Gas-Liquid Chromatographic Determination of T-2 Toxin in Plasma

STEVEN P. SWANSON, VENKATACHALAM RAMASWAMY, VAL R. BEASLEY, WILLIAM B. BUCK, and HAROLD H. BURMEISTER
University of Illinois, Department of Veterinary Biosciences, Urbana, IL 61801

A gas-liquid chromatographic method for the determination of T-2 toxin in plasma is described. The toxin is extracted with benzene, washed with aqueous sodium hydroxide, and chromatographed on a small Florisil column; the heptafluorobutyryl derivative is prepared by reaction with heptafluorobutyrylimidazole. The T-2 HFB derivative is chromatographed on OV-1 at 230°C and measured with an electron capture detector. Iso-T-2, an isomer of T-2 toxin, is added to samples as an internal standard before extraction. Recoveries averaged 98.0 ± 5.5% at levels ranging from 50 to 1000 ng/mL. The limit of detection is 25 ng/mL.

T-2 toxin, a 12,13-epoxytrichothecene mycotoxin, is a secondary metabolite produced by various species of Fusarium (1, 2). This compound has been implicated in sporadic but serious and often lethal cases of toxicoses in farm animals, including fusariotoxicoses in Canada (3) and moldy corn toxicoses in the midwestern United States (4).

Studies on the distribution and excretion of radiolabeled T-2 toxin have been conducted in swine (5), poultry (6, 7), and a lactating cow (8); however, at the present time, no pharmacokinetic experiments have been reported. Such experiments, however, require methods which are not only sensitive but also use only a minimal sample volume.

Methods currently available for analysis of T-2 toxin include procedures which use thin layer chromatography (9-11), flame ionization gas chromatography (9, 10), electron capture gas chromatography (10, 12), radioimmunoassay (13, 14), and gas chromatography–mass spectrometry (15, 16). The majority of methods, however, are not suitable for pharmacokinetic studies in farm animals because they are not sensitive enough, they are not adaptable to biological fluids, or they require sophisticated instruments not available in many laboratories. Although radioimmunoassay methods appear promising, the required antibodies and tritium-labeled T-2 toxin are not commercially available.

In the interest of developing a procedure for plasma analysis, we elected to use gas-liquid chromatography (GLC) with electron capture detection (ECD). The available GLC-ECD methods were originally developed for analysis of feeds and, although sensitive for these substrates, were not suitable for analysis of plasma without major modifications in extraction and cleanup.

The method presented here incorporates a modified heptafluorobutyryl derivatization technique and GLC-ECD steps reported by Romer et al. (12). The use of iso-T-2 as a true internal standard was also incorporated into the procedure, providing greater precision and reproducibility. Efficient and simple cleanup steps were then developed to complement the GLC-ECD final determination steps.

METHOD

Apparatus

(a) Gas chromatograph.—Hewlett-Packard Model 5840A equipped with 1.8 m × 2 mm id glass column packed with 3% OV-1 on 100-120 mesh Supelcoport. 63Ni electron capture detector, and electronic integrator.

(b) Tube shaker.—Thermolyne Speci-Mix.

(c) Chromatographic column.—Polypropylene column with 30 mL reservoir (Supelco 5-8101, Bellefonte, PA 16823).

(d) Test tubes.—13 × 100 and 16 × 125 mm glass screw-cap tubes with Teflon-lined caps.

Reagents

(a) Florisil.—60–100 mesh (Fisher Scientific). Activate by heating 3 h at 120°C.

(b) Heptafluorobutyrylimidazole (HFBi).—Pierce Chemical Co., Rockford, IL 61105.

(c) Sodium bicarbonate.—5% w/v solution.

(d) Potassium dihydrogen phosphate buffer.—10% w/v solution.

(e) Sodium hydroxide.—0.05N NaOH with 4.0% KCl added.

(f) Solvents.—Benzene, hexane, chloroform, methanol, dichloromethane. All distilled in glass.

(g) T-2 toxin.—Mycolab Co., PO Box 321, Chesterfield, MO 63107 (Figure 1).

(h) Iso-T-2.—Synthesize from HT-2 as described (17). T-2 is hydrolyzed to HT-2 with

[1 Northern Regional Research Center, Agricultural Research Service, Peoria, IL 61604.
Received June 18, 1982. Accepted November 24, 1982.]
ammonium hydroxide in methanol. HT-2 is partially acetylated with acetic anhydride and pyridine to yield iso-T-2. Prepare working standard containing 25 μg/mL ethanol.

Sample Preparation

Centrifuge all blood samples immediately after collection and store plasma at -20°C until day of analysis. Thaw and mix samples thoroughly before extraction.

Extraction and Cleanup

Add 2.5 mL plasma to 13 × 100 mm test tube containing 0.5 mL phosphate buffer and 50 μL iso-T-2 internal standard. (If amount of T-2 detected is beyond linear range, re-extract smaller aliquots of plasma and dilute with water to give final volume of 2.5 mL.) Transfer 4.0 mL benzene to tube, cap, and mix 10 min on tube shaker. Centrifuge 5 min at 2000 rpm. Pipet benzene extract into clean 16 × 125 mm test tube. Repeat extraction of plasma with additional 4 mL benzene and combine extracts. Add 2 mL NaOH to benzene extracts and mix 1 min on tube shaker. Transfer benzene layer to clean test tube. Repeat partition base with additional 2 mL benzene, combine benzene extracts, and add 4 mL hexane.

Florisil Chromatography

Prepare Florisil column as follows: Tamp small ball of glass wool at bottom of column and add, in order, 1 cm anhydrous sodium sulfate, 1 g Florisil, and 1 cm anhydrous sodium sulfate. Columns can be prepared in advance and stored at least 1 week in desiccator.

Prewash column immediately before use with 10 mL chloroform-methanol (95 + 5) followed by 10 mL hexane. Do not let column run dry at any time. When hexane is 1 cm above sodium sulfate, add sample extract. Wash column with 10 mL dichloromethane of which 2 mL is used to rinse the test tube. Elute T-2 with 15 mL chloroform-methanol (95 + 5) into 16 × 125 mm tube. Concentrate to dryness under stream of nitrogen and gentle heat ca <50°C.

Derivatization

Redissolve residue in 1 mL toluene. Add 50 μL HBFBL cap, and mix 30 s. Add 1 mL bicarbonate and mix until toluene layer is clear. Add 4.0 mL hexane, mix, and centrifuge 5 min at 2000 rpm.

Gas Chromatography

Inject 4 μL organic layer into gas chromatograph, using following conditions: column 230°C; injector 250°C; detector 325°C; argon-methane (95 + 5) carrier gas flow 22 mL/min. Inject standard and blank samples daily. With procedure described above, 2.0 μL equivalents of plasma are injected into gas chromatograph. Retention times are 8.60 and 9.49 min for T-2 and iso-T-2 toxin, respectively.

Results and Discussion

Initial studies used ethyl ether and ethyl acetate as extraction solvents for partitioning T-2 toxin from plasma. Although extraction efficiencies of 85-90% were observed, a large amount of impurities was also co-extracted. The use of benzene as an extraction solvent provided efficiencies similar to that obtained with ethyl ether; however, the resulting extract contained fewer interferences.

Base partitioning has previously been used as a cleanup step before determination of T-2 toxin and diacetoxyscirpenol in animal feeds (12). The base wash was simple and rapid, and efficiently removed acidic components present in the plasma extracts. Because impurities were detected in the base solution, the sodium hydroxide was partitioned once with equal volumes of benzene before use. No loss of either T-2 toxin or the internal standard iso-T-2 was observed at this step.

The Florisil column was reduced in size to minimize the solvents required, the time involved, and the total cost of analyses. One g Florisil was sufficient to provide the necessary cleanup of sample extracts. The polypropylene columns were inexpensive enough to be disposable, yet adequately durable so they could be washed and reused if desired. Polarity and volume of the elution solvent were adjusted to provide close to 100% recovery from the column.

Figure 1. Chemical structures of T-2 toxin (top) and iso-T-2 toxin (bottom).
Table 1. Recovery of T-2 toxin added to plasma

<table>
<thead>
<tr>
<th>Amt added, ng/mL</th>
<th>N</th>
<th>Recovery, % (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>5</td>
<td>100.5 ± 9.1</td>
</tr>
<tr>
<td>100</td>
<td>6</td>
<td>100.1 ± 5.3</td>
</tr>
<tr>
<td>250</td>
<td>7</td>
<td>97.0 ± 2.9</td>
</tr>
<tr>
<td>500</td>
<td>4</td>
<td>96.3 ± 9.8</td>
</tr>
<tr>
<td>1000</td>
<td>7</td>
<td>96.4 ± 3.5</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>98.0 ± 5.5</td>
</tr>
</tbody>
</table>

in a fraction (15 mL) collected directly into a test tube suitable for derivatization. This decreased the time of analysis by eliminating one transfer step. The Florisil columns could be prepared in advance and stored in a desiccator for at least one week without affecting elution profiles.

Preliminary work used benzene as the derivatizing solvent; however, toluene worked equally well and was substituted in later analyses. Derivatization was quantitative with 50 µL HFB.

The electron capture detector is very sensitive to the heptafluorobutyrate of T-2 toxin. Romer et al. (12) described a minimum sensitivity of 150 pg T-2 toxin, using an electron capture detector. By using a higher column temperature and diluting the sample extract less than previously reported, 25 pg standard T-2 could be detected. In actual plasma samples, however, 50 pg was required for identification.

Detector response to T-2 heptafluorobutyrate was linear up to 2.0 ng injected, using peak areas determined by electronic integration. Detector response varies slightly from day to day, so standards must be injected daily. Using the method as described, up to 2.0 µL equivalents of plasma are injected into the gas chromatograph, resulting in a detection limit of 25 ng/mL.

Initial recovery experiments were performed by adding T-2 toxin to plasma and quantitating by using an external standard. Using this method, absolute recoveries of T-2 toxin averaged 83.6% over a range of 100–1000 ng/mL, and recoveries of the internal standard iso-T-2 averaged 85.2%. By adding iso-T-2 directly to the plasma before extraction and using the internal standard method of quantitation, recoveries of T-2 toxin (normalized to the recovery of the internal standard iso-T-2) averaged 98.0% (Table 1). The coefficient of variation was also good (5.5%) with the use of this internal standard for quantitation. Because iso-T-2 and T-2 toxin are isomers, their chemical properties such as extraction efficiency and Florisil column elution profiles are nearly identical. The 2 compounds, however, still separated sufficiently under the described gas chromatographic conditions for accurate quantitation. Because of similar chemical behavior between these trichothecenes, small variations in recovery from sample to sample did not reduce the precision of the assay, as indicated by the low coefficient of variation.

The gas chromatographic portion of the anal-
ysis could be completed within 12 min per sample. Higher column temperatures could be used to reduce the analysis time; however, at higher temperatures T-2 toxin did not separate as well from the iso-T-2 internal standard. A column temperature of 230°C provided the best compromise between resolution of T-2 and iso-T-2, and the total time required for GC analysis.

Extracts of plasma were colorless and control samples produced chromatograms without interferences at the retention time of either T-2 or iso-T-2 (Figure 2). In addition, no late-eluting peaks were observed which could interfere with subsequent injections.

Two variables were critical to obtain chromatograms free from interfering peaks. It was necessary that plasma samples be clean and obtained from unhemolyzed blood. Compounds present in hemolyzed blood produced peaks which eluted just prior to T-2 toxin. If the blood was sufficiently hemolyzed, the interfering peak was large enough to interfere with quantitation.

The derivatizing agent was also very important. Heptafluorobutyrylimidazole which was old or which contained a pinkish color did not actually interfere with derivatization, although it did provide extraneous peaks on the chromatograms which sometimes affected quantitation.

The method described was designed for analysis of blood samples for investigations on the toxicokinetics and of T-2 toxin in swine and cattle. Such studies require a sensitive analytical method which can be used with relatively small sample sizes. The method developed fulfilled the above requirements. Studies on the toxicokinetics and toxicodynamics of T-2 toxin in swine and cattle are currently in progress and will be described in a subsequent paper.