and may fail to confirm exposure. The major free (unconjugated) metabolites in bile and urine were 3′-OH HT-2 and T-2 triol. These two metabolites, however, represent at most only 24% of the metabolite residues in urine and 15% in bile.

The major conjugated metabolites in bile and urine were glucuronides of HT-2, 3′-OH T-2, 3′-OH HT-2, and T-2 toxin. The glucuronide of T-2 toxin, however, represented a much higher percentage of the metabolite residues in bile (approximately 42%) than in urine (approximately 11%). If swine intestinal microflora are found to hydrolyze glucuronide conjugates of T-2 toxin and its metabolites, then a large amount of the parent compound in addition to several metabolites will be available for reabsorption and possibly enterohepatic recirculation.

Since the identification of glucuronide conjugates in this study was accomplished by analysis of metabolites following enzymatic hydrolysis, the actual location(s) of the glucuronic acid moiety is (are) unknown. The parent compound, however, has only one hydroxyl group located at the C-3 position. Glucuronide conjugation is, therefore, likely to occur at this position for T-2 toxin. The distribution of T-2 toxin and its metabolites in the tissues of these pigs will be reported later.

ACKNOWLEDGMENT

Registry No. T-2 toxin, 21259-20-1; 3′-OH HT-2, 78368-54-4; T-2 triol, 2270-41-9; HT-2 toxin, 26934-87-2; 3′-OH T-2 toxin, 84474-35-1; NEO, 36519-25-2; 4-DN, 74833-39-9; T-2 tetraol, 34114-99-3; T2 toxin glucoronide, 98760-43-1.

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