A simple procedure was developed for in vitro synthesis and characterization of aflatoxin B1-lysine adduct using aflatoxin B1, N-\(\alpha\)-acetyl lysine and \(m\)-chloroperbenzoic acid (MCPBA). At a molar ratio of 1:16 (aflatoxin B1: N-\(\alpha\)-acetyl lysine), the recovery of adduct was 62\%. Analysis of the adduct by thin-layer chromatography showed a single spot (Rf = 0). Absorption spectra of the adduct showed 2 peaks at 275 and 335 nm. Liquid chromatographic (LC) analysis of the AFB1-lysine adduct showed a relative retention time of 2.1 min. Using the same epoxidation procedure, BSA-AFB1 adduct and ovalbumin-AFB1 adduct were synthesized for production of antibodies and as coating antigen, respectively. Control rat serum, spiked with AFB1-lysine adduct and subjected to LC analysis showed a retention time of 2.1 min, which is similar to that of AFB1-lysine reference standard, synthesized. Further, enzymatically hydrolyzed, control rat serum spiked with BSA-AFB1 adduct showed 2 peaks with retention times of 2.1 and 2.7 min. Based on the LC analysis, recovery of BSA-AFB1 in terms of AFB1-lysine adducts was 67 ± 5%. The major peak (2.1 min) accounted for 72% of the adduct; the second minor peak (2.7 min) accounted for 28% of the total AFB1-lysine adducts formed. Stability studies on the AFB1-lysine adduct synthesized, indicated that it was stable for 1 month. Antibody capture assay showed an absorbance of 0.9 to 1.0 at a dilution of 1:50 000 when ovalbumin-AFB1 was used as a coating antigen. Indirect competitive ELISA showed 50% displacement (IC50) of the antibodies at a concentration of 13 ng AFB1-lysine, whereas the IC50 for AFB1 was 7 ng. The recovery of AFB1-lysine adduct spiked to control rat serum followed by enzymatic hydrolysis and immunoanalysis (indirect ELISA) was 93 ± 6%. The enzyme immunoassay was validated by a rodent model, in which the animals were exposed to aflatoxin B1 (20 \(\mu\)g AFB1/kg body mass/day). The level of AFB1-lysine adduct in the rat serum was 27.3 ± 4.37 \(\mu\)g/mg albumin.

Aflatoxins are toxic secondary metabolites, produced by the fungus \textit{Aspergillus flavus} and \textit{A. parasiticus} (1, 2). They are common contaminants of agricultural commodities such as maize and oil seeds. Aflatoxin B1 (AFB1) is one of the most potent naturally occurring carcinogens and is classified as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC; 3). Epidemiological studies provided evidence of an association between dietary aflatoxin exposure and diseases such as aflatoxin hepatitis, primary hepatocellular carcinoma (PHCC), Indian Childhood cirrhosis, Reye’s Syndrome, Kwashiorkor, and colo-rectal cancer (3, 4).

Aflatoxin B1 is metabolized in the body to a highly reactive AFB1-8,9-epoxide which form adducts with guanine moiety of DNA and \(\varepsilon\)-amino groups of lysine residues of protein (5–9). Aflatoxin B1-8,9-epoxide has been successfully synthesized with chemical oxidants such as \(m\)-chloroperbenzoic acid (MCPBA) and dimethyl dioxirane (10–12). Dimethyl dioxirane was highly unstable and had a low shelf life, whereas MCPBA was a stable oxidant that generated AFB1-8,9 epoxide (11). This chemical oxidant had been used to synthesize CT-DNA (calf thymus DNA), oligodeoxynucleotide, and guanine adduct of AFB1 (5, 12–14).

Earlier, AFB1-lysine was synthesized with AFB1-8,9-epoxide which form adducts with guanine moiety of DNA and \(\varepsilon\)-amino groups of lysine residues of protein (15). This report describes the chemical synthesis of AFB1-lysine using N-\(\alpha\)-acetyl lysine and AFB1-8,9-epoxide. The aflatoxin epoxide was generated with MCPBA as a chemical oxidant, in a manner similar to in vivo activation of AFB1. The synthesized AFB1-lysine adduct was also characterized immunologically and by liquid chromatographic (LC) analysis. An enzyme immunoassay for quantitation of AFB1-lysine adduct in serum is also described and validated.

**Experimental**

**Apparatus**

(a) **Precoated thin-layer chromatographic (TLC) plates.**—Precoated polyester silica gel G TLC plates (size 20 \(\times\) 20 cm; particle size 2–25 \(\mu\)m; Sigma Chemical Co., St. Louis, MO).
Microtiter plates were coated with 50 μL coating buffer (0.1M carbonate buffer, pH 9.6) per well containing (100 ng) of ovalbumin-AFB$_1$.

The plate was dried overnight at 37°C in ELISA incubator.

The antigen coated plate was washed 3x with washing buffer (0.01M sodium phosphate buffer, pH 7.2, with 0.05% Tween-20, 0.85% NaCl).

The wells were blocked for nonspecific binding with 50 μL per well of blocking buffer (0.01M sodium phosphate buffer, pH 7.2, with 0.1% fish gelatin, 0.85% NaCl) for 30 min at 37°C.

The plate was washed (3x) with washing buffer and 25 μL 1:25 000 diluted antisera in diluent buffer (0.02M sodium phosphate buffer, pH 7.2, with 1.7% NaCl and 0.02% BSA) per well along with different concentrations of standard AFB$_1$-lysine (100 pg–1000 ng) in 25 μL 10% methanol. The plate was incubated at 37°C for 2 h.

After 2 h the plate was washed (3x) and incubated at 37°C for 1 h with 50 μL per well of 1:5000 diluted alkaline phosphatase-labeled anti-rabbit IgG raised in goat in PBS.

After 1 h incubation, the plate was washed (3x), and 150 μL per well of substrate buffer (1.25 mg p-nitrophenyl phosphate in 1 mL 10% diethanolamine–HCl buffer, pH 9.6, containing 0.05mM MgCl$_2$) was added.

The reaction was terminated after 45 min by adding 100 μL per well of stopping reagent (5N NaOH).

Absorbance at 405 nm was recorded using an ELISA reader along with the reagent blank.

**Figure 1. Flow chart of indirect competitive ELISA for AFB$_1$-lysine adduct.**
ences, National Institute of Nutrition, Hyderabad, India) for production of polyclonal antibodies to BSA-AFB$_1$ adduct.

(b) Rats.—Fisher 344 strain: male, 180–220 g body mass (National Center for Laboratory Animal Sciences).

**Biosafety**

AFB$_1$ and many of its derivatives are potently carcinogenic. Great care should be exercised to avoid personnel exposure. When handling the pure compound in the crystalline form, disposable cotton gloves are recommended. General procedures for minimizing exposure to chemical carcinogens and for reducing the risk of laboratory contamination were followed (16).

**Preparation of AFB$_1$-Lysine Adduct**

All reactions were performed under subdued light to minimize formation of photo products. Aflatoxin B$_1$-lysine conjugate was synthesized in 2 steps. In the first step AFB$_1$-8,9-epoxide was generated from AFB$_1$ (100 µg, 0.32 µmol) by using MCPBA (60% pure) as the chemical oxidant (11, 12). The epoxide was allowed to react with N-$\alpha$-acetyl lysine (1 mg, 5.32 µmol) in a biphasic reaction mixture containing dichloromethane and 0.1M phosphate buffer (pH 7.2). In 250 µL dichloromethane, 3.53 mg MCPBA (12.26 µmol) was dissolved. The mixture was washed 3 times with 500 µL 0.1M phosphate buffer, pH 7.2. In another 250 µL dichloromethane, 588 µg (1.88 µmol) AFB$_1$ was dissolved and added to the MCPBA in dichloromethane. The reaction was continued with gentle stirring for 100 min at 5°C. After 100 min, 5 mg BSA (75 nmol) dissolved in 250 µL 0.1M phosphate buffer,

<table>
<thead>
<tr>
<th>Mole to mole ratio of AFB$_1$ to N-$\alpha$-acetyl lysine</th>
<th>% Recovery of AFB$_1$-lysine</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5:1</td>
<td>12</td>
</tr>
<tr>
<td>1:3</td>
<td>4</td>
</tr>
<tr>
<td>1:16</td>
<td>62</td>
</tr>
</tbody>
</table>

$^a$ n = 4.

**Synthesis of BSA-AFB$_1$ Immunogen**

BSA-AFB$_1$ was synthesized to raise polyclonal antibodies by epoxidation. In the first step, AFB$_1$-8,9-epoxide was generated from AFB$_1$ by using MCPBA (60%) as the chemical oxidant (11, 12). The epoxide was allowed to react with BSA (5 mg, 75 nmol) in a biphasic reaction mixture containing dichloromethane and 0.1M phosphate buffer (pH 7.2). In 250 µL dichloromethane, 3.53 mg MCPBA (12.26 µmol) was dissolved. The mixture was washed 3 times with 500 µL 0.1M phosphate buffer, pH 7.2. In another 250 µL dichloromethane, 588 µg (1.88 µmol) AFB$_1$ was dissolved and added to the MCPBA in dichloromethane. The reaction was continued with gentle stirring for 100 min at 5°C. After 100 min, 5 mg BSA (75 nmol) dissolved in 250 µL 0.1M phosphate buffer,
pH 7.2, was added and the reaction was allowed to proceed at 5°C for 60 min. The ratio of BSA to AFB$_1$ is 1:20. After the reaction, the reaction mixture was centrifuged at 10 000 rpm for 5 min in a microfuge. The dichloromethane fraction was separated and the buffer fraction containing the BSA-AFB$_1$ conjugate was washed 3 times with dichloromethane to remove unreacted AFB$_1$. Both aqueous and organic phases were characterized for the presence of adduct and unreacted AFB$_1$ and its derivatives by TLC.

The mole to mole ratio of ovalbumin-AFB$_1$ and BSA-AFB$_1$ was estimated by TNBS assay (17), in which the concentration of free ε-amino groups of carrier protein was determined before and after conjugation with AFB$_1$. Briefly, to 1 mL BSA/ovalbumin or BSA-AFB$_1$/ovalbumin-AFB$_1$ (100 μg) solution, 1 mL 4% NaHCO$_3$, pH 8.5 and 0.01% freshly prepared TNBS reagent were added. The reaction was performed at 42° ± 2°C for 2 h followed by addition of 1 mL 10% sodium dodecyl sulfate and 0.5 mL 1N HCl solution. Absorbance was read at 335 nm in a spectrophotometer. Conjugation of aflatoxin to protein was calculated with the following equation:

\[
\text{Conjugation, } \% = \frac{\text{conc. } \varepsilon \text{- amino groups in conjugate} - \text{conc. } \varepsilon \text{- amino groups in carrier protein}}{\text{conc. } \varepsilon \text{- amino groups in carrier protein}} \times 100
\]

**Characterization by TLC**

Polyester silica gel TLC plates were used for analysis of buffer and organic fraction. The plate was developed in a chloroform–acetone (9 + 1) solvent system. The fluorescence of the resulting compound was visualized under longwave UV light (365 nm) in a UV cabinet. To calculate recoveries, the plate was scanned fluorodensitometrically (18). Concentration of the analyte was calculated based on the densities (measured as peak areas).

**Spectral Analysis**

Spectral analysis of the adduct was performed in spectro-grade methanol using a Quant-I soft pack module in a Beckman DU-50 recording spectrophotometer. The sample was scanned from 200–500 nm using methanol as a blank.

**Characterization by LC**

The adduct AFB$_1$-lysine was characterized by an LC system (Shimadzu) using a reversed-phase column (Shimadzu C$_{18}$, 250 × 4.6 mm id, particle size, 10 μm) linked to a diode array detector (SPD-M10 AV) and fluorescence detector (RF-10 AXL). For the diode array detector, the wavelength was set at 254 and 360 nm. For fluorescence detector, the excitation wavelength was set at 360 nm, and emission wavelength was set at 440 nm. The mobile phase was water–acetonitrile–methanol (70 + 15 + 15) at a flow rate of 1 mL/min (19). Before loading, AFB$_1$, N-α-acetyl lysine, and...
the adduct were treated with trifluoroacetic acid (TFA), and kept at 50°C for 5–10 s. The samples were dried under nitrogen and reconstituted with methanol. A 20 µL amount of sample was loaded onto the column. Samples without TFA treatment were also analyzed by LC.

**Stability Studies**

The reaction mixture was aliquoted (50 µL) and lyophilized. The stability of AFB₁-lysine adduct was checked by LC every week for 1 month (30 days).

**Albumin Hydrolysis**

The control rat serum sample was spiked with 10 µg BSA-AFB₁ (1 µg AFB₁) or 5.68 µg AFB₁-lysine. The enzymatic hydrolysis of the spiked rat serum was performed by the method described earlier (20, 21). The rat serum (4 mg protein) was incubated in 500 µL 0.1M sodium phosphate buffer, pH 7.4, with 2 mg protease (pronase-E) for 16 h at 37°C, with total protein to enzyme ratio at 2:1. After 16 h, 10 mg BSA was added to improve precipitation of protease and any remaining incompletely hydrolyzed albumin. Proteins were then precipitated by addition of 2 volumes of cold ethanol and placed at −20°C for 2 h. Precipitated proteins were removed by centrifugation and the aqueous fraction containing ethanol was left at 37°C overnight. The aqueous fraction was used for LC and ELISA analysis. The extent of albumin hydrolysis was checked by estimating the liberated α-amino nitrogen at different time points (0, 1, 2, 4, 8, 12, 16, and 24 h; 22). The protein content was estimated by the Lowry et al. method (23).

**Immunization**

Polyclonal antibodies against BSA-AFB₁ (1:20 mole to mole ratio) were produced in 2 rabbits (body mass, 2.5–3 kg). A primer dose of 92 µg equivalent of AFB₁/kg body mass was given by multiple-site epidermal injection. The antigen was prepared under aseptic conditions, using a laminar flow system. The filter sterilized (0.45 µm; Millipore Corp., Bedford, MA) antigen was redissolved in sterile saline and emulsified with Freund’s complete adjuvant in 1:1 ratio. For subsequent boosters, Freund’s incomplete adjuvant was used. The boosters were given by intramuscular route. The antigen dose for the first booster was 40 µg equivalent of AFB₁/kg body mass. Each booster was spaced by 10–12 days. On day 30 of the primer and on day 10 of each booster, blood was collected in heparinized capillary tubes from the retro-orbital plexus. At the end of the immunization schedule, the animals were sacrificed and blood was collected by cardiac puncture. Serum was separated, lyophilized, and stored at −20°C until further use.

Serum from each animal was analyzed for specific antibodies by Ouchterlony double diffusion technique (24), followed by antibody capture assay (13).

**Affinity Purification of Antisera**

Polyclonal antisera raised against BSA-AFB₁ was affinity-purified by using commercial Protein-G column. The column was equilibrated with 10 bed volumes of 0.02M phosphate buffer, pH 7.0. Later, 1 mL antisera in 1 mL

The flow rate of the column was maintained at 0.5 mL/min.
Titer Determination of Antisera by Antibody Capture Assay

Antisera titers were determined by checkerboard analysis using antibody capture assay. Ovalbumin-aflatoxin B1 adduct was used as a coating antigen to avoid interference by antibodies specific to the carrier protein BSA. A Microtiter plate was coated with 50 µL coating buffer (0.1M carbonate buffer, pH 9.6) per well containing different concentration of ovalbumin-AFB1 (10, 50, 100, 500 ng protein equivalent). The plate was dried overnight at 37°C in an ELISA incubator. The plate was washed 3 times with washing buffer (0.01M PBS, pH 7.2, containing 0.05% Tween-20). The wells were blocked for nonspecific binding with 50 µL blocking buffer (0.01M PBS containing 0.1% fish gelatin) per well for 30 min at 37°C. The plate was washed 3 times with washing buffer, and 50 µL/well of different dilutions of antisera (in 0.02M phosphate buffer, 1.7% saline, pH 7.2, containing 0.02% BSA) raised against BSA-AFB1 was added. The plate was incubated at 37°C for 2 h, washed 3 times, and incubated at 37°C for 1 h with 50 µL 1:5000 diluted alkaline phosphatase-labeled anti-rabbit IgG raised in goat. After incubation, the plate was washed 3 times, and 150 µL/well of substrate buffer [1.25 mg, p-nitrophenyl phosphate/mL of 10% diethanolamine–HCl buffer (pH 9.6) containing 0.05mM MgCl2] was added. The reaction was terminated by addition of 100 µL/well of stopping reagent. The absorbance was determined at 405 nm using an ELISA reader. The percent binding of antibodies versus concentration of the analyte (AFB1-lysine) was used to generate an inhibition plot, based on linear regression analysis. The flow chart for indirect competitive ELISA is given in Figure 1. The antibodies against BSA-AFB1 were also tested for its reactivity with AFB1 by indirect competitive ELISA, as described above.

Indirect Competitive ELISA for Quantitation of AFB1-Lysine Adduct

Microtiter plate wells were coated with 100 ng (10 ng AFB1) ovalbumin-AFB1 and the plate was incubated overnight at 37°C. The plate was washed with PBS-T (3 times). The wells were blocked with 0.1% fish gelatin in PBS (50 µL/well) and incubated at 37°C for 30 min. The plate was washed 3 times with washing buffer and 50 µL/well of different concentration of AFB1-lysine (100 pg–1000 ng or sample) plus affinity purified antibody (1:25 000) was added and incubated for 2 h at 37°C. The plate was washed (3 times) and incubated for 1 h with 50 µL/well of 1:5000 diluted enzyme-labeled antibody (alkaline phosphatase-labeled anti-rabbit IgG raised in goat). The plate was washed 3 times and 150 µL/well of substrate buffer [1.25 mg, p-nitrophenyl phosphate/mL of 10% diethanolamine–HCl buffer (pH 9.6) containing 0.05mM MgCl2] was added. The reaction was terminated by addition of 100 µL/well of stopping reagent. The absorbance was determined at 405 nm using an ELISA reader. The percent binding of antibodies versus concentration of the analyte (AFB1-lysine) was used to generate an inhibition plot, based on linear regression analysis. The flow chart for indirect competitive ELISA is given in Figure 1. The antibodies against BSA-AFB1 were also tested for its reactivity with AFB1 by indirect competitive ELISA, as described above.

Animal Studies

Male Fisher 344 rats were divided into 2 groups of 4 rats each. The animals were fed on 20% protein-based commercial diet (containing 65% starch, 5% fat, and 1% vitamin and 4% mineral mix). All the animals were maintained in 12 h light/12 h dark cycle with water ad libitum. Group I animals received normal diet, ad libitum along with 20 µg pure AFB1/kg body mass/day dissolved in peanut oil through oral gavage. Group II animals received peanut oil vehicle along with ad libitum diet.

The animals were sacrificed at the end of 6 weeks. Blood was collected by cardiac puncture. Serum was separated and stored at −20°C until further use. Protein was estimated by the method of Lowry et al. (23). Serum samples were subjected to enzymatic hydrolysis and used for analysis of AFB1-lysine adduct by indirect competitive ELISA and LC as described earlier.

Table 2. Relative retention time of LC separated compounds a

<table>
<thead>
<tr>
<th>Compound</th>
<th>254 nm</th>
<th>360 nm</th>
<th>Fluorescence detector (λEx = 360 nm; λEm = 440 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Diode array detector</td>
</tr>
<tr>
<td>AFB1</td>
<td>9.89</td>
<td>9.89</td>
<td>9.89</td>
</tr>
<tr>
<td>Lysine</td>
<td>2.00</td>
<td>ND b</td>
<td>ND</td>
</tr>
<tr>
<td>Nα-acetyl lysine</td>
<td>1.97</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>AFB1-lysine</td>
<td>2.10</td>
<td>2.10</td>
<td>2.10</td>
</tr>
<tr>
<td>Rat serum + AFB1-lysine (hydrolyzed)</td>
<td>Numerous peaks</td>
<td>2.10</td>
<td>2.10</td>
</tr>
<tr>
<td>Rat serum + BSA-AFB1 (hydrolyzed)</td>
<td>Numerous peaks</td>
<td>2.10 (Major peak)</td>
<td>2.10 (Major peak)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.70 (Minor peak)</td>
</tr>
</tbody>
</table>

a LC solvent system = water–acetonitrile–methanol (70 + 15 + 15).
b ND = not detected.
Results and Discussion

The carcinogenicity of AFB_1 is due to the formation of the AFB_1-8,9-epoxide by hepatic cytochrome P_450-dependent mixed function oxidases. This highly reactive AFB_1-8,9-epoxide binds to DNA through N_7 position of guanine residues in DNA (14) and ε-amino group lysine of albumin (25). Measurements of protein adduct are useful because they give information about the long-term exposure of AFB_1 (9). Albumin is a major serum protein which covalently binds to AFB_1 (15). In experimental animals, 1–3% of single dose of AFB_1 is bound to albumin which reflects the degree of DNA damage in the liver, the target organ (7, 15, 26). Groopman et al. (27) showed good correlation between DNA-AFB_1 adducts and BSA-AFB_1 adduct in the serum. Serum albumin adduct has been isolated and structurally identified (15). Several analytical methods such as radioimmunoassay (28), ELISA (21), and LC (20, 29) have been developed to measure this adduct.

Sabbioni et al. described a method to synthesize the AFB_1-lysine adduct by using AFB_1-dibromide (15). Recently, we synthesized a reference standard AFB_1-guanine, wherein AFB_1-8,9-epoxide was generated by using a chemical oxidant MCPBA. Later, free guanine was conjugated with the AFB_1-epoxide generated (13). This single step procedure was further adopted for synthesis of the AFB_1-lysine adduct, a procedure which is simple and less time consuming than the earlier method (15).

Characterization by TLC

TLC analysis of the buffer fraction of the reaction mixture showed a single fluorescent spot at the base with zero R_f value, indicating the presence of AFB_1-lysine. Free, unreacted AFB_1 was not detected. The recoveries of AFB_1-lysine adduct synthesized, using different molar ratios of N-α-acetyl lysine and AFB_1, were quantitated by fluorodensitometric method (18). Table 1 shows recoveries of the adduct at different molar ratios. At 1:16 molar ratio of AFB_1 and N-α-acetyl lysine the recovery of the adduct was 62% (Table 1).

Similarly, TLC analysis of BSA-AFB_1 and ovalbumin-AFB_1 showed the presence of a single fluorescent spot at the origin, suggesting formation of the adduct. The dichloromethane fraction showed unreacted AFB_1 (R_f = 0.84) with other fluorescent spots possibly corresponding to aflatoxin-diols and hydroxy esters. Figure 2 shows the characterization of buffer fraction and DCM fraction by TLC. The mole to mole ratio of ovalbumin-AFB_1 and BSA-AFB_1 estimated by TNBS assay was 1:15 and 1:20, respectively. In an earlier study, conjugates with molar ratio in the range of 1:9 to 1:25 showed a better binding capacity to polystyrene microtiter plates (30). Estimation of protein in the buffer fraction of BSA-AFB_1 and ovalbumin-AFB_1 showed a recovery of 90 and 60%, respectively. Lower recovery of ovalbumin-AFB_1 conjugate can be attributed to the lower solubility of the protein in buffer than BSA.

Figure 5. Alpha-amino nitrogen liberated during enzymatic hydrolysis of BSA.
Spectral Analysis

The UV absorption spectrum (Figure 3) of AFB₁-lysine adduct showed 2 peaks (275 and 335 nm), and was similar to the spectrum of the adduct synthesized by Sabbioni et al. (15).

LC Analysis

Lysine and N-α-acetyl lysine showed peaks with a relative retention time of 2.0 and 1.97 min at 254 nm with diode array detector; no peaks were observed at 360 nm. The separation profile of AFB₁-lysine adduct showed a relative retention time of 2.1 min with diode array detector, both at 254 and 360 nm. The relative retention time of AFB₁-lysine with fluorescence detector was 2.1 min, whereas that of TFA-treated AFB₁ (detected as AFB₂a) was 9.89 min (Figure 4A). TFA treatment did not alter the retention time (2.1 min) of the AFB₁-lysine adduct. When control rat serum was spiked with BSA-AFB₁ and enzymatically hydrolyzed, 2 peaks were observed at 360 nm (2.1 and 2.7 min) and many more peaks were obtained at 254 nm in addition to these 2 peaks (2.1 and 2.7 min). Similarly, with fluorescence detector 2 peaks with relative retention times of 2.1 and 2.7 min were observed (Figure 4B). This indicated the presence of lysine covalently linked to aflatoxin B₁, as the fluorescence can be attributed only to the presence of AFB₁. Table 2 shows the relative retention time of LC separated compounds. A similar LC profile was observed when control rat serum was spiked with chemically synthesized AFB₁-lysine adduct (Figure 4C).

Based on the LC analysis, the recovery of BSA-AFB₁ in terms of AFB₁-lysine adducts was 67 ± 5% (as accounted by the peaks recorded at 2.1 and 2.7 min; Table 2). The LC separation profile of AFB₁-lysine adducts indicates that the major peak corresponding to 2.1 min accounted for 72% of the AFB₁-lysine adduct, while the second minor peak (2.7 min) accounted for 28% of the total lysine adducts formed after enzymatic hydrolysis. These studies clearly establish that the enzymatic hydrolysis of BSA-AFB₁ adduct results in 2 pools of AFB₁-lysine adducts, as evidenced by the LC separation profile (Figure 4B). The stability studies on the AFB₁-lysine adduct synthesized also indicated that the adduct was stable for 1 month, under lyophilized condition.

Albumin Hydrolysis

Earlier methods reported substantial losses of the adduct during chromatographic purification of albumin (28, 29, 31).

Figure 6. Standard displacement plot for AFB₁-lysine as determined by indirect competitive ELISA \[y = 66.78 + (-10.88)x; r = -0.957\]. Data points are mean ± standard deviation.
These reported methods involved numerous experimental steps, resulting in significant losses of adduct in the range of 35–95%. As shown earlier (20, 26), enzymatic hydrolysis of serum protein enhances the detectibility of AFB1-protein adduct by radioimmunoassay (RIA), presumably as a result of the release of AFB1-adducts from proteins containing them. Earlier studies have indicated that human serum could be processed for AFB1-lysine analysis by direct enzymatic digestion without requiring prior albumin purification (20). Isolation of albumin using Reactive Blue-2 Sepharose showed that only the albumin fraction contained a detectable amount of aflatoxin-protein adduct.

In the present investigation, systematic studies were undertaken with in vitro-synthesized AFB1-lysine adducts, which were spiked to control rat serum, followed by enzymatic hydrolysis. To assess the completion of protein hydrolysis, pure BSA was also subjected to enzymatic digestion. Estimation of α-amino nitrogen at different times during albumin hydrolysis showed that hydrolysis was maximal at 12 h. Figure 5 shows the liberated α-amino nitrogen used as an index of albumin hydrolysis at different times. The spiking studies, revealed that the recovery of the AFB1-lysine adduct, as analyzed by ELISA, was 93 ± 6%. The results of these experimental studies suggest that prior purification of albumin and immunoaffinity purification of AFB1-lysine adduct after enzymatic hydrolysis are not required.

**Characterization of Polyclonal Antibodies Raised Against BSA-AFB1**

Both animals responded well to the injected immunogen (BSA-AFB1). The antisera gave a titer of 1:4 in rabbit No. 1 and a titer of 1:6 in rabbit No. 2, after second booster, as determined by Ouchterlony double diffusion technique. Titer determination by checkerboard analysis (based on antibody capture assay) at different concentrations of ovalbumin-AFB1 (10, 50, 100, 500 ng) used as coating antigen showed that a dilution of 1:50 000 of antiserum (rabbit No. 2) at a concentration of 100 ng ovalbumin-AFB1/well gave an absorbance of 0.9–1.0 at 405 nm, when alkaline phosphatase was used as an enzyme label with p-nitrophenyl phosphate as an enzyme substrate. The ovalbumin-AFB1 conjugate was used for the immunoassay to reduce the nonspecific binding of antibodies raised against BSA-AFB1 immunogen, thus enhancing the assay sensitivity.

**Indirect Competitive ELISA**

The standard displacement curve for AFB1-lysine adduct is depicted in Figure 6. Assay sensitivity ranged from 100 pg to 1000 ng. At an inhibitory concentration (IC_{50}) of 13 ng AFB1-lysine, 50% displacement of antibodies was achieved. The IC_{50} value of pure AFB1 was 7 ng as compared to 13 ng for AFB1-lysine adduct.

Because of this nearly 2-fold difference in the IC_{50} value with respect to AFB1 (7 ng) and AFB1-lysine (13 ng), it is appropriate to use AFB1-lysine adduct as a reference standard for immunoassays (ELISA) for quantitating aflatoxin B1-protein adducts (as AFB1-lysine) rather than pure AFB1. If this experimental observation is overlooked, then quantitated values of the AFB1-lysine adduct in the biological samples would be grossly underestimated.

**Method Validation**

Serum samples collected from rats dosed with 20 µg AFB1/kg body mass/day considerably displaced antibodies in the indirect competitive ELISA. The amount of AFB1-lysine adduct in serum was 27.3 ± 4.37 µg/mg albumin in Group I experimental animals; no adduct was detected in the control group (Group II) animals. LC analysis of the enzymatically hydrolyzed experimental Group I rat serum also confirmed the presence of AFB1-lysine adduct, with a retention time of 2.1 min along with a minor peak at 2.7 min. The recovery studies performed with AFB1-lysine adduct spiked to control rat serum was 93 ± 6% by the enzyme immunoassay method.

The method of chemical synthesis of AFB1-lysine adduct reported in the present study is a simple, single-step procedure and consumes less time than the earlier method. This method of synthesis can be used to prepare reference standards and can be successfully used in molecular epidemiological studies in relation to dietary aflatoxin exposure for quantitation of protein-bound aflatoxin. The experimental evidence provided in the present investigation suggests that the use of AFB1-lysine adduct as a reference standard over the pure AFB1 may avoid underestimation of aflatoxin-protein adducts in biological samples. The present enzyme immunoassay procedure may find application as a biochemical tool for the analysis of serum aflatoxin-albumin adducts in humans exposed to dietary aflatoxins, and in assessing the potency of natural and synthetic anticarcinogenic compounds in metabolic intervention studies with respect to reversing the toxic effects of dietary aflatoxins.

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**References**
