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The determination of fumonisin B_1 in human faeces: a short term marker for assessment of exposure

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Fumonisin B_1 (FB_1) is a compound that occurs frequently in rural foods and feeds, creating health hazards. When ingested, FB_1 does not appear to change in structure and is mostly excreted unchanged in faeces within 24 h. Twenty human stool samples obtained from rural school children of Vulamehlo, south of Durban (South Africa), were analysed for FB_1, as well as 23 urban control samples obtained from various households within the Durban metropolitan area. The samples were freeze-dried and ground to a fine powder. A fraction of each sample was extracted three times with aqueous ethylenediaminetetraacetic acid at pH 5.2. The pooled extracts were purified using reversed phase C_{18} solid phase extraction cartridges. Analytical high performance liquid chromatography was used to quantitate the amount of FB_1 as an *o*-phthaldialdehyde (OPA) derivative in the extracts. The rural (35%) and the urban samples (9%) showed the presence of FB_1 ranging from 790 to 19 560 ng g^{-1} of freeze dried stool. It was concluded that this method could be used as a routine biomarker for short term human exposure to FB_1 in contaminated food.

Keywords: fumonisin B_1, faeces, biomarker, exposure.

Introduction

Fumonisin B_1 (FB_1) is one of the related group of mycotoxins which is produced mainly by *Fusarium moniliforme*, which commonly occurs worldwide in maize-based foodstuffs and feeds. Other prominent producers include: *F. proliferatum, F. nygamai,* and *F. napiforme*. Some members of the genus *Alternaria* are also known to produce FB_1 (Chen *et al.* 1992). Fusaria infect maize and occasionally sorghum, millet and other grains grown throughout the world (Marasas *et al.* 1984). Several types of fumonisins have been isolated from *F. moniliforme* cultures: fumonisins A_1, A_2, B_1, B_2, B_3, B_4 and C_1 (Gelderblom *et al.* 1992a, Branham and Plattner 1993). Fumonisin B_1 is the most toxic and the most abundant representative of the known fumonisins. It has been associated with a number of animal diseases such as equine leukoencephalomalacia (ELEM) (Marasas *et al.* 1988), porcine pulmonary oedema (PPE) (Harrison *et al.* 1990), liver and kidney toxicity in rat (Riley *et al.* 1994a, Gelderblom *et al.* 1996), human oesophageal cancer in South Africa (Rheeder *et al.* 1992, Myburg 1998) and China (Chu and Li 1994) and immunosuppression in chickens (Marijanovic *et al.* 1991). There is no absolute evidence showing that FB_1 is a human carcinogenic, although it has been immunolocalized in human oesophageal tumours (Myburg 1998). However, it appears to be an initiator and promoter of carcinogenesis in rat (Gelderblom *et al.* 1992b) and has been classified as a '2B' carcinogen (IARC 1993).

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Its major toxic effects are based solely on its disruptive effects on sphingolipid biosynthesis, accumulation of sphingoid bases leading to cell membrane damage, as seen, for example, in kidney cells (Yoo *et al.* 1992).

The ubiquitous occurrence of FB_1 in maize and its products, sometimes at elevated levels, presents a potential threat to human and animal health and realistic tolerance levels need to be set. Hence, there is a need to develop effective methods of analysing food and to monitor human exposure using a suitable biomarker. The estimation of free FB_1 in blood is impractical due to its poor absorption and rapid excretion from the gut leading to low plasma levels (Prelusky *et al.* 1994). Furthermore, FB_1 does not seem to readily form conjugates like aflatoxin B_1 (AFB_1) with either DNA or plasma proteins like albumin. Hence methods based on measurement of sphingoid bases, sphinganine (Sa) and sphingosine (So) which accumulate due to the action of the toxin, have been developed (Riley *et al.* 1994b). This approach appears to give some hope of appraising human exposure to FB_1, over a time-scale approaching that given by AFB_1 adducts but a disadvantage seems to be poor and erratic recoveries of the sphingoid bases from physiological fluids and tissues.

As the fumonisins, including FB_1, mainly pass straight through the digestive tract and what is absorbed is mainly excreted in bile (Shephard *et al.* 1994a), it seems reasonable to look for FB_1 and its degradation products in faeces rather than in blood and urine. The added advantage of this is that faecal samples are taken as a matter of routine in hospitals and do not require sick patients to undergo further invasive techniques. Fumonisin B_1 analysis in the faeces of non-human primates and other animals has been carried out previously (Prelusky *et al.* 1994, Shephard *et al.* 1994b, Smith and Thakur 1996). The results showed that when ingested, less than 1% of the administered dose is absorbed from the gastrointestinal tract. This may explain the high levels of contamination required ($>5\ mg\ kg^{-1}$) to produce symptoms of illness (Dutton 1996). Fumonisin B_1 does not appear to undergo any significant biotransformation in the rat and is mostly excreted unmetabolized within 24 h, mainly in faeces (Shephard *et al.* 1992) at the rate of 80% with trace amounts in urine. However, there is evidence that at least three decomposition products exist: one fully hydrolysed form (HFB_1), and two partially hydrolysed forms of FB_1 ($PHFB_1$) (figure 1) (Jackson *et al.* 1996), which could be present in faeces. In this study, analysis of FB_1 in human faeces was carried out to monitor human dietary exposure to this toxin with a view of developing a routine short term marker.

Materials and methods

Study area and population

A total of 43 faecal samples were processed and analysed for FB_1. Twenty of the samples were from Vulamelho, a rural district school south of Durban and 23 other samples were collected from the Durban metropolitan area. The samples were randomly selected from 200 samples, which were collected in the ongoing study of the geohelminth infections in Kwazulu Natal. The geohelminth infection study was carried out at the MRC, Durban, South Africa. Samples for this study were selected after they were examined for helminthic infections. The selection of the samples did not take into consideration whether the included samples were positive or negative for the geohelmiths. A laboratory list of numbered samples was used for selection.

Reagents and materials

All reagents were of analytical grade.

(a) *SPE C_{18} cartridges:* 10 ml capacity (Varian Bond-Elut from Analytichem, Harbour City, CA 90710) containing 500 mg sorbent.

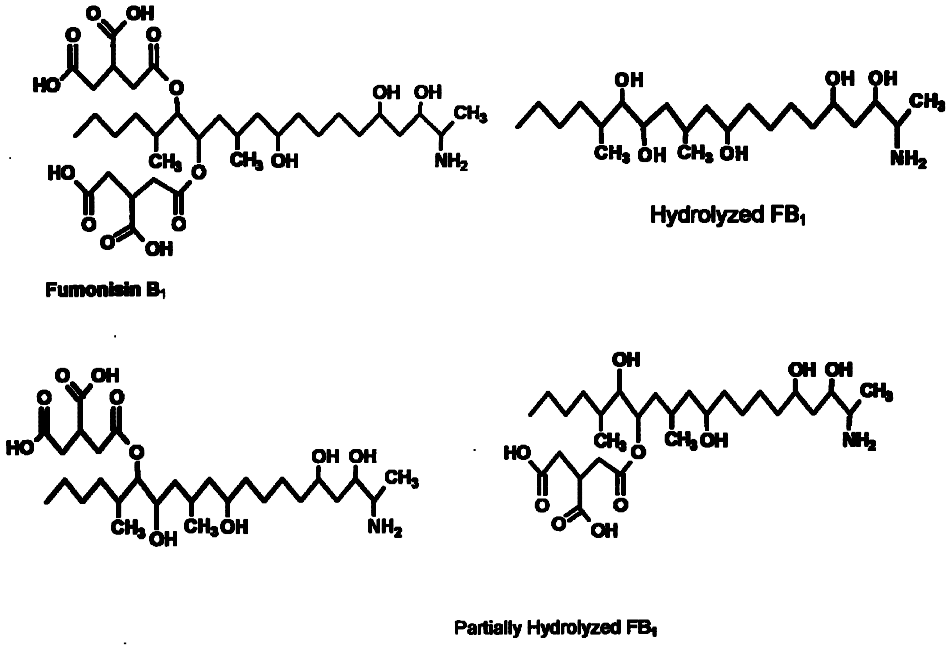

Figure 1. Fumonisin B_1 and its hydrolysed derivatives

(b) *Mobile phase:* Methanol: 0.1 M sodium dihydrogen phosphate (80:20,v/v) adjusted to pH 3.4 with orthophosphoric acid and pumped at a flow rate of 1 ml min^{-1}.

(c) o-*Phthaldialdehyde (OPA) reagent:* 40 mg of OPA dissolved in 1 ml of methanol and diluted with 5 ml of 0.1 M sodium tetraborate and mercapthoethanol (50 µl).

(d) *Solvents:* Acetonitrile/water (1:1, v/v), butanol, acetic acid and methanol, both obtained from BDH Chemicals, Poole, UK.

(e) *o-Phosphoric acid* (concentration >85%), obtained from BDH Chemicals, Poole, UK.

(f) *Anisaldehyde spray :* This was prepared by mixing 70 ml of methanol and 10 ml of acetic acid. Concentrated H_2SO_4 (5 ml) was added followed by 0.5 ml anisaldehyde.

(g) *Fumonisin B_1 standard.* (1 mg ml^{-1} and 50 mg ml^{-1}) dissolved in acetonitrile/water (1:1 v/v). The standard was obtained from PROMEC, Cape Town, South Africa.

(h) *Silica 60 aluminium backed TLC plates:* Obtained from Merck, Darmstadt, Germany.

Extraction and clean-up of samples

Twenty faecal samples obtained from school-children (aged between 6 and 12 years) of Vulamehlo, a rural district south of Durban (South Africa), were analysed for FB_1. The 23 adult volunteers from the Durban metropolitan area were aged between 12 and 60 years. Frozen faecal samples were first lyophilized and then ground to a fine powder. A fraction (1.5 g) of the sample was extracted thrice by vortexing for 1 min in a capped tube with 15 ml of 0.1 M ethylenediaminetetraacetic acid (pH 5.2). The mixture was centrifuged at 2000 *g* for 10 min at 4 °C, the supernatant removed and the extraction repeated a further two times. The supernatants were combined, acidified to pH 2.9–3.2 with 5 M hydrochloric acid and centrifuged at 4000 *g* for 10 min. A supernatant aliquot of 10 ml was applied to a Bond-Elut C_{18} cartridge previously conditioned with 5 ml methanol and 5 ml of water. The sorbent was firstly washed with 5 ml water, followed by 5 ml methanol:water (1:3, v/v) and finally with 3 ml of methanol:water (1:1, v/v). Fumonisin B_1 was eluted with 15 ml of methanol and the solvent evaporated under a stream of nitrogen at 60 °C.

Standard recoveries on spiked samples

Three stool samples (1.5 g) which showed no detectable FB_1 were each spiked with 50 mg of FB_1 in 3 ml of methanol. They were left to dry overnight at room temperature in a fume cupboard and were then extracted as described above.

Thin-layer chromatography (TLC)

The dried extracts were dissolved in 200 µl of acetonitrile:water (1:1, v/v) and an aliquot of 20 µl spotted on silica thin layer chromatography (TLC) plates (10 × 10 cm). Standards (10 µl) of known concentration (5, 10 and 100 µg ml^{-1}) were also spotted on the plate and developed unidimensionally in butanol:water:acetic acid (12:5:3, v/v); dried and sprayed with anisaldehyde. The plates were heated briefly for 3 min at 110 °C. Resolution factors (R_f) were noted and the quantity of FB_1 in the extracts was deduced by comparing the intensity of the purple coloured spots of the samples with those of the known standards.

High performance liquid chromatography (HPLC)

Extracts were further analysed by a high performance liquid chromatography (HPLC) system with Spectra SYSTEM P2000 manual injector pump, Nova-Pak 4 mm C_{18} reversed phase analytical column (150 × 3.9 mm i.d., from Waters, Milford, MA, USA) Spectra SYSTEM FL2000 fluorescent detector. Detector excitation and emission wavelengths were set at 335 and 440 nm respectively.

A sample (25 µl) or standards (50 µg ml^{-1}) were pipetted into a tube and 225 µl of OPA was added and mixed. An aliquot of 20 µl of derivatized sample or standard was injected into the column within 1 min of adding OPA. The mobile phase, methanol:sodium dihydrogen phosphate (80:20, v/v), was run isocratically at the rate of 1 ml min^{-1}. Fumonisin B_1 was identified by its constant retention time. Quantities were deduced by comparing the peak areas of the standards with those of the samples.

Results

Thin layer chromatography

Fumonisin B_1 standards and spiked samples showed a purple visible colour with an R_f of 0.59. Some of the samples had purple spots at R_f 0.63. All the samples had yellow and brown pigments with R_f values of 0.75 and 0.78 respectively. Partial clean up of FB_1 using C_{18} did not remove most of the impurities making the TLC readings difficult. Some samples which appeared positive on TLC were negative on HPLC. This could be due to anisaldehyde spray, which is not specific for fumonisins. Because of these problems, sample analysis by TLC was not regarded as being reliable and was always confirmed by HPLC.

High performance liquid chromatography

The high performance liquid chromatography results (table 1) show that seven out of 20 rural samples had varying levels of FB_1 which ranged from 6.0 to 19.56 mg g^{-1} while the urban samples had only two positive samples (3.5 and 16.2 mg g^{-1}). The detection limit of the method was 0.05 mg g^{-1} and three typical chromatograms are presented in figure 2.

Recovery experiments on spiked faeces showed that 74% of the added FB_1 could be obtained on three extractions with aqueous EDTA. It took nine such extractions to increase the total to 90% (table 2).

Discussion

This is the first report showing the presence of FB_1 in human faeces. The peak period of excretion is not known but it can be concluded that the amount of FB_1 excreted decreases with time. However, the amount of food taken and the frequency of bowel movements may play a role in transit time (clearance) of FB_1 from the body. It would not be possible therefore, to obtain the same concentration value of FB_1 from the samples collected at different times of the day. The HPLC method used in this investigation was developed by Shephard *et al.* (1994b) with

Table 1. Concentration of fumonisin B_1 detected by HPLC in faecal samples of rural school-children from Vulamehlo (V1–V20) and samples collected from the Durban Metropolitan area (T1–T23).

Rural samples	FB_1 (μg g^{-1})	Urban samples	FB_1 (μg g^{-1})
V1	ND	T1	ND
V2	ND	T2	16.2 (± 4.6)
V3	ND	T3	ND
V4	ND	T4	ND
V5	ND	T5	ND
V6	10.0 (± 4.0)	T6	ND
V7	11.0 (± 4.7)	T7	ND
V8	ND	T8	ND
V9	ND	T9	ND
V10	0.79 (± 0.2)	T10	ND
V11	ND	T11	ND
V12	18.4 (± 5.7)	T12	ND
V13	ND	T13	ND
V14	19.56 (±2.8)	T14	ND
V15	ND	T15	ND
V16	14.7 (± 4.4)	T16	3.5 (± 0.9)
V17	0.6 (± 0.1)	T17	ND
V18	ND	T18	ND
V19	ND	T19	ND
V20	ND	T20	ND
		T21	ND
	T22	ND	
	T23	ND	

ND: Not Detected (below the detection limit of 50 ng g^{-1}).

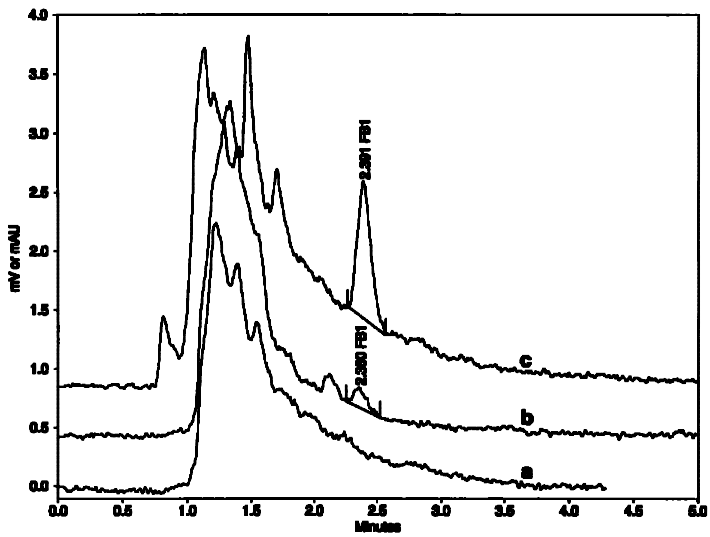

Figure 2. HPLC chromatograms of (a) control freeze dried faeces (1.5 g); (b) FB_1 from contaminated faeces (1.5 g), and (c) FB_1 (50 mg) recovered from 1.5 g freeze dried faeces.

minor modifications. In this procedure, monkey faeces required at least six extractions to obtain approximately full recovery (Shephard *et al.* 1994b) and nine extractions in case of rat and bovine faeces (Shephard *et al.* 1992, 1995, Smith and Thakur 1996). While high recovery is desirable, in this case it resulted in long

Table 2. Recoveries obtained from spiking stool samples (1.5 g) with 50 μg of fumonisin B_1 (50 μg). Analysis was carried out using HPLC.

Sample[a]	Amount recovered (mg)	%Recovery
Extract 1 (3 times)	37.0 (± 2.3)	74
Extract 2 (6 times)	42.5 (± 3.6)	85
Extract 3 (9 times)	45.5 (± 6.2)	91

[a] Three different samples.

extraction procedures and increased expense in both time and the amount of solvent used. In our study, extraction of spiked faecal samples showed that 74% of FB_1 could be recovered in three extractions (table 2). This number of extractions was adopted for all the samples. This had the positive effect of minimizing the amount of contaminating materials also being extracted. It was also observed that addition of methanol into the extraction solvent (Shephard *et al.* 1995) did not improve the recovery while it extracted more impurities, making the analytical procedure difficult. Thin layer chromatography was found not to be very useful as a tool for FB_1 analysis in faeces since the extracts had bile pigments which could have reacted with anisaldehyde spray and had the same R_f as that of FB_1. This led to unnecessary confusion.

Attention has to be given to the possibility of the formation of fumonisin degradation products, which is likely due to the acidic nature of the stomach and the microbial activity of the lower gastrointestinal tract (GIT). Little at the moment is known about the gastrointestinal absorption of FB_1. A limited amount of FB_1 is absorbed into the bloodstream in its intact form. However when it loses the tricarballylic acid side chains by hydrolysis, absorption is enhanced (Hopmans *et al.* 1997) and this may explain the greater toxicity of HFB_1 in comparison to FB_1. One school of thought is that FB_1 is polyanionic and may interfere with its own absorption by binding cations such as sodium, potassium, and other large molecules which are required for active transport across the intestinal membrane. Polycations have been known to inhibit active transport of sugars and amino acids across the intestinal membrane in rats (Elsenhans *et al.* 1983).

Although $PHFB_1$ and HFB_1 (FB_1 degradation products, ffigure 1) have been analysed alongside FB_1, in monitoring FB_1 exposure in animals (Shephard *et al.* 1994b, Smith and Thakur 1996), their concentration in faeces is relatively small in comparison to that of FB_1 and their standards are not readily available. In addition, different chromatographic conditions from those used for FB_1 analysis in this study are necessary in order to separate $PHFB_1$ from FB_1 since they co-elute (Shephard *et al.* 1994b). These render the technique unnecessarily lengthy. In this case, analysis of FB_1 alone was found to be satisfactory.

The results (table 2), whilst encouraging from the development of a biomarker point of view, are quite alarming. Thirty five percent of the rural samples were positive, although it might be argued that the highest concentration of about 20.0 mg g^{-1} of dry faeces is nothing to cause concern. What this represents in terms of dietary uptake, however, is unknown. Clearly, the rural population have greater exposure as they consume locally produced maize which is often contaminated with FB_1 since maize and its products are not subject to any regulatory restrictions in South Africa at the moment. Considering the losses due to degradation during digestion, recovery, dilution effects and the time interval to when food was

consumed, the quantities detected might be equivalent to considerable amounts of fumonisins in the food. Clearly the children from the rural area, who were not regarded as suffering from any disease, are exposed routinely to FB_1, which is resident in their GIT for considerable periods of time, depending upon personal habits. This situation cannot be conducive to good health in later life.

A more interesting result was found in the samples from the urban area in Durban. If positive results were to be found, it was predicted that these would be from the rural population since maize is the staple food. Surprisingly, two volunteer subjects (9%, table 1) from the urban area were positive at levels in the higher range of contamination (3.5 and 16.2 mg g^{-1} respectively). Fisher's exact test showed that the rural group is six times more at risk to FB_1 exposure than the urban group. Whether this represents a real situation remains to be seen from further studies. It is possible that the positive volunteers could have travelled from the rural area to the city after ingesting contaminated food. It is usual, however, for urbanized black people to eat maize products, presumably from urban stores, and, therefore, may be complacent about its quality.

In conclusion, it would seem that the measurement of FB_1 in human faeces is the basis for a short term biomarker for exposure to FB_1. Ironically, it may very well become a way of determining the quality of maize being consumed by a particular population. Work currently being carried out in the rural Kwazulu Natal, which involves extensive sampling and comparison of biomarker methods, may resolve many of the questions raised in this study.

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