Occurrence of AFM1 in urine samples of a Brazilian population and association with food consumption

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Aflatoxin M1 (AFM1) is a hydroxylated metabolite of aflatoxin B1 (AFB1), which may be used as aflatoxin B1 exposure biomarker. The AFM1 analysis was performed using immunoaffinity clean-up and detection by high-performance-liquid chromatography with fluorescence detector. A total of 69 samples were analyzed and 45 of them (65%) presented contaminations >1.8 pg ml⁻¹, which was the limit of quantification (LOQ). Seventy-eight percent (n = 54) of the samples presented detectable concentrations of AFM1 (>0.6 pg ml⁻¹). The AFM1 concentration among samples above LOQ ranged from 1.8 to 39.9 pg ml⁻¹. There were differences in food consumption profile among donors, although no association was found between food consumption and AFM1 concentration in urine. The high frequency of positive samples suggests exposure of the populations studied to aflatoxins.

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1. Introduction

Human exposure to aflatoxins is a concern worldwide because aflatoxins are potent cancer-promoting agents, especially liver cancer (International Agency for Research Cancer (IARC) 1993). One way of evaluating aflatoxin exposure through diet is to estimate the aflatoxin concentration in foods and the level at which these foods are consumed. However, several other factors may impair this estimation such as heterogeneity in relation to food contamination, difficulties in measuring food consumption, seasonal variations associated with food contamination and effect of food preparation on contamination level, among others (Hall & Wild, 1994; Polychronaki, 2007).

An alternative for a more precise evaluation of total aflatoxin exposure is to measure the presence of aflatoxin metabolites in body fluids such as milk, urine and blood (Hall & Wild, 1994). The detection of several aflatoxin metabolites in body fluids has already been reported in the literature (Essigmann et al., 1977; Gorelick, 1990; Mykkänen et al., 2005; Sabbioni, Skipper, Buchi, & Tannenbaum, 1987). However, these metabolites can only be used as evidence of aflatoxin exposure after their validation as biomarkers. For aflatoxins, the presence of adducts, aflatoxin N7 guanine in urine, aflatoxin lysine in blood and aflatoxin M1 in urine is considered a valid alternative for aflatoxin exposure verification (Groopman & Kensler, 1999).

Aflatoxin M1 (AFM1) is a hydroxylated metabolite of aflatoxin B1 (AFB1) originated from animal and human metabolism after the ingestion of aflatoxin B1, which may be present in body fluids such as urine, milk, and blood (Groopman, 1994). Only from 1.2% to 2.2% of the aflatoxin present in the diet is excreted as AFM1 in human urine (Zhu et al., 1987).

The use of AFM1 present in urine as biomarker of human exposure to aflatoxins has already been reported in several world populations. In Taiwan, the analysis of urinary excretion performed by Hatch et al. (1993) with 250 people resulted in concentrations ranging from 0.003 to 0.108 ng ml⁻¹. Data obtained by Cheng, Root, Pan, Chen, and Campbell (1997) with people from China and Taiwan revealed 64–66% of positivity, with concentrations ranging from 3.2 to 108 ng for 12 h and from 2.7 to 17 ng for 12 h among people who live in China and Taiwan, respectively. In Sierra Leone, Africa, concentrations ranged from 0.1 to 374 ng ml⁻¹, according to a study by Jonsyn-Ellis (2000). In Ghana, concentrations reported by Jolly et al. (2006) reached maximum levels close to 12 ng mg⁻¹ of creatinine. In the Czech Republic, Malík et al. (2006) reported the occurrence of AFM1 in 57.6% of urine samples analyzed, with concentrations ranging from 0.019 to 19.219 ng g⁻¹ of creatinine.
In Brazil, there are several reports on the occurrence of aflatoxin in foods aimed at human and animal consumption. However, corn and peanut, as well as corn and peanut-based products are the main foods related to aflatoxin contamination (Caldas, Silva, & Oliveira, 2002; Gloria, Fonseca, & Souza, 1997; Gloria, Romero, Carvalho, Calori-Domingues, & Gonçalves, 2006; Kawashima & Valente-Soares, 2006; Rodriguez- Amaya & Sabino, 2002; Sabino et al., 2000). Milk and dairy products are also frequently reported as contaminated in Brazil with low aflatoxin levels (Gonçalez et al., 2005; Oliveira & Ferraz, 2007; Oliveira, Germano, Bird, & Pinto, 1997); Oliveira, Rosmaninho, & Rosim, 2006; Shundo & Sabino, 2006.

However, there are few investigations on the presence of aflatoxin exposure biomarkers in the Brazilian population. Scussel, Haas, Gong, Turner, and Wild (2004) analyzed blood samples from 50 blood donors in a hospital in the city of São Paulo, Brazil, and observed the presence of AFB1-lysine in 62% of the samples analyzed, with concentrations ranging from <3 to 57.3 pg mg⁻¹. Navas (2003) reported the analysis for AFM1 of samples of human breast milk collected in a hospital in the city of São Paulo as well. Only one sample presented a contamination level of 0.024 ng ml⁻¹. The detection/quantification limit for the methodology proposed by that author was 0.01 ng ml⁻¹.

This study aimed to conduct a survey on the occurrence of aflatoxin M1 in the urine of individuals that live in Piracicaba, São Paulo, Brazil. Donor feeding habits were evaluated by means of two types of food inquiries and consumption data were tested for correlation with contamination levels observed in urine samples.

This study has been approved by Comitê de Ética em Pesquisa (Research Ethics Committee), Faculdade de Odontologia de Piracicaba - Universidade Estadual de Campinas (Protocol No. 095/2005).

2. Material and methods

2.1. Donor selection

Sixty-nine individuals were randomly selected among residents from different regions of the city of Piracicaba, São Paulo, Brazil as urine donors.

2.2. Food Inquiries

The individuals selected as donors answered two types of food inquiries, namely a feeding frequency inquiry (FFI) on the monthly consumption by individuals, and a 24-h recollection (IR24), in which the consumption of all foods ingested on the day before urine collection were recorded. Among the FFI component items and items reported by individuals in the IR24, foods were selected and grouped as corn-, peanut-, and milk-based foods, whose contamination by aflatoxins has been more frequently reported in the scientific literature.

2.3. Sampling

Each donor collected an early-morning urine sample using a 300 ml polyethylene flask. The sample volume ranged from 50 to 300 ml among donors. After collection, samples were identified and immediately sent to the laboratory, and were stored at −18 °C until analysis.

2.4. Aflatoxin M1 analysis in urine

The extraction and purification of urine samples for AFM1 determination were performed as recommended by Kussak, Andersson, and Andersson (1995), with some modifications. A 30 ml volume from the urine sample was filtered through a glass microfiber filter paper (Whatman Schleider & Schuell, Maidstone, England, product number 934-AH). Later, 20 ml of filtered extract was transferred to a 50 ml capacity vial and 20 ml of sodium acetate buffer (pH 5.0) was added. The pH of the mixture was measured and corrected to 5.0 using an appropriate volume of a 0.1 M glacial acetic acid solution whenever necessary. The mixture was directly passed through an immunoaffinity column (Neocolumn®, Neogem Europe, UK) at a flow rate of approximately 1.0–1.5 ml min⁻¹. After adding the mixture the column was washed with 40 ml of ultrapure water (Milli Q®, Millipore, Bedford, MA, USA). The column was dried by applying positive pressure with a syringe and bound AFM1 was eluted with 2.0 ml of HPLC-methanol which was recovered in a 4 ml vial previously treated with acid. The eluate was evaporated under nitrogen gas and reconstituted with 500 µl of the mobile phase before liquid chromatograph analysis.

Detection and quantification of sample extracts were performed by high-performance-liquid chromatography (HPLC) with a liquid chromatography system equipped with a LC-10AT Shimadzu pump (Kyoto, Japan), a Shimadzu RF-10AXL fluorescence detector (excitation 365 nm and emission 460 nm), an injection volume of 100 µl, and a reverse phase column (250 × 4.6 mm, particle size of 3 µm) and precolumn (Synergi Fusion®, Phenomenex Inc., Torrance, CA, USA) kept at room temperature. The mobile phase consisted of an isocratic mixture of water and acetonitrile at a volume ratio of 75:25 and a flow rate of 1.0 ml min⁻¹. A calibration curve was prepared using standard AFM1 solutions in mobile phase at concentrations of 0.005, 0.01, 0.02, and 0.03 ng ml⁻¹. The standard obtained [Sigma, St. Louis, MO, USA, product code 6428, 10 µg] as purified crystalline AFM1 was dissolved in HPLC-grade acetonitrile and its concentration was determined by spectrophotometry according to Trucksess (2005, chap. 49).

2.5. Aflatoxin M1 identity confirmation

The AFM1 identity was confirmed by the formation of AFM1-hemiacetal derivative (AFM1Ac). The sample extract was evaporated under nitrogen gas and re-dissolved with 200 µl of HPLC-n-hexane and 200 µl of trifluoroacetic acid (TFA). The mixture was vortexed during 1 min and left to rest for 10 min. The mixture was evaporated under nitrogen gas and re-suspended in 500 µl of mobile phase before injection into the liquid chromatography system. The disappearance or reduction of the original AFM1 peak and the appearance of a new peak at an earlier retention time confirmed the presence of AFM1 in the sample.

2.6. Method evaluation

The limit of detection (LOD) was considered as the lowest AFM1 concentration that could be reliably detected (>3:1 signal to noise ratio) in blank urine sample chromatograms. The quantification limit (LOQ) was considered as three times the LOD.

The method was studied for its recovery rate using human urine sample as blank and for this a non detect AFM1 urine sample was used. Seven replicates were made. AFM1 standard solution was added to samples immediately before analyze to give the concentration of 4.0 pg ml⁻¹ of AFM1. The recovery rate was considered as the concentration of AFM1 quantified over the concentration added. The repeatability measured by relative standard deviation was checked over replicates of urine samples used in the recovery study.

2.7. Statistical analysis

The results obtained were analyzed via the LSD Means Comparison Test (p < 0.05) between the individuals total and the 90 percentile of AFM1 concentrations in urine. The correlation between...
consumption of food groups and AFM1 concentrations in urine was tested via Spearman’s correlation Test. For statistical analysis purposes, AFM1 contamination results lower than the limit of detection of the methodology employed were considered numerically equal to zero, while values between the limits of quantification (1.8 pg ml\(^{-1}\)) and the detection (0.6 pg ml\(^{-1}\)) were considered as the mean between those extremes, that is, 1.2 pg ml\(^{-1}\). Statistical analyses were performed using Statistical Analysis System software (SAS, 2004).

3. Results and discussion

3.1. Method evaluation

The AFM1 retention time considering the liquid chromatographic conditions used was 14.0 min. The linearity of the calibration curve was 0.9907. The limit of detection (LOD) considering a final sample extract of 500 µl and an injection volume of 100 µl was 0.6 pg ml\(^{-1}\) and the limit of quantification (LOQ) was 1.8 pg ml\(^{-1}\). The recovery rates ranged from 73% to 97% with a mean value of 83% and relative standard deviations for seven spiked samples (4 pg ml\(^{-1}\)) of 20.7%. The AFM1 identity confirmation method by TFA described showed that the original AFM1 peak in the positive sample disappeared and a new peak appeared at a shorter retention time of 5.1 min.

3.2. Occurrence of aflatoxin M1

The AFM1 levels detected in urine samples are presented in Table 1. From 69 samples analyzed, 54 (78%) presented detectable AFM1 concentrations. Forty-five samples (65%) had concentrations between 1.8 and 9.7 pg ml\(^{-1}\) while the other 90% showed values lower than the limit of quantification.

3.3. Food intake

The FFI was used to estimate food intake habits among the individuals sampled and confirmed that the foods generally reported as contaminated with aflatoxins in Brazil were also part of the regular diet of donors. Milk and milk-based products were the foods consumed at the highest frequencies and quantities, and were consumed by all individuals (69), followed by corn and corn-based products (67) and peanut and peanut-based products (39).

IR24 was used to obtain the ingestion of foods on the day that preceded collection of the urine sample. Consumption of milk and milk-based products were the most frequent among the 69 individuals, and was reported by 61 individuals. Corn and peanut consumption had lower frequencies, and were reported by 12 and 14 individuals, respectively (Table 2).

The consumption of corn and corn-based product food group significantly differed from peanut and peanut-based products, as well as from milk and milk-based product in the FFI I and II IR24 inquiries. In IR24 I, corn and corn-based products and peanut and peanut-based products did not differ from each other, but were both different on average from milk-based products.

The comparison between means for all individuals and only those that reported consumption (Table 2) indicates that for FFI the mean of all individuals could be used as an instrument representative of the group. For IR24, the corn and peanut groups had significantly higher means when the analysis was made only between individuals that consumed those foods, demonstrating that for the evaluation of exposure to aflatoxins via those foods, it would also be important to consider consumption means only between individuals that reported some level of consumption.

3.4. Association between consumption of risk foods and urine AFM1

The correlation analysis for food intake and detected AFM1 levels, obtained by Spearman’s correlation Test, showed statistically non-significant \( r \) values (Table 3), indicating that no correlation trend exists between those factors. Consequently, in spite of the differentiated consumption of donors in relation to food groups, no association was found between the specific consumption of any of those food groups and AFM1 concentrations in the urine of individuals, as well as between the total consumption of those food groups and AFM1.

A correlation analysis between consumption AFM1 levels was also performed; comparisons were made for the group of donors with the highest AFM1 levels observed and all other donors. To accomplish that, the 90 percentile for AFM1 concentration in urine showed that 10% of the highest AFM1 concentrations in urine ranged from 9.8 to 39.9 pg ml\(^{-1}\) while the other 90% showed values lower than 9.7 pg ml\(^{-1}\). The means comparison between both groups of individuals considering the 90 percentile of contamination did not detect significant differences in food intake between individuals, although the mean AFM1 contamination values were significantly different between groups.

### Table 1

<table>
<thead>
<tr>
<th>AFM1 (pg ml(^{-1}))</th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.6</td>
<td>15</td>
<td>21.7</td>
</tr>
<tr>
<td>0.6–1.7</td>
<td>9</td>
<td>13.1</td>
</tr>
<tr>
<td>1.8–9.7</td>
<td>37</td>
<td>53.6</td>
</tr>
<tr>
<td>9.8–39.9</td>
<td>8</td>
<td>11.6</td>
</tr>
<tr>
<td>Mean(^*) (standard deviation): 5.96 (±6.39)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median(^*): 3.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coefficient of variation(^*): 107%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^*\) Values for the interval between 1.8 and 40.0 pg ml\(^{-1}\).

### Table 2

Comparison between consumption means reported in the FFI and IR24 inquiries for food groups considered as risk groups for contamination by aflatoxins.

<table>
<thead>
<tr>
<th>Food groups</th>
<th>Mean intake in inquiries (g/day)</th>
<th>FFI I</th>
<th>FFI II</th>
<th>IR24 I</th>
<th>IR24 II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn and corn-based products</td>
<td>25.3(^b)A, 23.2(^a)A, 22.9(^b)A</td>
<td>5.1(^a)A</td>
<td>9.1(^b)A</td>
<td>7.1(^a)A</td>
<td>35.0(^b)B</td>
</tr>
<tr>
<td>Peanut and peanut-based products</td>
<td>5.1(^b)A, 23.9(^a)A</td>
<td>0.1(^b)A</td>
<td>0.9(^b)A</td>
<td>13.3(^a)A</td>
<td>76.7(^b)B</td>
</tr>
<tr>
<td>Milk and milk-based products</td>
<td>286.1(^a)A, 286.1(^a)B</td>
<td>307.7(^a)A</td>
<td>348.0(^b)A</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^*\) In both inquiries, I corresponds to the mean of all individuals and II corresponds to the mean of individuals that reported some level of intake only.

\(^**\) The same lower case letters in the same column indicate that the means are not different from each other. Each in inquiry, equal upper case letters on the same row indicate that the means are not different from each other (LSD Test, \( p < 0.05 \)).

### Table 3

Spearman’s correlation Test between AFM1 and consumption reported in the FFI and IR24 inquiries for food groups.

<table>
<thead>
<tr>
<th>Food groups</th>
<th>Spearman’s correlation Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FFI I</td>
</tr>
<tr>
<td>Corn and corn-based products</td>
<td>−0.0174</td>
</tr>
<tr>
<td>Peanut and peanut-based products</td>
<td>0.1774</td>
</tr>
<tr>
<td>Milk and milk-based products</td>
<td>0.0876</td>
</tr>
</tbody>
</table>

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The lack of association between AFM1 levels present in urine and consumption of certain types of food investigated in this study shows that those foods do not make an important or expressive contribution toward exposure to aflatoxins through the diet. Perhaps in association with other foods they may contribute partially and fortuitously toward total exposure. However, the possibility that the source of exposure to aflatoxins here detected comes from a particular type of food or food group not addressed in the food inquiries formulated in this study cannot be ruled out (see Table 4).

Studies conducted in regions of Asia and Africa were successful in obtaining correlations between food ingestion and excretion of biomarkers for aflatoxins or with aflatoxicosis signals (Azziz-Baumgartner et al., 2005; Gan et al., 1988; Lewis et al., 2005; Ly et al., 1995; Zhu et al., 1987). However, it is observed that those regions are greatly susceptible to the occurrence of aflatoxins in foods, providing greater food exposure. In regions of China, it is estimated that the daily ingestion of AFB1 from corn consumption is 184.1 μg; however, AFB1 ingestion from total diet is even higher, estimated between 6.5 and 2027 ng kg⁻¹ d⁻¹. In regions of Africa, the ingestion of aflatoxins by peanut consumption ranges from 9.9 to 99.2 ng kg⁻¹ d⁻¹, but exposure via total consumption may reach from 3.5 to 183.7 ng kg⁻¹ d⁻¹ (Williams et al., 2004). There are few studies in Brazil dealing with human exposure estimation resulting from the presence of aflatoxins in foods. Oliveira, Germano, Bird, and Pinto (1997) estimated exposure to AFM1 in 4-month-old children and obtained values of 3.7 ng kg⁻¹ d⁻¹ for those individuals after analyzing milk samples for infant consumption in day-care centers in São Paulo, Brazil. Similarly, in a study on the occurrence of aflatoxins in foods, Amaral, Nascimento, Sekiyama, Janeiro, and Machinski (2006) estimated exposure via aflatoxin concentrations obtained in corn flour and consumption data available in the scientific literature, and obtained an exposure value of 0.14 ng kg⁻¹ d⁻¹ provided by the consumption of that corn-based food.

The AFM1 values in urine observed in this study were lower than those previously reported for other countries however, the number of positive samples observed was within the same range (Cheng et al., 1997; Hatch et al., 1993; Jolly et al. 2006; Jonsyn-El lis, 2000; Malir et al., 2006). It can be seen that although AFM1 in urine is a validated biomarker to evaluate exposure to aflatoxins, in this study the collection of a punctual urine sample that was not representative of a 12 or a 24 h period, prevented the estimation of ingestion of aflatoxins and, consequently, a correlation between total AFM1 excretion per time period with food consumption in the previous day. The possibility of AFM1 glucoronide and sulphate (Eaton, Ramsdell, & Neal, 1994) conjugated excretion must be considered with an additional factor which can affect the correlation (Mykkänen et al., 2005) despite free AFM1 be a validated biomarker. Nevertheless, the high rate of positive samples observed constitutes important information because it shows that those populations are exposed to aflatoxin.

Since the presence of AFM1 in urine is considered a short-term AFB1 exposure biomarker (Gropman, 1994) and aflatoxin contamination level is heterogeneous and could be seasonal (Council for Agriculture Science & Technology (CAST), 2003) perhaps a 24 h urine sample and a different sampling frequency could more adequately show AFM1 contamination in the urine of those populations.

### 4. Conclusions

This study is the first to report AFM1 in urine samples from Brazilian populations. In the Brazilian populations mentioned here, concentration levels were not as high as those reported for other countries but their frequencies, resulting from the low AFM1 detection limit achieved in this study, were as high as or even higher than those, showing that most donors were exposed to aflatoxin. The lack of association between consumption of the foods investigated in this study, considered as normally contaminated with aflatoxins in Brazil, and AFM1 levels in urine suggest that exposure of this population via their diet did not have those foods individually as the main sources of exposure. Further AFM1 biomarker studies are required to better evaluate the true levels of aflatoxin exposure over a long period, with urine collections that would quantitatively represent AFM1 excretion among individuals.

Since the city of Piracicaba is considered a region with high social and economic indicators (Instituto Brasileiro de Geografia e Estatística – IBGE (Internet), 2007), the observed high frequencies of aflatoxin exposure suggests that maybe other less developed regions of the country present disturbing levels of aflatoxin exposure, corroborating the need for new studies to evaluate individual exposure to aflatoxins in Brazil.

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