Use of an Improved Method for Analysis of Urinary Aflatoxin M1 in a Survey of Mainland China and Taiwan

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Abstract

An improved monoclonal antibody immunoaffinity chromatography/high-pressure liquid chromatography/fluorescence detection method was developed to measure aflatoxin (AF) exposure by quantifying AFM1 in human and rat urine samples.

Analysis of different amounts of various AF metabolites showed that the immunoaffinity resin was highly selective for aflatoxin B1 (AFB1), AFB2, and AFM1. Recovery of added AFs increased with the amount of immunoaffinity resin and was virtually complete within the range of 0.01–10 ng of AFM1 by using 7 ml of resin. The detection limit of this method is 0.5 pg/ml urine.

Rats dosed with tritiated AFB1 excreted in their urine tritiated AFM1, among other AF metabolites, as indicated by chemical derivative confirmation and cochromatography with authentic AFM1 and agreement of radioactivity and fluorescence quantitation. A linear dose-response relationship was found over the range of 0.05–50 μg/kg of body weight/day. Two humans dosed with 1.0 μg of pure AFB1 excreted 6–7% of the dose as urinary AFM1 over 5–7 days.

Pooled urine samples from 30 men from each of 69 rural counties in mainland China and 16 survey areas in Taiwan, with two villages per county or area, were analyzed with this improved method (170 villages total). The correlation coefficient of urinary excretion of AFM1 compared between villages within all 85 survey areas was 0.50 ($P < 0.001$). Sixty-five % of the samples contained detectable concentrations of AFM1 with an average excretion of 3.1 ng/12 h. Assuming an excretion rate of 2–6%, this AFM1 excretion corresponds to a very low average daily AF consumption of 0.1–0.3 μg/day (possible range, 0–11 μg/day). Patterns of urinary excretion of AFM1 were similar in mainland China and Taiwan.

Introduction

AFs[2] are a group of toxic secondary metabolites produced by the fungus *Aspergillus flavus/parasiticus*, which may contaminate a variety of human and animal food crops (1). The most common and most biologically active of these fungal metabolites is AFB1 (2). Upon ingestion and absorption of AFB1, a series of metabolites is produced, primarily in the liver. These include $AF_{epoxide}$, AFM1, AFP1, AFQ1, AFB2a, and aflatoxicol (3).

AF has been declared a human carcinogen by an IARC working group (4). In contrast to several earlier ecological studies (5–9), our comprehensive ecological study of 48 counties in rural China reported no relationship ($r = -0.17$) between AF exposure and a wide range of primary liver cancer mortality rates (10).

This conclusion was challenged (11, 12) on grounds that the methodology (13) to analyze the urine samples for oxidized AFB1 metabolites as a means of assessing AF exposure was inadequate. Because of this criticism, we developed a much more sensitive and specific method using monoclonal immunoaffinity column resin and HPLC to measure AFM1 in a more recent collection of urine samples from 16 survey sites of Taiwan and 69 rural counties in mainland China (including samples from the original 48 counties reported earlier).

Materials and Methods

Experimental Design of Mainland China Ecological Study. The study design for the cross-sectional ecological survey performed in 1983 was described by Chen et al. (14). The 1989 study was essentially identical to the 1983 study, with a few important differences. Only those details relevant to the data presented in this report are described here. The original 65 survey counties were chosen in 1983 by a multistage sampling procedure in order to obtain a wide geographical scatter and to encompass the full range of mortality rates for seven of the more common cancers in China. These counties were located in 25 provinces and autonomous regions and widely dispersed throughout the mostly rural regions of mainland China. In 1989, an extra four counties from the more remote regions of China were added to the study (two from Xinjiang Autonomous Region and two from Ningxia Autonomous Region), bringing the total to 69 counties. The additional counties contained high percentages of national minority groups and extended the breadth of dietary practices for the entire survey. In 1983 two xiangs (subcounties) were randomly selected within each county, and within each xiang, one village was randomly chosen, yielding a total of 130 villages. Survey xiangs were restricted to those within 4 h travel time of the county laboratory. Within each of the villages, 25 households were randomly

selected from an official registry of residences. From a random one-half of the households, one male, age 35–64 years, was then invited to participate in the study. If the selected subject was absent or declined to participate (<1% of those initially identified), then another subject in a neighboring household was selected. The same individuals participating in the 1983 survey were invited to participate in the 1989 survey. If the subjects were no longer available (by reason of death, illness, translocation, unwillingness to participate, or falling outside the age group of the study), then new participants were randomly selected. Five additional households per village were randomly selected for inclusion so that from each county 60 male subjects participated, yielding a total of 4140 subjects (69 counties, 30 males from each of 2 villages per county). This study was approved by the Cornell University Committee on Human Subjects.

Experimental Design of 1989 Taiwan Ecological Study. A stratified multistage random sampling procedure was used to select 16 survey areas in Taiwan. The sampling procedure was based on the level of urbanization, ethnicity, crude cancer mortality rates, population size, and geographical location. These survey areas included coastal, inland, mountain, and plains areas. In each survey area, two xiangs, in each xiang, two villages, and in each village, 33 males, ages 35–64, were chosen randomly. If the number of subjects was not enough to meet the intended number, then additional subjects in neighboring households were selected. The total number of participating male subjects was 1056 (16 survey areas, 33 males from each of two xiangs). When added to the survey subjects of mainland China, a total of 5196 adult males were included, compared to 3250 adult males in the 1983 survey, most of whom participated in both surveys.

Human Urine Collection and Storage. Each male was invited to donate a urine sample. In 1983 this was a 4 h collection after an oral dose, for other purposes, of riboflavin and ascorbic acid. In 1989, an overnight 12 h urine sample was collected from 7 p.m. to 7 a.m. A 5% aliquot of each sample was combined into a pool for each xiang, then was stored at −20° within 4 h of collection of the last sample. The samples were shipped on dry ice either to the Institute of Nutrition and Food Hygiene in Beijing or to Academia Sinica in Taipei, where the samples were stored at −30°C. In 1991 and 1992, the pooled samples from 1989 were shipped to Cornell University (Ithaca, NY), and were stored at −80°C.

Human AFB₁ Dosing. This experiment was designed to determine the sensitivity of the new urinary AFM₁ method in detecting small levels of AFB₁ intake. After collecting a complete 24 h urine sample, two men were administered 1.0 μg AFB₁ in 100 μl of tricaprylin oil with a meal. Complete daily urine collections were taken over the next 10 days. The subjects consumed no peanut products during the collection period. Urine volumes were recorded, and urine specimens were frozen at −20°C. Within 1 week the samples were defrosted and analyzed for urinary AFM₁. Other random urine samples, collected before and after this period, contained no detectable AFM₁. This procedure was approved by the Cornell University Committee on Human Subjects.

Urinary AFM₁ Extraction. Urinary AFM₁ was analyzed by a method resulting from major modifications of the method of Groopman *et al.* (15). All procedures were carried out at room temperature. The preparation of monoclonal immunoaffinity resin was described by Groopman *et al.* (16). A stored urine sample was defrosted and mixed well. Ten ml were removed and adjusted to pH 4 by using 0.1 mol/liter HCl. Individual C_{18}

Sep-Pak cartridges (Millipore Corp.) were prepared by sequential elution with 5 ml each of 5, 80, 100, and 5% methanol in water. The urine was applied to the prepared Sep-Pak cartridge at a flow rate of 5 ml/min, and the effluent was discarded. The Sep-Pak cartridge was then rinsed with 5 ml of 5% methanol. The AFs were eluted with 10 ml of 80% methanol, and the eluate was collected in a glass conical 15-ml centrifuge tube and evaporated under nitrogen in a 50°C water bath. After the eluate was evaporated to 0.3–0.5 ml, 0.3 ml of 0.1 mol/liter HCl was added, mixed, and cooled to room temperature, and then 0.5 ml of 1 mol/liter ammonium formate, pH 4.5, was added. The volume was adjusted to 10 ml with water, and the sample was applied to a 7 ml monoclonal immunoaffinity column and allowed to elute under gravity. For the subsequent elutions, a small aquarium pump was used to apply slight pressure to the top of the column. The column was washed with two 14-ml volumes of PBS, pH 7.4, and then 14 ml of water. The AFs were eluted with 14 ml of 70% DMSO in water; this was followed with two more washes of 14 ml PBS. The DMSO fraction and the two following PBS washes were combined and diluted to 84 ml with water to dilute out the DMSO. This sample was applied to a conditioned Sep-Pak cartridge, prepared as described above, to remove DMSO. The AFs were eluted with 8 ml of 80% methanol, collected in a glass conical 15 ml-centrifuge tube, and then evaporated under nitrogen in a 50°C water bath to 0.1 ml. To this solution 100 μl of 0.1 mol/liter HCl was added, which was then mixed and adjusted to pH 3.0 with 200 μl of 1.0 mol/liter ammonium formate, pH 4.5. The sample was then adjusted to 0.5 ml with 0.01 mol/liter TEAF and submitted to HPLC analysis.

Urinary AFM₁ HPLC Analysis. The HPLC system was a gradient model 334 from Beckman Instruments (Fullerton, CA) with a model FL-750BX fluorescence detector (McPherson Division of Schoeffel Instruments Corporation, Acton, MA). The excitation wavelength was 365 nm with an interference UT-1 filter. The emission filter was a long-band pass filter. The column was an Ultramex 5 μm C_{18} 250 × 4.6 mm column (Phenomenex, Inc., Torrance, CA) and was heated with a column temperature controller (Timberline, Inc., Boulder, CO). A model SP 4290 integrator (Spectra Physics, San Jose, CA) was attached. The mobile phase was 13% ethanol and 3% methanol in TEAF, and the flow rate was 0.6 ml/min. A typical system pressure of 50–70 bar was observed. A 0.2 μm precolumn filter (Upchurch Scientific, Oak Harbor, WA) was changed daily. Authentic AF standards were obtained from Sigma Chemical Co. (St. Louis, MO). AFN⁷G was a gift from Dr. John Groopman (Johns Hopkins University, Baltimore, MD).

For each human urine sample, two subsamples of 0.25 ml each were prepared for HPLC analysis. One subsample was run as is, and a second was run after adding an amount of AFM₁ standard approximately equal to that which appeared in the first chromatogram. The retention time of AFM₁ was about 12 min.

Calculations were made by comparing the integrated peak areas for AFM₁ external standards with peak areas for the sample AFM₁ areas. Excretion of AFM₁ per 12 h was calculated by multiplying by the appropriate volume of the 12 h urine pools.

Rat Experimental Protocol. Male Fisher-344 rats were purchased at 6 weeks of age from Charles River Breeding Laboratories, Inc. (Wilmington, MA) and housed singly in wire-bottomed metabolic cages in a temperature- and humidity-controlled environment with a 12-h-light/12-h-dark photoperiod. Food and water were available *ad libitum*. Animals

Table 1   Recovery of AF metabolites (50 ng) in 10 ml of water (% ± SD) from 4 ml of the immunoaffinity column

AF	Void volume elution	PBS and water elution	DMSO elution	Total elution
AFB_1	0	0	95 ± 7	95 ± 7
AFB_2	0	0	109 ± 4	109 ± 4
AFM_1	0	0	97 ± 1	97 ± 1
AFG_1	0	78 ± 3	25 ± 4	103 ± 5
AFG_2	0	94 ± 4	0	94 ± 5
AFP_1	0	66 ± 2	24 ± 1	90 ± 4
AFQ_1	44 ± 5	54 ± 5	0	98 ± 1
AFN^7G	31 ± 1	72 ± 4	0	103 ± 7

were fed AIN-76A diet (17), and at 7 weeks of age had attained body weights between 160 and 180 g. They were then randomly assigned into seven groups of five animals per group. Six groups were dosed daily Monday through Friday by oral gavage for 2 weeks and then killed. One group was dosed with AFB_2, AFG_1, and AFG_2 for 1 week each on Monday through Friday, and after 3 weeks they were killed. The dose for each of these AFs was 50 $\mu g/kg$ of body weight/day. ^3H-AFB_1 (Moravek Biochemicals, Brea, CA) and unlabeled AFB_2, AFG_1, and AFG_2 were administered in tricaprylin oil. Among the six groups dosed for 2 weeks, a control group of rats was treated with only tricaprylin oil, whereas the other five were treated with ^3H-AFB_1 at dosages of 0.005, 0.05, 0.5, 5, and 50 $\mu g/kg$ of body weight/day.

Rat Urine Collection. Quantitative collection of urine samples was accomplished by washing down the metabolic cage surfaces with 10 ml of water each day. The average daily sample contained approximately 10 ml of water and 10 ml of urine. Three-day urine samples from 9:00 a.m. Wednesday morning through 9:00 a.m. Saturday morning of each week were pooled and kept frozen at −80°C. Generally, 1 ml of pooled urine was adjusted to pH 4 with 0.1 mol/liter HCl. The volume was adjusted to 10 ml by adding water and analyzed for urinary AFM_1 as described above. Radioactivity was quantitated with Liquiscint scintillation fluid (National Diagnostics, Somerville, NJ) in a model LS 7000 liquid scintillation counter (Beckman Instruments).

Confirmatory Test for Urinary AFM_1. A chemical derivative confirmatory test for AFM_1 was performed on several human and rat urine samples to confirm the identity of AFM_1 by this new method. This test was originally developed to identify AFB_1 in food (18), although later it was used to identify AFM_1 in human urine (19). In the present study, 10 suspected AFM_1-positive urine samples from mainland China with different levels of AFM_1 and 1 rat urine sample from each of the AFB_1-dosed groups were selected for AFM_1 confirmation. When the urine was ready for HPLC injection, it was adjusted from 0.50 to 0.75 mL with 0.01 mol/liter TEAF. A 0.25 ml subsample was evaporated just to dryness in a 50°C water bath under nitrogen to remove residual water. Ethanol (0.25 ml) was added, and the vial was placed in a 50°C water bath for 30 min to evaporate the ethanol under nitrogen. Concentrated formic acid (0.2 ml) and 1 drop of thionyl chloride were added to the residue. The mixture was allowed to stand at room temperature for 5 min and then was evaporated to dryness under the same conditions as described above. The residue was dissolved in 0.25 ml 0.01 mol/liter TEAF and injected onto the HPLC column. Authentic AFM_1 standard dissolved in ethanol also was treated with formic acid and thionyl chloride in the same manner as the test samples for HPLC analysis.

Table 2   Recovery of variable amounts of added AFM_1 in 10 ml of urine (% ± SD) from the immunoaffinity column of variable sizes

Volume of antibody (ml)	AFM_1 added (ng)	No. of replicates	Recovery (%)
4	50	6	89 ± 2
4	20	2	80 ± 4
4	10	15	39 ± 3
6	10	2	84 ± 4
7	10	15	98 ± 5
7	5	1	90
7	2	2	108 ± 3
7	1	7	88 ± 4
7	0.02	9	96 ± 7

Statistical Methods. Minitab statistical software, Release 8 (Minitab Inc., State College, PA) was used to assess simple statistic parameters.

Results

Development of the AFM_1 Method. This newly modified method was tested initially by examining the recovery of various AFs added to water (Table 1). Using the 4-ml immunoaffinity column recommended in the original method of Groopman *et al.* (15), only AFB_1, AFB_2, and AFM_1 bound well to the resin and were eluted by DMSO. Recoveries of AFG_1 and AFP_1 in the DMSO fraction were poor and only reached 25%. Other AF metabolites, including AFN^7G, were completely eluted before application of the DMSO. Recovery of added AFM_1, using three different column volumes, is shown in Table 2. In 4 ml of resin, percentage of recovery decreased when decreasing amounts of AFM_1 were added to the column. In contrast, recovery improved when increasing amounts of resin were used. Recovery of AFM_1 reached 96% when using 7 ml of resin and as little as 10 pg of AFM_1 per 10-mL urine sample. Subsequently, 10 ml of urine and 7 ml of resin were used in all analyses, unless otherwise stated.

A HPLC chromatogram of 10 pg AFM_1 standard is shown in Fig. 1. Although this very small quantity of pure AFM_1 standard was clearly measurable, urine samples, in contrast, contained considerable amounts of interfering fluorescent material. To minimize the background fluorescence, the urine sample volume was reduced from the original 25 to 10 ml. With these conditions, the detection limit of this method was about 2.5 pg of AFM_1 per injection, or about 0.5 pg/ml of urine.

Among every 20 human subjects, one human control urine sample spiked with 10 pg AFM_1 was analyzed. A total of 10 spiked samples showed a mean recovery of 96% (87–109%). In addition, 12 human samples from China containing varying
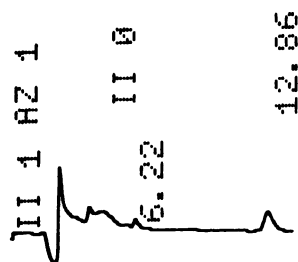
Fig. 1. Test for AFM₁ standard. AFM₁ standard was dissolved in 0.25 ml ethanol and injected on the HPLC. The peak was obtained at 12.86 min of retention time. The amount of standard injected was 10 pg.

amounts of AFM₁, from undetectable to 108 ng/12 h, were analyzed in duplicate. The analysis was performed blind. Amounts of AFM₁ between first and second runs were highly correlated (r = 0.96), with a coefficient of variation of about 10%.

Rat Urinary Excretion of AFM₁. No potentially interfering peaks were detected in the urine samples of the control animals or in the urine samples from animals dosed with AFB₂, AFG₁, or AFG₂. About 10% of the administered radioactivity was excreted in the urine for each of the [³H]AFB₁-dosed groups. Between 37 and 68% of the urinary radioactivity eluted in the void and wash volumes, reflecting the nonretention of many of the AFB₁ metabolites by the resin (see Table 1).

AFM₁ was detected in the urine samples of all AFB₁-dosed rats except those in the lowest dosage group. Approximately 1–2% of dietary AFB₁ was excreted as urinary AFM₁. There was a linear relationship between the logarithm of the dose between 0.05–50 μg/kg of body weight/day and the logarithm of urinary AFM₁ in ng/day (r = 0.91; P < 0.001). The suspected AFM₁ peak of the rat urine samples was chromatographically identical to the suspected AFM₁ in the human urine samples and to the AFM₁ standard. Moreover, added AFM₁ standard cochromatographed as one peak with the suspected AFM₁. And finally, quantitation of AFM₁ either by fluorescence detection or by radiotracer content in the collected peak was also in good agreement.

Confirmatory Tests for AFM₁ in Human Urine. Selected human urine samples prepared for HPLC analysis were each divided into three subsamples. The first untreated subsample was chromatographed, and a peak with a retention time similar to authentic AFM₁ standard was observed (Fig. 2). In the second subsample, added AFM₁ standard cochromatographed with the suspected AFM₁ peak (Fig. 3). The suspected AFM₁ peak of the third subsample was selectively derivatized by thionyl chloride treatment (Fig. 4). When this procedure was performed on the radiolabeled rat samples, not only did the fluorescent suspected AFM peak disappear, but most (about 93%) of the suspected AFM₁ radioactivity was not recovered at the expected retention time. The results of these various confirmatory tests provided evidence that the suspected AFM₁ peak of the urine samples was the same compound as the standard AFM₁.

Excretion of AFM₁ by Dosed Humans. Two adult human males were administered 1.0 μg AFB₁, and urine was collected over the next 10 days until no further AFM₁ was detected. One subject excreted 5.6% of the initial AFB₁ as AFM₁ over a period of 5 days, whereas the second subject excreted 6.6% of the AFB₁ dose as AFM₁ over a period of 7 days (Table 3). Maximum excretion occurred during the second day after dos-
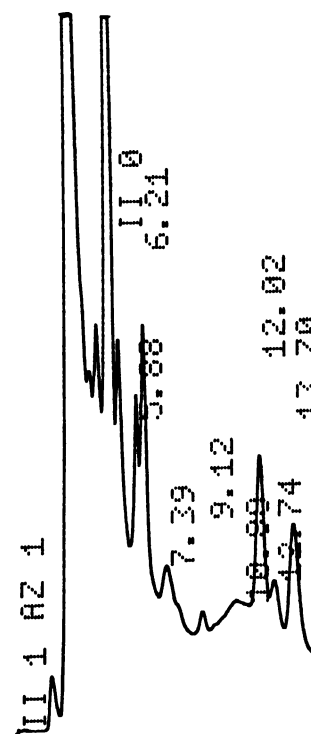

Fig. 2. Confirmatory tests for AFM₁ in human urine. A suspected AFM₁ peak was eluted at 12.74 min. The original peak contained 16 pg AFM₁.

ing. Total body clearance half-lives were approximately 0.9 days. Urine samples collected before and after this period contained no AFM₁.

Analysis of AFM₁ in Human Urine. A total of 138 urine samples from mainland China and 32 from Taiwan representing pools for each village were analyzed (Fig. 5). Eighty-eight pooled samples (64%) from mainland China and 21 (66%) from Taiwan showed measurable levels of urinary AFM₁. The mean and highest levels of AFM₁ excretion rates in mainland China was 3.2 and 108 ng/12 h, respectively, whereas the mean and highest levels in Taiwan were 2.7 and 17 ng/12 h, respectively. Urinary AFM₁ excretion rates were not significantly different between mainland China and Taiwan.

With the exclusion of the single highest outlier value, the correlation between urinary AFM₁ measurements from the two xiangs within each of the 69 counties in rural China was r = 0.50 (P < 0.001), thus indicating considerable homogeneity of AF exposure within each survey site and providing validation of the AF exposure procedures. However, AF exposure in 1989 (as urinary AFM₁) was not correlated with AF exposure in 1983 (as urinary oxidized AF metabolites) (r = −0.04), thus reflecting either a considerable variability of AF exposure over time or the modification in methodology.

Discussion

The immunoaffinity column method did not perform as well as previously indicated (15, 16, 20). Not all metabolites were retained on the resin; some (AFN⁷G and AFQ₁) were eluted either in the void volume or with the PBS and water eluants (Table 1). However, AF binding patterns, as indicated by elution order, still reflected the sequence of binding constants
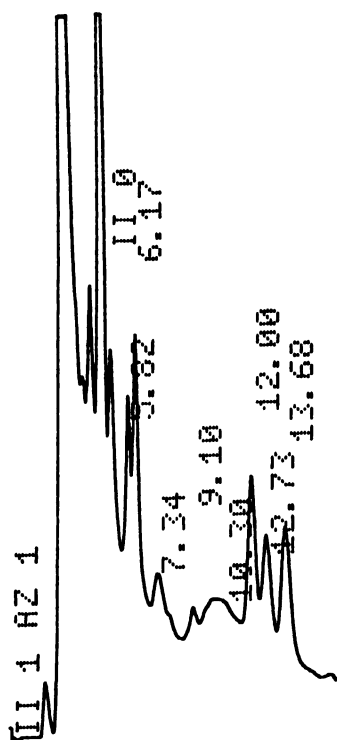
Fig. 3. Confirmatory tests for AFM₁ in human urine. To a second aliquot, 50 pg AFM₁ standard was added, and then the aliquot was analyzed by HPLC.

Fig. 4. Confirmatory tests for AFM₁ in human urine. The third aliquot was treated with formic acid and thionyl chloride and injected on the high-performance liquid chromatograph.

observed for the "2B11" antibody originally used in the preparation of the column (16). AFB_1, AFM_1, and AFB_2 bound well and were eluted quantitatively with DMSO. The difference in AFN^7G binding between this and previous studies suggests that slight changes in procedure or in batches of resin material may be responsible for major alterations in performance of the immunoaffinity column procedure.

In light of the previous demonstration that urinary AFM_1 is highly correlated with AFB_1 intake (15, 19–21), we proceeded to explore and to adopt four major changes in the methodology earlier used in studies in Guangxi (20), The Gambia (15), and Shanghai (22). These changes included the following: First, the chief analyte of interest was changed from AFN^7G to AFM_1. In addition to the poor recovery of AFN^7G from the immunoaffinity column, AFM_1 was also chosen because of its later retention on the HPLC column, thus minimizing the interference resulting from the earlier eluting peaks; Second, the earlier HPLC method relied on a mobile phase gradient. This modified method, which was less encumbered with early peak interference, employed isocratic elution, which typically yields more reproducible peak areas and shorter analysis times; Third, the immunoaffinity column volume was increased from 4 to 7 ml, thus obtaining reproducible recovery of the extremely low levels of AFM_1 usually found in urine samples; and fourth, AFM_1, which is more fluorescent than AFN^7G and which is present at about twice the concentration of AFN^7G in human urine (15, 20), was analyzed by a specialized McPherson model FL-750BX fluorescence detector, which uses a 200-W xenon-mercury lamp that had a strong emission peak at about the 365 nm absorption maximum that is characteristic of AF metabolites. The matching of this emission peak and the absorption maximum of AFs contributed to the
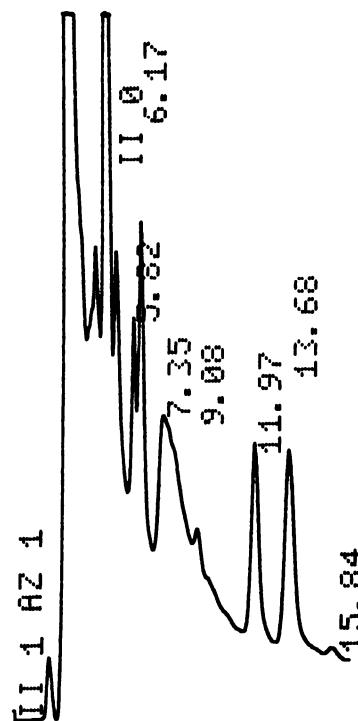
Table 3 Excretion of AFM_1 by two men after dosing with 1.0 μg AFB_1

Day	% of administered dose	
	Subject 1	Subject 2
1	0.59	0.52
2	2.77	3.14
3	1.12	0.92
4	0.77	0.75
5	0.36	0.86
6	ND[a]	0.27
7	ND	0.13
8	ND	ND
Total	5.61	6.59

[a] ND, not determined.

enhancement by more than 100-fold of the detection limit of this method when pure standards between this and earlier methods were compared (22). In studies validating the use of AFN^7G as a biomarker of AFB_1 intake (15, 19, 20), daily AFM_1 excretion also exhibited a relatively strong and highly significant correlation (r = 0.55–0.66) with AFB_1 intake, although this correlation was somewhat less than that for AFN^7G. All of these considerations and modifications served to enhance the sensitivity of the present method to assess AF exposure, particularly at low doses.

The detection limit of the method used for studies validating AFN^7G and AFM_1 excretion as biomarkers of AFB_1 intake in Africa and China was approximately 40 pg/ml urine for both metabolites (15, 20, 22). However, Weaver and Groopman (23) recently developed still-newer methods for urinary AFN^7G based on immunoaffinity column sample preparation
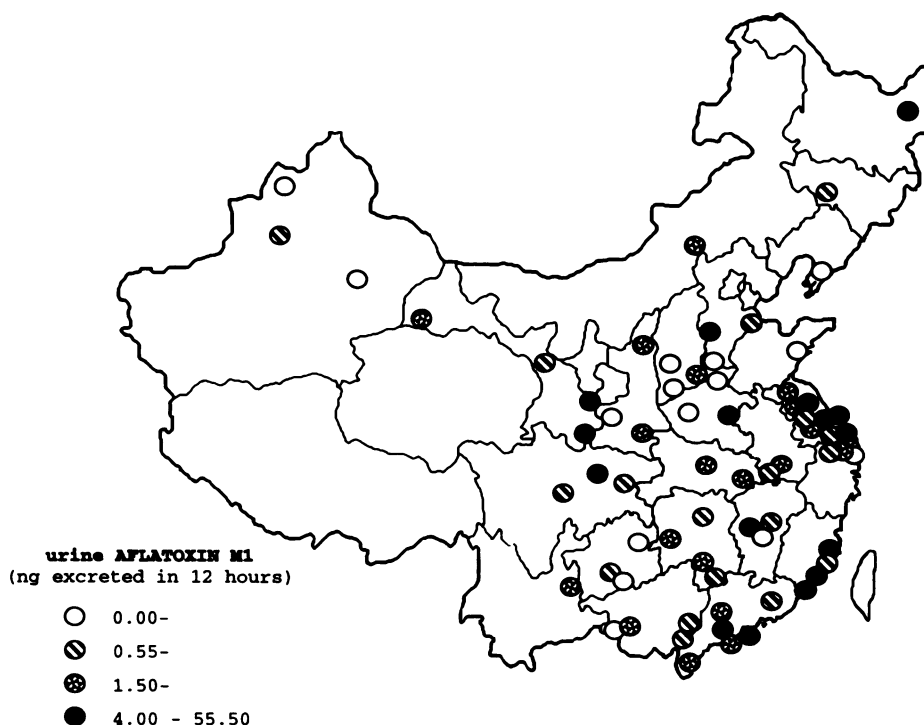
Fig. 5. Distribution of average urinary AFM concentrations in counties in China.

urine **AFLATOXIN M1**
(ng excreted in 12 hours)

○   0.00-

⊘   0.55-

⊛   1.50-

●   4.00 - 55.50

and either synchronous fluorescence spectroscopy or HPLC quantitation. For HPLC quantitation, the AFN⁷G-containing fraction was collected from one HPLC run, then subjected to acid hydrolysis and subsequent quantitation as AFB₁-diol by another HPLC run. The detection limit of this method for pure AFN⁷G standard was about 1.5 pg/ml. Our method, when comparing standard solutions of AF in water, exhibited a detection limit of 0.2 pg/ml. In another recent method also using an immunoaffinity column and HPLC but using postcolumn derivatization, the detection limit was 6.8 pg/ml (24).

Our study appears to be the first in which humans were intentionally dosed with pure AFB₁. The enhanced sensitivity of this new method allowed for very small doses of AFB₁ to be consumed. The quantity consumed is equivalent to the amount of AFB₁ found in a sandwich made with peanut butter containing the maximum legal limit of AFB₁ in the United States (20 ppb). The two adult male subjects (Z. C. and M. R.) who consumed AFB₁ gave similar urinary excretion patterns. The percentage of the original dose of AFB₁ found in the urine was 6–7% over a period of 5–7 days, which is higher than the 1–4% earlier estimated by us and others (15, 19, 21) for shorter collection periods. Randomly collected urine samples from these same two subjects showed no evidence of AFB₁ exposure.

The purpose of the rat experiment was to confirm the biological source, the chemical identity, and the quantitation of AFM₁ in urine. Based on the lack of interfering peaks from AFB₂, AFG₁, or AFG₂ and the dose-response relationship between AFB₁ dose and AFM₁ excretion, we are confident that the source of the suspected AFM₁ peak is derived from the dose of AFB₁ and is not from other AF metabolites. The percentage of administered radiolabel derived from the ingested AFB₁ found in the urine, the percentage of urinary AF that binds to the affinity column, and the percentage of dosed AFB₁ excreted as urinary AFM₁ all confirm similar percentages found in

earlier studies (13, 16). Fluorescence detection and radioactivity measurements provided similar estimates of AFM₁ concentrations; moreover, formic acid-thionyl chloride derivatization of the peak provided additional confirmation of its identity.

AF contamination of foods (1) observed over time has long been known to be exceptionally variable. Thus, it would be expected that urinary excretion of AF metabolites by individuals consuming those foods also would be unusually variable, although somewhat less so. With a biological half-life of about 1 day, the urinary excretion of biomarkers such as AFM₁ could reflect AFB₁ intake averaged over 1–3 days. In the ecological study described herein, 170 pooled urine samples, each comprising 30 men, were analyzed, thus yielding approximately 30–90 man-days of integrated exposure for each data point, or approximately 5,100–15,300 total man-days of exposure for the entire survey. This represents substantial improvement in sampling stability over previous studies, although seasonal and yearly changes in AF exposure still remain underrepresented. It is difficult to predict how these results would compare to surveys performed in other seasons or years. The sensitivity of the method reported herein allows for the detection of 1 subject out of 30 in a pool consuming about 1 μg of AFB₁ once during the 3 days prior to urine sampling and pooling. Thus, the combination of sampling stability and analytical method sensitivity greatly improves the ability to assess AF exposure.

In the survey in mainland China and Taiwan, the urinary excretion of AFM₁ was very low, with one-third of the samples below the detection limit. Assuming that the 12 h collection represents one-half of the daily excretion and that 2–5% of AFB₁ consumed in the diet is excreted as AFM₁, the average intake of AFB₁ in mainland China and Taiwan was about 0.1–0.3 μg/day; the highest estimated level of intake was about 11 μg/day. Most of these intakes are considerably below those from high-primary liver cancer areas such as The Gambia, Guangxi, and the Philippines (15, 19, 20), where average in-

takes have been reported to range from 12 to 77 μg/day. They are also below previous reports for Taiwan of total urinary aflatoxin excretion (25).

Because these low estimates of AFB$_1$ intake were puzzling, we reanalyzed a collection of samples blind about 1 year later to confirm our earlier analyses. Correspondence between replicates was excellent. As noted earlier, validation of this method also was examined by several other analytical procedures, including assessment of AFM$_1$ excretion by rats and humans. A possible explanation of these low levels is deterioration of the AFM$_1$ during the 4 years between collection and analysis. No direct evidence is available on this point. Wild et al. (26) contend that AF-albumin adducts in sera are stable when kept frozen at $-70°C$ for as long as 15 years. Our own experience with the samples was that no decay occurred over 1 year between the first and second testing of a subset of samples. Thus, we are confident that these estimates of AF intake are reliable. It also should be noted that this estimate of relatively low AF intakes corresponds to the findings of two other reports from China (27, 28), both of which used an ELISA method to directly measure AFB$_1$ intake and which gave mean intakes of 0.1 and 3.4 μg/day for subjects residing in Fusui county. In a study of aflatoxin exposure in The Gambia, Wild et al. (29) found an average daily intake of 1.4 μg/day. In a survey of chemical contaminants of food in four areas of China, the average intake of AFB$_1$ was 0.15 μg/day (30). In our survey county, corn consumption, a common source of AFB contaminants in food, decreased about 40% between 1983 and 1989, according to preliminary dietary intake data from our ecological survey. We have no further explanation for the relatively high estimates of AFB$_1$ intake from other studies except to suggest that the populations under investigation may have been biased toward very high intake areas. Although the intakes from this survey are unusually low, the highly significant correlation between villages within the same counties supports the geographic diversity of AFB exposure.

In conclusion, a reliable, sensitive, selective, reproducible method for measuring urinary AFM$_1$ has been developed. Animal studies confirm the source, identity, and quantitation of the AFM$_1$. The excretion rate from two intentionally dosed humans was 5–6%. AFM$_1$ excretion across mainland China and Taiwan was very low (3.1 ng/12 h), with good agreement between villages from the same county and similar results from mainland China and Taiwan. AFB$_1$ intake was calculated to average 0.1–0.3 μg/day (possible range, 0–11 μg/day).

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References

1. Detroy, R. W., Lillehoj, E. B., and Ciegler, A. Aflatoxin and related compounds. In: A. Ciegler, S. Kadis, and S. J. Ajil (eds.), Microbial Toxins, Vol. IV, pp. 3–178. New York: Academic Press, 1971.

2. Wogan, G. N., Edwards, G. S., and Newberne, P. M. Structure-activity relationships in toxicity and carcinogenicity of aflatoxins and analogs. Cancer Res., 31: 1936–1942, 1971.

3. Wogan, G. N. Aflatoxin carcinogenesis. In: H. Busch (ed.), Methods in Cancer Research, pp. 309–344. New York: Academic Press, 1973.

4. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. some naturally occurring substances: food items and constituents. In: Heterocyclic Aromatic Amines and Mycotoxins, pp. 245–395. Lyon, France: IARC, 1993.

5. Shank, R., Bhamarapravati, N., Gordon, J. E., and Wogan, G. N. Dietary aflatoxins and human liver cancer. IV. Incidence of primary liver cancer in two municipal populations in Thailand. Food Cosmet. Toxicol., 10: 171–179, 1972.

6. Peers, F. G., and Linsell, C. A. Dietary aflatoxins and liver cancer: a population-based study in Kenya. Br. J. Cancer, 27: 473–484, 1973.

7. Van Rensburg, S. J., Van der Watt, J. J., Purhase, I. F. H., Coutinho, L. P., and Markham, R. Primary liver cancer rate and aflatoxin intake in a high cancer area. S. Afr. Med. J., 48: 2508a–2508d, 1974.

8. Peers, F. G., Gilman, G. A., and Linsell, C. A. Dietary aflatoxins and human liver cancer. A study in Swaziland. Int. J. Cancer, 17: 167–176, 1976.

9. Van Rensburg, S. J., Cook-Mozaffari, P., Van Schalkwyk, D. J., Van Der Watt, J. J., Vincent, T. J., and Purchase, I. F. Hepatocellular carcinoma and dietary aflatoxin in Mozambique and Transkei. Br. J. Cancer, 51: 713–726, 1985.

10. Campbell, T. C., Chen, J., Liu, C., Li, J., and Parpia, B. Nonassociation of aflatoxin with primary liver cancer in a cross-sectional ecologic survey in the People's Republic of China. Cancer Res., 50: 6882–6893, 1990.

11. Wild, C. P., and Montesano, R. Re: "Nonassociation of aflatoxin with primry liver cancer in a cross-sectional ecological survey in the People's Republic of China" (Letter). Cancer Res., 51: 3825, 1991.

12. Campbell, T. C., Parpia, B., Chen, J., Liu, C., and Li, J. Re: "Nonassociation of aflatoxin with primary liver cancer in a cross-sectional ecological survey in the People's Republic of China" (Reply). Cancer Res., 51: 3826–3827, 1991.

13. Groopman, J. D., Trudel, L. J., Donahue, P. R., Marshak-Rothstein, A., and Wogan, G. High affinity monoclonal antibodies for aflatoxins and their application to solid-phase immunoassays. Proc. Natl. Acad. Sci. USA, 81: 7728–7731, 1984.

14. Chen, J., Campbell, T. C., Li, J., and Peto, R. Diet, life-style and mortality in China. A study of the characteristics of 65 Chinese counties. Oxford, United Kingdom: Oxford University Press, 1990.

15. Groopman, J. D., Hall, A. J., Whittle, H., Hudson, G. J., Wogan, G. N., Montesano, R., and Wild, C. P. Molecular dosimetry of aflatoxin-N^7-guanine in human urine obtained in The Gambia, West Africa. Cancer Epidemiol., Biomarkers & Prev., 1: 221–227, 1992.

16. Groopman, J. D., Hasler, J. A., Trudel, L. J., Pikul, A., Donahue, P. R., and Wogan, G. N. Molecular dosimetry in rat urine of aflatoxin-N7- guanine and other aflatoxin metabolites by multiple monoclonal antibody affinity chromatography and immunoaffinity/high performance liquid chromatography. Cancer Res., 52: 267–274, 1992.

17. Bieri, J. G., Stowesand, G. S., Griggs, G. M., Phillips, R. W., Woodard, J. C., and Knapka, J. J. Report of the American Institute of Nutrition Ad Hoc Committee on Standards for Nutritional Studies. J. Nutr., 107: 1340–1348, 1977.

18. Andrellos, P. J., and Reid, G. R. Confirmatory tests for aflatoxin B1. J. Assoc. Off. Agric. Chem., 47: 801–803, 1964.

19. Campbell, T. C., Caedo, J. P., Jr., Bulatao-Jayme, J., Salamat, L., and Engel, R. W. Aflatoxin M$_1$ in human urine. Nature (Lond.), 227: 403–404, 1970.

20. Groopman, J. D., Zhu, J., Donahue, P. R., Pikul, A., Zhang, L., Chen, J., and Wogan, G. N. Molecular dosimetry of urinary aflatoxin-DNA adducts in people living in Guangxi Autonomous Region, People's Republic of China. Cancer Res., 52: 45–52, 1992.

21. Zhu, J., Zhang, L., Hu, X., Xiao, Y., Chan, J., Xu, Y., Fremy, J., and Chu, F. S. Correlation of dietary aflatoxin B$_1$ levels with excretion of aflatoxin M$_1$ in human urine. Cancer Res., 47: 1848–1852, 1987.

22. Qian, G-S., Ross, R. K., Yu, M. C., Yuan, J-M., Gao, Y-T., Henderson, B. E., Wogan, G. N., and Groopman, J. D. A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in Shanghai, People's Republic of China. Cancer Epidemiol., Biomarkers & Prev., 3: 3–10, 1994.

23. Weaver, V., and Groopman, J. D. Fluorescence quantification of aflatoxin N^7-guanine adducts. Cancer Epidemiol., Biomarkers & Prev., 3: 669–674, 1994.

24. Kussak, A., Andersson, B., and Andersson, K. Immunoaffinity column clean-up for the high-performance liquid chromatographic determination of aflatoxins B$_1$, B$_2$, G$_1$, G$_2$, M$_1$ and Q$_1$ in urine. J. Chromatogr., 672: 253–259, 1995.

25. Hatch, M. C., Chen C-J., Levin, B., Ji, B-T., Yang, G-Y., Hsu, S-W., Wang, L-W., Hsieh, L-L., and Santella, R. M. Urinary aflatoxin levels, hepatitis-B virus infection and hepatocellular carcinoma in Taiwan. Int J. Cancer, 54: 931–934, 1993.

26. Wild, C. P., Jiang, Y. Z., Allen, J. S., Jansen, L. A. M., Hall, A. J., and Montesano, R. Aflatoxin-albumin adducts in human sera from different regions of the world. Carcinogenesis (Lond.), 11: 2271–2274, 1990.

27. Zhang, M., Yu, S., Guo, H., Wang, H., Shao, Y., and Huang, Z. Aflatoxins and primary liver cancer: a population based case-control study. Chinese J. Prev. Med., 26: 331–333, 1993.

28. Cheng, Z., Liu, Y., Yu, S., Zao, Y., Huang, Z., and Yan, W. An epidemiological survey on the relationship between aflatoxins and primary liver cancer. Acta Academiae Medicinae, 17: 153–156, 1990.

29. Wild, C. P., Hudson, G. J., Sabbioni, G., Chapot, B., Hall, A. J., Wogan, G. N., Whittle, H., Montesano, R., and Groopman, J. D. Dietary intake of aflatoxin and the levels of albumin-bound aflatoxin in peripheral blood in the Gambia, West Africa. Cancer Epidemiol., Biomarkers & Prev., 1: 229–234, 1992.

30. Chen, J., and Gao, J. The Chinese total diet study of 1990. Part I. Chemical contaminants. J. AOAC Int., 76: 1193–1200, 1993.