Journal of Chromatography, 248 (1982) 456–460
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,142

Note

Gas chromatographic method for the determination of diacetoxyscirpenol in swine plasma and urine

S. P. SWANSON*, L. TERWEL, R. A. CORLEY and W. B. BUCK
University of Illinois, Department of Veterinary Biosciences, 1101 West Peabody, Urbana, IL 61801 (U.S.A.)
(Received June 28th, 1982)

Diacetoxyscirpenol (DAS) belongs to a group of mycotoxins called the 12,13-epoxytrichothecenes which consist of closely related sesquiterpenoids produced by several imperfect fungi[1]. The chemical structure of DAS is given in Fig. 1. DAS was first isolated from cultures of *Fusarium scirpi*, *Fusarium equisetti*, and *Gibberella intricans*[2]. At present, about 45 trichothecene derivatives are known of which only a few have actually been associated with natural outbreaks of mycotoxicoses. T-2 toxin[3,4], DAS[4], nivalenol[5], and deoxynivalenol[4–7] are the trichothecenes most commonly found in naturally contaminated feed. The signs of trichothecene *Fusarium* intoxication include: emesis, lack of weight gain, lethargy, diarrhea, feed refusal, necrosis, hemorrhaging, and death[1,3,6–8,11]. DAS has been found at levels of 400 to 500 ppb* in a mixed feed associated with hemorrhagic bowel syndrome in swine[4].

Until now, little has been known about the absorption, distribution, and elimination of DAS in animals. In order to conduct toxicokinetic studies in domestic animals, sensitive methods for detection of DAS in blood, urine, and tissues are required. Methods have been developed for analysis of DAS in feedstuffs[9–15]; however, no methods are available for detection of DAS in biological samples such as tissue and body fluids. This paper describes a method for detection of DAS in swine blood and urine.

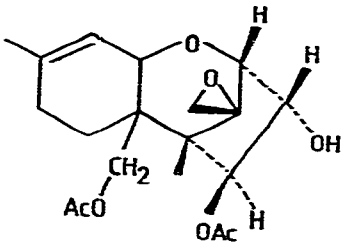

Fig. 1. Chemical structure of DAS.

* Throughout this article the American billion (10^9) is meant.

0021-9673/82/0000–0000/$02.75

EXPERIMENTAL

Chemicals

DAS was obtained from MycoLab (Chesterfield, MO, U.S.A.). Heptafluorobutyrylimidazole (HFBI) was purchased from Pierce Chemical (Rockford, IL, U.S.A.). The internal standard methoxychlor (MXCL) was a gift from the US Environmental Protection Agency. All solvents were "distilled in glass" quality (Burdick & Jackson Labs., Muskegon, MI, U.S.A.).

Florisil (60–100 mesh) was activated for 12 h at 120°C.

Apparatus

A Hewlett-Packard 5840A gas chromatograph equipped with a 1.8 m × 2 mm I.D. glass column packed with 3% OV-1 on 100–120 mesh Supelcoport and a ^{63}Ni electron-capture detector was used. The gas chromatographic operating conditions were as follows: column 215°C, injector 220°C, detector 325°C, argon–methane (95:5) carrier-gas flow-rate at 22 ml/min.

A Vac Elut extraction manifold (Analytichem International, Harbor City, CA, U.S.A.) was used with 3 ml reversed-phase C_{18} cartridges containing 200 mg packing. Florisil columns were prepared by packing 7 ml polypropylene columns which have 15-ml reservoirs (Supelco, Bellefonte, PA, U.S.A.) with 1 g Florisil and 1 cm anhydrous sodium sulfate on top and bottom.

Extraction and cleanup

A C_{18} cartridge was inserted into the vacuum manifold and rinsed in sequence with 2 column volumes of methanol followed by 2 column volumes of water. A 1-ml volume of water was pipetted onto the cartridge followed by 2.0 ml plasma (or 0.5 ml urine). The cartridge was rinsed with 3 ml water followed by 2 ml methanol–water (3:7, v/v). The stainless-steel needles were wiped clean and DAS was then eluted with 2 × 0.6 ml methanol–water (8:2, v/v). One ml aqueous sodium chloride solution (7.5%, w/v) was added to the eluate, and the mixture partitioned 3 times (4 + 2 + 2 ml) with toluene–ethyl acetate (9:1, v/v). A 10-ml volume of hexane was added to the combined toluene–ethyl acetate layers, and the mixture transferred to a Florisil column. Florisil columns were prerinsed with 10 ml chloroform–methanol (95:5) and 10 ml hexane immediately prior to use. At no time was the column allowed to run dry. The Florisil column was rinsed with 4 ml dichloromethane followed by 8 ml chloroform–acetone (97:3). DAS was eluted with 15 ml chloroform–methanol (95:5) into a 17-ml screw-top test tube. The eluate was concentrated to dryness under nitrogen and gentle heat and the residue redissolved in 0.5 ml toluene.

Derivatization

A 50-μl volume of HFBI was added to the toluene solution. After mixing 0.5 ml internal standard solution (1 μg MXCL/ml toluene), 7.0 ml hexane and 1 ml bicarbonate solution (5% $NaHCO_3$) were added, and the mixture vortexed until the top layer was clear. Samples were centrifuged for 5 min at 1000 rpm and 4 μl of the top layer (equivalent to 1.0 μl plasma or 0.25 μl urine) were injected into the gas chromatograph.

RESULTS AND DISCUSSION

Initial experiments were conducted using a benzene extraction, base partitioning, and Florisil chromatography prior to gas chromatography as described for the analysis of T-2 in plasma[13]. This procedure, however, did not provide adequate cleanup of extracts. A compound was detected in control samples which eluted just after but could not be separated completely from DAS. This peak was variable in amount and interfered with accurate quantitation.

The use of C_{18} reversed-phase silica cartridges, which provide simultaneous extraction and cleanup was then employed. Recovery experiments demonstrated that DAS was efficiently retained from urine and plasma samples by the reversed-phase C_{18} silica cartridge. No loss of DAS occurred during the water or 30% methanol wash and nearly 100% of DAS was recovered in the 80% methanol eluate. The use of C_{18} cartridges not only decreases the time of analysis but also efficiently removed the interfering compound present in benzene extracts.

All samples were centrifuged immediately prior to analysis in order to remove any precipitate present which impeded flow of solvent through the column. Urine contained larger amounts of sample interferences than plasma samples. These interfering compounds overloaded the C_{18} cartridge when urine volumes of 1 ml or larger were analyzed, resulting in decreased recoveries proportional to the concentration of DAS present in the samples. Recoveries of only 55% were observed at concentrations of 500 and 1000 ng/ml when sample sizes of 1 ml were analyzed. This effect was not observed when a urine sample size of 0.5 ml was used.

Initially, the C_{18} 80% methanol eluates were concentrated to dryness and residue redissolved in chloroform prior to Florisil column chromatography. This procedure, however, gave inconsistent recovery with plasma and urine, although recoveries of DAS-spiked water blanks were usually good. The inconsistency in recovery was due to a small amount of residue remaining after the concentration step which was insoluble in chloroform. This insoluble residue prevented complete transfer of DAS to the Florisil column resulting in poor recovery. The elimination of this concentration step and the substitution of a partitioning step resulted in improved

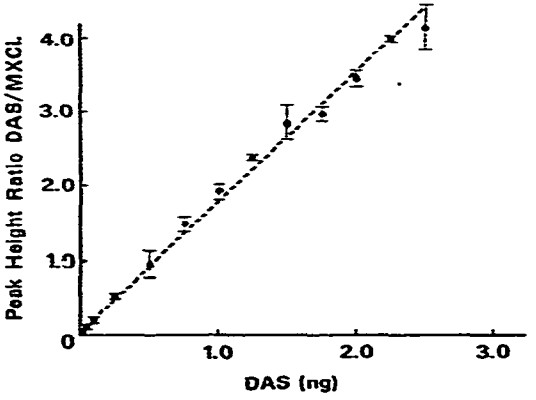

Fig. 2. Standard curve plot of DAS amount (ng) in sample injected *vs.* ratio of HFB-DAS/MXCL peak heights.

TABLE I

RECOVERY OF DIACETOXYSCIRPENOL FROM SWINE PLASMA AND URINE

Sample	*Added (ng/ml)*	*n*	*Recovery (%)*	*S.D.*
Plasma	25	6	90.9	8.0
	100	6	95.5	5.4
	250	8	88.7	6.6
	500	6	87.4	9.0
	1000	6	88.4	9.3
Urine	100	5	95.5	6.7
	500	5	92.2	4.6
	1000	5	89.2	8.0
Total		47	91.0	7.2

recovery and reproducibility. The addition of sodium chloride to the eluate followed by 3 extractions with toluene–ethyl acetate (9:1) gave virtually complete recovery of DAS. Adding hexane to the toluene–ethyl acetate extract eliminated the need to concentrate the extracts prior to Florisil chromatography, reducing the overall time of analysis. The gas chromatograph and derivatization steps were derived from a method originally developed for analysis of T-2 toxin and DAS in animal feeds by Romer *et al.*[9] and revised by Swanson *et al.*[10] for analysis of T-2 toxin in plasma. Complete derivatization of DAS in the sample extracts was accomplished with 50 μl HFBI in less than 30 sec at room temperature. Methoxychlor was added as a gas

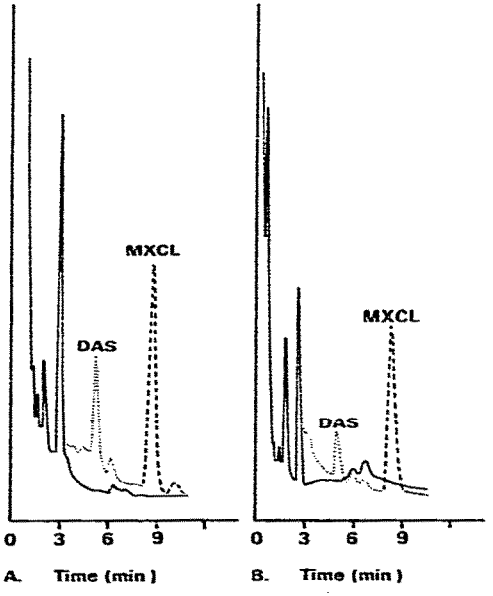

Fig. 3. Gas chromatograms of (A) plasma spiked with 250 ng/ml DAS and (B) urine spiked with 500 ng/ml DAS. MXCL was added as an internal standard. Solid lines refer to responses of blanks and dotted lines the spiked samples.

chromatographic internal standard, the amount injected was equivalent to 250 pg. The minimum amount of standard DAS detectable per injection was 15 pg.

The standard curve of DAS is presented in Fig. 2. Responses were calculated by peak height of the DAS peak relative to the peak height of methoxychlor. The upper limit of the linear range for DAS was 2.25 ng injected. Analyses of the data points by linear regression gave a correlation coefficient of 0.995.

Gas chromatographic conditions were adjusted to provide optimal separation in a minimal amount of time. At a column temperature of 215°C, DAS and methoxychlor eluted at 5.38 and 8.72 min, respectively. Total gas–liquid chromatography analysis time was less than 12 min per sample.

Recoveries of DAS added to plasma at concentrations ranging from 25 to 1000 ng/ml averaged 90.1 $\pm$ 7.8% and recoveries from urine averaged 92.3 $\pm$ 6.7% at concentrations from 100 to 1000 ng/ml. The combined overall recovery (Table I) for both urine and plasma was 91.0%.

Gas chromatograms of swine plasma and urine extracts are given in Fig. 3. These chromatograms represent the equivalent of 1.0 μl of plasma and 0.25 μl of urine. No interfering peaks were detected at the retention times of DAS or methoxychlor nor were any late eluting peaks observed, which could interfere with subsequent detection. Detection limits for DAS in plasma were 20 ng/ml and for urine 80 ng/ml. If greater sensitivity is required, the use of larger C_{18} reversed-phase cartridges (500 mg packing *vs.* 200 mg) would probably allow the extraction of larger sample sizes resulting in a corresponding reduction in the detection limit.

The method as described has also been used for analysis of plasma from cattle with no differences found when compared to swine samples. We therefore anticipate no difficulties in application of this method to other animals.

At present, our laboratory is involved in a toxicokinetic study of DAS in swine and cattle. Results of these experiments will be presented in a separate publication.

REFERENCES

1 S. V. Pathre and C. J. Mirocha, *J. Amer. Oil Chem. Soc.*, 56 (1979) 820–823.
2 P. W. Brian, A. W. Dawkins, J. F. Grove, H. G. Hemming, D. Lowe and G. L. F. Norris, *J. Exp. Botany*, 12 (1961) 1–12.
3 I. C. Hsu, E. B. Smalley, F. M. Strong and W. E. Ribelin, *Appl. Microbiol.*, 24 (1972) 684–690.
4 C. J. Mirocha, S. V. Pathre, B. Schauerhamer and C. M. Christensen, *Appl. Envir. Microbiol.*, 32 (1976) 553–556.
5 N. Morooka, N. Uratsuji, T. Yoshizawa and H. Yamamoto, *Jpn. J. Food Hyg.*, 13 (1972) 368–375.
6 R. F. Vesonder, A. Giegler and A. H. Jensen, *Appl. Microbiol.*, 26 (1973) 1008–1010.
7 H. L. Trenholm, W. P. Cochrane, H. Cohen, J. I. Elliot, E. R. Farnsworth, D. W. Friend, R. M. G. Hamilton, G. A. Neish and J. F. Standish, *J. Amer. Oil Chem. Soc.*, 58 (1981) 992A.
8 G. A. Weaver, H. J. Kurtz, C. J. Mirocha, F. Y. Bates and J. C. Behrens, *Can. Vet. J.*, 19 (1978) 267–271.
9 H. Kamimura, M. Nishijima, K. Yasuda, K. Saito, A. Ibe, T. Nagayama, H. Ushiyama and Y. Naoi, *J. Ass. Offic. Anal. Chem.*, 64 (1981) 1067–1073.
10 T. Romer, T. M. Boling and J. L. MacDonald, *J. Ass. Offic. Anal. Chem.*, 61 (1978) 801–808.
11 R. Schmidt, E. Ziegenhagen and K. Dose, *J. Chromatogr.*, 212 (1981) 370–373.
12 Cs. Szathmáry, J. Galácz, L. Vida and G. Alexander, *J. Chromatogr.*, 191 (1980) 327–331.
13 S. Swanson, V. Ramaswamy, V. R. Beasley, W. Buck and H. H. Burmeister, *J. Ass. Offic. Anal. Chem.*, submitted for publication.
14 H. M. Stahr, A. A. Kraft and M. Schuh, *Appl. Spectroscopy*, 33 (1979) 294–297.
15 Y. Takeda, E. Isohata, R. Amano and M. Uchiyama, *J. Ass. Offic. Anal. Chem.*, 62 (1979) 573–578.