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RAPID SCREENING PROCEDURE FOR THE DETECTION OF TRICHOTHECENES IN PLASMA AND URINE

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SUMMARY

A rapid and easy procedure to screen for trichothecenes in plasma and urine is presented. The toxins are extracted using a Clin-Elut column, hydrolyzed to their corresponding parent alcohols and cleaned up with a silica cartridge followed by derivatization for gas chromatographic analysis. The detection of any of the parent alcohols in plasma or urine would indicate an exposure to trichothecenes. Recoveries in urine are between 78 and 119% at levels of 50—1000 ng/ml and recoveries in plasma are between 80 and 116% at levels of 50—500 ng/ml. The limit of detection is better than 25 ppb.

INTRODUCTION

Trichothecenes are a series of mycotoxins produced by several species of the fungi *Fusarium* which commonly grow on agricultural products such as corn and wheat [1- 5]. Consumption of *Fusarium*-infected feedstuffs containing these mycotoxins has been associated with a number of adverse health effects. Specific signs of toxicoses include emesis, diarrhea, lethargy, reduced weight gain and in some cases death [6—10].

The primary trichothecenes of concern in this paper are T-2 and diacetoxyscirpenol (DAS) along with their respective metabolites, and deoxynivalenol (DON, vomitoxin). Many metabolites of T-2 and DAS have been found in the blood, urine, feces, bile and tissues after exposure in a number of species including cattle, swine, chickens, rats and mice [11—23]. These metabolites include HT-2, T-2 triol, T-2 tetraol, 4-acetyl tetraol, 8-acetyl tetraol, neosolaniol, 4-deacetyl neosolaniol, 3'-OH T-2, 3'-OH HT-2 in the T-2 group [11—14, 17, 18, 24] and monoacetoxyscirpenol and scirpenetriol in the DAS

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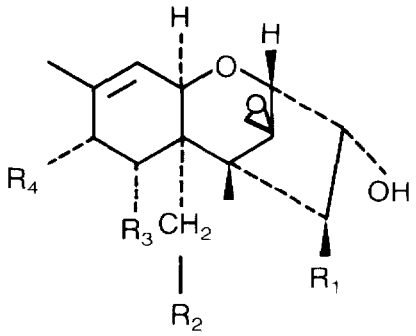

Fig. 1. Structures of various trichothecenes.

Compound	R_1	R_2	R_3	R_4
T-2 group				
T-2 toxin	OAc	OAc	H	$OCCH_2CH(CH_3)_2$ (O)
HT-2	OH	OAc	H	$OCCH_2CH(CH_3)_2$ (O)
T-2 triol	OH	OH	H	$OCCH_2CH(CH_3)_2$ (O)
3'-OH T-2	OAc	OAc	H	$OCCH_2C(CH_3)_2$ (O, OH)
3'-OH HT-2	OH	OAc	H	$OCCH_2C(CH_3)_2$ (O, OH)
T-2 tetraol	OH	OH	H	OH
Neosolaniol	OAc	OAc	H	OH
4-Deacetyl neosolaniol	OH	OAc	H	OH
4-Acetyl tetraol	OAc	OH	H	OH
8-Acetyl tetraol	OH	OH	H	OAc
DAS group				
Diacetoxyscirpenol	OAc	OAc	H	H
Monoacetoxyscirpenol	OH	OAc	H	H
Scirpenetriol	OH	OH	H	H
Deoxynivalenol (DON)	H	OH	OH	O

group [21–23] (Fig. 1). There have been few metabolism studies with DON, therefore, metabolites of DON have not been identified with the exception of a novel de-epoxide metabolite [25]. Collectively, these compounds exhibit a wide range of chemical behavior owing to the varied number and types of side groups. Current methods to analyze for T-2, DAS and their metabolites, or DON in biological samples can be lengthy, difficult or costly. Standards for many of the metabolites of T-2 toxin and DAS are not readily available and, if available, they are often expensive. This paper outlines a simple and rapid screening procedure that utilizes readily available standards, and aids in the determination of DON, DAS and T-2 exposure.

EXPERIMENTAL

Materials

All solvents (HPLC grade) and Prep-Sep silica cartridges were purchased from Fisher Scientific (Itasca, IL, U.S.A.).

Clin-Elut 1010 columns were purchased from Analytichem International (Harbor City, CA, U.S.A.).

Trifluoroacetic anhydride (TFAA) and 4-dimethylaminopyridine (DMAP) were purchased from Sigma (St. Louis, MO, U.S.A.).

DON and DAS were obtained from Myco Labs. (Chesterfield, MO, U.S.A.). T-2 tetraol and scirpenetriol were prepared in our laboratory by the alkaline hydrolysis of T-2 toxin and DAS, respectively [26]. The T-2 was produced in our laboratory from fungal cultures.

All other reagents were analytical-reagent grade.

Extraction

A 1-ml volume of saturated sodium chloride (aqueous) followed by 8 ml of centrifuged plasma or urine were added to a Clin-Elut 1010 column. After 2–3 min, when the sample had adsorbed onto the packing material, 2 × 40 ml of ethyl acetate were added to the column. The eluate was collected in a boiling flask and concentrated to dryness. A quantitative aliquot may be taken and saved for further confirmatory analysis.

Hydrolysis

The residue was transferred to a 8-ml screw-cap test tube with methanol then concentrated to dryness. A 200-μl volume of 0.25 *M* sodium hydroxide in 90% methanol was added, the test tube tightly capped and then vortexed. The solution was heated for 5 min at 60°C and then cooled at room temperature for 10 min. Immediately following the 10-min cooling period, 200 μl of 0.25 *M* acetic acid in toluene were added to the test tube and vortexed. The solvent was concentrated to dryness under a stream of dry nitrogen and gentle heating in a 50°C water bath.

Silica cartridge clean-up

A silica cartridge was preconditioned with one column volume of chloroform–acetone (1:3) followed by an equivalent volume of chloroform. The hydrolysis residue was transferred to the cartridge with 2 × 2 ml of chloroform–acetone (9:1). After the rinse solvent had reached the top of the packing, an 8-ml screw-cap test tube was placed beneath the cartridge. A 200-μl volume of absolute ethanol was added to the test tube originally containing the hydrolysis residue, then followed with 1 ml of chloroform. The chloroform–ethanol mixture was transferred to the cartridge. To the test tube containing the original hydrolysis residue, 1 ml of chloroform–acetone (1:3) was added, vortexed and then added to the cartridge when the last portion of the chloroform–ethanol mixture had reached the top of the cartridge packing. Immediately after adding the first 1 ml of chloroform–acetone (1:3), 4 ml of chloroform–acetone (1:3) were added directly to the cartridge. All of the eluates including the chloroform–ethanol mixture were collected in the same

8-ml screw-cap test tube for a total volume of approximately 6 ml. The solvent was evaporated to dryness under a stream of dry nitrogen and gentle heating in a 50°C water bath.

Derivatization

To the residue, 1 ml of toluene–acetonitrile (9:1) containing 2 mg/ml DMAP was added followed by 50 μl of TFAA. The test tube was tightly capped and the sample was heated for 20 min at 60°C. After cooling, 1 ml of a 5% aqueous sodium bicarbonate solution was added. The sample was vortexed until the top layer was clear, a 100-μl aliquot of the top layer taken and diluted to 4 ml with hexane for gas chromatography.

Gas chromatography

A 2-μl volume was injected into a Hewlett-Packard 5840A gas chromatograph equipped with a Ni^{63} electron-capture detector, a Hewlett-Packard 7672A autosampler and a 1.8 m × 2 mm glass column packed with 3% OV-17 on 100–120 mesh Supelcoport. The GC conditions were as follows. oven temperature 165°C, injector temperature 275°C, detector temperature 300°C, flow-rate of helium 35 ml/min.

RESULTS AND DISCUSSION

The method presented in this paper is a rapid and simple procedure to screen for trichothecenes in plasma and urine. About twenty samples can be easily prepared for GC analysis in 8 h. The method requires only three standards (deoxynivalenol, T-2 tetraol and scirpenetriol), which are commercially available or readily prepared by alkaline hydrolysis [26], to screen for at least fifteen different trichothecene mycotoxins. This is advantageous since T-2 toxin and DAS are metabolized to a large variety of different metabolites by exposed animals [11–24].

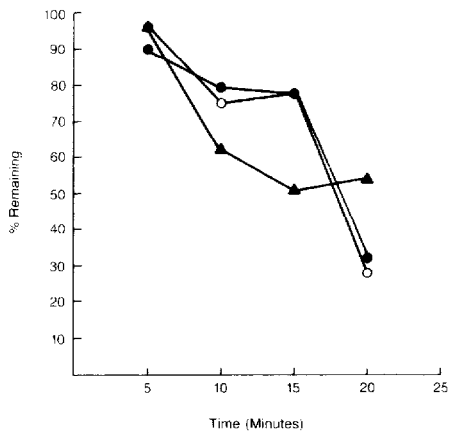

Fig. 2. Stability of T-2 tetraol, scirpenetriol and deoxynivalenol under hydrolysis conditions. The *y*-axis shows percentages of T-2 tetraol (•), scirpenetriol (▲) and deoxynivalenol (○) remaining after incubation of T-2, diacetoxyscirpenol and deoxynivalenol, respectively, with 0.25 *M* sodium hydroxide in 90% methanol at 60°C.

T-2 and its metabolites (HT-2, T-2 triol, 4-acetyl tetraol, 8-acetyl tetraol, neosolaniol, 4-deacetyl neosolaniol, 3′-OH T 2, 3′-OH HT-2) are all converted to T-2 tetraol with the hydrolysis of the acetyl and isovaleryl ester groups by sodium hydroxide. DAS and its metabolite monoacetylscirpenol (MAS) was subject to the same type of hydrolysis except the final product was scirpenetriol. T-2 tetraol and scirpenetriol, if present, undergo no change under the hydrolysis conditions utilized. DON was not hydrolyzed, however, DON was susceptible to degradation under basic conditions that are slightly more severe than the hydrolysis conditions in this procedure. The hydrolysis conditions chosen were optimized to achieve the complete conversion of T-2 to T-2 tetraol and DAS to scirpenetriol with minimal loss of DON. A reduced time and/or temperature parameter to insure the structural integrity of DON may not be adequate to insure the complete conversion to T-2 tetraol or scirpenetriol from their respective precursors. Heating times beyond the optimized conditions lead to the loss of DON, scirpenetriol and T-2 tetraol (Fig. 2). With pure standards of T-2, DAS and DON, heating for 5 min at 60°C (followed by immediate neutralization with acid) was sufficient for the complete conversion to T-2 tetraol and

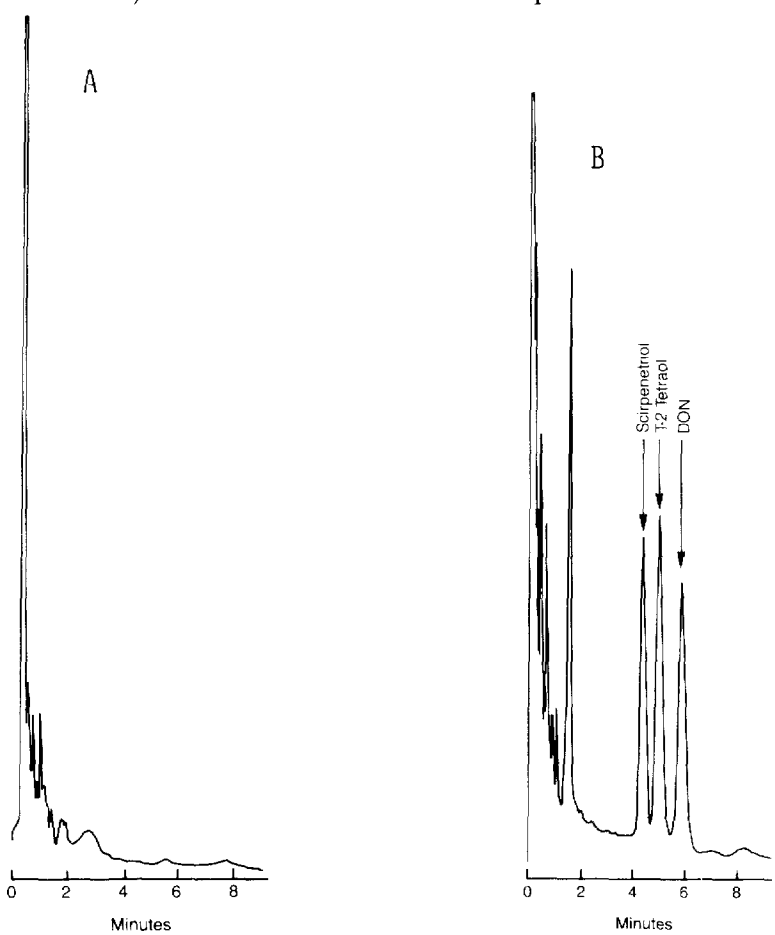

Fig. 3. Gas chromatograms of control pig plasma (A) and spiked pig plasma (B) at 250 ppb of T-2, diacetoxyscirpenol and deoxynivalenol.

scirpenetriol with a minimal loss of DON. However, in the presence of residues from plasma or urine, a slightly longer incubation period was necessary for the total conversion to T-2 tetraol and scirpenetriol. An additional 10-min incubation at room temperature immediately following 5 min of heating at 60°C was sufficient to achieve the full conversion with minimal loss of DON in the presence of sample residues. The percentage of T-2 tetraol, scirpenetriol and DON remaining after 5 min at 60°C plus 10 min room temperature incubation with biological samples were nearly identical to a 5-min heating at 60°C with pure standards (90% T-2 tetraol, 95% scirpenetriol, 96% DON).

Chromatograms of control swine plasma and urine contained no interfering peaks (Figs. 3 and 4). Bovine urine gave slightly more complex chromatograms but accurate quantitation of the toxin peaks was not affected (Fig. 5). Baseline separation of T-2 tetraol, scirpenetriol and DON was achieved with a sensitivity level better than 25 ppb facilitating a multi-toxin analysis. Since both T-2 and DAS plus their respective metabolites are all converted to a single polar form (i.e., T-2 → T-2 tetraol, DAS → scirpenetriol), a cumulative effect results leading to a greater ability to detect trichothecene exposure. Upon derivatization to their corresponding trifluoroacyl derivatives, T 2 tetraol and scirpenetriol have

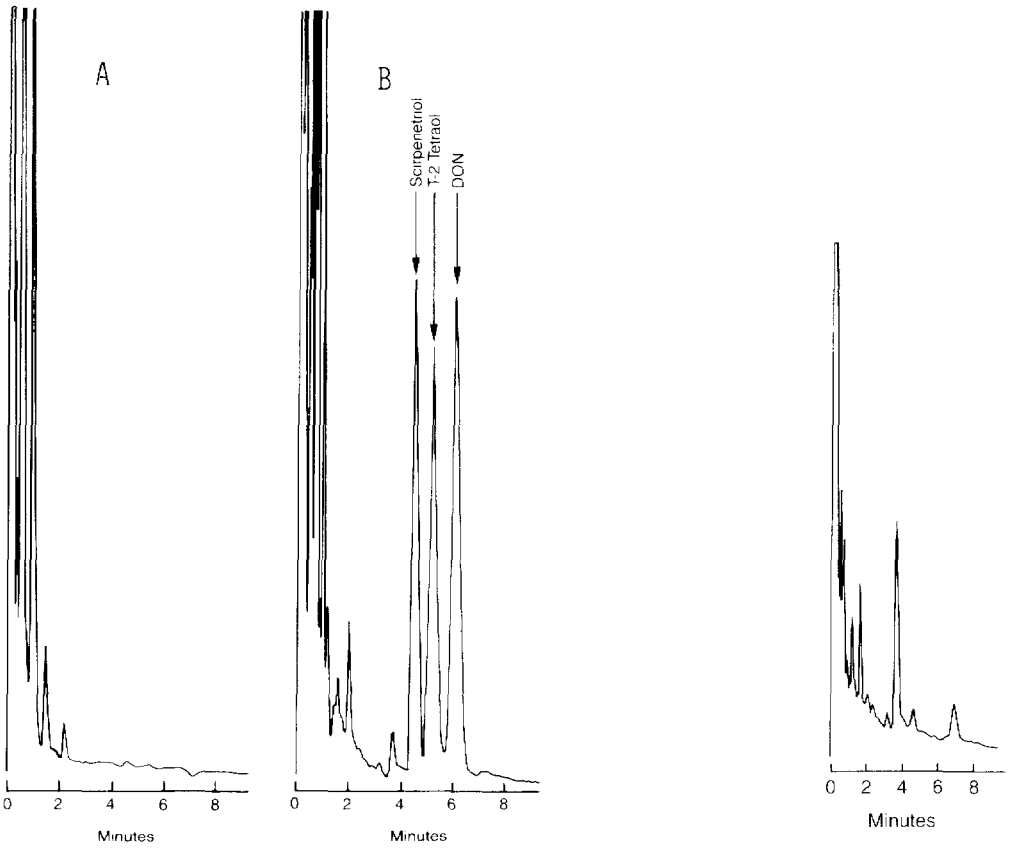

Fig. 4. Gas chromatograms of control pig urine (A) and spiked pig urine (B) at 500 ppb of T-2, diacetoxyscirpenol and deoxynivalenol.

Fig. 5. Gas chromatogram of control bovine urine.

greater response factors with an electron-capture detector than the parent compounds, therefore, resulting in increased sensitivity. Trichothecene exposure may therefore be determined with greater sensitivity. We have utilized this procedure to analyze urine samples from swine orally dosed with T-2. In previous examinations, T-2 and its individual metabolites were not detected (detection limit 50 ppb). However, when several of these samples were analyzed using this screening procedure, levels of 100—300 ppb of T-2 tetraol were found.

Recoveries were checked by spiking swine urine and plasma with DON, T-2 and DAS at several levels prior to extraction. The standard solutions in absolute ethanol (volume, 50 μl) were added to the Clin-Elut column immediately prior to the ethyl acetate extraction. The recoveries are given in Tables I and II. The recovery of T-2 tetraol directly from a Clin-Elut column is low (10- 20%), however, current studies in cattle, swine, chickens, rats and mice have shown that T-2 tetraol is a minor metabolite in exposed animals [17, 19, 27]. Plasma and urine extracts contain some residue upon concentration after hydrolysis, and to achieve good recoveries, a total transfer of this residue to the silica cartridge was necessary. The relatively non-polar portion of the residue was transferred then eluted with the chloroform—acetone (9:1). The 200 μl of absolute ethanol was necessary to transfer the more polar portion of the residue, and the T-2 tetraol, scirpenetriol and DON which do not completely transfer with the chloroform—acetone (9:1). The 1 ml of chloroform—acetone (1:3) insured a complete transfer of any remaining residue and trichothecene compounds.

TABLE I

DAS, DON AND T-2 RECOVERIES IN PLASMA AFTER HYDROLYSIS

Level (ppb)	*n*	Recovery (mean* ± S.E.) (%)		
		DAS	DON	T-2
50	10	94 ± 4	93 ± 8	80 ± 6
250	10	96 ± 4	94 ± 3	80 ± 3
500	10	116 ± 7	114 ± 3	99 ± 5

*Molar percent.

TABLE II

DAS, DON AND T-2 RECOVERIES IN URINE AFTER HYDROLYSIS

Level (ppb)	*n*	Recovery (mean* ± S.E.) (%)		
		DAS	DON	T-2
50	10	88 ± 5	78 ± 4	105 ± 10
250	4	119 ± 1	86 ± 1	97 ± 2
500	6	112 ± 3	95 ± 4	111 ± 4
1000	10	110 ± 4	93 ± 4	106 ± 9

*Molar percent.

Kinetic studies have revealed that the half-life of T-2 and DAS was approximately 10–20 min in the blood of pigs following intravascular administration. At lethal intravenous doses, neither parent compound was found in the blood 1 h post-dosing, and at lethal oral doses, no parent compound was found 2 h post-dosing [28]. For this reason, analysis of plasma and urine for the parent compounds, T-2 or DAS, may be difficult and in many cases of minimal benefit. However, many metabolites of T-2 and DAS are found in the plasma and urine after the parent compounds are at low levels or no longer can be detected [11–13, 17, 28]. The screening procedure outlined in this paper would be of benefit especially if analysis was desired after initial exposure to the source of trichothecenes — the parent compound would be undetectable, but its metabolites could still be present and available for hydrolysis to T-2 tetraol or scirpenetriol.

Analysis of plasma or urine by this method for the confirmation of trichothecene exposure in animals may be used as a complimentary procedure to the direct analysis of suspect feed. Analysis of feed suspected to be contaminated by trichothecenes may not always give an accurate account of the actual exposure levels for several reasons. The feed sample may not be available for analysis or may have been entirely consumed by the animals. Sample handling techniques between exposure and subsequent analysis may have contributed to a decrease or increase of the toxin levels present especially if the feed samples were not dried properly. Also, the sample taken for analysis may not be a representative of the portion eaten. Analysis of blood and/or urine from the suspected animal for T-2 tetraol, scirpenetriol or DON would give a better indication of actual trichothecene exposure provided the urine and plasma samples were taken as soon as exposure was suspected.

This screening procedure provides a rapid and simple means of determining T-2, DAS and DON exposure by analysis of plasma and urine for trichothecene hydrolysis products. Also, high sensitivity and the availability of the necessary standards are added benefits.

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