

RESEARCH ARTICLE

The kinetics of urinary fumonisin B₁ excretion in humans consuming maize-based diets

Ronald T. Riley¹, Olga Torres², Jency L. Showker¹, Nicholas C. Zitomer¹, Jorge Matute², Kenneth A. Voss¹, Janee Gelineau-van Waes³, Joyce R. Maddox³, Simon G. Gregory⁴ and Allison E. Ashley-Koch⁴

¹ USDA – ARS, Toxicology and Mycotoxin Research Unit, R.B. Russell Research Center, Athens, GA, USA

² Centro de Investigaciones en Nutrición y Salud, Guatemala City, Guatemala

³ School of Medicine, Creighton University, Omaha, NE, USA

⁴ Duke University Medical Center, Durham, NC, USA

Scope: Fumonisin (FB) are mycotoxins found in maize. The purpose of this study was to (i) determine the relationship between FB₁, FB₂, and FB₃ intake and urinary excretion in humans, (ii) validate a method to isolate urinary FB on C₁₈-SPE cartridges for international shipment, and (iii) test the method using samples from Guatemala.

Methods and results: Volunteers ($n = 10$) consumed 206 grams/day of tortillas and biscuits prepared from masa flour and a product containing maize flour. Volunteers estimated their daily urine output and samples were analyzed for FB₁, FB₂, and FB₃ and hydrolyzed FB₁. Only FB₁ was detected in urine suggesting lower absorption of FB₂ and FB₃. Excretion was highly variable peaking soon after consumption began and decreasing rapidly after consumption stopped. Within 5 days after consumption ended, FB₁ was not detected in urine. In a study with eight volunteers, the average total urinary FB₁ was 0.5% of the intake. FB₁ was detected in 61% (107/177) of the samples collected in Guatemala.

Conclusion: The results support the use of urinary FB₁ to assess ongoing exposure in population-based studies. However, relating the FB₁ concentration in urine to dietary intake of FB by individual subjects will be complicated due to interindividual variability and the rapidity of clearance.

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1 Introduction

Fusarium verticillioides is a fungal pathogen of maize that produces FB, potent inhibitors of ceramide synthases [1]. FB cause animal diseases (reviewed in [2]), and are implicated in human carcinogenesis [3], neural tube defects [4], and stunting in children [5]. While there are many forms of FB, those most common in maize are FB₁, FB₂, and FB₃ [6, 7]. Where maize is a dietary staple, the probable daily intake of FB indicates that many maize consumers will exceed

the provisional maximum tolerable daily intake (PMTDI) of 2 µg/kg b.w. (FB₁, FB₂, and FB₃ alone or in combination) recommended by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [8].

In the USA, Mexico, and Central America, maize-based foods are eaten in large amounts and are often produced through a process called nixtamalization [9]. The process of nixtamalization involves alkaline treatment of maize prior to cooking and reduces the total FB and increases the hydrolyzed FB (HFB); FB lacking both of the tricarballic acid side chains [10]. HFB₁ is less toxic in animals compared to the parent compound [11, 12]. Nonetheless, in areas of Central America where conditions are conducive to growth of *F. verticillioides*, there is considerable potential for human exposure to high levels of FB because maize consumption is high [13] and even after nixtamalization there is still significant amounts of FB in the masa flour.

In humans, FB₁ is excreted in both feces and urine [14, 15]. Studies in areas of the world where maize is consumed in

Correspondence: Dr. Ronald T. Riley, Toxicology and Mycotoxin Research Unit, R.B. Russell Research Center, USDA-ARS, 950 College Station Road, Athens, GA 30605, USA

E-mail: ron.riley@ars.usda.gov

Fax: +1-706-546-3116

Abbreviation: FB, fumonisins; FB₁, fumonisin B₁; FB₂, fumonisin B₂; FB₃, fumonisin B₃; HFB₁, hydrolyzed fumonisin B₁; PMTDI, provisional maximum tolerable daily intake.

large amounts have used urinary FB₁ to evaluate human exposure. These studies show that even though the concentration of FB₁ in the urine is low, the marker is useful for demonstrating the correlation between the amount of maize-based food consumed and levels of urinary FB₁ [15]. It is also useful for identifying high and low exposure populations [16], and for validating intervention strategies including sorting and washing of the maize [17] and ingestion of calcium montmorillonite (NovaSil) [18].

Little is known about the kinetics of absorption and excretion of FB in humans. Nonetheless, urinary FB₁ has been used to estimate intake in humans using assumptions about absorption and the kinetics of excretion based on studies in laboratory and farm animals. In animals, FB₁ is rapidly but poorly absorbed from the gastrointestinal tract [19–21]. Once absorbed, there is no evidence that FB are metabolized. The majority of the FB₁ ingested is excreted in the feces unchanged or as the fully hydrolyzed or partially hydrolyzed form; lacking one of the two tricarballic acid side chains. Relative to feces, a much smaller amount of FB₁ is excreted in urine (< 2%) and the little that is excreted in urine is the parent compound.

Based on the few studies conducted in humans, it is likely that much of what is known about excretion in animal studies is also true in humans. For example, in humans very little FB₁ is detected in urine [15–18] relative to what has been reported in feces [14]. In one study where the transfer to urine could be indirectly estimated, the percent FB₁ was calculated to be 0.075% (0.054–0.104%) [17]. All of the studies of urinary excretion in humans have analyzed only FB₁ [15–18]. Thus, there is no published human information documenting urinary excretion of FB₂ and FB₃.

The specific objectives of this study were (i) determine the quantitative relationship between FB₁, FB₂, and FB₃ dietary intake and urinary excretion in humans consuming maize-based foods in amounts approximating consumption where maize is a dietary staple, (ii) develop and validate a method to isolate urinary FB₁, FB₂, and FB₃ on C₁₈-SPE cartridges for international shipment for analysis by LCMS, and (iii) test the method using urine samples collected from humans in Guatemala.

2 Materials and methods

2.1 Chemicals and standards

Acetonitrile (ACN) and water were HPLC grade and formic acid was reagent grade. FB₁, FB₂, and FB₃ (>95% pure) and also qualitative standards of HFB₂ and HFB₃ were provided as a gift from Ronald Plattner, National Center for Agricultural Utilization Research, USDA-ARS, Peoria, IL, USA. HFB₁ was prepared from pure FB₁ by hydrolysis in 2 N KOH (70°C overnight) followed by cleanup over a 35 cc C₁₈ SepPak[®] column (Waters, Milford, MA USA) [10]. N-(1-deoxyfructos-1-yl) FB₁ was a gift from Lauren S. Jackson

USFDA, Summit-Argo, IL USA). A qualitative standard of fumonisin C₁ was a gift from Jennifer Tonos (USDA, ARS, Crop Genetics and Production Research Unit, Stoneville, MS). U-[¹³C₃₄]-FB₁-solution (25 µg/mL) OEKANAL[®] was purchased from Sigma-Aldrich Laborchemikalien, Seelze, Germany.

2.2 Liquid chromatography/ mass spectrometry (LCMS) quantitation of FB

The LCMS method for quantitation of FB₁, FB₂, and FB₃ was similar to that described previously [22, 23]. Additional details about the validation of the analysis of the uncooked and cooked maize-based foods and modifications of the LCMS method are described in the Supporting Information Tables S1, S2, and S3 and Figure S1.

2.3 Purchase of masa flour and maize-based foods in the USA and extraction and isolation of FB

Maize masa flour (produced in the USA, *n* = 11, 2 kg each) and a maize-based hot cereal/atol (traditional maize flour-based Central American product produced in Guatemala, *n* = 9, 0.45 kg each) were purchased from grocery stores in the USA in 2007 and 2009 and analyzed for FB. Samples were removed directly from the manufacturer's packages without additional mixing and extracted and processed using C₁₈-SPE cartridges (Sep-Pak[®] Classic C₁₈ cartridges, Waters Corporation, Milford, MA, USA) following the procedure described for shelled maize [24]. The recoveries from the uncooked and cooked foods were determined experimentally (Supporting Information Table S1). The recovery of U-[¹³C₃₄]-FB₁ internal standard closely approximated the recoveries of FB₁, FB₂, and FB₃ (Supporting Information Table S1). Thus, the quantitation of FB₂ and FB₃ based on the recovery of U-[¹³C₃₄]-FB₁ provides a reasonably accurate estimate of the FB₂ and FB₃ in the tortillas and biscuits. Analysis of maize masa flour and maize-based hot cereal/atol purchased for this study revealed that all contained detectable levels of FB₁, FB₂, and FB₃ at a ratio, on average, of 1.00:0.30:0.15 and 1.00:0.25:0.09 in the masa flour and the hot cereal/atol, respectively (Supporting Information Table S4).

2.4 Preparation of tortillas and maize biscuits and FB analysis

Tortillas were prepared according to the recipe provided by the manufacturer of the masa flour. The only ingredients added were salt and water (0.67 g table salt and 133.6 mL tap water/100g masa flour). The tortillas were formed using a tortilla press and cooked on a griddle at medium high for approximately 2 min/side. The maize flour biscuits were prepared using the maize-based hot cereal/atol following a recipe from the North American Millers Association. The only

ingredients added were baking powder, sugar, almond extract, and water (4 g baking powder, 24 g cane sugar, 1.25 mL almond extract, and 105 mL water/ 100g maize-based hot cereal/atol). The biscuits were baked at 163°C for 20 to 25 min. Tortillas and biscuits were cooled to ambient temperature and then stored in sealed plastic bags at 4°C.

Samples of the uncooked masa flour and maize-based hot cereal/atol and the cooked tortillas and biscuits were lyophilized and weighed. The cooked materials were ground into a fine powder using a high-speed electric blender to the same consistency (based on visual appearance and tactile properties) as the uncooked materials. The average dry weight of tortillas and biscuits was $19.2 \text{ g} \pm 0.7 \text{ g}$ ($\pm \text{SD}$, $n = 19$) and $18.2 \text{ g} \pm 0.4 \text{ g}$ ($\pm \text{SD}$, $n = 18$), respectively. After mixing, a total of 150 ng of U-[$^{13}\text{C}_{34}$]-FB₁ was added to samples (2.5 g to 10 g) of each material which was again mixed and then allowed to dry under vacuum overnight before extracting. The dried samples were extracted and processed using C₁₈-SPE cartridges, and analyzed by LCMS as described in the online Supporting Information Tables S5 and S6. Samples were analyzed for U-[$^{13}\text{C}_{34}$]-FB₁, FB₁, FB₂ and FB₃ and HFB₁, HFB₂

and HFB₃. Unless stated otherwise, the quantitation of all FB was based on the recovery of the U-[$^{13}\text{C}_{34}$]-FB₁ internal standard.

2.5 Studies in humans in the USA and Guatemala

Table 1 summarizes the studies conducted in humans and briefly outlines the specific objectives of each study. A total of four studies were conducted and are described in detail below. Briefly, three studies (*Study 1*, *Study 2*, and *Study 3*) were conducted in the USA under controlled conditions and *Study 4* was conducted in Guatemala, a country where maize is a dietary staple consumed in large amounts and frequently containing FBs [13]. *Study 1*, *2*, and *3* were intended to model for a brief period (3 or 6 days) the levels of maize consumption and FB intake that are common in Guatemala [13]. In Guatemala, maize is a dietary staple and people consume maize-based foods every day at every meal over their entire life. The levels of FB in the commercial products purchased in the USA (Supporting Information Table S4) and the cooked

Table 1. Summary of the studies conducted and the primary objectives of each study

Study name	Duration (Days abstain/ consume/abstain)	Number of volunteers (Number of urine samples expected to be collected)	Objectives
<i>Study 1</i>	11 days (3/3/5)	$n = 1$ ($n = 54$, 1 PM sample during 3 abstaining days before consuming and then every urination during 3 days consuming and the following 5 days abstaining)	1. Collect urine for methods development. 2. Confirm ability to detect FB ₁ , B ₂ , and B ₃ . 3. Obtain preliminary 3-day kinetic data.
<i>Study 2</i>	14 days (3/6/5)	$n = 1$ ($n = 25$, 1 PM sample during 3 abstaining days before consuming and then 1 AM and 1 PM during 6 days consuming and the following 5 days abstaining)	1. Determine FB ₁ , B ₂ , and B ₃ in AM and PM spot urine samples. 2. Obtain 6-day kinetic data to compare to <i>Study 1</i> .
<i>Study 3</i>	11 days (3/3/5)	$n = 8$ ($n = 88$, 1 sample/day/volunteer)	1. Determine interindividual variability. 2. Confirm clearance kinetics from <i>Studies 1 and 2</i> . 3. Confirm ability to detect FB ₁ , B ₂ , and B ₃ . 4. Confirm spot urine sample use to estimate intake.
<i>Study 4</i> ^{a)}	Daily (all consuming)	$n = 177$ ($n = 177$, 1 sample/volunteer)	1. Confirm method in naturally exposed population. 2. Confirm detection of FB ₁ , B ₂ , and B ₃ in naturally exposed population. 3. Confirm spot urine sample use to estimate intake.

a) See Supporting Information Table S7 for demographic information.

tortillas and biscuits (Supporting Information Tables S5 and S6) prepared from those commercial products are similar to what is seen in Guatemala [13].

2.6 Urinary FB excretion in humans consuming known amounts of FB in controlled studies (Study 1, 2 and 3)

Ten healthy (self described) volunteers (two females and eight males) were recruited in Athens, Georgia, USA to participate in studies to determine the kinetics of urinary excretion and to develop and validate a method for isolating urinary FB₁, FB₂, and FB₃ using C₁₈-SPE cartridges. The human subjects research protocol and informed consent forms were approved by the University of Georgia Human Subjects Office Institutional Review Board (Project Number 2009-10769-2). All consenting volunteers were asked to do the following: (i) answer questions about their health, age, weight, and activity level, (ii) receive instructions about maize-based foods to avoid during the periods requiring abstaining from maize-based foods, and (iii) participate in training on the procedure for collecting daily urine samples and estimating daily urine output. The average age of volunteers was 45.8 years old (range 24–64 years) and the average height and weight was 178 cm (157–191 cm) and 91 kg (73–132 kg), respectively.

Study 1 was conducted to obtain urine and preliminary kinetic data for methods development. A healthy consenting volunteer ($n = 1$) was asked to provide urine samples after abstaining from eating maize-based foods for 3 days. The volunteer was asked to consume six tortillas and five biscuits per day for 3 days followed by 5 days abstaining from any maize-based foods. The volunteer was asked to collect urine samples for FB analysis at each urination during 3 days of consuming and the following 5 days of abstaining. Urine samples for FB analysis were collected and the urine volume measured at each urination during the entire 3 days of consuming and the following 5 days of abstaining. FB intake was based on the concentration of FB in the cooked tortillas and biscuits (Supporting Information Table S5).

In *Study 2*, a single ($n = 1$) consenting volunteer was asked to do the same as described in *Study 1* but with 6 days consuming tortillas and biscuits (3 days abstaining, 6 days consuming, 5 days abstaining). This volunteer was asked to measure the volume of every urination and to collect a urine sample in the morning (AM sample) and again in the evening (PM sample) at least 2 h after consuming the evening meal for FB analysis. Urinary creatinine was measured using the Jaffe's Method [25] (http://www.searo.who.int/en/Section10/Section17/Section53/Section481_1755.htm). FB intake was based on the concentration of FB in the cooked tortillas and biscuits (Supporting Information Table S5).

In *Study 3*, eight volunteers ($n = 8$) were asked to abstain from eating any maize-based foods for 3 days followed by 3 days consuming six tortillas and five biscuits per day followed

by 5 days abstaining from maize-based foods and to collect daily urine samples and estimate and record the total volume of urine produced each day for the entire 11 days of the study. In all studies (*Study 1*, 2 and 3), the volunteers were instructed to space the consumption of foods so they would be consumed in the morning, afternoon, and evening of each day. Samples of uncooked materials were analyzed for FB before the study began and cooked materials were analyzed after the study was completed (Supporting Information Table S6).

2.7 Urinary FB in humans consuming unknown amounts of FB in Guatemala (Study 4)

In order to test the method for collecting, processing, and shipping, the C₁₈-SPE extracts of the urine from Guatemala to the USA a total of 101 males and 76 female consenting volunteers between the ages of 18 and 70 years old were recruited in the Departments of Chimaltenango (male, $n = 54$; female, $n = 40$) and Escuintla (male, $n = 47$; female, $n = 36$). The human subjects research protocol and informed consent forms were approved by the Comité Institucional de Ética of the Instituto de Nutrición de Centro América y Panamá (Project Number CIE REV003/2010). Consenting volunteers were asked to complete a questionnaire concerning their consumption of maize-based foods. Individuals consuming ≥ 410 grams of maize-based food/day and whose self-reported health was good or "average" were invited to participate in the study and to provide a urine sample. The height, weight, age, and information about maize consumption were recorded as part of the questionnaire. All volunteers reported they ate maize-based foods every day and tortillas comprised over 96% of the food consumed and over 72% of the volunteers consumed maize-based food only a few hours before providing the urine sample. Demographic information is summarized in Supporting Information Table S7. The urine sample was stored on ice until it could be frozen at -20°C . The urine was processed and FB were isolated on C₁₈-SPE cartridges as described above and in the online Supporting Information materials except that after the final wash of the loaded cartridge, the excess solvent was removed and the cartridges were wrapped with ParafilmTM and express shipped to the USDA laboratory in Athens. Maize being sold for human consumption was collected from vendors in both Departments at the same time as the urine sampling and analyzed for FB₁, FB₂, and FB₃. For additional details on FB stability, processing, and shipping of the C₁₈-SPE cartridges see Riley et al. [24].

2.8 Statistical analysis

Statistical analysis was performed using SigmaStat software (Jandel Scientific, San Rafael, CA, USA). When many groups were compared, one-way analysis of variance was used, followed by posthoc multiple comparisons unless the data failed

normality or was of unequal variance in which case the Mann–Whitney rank sum test was used. When only two groups were compared, a Student's *t*-test or Mann–Whitney rank sum test was used. The Chi Square test was used to test for differences in incidence or frequency. All data are expressed as mean \pm SD, and differences among or between means was considered to be significant if the probability (*p*) was < 0.05 .

3 Results

All volunteers in *Study 1*, 2, and 3 conducted in the USA abstained from eating maize-based foods for 3 days before consuming the tortillas and biscuits. A spot urine sample was collected on each of the three abstaining days and FB₁ was never detected in any of the samples that were analyzed.

3.1 FB₁ detection in urine (*Study 1*)

In *Study 1*, the single volunteer consumed 206 grams (daily total) of maize-based food (six tortillas and five biscuits) in three meals (morning, afternoon, evening) for 3 days (72 h). The concentration of FB₁ in the tortillas and biscuits consumed was 1.37 $\mu\text{g/g}$ and 1.63 $\mu\text{g/g}$, respectively (Supporting Information Table S5). Compared to the uncooked starting material, the tortillas and biscuits contained significantly less FB₁ and total FB (FB₁+FB₂+FB₃) (Supporting Information Table S5). Based on the cooked foods, the single volunteer consumed 306 μg of FB₁/day and the FB₁ intake was 4 $\mu\text{g/kg}$ b.w./day.

Out of eight urine samples analyzed over the course of 26 h, all were positive for FB₁ (8/8) but there was no FB₂ or FB₃ detected (0/8). The absence of FB₂ and FB₃ in the spot urine samples in the preliminary study was unexpected. It was hypothesized that the SPE cartridges were not binding or releasing FB₂ and FB₃. To test this hypothesis, eight urine samples were spiked with FB₃ and it was confirmed that the C₁₈-SPE were effectively binding FB₃ (Fig. 1). During the development of the method (*Study 1* and 2), it was found that recoveries of FB₂ and FB₃ were slightly, but not significantly, greater (8% and 23%, respectively) than FB₁ (Supporting Information Table S8) a result consistent with the estimated LODs for FB₁, FB₂, and FB₃ (Supporting Information Table S3) which showed that the LODs were similar for all three FBs.

3.2 Time course of FB₁ urinary excretion (*Study 1* and *Study 2*)

In *Study 1*, the first meal of two tortillas and two biscuits was at 07:30 and the first urination was at 10:15 at which time FB₁ was detected in the urine sample. The FB₃ added to the urine samples before extraction (20 ng total/sample) was used as an internal standard for quantification of FB₁.

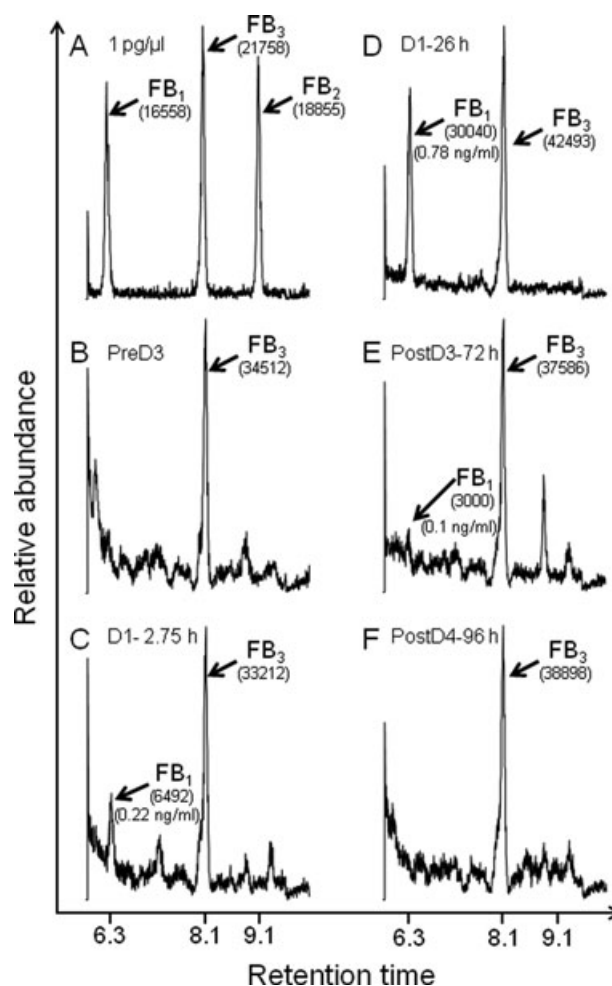


Figure 1. (A) Chromatogram showing the separation, retention times, and area (number in parentheses) under the peaks for a standard solution containing 1 $\mu\text{g}/\mu\text{L}$ of FB₁, FB₂, and FB₃. In all the other panels (B, C, D, E, and F) the chromatograms are from urine samples spiked with FB₃ before C₁₈-SPE clean up. In the FB₁ positive samples (C, D, and E), the areas and the ng FB₁/mL urine are shown in parentheses. (B) A urine sample after 3 days of not consuming any maize products (PreD3). (C) The urine sample at 2.75 h after consuming two tortillas and two biscuits containing FB₁, FB₂, and FB₃ (D1-2.75 h). (D) The urine sample at 26 h after consuming four additional tortillas and three additional biscuits (D1-26 h) and (E) the urine after 72 h and (F) 96 h abstaining from consuming any additional maize-based foods (PostD3 and PostD4). None of the peaks eluting near the retention time of FB₂ had the base peak or mass spectra of FB₂ (data not shown). The urinary FB₁ levels for each 24-h interval calculated based on the recovery of FB₃ are shown in Supporting Information Table S9.

Based on the FB₃ recovery, the concentration of FB₁ at 10:15 on day 1 of dosing (Fig. 1C, D1-2.75 h) was 0.217 ng FB₁/mL and at 26 h after the first meal the level of FB₁ in the urine was 0.78 ng FB₁/mL (Fig. 1D, D1-26 h). Three days after the last meal (Fig. 1E, PostD3) only a trace (0.1 ng/mL) of FB₁ was detectable, and 4 days post dosing (Fig. 1F, PostD4) no FB₁ was detected. Based on the total FB₁ intake (918 μg over

3 days), it was estimated that the cumulative percent FB₁ excreted in the urine was 0.78% (Supporting Information Table S9).

Study 2 also used a single volunteer ($n = 1$) and was conducted similar to *Study 1* described above except that the tortillas and biscuits were consumed for 6 days. Urine samples were collected in the morning and in the evening and the urine volume for every urination was measured throughout the period of consumption and for 5 days postconsumption. As in *Study 1*, no FB₂ or FB₃ was detected in any of the FB₁ positive urine samples (0/18). The total FB₁ excreted in the urine per day (Fig. 2A) and the cumulative FB₁ intake and cumulative FB₁ excreted in the urine (Fig. 2B) was calculated based on the average of the AM and PM FB₁ concentration in the urine (Fig. 3A) and the total daily urine volume. In total, 0.42% of FB₁ consumed was excreted in the urine (Fig. 2B, inset). The first evening urine sample collected after consuming the first three meals (D1–11.5 h) contained 0.82 ng FB₁/mL (Fig. 3A), a value similar to that seen in the 26 h sample in the preliminary study (Fig. 1D). The highest urinary FB₁ concentration was 1.40 ng/mL in the urine sample collected in the evening on consuming day 5 (Fig. 3A). After four days of not consuming tortillas or biscuits, FB₁ was not detected in the urine (Fig. 3A).

3.3 Levels of FB₁ in morning and evening spot urine samples (*Study 2*)

The urinary FB₁ in the AM and PM samples were similar although during the period of tortilla and biscuit consumption, the PM levels were generally higher and postconsumption, the PM levels were generally lower (Fig. 3B). This was apparent regardless of whether FB concentration was normalized to urine volume or creatinine. The difference between PM versus AM samples was statistically significant ($p < 0.05$, $n = 6$) in samples collected on days 1 to 6 when normalized to urine volume (Fig. 3B).

3.4 Interindividual variability in FB₁ excretion (*Study 3*)

A third study, *Study 3*, using eight volunteers ($n = 8$) was conducted that was similar to *Study 1* and *Study 2* described above, except that urine samples were collected once per day in the evening. The tortillas and biscuits were consumed for 3 days. The mean concentration of FB₁ in tortillas and biscuits was 1.52 $\mu\text{g/g}$ and 0.89 $\mu\text{g/g}$, respectively (Supporting Information Table S6) and the calculated intake was 256 μg of FB₁/day/volunteer resulting in an average intake of 2.94 ± 0.55 $\mu\text{g/kg b.w./day}$ (\pm SD, $n = 8$).

The total FB₁ excreted in the urine (Fig. 4A) and the percent of the total FB₁ intake excreted in the urine (Fig. 4A, inset) was calculated based on the single FB₁ concentration in the urine in the PM sample and the total estimated daily

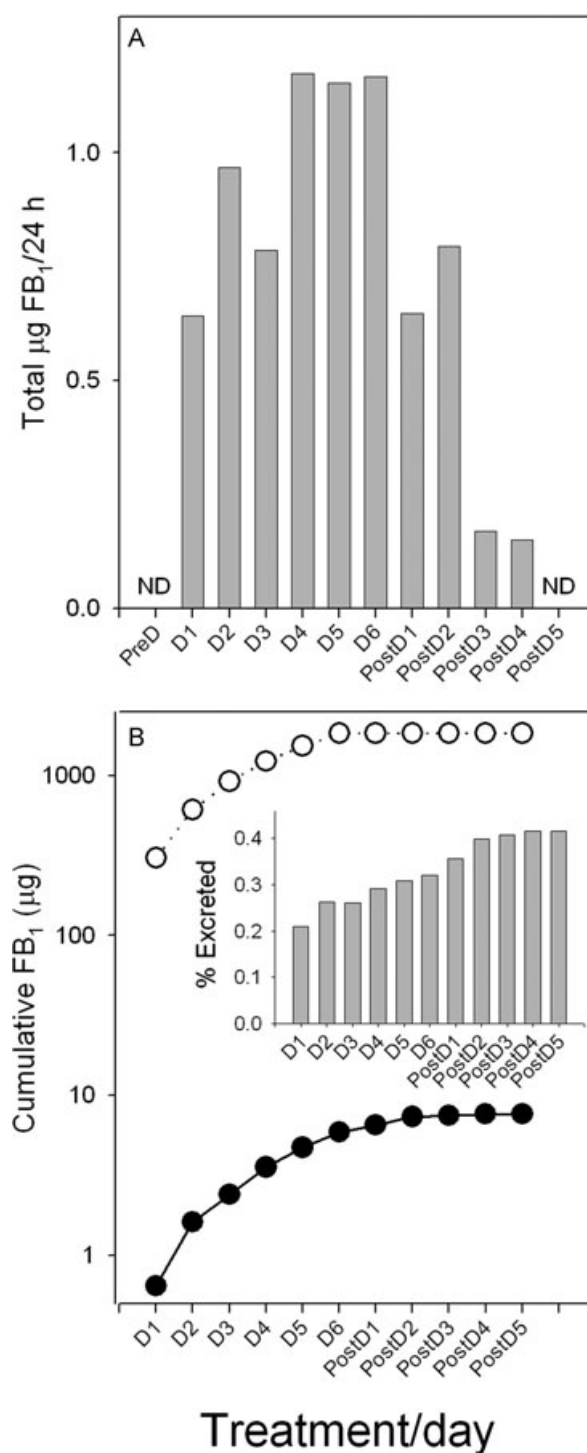


Figure 2. (A) Total daily urinary FB₁ excreted ($\mu\text{g}/24$ h) by a volunteer abstaining from consuming maize-based foods for 3 days (PreD), 6 days consuming six tortillas and five biscuits/days (D1–D6) followed by 5 days abstaining (PostD1–PostD5). (B) Cumulative FB₁ intake (open circles) and cumulative excretion in the urine (solid circles) over the entire period of consuming (D1–D6) and postconsuming (PostD1–5). Inset in (B) is the percentage of the cumulative FB₁ intake excreted in the urine at each time period. ND = not detected.

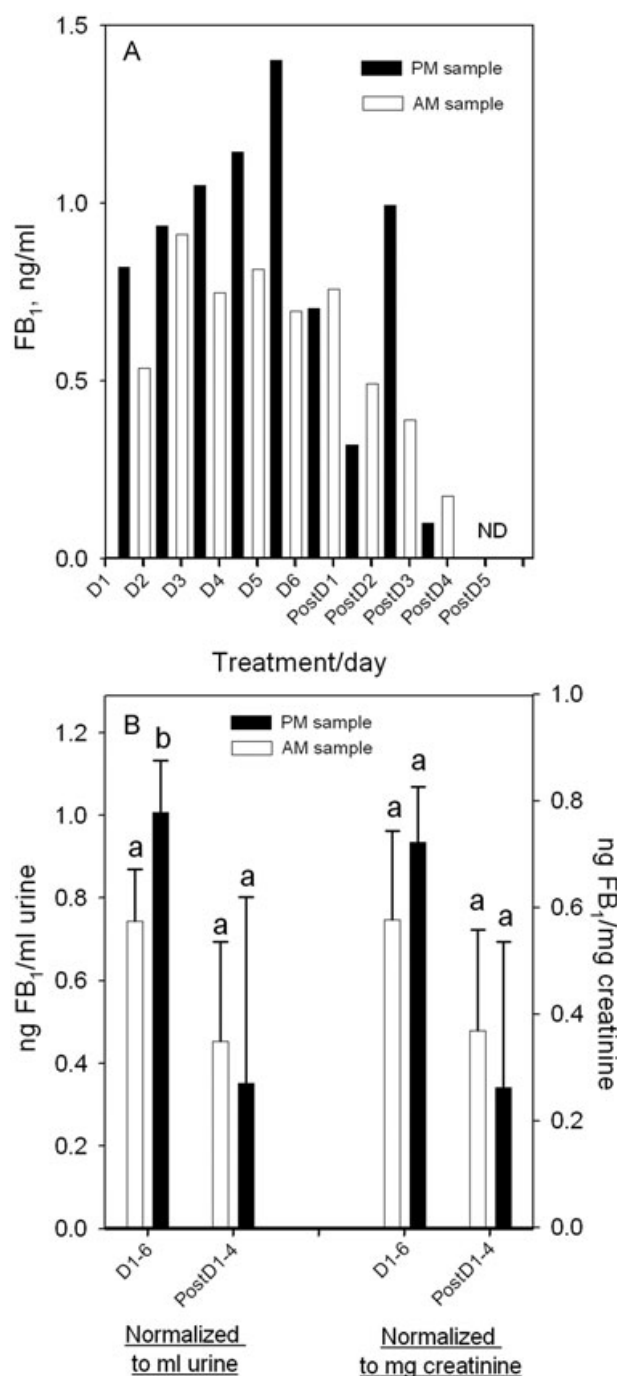


Figure 3. (A) FB₁ in evening (PM, shaded bars) and morning (AM, light bars) urine samples from a single volunteer during 6 days of consuming 206 g of tortillas and biscuits (D1–D6) followed by 5 days abstaining from maize foods (PostD1–5). (B) Comparison of the FB₁ concentration expressed as ng/mL urine or ng/mg creatinine in AM and PM urine samples while consuming (D1–6, $n = 6$) or abstaining (PostD1–5, $n = 4$) from maize foods. Pairs of bars with differing superscripts are significantly different ($p < 0.05$).

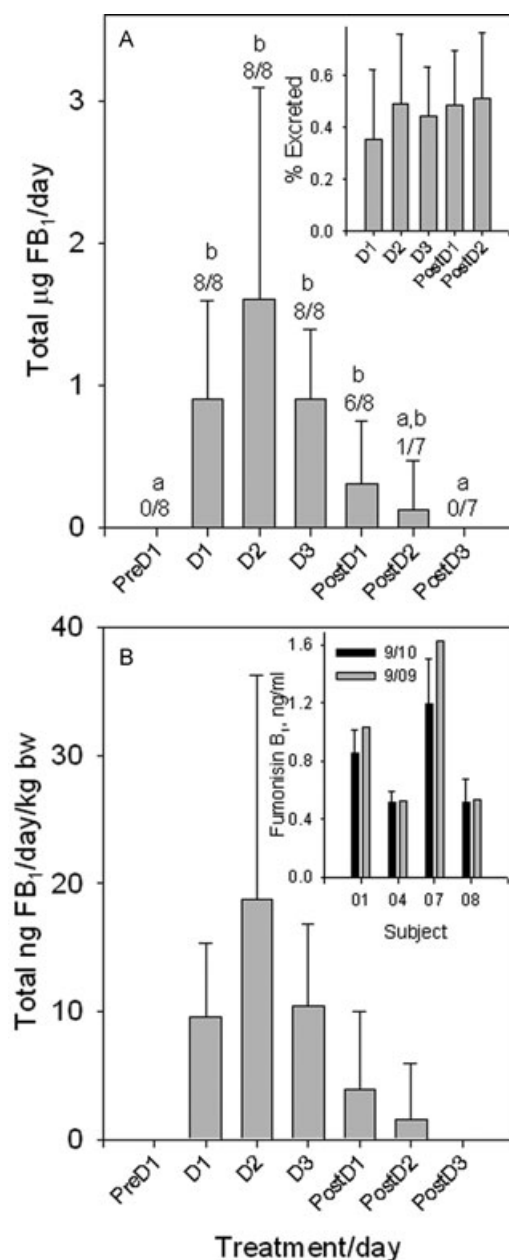


Figure 4. (A) Mean urinary FB₁ excreted per day ($\mu\text{g}/24 \text{ h}$) by eight volunteers abstaining from consuming maize-foods for 3 days (PreD1), 3 days consuming six tortillas and five biscuits/days (D1–D3) followed by 3 days abstaining (PostD1–PostD3) (The PostD4 and PostD5 urine samples were not analyzed and samples for PostD2 and 3 were not available for one volunteer). The number of volunteers that were FB₁ positive over the total urine samples analyzed is shown above each error bar. Mean values with differing superscripts are significantly different. Inset in (A) is the mean cumulative FB₁ excreted in the urine as a percentage of the cumulative FB₁ intake. (B) FB₁ concentration (ng/mL) normalized to body weight (The results of the statistical analysis was the same as in (A)). Inset in (B) is the result of the reanalysis ((September 2010 = 9/10, $n = 3$ replicates) of urine samples from four volunteers stored frozen and analyzed 1 year after the initial analysis (September 2009 = 9/09, $n = 1$).

urine volume. In total, $0.50\% \pm 0.24\%$ (\pm SD, $n = 8$) of the FB₁ consumed was excreted in the urine. All subjects had detectable levels of urinary FB₁ on consuming days 1, 2, and 3 and because of the high variability, there was no significant difference in the mean total urinary FB₁ on any of the consuming days or post dosing days 1 and 2. By post dosing day 3, there was no FB₁ detected in the urine. Normalization of total urinary FB to the weight of each volunteer did not reduce the variability (Fig. 4B). The average maximum concentration of FB₁ in the urine samples ($1.36 \text{ ng/mL} \pm 0.76 \text{ ng/mL}$; \pm SD, $n = 8$) was similar to the highest concentration (1.40 ng/mL) found in *Study 2*, the 6-day study described above (Fig. 3A). FB₂ and FB₃ were not detected in any of the FB₁ positive spot urine samples analyzed (0/26).

3.5 Stability of FB₁ in stored urine samples

Collecting of spot urine samples was used in sampling urine in Guatemala and those samples were stored frozen for up to a month after collection. Samples of the urine collected and analyzed from four volunteers in September of 2009 were reanalyzed in September of 2010 (Fig. 4B, inset). The results show that FB₁ concentration in the urine stored frozen at -20°C for 1 year was very similar to that in the original urine sample. Thus, FB₁ was stable in the frozen urine for up to 1 year.

3.6 FB₁ levels in urine of maize consumers in Guatemala (*Study 4*)

A method for assessing FB contamination of maize in Guatemala by express shipping maize extracts processed in Guatemala for analysis at the USDA laboratory in Athens has been used successfully for several years [13, 24]. In order to test if the modified process would work equally well with human urine, volunteers were recruited from two Departments in Guatemala (*Study 4*). Healthy volunteers (male and female) from these two communities were recruited in March of 2011 and asked to complete a questionnaire and if their consumption of maize-based foods, based on recall, was greater than or equal to 410 g/day , then they were asked to provide a urine sample after giving informed consent. Samples of maize (1–2 kg) sold for human consumption in the local market were collected at the same time in March 2011. Analysis of the maize showed low levels of FB in the maize (Fig. 5A). The average level of FB₁ in the maize purchased in Chimaltenango and Escuintla was $0.39 \pm 0.34 \text{ mg/kg}$ ($n = 5$) and $0.33 \pm 0.42 \text{ mg/kg}$ ($n = 10$), respectively, differences that were not significantly different. Similarly, the mean consumption of maize-based foods was not significantly different between Chimaltenango and Escuintla or among males and females in both communities (Fig. 5B). The only statistically significant difference in urinary FB₁ was that males in Escuintla had higher mean levels and a higher frequency of positive samples compared to females in either Chimaltenango or

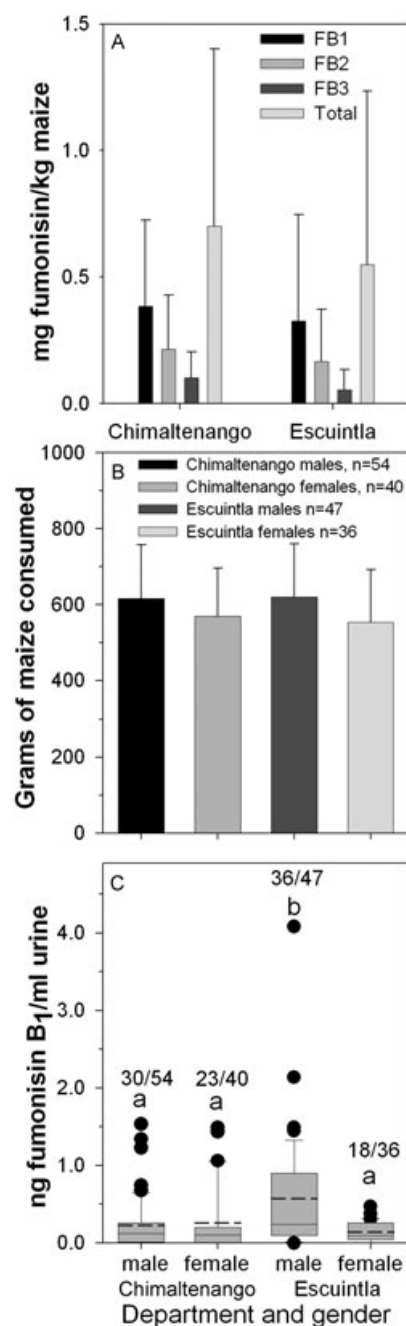


Figure 5. (A) The average (\pm SD) concentration ($\mu\text{g/g}$) of FB₁, FB₂, FB₃ and total FB (FB₁+FB₂+FB₃) in maize samples collected in Chimaltenango ($n = 5$) and Escuintla ($n = 10$) at the same time that urine samples were collected. (B) The average consumption (\pm SD) of maize-based foods by males and females. The estimated grams of dry maize equivalents are given in Supporting Information Table S7. (C) The box plot of the urinary FB₁ concentration (ng/mL) in the spot urine samples from males and females in Chimaltenango and Escuintla. The numbers above each bar in (C) are the total FB₁ positive urine samples over the total urine samples analyzed. The dashed line associated with each box is the mean value and the solid line is the median. The top of the error bars is the 95% confidence limit and the solid circles are outliers.

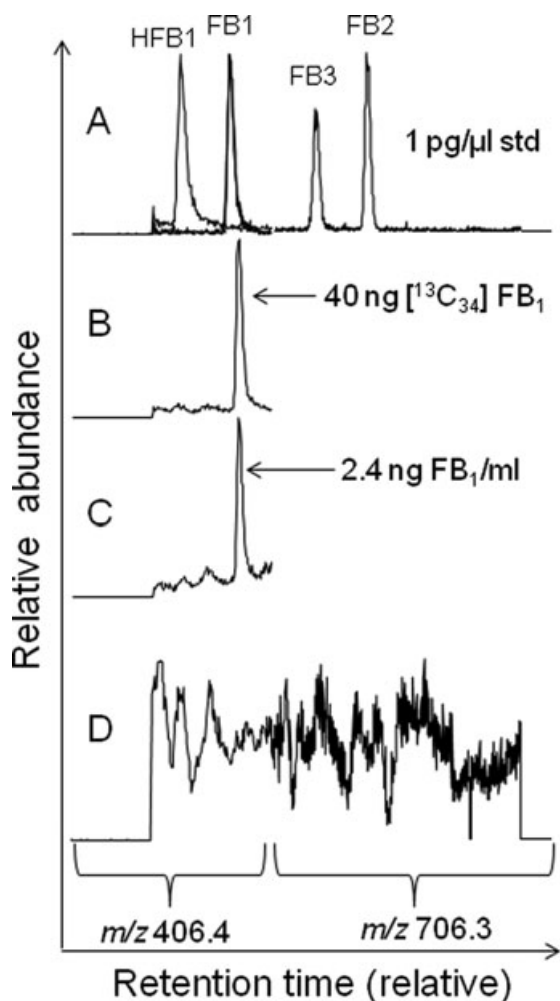


Figure 6. Example of a urine sample from Guatemala showing clear evidence of FB₁ but no evidence of FB₂, FB₃, or hydrolyzed FB₁. (A) A composite chromatogram showing the separation and retention time of a 1 pg/μL standard of HFB₁, U-[¹³C₃₄] FB₁ and FB₁, FB₂, and FB₃. (B) U-[¹³C₃₄] FB₁ in a 9 mL urine sample spiked with 40 ng of U-[¹³C₃₄] FB₁ before extraction and clean-up on the C₁₈-SPE cartridge. (C) The FB₁ in the urine sample calculated to contain 2.4 ng FB₁/mL based on recovery of U-[¹³C₃₄] FB₁. (D) TIC scanning at 406.4 m/z and 706.3 m/z showing there are no definitive peaks at the correct retention times (see (A)) or mass spectra (data not shown) for HFB₁ (m/z 406.4) or FB₂ or FB₃ (m/z 706.3).

Escuintla or males in Chimaltenango (Fig. 5C). A total of 177 spot urine samples were analyzed from Chimaltenango and Escuintla and 107 were positive for FB₁, but none contained detectable levels of FB₂ or FB₃ or hydrolyzed FB₁ (Fig. 6A–D).

4 Discussion

The kinetic studies in humans (*Study 1, 2, and 3*) showed that FB₁ was rapidly absorbed following oral exposure. Once absorbed, FB₁ levels remained elevated in the urine but the lev-

els rapidly decreased after consumption of foods containing FB₁ ceased. For example, after consuming FB contaminated diets for 3 or 6 days FB₁ was not detectable in urine 5 days after intake ceased. Thus, urinary elimination is rapid with an apparent half-life (visually approximated) of less than 48 h when consuming for 3 consecutive days and greater than 48 h but less than 72 h when consuming for 6 consecutive days.

The total urinary excretion of FB₁ was less than 1% of the cumulative dose ranging from 0.12 to 0.90% (*n* = 10) which is greater than that reported in van der Westhuizen et al. [17] but at the lower range of values (0.25–2%) reported in animals (reviewed in [19–21]). FB₁ was excreted in urine much more efficiently than FB₂ or FB₃. FB₂ and FB₃ were never convincingly detected in human urine samples. The LOD for FB₂ in the urine was less than 0.04 ng/mL urine. In the maize and maize-based foods analyzed in this study, the FB₂ concentration was between 20% and 50% of the FB₁ concentration (Fig. 5A and Supporting Information Tables S4, S5, and S6). Thus, based on the LOD for FB₁ and FB₂, if FB₁ and FB₂ were absorbed and excreted with equal efficiency, then FB₂ should have been detected in urine samples that contained greater than 0.2 ng/mL of FB₁. Assuming that fumonisins B₁, B₂ and B₃ are absorbed equally well from the gastrointestinal tract, then FB₁ would appear to be transferred to the urine much more efficiently than FB₂ or FB₃. Alternatively, relative to FB₁, FB₂ and FB₃ may not be as well absorbed as suggested by the absence of toxicity or disruption of sphingolipid metabolism in liver of mice-fed pure FB₂ or FB₃ [11]. Reduced absorption is also consistent with the observation that in rat serum, liver, and kidney FB₂ and FB₃ were detected in lower levels than expected based on the levels of FB₁, FB₂, and FB₃ in the diets [26]. Hydrolyzed FB₁ was also never clearly detected in urine samples from Guatemala even when the urinary FB₁ concentration was as high as 4 ng/mL (Fig. 5C). This result is also consistent with the reduced toxicity of hydrolyzed FB₁ in animal models [11, 12] and may also reflect less efficient absorption from the gastrointestinal tract. This is of importance from the point of view of risk managers since the current established group PMTDI is for FB₁, FB₂, and FB₃, alone or in combination [8].

The percentage of the predicted FB₁ intake excreted in the urine of the Guatemalan consumers was similar to that seen in the controlled studies in the USA. Assuming that the mean levels of FB₁ in the Guatemalan maize (Fig. 5A) are representative of the maize being used to produce nixtamalized maize-based foods and that traditional Mayan nixtamalization reduced the total fumonisins (FB) by 50% [10] and that FB₁ constitutes 50% of the remaining FB [10], then the calculated estimated mean FB₁ intake for people in Chimaltenango and Escuintla would be 0.45 μg/kg b.w./day ((0.356 μg FB₁/g maize × 337 g maize/day)/67 kg). The mean urinary FB₁ for the Guatemalan consumers was 0.30 ng/mL. Assuming daily urine output of 1000 mL, then on average the total amount of FB₁ excreted in the urine was 0.3 μg/day. The calculated average FB₁ intake/day/person is

30.0 µg (0.45 µg/kg b.w./day × 67 kg). Thus, 1.0% of the FB₁ intake was excreted in the urine. For comparison, the percentage of the FB₁ intake excreted in the urine by the volunteers in the controlled studies in the USA was 0.50% ± 0.24% ($n = 8$).

The significantly higher level of urinary FB₁ in males in Escuintla is difficult to explain since there was no difference between males and females in Chimaltenango and the amount of maize-based food consumed was the same. One possibility is that the source of the maize being consumed by males in Escuintla is not the same as for the females. One difference between Chimaltenango and Escuintla is that Escuintla is a much more rural population (Supporting Information Table S7) with many large sugar cane plantations. It is possible that some of the males recruited in Escuintla were migrant workers and were eating maize-based foods containing higher levels of FB₁ than the women living in Escuintla.

In conclusion, the levels of FB₁ in the urine, even under controlled conditions, are highly variable suggesting that the processes regulating excretion of FB are complex. Nonetheless, the results provide support for the usefulness of urinary FB₁ as a marker to assess ongoing exposure in population-based studies. In individuals consuming maize every day (as in Guatemala), the difference between AM and PM spot urine samples should be minimal if the level of exposure is relatively constant. However, relating the urinary FB₁ concentration in spot urine samples to dietary intake of FB in individuals will be complicated due to interindividual variability and the rapidity of clearance. Regardless, the monitoring of urinary FB₁ levels in combination with more mechanism-based biomarkers has the potential for testing the likelihood of FB as a contributing factor in human diseases in areas of the world where maize is consumed in large amounts and FB exposure is likely.

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