ELSEVIER

Journal of Chromatography A, 692 (1995) 39–43

JOURNAL OF CHROMATOGRAPHY A

Liquid chromatographic determination of the mycotoxin fumonisin B_2 in physiological samples

G.S. Shephard*, P.G. Thiel, E.W. Sydenham

Programme on Mycotoxins and Experimental Carcinogenesis, Medical Research Council, P.O. Box 19070, Tygerberg 7505, South Africa

Abstract

The fungus *Fusarium moniliforme* produces a group of mycotoxins, the fumonisins, of which the most abundant are fumonisins B_1 (FB_1) and B_2 (FB_2). Previously developed analytical methods for the determination of FB_1 in physiological samples have been modified for the determination of FB_2 by the use of less polar extraction solvents. Plasma and urine extracts were purified on strong anion-exchange solid-phase extraction cartridges and fecal extracts on reversed-phase (C_{18}) cartridges. FB_2 in purified extracts was determined by reversed-phase HPLC with fluorescence detection using preformed *o*-phthaldialdehyde derivatives. These methods were reproducible (R.S.D. of less than 6%) with recoveries greater than 85%. In a short preliminary study, they have been applied to the determination of the fate of FB_2 dosed to rats by gavage. Of the dose given to the animals, over 90% was recovered unmetabolised in the feces within 48 h.

1. Introduction

The fumonisin mycotoxins are a group of structurally related secondary metabolites produced by the fungus *Fusarium moniliforme* Sheldon, a common contaminant of corn worldwide [1]. Of the fumonisins currently identified, fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2) are the major compounds produced in culture and found in contaminated feed and foodstuffs [2,3]. Both toxins are diesters of propane-1,2,3-tricarboxylic acid and similar long-chain polyhydroxyamines [1] (Fig. 1).

FB_1 has been shown to cause equine leukoencephalomalacia [4] and porcine pulmonary edema [5]. It is also hepatocarcinogenic and hepatotoxic in rats [1,6] and has been statistically associated with an increased risk of esophageal cancer in man [7]. As FB_2 is generally found at significant levels together with FB_1 in contaminated food [3,8], and since it possesses similar cancer initiating and cytotoxic properties to FB_1

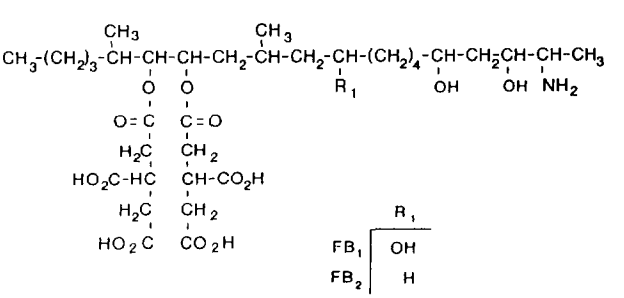

Fig. 1. Structures of fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2).

* Corresponding author.

0021-9673/95/$09.50 © 1995 Elsevier Science B.V. All rights reserved
SSDI 0021-9673(94)00559-R

[9], it may also be expected to have importance to human health. Toxicokinetic studies have been undertaken with FB_1 in laboratory animals [10–15]. In order to extend these studies to FB_2, analytical methods, developed for FB_1 [14,16], have been modified and validated for the determination of FB_2 in plasma, urine and feces. This paper reports the development and validation of these methods and their application to a short preliminary study on the excretion of FB_2 by experimental rats.

2. Experimental

2.1. Reagents

Bond-Elut strong anion-exchange (SAX) and reversed-phase (C_{18}) solid-phase extraction cartridges (3 ml capacity containing 500 mg sorbent) were purchased from Varian (Harbor City, CA, USA). FB_2 was isolated from *F. moniliforme* MRC 826 cultures [17]. ^{14}C-labelled FB_2 ([^{14}C]FB_2; 64 μCi mmol^{-1}) was prepared by spiking a growing corn culture of *F. moniliforme* MRC 826 with L-[methyl-^{14}C]methionine (>50 mCi mmol^{-1}; Amersham, UK) [18]. The radiochemical purity of the [^{14}C]FB_2 was assessed by TLC [17]. The FB_2 spot on the TLC plate contained 92% of the applied radioactivity. All other reagents and solvents were analytical grade from Merck (Darmstadt, Germany).

2.2. Sample collection

Physiological samples (blood, urine and feces) were obtained from rats and monkeys bred in the animal unit of the Medical Research Council (Tygerberg, South Africa). Blood samples were collected in tubes containing tripotassium EDTA as anticoagulant. Plasma was obtained by centrifugation at 1200 *g* for 10 min at 4°C. In a short preliminary study on the excretion of [^{14}C]FB_2 by rats, two male Wistar rats (approximately 100 g body mass) were each dosed by gavage with 0.12 mg FB_2 in a water solution and confined in a single metabolic cage for the collection of urine and feces daily over a 2-day period.

Samples for method development were obtained by spiking plasma, urine, and fecal extracts with FB_2 standard to a level of between 3 and 5 μg ml^{-1}. Fecal samples obtained from the preliminary study on excretion were used to investigate the extraction of FB_2 from rat feces. The radioactivity present in samples was determined by liquid scintillation counting after solubilization [14].

2.3. Determination of FB_2 in plasma

A 500-μl aliquot of plasma was diluted with 1 ml of water and deproteinised with 1.5 ml of acetonitrile. After centrifugation at 1200 *g* for 10 min at 10°C, a 2-ml aliquot of the supernatant was purified on a SAX cartridge which had been conditioned with 5 ml of acetonitrile and 5 ml of acetonitrile–water (1:1, v/v). The sorbent was immediately washed with 5 ml of acetonitrile–water (1:1, v/v) and 5 ml of methanol. FB_2 was eluted with 10 ml of 5% acetic acid in methanol. The flow-rate through the cartridge was maintained at around 1–1.5 ml min^{-1}. The eluate was dried under a stream of nitrogen at 60°C and the residue was redissolved in 200 μl of 0.1 *M* sodium borate prior to derivatization and HPLC analysis.

2.4. Determination of FB_2 in urine

A 250-μl aliquot of rat urine was diluted with 750 μl of water and 3 ml of methanol. This diluted sample was purified on a SAX cartridge, conditioned with 5 ml of methanol and 5 ml of methanol–water (3:1, v/v). After washing the sorbent with 5 ml of methanol–water (3:1, v/v) and 5 ml of methanol, the FB_2 was eluted and dried as described above for plasma extracts. The residue was dissolved in 200 μl of methanol prior to HPLC analysis.

2.5. Determination of FB_2 in feces

Feces were freeze-dried after collection and then ground to a powder. A subsample (1.5–2.5

g) was extracted by vortexing for 1 min in a capped tube with 15 ml 0.1 *M* EDTA (pH 5.2)–methanol (4:1, v/v). The mixture was centrifuged at 2000 *g* for 10 min at 4°C, the supernatant removed and the extraction repeated a further 9 times. The supernatants were combined, centrifuged at 4000 *g* for 10 min. and then an aliquot was acidified with 5 *M* hydrochloric acid to pH 3.1–3.2. Immediately thereafter a 3-ml aliquot was applied to a Bond-Elut C_{18} cartridge which had been conditioned with 5 ml of methanol and 5 ml of water. The sorbent was washed with 5 ml of water, 5 ml of methanol–water (1:3, v/v) and 3 ml of methanol–water (1:1, v/v). FB_2 was eluted with 15 ml of methanol and the solvent evaporated under a stream of nitrogen at 60°C. The residue was dissolved in 0.5 ml of methanol prior to HPLC analysis.

2.6. Chromatographic analysis

FB_2 in the purified extracts was quantified by HPLC of preformed *o*-phthaldialdehyde (OPA) derivatives on a Phenomenex (Rancho Palos Verdes, CA, USA) C_{18} reversed-phase IB-SIL column (50 × 4.6 mm I.D.) packed with material of 3 μm particle size and eluted at 1 ml min^{-1} with methanol–0.1 *M* sodium dihydrogenphosphate (72:28, v/v) adjusted to pH 3.4 with orthophosphoric acid. The remainder of the HPLC system and the preparation of OPA derivatives were as previously described [16].

3. Results and discussion

The analytical methods described here for the determination of FB_2 in physiological samples were developed by modification of previously published methodology for FB_1 [14,16]. Since FB_2 is less polar than FB_1, consideration was given to the use of less polar extraction solvents. In the extraction of FB_2 from plasma, use of methanol for deproteinisation resulted in recoveries of 80.4% ± 4.5% R.S.D. ($n = 5$). Substitution of acetonitrile for methanol resulted in an increase in recovery to 90.3% ± 2.5% R.S.D. ($n = 8$) at the 4.3 μg ml^{-1} level. Six replicate analyses of a single sample of plasma gave precision of 2.1% R.S.D. at this level.

The analytical method developed for the determination of FB_1 in urine was found to be equally valid for FB_2. Analytical recovery of FB_2 was 101.5% ± 2.2% R.S.D. ($n = 6$) at the 5.0 μg ml^{-1} level and replicate analyses of a single urine sample gave a precision of 2.9% R.S.D. ($n = 6$).

It has previously been shown that FB_1 is strongly retained within the fecal matrix of rats and, to a lesser extent, also of monkeys [14]. Similarly, FB_2 was found to be strongly bound within this matrix and repeated extractions were required to achieve adequate analytical recovery of the toxin. Methanol was again inadequate as an extraction solvent. The efficiency of 0.1 *M* EDTA (pH 5.2), previously used for fecal extractions, was slightly improved by the addition of 20% methanol, although nine extractions were required to achieve extraction efficiencies greater than 90% (Fig. 2). The fecal extracts were combined and the pH was adjusted to 3.2 prior to clean-up on C_{18} solid-phase extraction cartridges. At pH values below 3.1 considerable precipitation of EDTA occurred and the recovery of FB_2 was deleteriously affected. Analytical recovery from the fecal extract deter-

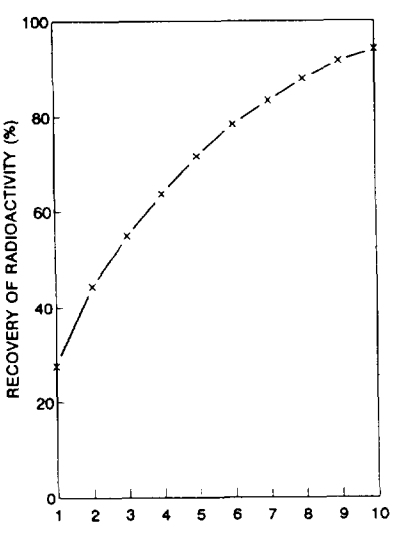

Fig. 2. Cumulative extraction recovery of [^{14}C]FB_2 from rat feces as a percentage of the radioactivity in the sample.

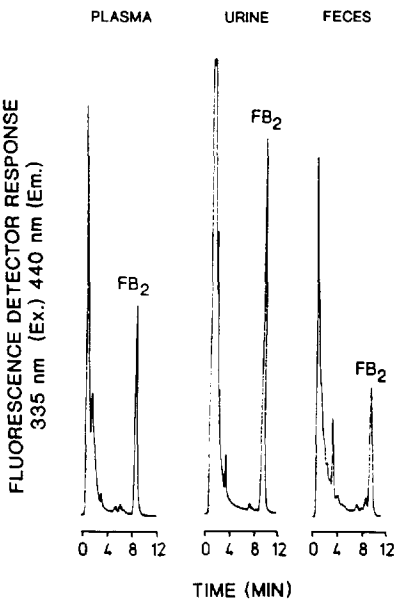

Fig. 3. Chromatograms of OPA-derivatised plasma, urine and fecal samples. The amount of FB_2 injected ranged from 20 to 45 ng.

mined at the 3.1 μg ml^{-1} level was 86.4% ± 4.6% R.S.D. ($n = 6$) and the precision of FB_2 determination from a single extract was 4.6% R.S.D. ($n = 6$).

Fig. 3 shows chromatograms obtained from each of the three physiological samples. In all analyses, FB_2 was separated from other components which mainly elute near the front-end of the chromatogram. The absence of co-eluting impurities was demonstrated in all cases by analysis of blank samples.

Table 1 shows the results of a preliminary study of excretion of [^{14}C]FB_2 by two rats dosed by gavage. Most of the dosed toxin was recovered unmetabolised in a 48-h period, mainly from the feces with trace amounts in urine. The levels of FB_2 were corrected for extraction and analytical recoveries and show a close correspondence to the radioactivity levels determined in the samples.

The methods reported here for the determination of FB_2 have been shown to be reproducible with good recoveries. As in the case of FB_1, FB_2 was strongly retained in feces and required multiple extraction steps to achieve acceptable recoveries. The preliminary study of the excretion of FB_2 by rats has indicated that the methods described here will yield further insights into the toxicokinetics of the fumonisins in experimental animals.

Acknowledgements

The authors wish to thank J.V. Seier, P.W. De Lange and H.J.B. Joubert for technical assistance in the handling of laboratory animals.

Table 1
Excretion of [^{14}C]FB_2 by rats

Day	Urine		Feces	
	FB_2 [a] (% of dose)	^{14}C [b] (% of dose)	FB_2 [a] (% of dose)	^{14}C [b] (% of dose)
1	0.2	0.7	72.9[c]	68.4
2	<0.1	0.3	23.1[c]	30.5
Total	0.2	1.0	96.0	98.9

[a] Determined by HPLC.
[b] Determined by liquid scintillation counting.
[c] Corrected for extraction and analytical recovery.

References

[1] W.C.A. Gelderblom, K. Jaskiewicz, W.F.O. Marasas, P.G. Thiel, R.M. Horak, R. Vleggaar and N.P.J. Kriek, *Appl. Environ. Microbiol.*, 54 (1988) 1806.

[2] P.G. Thiel, W.F.O. Marasas, E.W. Sydenham, G.S. Shephard, W.C.A. Gelderblom and J.J. Nieuwenhuis, *Appl. Environ. Microbiol.*, 57 (1991) 1089.

[3] E.W. Sydenham, G.S. Shephard, P.G. Thiel, W.F.O. Marasas and S. Stockenström, *J. Agric. Food Chem.*, 39 (1991) 2014.

[4] T.S. Kellerman, W.F.O. Marasas, P.G. Thiel, W.C.A. Gelderblom, M. Cawood and J.A.W. Coetzer, *Onderstepoort J. Vet. Res.*, 57 (1990) 269.

[5] L.R. Harrison, B.M. Colvin, J.T. Greene, L.E. Newman and J.R. Cole, *J. Vet. Diagn. Invest.*, 2 (1990) 217.

[6] W.C.A. Gelderblom, N.P.J. Kriek, W.F.O. Marasas and P.G. Thiel, *Carcinogenesis*, 12 (1991) 1247.

[7] E.W. Sydenham, P.G. Thiel, W.F.O. Marasas, G.S. Shephard, D.J. Van Schalkwyk and K.R. Koch, *J. Agric. Food Chem.*, 38 (1990) 1900.

[8] P.F. Ross, L.G. Rice, G.D. Osweiler, P.E. Nelson, J.L. Richard and T.M. Wilson, *Mycopathologia*, 117 (1992) 109.

[9] W.C.A. Gelderblom, M.E. Cawood, S.D. Snyman, R. Vleggaar and W.F.O. Marasas, *Food Chem. Toxicol.*, 31 (1993) 407.

[10] G.S. Shephard, P.G. Thiel and E.W. Sydenham, *Food Chem. Toxicol.*, 30 (1992) 277.

[11] G.S. Shephard, P.G. Thiel, E.W. Sydenham, J.F. Alberts and W.C.A. Gelderblom, *Toxicon*, 30 (1992) 768.

[12] G.S. Shephard, P.G. Thiel, E.W. Sydenham and J.F. Alberts, *Food Chem. Toxicol.*, 32 (1994) 489.

[13] W.P. Norred, R.D. Plattner and W.J. Chamberlain, *Nat. Toxins*, 1 (1993) 341.

[14] G.S. Shephard, P.G. Thiel, E.W. Sydenham, R. Vleggaar and J.F. Alberts, *Food Chem. Toxicol.*, 32 (1994) 23.

[15] G.S. Shephard, P.G. Thiel, E.W. Sydenham, J.F. Alberts and M.E. Cawood, *Toxicon*, in press.

[16] G.S. Shephard, P.G. Thiel and E.W. Sydenham, *J. Chromatogr.*, 574 (1992) 299.

[17] M.E. Cawood, W.C.A. Gelderblom, R. Vleggaar, Y. Behrend, P.G. Thiel and W.F.O. Marasas, *J. Agric. Food Chem.*, 39 (1991) 1958.

[18] J.F. Alberts, W.C.A. Gelderblom, R. Vleggaar, W.F.O. Marasas and J.P. Rheeder, *Appl. Environ. Microbiol.*, 59 (1993) 2673.