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The Kinetics of Urinary Fumonisin B_1 Excretion in Humans Consuming Maize-Based Diets

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Abstract

Fumonisins (FB) are mycotoxins found in maize. The purpose of this study was to 1) determine the relationship between FB_1, FB_2 and FB_3 intake and urinary excretion in humans, 2) validate a method to isolate urinary FB on C_{18}-SPE cartridges for international shipment, and 3) test the method using samples from Guatemala. 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 FB_1, FB_2 and FB_3 and hydrolyzed FB_1. Only FB_1 was detected in urine suggesting lower absorption of FB_2 and FB_3. Excretion was highly variable peaking soon after consumption began and decreasing rapidly after consumption stopped. Within five days after consumption ended FB_1 was not detected in urine. In a study with eight volunteers, the average total urinary FB_1 was 0.5% of the intake. FB_1 was detected in 61% (107/177) of the samples collected in Guatemala. The results support the use of urinary FB_1 to assess ongoing exposure in population based studies. However, relating the FB_1 concentration in urine to dietary intake of FB by individual subjects will be complicated due to inter-individual variability and the rapidity of clearance.

Keywords

Fumonisin; *Fusarium verticillioides*; Urinary fumonisin B_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 FB_1, FB_2 and FB_3 [6, 7]. Where maize is a dietary staple, the probable daily intake of FB indicate that many maize consumers will exceed the provisional maximum tolerable daily intake (PMTDI) of 2 μg/kg

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b.w. (FB_1, FB_2 and FB_3 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 tricarballylic acid side chains [10]. HFB_1 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, FB_1 is excreted in both feces and urine [14, 15]. Studies in areas of the world where maize is consumed in large amounts have used urinary FB_1 to evaluate human exposure. These studies show that even though the concentration of FB_1 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 FB_1 [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 FB_1 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, FB_1 is rapidly but poorly absorbed from the gastrointestinal tract [19, 20, 21]. Once absorbed there is no evidence that FB are metabolized. The majority of the FB_1 ingested is excreted in the feces unchanged or as the fully hydrolyzed or partially hydrolyzed form; lacking one of the two tricarballylic acid side chains. Relative to feces, a much smaller amount of FB_1 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 FB_1 is detected in urine [15, 16, 17, 18] relative to what has been reported in feces [14]. In the one study where the transfer to urine could be indirectly estimated the percent FB_1 was calculated to be 0.075% (0.054–0.104%) [17]. All of the studies of urinary excretion in humans have analyzed only FB_1 [15, 16, 17, 18]. Thus, there is no published human information documenting urinary excretion of FB_2 and FB_3.

The specific objectives of this study were 1) determine the quantitative relationship between FB_1, FB_2 and FB_3 dietary intake and urinary excretion in humans consuming maize-based foods in amounts approximating consumption where maize is a dietary staple, 2) develop and validate a method to isolate urinary FB_1, FB_2 and FB_3 on C_{18}-SPE cartridges for international shipment for analysis by LCMS, and 3) test the method using urine samples collected from humans in Guatemala.

MATERIALS AND METHODS

Chemicals and standards

Acetonitrile (ACN) and water were HPLC grade and formic acid was reagent grade. FB_1, FB_2 and FB_3 (>95% pure) and also qualitative standards of HFB_2 and HFB_3 were provided as a gift from Ronald Plattner, National Center for Agricultural Utilization Research, USDA-ARS, Peoria, IL, USA. HFB_1 was prepared from pure FB_1 by hydrolysis in 2 N

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KOH (70°C overnight) followed by cleanup over a 35 cc C_{18} SepPak® column (Waters, Milford, MA USA) [10]. N-(1-deoxyfructos-1-yl) FB_1 was a gift from Lauren S. Jackson USFDA, Summit-Argo, IL USA). A qualitative standard of fumonisin C_1 was a gift from Jennifer Tonos (USDA, ARS, Crop Genetics and Production Research Unit, Stoneville, MS). U-[$^{13}C_{34}$]-FB_1-solution (25 μg/ml) OEKANAL® was purchased from Sigma-Aldrich Laborchemikalien, Seelze, Germany.

Liquid chromatography/ mass spectrometry (LCMS) quantitation of FB

The LCMS method for quantitation of FB_1, FB_2 and FB_3 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.

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 manufactures packages without additional mixing and extracted and processed using C_{18}-SPE cartridges (Sep-Pak® Classic C_{18} 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-[$^{13}C_{34}$]-FB_1 internal standard closely approximated the recoveries of FB_1, FB_2 and FB_3 (Supporting Information Table S1). Thus, the quantitation of FB_2 and FB_3 based on the recovery of U-[$^{13}C_{34}$]-FB_1 provides a reasonably accurate estimate of the FB_2 and FB_3 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 FB_1, FB_2 and FB_3 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)

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 minutes/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 g±0.7 g (±SD, n=19) and 18.2 g±0.4 g (±SD, n=18), respectively. After mixing, a total of 150 ng of U-[$^{13}C_{34}$]-FB_1 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 C_{18}-SPE cartridges, and analyzed by LCMS as described in the online Supporting Information Tables

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S5 and S6. Samples were analyzed for U-[$^{13}C_{34}$]-FB_1, FB_1, FB_2 and FB_3 and HFB_1, HFB_2 and HFB_3. Unless stated otherwise, the quantitation of all FB was based on the recovery of the U-[$^{13}C_{34}$]-FB_1 internal standard.

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

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

Ten healthy (self described) volunteers (2 females and 8 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 FB_1, FB_2 and FB_3 using C_{18}-SPE cartridges. The human-subjects research protocol and informed consent form 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: 1) answer questions about their health, age, weight and activity level, 2) receive instructions about maize-based foods to avoid during the periods requiring abstaining from maize-based foods, and 3) 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 to 64 yr) and the average height and weight was 178 cm (157 to 191 cm) and 91 kg (73 to 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 three 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 the 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 six 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 hours 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).

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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 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).

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 C_{18}-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 form was approved by the Comité Institucional de Etica 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 C_{18}-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 Parafilm™ 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 FB_1, FB_2 and FB_3. For additional details on FB stability, processing and shipping of the C_{18}-SPE cartridges see Riley et al. [24].

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 post-hoc 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 *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 ±SD, and differences among or between means was considered to be significant if the probability (p) was <0.05.

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 FB_1 was never detected in any of the samples that were analyzed.

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FB_1 Detection in Urine (Study 1)

In *Study 1*, the single volunteer consumed 206 grams (daily total) of maize-based food (6 tortillas and 5 biscuits) in three meals (morning, afternoon, evening) for 3 days (72 h). The concentration of FB_1 in the tortillas and biscuits consumed was 1.37 μg/g and 1.63 μg/g, respectively (Supporting Information Table S5). Compared to the uncooked starting material, the tortillas and biscuits contained significantly less FB_1 and total FB (FB_1+FB_2+FB_3) (Supporting Information Table S5). Based on the cooked foods the single volunteer consumed 306 μg of FB_1/day and the FB_1 intake was 4 μg/kg b.w./day.

Of eight urine samples analyzed over the course of 26 h, all were positive for FB_1 (8/8) but there was no FB_2 or FB_3 detected (0/8). The absence of FB_2 and FB_3 in the spot urine samples in the preliminary study was unexpected. It was hypothesized that the SPE cartridges were not binding or releasing FB_2 and FB_3. To test this hypothesis, the eight urine samples were spiked with FB_3 and it was confirmed that the C_{18}-SPE were effectively binding FB_3 (Fig. 1). During the development of the method (*Study 1* and *2*) it was found that recoveries of FB_2 and FB_3 were slightly, but not significantly, greater (8% and 23%, respectively) than FB_1 (Supporting Information Table S8) a result consistent with the estimated LODs for FB_1, FB_2 and FB_3 (Supporting Information Table S3) which showed that the LOD were similar for all three FBs.

Time-Course of FB_1 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 FB_1 was detected in the urine sample. The FB_3 added to the urine samples before extraction (20 ng total/sample) was used as an internal standard for quantification of FB_1. Based on the FB_3 recovery, the concentration of FB_1 at 10:15 on day 1 of dosing (Fig. 1C, D1-2.75 h) was 0.217 ng FB_1/ml and at 26 h after the first meal the level of FB_1 in the urine was 0.78 ng FB_1/ml (Fig. 1D, D1-26 h). Three days after the last meal (Fig. 1E, PostD3) only a trace (0.1 ng/ml) of FB_1 was detectable, and 4 days post dosing (Fig.1F, PostD4) no FB_1 was detected. Based on the total FB_1 intake (918 μg over 3 days) it was estimated that the cumulative percent FB_1 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 post-consumption. As in *Study 1*, no FB_2 or FB_3 was detected in any of the FB_1 positive urine samples (0/18). The total FB_1 excreted in the urine per day (Fig. 2A) and the cumulative FB_1 intake and cumulative FB_1 excreted in the urine (Fig. 2B) was calculated based on the average of the AM and PM FB_1 concentration in the urine (Fig. 3A) and the total daily urine volume. In total, 0.42 % of FB_1 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 FB_1/ml (Fig. 3A), a value similar to that seen in the 26 h sample in the preliminary study (Fig. 1D). The highest urinary FB_1 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, FB_1 was not detected in the urine (Fig. 3A).

Levels of FB_1 in Morning and Evening Spot Urine Samples (Study 2)

The urinary FB_1 in the AM and PM samples were similar although during the period of tortilla and biscuit consumption the PM levels were generally higher and post consumption 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

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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).

Inter-individual Variability in FB_1 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 FB_1 in tortillas and biscuits was 1.52 μg/g and 0.89 μg/g, respectively (Supporting Information Table S6) and the calculated intake was 256 μg of FB_1/day/volunteer resulting in an average intake of 2.94±0.55 μg/kg b.w./day (±SD, n=8).

The total FB_1 excreted in the urine (Fig. 4A) and the percent of the total FB_1 intake excreted in the urine (Fig. 4A, inset) was calculated based on the single FB_1 concentration in the urine in the PM sample and the total estimated daily urine volume. In total, 0.50% ± 0.24% (±SD, n=8) of the FB_1 consumed was excreted in the urine. All subjects had detectable levels of urinary FB_1 on consuming days 1, 2 and 3 and because of the high variability there was no significant difference in the mean total urinary FB_1 on any of the consuming days or post dosing days 1 and 2. By post dosing day 3 there was no FB_1 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 FB_1 in the urine samples (1.36 ng/ml ± 0.76 ng/ml; ±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). FB_2 and FB_3 were not detected in any of the FB_1 positive spot urine samples analyzed (0/26).

Stability of FB_1 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 FB_1 concentration in the urine stored frozen at −20°C for 1 year was very similar to that in the original urine sample. Thus, FB_1 was stable in the frozen urine for up to 1 year.

FB_1 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 [24, 13]. 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 to 2 kg) sold for human consumption in the local market was 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 FB_1 in the maize purchased in Chimaltenango and Escuintla were 0.39±0.34 mg/kg (n=5) and 0.33±0.42 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 FB_1 was that males in Escuintla had higher mean levels and a higher frequency of positive samples compared to females in either Chimaltenango or Escuintla or males in Chimaltenango (Fig. 5C). A total of 177 spot urine samples were analyzed from Chimaltenango and Escuintla and

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107 were positive for FB_1, but none contained detectable levels of FB_2 or FB_3 or hydrolyzed FB_1 (Fig. 6 A–D).

DISCUSSION

The kinetic studies in humans (*Study 1, 2 and 3*) showed that FB_1 was rapidly absorbed following oral exposure. Once absorbed, FB_1 levels remained elevated in the urine but the levels rapidly decreased after consumption of foods containing FB_1 ceased. For example, after consuming FB contaminated diets for 3 or 6 days FB_1 was not detectable in urine 5 days after intake ceased. Thus, urinary elimination is rapid with an apparent half-life (visually approximated) of < 48 h when consuming for 3 consecutive days and > 48 h but < 72 h when consuming for 6 consecutive days.

The total urinary excretion of FB_1 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 to 2%) reported in animals (reviewed in [19, 20, 21]. FB_1 was excreted in urine much more efficiently than FB_2 or FB_3. FB_2 and FB_3 were never convincingly detected in human urine samples. The LOD for FB_2 in the urine was less than 0.04 ng/ml urine. In the maize and maize-based foods analyzed in this study the FB_2 concentration was between 20% and 50% of the FB_1 concentration (Figure 5A and Supporting Information Tables S4, S5 and S6). Thus, based on the LOD for FB_1 and FB_2, if FB_1 and FB_2 were absorbed and excreted with equal efficiency then FB_2 should have been detected in urine samples that contained greater than 0.2 ng/ml of FB_1. Assuming that fumonisins B_1, B_2 and B_3 are absorbed equally well from the gastrointestinal tract, then FB_1 would appear to be transferred to the urine much more efficiently than FB_2 or FB_3. Alternatively, relative to FB_1, FB_2 and FB_3 may not be as well absorbed as suggested by the absence of toxicity or disruption of sphingolipid metabolism in liver of mice fed pure FB_2 or FB_3 [11]. Reduced absorption is also consistent with the observation that in rat serum, liver and kidney FB_2 and FB_3 were detected in lower levels than expected based on the levels of FB_1, FB_2 and FB_3 in the diets [26]. Hydrolyzed FB_1 was also never clearly detected in urine samples from Guatemala even when the urinary FB_1 concentration was as high as 4 ng/ml (Fig. 5C). This result is also consistent with the reduced toxicity of hydrolyzed FB_1 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 FB_1, FB_2 and FB_3, alone or in combination [8].

The percentage of the predicted FB_1 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 FB_1 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 by 50% [10] and that FB_1 constitutes 50% of the remaining fumonisins [10] then the calculated estimated mean FB_1 intake for people in Chimaltenango and Escuintla would be 0.45 μg/kg b.w./day ((0.356 μg FB_1/g maize × 337 g maize/day)/67 kg). The mean urinary FB_1 for the Guatemalan consumers was 0.30 ng/ml. Assuming daily urine output of 1000 ml then on average the total amount of FB_1 excreted in the urine was 0.3 μg/day. The calculated average FB_1 intake/day/person is 30.0 μg (0.45 μg/kg b.w./day×67 kg) which is 1.0% of the calculated FB_1 intake. For comparison the percentage the FB_1 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 FB_1 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

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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 FB_1 the women living in Escuintla.

In conclusion, the levels of FB_1 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 FB_1 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 FB_1 concentration in spot urine samples to dietary intake of FB in individuals will be complicated due to inter-individual variability and the rapidity of clearance. Regardless, the monitoring of urinary FB_1 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

FB	fumonisins
FB_1	fumonisin B_1
FB_2	fumonisin B_2
FB_3	fumonisin B_3
HFB_1	hydrolyzed fumonisin B_1
PMTDI	provisional maximum tolerable daily intake.

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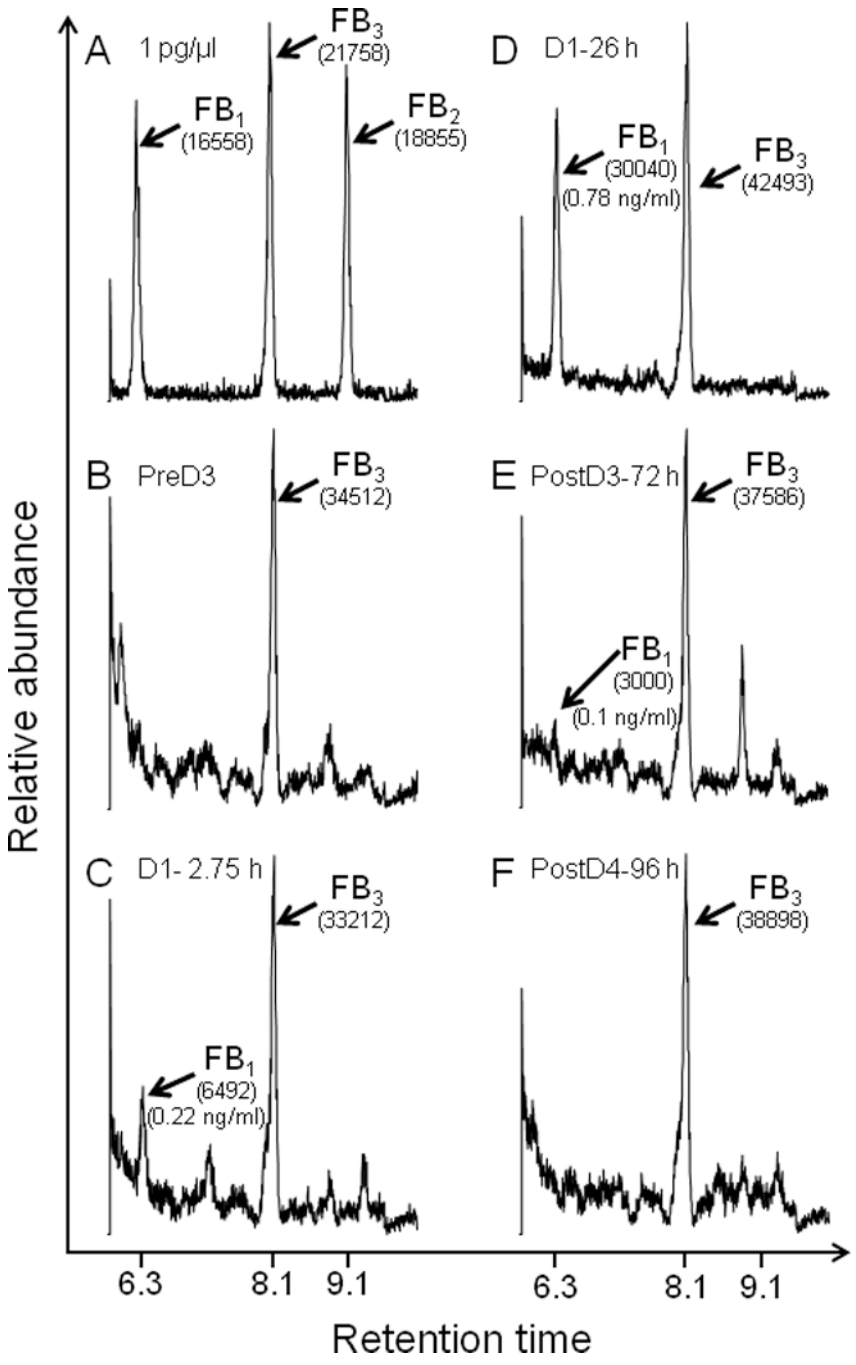

Figure 1.

(A) Chromatogram showing the separation, retention times, and area (number in parentheses) under the peaks for a standard solution containing 1 pg/µl of FB_1, FB_2 and FB_3. In all the other panels (B, C, D, E, and F) the chromatograms are from urine samples spiked with FB_3 before C_{18}-SPE clean up. In the FB_1 positive samples (C, D and E) the areas and the ng FB_1/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 FB_1, FB_2 and FB_3 (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

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foods (PostD3 and PostD4). None of the peaks eluting near the retention time of FB_2 had the base peak or mass spectra of FB_2 (data not shown). The urinary FB_1 levels for each 24 h interval calculated based on the recovery of FB_3 are shown in Supporting Information Table S9.

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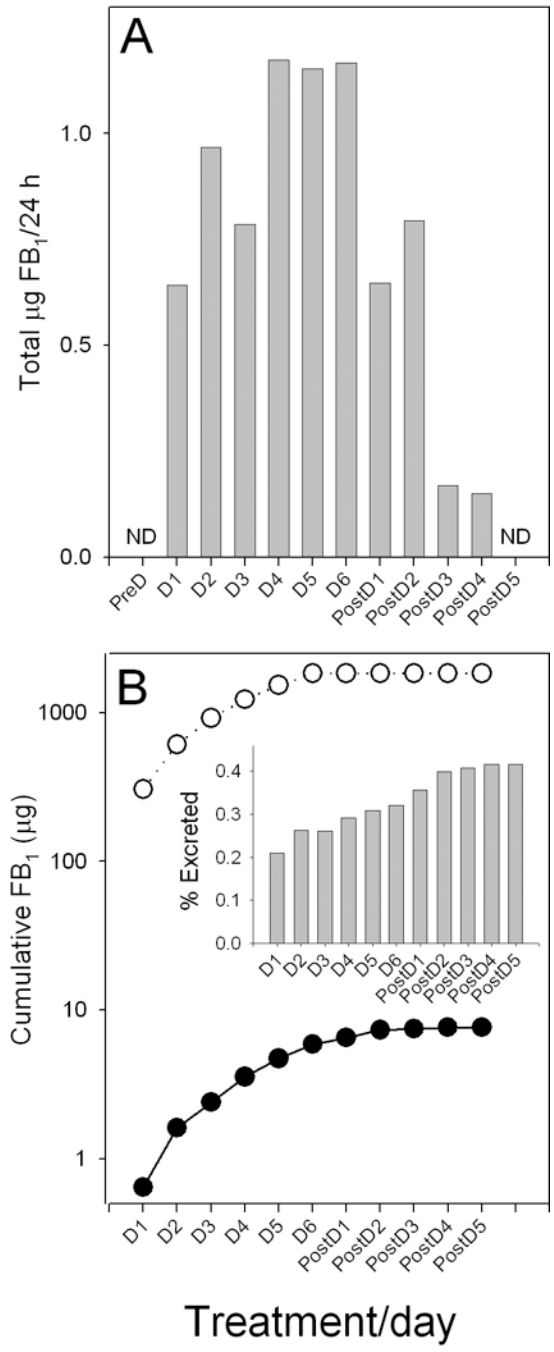

Figure 2.

(A) Total daily urinary FB_1 excreted (μg/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 FB_1 intake (open circles) and cumulative excretion in the urine (solid circles) over the entire period of consuming (D1-D6) and post-consuming (PostD1-5). Inset in (B) is the percentage of the cumulative FB_1 intake excreted in the urine at each time period. ND= not detected.

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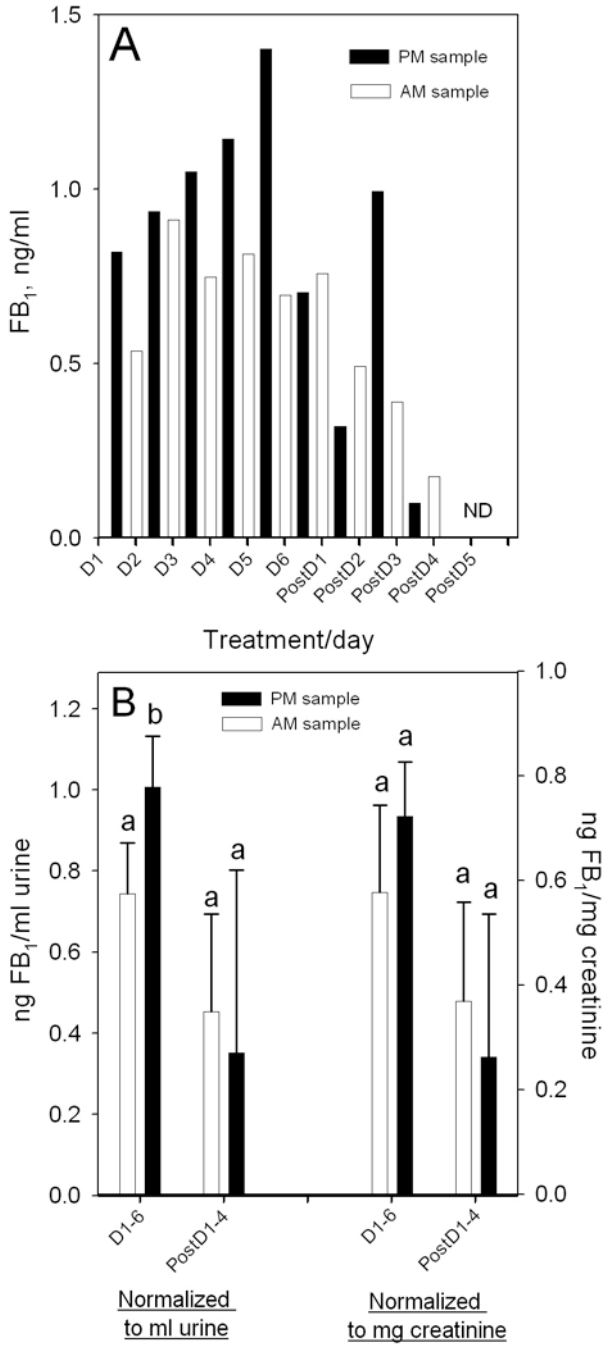

Figure 3.

(A) FB_1 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 FB_1 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 ($p<0.05$).

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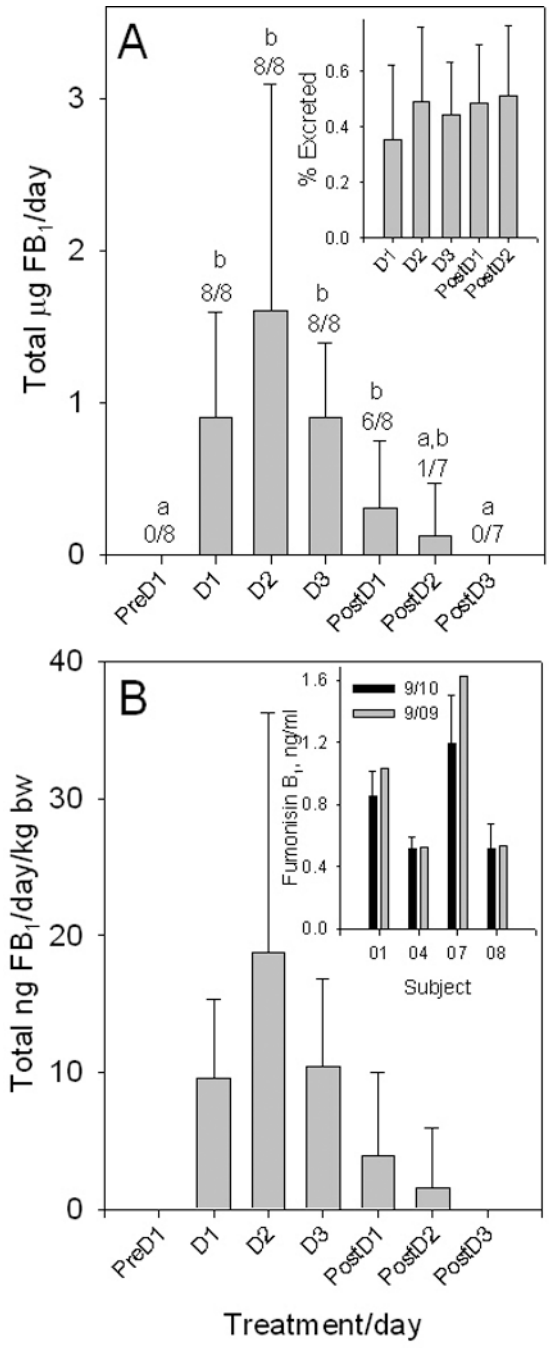

Figure 4.

(A) Mean urinary FB_1 excreted per day (µg/24 h) by eight volunteers abstaining from consuming maize-foods for 3 days (PreD1), 3 days consuming 6 tortillas and 5 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 FB_1 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 FB_1 excreted in the urine as a percentage of the cumulative FB_1 intake. (B) FB_1 concentration (ng/ml) normalized to body weight (The results of the statistical analysis was the same as in (A)). Inset in (B) is the

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results of the re-analysis ((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).

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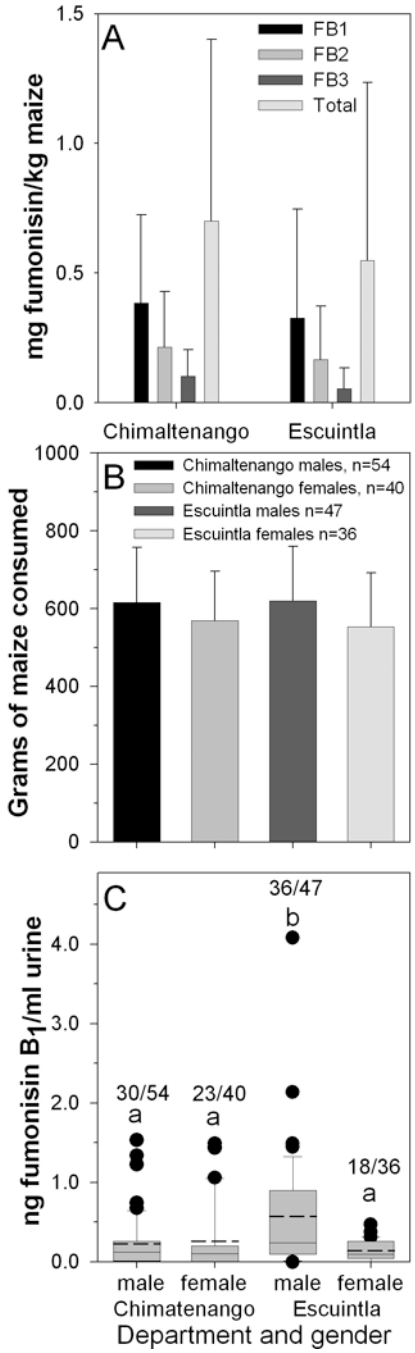

Figure 5.

(A) The average (±SD) concentration (μg/g) of FB_1, FB_2, FB_3 and total FB (FB_1+FB_2+FB_3) 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 (±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 FB_1 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 FB_1 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.

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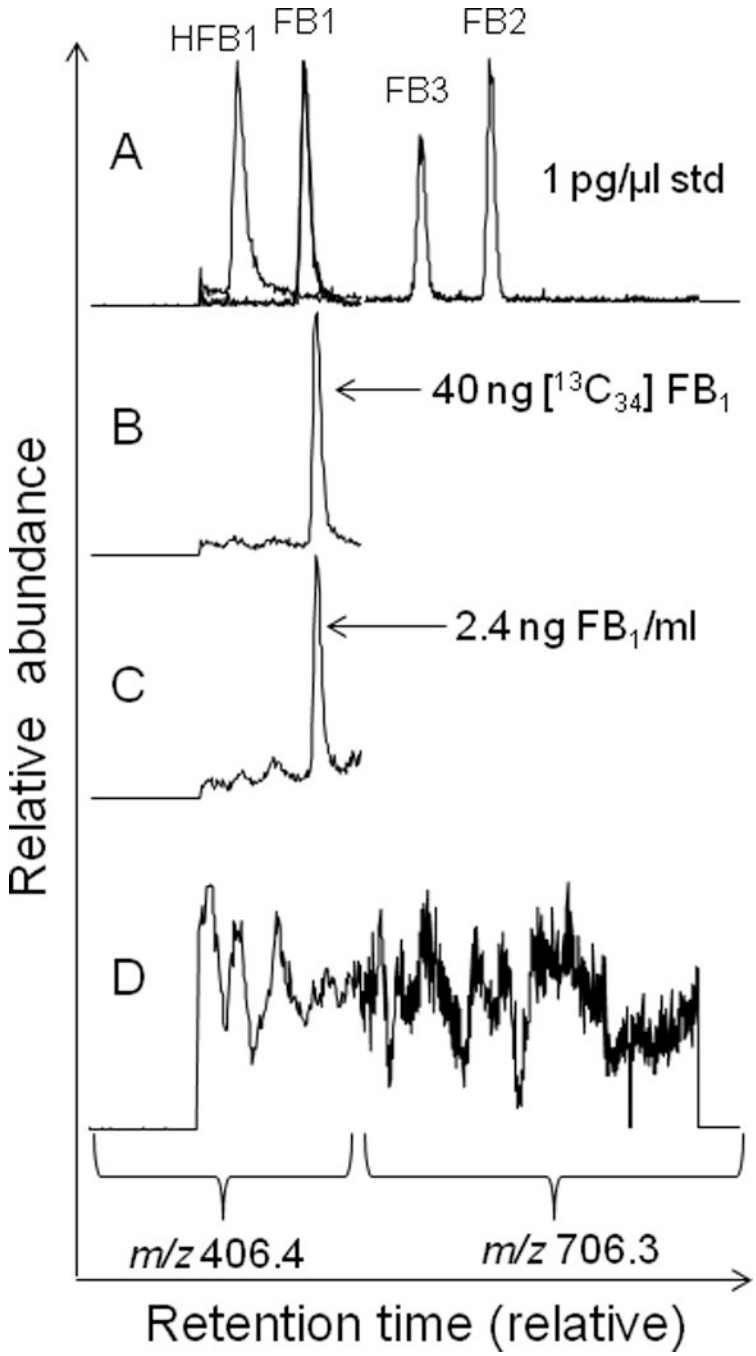

Figure 6.

Example of a urine sample from Guatemala showing clear evidence of FB_1 but no evidence of FB_2, FB_3 or hydrolyzed FB_1. (A) A composite chromatogram showing the separation and retention time of a 1 pg/µl standard of HFB_1, U-[$^{13}C_{34}$] FB_1 and FB_1, FB_2 and FB_3. (B) U-[$^{13}C_{34}$] FB_1 in a 9 ml urine sample spiked with 40 ng of U-[$^{13}C_{34}$] FB_1 before extraction and clean-up on the C_{18}-SPE cartridge. (C) The FB_1 in the urine sample calculated to contain 2.4 ng FB_1/ml based on recovery of U-[$^{13}C_{34}$] FB_1. (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 HFB_1 (*m/z* 406.4) or FB_2 or FB_3 (*m/z* 706.3).

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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
Study 1	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.
Study 2	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 *Study 1*.
Study 3	11 days (3/3/5)	n=8 (n=88, 1 sample/day/volunteer)	**1** Determine inter-individual variability. **2** Confirm clearance kinetics from *Studies 1&2*. **3** Confirm ability to detect FB_1, B_2 and B_3. **4** Confirm spot urine sample use to estimate intake.
Study 4	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 Supplemental Table 7.