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Fumonisin mycotoxins in human hair

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This study shows for the first time the accumulation of fumonisin mycotoxins in human hair of population clusters exposed to contaminated maize, and thus the feasibility of human hair analysis for the assessment of past fumonisin exposure. Composite hair samples were obtained from the Bizana, Butterworth and Centane districts within the Transkei region of the Eastern Cape Province of South Africa. Following methanol extraction and strong anion exchange clean up, the fumonisins FB₁, FB₂ and FB₃ were detected using high performance liquid chromatography coupled to electrospray ionization-mass spectrometry (HPLC-ESI-MS). Hair from Centane and Butterworth showed mean levels of FB₁ of 26.7 and 23.5 $\mu g \, kg^{-1}$ hair, respectively. FB₂ was only detected in hair from Centane and in one sampling point in Butterworth, with mean levels of 6.5 and 5.7 $\mu g \, kg^{-1}$ hair, respectively. Hair samples from Bizana, on the other hand, were found to contain higher levels of FB₁ (mean 33.0 $\mu g \, kg^{-1}$ hair) and FB₂ (mean 11.1 $\mu g \, kg^{-1}$ hair). No samples contained more than trace levels of FB₃. Recoveries from spiked hair samples using this method ranged from 81% to 101%, demonstrating the applicability of hair analysis in assessing human exposure to fumonisin mycotoxins.

Keywords: fumonisins, human hair, biomarker, chronic exposure, Fusarium verticillioides, high performance liquid chromatography-tandem mass spectroscopy.

Introduction

The fumonisin mycotoxins are toxic and carcinogenic secondary metabolites produced primarily by the seed-borne fungus *Fusarium verticillioides* (Sacc.) Nirenberg (formerly known as *F. moniliforme* Sheldon) that commonly infects corn throughout the world (Gelderblom *et al.* 1988). Human exposure to the fumonisins occurs mainly by the consumption of contaminated corn or corn-based foods. The fumonisins have been shown to affect several target tissues in animals, resulting in disease syndromes such as equine leukoencephalomalacia and porcine pulmonary oedema as well as causing nephrotoxicity, hepatotoxicity and hepatocellular carcinoma in rats (Marasas 1995). The International Agency for Research on Cancer (IARC) has classified the toxins produced by *F. moniliforme* as possible human carcinogens (group 2B) (Vainio *et al.* 1993). The B series of the fumonisin analogues consists mainly of fumonisin B₁ (FB₁), fumonisin B₂ (FB₂) and fumonisin B₃ (FB₃). These have similar toxicological profiles, although FB₁ is the most prevalent analogue in naturally contaminated corn.

The consumption of fumonisin-contaminated corn has been statistically associated with the development of hyperendemic levels of human oesophageal cancer in the Transkei region of the Eastern Cape Province of South Africa

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(Rheeder *et al.* 1992) and in the Hebei and Henan Provinces in China (Chu and Li 1994). Furthermore, recent work has also shown an association with primary liver cancer in the Henan Province (Ueno *et al.* 1997). In addition to cancer, fumonisin exposure has also been inferred as a possible contributory risk factor for neural tube defects (NTDs) in the Lower Rio Grande Valley along the Texas–Mexico border (Hendricks 1999), and may play a role in the high NTD rates in the Transkei region (Ncayiyana 1986) and in the Hebei Province (Moore *et al.* 1997). Corn is a main staple of the diet and elevated levels of fumonisins have been demonstrated in corn-based foodstuffs in all of these areas (Sydenham *et al.* 1990, Chu and Li 1994).

These concerns increase the importance of assessing exposure levels in humans; attempts to do so have not been very successful due to the lack of a suitable biomarker indicative of past fumonisin exposure. The results of toxicokinetic studies of FB_1 , summarized by Shier (2000), indicate that it has a very low oral bioavailability in rats, pigs, chickens, cows and monkeys. In addition, almost all FB_1 administered by the oral, intragastric, intraperitoneal or intravenous route is rapidly excreted largely unchanged, with only traces being found in the liver and kidney. While the detection of FB_1 in human faeces (Chelule *et al.* 2000) and urine (Shetty and Bhat 1998) has been reported, the detection time window is short (<3 days), and therefore only recent exposures can be detected. Surrogate dose monitors such as altered sphingolipid biosynthesis potentially provide a useful short-term biomarker (Riley et al. 1994), which is best expressed as an elevation in the ratio of the sphingoid bases, sphinganine and sphingosine. However, the stability, sensitivity and specificity of this marker to past fumonisin exposure remains unresolved (Van der Westhuizen et al. 1999). Nevertheless, the multitude of effects in animals, the serious nature of the possible effects in humans and intermittent ongoing dietary exposure stress the need to monitor fumonisin exposure.

Analysis of hair for toxic substances has increased in popularity in recent decades, and this is reflected in the numerous publications emerging on this topic. Interest in the hair matrix has arisen due to its ease of sampling and longer detection time window, depending on hair length. This, coupled with the progress made in separation techniques and detection sensitivity and selectivity, has made possible the detection of drugs in hair at levels of pmol mg⁻¹ hair (Nakahara 1999). More than a hundred pharmaceuticals, drugs of abuse and their metabolites or doping agents have been reported to be detectable in human hair (Gaillard and Pépin 1999).

We recently demonstrated the possibility of using hair from non-human primates (vervet monkeys, *Cercopithecus aethiops*) and rats as a non-invasive matrix for assessing chronic exposure to fumonisins (Sewram *et al.* 2001). The aim of the current study was to analyse human hair from potentially exposed subjects for the presence of fumonisin mycotoxins in order to determine the extent to which these toxins accumulate, and hence the feasibility of hair analysis as a means of assessing fumonisin exposure in humans. It needs to be emphasized that the aim of this study was not to provide data from specific individuals, but rather to demonstrate the possibility of determining fumonisins in human hair and hence the possible use of human hair in fumonisin exposure assessment. We now report for the first time on the discovery of these mycotoxins in human hair.

Materials and methods

Hair samples

Composite bulk hair samples (one from each) were obtained from five barber shops in three districts of Transkei: Bizana in the north-eastern region (one bulk sample), and Butterworth and Centane in the south-western region (two bulk samples each). The hair samples collected included both distal and proximal segments, and were a mixture of grey and black hair removed using a hand-held cutter.

The cultivation of corn is the main agricultural practice and provides the major source of food for survival in these regions. The majority of the residents are black Xhosa-speaking Africans, and recent statistics have confirmed a high incidence of oesophageal cancer among these populations.

Chemicals and solvents

Pure standards of FB_1 , FB_2 and FB_3 were prepared from culture material of *F* verticillioides, as previously described (Cawood *et al.* 1991). Formic acid (analytical grade), sodium hydroxide pellets, hexane, ethanol and methanol were obtained from Merck (Darmstadt, Germany). Acetonitrile (high performance liquid chromatography [HPLC] grade) was obtained from Romil (Cambridge, England), while water for general laboratory use and the HPLC mobile phase was deionized using a Milli-Q water purification system (Millipore, Bedford, Massachusetts, USA).

Extraction of hair samples

Each hair sample was stirred in ethanol at room temperature for 15 min to remove external sources of fumonisin contamination as well as possible fungal and bacterial contaminants. The hair was then dried at 45° C for 3 h and finely ground under liquid nitrogen in a mortar. The ground hair was shaken for 5 min in a bag filled with hot air to evenly distribute the finely ground segments throughout the sample. The sample was then further dried in an oven at 45° C for 5 h. Five test portions (700–1000 mg) from each population test sample were weighed into extraction flasks and refluxed with methanol (150 ml) for 5 h. The extracts were subsequently centrifuged on a Sorvall[®] RC-3B refrigerated centrifuge (DuPont, Connecticut, USA) at 4°C at 4000 g for 5 min and filtered (Whatman No. 4 filter paper). The methanol was evaporated under reduced pressure and the extract reconstituted in 20 ml of methanol–water (70:30). Hexane (5 ml) was then added to the extract in order to remove the lipophilic layer using a separatory funnel. The methanol–water solution was then adjusted to pH 6.0 with 1 M NaOH, and clean-up performed on BondElut LRC[®] strong anion exchange (SAX) solid phase extraction cartridges containing 500 mg of sorbent (Varian, Harbor City, California, USA) according to the method of Sydenham *et al.* (1996). The eluate from the SAX cartridge was evaporated to dryness at 60°C under nitrogen, and the residue reconstituted into 750 µl of HPLC mobile phase prior to HPLC injection.

HPLC-mass spectrometry (MS) analysis

HPLC-MS analysis was performed using a SpectraSERIES P2000 HPLC pump equipped with an AS 1000 autosampler (Thermo Separation Products Inc., Riviera Beach, Florida, USA) containing a 20 µl injection loop. The HPLC column was attached on-line to a Finnigan MAT LCQ ion trap mass spectrometer (San Jose, California, USA) set up for positive ion electrospray ionization (ESI). The test samples were filtered through a 0.45 µm syringe filter (Millipore, Yonezawa, Japan) prior to injection. Binary gradient reversed-phase HPLC was performed on a 150×4.6 mm internal diameter Luna C₁₈ column (Phenomenex, Torrance, California, USA) packed with 5 μm ODS-2. Solvents A and B consisted of water, acetonitrile and formic acid in the ratios 90:10:0.1 and 10:90:0.1, respectively, and were pumped at 0.7 ml min⁻¹. The initial composition of 80% A and 20% B was adjusted linearly over a 24 min period to 72% A and 28% B, held for 1 min, then re-adjusted linearly over 2 min back to 80% A and 20% B and held for 8 min, resulting in a total run time of 35 min. MS parameters were optimized separately for FB₁, FB₂ and FB₃ by direct infusion of a solution of each toxin (20 μ g ml⁻¹) into the source at a rate of 5 μ l min⁻¹. Full scan tandem mass spectrometry (MS-MS) between a mass to charge ratio (m/z) of 330 and 730 was undertaken to monitor FB₁, FB₂ and FB₃. A collision energy of 32% was used to fragment the protonated molecular ions, and the resulting product ions for each toxin were monitored as diagnostic indicators for the presence of these toxins in human hair. The HPLC eluate entered the mass spectrometer without splitting at a source voltage of 4.5 kV and a capillary voltage of 40 V. The heated capillary temperature was maintained at 220°C and the sheath-to-auxiliary gas ratio was set at 4:1.



Figure 1. Total ion chromatograms showing the presence of fumonisin B analogues in human hair extracts obtained from the samples collected in the district of Bizana.

Results

Typical mass chromatograms obtained for extracts of hair from Bizana can be seen in Figure 1. The two chromatograms superimposed on the time scale correspond to the highest and lowest concentration of toxins found in the population test sample.

With the MS-MS mode it was possible to selectively excite the protonated molecular ions of the fumonisin B analogues resident in the ion trap to produce diagnostic fragment ion spectra characteristic of these toxins. This two-stage filtering process allowed for increased specificity of detection. The corresponding mass spectra shown in Figure 2, produced by collision-induced dissociation of the protonated molecular ions, demonstrated typical fumonisin fingerprint profiles and hence provided unequivocal confirmation for each toxin in human hair. The fragments corresponded to sequential losses of water and tricarboxylic acid side chains from the alkyl backbone, whilst the analogues FB_2 and FB_3 , being isobaric, produced equivalent product ions 16 atomic mass units lower than the corresponding FB_1 fragments.

The on-column detection limit for the fumonisins was found to be 25 pg at a signal-to-noise ratio (S:N) of 2, while the limit of quantification (S:N = 10) was found to be 60 pg. The linearity of response was determined from the limit of quantification up to 100 μ g L⁻¹ for each analyte, and all the calibration plots



Figure 2. Product ion mass spectra produced by collision-induced dissociation of the protonated molecular ions of FB₁, FB₂ and FB₃ serving as diagnostic indicators for these mycotoxins in human hair. TCA, tricarboxylic acid.

showed a coefficient of determination $(R^2) > 0.99$. The precision of the measurements for each analyte was determined by performing triplicate injections under identical conditions; the relative standard deviation was found to be 3–9% at the 3 µg L⁻¹ level. Recoveries from spiked hair samples ranged from 81% to 101% with methanol extraction at the 0.6 µg ml⁻¹ level.

The mean concentrations of FB₁ and FB₂ and their ranges in hair samples obtained from the five areas are shown in Table 1. Five subsamples from each sampling location were tested. FB₁ levels in hair from both the sampling points in Centane were comparable (approximately 27.0 μ g kg⁻¹ hair). Similar results were found in hair obtained from the two sampling points in Butterworth. In contrast, hair samples from Bizana were found to contain higher levels of FB₁ (mean 33.0 μ g kg⁻¹ hair). The relative proportions of the fumonisin analogues detected in hair were similar to those generally observed in naturally contaminated maize.

Based on previously published experimental levels of FB₁ and FB₂ in the hair of non-human primates exposed to varying quantities of fumonisins (Sewram *et al.* 2001) and the current levels of fumonisins found in human hair, an attempt was made, purely on an exploratory basis, to extrapolate from the data to the levels of human exposure to fumonisin. In the absence of human data, the long-term study on non-human primates by Gelderblom *et al.* (2001) represents the closest available model. This was expected to depict a steady-state level of fumonisin concentration in hair at the time of hair analysis on termination of the experiment, and is the best approximation to humans as the subjects were exposed to consistent levels of fumonisins on a daily basis. Extrapolation from this data revealed that, on average, people could have been exposed to levels as high as 20.4 µg total fumonisin kg⁻¹ body weight day⁻¹ (range 18.4–27.0 µg total fumonisin kg⁻¹ body weight day⁻¹).

	Level of fumonisins detected ($\mu g k g^{-1} hair$)					Extrapolated human exposure level (μg total fumonisins kg ⁻¹ body weight day ⁻¹)
Sample	Mean	Range	Mean	Range	Mean	-
Centane 1	26.5	Trace-84.7	Trace		ND	21.3
Centane 2	26.9	6.3-56.8	6.5	Trace-16.6	ND	21.6
Butterworth 1	22.2	Trace-56.3	5.7	ND-10.9	Trace	18.4
Butterworth 2	24.7	6.6-61.0	ND		ND	20.0
Bizana 1	33.0	Trace-93.5	11.1	ND-23.5	Trace	27.0
Mean						20.4

 Table 1. Fumonisin levels detected in human hair from three districts within the Transkei region of the Eastern Cape Province of South Africa.

ND, not detected ($< 2.5 \ \mu g \ kg^{-1}$ hair); trace, $< 4.0 \ \mu g \ kg^{-1}$ hair.

Discussion

This study reports for the first time the accumulation of fumonisin mycotoxins in the hair of human populations exposed to contaminated maize, and assesses the potential of hair analysis as a means of determining human exposure to such toxins. The data obtained represents the average results obtained from scalp hair irrespective of the precise region of growth and demonstrates the power of hair analysis for determining the exposure status amongst human population groups. Since environmental chemical exposures in people are generally first explored by surveys in which samples are obtained from potentially exposed populations, it was decided in this case to obtain composite samples of hair from barber shops, sampling points considered to be appropriate for obtaining samples that best reflected the average exposure levels of the people living in these areas.

Previous studies in the Transkei region have shown a high incidence of F. verticillioides infection in home-grown corn, accompanied by high levels of fumonisin contamination (Rheeder et al. 1992). Using the mean total fumonisin level in 'healthy' corn from the 1985 season (2100 μ g kg⁻¹), the probable daily intake (PDI), based on the assumption that a 70 kg person reliant on maize as a staple diet consumes 460 g maize each day (Langenhoven et al. 1988), was calculated as 13.8 μ g total fumonisin kg⁻¹ body weight. The high PDI value estimated from the hair analysis (20.4 μ g total fumonisin kg⁻¹ body weight) could possibly arise from consumption of not only 'healthy' corn, but also mouldy corn used in the brewing of traditional beer. A credible estimation of exposure to FB_1 can only be drawn if extensive data on the intake of various food ingredients by the population, and on the contamination of these ingredients with FB₁, are available. Furthermore, the quantity of maize consumed may vary considerably, together with substantial variation in fumonisin levels among maize samples as well as seasonal variations. This study therefore demonstrates the power of human hair analysis to measure the cumulative exposure to fumonisins, and in particular to FB₁, irrespective of intake profiles and sources of contamination, which may be extremely variable. Moreover, hair sampling is non-intrusive and non-invasive.

The analysis of fumonisin levels in hair requires careful consideration of certain factors, including passive deposition by fungal spores, possible changes induced by cosmetic treatments of hair, the distribution of the toxins along the hair shaft, and the dose-to-concentration correlation. As seen from the results presented here, the quantity of fumonisins detected can vary widely within each of the test samples. This wide range can be attributed to the degree of interindividual variability, since a number of the factors known to affect analytes in hair are heterogeneous. These include hair growth rate, ethnicity, toxin metabolism, colour of hair, bioavailability, age and gender. Nevertheless, some evaluation is possible between detected and exposure levels in terms of high, medium and low exposure. The specificity of detection of fumonisins using MS-MS makes it unlikely that the results were influenced by external contaminants. The ethanol wash solvents were subsequently analysed for fumonisins, and minimal losses were observed (FB₁ < 4 μ g kg⁻¹). However, even though the possibility of underestimation of the results does exist, it is nevertheless extremely alarming to note that the people in the communities we investigated were likely to have been exposed to levels as high as 10 times the provisional maximum tolerable daily intake (PMTDI) for nephrotoxicity, as set by the 56th Joint FAO/WHO Expert Committee on Food Additives (WHO 2002) (2 μ g total fumonisin kg⁻¹ body weight). In addition, in relation to the tolerable daily intake (TDI) of 0.8 μ g total fumonisin kg⁻¹ body weight with respect to cancer induction based on a no observed effect level (NOEL) of 25 mg FB₁ kg⁻¹ diet in rats (Gelderblom et al. 1996), the population studied was exposed to levels approximately 25 times more than the TDI level for carcinogenicity. These results are consistent with the observation that the rural population in Transkei, who have the highest rates of oesophageal cancer and NTDs in South Africa, have the highest daily corn intakes, and consume home-grown corn containing the highest levels of fumonisins.

Conclusion

Correlation of maize consumption patterns with the level of fumonisin exposure remains a major challenge in establishing links with a human disease. The present study tested the sensitivity and specificity of the analytical method for fumonisin in hair, and provides an important link in translating information from experimental studies to an assessment of the presence and magnitude of previous exposure and risk in humans. This newly developed technique utilizing hair to measure mycotoxin exposure of human population groups at risk heralds a new and exciting phase of human mycotoxicology, since this is the first time that a mycotoxin has been discovered in human hair. This methodology was sufficiently sensitive to detect fumonisin exposure in populations subjected to naturally contaminated food at potentially hazardous levels. Moreover, the specificity of the analysis has also been established, since no other environmental toxin is known to produce similar chemical fingerprints following mass spectral fragmentation.

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