Measurement of Aflatoxin and Aflatoxin Metabolites in Urine by Liquid Chromatography–Tandem Mass Spectrometry*

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Abstract

Automated immunoaffinity solid-phase extraction followed by liquid chromatography–tandem mass spectrometry and chemical analogue internal standardization is employed to detect and quantify the aflatoxins AFB1, AFB2, AFG1, AFG2, and the metabolites AFM1 and AFP1 in urine. The dynamic range of the method is nearly three orders of magnitude with limits of detection in the low femtogram on column range. The method was validated over a 12-day period by eight analysts. This method is suitable for agricultural, forensic, and public health laboratories during an accidental outbreak or a chemical terrorism event where a rapid and accurate diagnosis of aflatoxicosis is needed.

Introduction

Because of the ever increasing threat of terrorist attacks around the globe and, more specifically, the threat of a chemical terrorism attack, analytical chemistry laboratories that would aid in forensic investigations and public health domains must be prepared to provide quality laboratory results quickly and efficiently. In such an event, the number of victims could be large and the type of warfare agent may not be immediately obvious. To this end, analytical methods that can provide rapid and sensitive confirmation and quantitation of the agent are vital in determining which agent is used, each individual’s degree of exposure, and the extent of the population that is exposed (1).

Of the many toxins that could be used in an attack, those previously weaponized are of particular interest. One example is aflatoxins, which were weaponized by the Iraq government during the first Gulf War. According to a United Nations Special Commission (UNSCOM) report, the Salman Pak weapons facility in Iraq produced 2200 L of aflatoxins loaded in 122-mm rockets, 400-lb bombs, and SCUD missiles (2).

Aflatoxins are secondary metabolites of the fungi Aspergillus flavus, from which their name (A. fla.) is derived. The predominant aflatoxins AFB1, AFB2, AFG1, and AFG2 are designated B and G because of their blue and green fluorescent color observed under UV illumination. The most studied and most hepatotoxic aflatoxin is AFB1 (LD50 1.16 mg/kg in rat) (3), for which the World Health Organization (WHO) suggests there is no safe dose (4). Moreover, these compounds are known to be mutagenic and teratogenic. Clinical symptoms of aflatoxin exposure include abdominal pain, rash, and gastrointestinal bleeding (5,6). The commonality of these symptoms with those seen in other illnesses prevent them from providing unambiguous identification of their cause, which further emphasizes the need for an analytical method that provides more definitive information and enables a conclusive diagnosis.

The work presented here builds upon previous investigations of aflatoxins in urine (7,8) in an effort to improve both the sensitivity and dynamic range of those methods. To improve upon the speed of previous methods, an automated immunoaffinity solid-phase extraction (SPE) method has been developed in conjunction with liquid chromatography–tandem mass spectrometry (LC–MS–MS) analysis to take advantage of the sensitivity, specificity, and ease of quantitation the technique provides. Figure 1 shows the structures for the four parent aflatoxins of interest and the metabolites of AFB1 that were chosen for this study.

* Results from this research were presented at the 54th ASMS Conference on Mass Spectrometry.
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Experimental

Chemicals and materials
High-performance liquid chromatography (HPLC) grade methanol, acetonitrile, and formic acid were purchased from Fisher Scientific (Fairlawn, NJ). Deionized water was purified in house to yield organic-free 18.3 M | cm water using an E-pure purification system (Barnstead International Dubuque, IA). Aflatoxin reference standards (AFB1, AFB2, AFG1, AFG2, AFM1, and AFP1) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO).

Standard preparation and characterization
Aflatoxins and the internal standard AFB2 were dissolved in acetonitrile and diluted in 85:15 MeOH/H2O (v/v) to a final concentration of 1 ng/μL. Eight calibration standards, low and high quality controls (QCs), and a urine blank containing internal standard were stored in 50-mL polypropylene conical tubes at 4°C. Calibration standards were prepared in 1 mL of pooled human urine spiked with 25 μL of the stock solution of AFB2. To all urine samples (unknowns, QCs, blanks, and standards) an equal amount of 85:15 MeOH/H2O was added. For example, the highest concentration standard was made by mixing (per 1 mL of urine) 250 μL of the 1 ng/μL aflatoxin mixture, and 25 μL of the 1 ng/μL internal standard for a total volume of 1.275 mL. To the other samples the same amount of urine and internal standard were used, but with varying amounts of aflatoxin standard and 85:15 MeOH/H2O while maintaining a total volume of 1.275 mL. The final concentrations of the urine standards were: 0.392, 0.784, 3.92, 7.84, 19.6, 58.8, 118, and 196 ng/mL for the calibration standards, 1.96 and 157 ng/mL for the QC low and high, respectively, and the internal standard concentration in all samples was 19.6 ng/mL.

Extraction
Urine (1 mL) and water (1 mL) were added to glass tubes (10 × 75 mm), and empty tubes of the same size (for elution) were inserted into the Gilson 215 Liquid Handler (Middleton, WI) for automated extraction. Custom-made immunoaffinity columns, (3-mL barrel, 400 ng aflatoxin equivalent binding capacity, Vicam, Watertown, MA) were used for the extraction. The buffer was discarded following by rinsing the column twice with water, leaving a small amount of water on top of the resin. The columns were then conditioned with water (2 × 2 mL). After the diluted urine sample was loaded, the column was washed with water (2 × 2 mL), followed by an air push (3 s) using the Gilson solenoid valve. The analytes were eluted from the columns with 85:15 MeOH/H2O with 1% (v/v) formic acid (2 × 0.5 mL), followed by an air push (30 s). The extract was then transferred to an autosampler vial for LC–MS–MS analysis.

LC–MS–MS
Chromatography was performed with an Agilent 1100 HPLC (Wilmington, DE) equipped with a 3-μm, 2.0- × 150-mm phenyl-hexyl column (Phenomenex, Torrance, CA) at 50°C. Injections of the extract (1 μL) were made on the HPLC using a mobile phase consisting of H2O (0.1% formic acid) (solvent A) and acetonitrile (0.1% formic acid) (solvent B). The mobile phase gradient is given in Table I.

Samples were analyzed by positive ion electrospray-MS–MS spectrometry operating in multiple reaction monitoring (MRM) mode on an API 4000 LC–MS–MS system (Applied Biosystems, Foster City, CA). The MS settings are listed in Table II. Individual compound specific parameters (i.e., declustering potentials, entrance potentials, and collision cell exit potentials) were optimized for each analyte.

Data analysis and recovery
The product ion abundances of the analyte and internal standard were used to calculate analyte/internal standard ratios for quantitation. Linear regression analysis with “1/x” weighting was used for curve fitting. All data processing was performed

Table I. Instrument Parameters for LC

<table>
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<td>30</td>
</tr>
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<td>30</td>
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Table II. Instrument Parameters for MS

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</tr>
<tr>
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</tr>
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</tr>
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automatically using the Analyst 1.4 software (Applied Biosystems). Extraction efficiencies were calculated as a percentage of the ratio of extracted analyte peak area and the non-extracted standard peak area. Four extractions over a period of two days were performed for this study, with all at a concentration of 7.84 ng/mL. Four injections of non-extracted standard at an equal concentration were also made. The peak areas for the extracted and non-extracted samples were averaged and this average was used to calculate the ratio for each analyte.

Animal study
Urine samples were obtained from two male F344 rats (173–176 g body weight). AFB1 (91 µg/kg body weight) or the vehicle (dimethylsulfoxide) were administered by intraperitoneal injection (150 µL) on two consecutive days, and the rats were housed in metabolic cages. Urine was collected for approximately 18 h after the second dose and stored at −20°C. Urine aliquots (1 mL) were treated with 250 µL of 85:15 MeOH/H2O, and 1 mL of this mixture was then extracted. The animal study was conducted in accordance with Johns Hopkins University’s Animal Care and Use Committee requirements, which comply with the National Research Council’s Guide for the Care and Use of Laboratory Animals.

Results and Discussion
The parent aflatoxins AFB1, AFB2, AFG1, and AFG2, and two phase I oxidative metabolites of AFB1, AFM1, and AFP2 (9), were selected for analysis. Both AFP1 and AFM1 are excreted in human urine (10) and studies have demonstrated that AFM1 is the most abundant AFB1 metabolite found in the urine of rats and humans (11). Furthermore, research involving AFM1 has shown that urinary AFM1 levels reflect exposure in humans (12). Metabolites of AFB2, AFG1, and AFG2 were not included in this study because these three compounds are generally not observed in the absence of AFB1 and AFB1 is the most common aflatoxin found in food (13).

Urine (vs. blood or serum) was chosen as the sample matrix primarily due to its ease of collection. If victims are in reasonably stable condition, they can provide a urine sample without the assistance of a medical professional. Minimizing the workload of hospital staff is critical in a scenario such as a terrorist attack when hospitals will likely be overwhelmed.

AFB2 was chosen as a chemical analogue internal standard because it behaves similarly to the other aflatoxins both in the immunoaffinity column (IAC) and in the analytical method. In addition, AFB2 is chromatographically well-resolved from the other analytes and less toxic than AFB1 and AFG1. Chemical analogue internal standardization was employed for two reasons. First, isotopically labeled standards were only available for AFB2 and were prohibitively expensive (~ $2000 for 8.3 µg AFB2 3H). Second, the upper limit of linearity (ULOL) was determined by occupying the available binding sites of antibody in the column. The use of isotopically labeled internal standards for each aflatoxin would result in more aflatoxin being added to the immunoaffinity column, which would lower the ULOL and, therefore, decrease the dynamic range of the method.

If AFB2 were in a real world sample, a t-test could be performed to determine if the internal standard peak areas in unknown samples are significantly higher than the mean AFB2 peak area in the calibration standards. If this were the case, then because all of the compounds of interest are equally suitable as chemical analogue internal standards, an aflatoxin not present in the sample would be added to a separate aliquot of unknown and then re-extracted. A second alternative would be to measure the concentration of AFB2 by the method of standard additions. However, both of these methods would be difficult if the concentration of AFB2 was at or near the limit of detection (LOD), so a third alternative, analyzing each unknown in duplicate (one with internal standard, one without), could be employed.

Immunoaffinity extraction of aflatoxins in various matrices has been reviewed (14) and involves non-covalent binding of the toxins to monoclonal antibodies in aqueous environment followed by release upon denaturing of the antibodies using high organic content solvents. After a comparison with C18 SPE in this laboratory, the immunoaffinity method was chosen because of its increased recovery, selectivity, and cleanliness of extracts. Because the extraction columns were originally manufactured for food analysis, the antibody used was designed to target only the parent aflatoxins; however, because of the structural similarities between the parent and metabolites, the metabolites were efficiently extracted as well. For this reason, the columns could readily be used for clinical samples.

<table>
<thead>
<tr>
<th>Table III. Extraction Efficiency for all Five Analytes Measured at 7.84 ng/mL in Urine (n = 4)</th>
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<tbody>
<tr>
<td><strong>Analyte</strong></td>
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<tr>
<td>-----------------</td>
</tr>
<tr>
<td>AFB1</td>
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<tr>
<td>AFM1</td>
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<tr>
<td>AFB2</td>
</tr>
<tr>
<td>AFG1</td>
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<td>AFG2</td>
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Figure 2. MRM extracted ion chromatogram of a 19.6 ng/mL urine extract using the transitions shown in Table II.
Automated extraction was utilized as a means to increase sample throughput. The extraction took 1.67 min/sample, corresponding to 36 samples/h. The efficiency of the automated extraction was measured at a concentration of 7.84 ng/mL in urine. The extraction efficiencies for each of the five compounds are shown in Table III. The results ranged from 80 to 93% and were in agreement with manufacturer specifications, which were defined for food matrices.

The best chromatographic resolution was obtained using a phenyl-hexyl column. Figure 2 depicts the separation of all six aflatoxins used in this study extracted from human urine at a concentration of 19.6 ng/mL. The MS–MS fragmentations of the aflatoxins are shown in Figure 3. It can be seen that both B aflatoxins and the AFB1 metabolites fragment by losing the carbonyl on the cyclopentanone group, and that AFM1 further breaks down by losing the two adjacent CH2 groups on the

![Figure 3](https://academic.oup.com/jat/article-abstract/31/3/150/712186)}
The G aflatoxins differ by containing a lactone group in place of the pentanone ring structure. These compounds fragment by the loss of water and the further loss of the outer carbonyl group and adjacent portion of the ring, as well as the loss of H₂. The fragmentation patterns suggest that the site of protonation for each aflatoxin was the inner carbonyl group. These fragmentation patterns were based upon interpretation of the MS–MS spectra because isotopically labeled experiments were not performed for reasons stated previously.

The instrument LOD (S/N = 3:1) for each compound is given in Table IV. LOD values were reported in femtograms (fg) on column to avoid ambiguity inherent in using units of concentration such as parts per billion (ppb). This ambiguity made it difficult to determine a relative LOD in papers where the pertinent information to calculate the LOD on column was not included (15,16). By providing the LOD as fg on column, the values reported in this manuscript are independent of the volume of urine extracted and/or injection volume, and they reflect the amount of sample that was detected by this method. Table IV shows the LOD for each analyte in terms of grams on-column and in moles. An LOD of 100 fg is equivalent to a 1-μL injection of non-extracted standard at 100 fg/μL. These values meet the goal of developing a sensitive method as these detection limits are more than 10 times lower than those previously reported (17,18). The limit of quantitation (LOQ) (S/N = 10:1) was calculated in urine using the same procedure stated previously. The LOQ was determined to be 392 fg on-column for all analytes.

A wide dynamic range is needed in the analysis of chemical warfare agents for two reasons. First, because there are no studies in the literature showing the range of concentrations in a victim exposed to weaponized aflatoxin, having a dynamic range of nearly three orders of magnitude increases the likelihood that this method will cover relevant concentrations. Second, the exposure of victims after an event will not be uniform because of their different proximities to the attack epicenter. To achieve this goal, custom-made extraction columns with a larger bed size were employed. The dynamic range of the method was from the LOQ at 0.392–196 pg on-column for each analyte. Procedures that required time consuming concentration steps, which may allow for the detection of lower aflatoxin levels, were avoided to increase assay throughput [i.e., 1 mL of urine was extracted into 1 mL of MeOH/H₂O with 1% (v/v) formic acid]. Figure 4 shows a calibration curve for all five toxins in human urine. The dynamic range presented here meets the aforementioned goal of covering a wider range than those previously reported (8,17).

As seen in Figure 4, a slight deviation from linearity begins to occur at the highest point. This is due to the large aflatoxin/antibody ratio at this concentration. This was examined by analyzing non-extracted standards that showed increased linearity at this same concentration and at even higher concentrations (data not presented), which verified that our ULOL was dictated by the binding capacity of the extraction column and not by the instrument. At concentrations above the ULOL, the number of...
binding sites became a limiting factor and some unbound aflatoxin was removed during the wash step of the extraction. This was an important consideration when determining the ULOL of an extraction method using immunoaffinity columns.

The method was validated by analyzing a calibration curve, two quality controls (low and high), and a urine blank spiked with internal standard. This experiment was repeated 20 times over a period of 12 days with no more than 2 sets being analyzed in a single day. Eight analysts conducted the experiments during the 12-day period. Linear regression with “1/x” weighting was used for each analyte to account for heteroscedasticity in the data. The calibration standards were analyzed in a random order and the curves for each aflatoxin had an average correlation coefficient ≥ 0.995. The quality of the method was represented by quality control low and high plots of AFG₁ (Figure 5). Similar results were obtained for the four other toxins. The results of the accuracy and precision of the method for each analyte are shown in Table V. The range of the percent accuracy of the means for all five analytes was from 97.0 to 105.6%, and the highest % relative standard deviation (RSD) found was 6.67%. In all but one case, the mean was ≤ one standard deviation away from the true value. No significant contributions from carryover were seen in the blank samples.

After validation, the method was further tested by analyzing the urine of an AFB₁ exposed rat. The dose administered to the rat (91 μg/kg body weight) was well below the LD₅₀ for rats and corresponded to a dose of several milligrams for a human adult. A dose of 2–6 mg/day was observed during an outbreak of aflatoxicosis in western India (19), and similar amounts were consumed in a recent outbreak in Kenya (20). It was expected that roughly equal amounts of the two metabolites AFM₁ and AFP₁, and a small amount of unmetabolized AFB₁, would be excreted. No aflatoxins other than the internal standard were detected in the negative control rat urine sample. AFB₁, AFM₁, and AFP₁ were detected in urine from the AFB₁-dosed rat at 1.38, 48.8, and 41.4 ng/mL, respectively; 4 mL of urine was collected and indicated total excretion of 5.52, 195.2, and 165.6 ng, respectively, during the 18-h period. The creatinine levels of the two urines were not measured; thus, no comparison of the metabolite concentrations with literature values were made. However, relative to AFP₁, the amount of AFM₁ was more abundant, which is consistent with previous studies of AFB₁ exposure in rats (11,21). Figure 6 shows the chromatograms for the negative control and AFB₁ dosed rat urine samples.

It was also expected that other metabolites of aflatoxin would be present in the dosed rat urine. Precursor and product ion masses of other AFB₁ metabolites (18) were used to build a separate MRM method to detect the presence of these compounds. Metabolites such as AFB₁-diol and AFQ₁ were found. The presence of AFQ₁ was further confirmed by matching the retention time with a chromatogram obtained previously when AFQ₁ was commercially available (data not presented). This method was suitable for monitoring other metabolites on a qualitative level, but quantitative analysis was difficult because AFM₁ and AFP₁ are the only commercially available AFB₁ metabolite standards.

### Conclusions

The LC-MS–MS method described uses less urine and has lower limits of detection than previously reported methods.
Other advantages of this approach are the specificity of both immunoaffinity extraction and tandem MS and no requirement for derivatization. The dynamic range of 0.392–196 pg on column, combined with an automated extraction, yield rapid results over a wide range of exposure. This method is well suited to aid forensic and public health laboratories during the investigation of a terrorist attack by providing confirmation of military or civilian exposure to weaponized aflatoxin. In addition, the method presented here can be utilized to diagnose aflatoxicosis (22) caused by consumption of a food supply contaminated intentionally by the hands of terrorists or accidentally during an outbreak (19,23,24).

Acknowledgments

The authors would like to thank Erin Carson, Mike Martin, Chris Nixon, Jeff Snow, Shane Wyatt, and Jessica Zuckschwerdt, who contributed to the validation process, and Janet Pruitt for the insightful discussions. The authors also like to thank Dr. Tom York for his comments to the manuscript. Funding for the analytical work (R.E., C.C., D.Z., and T.C.) was funded by the Centers for Disease Control and Prevention grant #U90/CCU317014. The animal study (P.S. and J.G.) work was funded by NIH grants #P01 ES06052 and #P30 ES03819.

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