T-2 Metabolites in the Excreta of Broiler Chickens Administered \(^{3}\)H-Labeled T-2 Toxin†

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A method for the detection of T-2 metabolites was developed and applied to analysis of metabolites in excreta of broiler chickens administered \(^{3}\)H-labeled T-2 toxin. The method used acetonitrile extraction and partitioning with petroleum ether followed by chromatography on Amberlite XAD-2, Florisil, and Sep-Pak C18. The recovery of T-2 toxin added to the chicken excreta was 73% at a concentration of 0.2 μg/g. About 80% of orally administered \(^{3}\)H-labeled T-2 toxin was rapidly metabolized to more polar derivatives and eliminated in the excreta within 48 h. T-2 toxin, HT-2 toxin, neosolaniol, and T-2 tetraol were detected at 0.06 to 1.13% of the total dose, 48 h after administration. Eight unknown derivatives, named TB-1 to TB-8, were quantitatively more significant than the metabolites above. TB-3 and TB-8 represented about 12 and 25% of the total dose, respectively. One of the metabolites (TB-6), 1.5% of the total dose, was identified as 4-deacetylneosolaniol (15-acetyl-3α, 4β, 8α-trihydroxy-12, 13-epoxytrichoc-9-ene).

T-2 toxin (I, Fig. 1), 4β,15-diacetoxy-8α-(3-methylbutyryloxy)-3α-hydroxy-12, 13-epoxytrichothec-9-ene, is a toxic metabolite produced by Fusarium spp. and possess various kinds of toxic effects in laboratory and farm animals. T-2 toxin, as well as diacetoxycepinol, deoxyximenalol, and nivalenol, are some of the most important trichothecene mycotoxins occurring naturally in agricultural products (4, 5, 10, 12, 16, 18, 20). Serious mycotoxicoses, including moldy corn toxicosis in the north central United States (6) and Fusario-toxicosis in Canada (12) have been attributed to T-2-contaminated feed. The toxin is possibly involved in the bean-hull toxicosis of farm animals in the northern part of Japan (16). In addition, alimentary toxic aleukia, which had been a human health problem in Russia, was found to be associated primarily with ingestion of moldy cereals infected with T-2-producing strains of Fusarium poae and Fusarium sporotrichioides (7, 16). Consequently, residues of T-2 toxin and its metabolites in animal products appeared to be an important human health problem. Recently, Chi et al. (2) estimated (based on radioactivity) that the muscle of broiler chickens contained 0.06 and 0.04 μg of T-2 toxin and its metabolites per g at 24 and 48 h, respectively, after dosing the birds with 0.5 mg of \(^{3}\)H-labeled T-2 toxin per kg of body weight. Chi et al. (1) also investigated the transmission of radioactivity into eggs from laying hens administered \(^{3}\)H-labeled T-2 toxin. Robison et al. (13) reported the transmission of T-2 toxin into milk of a lactating cow intubated with T-2 toxin.

Because of the high possibility of transmission of T-2 toxin and its metabolites into edible tissue of farm animals, the development of an analytical method for the detection of these mycotoxins in animal tissue and excreta is a prerequisite to not only metabolism studies, but also safety evaluation of animal foods for human health.

In this paper, we describe the development of an analytical method for the detection of T-2 toxin and its metabolites and application of this method to analysis and identification of metabolites in the excreta of broiler chickens administered \(^{3}\)H-labeled T-2 toxin.

MATERIALS AND METHODS

Animal treatment. \(^{3}\)H-labeled T-2 toxin (radio-purity, <99%; specific activity 100.6 μCi/mg) was synthesized by the method described by Wallace et al. (19) and was administered orally in a single dose of 1.6 mg/kg of body weight (3.53 × 10⁶ dpm/kg) to 47-day-old broiler chickens, which had been fed a ration containing 10 μg of nonradioactive T-2 toxin per g for 5 days before intubation of labeled T-2 toxin. Excreta were collected as described previously (2), flash frozen in liquid nitrogen, and stored at -20°C until required.

Extraction and fractionation. The sample (25 g) was extracted twice with 100 ml of acetonitrile in a blender and filtered by suction. The combined filtrate was partitioned with an equal volume of petroleum ether (bp 60 to 70°C) to defat, and the acetonitrile
layer was evaporated to dryness. The residue was dissolved in 10 ml of methanol, and 50 ml of water was added later. The mixture was concentrated to about 20 ml and chromographed on XAD and Florisil columns by a modified procedure of Kamimura's (8) method (Fig. 2). The aqueous solution was charged onto an Amberlite XAD-2 resin column (1 by 14 cm, 20-50 mesh, Mallinkrodt Inc., Paris, Ky.). The column was rinsed with 100 ml of water, followed by 100 ml of 90% methanol in water. The methanol eluate was concentrated to dryness in vacuo, dissolved in 10 ml of chloroform-methanol (3:1, vol/vol), and introduced onto a column containing Florisil (10 g, 1.5 by 12 cm, 60-100 mesh, Fisher Scientific Co., Pittsburgh, Pa.), packed in chloroform-methanol (3:1, vol/vol), and topped with a layer of anhydrous sodium sulfate (5 g). The column was eluted with an additional 90 ml of chloroform-methanol (3:1, vol/vol) and then with 100 ml of methanol. The chloroform-methanol eluate was concentrated, dissolved in 2 ml of water, and introduced onto a Sep-Pak C18 cartridge (Waters Associates Inc., Milford, Mass.). The cartridge was developed with a step gradient which involved in sequence (2 ml each): water, 20, 50, and 70% methanol in water, and methanol.

TLC analysis. Aliquots of the individual C18 eluates were spotted onto half precoated silica gel 60 chromatoplates (5 by 20 cm, 0.25 mm gel thickness), and standard compounds were spotted onto the opposite half of the plates. The C18-50% and 70% MeOH fractions were developed in chloroform-methanol (9:1, vol/vol), whereas the C18-20% MeOH fraction was developed in chloroform-methanol (5:1, vol/vol). After development, the radioactive region of the plate containing the sample was covered with a glass plate, and the standards were sprayed with 20% sulfuric acid in methanol, and heated and made visible by means of an ultraviolet lamp. The bands corresponding to the following Rf values were scraped from the thin-layer chromatography (TLC) plates (Rf values in parentheses): T-2 toxin (0.68), TB-1 (ca. 0.62), HT-2 toxin (0.45), and deacyl HT-2 toxin (0.30) from the C18-70% MeOH eluate; TB-2 (ca. 0.62), neosolaniol (0.51), TB-3 (ca. 0.38), TB-4 (ca. 0.30), and TB-5 (ca. 0.22) from the C18-50% MeOH eluate; TB-6 (ca. 0.45), T-2 tetraol (0.30), TB-7 (ca. 0.15), and TB-8 (0.00) from the C18-20% MeOH eluate. The bands named TB-1 to TB-8 did not correspond to any known T-2 metabolites (Fig. 3).

To obtain radiochromatographic profiles of the individual fractions extracted, aliquots of sample solutions were placed on high-performance silica gel TLC plates (200 μm gel thickness, Whatman Inc., Clifton, N.J.) and developed in the same solvent systems as described above. For each sample, 25 bands (3 mm width) were scraped from the TLC plates directly into scintillation vials, each containing a few drops of water. A 1-ml volume of ethanol was then added to the vials followed by 10 ml of Aquasol 2 (New England Nuclear Corp., Boston, Mass.). The counting of radioactivity was carried out in a Beckman LS-8000 liquid scintillation counter. All data were corrected for background, dilution, quenching, and counting efficiency.

GLC and GC-MS analysis. Constituents of the extracts were derivatized to trimethylsilyl (TMS) ethers or trifluoroacetic acid (TFA) esters with Tri-Sil BT or MBTFA (Pierce Chemical Co., Rockford, Ill.), respectively, before analyses by gas-liquid chromatography (GLC) and gas chromatography-mass spectroscopy (GC-MS). GLC analysis was performed on a Hewlett-Packard model 5710A gas chromatograph equipped with a hydrogen flame detector under the following conditions: 1 m by 3 mm stainless-steel column packed with 3% OV-17 on 100-120 mesh Gas Chrom Q; temperatures: injection 300°C, detection 300°C, oven programmed at 150 to 290°C at 8°C/min. Mass spectroscopy was carried out at 20 eV in an LKB-9000 GC-MS system equipped with a PDP-8E minicomputer based data system, selected ion monitoring mode.

RESULTS AND DISCUSSION

Extraction and fractionation of 3H residues from chicken excreta. Table 1 shows the recovery of 3H of the various fractions extracted from the chicken excreta. About 70% of
the $^3$H residue was recovered from the excreta at 48 h after administration. About 10% of the administered radioactivity (dpm) remained in the extracted residue. The extraction efficiency with acetonitrile was approximately 83 to 90%. Most of the radioactivity remaining in the residue was extractable with more polar solvents such as aqueous methanol and water (data not shown). An insignificant amount of $^3$H was found in the petroleum ether extract and the acetonitrile distillate, indicating that $^3$H located in the C-3 position of the trichothecene nucleus was stable in biological systems. About 86 to 90% of the radioactivity was concentrated in the XAD-90% MeOH eluate, and only a small amount of the activity was found in the XAD-H$_2$O eluate. When the XAD-90% MeOH eluate was chromatographed on Florisil columns, the majority (56 to 60%) of the extracted radioactivity was eluted with chloroform-methanol (3:1), whereas 17 to 24% was eluted with the more polar solvent, methanol. Radiochromatographic profiles of both eluates on TLC plates indicated that most of the radioactivity in the methanol eluate remained at the origin of plates and less polar metabolites such as T-2 toxin and partially deacetylated metabolites were found in the chloroform-methanol eluate.

When the chloroform-methanol eluates were separated on C$_{18}$ columns, the majority of the radioactivity was concentrated in the Sep-Pak C$_{18}$-50% MeOH (31 to 40%) and C$_{18}$-70% MeOH (5 to 12%) eluates, whereas less than 10% of the radioactivity extracted was found in the C$_{18}$-20% MeOH fraction. When $^3$H T-2 toxin was added to control excreta at 0.2 and 3.1 µg/g, it was concentrated in the C$_{18}$-70% MeOH fraction, and 77.5 and 78%, respectively, was recovered by the method outlined in Fig. 2. Overall recovery, after TLC analysis, of T-2 toxin added to control excreta was 73.6%.

Quantification of T-2 metabolites in the
were unknown. Collected in the fractions, whereas T-2 toxin acted on the radioactivity remained at the origin and this $R_f$ value corresponds to no known trichothecene. We have named these polar metabolites TB-8.

T-2 toxin administered orally to animals is eliminated primarily into the intestine from the liver through the bilial excretion system (2, 9).

The result (Table 2) indicates that T-2 toxin was rapidly metabolized to the more polar derivatives and about 80% of the radioactivity administered was eliminated in the chicken excreta within 48 h. Ellison and Kotsonis (3) reported that bovine and human liver homogenates were capable of metabolizing T-2 toxin into HT-2 toxin. Matsumoto et al. (9) detected a considerable amount of T-2 toxin, HT-2 toxin, and neosolaniol from excreta of rats orally administered with $^3$H-labeled T-2 toxin. The data in the present study, however, indicate that known derivatives such as HT-2 toxin, neosolaniol, T-2 tetraol, and deacetyl HT-2 toxin were minor metabolites, whereas unknown metabolites named TB-1 to TB-8 were more quantitatively significant metabolites in broiler chickens.

Identification of T-2 metabolites in the chicken excreta. Because attempts at isolation and characterization of metabolites in radioactive excreta were unsuccessful, the excreta were collected from birds that were fed a ration containing 10 μg of nonradioactive T-2 toxin per g for 3 days to acquire appreciable amounts of metabolites for structural confirmation. Individual $C_{18}$ eluates were analyzed by GC-MS (selected ion monitoring mode) after derivatization to TMS ethers. Concentrations of known metabolites, namely T-2 toxin (I), HT-2 toxin (II), neosolaniol (IV), and T-2 tetraol (VI) were estimated as 5.5, 1.4, 1.25, and 0.2 ppm, respectively.

To obtain samples suitable for full-scan mass spectra, the $C_{18}$-20% MeOH eluate fraction was purified on silica gel TLC plates developed in chloroform-methanol (5:1, vol/vol), and bands corresponding to T-2 tetraol and TB-6 were individually scraped and eluted. After derivatization with TMS and TFA reagents, the T-2 tetraol band revealed a peak having a similar retention time on GLC to that of the standard T-2 tetraol: 7.56 min (TMS ether) and 2.72 min (TFA ester). GC-MS of the peak (TMS ether) revealed the following diagnostic fragments ($m/e$: 586 [M$^+$], 494 [M-90], 365, 275, 193, 147, 103, and 73 [Fig. 4A]). The fragmentation pattern of
Table 1. Chromatographic separation of \(^3\)H residues recovered from excreta of broiler chickens administered \(^3\)H-labeled T-2 toxin

<table>
<thead>
<tr>
<th>Fraction</th>
<th>(^3)H-labeled T-2 standard</th>
<th>4 h (^b)</th>
<th>12 h (^b)</th>
<th>48 h (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dpm/administered</td>
<td>0.173 ± 0.004</td>
<td>5.24 ± 0.40</td>
<td>5.56 ± 0.15</td>
<td>5.73 ± 0.16</td>
</tr>
<tr>
<td>dpm/CH(_3)CN-extracted (× 10(^a)) (of administered)</td>
<td>0.174 ± 0.006</td>
<td>0.89 ± 0.53</td>
<td>1.37 ± 0.35</td>
<td>4.01 ± 0.95</td>
</tr>
<tr>
<td>dpm/CH(_3)CN-residue (× 10(^a)) (of administered)</td>
<td>(100.67 ± 3.70)</td>
<td>(17.26 ± 10.90)</td>
<td>(24.93 ± 8.84)</td>
<td>(70.02 ± 17.19)</td>
</tr>
<tr>
<td>% of administered</td>
<td>(2.05 ± 1.41)</td>
<td>(5.23 ± 2.73)</td>
<td>(10.17 ± 4.45)</td>
<td></td>
</tr>
<tr>
<td>dpm/excreted, % of administered</td>
<td>19.31 ± 11.98</td>
<td>29.95 ± 9.03</td>
<td>80.18 ± 14.20</td>
<td></td>
</tr>
<tr>
<td>Extraction efficiency (%)</td>
<td>89.97 ± 4.17</td>
<td>83.07 ± 5.35</td>
<td>86.61 ± 6.66</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Each value is the average of three replicate samples ± standard error.
\(^b\) Time after administration of \(^3\)H-labeled T-2 toxin. Each value is the average of six replicate samples ± standard error.

Table 2. Thin-layer chromatographic quantification of metabolites in the excreta of broiler chickens administered \(^3\)H-labeled T-2 toxin

<table>
<thead>
<tr>
<th>Metabolites(^a)</th>
<th>Conc (% of administered dpm) at:</th>
<th>4 h (^b)</th>
<th>12 h (^b)</th>
<th>48 h (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-2 toxin(^c)</td>
<td>0.02 ± 0.01</td>
<td>0.69 ± 1.08</td>
<td>0.06 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Neosolanil(^c)</td>
<td>0.07 ± 0.06</td>
<td>0.16 ± 0.09</td>
<td>0.36 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>HT-2 toxin(^c)</td>
<td>0.76 ± 0.46</td>
<td>0.51 ± 0.35</td>
<td>1.13 ± 0.35</td>
<td></td>
</tr>
<tr>
<td>Deacetyl HT-2</td>
<td>0.25 ± 0.18</td>
<td>0.44 ± 0.46</td>
<td>0.52 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>T-2 tetraol(^c)</td>
<td>0.13 ± 0.09</td>
<td>0.26 ± 0.07</td>
<td>0.91 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>TB-1</td>
<td>0.22 ± 0.15</td>
<td>0.33 ± 0.13</td>
<td>0.39 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>TB-2</td>
<td>0.08 ± 0.08</td>
<td>0.13 ± 0.08</td>
<td>0.24 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>TB-3</td>
<td>2.49 ± 1.97</td>
<td>3.36 ± 1.96</td>
<td>12.25 ± 3.91</td>
<td></td>
</tr>
<tr>
<td>TB-4</td>
<td>1.26 ± 0.82</td>
<td>1.24 ± 0.62</td>
<td>5.31 ± 2.44</td>
<td></td>
</tr>
<tr>
<td>TB-5</td>
<td>1.01 ± 0.76</td>
<td>1.06 ± 0.62</td>
<td>4.94 ± 0.95</td>
<td></td>
</tr>
<tr>
<td>TB-6(^d)</td>
<td>0.03 ± 0.04</td>
<td>0.14 ± 0.12</td>
<td>1.53 ± 0.32</td>
<td></td>
</tr>
<tr>
<td>TB-7</td>
<td>0.22 ± 0.20</td>
<td>0.49 ± 0.24</td>
<td>2.09 ± 0.46</td>
<td></td>
</tr>
<tr>
<td>TB-8</td>
<td>0.24 ± 0.26</td>
<td>0.31 ± 0.12</td>
<td>1.72 ± 0.35</td>
<td></td>
</tr>
<tr>
<td>(3.62 ± 1.95)(^d)</td>
<td>(4.66 ± 1.86)(^d)</td>
<td>(25.47 ± 2.93)(^d)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) \(R_s\) values of individual metabolites on TLC plates are shown in the text.
\(^b\) Time after administration of \(^3\)H-labeled T-2 toxin. Each value is the average of six replicate samples ± standard error.
\(^c\) These metabolites were confirmed by GC-MS (SIM-monitoring) analysis after derivatization with TMS and TFA reagents.
\(^d\) Percent of administered dpm calculated by adding TB-8 to the Florisil-methanol eluate.

The mass spectrum was identical to those of the standard T-2 tetraol.

The band corresponding to TB-6 gave a prominent peak at a retention time of 9.33 min (TMS ether) and at 4.13 min (TFA ester) on GLC. As shown in Fig. 4B, analysis by GC-MS of the TMS ether gave a molecular ion at m/e 556, indicating that TB-6 is a monoacetate of T-2 tetraol, and an ion at m/e 466 (M+90), indicating the elimination of a trimethylsilyloxy fragment.
from the C-8 position of the trichothecene nucleus. Ions which appeared at m/e 335, 275, 247, 193, 185, 157, and 117 were diagnostic fragments derived from a trichothecene nucleus. From this information, TB-6 should have an acetoxyl group at C-3, C-4, or C-15 position. Both C-3 and C-4 monoacetate (chemically synthesized), however, gave different retention times on GLC. Therefore, the C-15 monoacetate (V, Fig. 1) [15-acetoxy-3α,4β,8α-trihydroxy-12,13-epoxytrichothece-9-ene, 4-deacetyl-neosolaniol] was tentatively proposed for the chemical structure of TB-6. This compound was assumed to be an intermediate metabolite in the transformation of HT-2 toxin into T-2 tetraol. Further study on the structural elucidation of T-2 metabolites in chicken excreta and edible tissue will be described in a subsequent paper.

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LITERATURE CITED