Fluorescence Quantification of Aflatoxin N^7-Guanine Adducts

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Abstract

Increasingly sensitive assays are needed to understand and evaluate the effects of chemical exposures on individuals and populations. Several assays have been developed to measure the environmental dietary carcinogen, aflatoxin, and its metabolites in biological specimens. One, the 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy-aflatoxin B_1 nucleic acid adduct, has been shown to be both highly correlated with exposure and a strong predictor of carcinogenic outcome. Assays with increased sensitivity for this chemical adduct would be beneficial. Therefore, we have developed a hydrolysis reaction for the adduct found in urine, utilizing HCl acid and heat. Subsequently, quantification of the flourescent metabolites produced can be obtained by either synchronous fluorescence spectrophotometry or high pressure liquid chromatography with fluorescence detection. The detection of lower levels of the adduct could prove helpful in the evaluation of risk in populations with lower exposures, such as those in chemoprotection trials or occupationally exposed groups.

Introduction

AFB_1[3] is one of the most potent liver carcinogens and toxins known for experimental animals and is a frequent contaminant of the food supply as a result of mold spoilage (1). Consumption of contaminated foods by people living in some regions of Asia and Africa results in high intake of AFB_1. Several epidemiological studies have found strong positive associations between AFB_1 exposure and increased risk for hepatocellular carcinoma (2–4). Evidence from such studies justifies extensive research efforts undertaken by many organizations to minimize aflatoxin exposure.

Recent work in the molecular dosimetry of aflatoxin has focused on methods for DNA and protein adduct analysis and is reviewed in Groopman *et al.* (5). AFB_1 is metabolized by cytochrome P-450 enzymes producing an unstable, highly reactive 8,9-epoxide which can covalently interact with many nucleophilic centers in cellular macromolecules such as DNA and serum albumin. The two major macromolecular adducts identified are the AFB-N^7-Gua adduct in DNA (6) and the lysine adduct in serum albumin (7). Detoxification of AFB_1 is accomplished by the enzymatic conjugation of oxidized metabolites, which are then excreted (8). In addition, the 8,9-epoxide metabolite can be eliminated by conjugation with glutathione, resulting in a spectrum of thiol compounds (9). Thus, a major factor for assessing the biological hazard to a cell or organism from exposure to AFB_1 is the integrated balance between the activation and detoxification reaction pathways. The possible role for AFB_1-N^7-Gua adduct in the cancer initiation process provides the justification to study this agent as a molecular dosimeter in humans. Other molecular dosimetry methods for measuring aflatoxins have been developed using serum albumin adduct formation (10, 11). The data from the limited number of molecular dosimetry studies to date suggest that these methods are more reliable than dietary surveys to assign exposure and risk for aflatoxins. It is well known that dietary surveys are difficult to perform and have high statistical coefficients of variation (12). Thus, the use of DNA and protein adduct measurements is both mechanistically justified and, in practical terms, more feasible in population studies.

The AFB_1-N^7-Gua adduct, in particular, has been shown to be both highly correlated with exposure and a strong predictor of carcinogenic outcome (4, 13–15). This adduct is the urinary excretion product resulting from repair of the AFB_1-DNA adduct and its measurement is a surrogate for the biologically effective dose, which is the AFB_1-DNA adduct in liver. The ability to detect this biomarker at lower levels could be beneficial in the evaluation of exposed populations and in chemoprevention trials. Therefore, we developed a hydrolysis reaction which cleaves the AFB_1-N^7-Gua bond to produce highly flourescent derivatives and then evaluated the use of SFS and HPLC with fluorescence detection as analysis systems. Cleavage of this bond with measurement of released guanine has been performed previously by Essigmann *et al.* (6) in work defining the structure of the adduct and in work by Groopman *et al.* (16) to quantify the adduct. We wished to expand this research to systematically measure the flourescent products released in a simple quantitative assay.

Materials and Methods

Chemicals. AFB_1 was obtained from Aldrich Chemical Co. (Milwaukee, WI). All water used was purified by a MilliQ Water System (Millipore Corp., Bedford, MA). All other chemicals used were of the highest quality obtainable commercially.

Synthesis of AFB_1-diol. AFB_1-diol was synthesized by hydrolysis of the aflatoxin 8,9-epoxide with water. The ep-

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[3] The abbreviations used are: AFB_1, aflatoxin B_1; AFB_1-diol, 8,9-dihydro-8,9-dihydroxy-aflatoxin B_1; AFB_1-N^7-Gua, 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy-aflatoxin B_1; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline; SFS, synchronous fluorescence spectrophotometry; TEAF, triethylammonium formate.

oxide was prepared as described in Baertschi *et al.* (17). In this method, AFB_1 is converted to the 8,9-epoxide using dimethyldioxirane. The formation of the AFB_1-diol was confirmed by fast-atom bombardment mass spectroscopy. The AFB_1-diol standard was quantified using UV-Visible spectroscopy in a Beckman DU-7 spectrophotometer with a molar extinction coefficient of 21,800 [this is the extinction coefficient for AFB_1 which was used since it is more conservative than the value of 20,400 for AFB_{2a} (18)]. AFB_1-diol standards were stored frozen at −20°C in acidified water (approximately pH 4.0) under argon.

Preparation of AFB_1-N^7-Gua Standard. The standard was prepared as described previously by Groopman *et al.* (19). The AFB_1-N^7-Gua standard was quantified using UV-Visible spectroscopy in a Beckman DU-7 spectrophotometer with a molar extinction coefficient of 18,000. The standard was stored frozen at −20°C in water under argon.

Rat Experimental Protocols. Two F344 male rats, purchased from Harlan, were housed singly in wire-bottomed cages in temperature-, humidity-, and light-controlled conditions. Food and water were available *ad libitum*. The animals were fed an AIN-76 diet. The animals received a 0.8 mg/kg i.p. injection of AFB_1 in dimethyl sulfoxide (100 μg each). The rats were then placed in glass metabolic cages and the urine was collected for 42 h.

Preparation Procedure for Rat Urine. The urine obtained was combined and the pH was titrated to approximately 5 with 1 N HCl and 1 M ammonium formate, pH 5.3. The urine was centrifuged at 700 rpm for 10 min and 50% of the supernatant was applied at a flow rate of 0.3 ml/min (gravity) to a C_{18} Sep-Pak cartridge (Millipore Corp.). The cartridge had been activated by wetting with sequential washes containing 5 ml each of 5% methanol:water, 80% methanol:water, 100% methanol, and 5% methanol:water. The loaded Sep-Pak was then washed with 10 ml 5% methanol:water and eluted with 10 ml of 80% methanol: water. The eluate was evaporated to dryness in a Savant Speed Vac model SC210A (SAVANT Instruments, Inc. Farmingdale, NY). The pellets were reconstituted in 300 μl 0.1 N HCl acid and placed in a 50°C water bath for 10 min. The samples were cooled to room temperature and 500 μl of 1 M ammonium formate at pH 4.5 was added. The volume was then brought up to 2 ml with PBS. One hundred μl of this was added to 5 ml H_2O and applied to an immunoaffinity column containing a 4-ml bed volume of monoclonal aflatoxin antibodies bound to Sepharose, as described previously (20, 21). This column was sequentially washed with two 7-ml aliquots of PBS with 0.01% thimersol, followed by a 7-ml water wash. The aflatoxins were eluted with 7 ml of 70% dimethyl sulfoxide:water, followed by two 7-ml washes of PBS with 0.01% thimersol. The last 3 fractions, totalling 21 ml, were collected in a beaker, an equivolume of water was added, and the sample of approximately 42 ml was applied to an activated Sep-Pak. The aflatoxins were then eluted from the Sep-Pak with 8 ml of 80% methanol:water. The resulting sample was evaporated to approximately 1 ml in the Savant.

Chromatography. The HPLC used was a Varian 5020 ternary pump system with a Microsorb C_{18} 5-μm (25 cm x 4.6 mm) analytical column (Rainin Instrument Co., Woburn, MA). The column temperature was set at 55°C. Isocratic chromatography was performed in a mobile phase consisting of 19% reagent alcohol (Anachemia, Rouses Point, NY) in 5 mM TEAF, pH 3.0, eluted at a flow rate of 1 ml/min. Fluorescence detection was obtained with a Hewlett-Packard Model 1046A programmable fluorescence detector (Hewlett-Packard, Rockville, MD), set at 365 nm excitation and 428 nm emission. The lamp was set at 220 Hz, the amplification factor was set at 18, and the response time was set at 1 s. The excitation slit was 2 x 2 mm, resulting in a bandwidth of 25 nm, and the emission slits were 4 x 4 mm. The signal was quantified by a Hewlett-Packard Model 3390A integrator. The initial HPLC urine separation to collect the AFB_1-N^7-Gua adduct present used a 12% isocratic mobile phase consisting of a 1:1 mixture of acetonitrile: reagent alcohol with TEAF.

Synchronous Fluorescence Spectrophotometry. A Perkin Elmer Cetus luminescence spectrometer Model LS 50 was used (Perkin Elmer Cetus, Beaconsfield, Buckinghamshire, England). A 3-ml quartz fluorescence spectrophotometer cell was used for all samples with a standard sample size of 2 ml. Standard settings for synchronous scanning of hydrolyzed AFB_1-N^7-Gua samples at pH 8.4–8.6 included a $\delta\lambda$ (wavelength difference between the peak excitation and emission wavelength) of 50 nm, a range from 300 to 450 nm, excitation and emission band widths of 15 nm, and a scan speed of 500 nm. A $\delta\lambda$ of 74 nm was found to be optimal for AFB_1-diol at pH 4–4.3; the other settings remained the same. All spectra were collected and analyzed with Perkin Elmer Cetus software provided with the spectrophotometer. Fluorescence was measured by the area under the curve method after correction for background. The SFS technique has been described previously in detail (22). The optimal $\delta\lambda$ for each chemical was determined by serial scanning starting with a $\delta\lambda$ of 10 and increasing by increments of 4 to a final value of 100–118 after the approach of Harris *et al.* (23). The optimal value of the $\delta\lambda$ varies for the same chemical in different solvents and should be verified for each different situation.

Statistical Analysis. All analyses including correlation coefficients and coefficients of variation were performed in the software package Statgraphics (Ver. 3.0).

Results

The goal of this research was to develop a more sensitive method to quantify the major aflatoxin-nucleic acid adduct in biological samples. The AFB_1-N^7-Gua adduct is weakly flourescent compared to other aflatoxin derivatives, such as the AFB_1-diol. Since the hydrolysis products of AFB_1-N^7-Gua are guanine and the AFB_1-diol, the specific measurement of either of these products following hydrolysis would quantify the nucleic acid adduct. Previously, we had used the measurement of guanine release as confirmation of the nucleic acid adduct in urine samples (16). In this report we describe efforts to quantitatively release the highly flourescent AFB_1-diol from the nucleic acid adduct to attain a more sensitive assay for biological samples. Fig. 1 shows the entire method used to analyze the flourescent metabolites of the AFB_1-N^7-Gua adduct starting with urine. The previous method included the steps through HPLC separation with quantification of the adduct peak (13–15). The current proposed protocol involves two brief additional steps consisting of hydrolysis and HPLC separation with fluorescence detection and quantification.

Central to the success of this method was the development of hydrolysis conditions to release the AFB_1-diol from the nucleic acid adduct. The hydrolysis reaction was extensively optimized to achieve the highest fluorescence

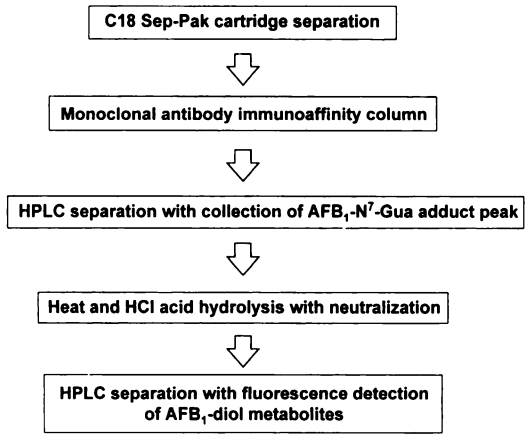

Fig. 1. Method used to analyze the flourescent metabolites of the AFB_1-N^7-Gua adduct in urine.

Table 1 Variables tested in optimization reactions

Acid	Base	Time (h)	Temperature (°C)	Neutralization
0.6 M HCl	0.75 M NH_4OH	0.25	50	Acetic acid and
1.1 M HCl	2.3 M NH_4OH	0.50	75	KOH to pH 4
2.1 M HCl	3.6 M NH_4OH	0.75	90	Formic acid and
2.9 M HCl	5 M KOH	1.00	95	KOH to pH 4
3.0 M HCl		1.50	100	Tris and KOH to pH
3.5 M HCl		2.00		8.5
4.7 M HCl		4.00		Ethylenediamine and
5.5 M HCl		6.00		KOH to pH 10.5
1.1 M $HClO_4$		8.00		Diethanolamine and
3.2 M $HClO_4$				KOH to pH 10.5
1.3 M H_3PO_4				
4.9 M H_3PO_4				

yield. A number of variables were evaluated as displayed in Table 1. Each hydrolysis chemical was tested at different times and concentrations; however, not all times and temperatures shown were evaluated for each chemical. In general, HCl was tested more extensively at a wider range of temperatures and times including very short hydrolysis times in minutes (data not shown). In addition, we attempted to use adduct methylation, as described by Essigmann *et al.* (6), in a effort to destabilize the aflatoxin-N^7 guanine bond, thus facilitating the release of the AFB_1-diol product. Finally, the neutralization reaction was optimized for specific chemicals and final pH.

Following these experiments, the optimal hydrolysis reaction of AFB_1-N^7-Gua was obtained by adding 32 µl 12.4 M HCl acid to 100-µl aliquots of the adduct (3 M final acid solution) and hydrolyzing in a 95°C heat block for 60 min. This reaction was neutralized with 25 µl of 16.7 M acetic acid and 95 µl 5 M KOH to a final pH of 4–4.3 for use with the HPLC. The neutralization for SFS used 100 µl of 1 M Tris and 73.5 µl of 5 M KOH with the addition of water to a final sample volume of 2 ml and a pH of 8.4–8.6. The flourescent yield of the reaction was equal to about 40% of that produced by equimolar amounts of AFB_1-diol.

UV HPLC analysis of the resultant hydrolysis products revealed a peak that coeluted with AFB_1-N^7-Gua standard, as well as a second peak that coeluted with AFB_1-diol

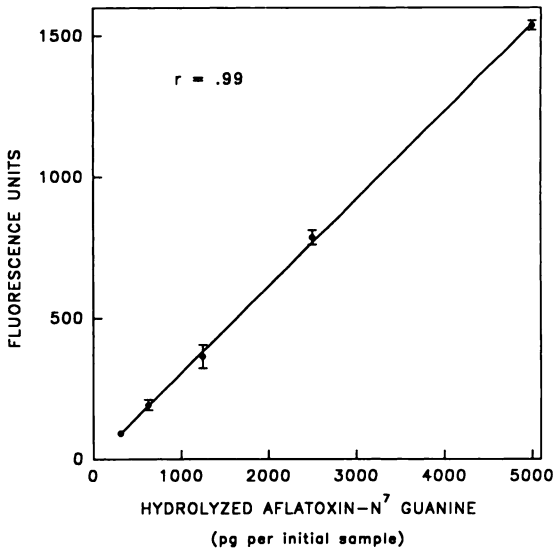

Fig. 2. Linear regression between the AFB_1-N^7-Gua adduct in pg per initial sample (total 2 ml final sample) and the fluorescence of the hydrolysis product measured by SFS on triplicate concentration curves. *Points*, mean; *bars*, SD.

standard. This second peak resolved into one main peak (coeluting with AFB_1-diol) with one smaller peak/shoulder on either side when gradient elution conditions were used (TEAF and 1:1 acetonitrile:reagent alcohol). The neutralization pH for optimal fluorescence was found to be 10–11 by SFS; however, certain basic buffers (ethylenediamine and diethanolamine) used to reproducibly achieve this pH resulted either in quenching or a lack of fluorescence enhancement. Therefore, the use of Tris buffer and KOH to achieve a final pH of 8.5 was found to be optimal in terms of fluorescence and reproducibility.

In optimizing this protocol, both SFS and HPLC with fluorescence detection were evaluated as analysis systems for the metabolites produced in the hydrolysis reaction. Fig. 2 shows the linear regression between the AFB_1-N^7-Gua adduct in pg per initial sample (total 2 ml final sample) and the fluorescence of the hydrolysis product measured by SFS on triplicate concentration curves. The correlation coefficient is 0.99. The limit of detection was set at a 3:1 signal-to-noise ratio, which occurs approximately at or below an area of 75. Ranges below this point are also associated with an unacceptable increase in the coefficient of variation. With these criteria, the method was sensitive down to 312 pg of the adduct pre-hydrolysis. The average coefficient of variation for the 5 points is 7.3%; the actual values by increasing concentration are 10.5, 9.8, 11.5, 3.4, and 1.1%.

SFS is useful to demonstrate other aspects of this protocol. The increase in fluorescence resulting from the hydrolysis reaction is illustrated in Fig. 3. Fig. 3*A* shows the synchronous fluorescence spectra of 5 ng (2.5 ng/ml) AFB_1-N^7-Gua after hydrolysis with neutralization to pH 8.5. The insert depicts the same concentration of AFB_1-N^7-Gua at pH 8.5 prior to hydrolysis, which, in distinction to prior publications (23–25), we found to be a poorly flourescent chemical. Fig. 3*B* displays the accompanying excitation and emission spectra for the hydrolyzed sample.

Final pH was shown to have a large impact on the fluorescence of the metabolites. Fig. 4*A* compares the syn-

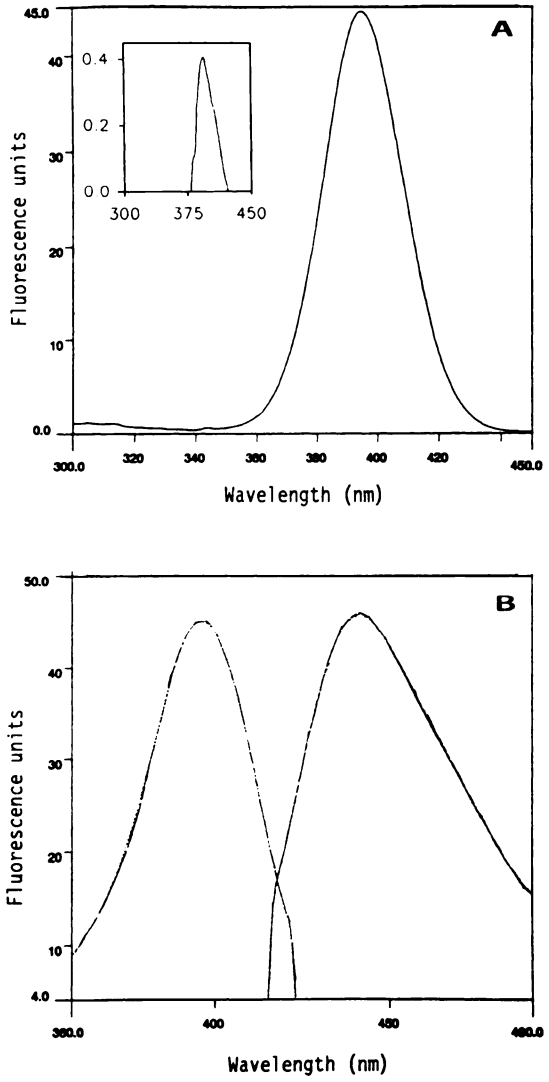

Fig. 3. Increase in fluorescence resulting from the hydrolysis reaction. (*A*) Synchronous fluorescence spectra of 5 ng (2.5 ng/ml) AFB_1-N^7-Gua after hydrolysis with neutralization to pH 8.5. The insert depicts the minimal fluorescence obtained on this sample prior to hydrolysis. (*B*) Accompanying excitation (left) and emission spectra for the hydrolyzed sample.

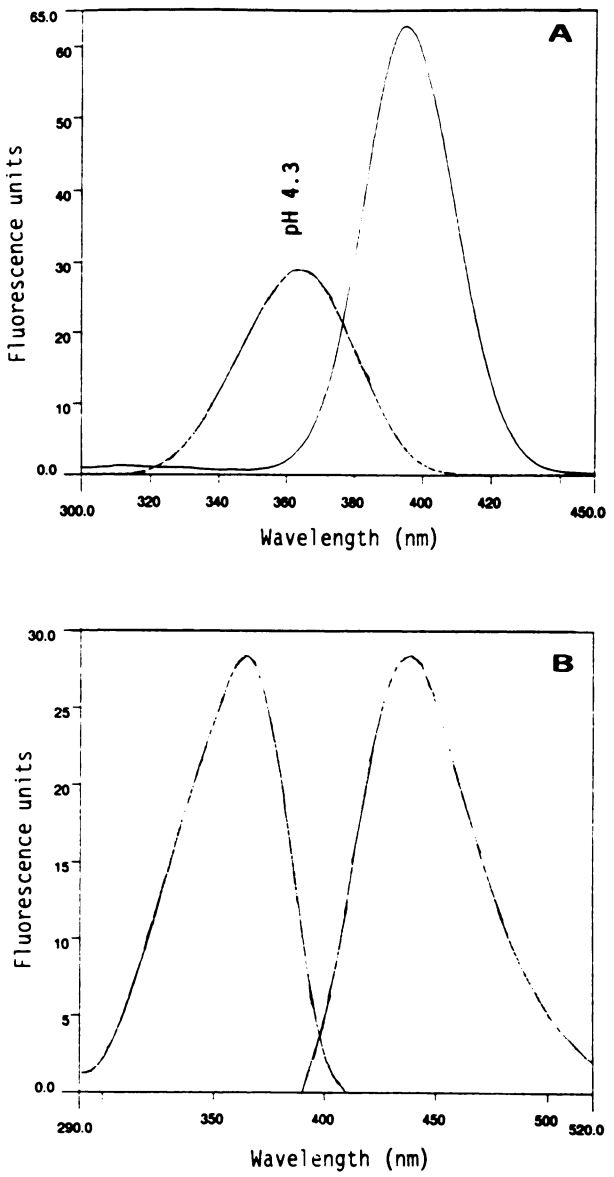

Fig. 4. (*A*) Comparison of the synchronous scan of 1 ng/ml of AFB_1-diol standard at pH 4.3 with the enhanced fluorescence seen in the corresponding scan at pH 8.4. (*B*) Accompanying excitation and emission spectra for the AFB_1-diol sample at pH 4.3.

chronous scan of 1 ng/ml (2-ml sample in cuvette) of AFB_1-diol standard at pH 4.3 with the enhanced fluorescence seen in the corresponding scan at pH 8.4. This additional fluorescence was a result of the combination of increased pH and the use of Tris buffer. Fig. 4*B* depicts the accompanying excitation and emission spectra for the AFB_1-diol sample at pH 4.3. These scans are similar to those seen for the hydrolyzed AFB_1-N^7-Gua adduct; it has been our experience and that of other researchers that aflatoxin metabolites appear similar via routine SFS (23).

The linear regression obtained between the AFB_1-N^7-Gua adduct (pg per initial sample) and the fluorescence of the hydrolysis products obtained via HPLC with fluorescence detection is displayed in Fig. 5 for quadruplicate samples. The error bars indicate the SD from the mean. The correlation coefficient is 0.99. The limit of detection was set at 39 pg, which approaches a 3:1 signal-to-noise ratio and occurs approximately at or below an area of 60,000. Samples below this point tend to have prominent shoulder peaks, and thus become an integration problem. Lower concentration curve points (39–158 pg) were affected to some extent by this problem as well, as evidenced by their increased variability. The average coefficient of variation for the 8 points is 11.8%; the actual values by increasing concentration are 14.1, 19.2, 18.3, 6.8, 9.6, 5.9, 14.9, and 5.5%.

Fig. 6*A* shows the UV chromatography from exposed rats. Rats were treated and urine was processed as discussed in "Materials and Methods." The peak labeled AFB_1-N^7-Gua was collected, dried down, resuspended in 100 μl of water, and hydrolyzed. Fig. 6*B* displays the subsequent fluorescence chromatography. Samples collected from

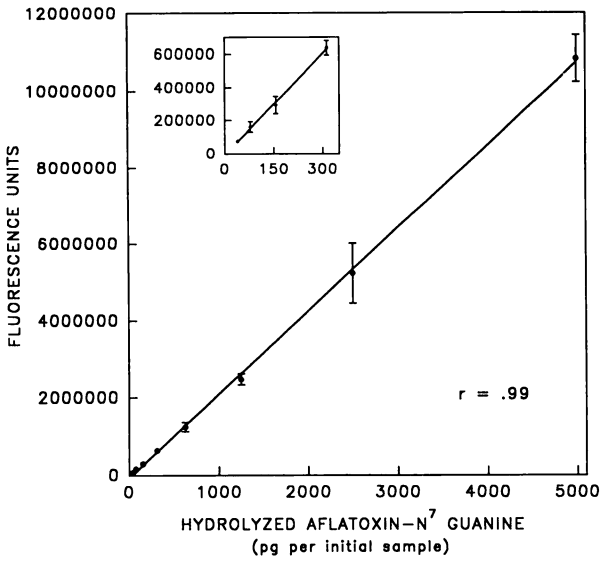

Fig. 5. Linear regression obtained between the AFB_1-N^7-Gua adduct and the fluorescence of the hydrolysis products obtained via HPLC with fluorescence detection. *Points,* mean; *bars,* SD.

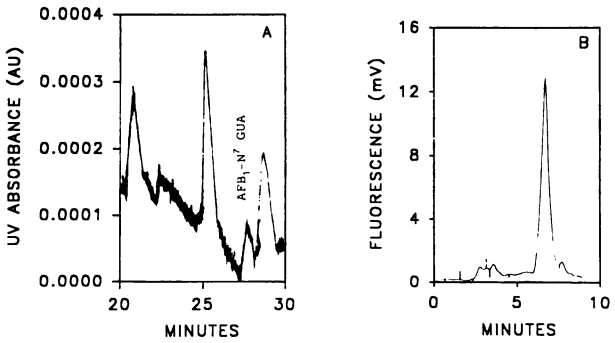

Fig. 6. (*A*) UV chromatography from exposed rats. Rats were treated and urine was processed as discussed in "Materials and Methods." The peak labeled AFB_1-N^7-Gua was collected, dried down, resuspended in 100 μl of water, and hydrolyzed. (*B*) Subsequent fluorescence chromatography.

baseline areas were compared as controls. No similar peaks were obtained (data not shown).

Discussion

The utilization of fluorescence detection to measure metabolites of the AFB_1-N^7-Gua adduct has been explored in an attempt to increase the sensitivity of the current methodology. The procedure involves acid hydrolysis followed by detection via either SFS or HPLC with fluorescence detection. The hydrolysis reaction produces a primary metabolite that coelutes with AFB_1-diol standard, which is the expected reaction product. The shoulder peaks may represent isomers of this chemical. The significant effect of pH and solvents on the resulting fluorescence is also an important consideration in optimization experiments for flourescent metabolites.

We found HPLC with fluorescence detection to be more sensitive than SFS, which is in distinction to Harris *et al.* (23). We did note a lower coefficient of variation for the SFS concentration curve points; however, these results depend on the careful subtraction of background blanks and any aberrancy in the blank can increase the variation. In addition, the increased variation for HPLC was found at lower concentration points which could not be detected with SFS.

We did not find unhydrolyzed AFB_1-N^7-Gua adduct to be flourescent enough to quantify without hydrolysis. It is our experience that careful separation techniques are needed to produce a single AFB_1-N^7-Gua peak in urine; past findings of fluorescence may have been related to coelution of AFB_1-N^7-Gua with another flourescent metabolite, a problem we experienced initially when trying to achieve a good separation. In addition, spontaneous hydrolysis may occur in AFB_1-N^7-Gua adduct standards, which would lead to some measurable fluorescence.

Acid hydrolysis has been used to release flourescent derivatives from other chemical adducts, resulting in assays with increased sensitivity. Manchester *et al.* (26) reported the use of acid hydrolysis to release *r*-7,*t*-8,*t*-9,*c*-10-tetrahydro-benzo(*a*)pyrene from placental *r*-7,*t*-8-dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydro-benzo(*a*)pyrene DNA adducts. This approach has been reported using lung tissue from smokers as well (27). The carboxylic acid ester linkage in several polycyclic aromatic hydrocarbon-hemoglobin adducts can also be cleaved with acid hydrolysis to release tetrols (28).

A number of problems were identified during this research which have reduced the sensitivity of the resulting assay. First, despite many optimization attempts as described above, we were unable to achieve a 100% yield. This may be related, in part, to the fact that harsh conditions are necessary to cleave the AFB_1-N^7-Gua bond, and these conditions lead to instability of AFB_1-diol. A second problem, of a technical nature, is that the optimal range for the Hewlett-Packard fluorescence detector is well below the nm range necessary for use with aflatoxin, thus limiting optimization of the fluorescence produced. Lastly, because the hydrolysis reaction produced more than one metabolite, resolution and integration of the products become limiting factors at low levels.

Despite these limitations, the proposed protocol increases the AFB_1-N^7-Gua adduct detection sensitivity by as much as 100-fold as assessed with the use of standards. The actual sensitivity will be less depending on the resolution time needed to achieve separation of the AFB_1-N^7-Gua adduct, which varies with columns, urine specimens, and HPLC systems. However, the HPLC assay could be further optimized by using a mobile phase at a higher pH (7.5) to increase to fluorescence as shown by SFS in Fig. 4*A*. The increased ability to measure low levels of the adduct in urine should be helpful in the evaluation of populations with lower exposures, such as in chemoprotection trials or in developed countries. This procedure may be beneficial for use with serum AFB_1-DNA adducts, as well.

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Fluorescence quantification of aflatoxin N7-guanine adducts.

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