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NEW, SENSITIVE THIN-LAYER CHROMATOGRAPHIC–HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR DETECTION OF TRICHOTHECENE MYCOTOXINS

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SUMMARY

Diphenylindenone sulphonyl (Dis) esters of trichothecene mycotoxins when sprayed with sodium methoxide showed fluorescent spots on a thin layer of silica gel when viewed under long-wavelength UV light. The detection limit for trichothecene esters in thin-layer chromatography (TLC) was 20–25 ng per spot for T-2 toxin, HT-2 toxin, diacetoxyscirpenol, T-2 triol, T-2 tetraol and iso-HT-2 toxin. A quantitative high-performance liquid chromatographic (HPLC) analysis of Dis-trichothecene esters was also developed using UV detection at 278 nm. The detection limit for the above esters varied between 30 and 50 ng per injection. This sensitive TLC–HPLC method is very useful for *in vivo* pharmacokinetic analyses of trichothecenes.

INTRODUCTION

During our investigation of the pharmacokinetic behaviour of 4β,15-diacetoxy-8α(3-methylbutyryloxy)-12,13-epoxytrichothec-9-en-3α-ol (T-2 toxin) and 15-acetoxy-8α-(3-methylbutyryloxy)-12,13-epoxytrichothec-9-ene-3α,4β-diol (HT-2 toxin) in dogs, a method of a high sensitivity was required for the analysis of these toxins and their metabolites in biological fluids. Procedures for the separation and detection of trichothecenes utilizing various chromatographic techniques including thin-layer (TLC), gas–liquid (GLC) and high-performance liquid (HPLC) chromatography are available. Detection on TLC plates was achieved by dipping or spraying the plate with sulphuric acid[1,2], aluminium chloride[3,4], *p*-anisaldehyde[5], 4-(*p*-nitrobenzyl) pyridine[6], nicotinamide–2-acetylpyridine[7] or chromotropic acid[8] followed by heating. Much more sensitive detection is achieved by derivatizing the trichothecenes with a silylating reagent[9–16], heptafluorobutyrylimidazole[17–19], trifluoroacetic anhydride[20] or heptafluorobutyric anhydride[21], and analyzing them by GLC using specific de-

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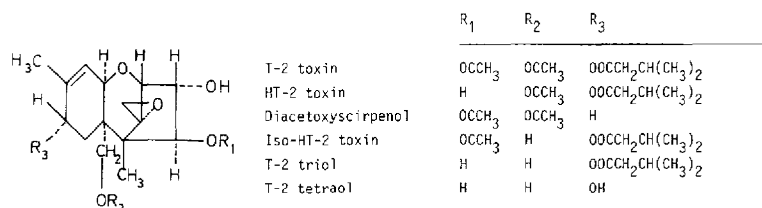
tectors. Recently the detection of T-2 toxin and HT-2 toxin by HPLC was carried out by using a differential refractometer[22]. The sensitivity of this method was very low in comparison with TLC and GLC methods.

In this paper we describe a new sensitive fluorescence TLC method for the detection of diphenylindenone sulphonyl (Dis) esters of trichothecenes. HPLC of these derivatives using UV detection resulted in a considerable improvement in the detection sensitivity of trichothecenes.

EXPERIMENTAL

Chemicals

Stock standard solutions of trichothecenes (Fig. 1) in acetone, were prepared at concentrations of 100 and 1 μg/ml. 2-*p*-Chlorosulphonyl-3-phenylindenone (diphenylindenone sulphonyl chloride, Dis-Cl) was synthesized and purified according to Ivanov[23] and an acetone stock solution at a concentration of 1 mg/ml was prepared. 4-Dimethylaminopyridine (Aldrich) was dissolved in acetone at a concentration of 1 mg/ml. All stock solutions were sealed and stored at 4°C.



	R_1	R_2	R_3
T-2 toxin	$OCCH_3$	$OCCH_3$	$OOCCH_2CH(CH_3)_2$
HT-2 toxin	H	$OCCH_3$	$OOCCH_2CH(CH_3)_2$
Diacetoxyscirpenol	$OCCH_3$	$OCCH_3$	H
Iso-HT-2 toxin	$OCCH_3$	H	$OOCCH_2CH(CH_3)_2$
T-2 triol	H	H	$OOCCH_2CH(CH_3)_2$
T-2 tetraol	H	H	OH

Fig. 1. Structures of trichothecene mycotoxins.

Precoated silica gel 60 TLC plates (layer thickness 0.2 mm) were purchased from E. Merck (Darmstadt, F.R.G.). Acetone for UV spectrophotometry was purchased from J. T. Baker (Phillipsburgh, NJ, U.S.A.). Ethyl acetate, light petroleum (b.p. 60–80°C), toluene, acetonitrile, benzene, diethyl ether and methanol (Frutarom, Israel) were distilled before use.

Apparatus

Liquid chromatographic analyses were performed on a Tracor Model 950 high-performance liquid chromatograph. A prepacked C_{18} (particle size 10 μm) column (25 cm × 4.6 mm I.D.) was obtained from Dr. H. Knauer KG (Bad Homburg, F.R.G.). The detection of the toxins was carried out by a variable-wavelength UV detector Model 970A (Tracor, Austin, TX, U.S.A.).

TLC

Appropriate volumes of trichothecene solutions and 200 μl of Dis-Cl solution were placed into centrifuge tubes, and the solvent was evaporated under a gentle stream of nitrogen. The residue was redissolved in 100 μl of the solution of 4-dimethylaminopyridine in acetone and the reaction was carried out in sealed tubes in an oven at 60°C for 30 min. The tubes were allowed to cool and the solvent was

evaporated. A 1-ml volume of ethyl acetate was added and the mixture was extracted with 2 ml of brine solution. Aliquots (5 μl) of the ethyl acetate layer were applied directly to the silica gel TLC plate. Alternatively, the ethyl acetate layer was separated, concentrated, redissolved in 10 μl ethyl acetate and then placed on the TLC plate. The TLC plate was developed with toluene–ethyl acetate–acetone (7:2:1). The developed TLC plate was dried in a stream of air and sprayed with sodium methoxide or butoxide (8 g sodium in 100 ml methanol or butanol). The fluorescent spots were examined under long-wavelength UV light while the plate was still wet.

HPLC

Appropriate amounts of T-2 toxin, diacetoxyscirpenol (DAS), T-2 triol and/or T-2 tetraol, 30 μl of Dis-Cl (1 mg/ml) and 30 μl of 4-dimethylaminopyridine in acetone (1 mg/ml) were mixed in a 5-ml glass tube and the volume was made up to 100 μl with acetone. The work-up was performed as described above. After the extraction with brine, the tubes were centrifuged at 2000 *g* for 1 min. The ethyl acetate layer was separated, concentrated to dryness, redissolved in a small amount of acetonitrile or methanol and injected into the HPLC system.

Quantitative preparation of Dis-trichothecenes

The general procedure for the synthesis of Dis-trichothecenes (exemplified for T-2 toxin) was as follows: 40 mg of T-2 toxin were reacted with 70 mg of Dis-Cl in the presence of 30 mg of 4-dimethylaminopyridine in 0.5 ml of pyridine at 60°C for 1 h. The reaction mixture was cooled, 1 ml of ethyl acetate was added and the pyridine was extracted twice with 2 ml of saturated aqueous sodium chloride.

An aliquot (1 μl) of the ethyl acetate layer was spotted on a TLC plate, developed with toluene–ethyl acetate–acetone (7:2:1), sprayed with sulphuric acid and heated. No starting material was detected. The ethyl acetate layer was passed twice through a 7-g silica gel column using 50 ml of ethyl acetate as an eluent. The eluent was concentrated and the Dis-trichothecene esters were isolated using medium-pressure liquid chromatography (silica gel 60 for column chromatography) and eluted with ethyl acetate–petroleum ether (40:60).

The product was crystallized from benzene–petroleum ether–diethyl ether or toluene–hexane. The yield of Dis-T-2 toxin before crystallization was 80%. Dis derivatives of DAS, HT-2 toxin, Iso-HT-2 toxin, T-2 triol and T-2 tetraol were prepared in the same way. The reaction products presented in Table I were identified by NMR, IR and mass spectral analyses.

RESULTS

Under the conditions described, the secondary alcohols of trichothecenes reacted with Dis-Cl producing Dis-trichothecene esters (Fig. 2). Fig. 3 shows a thin-layer chromatogram of six Dis-trichothecene esters. Fluorescent spots on the TLC plate were obtained after spraying the Dis derivatives with a sodium alkoxide. The chromatographic data for these compounds are summarized in Table I. By using toluene–ethyl acetate–acetone (7:2:1), TLC gave a good separation between T-2 toxin and its major metabolites. The limits of detection by means of visual inspection under long-wavelength UV light were between 20 and 25 ng per spot (Table I).

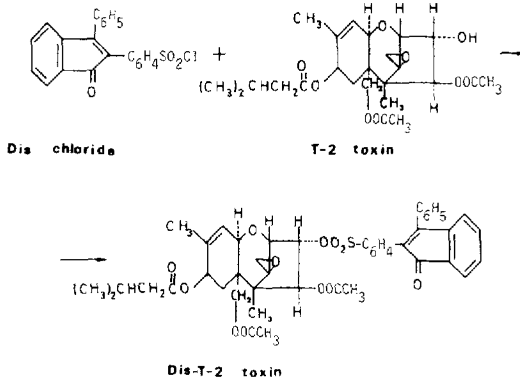


Fig. 2. Reaction of Dis-Cl with T-2 toxin.

HT-2 toxin, having two vicinal hydroxyl groups on carbons C-3 and C-4, produced two monoesters on reaction with Dis-Cl, suggesting steric hindrance between these hydroxyl groups (Fig. 3).

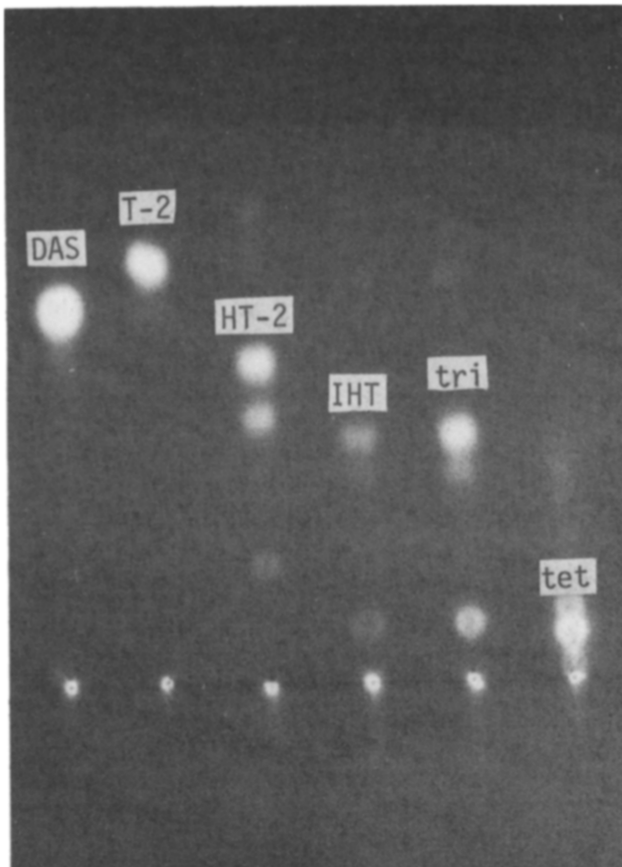


Fig. 3. Thin-layer chromatogram of trichothecene mycotoxins: DAS, T-2 toxin (T-2), HT-2 toxin (HT-2), Isto-HT-2 toxin (IHT), T-2 triol (tri) and T-2 tetraol (tet).

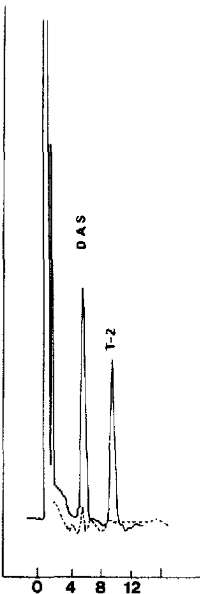
TABLE I

CHROMATOGRAPHIC DATA FOR DIS-TRICHOTHECENES USING TOLUENE-ETHYL ACETATE-ACETONE (7:2:1) AS SOLVENT

Compound	R_F ± *S.D. (n = 5)*	*Detection limit (ng per spot)*
Dis-T-2 toxin	0.64 ± 0.018	20
Dis-HT-2 toxin (upper spot)	0.48 ± 0.02	25
(lower spot)	0.39 ± 0.014	30
Dis-DAS	0.57 ± 0.011	20
Dis-Iso-HT-2 toxin*	0.37 ± 0.018	25
Dis-T-2 triol*	0.38 ± 0.013	25
Dis-T-2 tetraol*	0.085 ± 0.012	25

* R_F of the prominent spot was taken.

The detection limits of the HPLC method were 30 ng for T-2 toxin and for DAS (Fig. 4), 50 ng for T-2 triol and T-2 tetraol (Fig. 5) and 200 ng for HT-2 toxin. The retention times of DAS and T-2 toxin using acetonitrile-water (70:30) as a mobile phase were 5.6 and 9.6 min respectively (Table II). The retention times of T-2 tetraol and T-2 triol in methanol-water (75:25) were 6.2 and 10 min, respectively (Table II).



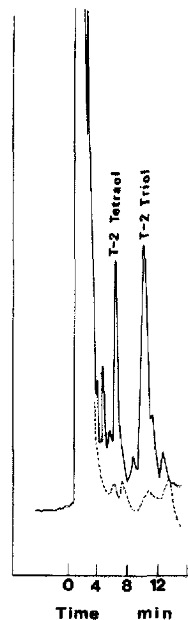


Fig. 4. HPLC chromatogram of Dis-T-2 toxin and Dis-DAS. The dotted line represents a control (an HPLC chromatogram of blank reaction mixtures). Mobile phase: 70% acetonitrile in water; flow-rate 3 ml/min. Detection: UV at 278 nm.

Fig. 5. HPLC chromatogram of Dis-T-2 triol and Dis-T-2 tetraol. The dotted line represents a control. Mobile phase: methanol-water (75:25); flow-rate 2 ml/min. Detection: UV at 278 nm.

TABLE II

RETENTION TIMES OF THE DIS ESTERS OF T-2 TOXIN, HT-2 TOXIN, T-2 TRIOL, T-2 TETRAOL AND DAS

Mobile phase	*Retention time (min)*				
	DAS	*T-2 toxin*	*HT-2 toxin*	*T-2 triol*	*T-2 tetraol*
Acetonitrile–water (70:30), flow-rate 3 ml/min	5.6	9.6	6.6		
Methanol–water (75:25), flow-rate 2 ml/min				10.0	6.2

DISCUSSION

Rapid, simple and sensitive detection methods are needed for the determination of mycotoxins in food and biological specimens. Because the samples may contain a variety of toxins in low concentrations, an ideal assay should be sensitive and highly specific. By derivatization of T-2 toxin and its major *in vivo* metabolites with Dis-Cl, detection limits of 20–25 ng (TLC) and 30–50 nmg (HPLC with UV detection) were achieved. There are three other sensitive TLC methods for detection of trichothecenes using 4-(*p*-nitrobenzyl)pyridine (NBP)[6], chromotropic acid (CA)[8] and nicotinamide–2-acetylpyridine (N-AP)[7]. Table III compares the sensitivity of the present method with those of the earlier assays.

TABLE III

SENSITIVITIES OF SOME TLC METHODS

NP = Not published.

Mycotoxin	*Detection limit (ng per spot)*			
	NBP	*CA*	*N-AP*	*Dis ester*
T-2 toxin	100	50	25	20
DAS	200	100	25	20
HT-2 toxin	20	50	NP	25
T-2 triol	NP	50	NP	25
T-2 tetraol	NP	50	NP	25

The TLC method described is more sensitive than that using NBP and CA and comparable to the N-AP method. Unlike the latter, the detection of fluorescent Dis-trichothecene esters is very simple and time saving. Due to the fact that HT-2 toxin, Iso-HT-2 toxin, T-2 triol and T-2 tetraol have free hydroxyl groups at positions other than C-3 of the trichothecene molecule, by-products may be formed, as shown in Fig. 3. However, these by-products are minimal (except for Dis-HT-2 toxin), accounting for about 10%; they therefore have little effect on the quantitative results.

Applying HPLC to the detection of various Dis-trichothecene esters allows more precise quantitative estimations of these trichothecenes. The TLC detection of Dis-trichothecene esters, and especially their HPLC assay, could be used for quantitation of trichothecenes in plasma and urine during metabolic studies *in vivo*. In order to study the pharmacokinetics of T-2 toxin and its metabolite HT-2 toxin in plasma from the dog, a special clean-up technique prior to GLC–electron-capture detection analysis was carried out[21,24]. The same clean-up method can be used prior to detection of their Dis derivatives by TLC. HPLC analysis may prove to be satisfactory for monitoring T-2 toxin and most of its metabolites in plasma and urine. However, the Dis ester of HT-2 toxin may be problematic as it is not as easily detected by HPLC. For this purpose the GLC–ECD method[21] is preferred. The analysis of T-2 triol and T-2 tetraol isolated from urine could easily be performed by HPLC of their Dis derivatives. The development of an assay for monitoring T-2 toxin metabolites in urine is in progress and will be described in a subsequent paper.

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