Measurement of Aflatoxin B₁, Its Metabolites, and DNA Adducts by Synchronous Fluorescence Spectrophotometry

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ABSTRACT

Sensitive and specific methods are needed for measuring human exposure to carcinogens. Synchronous fluorescence spectrophotometry can be used to measure fmo1 of aflatoxins, their metabolites, and DNA adducts. Computer-assisted analysis of spectra of these agents obtained by synchronous fluorescence spectrophotometry can be displayed as contour maps which are highly specific for each agent. Individual agents in mixtures, e.g., aflatoxins B₁ and M₁, can be identified by fourth derivative spectral analysis. This physical method should complement immunological and other methods to measure aflatoxin B₁, its metabolites, and nucleic acid adducts.

INTRODUCTION

Biochemical and molecular epidemiology is a multidisciplinary area that combines laboratory and epidemiological approaches of cancer research (1, 2). The primary goal is to identify individuals who are at high cancer risk by obtaining evidence of (a) pathological lesions in target cells caused by exposure to carcinogens and/or (b) increased susceptibility to carcinogens due to either inherited or acquired host factors. Carcinogen-DNA adducts are examples of pathological lesions considered to be important in the early stage, i.e., tumor initiation, and in a later stage, i.e., tumor conversion, of carcinogenesis. Recently, both immunological and nonradioactively labeled physical methods have been developed to measure adducts in macromolecules including DNA isolated from carcinogen-exposed tissues and cells (see review in Ref. 3). One of the advantages of these approaches is that they can be specific for both carcinogen and target tissue. Arguing that data obtained by 2 different analytical methods would be confirmatory and more convincing than measurements from a single technique, we describe here a refined physical method, SFS (4-7), to complement enzyme radioimmunoassays (8-11) for the measurement of aflatoxin B₁, its metabolites, and DNA adducts.

MATERIALS AND METHODS

Chemicals. Aflatoxins B₁, B₂, B₃, B₆, M₁, M₂, G₁, G₂, G₃, P₁, and Q₁, and aflatoxicol were obtained from Sigma Chemical Company, St. Louis, MO. Stock solutions were dissolved in chloroform and methanol and then diluted in 10 mM Tris-1 mM EDTA to working concentrations. δ-H-Aflatoxin B₁ (15 Ci/mmol) was obtained from Moravek Biochemicals, Brea, CA. AFQ₄ and AFM₃ were prepared by reacting AFQ₁ and AFM₁ with an equimolar amount of trifluoroacetic acid followed by hydrolysis with water as described previously by Buchi et al. (12). The product was dried in a vacuum. Preparation of AF₃β-N⁷-MGua was by the method described by Essigmann et al. (16) using dimethyl sulfate.

In Vivo Modified DNA. Male Fisher rats (150 g) were given i.p. injections of 50 μl of a mixture of 3H-AFB₁ and unlabeled AFB₁ at concentrations of 900, 138, or 62 μg/kg at a specific activity of 1.2 Ci/mmol in dimethyl sulfoxide. After 2 h, the rats were killed by CO₂ narcosis, and the livers were removed and frozen at -70°C.

Isolation of DNA. DNA was isolated from rat liver by a modification of the method of Marmur (13). Tissues were thawed and minced in 0.2X SSC buffer. After centrifugation at 1000 x g, the supernatant was decanted and the tissue was washed in 0.2X SSC and recentrifuged until the supernatant was clear. The tissue was then suspended in 5 ml of 0.2X SSC and the cells were separated in a Dounce homogenizer. After 3 washes in 0.2X SSC, the cell pellet was resuspended in 1 ml of 1X SSC, and 8 ml of 10 mM Tris (pH 7)-1 mM EDTA-1% sodium dodecyl sulfate were added to the pellet along with 10,000 units of Pronase in 1 ml. After 1 h incubation at 45°C, 10,000 additional units of Pronase were added and incubation was continued for 1 h. The digested tissue was phenol extracted 3 times and once with chloroform/isoamyl alcohol (24/1, v/v). After precipitation in ethanol and LiCl overnight at 4°C, the DNA was carefully spooled on a glass rod and washed 3 times in ethanol. The remaining ethanol was evaporated and the pellet was resuspended in 10 mM Tris-1 mM EDTA. The addition level of aflatoxin metabolites to DNA was determined by measurement of tritium bound to the purified DNA and, following hydrolysis, HPLC analysis.

Hydrolysis of DNA. Nucleic acids were adjusted to 0.15 N HCl and treated for 15 min at 90-95°C as detailed by Lin et al. (14). This procedure releases >90% of the covalently bound radioactivity from the modified DNA. Hydrolysates were rapidly cooled on ice, neutralized with 1 M ammonium formate (pH 5.0) and 1 N KOH, made 5% methanol, and applied to C₁₈-Sep-Pak (Waters Associated, Milford, MA). The Sep-Pak was washed with 5% methanol to remove unhydrolyzed DNA and other polar compounds and then eluted with 80% methanol to release the more lipophilic aflatoxin derivatives. The methanol was removed from these samples by rotary evaporation under reduced pressure and the resulting mixture was adjusted to 10% ethanol prior to HPLC.

Chromatography. Nucleic acid hydrolysates were analyzed by HPLC using an Ultrasphere-ODS C₁₈ 5-μm column (Beckman Instrument Co., Berkeley, CA) and a Beckman Model 322 liquid chromatograph equipped with Model 160 detector (365 nm). Gradient chromatography was performed at ambient temperature with an elution buffer of 10% redistilled ethanol-20 mM triethylammonium formate (pH 3.0) to 18% ethanol over 25 min at 1.0 ml/min. These procedures have been described previously (15). One-min fractions were collected and tritium quantified by liquid scintillation counting using an LKB Model 1211 beta counter.

Instruments. All spectra were recorded on a Perkin-Elmer Model 650/40 fluorescence spectrophotometer (Norwalk, CT). The synchronous scanning technique was performed by interlocking the excitation and emission monochrometers at a wavelength difference (Δλ) and scanning the sample over a range of wavelengths. The methodology of SFS has been described previously (4-7). Spectral data were collected and stored in a Perkin-Elmer Model 3600 data station which also corrected the spectra for variations in xenon lamp output and linearity during the scan. Limits of detection were defined as the minimum signal that was 3-fold above background noise.

Contour Maps. The data for contour maps were obtained by repeatedly scanning a sample from an excitation wavelength of 300 to 500 nm at different Δλ, typically starting with Δλ of 10 nm increasing in

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1 To whom requests for reprints should be addressed, at National Cancer Institute, Building 37, Room 2C07, Bethesda, MD 20892.
2 The abbreviations used are: SFS, synchronous fluorescence spectrophotometry; SSC, 0.15 M sodium chloride-0.0015 M sodium citrate, pH 7.0; AFB₁-N₁-Gua, 2,3-dihydro-2-(N₁-quany)-3-hydroxyaflatoxin B₁; AFB₁-N₃-MGua, 2,3,5,6-dihydro-2(N₃-formyl-2',5',6'-triamino-4'-oxo-N'-pyrimidyl)-3-hydroxyaflatoxin B₁; AFB₂, aflatoxin B₂ (other aflatoxins are similarly designated); HPLC, high pressure liquid chromatography.
increments of 4 to 118 nm. Collection of spectral data by multiple scanning can require up to 3 h/sample. Spectral data were then transferred to the NIH DEC-System-10 computer and analyzed using a program called MLAB (Modeling Laboratory) which provided the facilities to view the fluorescence spectra as contours. By projecting the data onto a 2-dimensional plane as a series of cross-sections of intensity levels, a map of excitation-emission spectra at multiple δλs can be viewed in a manner similar to that for topographic maps.

RESULTS

The fluorescence intensity was measured for each of the aflatoxins over several dilutions to determine the linearity of fluorescence and the spectral characteristics of the major DNA adducts and metabolites (Fig. 1). All synchronous scans were performed at a δλ of 34 nm which is optimal for AFBi-DNA. Each data point is the average of at least two scans in which the background fluorescence (nonadducted DNA) was removed by computer manipulation. A linear relationship exists between concentration and fluorescence intensity at and above concentrations of 100 fmol. Below this level linearity diminishes because of difficulties in accurately removing the background fluorescence. AFB1 shows a moderate amount of fluorescence at a δλ of 34 nm while AFB2α, which has a water molecule substituted across the 2,3 bond in the terminal furan ring, shows considerably more fluorescence at equal concentrations. AFB1 has been reported to bind to DNA through the oxidative metabolism of the 2,3 double bond (14, 16). The increased fluorescence from the derivatization of this bond can be seen in the standard curve of AFB1-DNA, although its activity is less than AFB2α (Fig. 1). AFB1-N7-Gua has a fluorescence level similar to that of AFB2α; however, AFB1-FAPyr, in which the guanine ring has opened, showed considerably less than the other DNA adducts and aflatoxin metabolites. All of these metabolites and adducts showed a fluorescence peak at an excitation wavelength of 402 nm and could not be distinguished from each other except by their relative intensity.

Contour Maps of Aflatoxins and Their Metabolites and Adducts. The contour maps shown in Fig. 2 were made by dividing the spectral data into 20 groups based on the fluorescence intensity and plotting the groups as a series of dashed lines. The shortest dashes represent the lowest intensity group and the longest dashes represent the highest. AFB1 and AFB2α (Fig. 2) can be easily distinguished as AFB1 shows its peak fluorescence at δλ 54 nm and AFB2α at δλ 38 nm. The differences between AFB2α and AFB1-DNA are not as obvious because their peak fluorescence is at similar positions, although the spectral pattern at lower intensities is noticeably different. Contour maps of AFB1-DNA are very similar to those for AFB2α-DNA. However, these maps show little resemblance to either AFB1 or AFB2α, while AFB2α is considerably different from AFB1 in 2-dimensional scans or contour maps.

Limits of Detection and Optimal δλs for Aflatoxin Adducts and Metabolites. Table 1 shows the results of measuring the various aflatoxin adducts and metabolites at the optimal δλ as determined by contour maps. By scanning the fluorescence of some aflatoxins at the optimal δλ, an increase in sensitivity of detection 1.5- to 10-fold can be seen over scanning at δλ 34 nm. The largest increases in detectability were seen with metabolites such as aflatoxicol in which the optimal δλ of 98 nm differed significantly from the δλ of 34 nm. The saturation of the double bond at the 2,3 position of the terminal furan ring caused an increase in fluorescence of the metabolites. This can be seen by comparisons of the limits of detection of AFB1, AFB2α, AFB2α, and AFQα, with their 2 and 2α metabolites.

In the interests of increasing the fluorescence detectability of the major product of AFB1 with DNA, AFB1-N7-Gua was further derivatized by methylation of position 9 of the guanine residue. This modification of AFB1-N7-Gua has been suggested previously to enhance fluorescence (16). As can be seen in Fig. 3, however, methylation of this adduct not only shifted the optimal δλ from 34 nm to 86 nm but also decreased the fluorescence of the chromophore as well as the excitation wavelength maximum. Thus, the formation AFB1-N7-mGua was not a viable means to enhance fluorescence.

Resolution of Mixtures Using Three Dimensional Scans. While contour maps allow discrimination of aflatoxin metabolites and DNA adducts in ways not possible with normal synchronous fluorescence, their ability to unravel mixtures is limited. An equimolar mixture of AFB1 and AFB2α have overlapping peaks which form a unique pattern. The resolution of the individual components can be determined by subtracting the fluorescence of one of the parts, but this depends on knowing at least one of the metabolites. To solve this problem, we made use of fourth derivative spectral analysis as described previously (17). Derivatives of the original spectra are calculated by shifting the original spectra by a set wavelength and subtracting this shifted spectra from the original curve. Repeating this operation three times results in a fourth derivative of the original spectra. This procedure emphasizes the individual components of the peaks and therefore allows overlapping spectra to be differentiated. As can be seen in Fig. 4, the components of the mixture can be distinguished following the fourth derivative analysis of the spectral data obtained from the mixture of AFB1 and AFB2α.

In Vivo Modification of Rat Liver DNA AFB1. In vivo AFB1-modified rat liver DNA showed a fluorescence spectrum indistinguishable from that of in vitro-modified DNA, despite the differences in AFB1-DNA adduct formation. The in vivo DNA samples had a range of modification of one AFB1 residue per 67,000 to one AFB1 residue per 15,200,000 nucleotides com-
Fig. 2. Contour maps and chemical structures of AFB₁, AFB₂a, AFB₁-DNA, AFB₁-N⁷-Gua (Adduct I), AFM₁, and AFM₁-DNA.
**Table 1** Optimal δλ for measurement of aflatoxins and their DNA adducts

<table>
<thead>
<tr>
<th>Sample</th>
<th>Optimum δλ</th>
<th>Excitation wavelength (nm)</th>
<th>Limit of detection (fmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB1</td>
<td>70</td>
<td>367</td>
<td>100</td>
</tr>
<tr>
<td>AFB2</td>
<td>38</td>
<td>402</td>
<td>100</td>
</tr>
<tr>
<td>AFB-DNA</td>
<td>34</td>
<td>402</td>
<td>100</td>
</tr>
<tr>
<td>AFQa</td>
<td>54</td>
<td>413</td>
<td>100</td>
</tr>
<tr>
<td>Aflatoxicol</td>
<td>98</td>
<td>340</td>
<td>100</td>
</tr>
<tr>
<td>AFMq</td>
<td>46</td>
<td>402</td>
<td>100</td>
</tr>
<tr>
<td>AFQq</td>
<td>50</td>
<td>417</td>
<td>100</td>
</tr>
<tr>
<td>AFB1</td>
<td>58</td>
<td>389</td>
<td>250</td>
</tr>
<tr>
<td>AFB2</td>
<td>66</td>
<td>362</td>
<td>250</td>
</tr>
<tr>
<td>AFB2</td>
<td>90</td>
<td>371</td>
<td>250</td>
</tr>
<tr>
<td>AFB2-N7-Gua</td>
<td>42</td>
<td>398</td>
<td>500</td>
</tr>
<tr>
<td>AFM1</td>
<td>46</td>
<td>366</td>
<td>500</td>
</tr>
<tr>
<td>AFB1</td>
<td>70</td>
<td>400</td>
<td>500</td>
</tr>
<tr>
<td>AFQ2</td>
<td>62</td>
<td>415</td>
<td>1,000</td>
</tr>
<tr>
<td>AFB-Diol</td>
<td>58</td>
<td>387</td>
<td>10,000</td>
</tr>
<tr>
<td>AFM-DNA</td>
<td>38</td>
<td>393</td>
<td>10,000</td>
</tr>
<tr>
<td>AFB1-FAPyr</td>
<td>42</td>
<td>395</td>
<td>100,000</td>
</tr>
</tbody>
</table>

**Table 2** Measurement of AFB1-DNA adducts in rat liver

<table>
<thead>
<tr>
<th>Dose (μg/kg)</th>
<th>Fluorescence</th>
<th>Radioactivity</th>
<th>HPLC pmol/mg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>62</td>
<td>0.2</td>
<td>0.1</td>
<td>ND</td>
</tr>
<tr>
<td>138</td>
<td>10.0</td>
<td>14.0</td>
<td>7.0*</td>
</tr>
<tr>
<td>900</td>
<td>45.0</td>
<td>78.0</td>
<td>58.6</td>
</tr>
</tbody>
</table>

* ND, not detected.

**DISCUSSION**

AFB1 is a mycotoxin produced by *Aspergillus flavus* and a potent carcinogen in experimental animals. AFB1 is also implicated as a risk factor in the etiology of human hepatocellular carcinoma (17–19). AFB1 and its metabolites have been measured by HPLC analysis and immunoassays in body fluids and tissues from people exposed to AFB1 in contaminated foods and beverages (for review, see Ref. 20). AFB1-N7-Gua adducts have...
also been measured in the urine from rats (21) and people (22, 23) exposed to AFB1.

Conventional excitation and emission spectra of aflatoxins contain many peaks. By scanning excitation and emission synchronously, i.e., SFS, with the proper fixed wavelength difference, only one peak emerges. The proper wavelength difference can be calculated from the 0-0 bands by determining the difference between the longest wavelength band of excitation and the shortest wavelength band of emission spectra (24). The sensitivities of detection of AFB1 by HPLC coupled with a fluorescence detector and by immunooassay are similar.

While SFS is a sensitive method for the detection of aflatoxins and aflatoxin-DNA adducts (Table 1), it ability to distinguish between closely related compounds is limited when measured at a single λ. For example, AFB2a differs from AFB1 in the addition of a water molecule across the double bond of the terminal furan ring, yet synchronous scanning of AFB1 and AFB2 at a λX of 34 nm produces almost identical spectra.

fluorescent molecules have been used successfully in their quantitation (25) and thus are presently under study in our laboratory.

REFERENCES

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