

Association between Tortilla Consumption and Human Urinary Fumonisin B1 Levels in a Mexican Population

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Abstract

Fumonisin is a mycotoxin produced by *Fusarium* spp. and commonly contaminate maize and maize products worldwide. Fumonisin is a rodent carcinogen and has been associated with human esophageal cancer. However, the lack of a valid exposure biomarker has hindered both the assessment of human exposure and the evaluation of disease risk. A sensitive liquid chromatography-mass spectrometry method to measure urinary fumonisin B1 (FB1) following extraction on Oasis MAX cartridges was established and applied to urine samples from women in a cohort recruited in Morelos County, Mexico. Urinary FB1 was compared with dietary information on tortilla consumption. FB1 recovery in spiked samples averaged 94% as judged by deuterium-labeled FB1 internal standard. Urinary FB1 was determined in 75 samples from women selected based on low, medium, or high consumption of maize-

based tortillas. The geometric mean (95% confidence interval) of urinary FB1 was 35.0 (18.8-65.2), 63.1 (36.8-108.2), and 147.4 (87.6-248.0) pg/mL and the frequency of samples above the detection limit (set at 20 pg FB1/mL urine) was 45%, 80%, and 96% for the low, medium, and high groups, respectively. Women with high intake had a 3-fold higher average FB1 levels compared with the "low intake" group ($F = 7.3$; $P = 0.0015$). Urinary FB1 was correlated with maize intake ($P_{\text{trend}} = 0.001$); the correlation remained significant after adjusting for age, education, and place of residence. This study suggests that measurement of urinary FB1 is sufficiently sensitive for fumonisin exposure assessment in human populations and could be a valuable tool in investigating the associated health effects of exposure. (Cancer Epidemiol Biomarkers Prev 2008;17(3):688-94)

Introduction

Fumonisin is a family of mycotoxins produced mainly by *Fusarium verticillioides* and *Fusarium proliferatum*, which frequently contaminate maize (corn) and maize products worldwide (1). Fumonisin B1 (FB1) is the most abundant of the naturally occurring fumonisins, which also include FB2 and FB3. FB1 results in liver and kidney tumors in rodents in addition to hepatotoxicity and nephrotoxicity in a range of animal species. FB1 causes leukoencephalomalacia in horses and pulmonary edema in swine (1, 2). Epidemiologic studies in South Africa and China reported associations between fumonisin levels in maize and esophageal cancer rates (3, 4), and a suggestion has also been made of a link with primary liver cancer (5). FB1 has been classified as "2B, possibly

carcinogenic to humans" by the IARC (1). In addition to its carcinogenic properties, neural tube defects have been associated with FB1 exposure in one study on the Texas-Mexico border (6).

Maize is the third most important cereal grain for human consumption in the world after wheat and rice and is a staple food in many regions. Mexico has one of the highest per capita consumptions of maize in the world, with a total production of ~15 million tons per annum. According to Food and Agriculture Organization data for the year 2005, per capita maize consumptions were 70, 104, and 120 kg in the United States, South Africa, and Mexico, respectively (7). In Mexico, 60% of the total maize production will be used for human consumption, and an average consumption of tortillas (the most common maize-based food) can be as high as 325 g/d per person (8).

Fumonisin has been found in maize samples worldwide, with levels of FB1 reaching >10 ppm in the United States and >100 ppm in parts of Africa (1). In Henan, China, FB1 levels of 18 to 155 ppm were reported in heavily mold-contaminated grains (3). In the former Transkei region of South Africa, human exposures to FB1 were calculated to be between 14 and 440 µg/kg body weight/d, whereas the exposure for consumers of maize in the United States was estimated as only 0.08 µg/kg body weight/d (1). Recognizing the carcinogenicity of FB1, and based on the observed renal toxicity in rats, the

Received 9/7/07; revised 12/10/07; accepted 12/14/07.

Grant support: National Institute of Environmental Health Sciences grant ES06052 (University of Leeds). The assembly and follow-up of the Mexican cohort was supported by Consejo Nacional de Ciencia y Tecnología de México grant CONACyT 31034-M; 41708; 13915.

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doi:10.1158/1055-9965.EPI-07-2534

WHO has recommended a provisional maximum tolerable daily intake of 2 µg/kg body weight (9) for FB1, FB2, and FB3 alone or in combination. Several countries worldwide have introduced recommended levels to control human exposure, with maximum levels set by the Food and Drug Administration at 4 ppm (total FB1, FB2, and FB3) for cleaned maize intended for masa production and 2 ppm (total FB1 and FB2) in the European Union for unprocessed maize [Commission Regulation (EC) No. 1881/2006; ref. 10]. However, in many developing countries where fumonisin exposure level is high, regulation is either absent or impractical to implement.

In terms of mechanism of action, fumonisins are structural analogues of sphingoid bases and have been shown to disrupt sphingolipid biosynthesis by inhibition of ceramide synthase (11, 12). This inhibition results in complex effects on cell signaling pathways, which may explain some of the toxicology of fumonisin. In addition to its carcinogenicity, fumonisins also exert effects on the immune system (13). Fumonisin has potential for neurodevelopmental toxicity in animals and humans due to disruption of sphingomyelin formation and folate transport (6, 14).

To date, there is a limited understanding of the role of fumonisin in human disease. This is at least partly due to the absence of a validated biomarker of fumonisin exposure (15). The heterogeneous nature of maize contamination (just one or two kernels in a cob may be contaminated; ref. 16) and the uniform nature of diets in many subsistence farming communities mean that neither food analysis nor dietary questionnaires provide reliable measures of exposure. In an analogous fashion to studies of another ubiquitous mycotoxin, aflatoxin (17), a validated biomarker is a critical component in efforts to explore the cancer risk associated with exposure.

Some attempts to develop a biomarker of exposure to fumonisin have been reported. Notably, based on the above-mentioned inhibition of sphingolipid biosynthesis, an alteration in the ratio of sphinganine/sphingosine can be detected in tissues, blood, and urine following fumonisin exposure (18, 19). In animal studies, this ratio has been shown to closely reflect the dose of fumonisin administered (12, 18). However, in studies in human populations, the altered ratio appears to have limited sensitivity to detect environmental levels of exposure (19, 20). We have therefore sought to develop an alternative approach to assess exposure to fumonisin by measuring the parent compound directly in urine samples. Fumonisin is eliminated quickly after exposure with the majority excreted unmetabolized or partially hydrolyzed in feces and urine (21, 22). The methodology for this approach is based on recent advances in the measurement of fumonisin in maize and various other food matrices using high-performance liquid chromatography with fluorescence or mass spectrometric detection (23-25). A method for extraction and analysis of fumonisin in human hair has also been developed (26).

We report here a liquid chromatography-mass spectrometry (LC-MS) method to detect FB1 in human urine samples. This biomarker was strongly correlated with maize consumption, a proxy for fumonisin exposure in Mexico where maize is a dietary staple.

Materials and Methods

Sample Collection. Spot urine samples were available from 996 women who were recruited previously in a cohort study of women of reproductive age (27) in one urban and three suburban municipalities in the state of Morelos, Mexico. The urine samples were collected at a baseline visit before pregnancy, at any time of the day. Seventy-five of these 996 women were selected for the current study according to their consumption of maize-based food as derived from a validated food frequency questionnaire (28). The two major variables determining maize intake for the majority of individuals were the frequency and the number of maize-based tortillas consumed. The product of these two variables was used to rank the 996 women from the highest to the lowest maize intake. Subsequently, the top 25 women were selected as the "high intake" group and the 25 from the bottom as the "low intake" group. A "medium intake" group of 25 women was chosen from those ranked around the median. The frequency of intake of other items containing maize (e.g., maize flakes, cereal, or gruel) were then considered to assess whether any subjects might have been misclassified; as a consequence, one subject (ID 388) was moved from the "low intake" group to "high intake" group because she had consumed a consistently high level of several other maize items despite a low consumption of tortillas.

Using this approach, 75 women ages 15 to 36 years formed the three maize intake categories. For each woman, information on age, body mass index (BMI), education, occupation, birth place, and place of residence (municipality) were also available. Ethical approval for the study was obtained from the Ethics Committee of the National Institute of Public Health in Mexico, with each woman giving informed consent before participation. For each of the 75 women, a urine sample of ~15 mL was available that had been stored at -80°C before urinary FB1 determination at the University of Leeds.

Urinary FB1 Analysis

Chemicals. FB1 was purchased from Sigma-Aldrich. Deuterium-labeled FB1 (FBd6) was isolated from *F. verticillioides* culture material as described previously (29). Both FB1 and FBd6 were initially dissolved in acetonitrile/water (1:1, v/v) as a stock solution and then were diluted in methanol/water (1:1, v/v) to serve as standard and internal standard, respectively, for the analytical work as this resulted in sharper peaks on the liquid chromatograph.

Urine Preparation. Urine samples (10 mL) were thawed and centrifuged at $675 \times g$ at 4°C to remove particulate matter. The urines were subsequently diluted with an equal volume of distilled water and internal standard FBd6 (2 ng) was added. The sample was then slowly (1 mL/min) loaded onto a 3CC Oasis MAX cartridge (Waters), which had been preconditioned with 2 mL methanol/water (1:1, v/v). After washing the cartridge with 2 mL of 5% ammonium hydroxide in water and 2 mL of 100% methanol consecutively, the FB1 was eluted with 2 mL of 2% formic acid in methanol at a flow rate of 1 mL/min. The eluate was dried in vacuum and reconstituted in 200 µL methanol/water (1:1, v/v) before injection onto high-performance liquid chromatography.

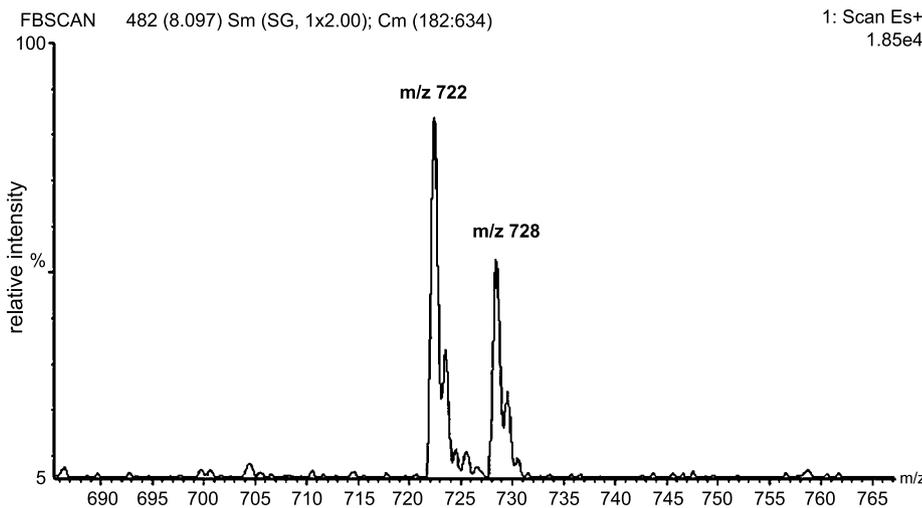
One negative and two positive quality controls were processed along with each batch of human urine samples. The quality controls comprised a UK urine sample either spiked or not with 2 ng FB1 and 2 ng FBd6.

LC-MS Analysis. A Waters 2795 high-performance liquid chromatography separation module (Waters) was coupled online with a Quattro Micro MS system (Waters) using electrospray ionization (capillary voltage 3 kV, cone voltage 40 V, and source temperature 140°C). A Luna C18 column (50 × 4.6 mm ID, 5 μm Phenomenex) was used for separation with a gradient starting with 75% mobile phase (A) water/acetonitrile/formic acid at 90%:10%:0.1% and 25% mobile phase (B) water/acetonitrile/formic acid at 10%:90%:0.1%, reaching 25% A and 75% B over 11 min. The flow rate was 1 mL/min. The reconstituted sample (25 μL) was injected onto the column. One fifth of the volume of the post-column eluate was directed into the electrospray ion source. The

mass spectrometer was operating in the selective ion monitoring mode with two functions, first with one channel ion scan at m/z 722.3 ($M + H^+$ for FB1) and second with one channel ion scan at m/z 728.2 ($M + H^+$ for FBd6). The FB1 concentration was quantified using Masslynx 4.0 (Micromass) software based on a standard curve using the internal standard, run daily. FBd6 contained a small amount (6%) of FB1, and this was subtracted when calculating the levels of urinary FB1. The standard curve was linear from 1 to 50 ng FB1/mL, equivalent to 20 to 1,000 pg/mL urine. In spiked human urine samples, the lowest FB1 concentration to give a clearly measurable peak with a signal-to-noise ratio >3, a recovery of ~100%, and a coefficient of variation of <20% was 20 pg/mL. Duplicate injections of each sample extract were done.

Urinary Creatinine. Urinary creatinine level was determined using the alkaline-picrate method (30) with

A



B

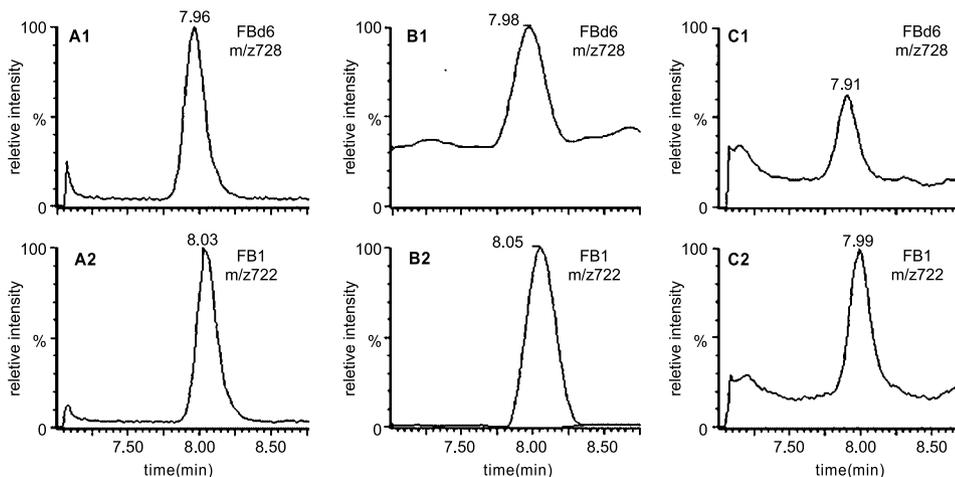


Figure 1. A. Electrospray ionization mass spectra of a mixture of FB1 (m/z 722) and FBd6 (m/z 728). **B.** LC-MS analysis of one standard and two representative urine samples. The mass spectrum was set in the selective ion monitoring mode with two scan functions, function 1 at m/z 722 for FB1 and function 2 at m/z 728 for FBd6. *A1* and *A2*, 10 ng/mL FBd6 and FB1 standards, respectively; *B1* and *B2*, a urine sample containing 400 pg/mL FB1 from the Mexican cohort; *C1* and *C2*, a UK urine sample spiked with 2 ng FBd6 and FB1.

Table 1. Characteristics of the 75 female subjects grouped according to maize intake

Characteristics	Maize consumption group*			
	Low (n = 24)	Medium (n = 25)	High (n = 26)	Total (n = 75)
Age (y), mean \pm SD [‡]	23.7 \pm 4.6	24.4 \pm 5.7	21.5 \pm 5.1	23.2 \pm 5.2
BMI, mean \pm SD	24.7 \pm 4.5	23.5 \pm 3.9	22.5 \pm 2.8	23.5 \pm 3.8
Education (y), mean \pm SD	13.4 \pm 3.8	12.0 \pm 3.0	8.7 \pm 2.7 [†]	11.3 \pm 3.7
Place of birth (%) [‡]				
Morelos state	42.0	24.0	35.0	33.0
Occupation (%) [§]				
Paid work	67.0	72.0	54.0	64.0
Municipality of residence (%)				
Urban	67.0	64.0	58.0	63.0
Suburban	33.0	36.0	42.0	37.0

*Based on tortilla consumption.

[†] $P = 0.001$ for ANOVA test.

[‡]Place of birth is either Morelos state or one of two other states.

[§]Occupation is categorized as either paid work or not.

^{||}Urban is Cuernavaca and suburban includes Emiliano Zapata, Puente de Ixtla, and Cuautla.

minor modifications to adapt the assay to a 96-well plate format. Urinary creatinine (mg/dL urine) was used to adjust the fumonisin concentration to control for inter-individual variation in urine concentration.

Statistical Analysis. Urinary FB1 levels were not normally distributed and values were therefore natural log transformed for statistical comparison between the different maize consumption groups using ANOVA and for correlation and regression analyses. Urine samples below the LOD (20 pg FB1/mL urine) were assigned a value of half the LOD (that is, 10 pg/mL) for statistical analysis. The data are presented in two ways: pg FB1/mL urine and pg FB1/mg creatinine. These two approaches gave similar overall results. A trend test was used to test the linearity between maize intake and urinary FB1. Multiple linear regression modeling was used to investigate the contribution of maize intake to urinary FB1 while considering other factors, such as age, education (a possible proxy of socioeconomic status), and place of residence. STATA version 9.0 was used for the analysis.

Results

The LC-MS technique provided a sensitive tool to determine urinary FB1. FB1 and FBd6 are ionized by the electrospray resulting in protonated molecules predominantly at m/z 722 and 728, respectively (see Fig. 1A). The availability of FBd6 as an internal standard contributes to the accurate calculation of FB1 by permitting adjustment of recoveries during the analysis. The method permits the detection of 20 pg FB1/mL urine in a 10 mL urine sample. The FB1 recoveries from six quality controls were 94%, with a coefficient of variation of 13%. Examples of the data obtained by LC-MS following Oasis MAX cartridge extraction are shown in Fig. 1B.

The women included in this study were generally young (being of childbearing age), had normal BMI, were well educated, and two-thirds were resident in Cuernavaca, an urban county (Table 1). There were no significant differences with respect to age, BMI, education, and place of residence between the 75 participating women compared with the full cohort (data not

included), although the percentage of women in paid employment was lower in the full cohort (64% in our subcohort versus 47% in full cohort). The average age, BMI, occupation, place of birth, and place of residence did not differ significantly among the women in the three maize intake groups, but the "high intake" group did have fewer years of education compared with the other groups ($F = 14.5$; $P < 0.001$).

Fifty six of the 75 (75%) samples had detectable urinary FB1. The geometric mean of the urinary FB1 was 70.1 pg/mL urine (range nondetectable to 9,312 pg/mL). The frequency of urine samples positive for FB1 increased with maize intake: 96% of the women in the "high intake" group had detectable urinary FB1, whereas only 45% of those with low intake did so ($\chi^2 = 17.3$; $P < 0.001$; Table 2). Only one subject in the high maize consumption group had nondetectable urinary FB1. The geometric mean urinary FB1 level was strongly correlated with maize consumption ($P_{\text{trend}} = 0.001$). Individuals in the high maize intake group had 3-fold higher mean urinary FB1 levels compared with the "low intake" group ($F = 7.3$; $P = 0.0015$). A similar trend is observed when creatinine-adjusted urinary FB1 levels are compared among the three groups (see Table 2).

Urinary FB1 was negatively correlated with age ($r = -0.40$; $P = 0.0003$) and with years of education ($r = -0.33$; $P = 0.0041$). BMI, occupation, and place of birth were not associated with urinary FB1 level. Women resident in the urban county had lower urinary FB1 levels than those resident in the suburban areas ($t = 2.76$; $P = 0.0073$). When age, education, BMI, and place of residence were considered in multiple regression models, maize intake remained a strong determinant of urinary FB1 levels (Table 3). When compared with the low maize intake group, the high group had 3.5-fold [$\text{Exp}(1.257)$; $P = 0.003$] higher urinary FB1 levels, whereas those in the "medium intake" group were 1.9-fold higher. Other factors positively related to urinary FB1 were young age and being resident in a suburban county.

Discussion

FB1 is classified by IARC as "2B, possibly carcinogenic to humans," reflecting the limited direct epidemiologic

Table 2. Urinary FB1 in relation to tortilla consumption

	Maize consumption group*		
	Low (n = 24)	Medium (n = 25)	High (n = 26)
Tortilla consumption			
No. tortillas consumed each meal, median (range)	2 (1-5)	3 (3-3)	10 (6-16)
No. meals per day when tortillas consumed, † median (range)	<1 (0-0.4)	2.5 (2.5-2.5)	2.5 (2.5-6)
Urinary FB1 levels			
% Samples above detection limit (FB1 > 20 pg/mL)	45	80	96 ‡
Urinary FB1 levels (pg FB1/mL urine), mean (95% confidence interval)	35.0 (18.8-65.2)	63.1 (36.8-108.2)	147.4 (87.6-248.0)§
Urinary FB1 levels (pg FB1/mg creatinine), mean (95% confidence interval)	44.0 (22.3-86.8)	92.3 (54.7-155.7)	133.9 (78.5-228.3)

*Based on tortilla intake.

†The number of meals per day when tortillas are consumed is defined as 0, never; 0.016, less than once a month; 0.066, one to three times a month; 0.132, once a week; 0.395, two to four times a week; 0.724, five to six times a week; 1, once a day; 2.5, two to three times a day; 4.5, four to five times a day; and 6, six times a day.

‡ $\chi^2 = 17.3$; $P < 0.001$.

§ANOVA test high versus low maize intake group, $F = 7.3$, $P = 0.0015$, and $P_{\text{trend}} = 0.015$.

||ANOVA test high versus low maize intake group, $F = 4.04$, $P = 0.0217$, and $P_{\text{trend}} = 0.001$.

evidence to support the causal association between dietary FB1 exposure and human cancer. The lack of a validated exposure biomarker is one important barrier to investigating such an association. This article describes a new biomarker of fumonisin exposure by measuring FB1 in human urine samples using a LC-MS-based methodology. The method was proven sensitive enough to detect exposure in the Mexican population at the individual level, and there was a strong correlation between the consumption of maize-based tortillas and the urinary FB1 biomarker level.

Tortillas are the major source of maize in the Mexican diet and can account for ~70% of the daily caloric intake in rural areas (31). The average tortilla consumption in this cohort of women from Morelos was around six tortillas per day, a figure close to the national average of seven (32). Therefore, tortillas would most likely be the major source of fumonisin exposure, and this was the reason for establishing the three maize consumption categories primarily based on tortilla consumption. The observation from this study that the daily consumption of tortillas was strongly correlated with urinary FB1 is therefore consistent with this measure reflecting fumonisin exposure. However, it should be noted that we did not have a direct measure of fumonisin intake, and as such, we cannot conclude that there is a quantitative relationship between fumonisin intake and urinary FB1. As only spot urines were available, we measured urinary creatinine to adjust for expected variations in urinary volume and FB1 levels were still strongly correlated with maize consumption when expressed in this way. In addition, we measured only FB1 in this study, although the tortillas are likely to also contain FB2 and FB3 as well as the hydrolyzed forms of FB1, FB2, and FB3 following the process of nixtamalization (33). Future consideration of other urinary fumonisins would be of value both in terms of exposure assessment but also to explore the absorption of different fumonisins in exposed people.

FB1 was detectable in 75% of the urine samples. Of the women in the high and medium maize intake groups, 96% and 80%, respectively, contained a detectable level of urinary FB1; levels were 3-fold higher in the "high intake" than the "low intake" group. The correlation

between maize intake and urinary FB1 was seen after adjustment for other factors such as age, education (also serves as a proxy of socio-economic status), occupation, and residence.

Although mean urinary FB1 was correlated with maize intake at the group level, there is clearly great interindividual variation within the groups. There are several possible explanations for this: the well-known heterogeneous contamination of maize may lead to a dissociation of maize consumption and fumonisin exposure (16); variations in food preparation methods can affect fumonisin level (34, 35); other sources of fumonisin exposure are not considered in the categorization of participants in this study; finally, due to the expected short half-life of urinary FB1 (22), the data will reflect recent exposure (that is, the previous 24- to 48-h exposure), whereas the food frequency questionnaire is summarizing typical maize consumption over the previous year.

Animal studies suggested that the majority of the FB1 ingested is excreted in feces, with only ~0.4% to 2% excreted in urine in rats, pigs, and nonhuman primates (22, 36-38). Assuming an average daily urine volume of 1.5 L and urinary excretion of 1% FB1 (based on the pig

Table 3. Determinants of urinary FB1 in the multiple regression model

Characteristics	Regression model for urinary FB1 level	
	Coefficient (95% confidence interval)	P
Age (y)	-0.085 (-0.147 to -0.0235)	0.008
Education (y)	0.014 (-0.086 to 0.113)	0.788
Place of residence*		
Suburban vs urban	0.664 (0.039-1.289)	0.038
Maize consumption †		
Medium	0.648 (-0.083 to 1.378)	0.081
High	1.257 (0.429-2.085)	0.003

*Urban is Cuernavaca and suburban includes Emiliano Zapata, Puente de Ixtla, and Cuautla.

†Based on tortilla intake; the "low intake" group was taken as the reference group to be compared with the "medium intake" and "high intake" groups.

and nonhuman primate data cited above), the mean level of 147 pg/mL urine in the high maize intake group would translate into a daily FB1 exposure of 368 ng/kg body weight (range, 0–23,311 ng/kg body weight/d) for an adult of body weight of 60 kg. This estimate has certain limitations due to the uncertainty concerning urinary FB1 excretion in humans. However, some of the subjects in the study do have estimated exposure levels of >2 µg/kg body weight, the current WHO provisional maximum tolerable daily intake for fumonisin. The calculated exposure is in a similar range to estimates of intake at the Texas-Mexican border where the women in the highest quartile of intake were estimated to consume 650 to 9,441 ng fumonisin/kg body weight/d (6). Our estimate is somewhat higher than intake levels estimated in the United States but lower than those in South Africa (39).

It is noteworthy that the nixtamalization process by which maize is boiled in a lime solution to produce masa for tortillas is known to reduce the fumonisin level up to 80% (34, 40, 41). According to De la Campa et al.'s (34) observation in four small scale tortilla companies, FB1 can be reduced to undetectable levels in tortillas made from maize contaminated with FB1 at 0.6 to 1.6 ppm. Therefore, intake of FB1 might be expected to be at a much higher level if maize is consumed in ways other than from tortillas.

In the multivariate model, age and place of residence were also associated with urinary FB1. Younger women had higher FB1 levels, but it is unclear whether this is due to a difference in maize intake or perhaps indicative of lower income and poorer diet in younger women. The current result also indicates that residents of an urban county may have lower levels of exposure to fumonisin than those living in the suburban municipalities, but the reasons for this could not be investigated in this study.

In summary, a LC-MS method for the detection of urinary FB1 has been developed with sufficient sensitivity to analyze urine samples from subjects exposed to fumonisin in Mexico. More than half the study population had detectable urinary FB1 and both the frequency of positive samples and the levels were associated with maize-based tortilla consumption. This biomarker therefore holds promise for future epidemiologic studies seeking to elucidate the role of these environmental toxins in human disease risk, including cancer and neural tube defects.

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Cancer Epidemiol Biomarkers Prev 2008;17:688-694.

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