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GAS CHROMATOGRAPHIC ASSAY WITH PHARMACOKINETIC APPLICATIONS FOR MONITORING T-2 AND HT-2 TOXINS IN PLASMA

BORIS YAGEN

Department of Natural Products, School of Pharmacy, Hebrew University, P.O. Box 12065, Jerusalem (Israel)

MEIR BIALER*

Department of Pharmacy, School of Pharmacy, Hebrew University, P.O. Box 12065, Jerusalem 91120 (Israel)

and

AMNON SINTOV

Department of Natural Products and Department of Pharmacy, School of Pharmacy, Hebrew University, P.O. Box 12065, Jerusalem (Israel)

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SUMMARY

A gas—liquid chromatographic (GLC) method for monitoring T-2 and HT-2 toxins in plasma was developed. The procedure involved extraction of the toxins with ethyl acetate, chromatography on a C_{18} reversed-phase column and derivatization with heptafluorobutyric anhydride (HFBA). The T-2 and HT-2 HFBA derivatives were chromatographed on OV-17 at various temperatures and measured with an electron-capture detector. Iso-T-2 toxin and iso-HT-2 toxin were used as internal standards. Recoveries averaged 95.1 ± 8.6% for T-2 toxin and 102.1 ± 5.2% for HT-2 toxin at levels ranging from 40 to 120 ng/ml. The limits of detection were 30 and 5 ng/ml of T-2 and HT-2 toxin, respectively. The range of the assay covers plasma concentrations at which toxicity becomes manifest. The pharmacokinetic application of this GLC method is illustrated by simultaneous monitoring of T-2 and HT-2 toxins levels in plasma obtained after intravenous administration of T-2 toxin to a dog.

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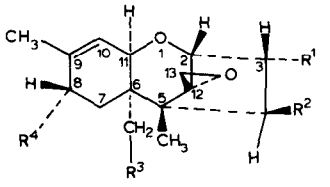
INTRODUCTION

T-2 toxin (I) [4β,15-diacetoxy-8α-(3-methylbutyryloxy)-12,13-epoxy-trichothec-9-en-3α-ol] is the principal toxin isolated from several strains of *Fusarium* fungi. Man and domestic animals can be seriously affected by T-2 toxin and its metabolites [1—5]. Alimentary toxic Aleukia (ATA), a disease caused by intoxication of T-2 toxin, reportedly killed hundreds of thousands of humans in the U.S.S.R. over four years [3, 6]. Numerous incidents of intoxication in animals reported from North America, Japan and South Africa were due to feed infested with *Fusarium* fungi [2, 4, 7, 8].



Trichothecene	R^1	R^2	R^3	R^4
T-2 toxin (I)	OH	CH_3COO	CH_3COO	$OCOCH_2CH(CH_3)_2$
HT-2 toxin (II)	OH	OH	CH_3COO	$OCOCH_2CH(CH_3)_2$

Recently, T-2 toxin and related trichothecenes were reported to be detected in samples of "yellow rain" resulting from chemical—biological warfare in Southeast Asia and Afghanistan [9, 10]. T-2 toxin and several of its immediate metabolites cause inhibition of protein and DNA synthesis in eukariotic cells, radiomimetic cellular injury to actively dividing cells, leukopenia, inflammation of the skin and vomiting in humans [11]. The assessment of the toxic potential of T-2 toxin and its metabolites has become an important step in studying their toxic mechanism of action. Studies on the distribution and excretion of radiolabelled T-2 toxin have been conducted in domestic animals and birds but no pharmacokinetic experiments have been performed [12–14].

In in vitro and in vivo experiments with T-2 toxin, it was shown that T-2 toxin is biotransformed very rapidly into HT-2 toxin (II) by non-specific carboxyesterases [15—17]. The concentration of HT-2 toxin found in the plasma of experimental animals treated with T-2 was very low. T-2 toxin plasma levels of 10—60 ppb (ng/ml) were found in a cow after oral administration of 180 mg/day (for 3 days) of T-2 toxin [14]. Levels of 18 ppb of T-2 toxin and 22 ppb of HT-2 toxin have been found in the blood of victims less than 24 h after exposure to "yellow rain" in Kampuchea [9].

In order to conduct pharmacokinetic studies, special sensitive methods for the determination of trichothecenes should be applied. These analytical methods should require a small amount of blood for each assay and permit the simultaneous determination of several trichothecenes appearing simultaneously in the blood during the in vivo metabolism of the parent toxin.

During the last decade, various methods for the determination of trichothecenes have been described. These methods include thin-layer chromatography (TLC), gas—liquid chromatography (GLC) and radioimmunoassays

[18—20]. The principal disadvantage of TLC with regard to pharmacokinetic studies is its limited sensitivity compared with radioimmunoassay. Immunological assays for trichothecenes are promising but have the disadvantage of misidentification because of cross-reactivity of several trichothecene metabolites. The use of GLC with electron-capture detection (ECD) for the determination of T-2 toxin in plasma was recently described by Swanson et al. [21].

The aim of this work was to develop a reliable, sensitive assay for the routine simultaneous quantitative determination of T-2 and HT-2 toxins in plasma. This assay is fundamental to pharmacokinetic and stability studies of T-2 toxin. Its in vivo applicability is illustrated by an experiment with dog plasma.

EXPERIMENTAL

Reagents and standards

Reference standards of T-2 and HT-2 toxins were purchased from Sigma (St. Louis, MO, U.S.A.). Experimental stock solutions of T-2 and HT-2 toxins were prepared by dissolving the pure substances separately in water—methanol (95:5) at a concentration of 1 μg/ml. Iso-T-2 and iso-HT-2 toxins were used as internal standards (I.S.) and the pure substances were dissolved in ethyl acetate at a concentration of 1 μg/ml. All solutions were stored at 4°C. Heptafluorobutyric anhydride (HFBA) was obtained from Aldrich (Milwaukee, WI, U.S.A.) and the octadecyl (C_{18}) HPLC sorbent from Sigma. The organic solvents (ethyl acetate, chloroform and dichloromethane) were obtained from Frutarom (Israel) and were all distilled in glass and dried over molecular sieves. Methanol, trimethylamine (TMA) and *n*-hexane (AnalaR grade) were purchased from BDH (Poole, U.K.).

Apparatus and conditions

A Packard Model 437 gas chromatograph (Packard, Downers Grove, IL, U.S.A.) equipped with a ^{63}Ni electron-capture detector and a Unicorder 225 recorder (Panto, Kyoto, Japan) was used. The glass column (180 cm × 2 mm I.D.) was packed with 3% OV-17 on 80—100-mesh Gas-Chrom Q. The following temperatures were used: injector, 280°C; detector, 325°C; column, 250°C for T-2 toxin detection and 220°C for HT-2 toxin detection. The flow-rate of the carrier gas (nitrogen) was 26 ml/min for both T-2 and HT-2 toxins.

Extraction and clean-up

Extraction of plasma. To 10 ml of dog plasma, aqueous solutions of the appropriate aliquots of T-2 and HT-2 toxins were added, followed by addition of 100 μl of iso-T-2 and iso-HT-2 toxins in ethyl acetate (internal standards) and 3.8 ml of ethyl acetate. The sample was vortexed for 20 sec and centrifuged at 2000 *g* for 3 min. From the ethyl acetate layer, 3.6 ml were transferred into another tube, concentrated to dryness by vacuum evaporation and redissolved in 0.2 ml of methanol.

Clean-up procedure. The reversed-phase C_{18} (octadecyl) column was prepared as follows: a small ball of glass-wool was tamped at the bottom of the column and 0.4 g of octadecyl (C_{18}) HPLC sorbent (10 μm) was added, followed by another ball of glass-wool. The column was rinsed in sequence with

10 ml of methanol and 10 ml of water. A 4-ml volume of water was then inserted into the column and the 0.2 ml of plasma extract was added. Another portion of 0.2 ml of methanol was used to rinse the tube and then pipetted into the column. After the volumes that accumulated in the column had passed through the C_{18} sorbent, T-2 and HT-2 toxins were eluted with 2 ml of methanol—water (8:2) into a 5-ml tube. The eluates were concentrated to dryness by vacuum evaporation and derivatized.

Derivatization

The residues in the tubes were dissolved in 0.4 ml of ethyl acetate—chloroform (1:3) and 100 μl of 0.05 *M* TMA in dried dichloromethane were added. A volume of 10 μl of HFBA was added to the tubes and the mixtures were vortexed for 5 sec and shaken for 10 min. The reaction mixtures were then concentrated to dryness by vacuum or under a nitrogen flow and redissolved in 0.3 ml of *n*-hexane. A 1-ml volume of 5% sodium hydrogen carbonate solution was added, the mixture vortexed and 3 μl of the top layer were injected into the gas chromatograph.

In order to determine the precision of the assay, 10 ml of dog plasma were spiked with the appropriate aliquots of the aqueous T-2 and HT-2 toxin stock solutions and stored at −20°C for 2 months. On different days, 1-ml samples were taken from the various stored samples and analysed by comparison with a fresh calibration graph constructed according to the extraction procedure on the same day. The entire procedure is summarized in a flow chart (Fig. 1).

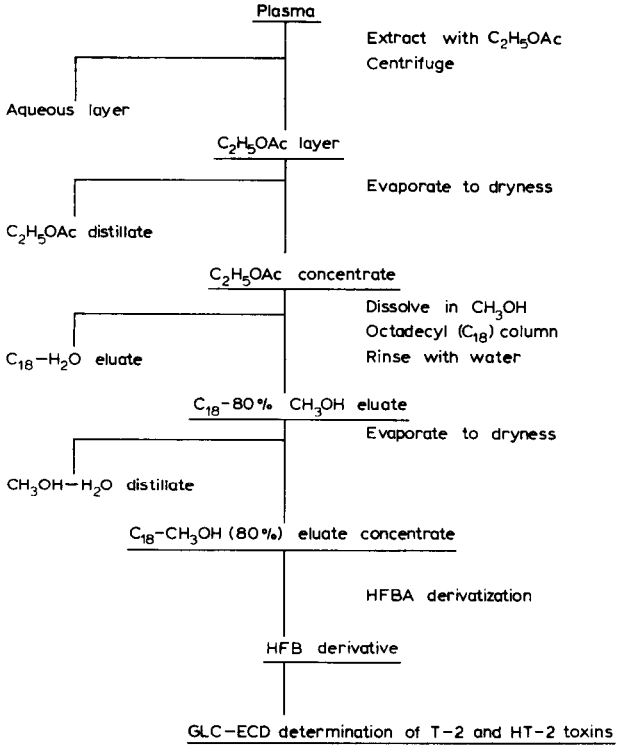


Fig. 1. Flow chart of the procedure for the determination of T-2 and HT-2 toxins in plasma.

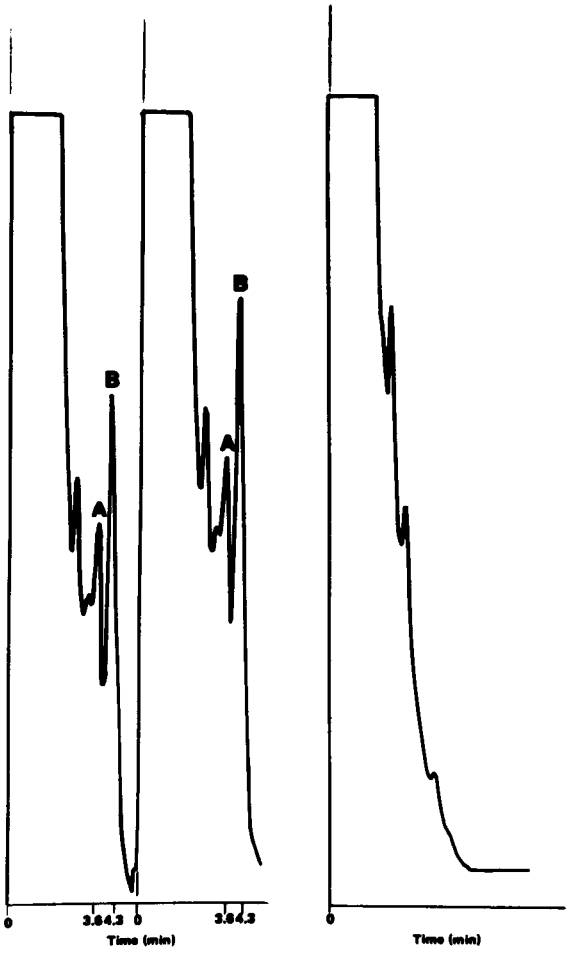


Fig. 2. Examples of chromatograms of T-2 toxin. Dog plasma blank; T-2 toxin (A) 50 ng/ml and iso-T-2 toxin (B) 100 ng/ml as I.S.

RESULTS AND DISCUSSION

Typical chromatograms of plasma extracts and drug-free plasma are presented in Figs. 2 and 3. Under the assay conditions used, the following retention times were obtained: T-2 toxin, 3.6 min; iso-T-2 toxin, 4.3 min; HT-2 toxin, 3.4 min; and iso-HT-2 toxin, 1.5 min.

There was no interference from endogeneous plasma compounds. Calibration graphs from the plasma extract showed a linear correlation between the peak-height ratio (y) (of T-2 or HT-2 toxin to their respective I.S.) and plasma concentration of the substances (x). The linear regression equations were $y = 0.0154x - 0.006$ ($r = 0.991$) for T-2 toxin and $y = 0.01355x + 0.0058$ ($r = 0.994$) for HT-2 toxin. The minimal detectable concentration (MDC) for T-2 toxin was 30 ng/ml and for HT-2 toxin 5 ng/ml (though the minimal concentration shown in Table I is 40 ng/ml). The range of linearity of the assay was between 5 and 5000 ng/ml for HT-2 toxin and between 30 and 5000 ng/ml for T-2 toxin.

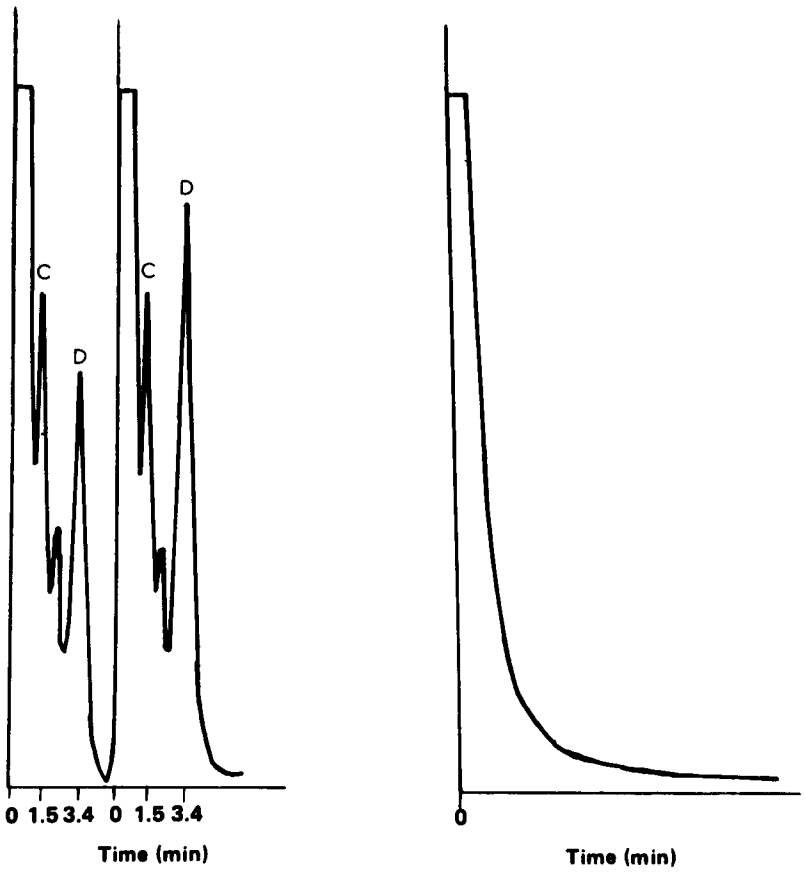


Fig. 3. Examples of chromatograms of HT-2 toxin. Dog plasma blank; HT-2 toxin (D) 80 and 100 ng/ml and iso-HT-2 toxin (C) 100 ng/ml as I.S.

Recoveries of the toxins were determined as follows. Various amounts of T-2 and HT-2 toxins were dissolved in 1 ml of drug-free plasma and the samples were analysed according to the extraction and clean-up procedures described above. In these instances, the I.S. solutions were added after the clean-up was terminated. A series of external standards were prepared by adding the same amounts of I.S. solutions to the various amounts of T-2 and HT-2 toxins, taken from the aqueous stock solutions. Prior to the determination of the toxins by GLC, the solutions were concentrated to dryness and derivatized as described above. Analytical recoveries were calculated by comparing peak-height ratios of the extracted standard to the external standards (Table I). The standard deviations from the recovery studies can serve as a good estimate of the reproducibility. The mean (± S.D.) recoveries of T-2 and HT-2 toxins were 95.1 ± 8.6% and 102.1 ± 5.2%, respectively (Table I).

The precision of the assay was determined by performing eight replicate analyses of five control samples containing 50, 70, 90, 110 and 120 ng/ml of the toxins on different days after a 2-month period. The results are shown in Table II. The observed values of the various concentrations of T-2 and HT-2 toxins were not statistically different from the added concentrations ($p > 0.05$).

TABLE I

RECOVERY AND REPRODUCIBILITY OF T-2 AND HT-2 TOXINS IN DOG PLASMA

T-2 toxin				HT-2 toxin			
Concentration (ng/ml)	Recovery (%)*	S.D. (%)	C.V. (%)	Concentration (ng/ml)	Recovery (%)*	S.D. (%)	C.V. (%)
40	87.5	3.8	4.3	40	107.2	1.4	1.3
50	101.6	1.5	1.5	50	107.2	6.9	6.4
70	93.1	6.9	7.4	70	98.0	9.4	9.6
80	85.2	3.4	4.0	80	106.2	9.1	8.6
90	89.8	7.0	7.8	90	97.5	9.4	9.6
110	99.7	10.9	10.1	120	97.0	9.4	9.7
120	109.0	11.0	10.1				
Mean	95.1	8.6	9.0		102.1	5.2	5.0

*Mean of four determinations.

TABLE II

PRECISION OF THE ASSAY OF T-2 AND HT-2 TOXINS IN DOG PLASMA

T-2 toxin				HT-2 toxin			
Concentration (ng/ml)	Concentration found* (ng/ml)	S.D. (ng/ml)	C.V. (%)	Concentration (ng/ml)	Concentration found* (ng/ml)	S.D. (ng/ml)	C.V. (%)
50	50.3	4.9	9.7	60	59.7	5.3	8.9
70	66.2	5.8	8.8	80	82.5	7.2	8.7
90	94.8	8.5	9.0	90	94.9	8.8	9.3
110	103.4	10.3	10.0	110	111.2	9.7	8.7
120	115.8	10.9	9.4	120	114.8	6.5	5.7

*Mean of eight determinations.

The ethyl acetate used in this assay procedure as the extraction solvent was shown to be a very efficient solvent by the high recovery of T-2 toxin and its metabolite HT-2 toxin. Owing to stability problems with the two toxins, the use of a base and a Florisil column was avoided. A C_{18} reversed-phase column was very efficient in the separation of interfering compounds extracted by ethyl acetate. The use of HFBA was preferred to HFBI (heptafluorobutyroimidazole), as the former was much more effective in the derivatization procedure.

A biomedical application of the GLC method is illustrated by the following preliminary pharmacokinetic study. In this study, T-2 toxin (0.4 mg/kg) was administered intravenously (i.v.) to a dog (mongrel, 20 kg). T-2 toxin was very rapidly biotransformed to HT-2 toxin and the plasma levels of the two toxins are shown in Figs. 4 and 5. In this study, T-2 was found to have a very short half-life of 2.1 min, while the terminal half-life of HT-2 toxin was 41.1 min. Although T-2 and HT-2 toxins are usually administered by oral or percutaneous routes, the i.v. administration is fundamental and essential for pharmacokinetic studies of the two toxins after any mode of administration.

The proposed method is very convenient and specific for the routine assay of

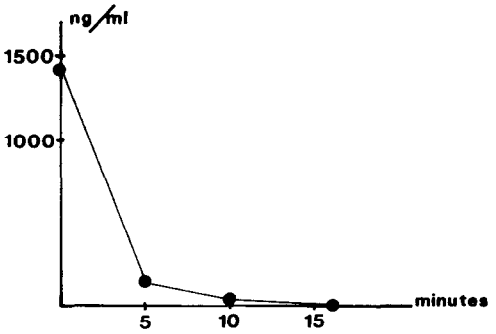


Fig. 4. Plasma levels of T-2 toxin obtained after i.v. administration of T-2 toxin (0.4 mg/kg) to a dog.

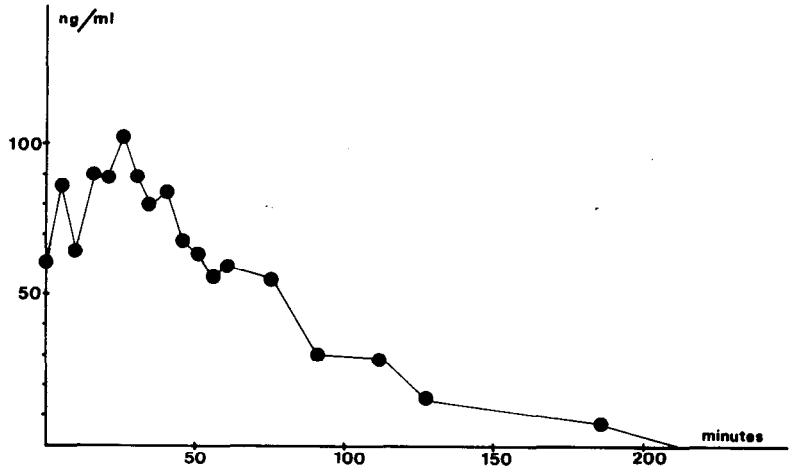


Fig. 5. Plasma levels of HT-2 toxin obtained after i.v. administration of T-2 toxin (0.4 mg/kg) to a dog.

T-2 and HT-2 toxins in plasma. This assay should be very useful in pharmacokinetic studies of these toxins. Using this assay the pharmacokinetic profile of T-2 toxin and its metabolite HT-2 toxin after intravenous administration of T-2 toxin to a dog has been measured for the first time.

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