Assessing Chronic Exposure to Fumonisin Mycotoxins: The Use of Hair as a Suitable Noninvasive Matrix

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Abstract

This study describes for the first time the accumulation of measurable levels of fumonisin mycotoxins in the hair of nonhuman primates (vervet monkeys, *Cercopithecus aethiops*) and rats exposed to contaminated feed. Hair was subjected to reflux with methanol, and the resulting extract was cleaned up on strong anion exchange (SAX) and C_{18} solid-phase sorbents. Fumonisins FB_1, FB_2, and FB_3 as well as their hydrolysis products commonly known as aminopolyols, AP_1 and AP_2, were detected in monkey hair using high-performance liquid chromatography coupled to electrospray ionization mass spectrometry (HPLC–ESI-MS). Despite matrix interferences, the two-stage mass spectrometric process (MS–MS) yielded product ion mass spectra, which served as diagnostic indicators thus providing unequivocal identification of FB_1, FB_2, and FB_3 as well as AP_1 and AP_2. In vervet monkeys, the levels of exposure related well to the levels of toxin detected in hair, and levels as high as 5.98 mg FB_1, 33.77 mg FB_1, and 65.93 mg FB_1/kg of hair were found in monkeys receiving control, low-dose, and high-dose contaminated diets, respectively. Hair was also analyzed from rats given either single gavage doses of 1 and 10 mg FB_1/kg body weight or contaminated feed (50 mg FB_1/kg), resulting in an exposure of approximately 4.25 mg FB_1/kg body weight/day based on the measured daily feed intake. Analysis of rat hair over a four-week period indicated that mean levels up to 34.50 mg/kg and 42.20 mg/kg were detectable by the fourth week in the rats treated by gavage (10 mg FB_1/kg body weight) and those receiving contaminated feed, respectively. This relationship indicates that hair can provide an easily applicable non-invasive matrix for assessing chronic exposure to fumonisin mycotoxins.

Introduction

The fumonisin mycotoxins are secondary metabolites of the fungus *Fusarium verticillioides* (Sacc.) Nirenberg (formerly known as *F. moniliforme* Sheldon) and are ubiquitous contaminants of maize worldwide (1). These metabolites, of which fumonisin B_1 (FB_1) is the most abundant, are of toxicological concern as they have been shown to cause various animal disease syndromes, which include equine leukoencephalomalacia (2,3), porcine pulmonary edema (4) and nephrotoxicity (5), and hepatotoxicity and hepatocellular carcinoma in rats (6). In addition, epidemiological surveys have indicated a possible relationship between the consumption of fumonisin-contaminated maize and the development of esophageal cancer in human populations in the Transkei region of the Eastern Cape Province of South Africa (7,8) and Cixian and Linxian counties in China (9). Fumonisins have also been implicated as a possible contributory risk factor for primary liver cancer in Haimen, China (10). Although the precise role and mechanisms of action of fumonisins in the context of human disease causation still need to be established, there are nevertheless several reasons for concern, thus increasing the fundamental importance of assessing human exposure levels.

Currently, exposure at the individual level can be assessed by monitoring fumonisins in the urine (11) of populations that are highly exposed to contaminated maize, but this would not be feasible with current methodologies in areas where levels of contamination or maize intake are relatively low. The detection of FB_1 in human feces has also been reported (12); however, this method can only detect relatively recent exposure because experimental studies in nonhuman primates have indicated that FB_1 is eliminated in feces over a five-day period after dosing (13). The altered sphinganine (Sa)/sphingosine (So) ratio in urine and in blood has also been investigated as a possible biomarker for fumonisin exposure in animals, including nonhuman primates (14,15). The fumonisins, which are structurally similar to the sphingoid bases, Sa and So, cause inhibition of a key sphingolipid biosynthetic enzyme, ceramide synthase, leading to an elevation of sphinganine levels in cells, thus resulting in an increase in the Sa/So ratio (16). In a recent paper (17), the Sa/So ratios were reported in the plasma and urine of residents consuming a staple diet of home-grown maize in the KwaZulu-Natal province and the Transkei region of the Eastern Cape province of South Africa. However, there were no significant differences found in the Sa/So ratios between subjects eating fumonisin-contaminated maize and those eating maize in which no fumonisin was detected (< 10 mg/kg). Thus, it is still unclear how sensitive this approach is with respect to detecting human exposure to fumonisins. In addition,

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the altered ratios are reversible once exposure stops (18), making assessment of past exposures difficult. Furthermore, complications arise when trying to determine the normal levels of a control group because all the studies conducted to date in humans and animals alike have demonstrated a considerable variation in the ratio between individuals and within an individual over time (17). In addition, toxicokinetic data have shown that fumonisin B_1 is rapidly eliminated from serum in vervet monkeys (13). This rapid elimination together with the low bioavailability and the lack of a known protein and/or DNA adduct makes direct measurement of fumonisins unfeasible as a biomarker of exposure in body fluids.

In recent years, human hair has received considerable attention as a toxicological specimen for evidence of chronic drug use in both clinical and forensic investigations and is now being recognized as the third fundamental biological specimen for drug testing besides urine and blood (19). More than 100 pharmaceuticals, drugs of abuse, their metabolites, and doping agents have been reported to be detectable in human hair (20). Toxins present in an individual's bloodstream can be transported to hair by capillaries, which perfuse the hair root and in this manner can become incorporated in the keratinized hair matrix. Other pathways include autocontamination from sweat and sebum (endogenous-exogenous pathway) and contamination from the external environment (exogenous pathway). Whereas the analysis of body fluids can detect only relatively recent toxin exposure, hair analysis has the potential for greatly expanding the detection time window. Furthermore, sample acquisition is less intrusive, and patients are not subjected to any of the pain or discomfort that is generally associated with the withdrawal of blood. Hence, this study was aimed at analyzing hair from nonhuman primates and rats chronically exposed to fumonisin mycotoxins, using high-performance liquid chromatography (HPLC) coupled to mass spectrometry (MS), 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.

Experimental

Reagents

Pure standards of FB_1, FB_2, and FB_3 were prepared from culture material of *F. verticillioides* as previously described (21). The fully hydrolyzed products of FB_1 and FB_2, referred to as aminopolyols AP_1 and AP_2, respectively, were prepared by alkaline hydrolysis of a 250-mg/mL solution of FB_1 and FB_2 (4 mL each) with 0.1M NaOH (50 mL) at 70°C for 3 h. Purification was subsequently done by readjusting the solutions to pH 2.5 with 0.1M HCl and passing them through BondElut C_{18} solid-phase extraction (SPE) cartridges containing 500 mg of sorbent (Varian, Harbor City, CA) according to the method of Sydenham et al. (22). Acetonitrile and methanol (HPLC grade) were obtained from BDH Chemicals (Poole, U.K.), and formic acid (analytical grade) was obtained from Merck (Darmstadt, Germany). Water for the HPLC mobile phase was purified in a Milli-Q system (Millipore, Bedford, MA).

Experimental animals

The subjects of this experiment were three adult female vervet monkeys (*Cercopithecus aethiops*) and 11 male Fischer rats, all of which were bred in the Primate Unit of the Medical Research Council (Tygerberg, South Africa). The vervet monkeys were subjects of a long-term study (14,23) on the chronic toxicological effects of ingestion of fumonisin-containing *F. verticillioides* MRC 826 culture material at levels ≤ 1% of a low-fat carbohydrate diet. The experimental group consisted of two monkeys (#711 and #712). Subject #711 received a low-dose contaminated diet with 0.5% culture material resulting in an exposure of approximately 0.3 mg total fumonisins/kg body weight/day, and subject #712 received a high-dose contaminated diet with 1% culture material resulting in an exposure of approximately 0.8 mg total fumonisins/kg body weight/day. The control monkey (#708) received a diet similar to the experimental group but with maize in place of *F. verticillioides* culture material added to the diet. At the time of hair sampling, the monkeys #708, #710, and #711 were 4990 days into the study and weighed 3.69, 4.48, and 5.71 kg, respectively.

The male Fischer rats were 11-weeks old with a mean weight of 175 g and were caged individually in a controlled environment (23–25°C) with a 12-h light-dark cycle. Rats #1 to #3 were given a single gavage dose of 1 mg FB_1/kg body weight (low-dose) while rats #4 to #6 were given a single gavage dose of 10 mg FB_1/kg body weight (high-dose). Rats #8 to #10 received the AIN-93M (American Institute of Nutrition) diet contaminated with 50 mg FB_1/kg of feed resulting in an exposure of approximately 4.25 mg FB_1/kg body weight/day based on the measured daily feed intake. Rats #7 and #11 were the control subjects and received uncontaminated AIN diet, as did the rats that had received a single gavage dose. The duration of the experiment was 28 days, and during this time, the rats had free access to feed and water and were weighed three times per week. The experimental protocols were approved by the Ethics Committee for Research on Animals (ECRA) of the Medical Research Council, Tygerberg, South Africa.

Hair removal

Hair was removed from the sacroileal region of the monkeys and predetermined regions of the rats by means of an electric shaver. Hair was removed from the rats prior to commencement of the experiment and thereafter on a weekly basis over a period of four weeks. On the last day of the experiment, samples of hair were removed from a previously uncut region as well as from the region from which hair was removed prior to dosing and this region was referred to as the regrowth region. The hair samples were washed with distilled water to remove external contaminants and then dried at 40°C.

Extraction of hair samples

Hair samples were finely ground under liquid nitrogen in a mortar and test portions (500–700 mg) weighed into an extraction flask and refluxed with methanol (50 mL) for 5 h. The extracts were subsequently centrifuged on a Sorvall® RC-3B refrigerated centrifuge (DuPont, CT) at 4°C at 4000 × *g* for 5 min and filtered (Whatman No. 4). The methanol was evaporated under reduced pressure, and the extract was reconstituted

in methanol/water (70:30, 20 mL). The solution was adjusted to pH 6.0 with 1M NaOH, and cleanup was performed on BondElut LRC® strong anion exchange (SAX) solid-phase extraction cartridges containing 500 mg of sorbent (Varian) according to the method of Sydenham et al. (24). In order to investigate the presence of the aminopolyols, the column eluates during sample addition and column washing were collected, the pH adjusted to 2.5 with 0.1M HCl, and passed through BondElut LRC C_{18} sorbents as outlined by the method of Sydenham et al. (22). This was necessary as the hydrolysis products are not retained on SAX sorbent. The hair samples obtained from the rats were also subjected to the described extraction and clean up, but required a partition step with hexane (20 mL) immediately after filtration of the sample. Water (10 mL) was added to the separatory funnel to promote phase separation. The final eluates from the SAX and C_{18} cartridges were then combined and evaporated to dryness under nitrogen and the composite residue reconstituted into 500 mL of HPLC mobile phase.

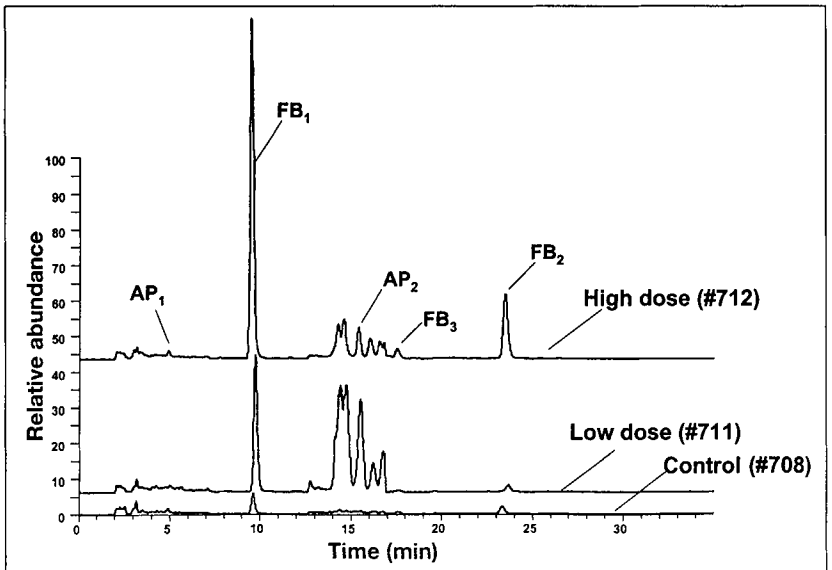

Figure 1. Total ion chromatograms of the hair extracts from nonhuman primates exposed to different levels of toxins showing fumonisin analogues present in hair matrix.

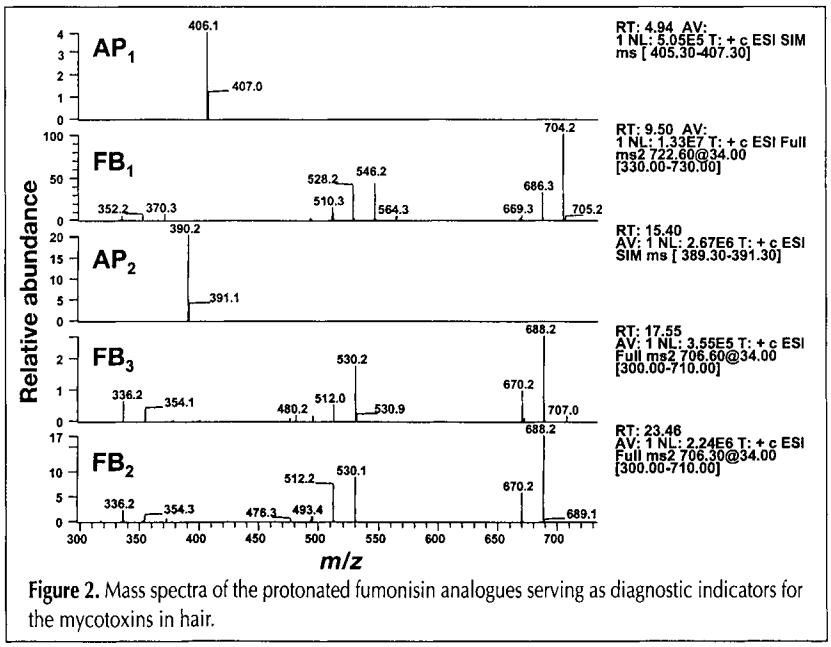

Figure 2. Mass spectra of the protonated fumonisin analogues serving as diagnostic indicators for the mycotoxins in hair.

Extraction of fumonisin B_1 from plasma

Monkey blood samples were collected by venipuncture into vacuum tubes containing tripotassium ethylenediaminetetraacetic acid (EDTA) as anticoagulant. Plasma was obtained by centrifugation at 1200 × *g* for 10 min at 4°C. The samples were prepared for analysis according to the method of Shephard et al. (25).

HPLC conditions

HPLC analysis was performed using a SpectraSERIES P2000 pump and an AS 1000 autosampler equipped with a 20-mL injection loop (Thermo Separation Products Inc, Riviera Beach, FL). Binary gradient reversed-phase HPLC was performed on a 150 × 4.6-mm i.d. Luna C_{18} column (Phenomenex, Torrance, CA) packed with 5 µm ODS-2. Solvents A and B consisted of water/acetonitrile/formic acid in the ratios 90:10:0.1 and 10:90:0.1, respectively. The mobile phase was pumped at a flow rate of 0.7 mL/min. The initial composition of 80% A and 20% B was adjusted linearly over a 24-min period to 72% A and 28% B and held for a period of 1 min. The composition was thereafter adjusted in a linear profile over 2 min to 80% A and 20% B and held for a period of 8 min resulting in a total run time of 35 min. The samples were filtered through a 0.45-µm syringe filter (Millipore, Yonezawa, Japan) prior to injections.

MS conditions

Positive ion electrospray ionization (ESI)-mass spectrometry was performed using a Finnigan MAT LCQ ion-trap MS (San Jose, CA). MS parameters were optimized separately for FB_1, FB_2, FB_3, AP_1, and AP_2 (each 20 mg/mL) by direct infusion of a solution of each toxin into the source at a rate of 5 µL/min. The aminopolyols, AP_1 and AP_2, were monitored in the single ion mode (SIM) by observing their protonated molecular ions at *m/z* 406 and *m/z* 390, respectively. Full-scan MS–MS between *m/z* 330 and *m/z* 730 was undertaken to monitor FB_1, FB_2, and FB_3. Collision energy of 32% was used to fragment the parent molecules and the product ions for each toxin were monitored as diagnostic indicators for the presence of these toxins in hair. The HPLC eluate entered the MS 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.

Results

HPLC–MS optimization

The choice of mobile phase gradient together with use of appropriate segments during chromatographic separation made mass spectral scanning at optimum conditions possible for each of the target analytes and hence the detection of each analogue in a single run. The on-column detection limits for the fumonisins and their hydrolyzed analogues were found to be 25 and 35 pg, respectively, at a signal-to-noise ratio (S/N) of 2, and the limits of quantitation (S/N = 10) were found to be 60 and 80 pg, respectively. The linearity of response was determined from the limit of quantitation up to the level of 100 µg/L for each analyte and all the calibration plots 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 and found to have a RSD between 3 and 9% at the 3-µg/L level.

Analysis of monkey hair

Analysis of monkey hair revealed that in addition to the fumonisin mycotoxins FB_1, FB_2, and FB_3, the aminopolyols, AP_1 and AP_2, were also present in trace quantities (Figure 1). Peak identities in the sample were confirmed by comparison of the retention times and the mass spectra (Figure 2) with those of the standards. The fragmentation pathway for the fumonisins following collision-induced dissociation (CID) consisted of sequential losses of water and tricarboxylic acid side chains from the alkyl backbone (Table I). The analogues FB_2 and FB_3 are isobaric and hence produced equivalent product ions 16 amu lower than the corresponding FB_1 fragments. Quantities of the fumonisins and aminopolyols detected in monkey hair, as well as corresponding levels of FB_1 in plasma are shown in Table II. The recoveries from spiked hair samples ranged between 81 and 101% with methanol extraction at the 0.6-µg/mL level.

Table I. Fragment Ions Observed for Fumonisin Analogues Under MS–MS of the Protonated Molecular Ion on the Ion-Trap Mass Spectrometer

Product ions	Mass-to-charge ratio (*m/z*)		
	FB_1	FB_2	FB_3
$[M+H-H_2O]^+$	704	688	688
$[M+H-2H_2O]^+$	686	670	670
$[M+H-TCA]^+$	546	530	530
$[M+H-H_2O-TCA]^+$	528	512	512
$[M+H-2TCA]^+$	370	354	354
$[M+H-2TCA-H_2O]^+$	352	336	336

Analysis of rat hair

Fumonisin B_1 was detected in the fourth week in hair of rats given a single gavage dose of 10 mg FB_1/kg body weight and those receiving a dietary dose of 4.25 mg FB_1/kg body weight/day (50 mg FB_1/kg feed) over 28 days. The mean levels detected in hair were 34.50 µg FB_1/kg (range 30.50–39.40 µg/kg, SD = 4.50) and 42.20 µg/kg of hair (range 39.20–46.60 µg/kg, SD = 5.2) in both groups, respectively. The mean FB_1 levels in hair from the regrowth region in both groups were found to be 40.0 µg/kg (range 21.30–48.40 µg/kg, SD = 15.5) and 47.40 µg/kg (range 40.40–54.30 µg/kg, SD = 9.9), respectively. The rats receiving contaminated feed were exposed to a mean cumulative level of 23.30 mg FB_1 for the entire study period as calculated from the measured daily feed intake. Fumonisin B_1 could not be detected, however, in the hair of rats that received a single gavage dose of 1 mg FB_1/kg body weight.

Discussion

This paper describes for the first time the accumulation of fumonisin mycotoxins in the hair of monkeys and rats exposed to contaminated feed and opens the possibility of hair analysis as a means of assessing chronic exposure to fumonisin mycotoxins. In a previous study conducted to investigate the potential of noninvasive biomarkers of exposure to fumonisins, hair and urine samples from mink exposed to *F. verticillioides* culture material containing FB_1, FB_2, and FB_3 were analyzed for changes in sphingolipids (26). Although marked increases in urinary free sphinganine and sphingosine were detected, the levels in hair remained unaltered. In the present study, however, it was observed that the mycotoxins themselves accumulate to a measurable extent in the hair matrix. The levels of the toxins found in hair from the three monkeys (Table II) relates to the differences in exposure levels, as well as the corresponding levels of FB_1 in plasma as determined by HPLC–MS–MS. Trace levels of fumonisin analogues were found in the hair of the control monkey #708 fed commercial maize with an exposure level of approximately 0.01 mg FB_1/kg body weight/day. This indicates that natural contamination of maize can also lead to detectable quantities of the toxins in hair as a result of long-term accumulation.

Table II. Levels of Fumonisin Analogues Detected in Hair from Nonhuman Primates Exposed to Different Doses of *Fusarium verticillioides* MRC 826 Culture Material in the Diet

Monkey #	Exposure to fumonisin mycotoxins	Quantity of fumonisin analogues detected in hair (µg/kg)					Concentration of FB_1 in plasma (µg/L)
		FB_1	FB_2	FB_3	AP_1	AP_2	
708 (Control)	~0.01 mg total fumonisins/kg body weight/day	5.98	2.96	0.80	0.10	0.31	1.80
711 (low dose)	~0.03 mg total fumonisins/kg body weight/day	33.77	9.06	1.01	0.25	9.32	6.70
712 (high dose)	~0.08 mg total fumonisins/kg body weight/day	65.93	17.10	2.07	1.72	1.88	14.30

The presence of hydrolyzed fumonisins indicates possible metabolic modification, and such aminopolyols have been previously detected in the gastrointestinal tract of vervet monkeys (27,28). Although a substantial quantity of the fumonisin B mycotoxins are excreted in feces, detectable levels are present in hair. The quantities detected in hair, however, may not represent the true quantity present in the circulatory system because, firstly, of possible endogenous-exogenous contamination from saliva and excreta and secondly, because different fumonisin analogues may partition within the body at different rates and their individual incorporation into hair can be influenced by factors such as their melanin affinity, lipophilicity, and basicity (pK_a) (29). Nevertheless, this study has demonstrated that a relationship is possible between concentrations of analytes in hair and the exposed dose in terms of high, medium and low concentrations. The levels of fumonisins to which the monkeys were exposed can be compared to the estimated fumonisin intakes by different human population groups in the high esophageal cancer area of the Transkei region, South Africa. It has been estimated that the probable daily intake (PDI) of fumonisins is 0.047 mg/kg/day for a person eating "healthy homegrown maize", but could rise to 0.355 mg/kg/day if exposed to moldy maize (30), making detection in hair possible.

The experiment in rats was undertaken to determine how soon after exposure, detection of FB_1 in hair would be possible. This toxin was detected after the fourth week only in the rats that received a gavage dose of 10 mg FB_1/kg body weight and contaminated feed containing 50 mg FB_1/kg of feed. The fact that FB_1 was only detectable after the fourth week may indicate a lag period for the incorporation of this toxin into hair. Furthermore, the incorporation rates of toxins into hair also depend on a multiplicity of different factors such as anatomical origin, interindividual variability, and growth rate (29). Analysis of hair from the regrowth region at the 28th day of sampling indicated that this time period was sufficient for accumulation of detectable levels of the toxin in newly grown hair. In this study, sampling areas on the body surface were the same for each rat and during the time span of the experiments, the daily feed intake remained consistent, and the rats gained weight in all three groups without any signs of toxicity being evident.

Most of the procedures devoted to drug detection in hair have been based on gas chromatography–mass spectrometry in the electron impact mode (20). However, HPLC–MS has recently achieved technical prominence, and its potential in forensic and clinical toxicology has been demonstrated (31). MS provides high selectivities of detection because of the ability to separate or filter ions according to their mass-to-charge ratios. This selectivity, in addition to the resolution of chromatographic separation, and in combination with selected ion monitoring (SIM) and the two-stage MS process, made possible highly structure-specific detection of the target analytes. Hair matrices are complex in their chemistry and require acceptable analytical methodology as in certain cases, matrix components may co-elute and interfere with analyte signals. Such may have been the case for the detection of AP_2 by monitoring of the protonated molecular ion. An interfering compound with the same molecular ion may have contributed to the signal resulting in an extremely high level of AP_2 for the low-dose monkey. However, with the two-step MS detection used for analysis of FB_1, FB_2, and FB_3, this problem would have been easily identifiable as the resulting full-scan MS–MS spectra served as a fingerprint for the fumonsin mycotoxins and impurities would have been easily recognized.

The results of this study have disclosed a new paradigm in assessing for biomarkers of fumonisin exposure. In the light of these results it is necessary for more basic studies on the relationship between fumonisin and its disposition in hair to be carried out. The pharmacodynamics and pharmacokinetics of uptake will need greater exploration so that hair matrix can occupy a meaningful alternative in exposure assessment along with urine and blood.

Conclusions

The results of this preliminary study indicate that fumonisin mycotoxins, FB_1, FB_2, and FB_3 as well as the aminopolyols AP_1 and AP_2, accumulate in the keratin matrix of hair and can hence provide for an ethically acceptable and easily applicable system for assessing chronic exposure to fumonisins. The relationship between fumonisin exposure and its incorporation in hair accords well, indicating its potential as a matrix for exposure assessment and the resulting possibility of evaluating past exposures and undertaking prevalence studies in populations consuming contaminated maize.

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