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ABSTRACT

Aflatoxin B_1 (AFB_1) was detected in serum samples of healthy Japanese males by radioimmunoassay and high-performance liquid chromatography. Blood samples were obtained from 20 subjects after fasting and from 80 subjects after lunch. The subjects ranged in age from 20 to 63 years of age. Measurement of AFB_1 in the samples was performed by radioimmunoassay and was confirmed by high-performance liquid chromatography using a μPorasil column and a C_{18}-μBondapak column after the conversion of AFB_1 to its water adduct AFB_2a.

AFB_1 was detected in 5 of 20 fasting blood samples [20 to 56 pg/ml of serum; 33.6 ± 14.6 (S.D.)] and in 29 of 80 serum samples taken after lunch (20 to 1169 pg/ml of serum; 218.1 ± 268.3). Mass spectral analysis of the material obtained after high-performance liquid chromatography separation from serum samples confirmed the presence of AFB_1.

INTRODUCTION

A high incidence of hepatoma is seen throughout Africa and Southeast Asia. Many epidemiological studies in these areas have suggested that the exposure to environmental hepatocarcinogens is, in part, related to cultural factor and that aflatoxin, a potent hepatocarcinogen elaborated by *Aspergillus flavus*, may play a role in primary liver cancer (8–10, 12, 13). The hepatocarcinogenicity of AFB_1[2] in various experimental animals indicates a causal relationship between exposure to AFB_1 and hepatocellular carcinoma in humans (10, 12). Recently, AFB_1 has been demonstrated in human tissues by HPLC and RIA (6, 9, 11, 14, 15).

We used RIA and HPLC for the analysis of AFB_1 in serum samples. This is the first report on the detection of AFB_1 in Japanese serum samples.

MATERIALS AND METHODS

Equipment. A Waters Model 440 liquid chromatograph was used with a Shimadzu RF 530 fluorescence monitor and a Waters Data Module. Two 300- x 4-mm (inside diameter) stainless steel HPLC columns were used, a μPorasil and a C_{18}-μBondapak (Waters Associates, Milford, MA). A 300- × 10-mm (inside diameter) glass column was used for acidic alumina column chromatography.

Reagents. Acidic alumina (Woelm Pharmaceuticals, West Germany) was prepared by adding 3% water (w/w), shaking for several min, and allowing the mixture to stand overnight to equilibrate. Crystalline aflatoxins B_1, B_2, G_1, and G_2 were purchased from Makor Chemicals, Ltd., Jerusalem, Israel. The elution solvents for HPLC were water-saturated chloroform:cyclohexane:acetonitrile-2-propanol (75:25.5:3:1.5) for μPorasil column chromatography and distilled water:methanol:acetonitrile (67:20:13) for C_{18}-μBondapak column chromatography (4).

All chemicals were of special reagent grade available from commercial sources.

Sample Preparation and Extraction. Serum samples (10 ml each) were obtained after fasting from 20 healthy men in Kobe. Serum samples (10 ml each) were obtained 2 to 3 hr after lunch from 2 groups: Group I, 30 healthy men in Kobe; Group II, 50 healthy men in Osaka, 40 km east of Kobe.

Before analysis, 2400 dpm of [^{3}H]AFB_1 (20 Ci/mmol; Moravek Biochemicals, Inc., City of Industry, CA) were added to each sample to obtain the recovery data.

Distilled water and methanol (10 ml, 9:1) were added to each serum sample. The samples were treated with 10 ml of hexane to remove lipids and were then extracted twice with 10 ml of chloroform. After acidic alumina chromatography (4), the solution was evaporated to dryness under nitrogen and dissolved in the mobile phase.

HPLC (4, 11). The sample components were separated on a μPorasil column at an elution rate of 1.0 ml/min at a normal pressure of 400 psi. The fractions with the same retention time as that of authentic AFB_1 were collected and evaporated to dryness under nitrogen. One-half of the sample solution was used for RIA. The radioactivity in one-tenth of each sample solution was counted to obtain the percentage recovery.

Trifluoroacetic acid (100 μl) and distilled water (100 μl) were added to each vial of standard or the sample in which AFB_1 was detected by RIA. The vials were heated in a steam bath at 50° for 30 min. Then the solution was evaporated and dissolved in the mobile phase. A 20-μl aliquot of the sample or standard was injected into the C_{18}-μBondapak column, and the AFB_2a peak was identified by comparing the retention time with that of the standard at an elution rate of 1.0 ml/min at a normal pressure of 1200 psi (4). The peak area and the retention time were determined by a Waters Data Module.

A Shimadzu RF 530 fluorescence monitor, with a 365 nm band pass filter in the excitation beam and a 425 nm long pass filter in the emission beam, was used for the detection of both AFB_1 and AFB_2a.

RIA (2, 5). One-half of the solution was used for RIA.

A 0.2-ml mixture containing an appropriately diluted antibody with 32,000 dpm [^{3}H]AFB_1 in Tris buffer (50 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, 0.3 M KCl, 0.25 M sucrose, and 10% horse serum, pH 7.5) was added to the dried samples and to standards containing 0, 25, 50, 100, 250, 500, 750, and 1000 pg of AFB_1 in duplicate. The contents were agitated on a Vortex mixer for 10 sec and then incubated at 4° overnight. After incubation, 0.2 ml of dextran-coated charcoal solution (1% activated charcoal and 0.1% dextran sulfate sodium salt in the buffer without sucrose) was added to all tubes except the total radioactivity controls. The tubes were agitated vigorously, allowed to stand for 10 min, and then centrifuged at 3000 rpm for 10 min. Next, 0.2 ml of the clear supernatant was pipeted into a counting vial containing 10 ml of scintillation fluid (10 g PPO and 40 ml of methanol in 2 liters of toluene) and then counted by a liquid scintillation counter.

Mass Spectral Analysis. The amount of each sample obtained after HPLC separation was not enough to be subjected to mass spectral analysis. Therefore, these samples and the other blood samples collected at Kobe University School of Medicine were separated by a μPorasil

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[2] The abbreviations used are: RIA, radioimmunoassay; HPLC, high-performance liquid chromatography; AFB_1, B_2, and G_1, aflatoxin B_1, B_2, and G_1; AFB_2a, water adduct of aflatoxin B_1.

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column. The total volume of serum samples was more than 3200 ml. Peaks containing AFB_1 were collected, pooled, evaporated to dryness, and separated again by HPLC. The samples were subjected to mass spectral analysis using a JEOL JMS-D100 mass spectrometer system and a JMA-2000 mass spectrum analyzer (Nihon Denshi, Japan). The analysis was performed with direct-sample inlet system at 300 μa and 36 eV.

RESULTS

Separation Using the μPorasil Column. The resolution of aflatoxins in the reference standards and the typical resolutions of samples are shown in Chart 1.

Each separated sample was collected for 1.5 min at the same retention time as the authentic AFB_1.

Separation Using the C_{18}-μBondapak Column. The aflatoxin standards and the collected samples which were used for the RIA detection of AFB_1 were evaporated, diluted with the eluting solvent, and injected into the C_{18}-μBondapak column after the conversion of AFB_1 (and AFG_1) to its water adduct AFB_2a (and AFG_2a). The resolution of aflatoxins in the reference standard and the typical resolutions of samples are shown in Chart 2.

The limit of detection was found to be 80 pg of AFB_2a at Range 4 of the monitor. The signal:noise ratio which was obtained at that sensitivity was 5.33.

RIA. In the anti-AFB_1 system, only 150 to 250 pg of AFB_1 were required for 50% inhibition, and as little as 20 pg could be detected. The results of the analyzed aflatoxin values of the samples are shown in Chart 3 and Table 1.

A significant positive correlation was found between the values obtained by HPLC and those obtained by RIA ($r = 0.73$; $p < 0.01$). The values obtained by the Waters Data Module were higher than those obtained by RIA ($Y = 1.26X + 14.16$).

Recovery Data. The percentage recovery of AFB_1 was calculated from the ratio of the added [^{3}H]AFB_1 radioactivity obtained before and after extraction.

The percentage of recovery of AFB_1 ranged between 47 and 72%.

Mass Spectral Analysis. Mass spectral analysis of the material obtained after HPLC separation confirmed the presence of AFB_1. A prominent peak at m/e 312 appeared at a temperature of 180° (Chart 4).

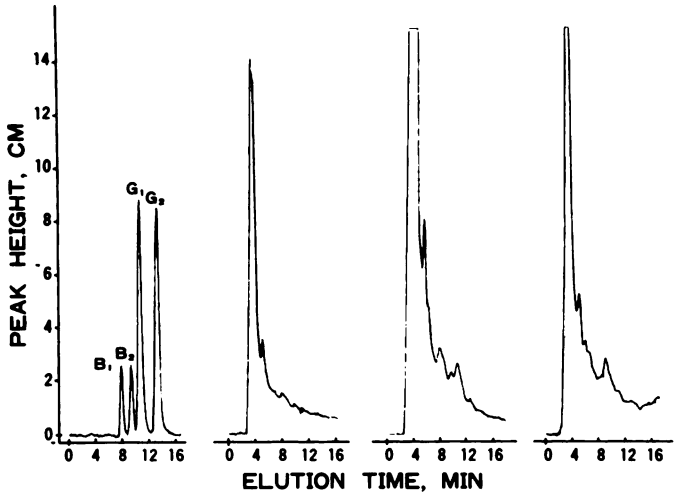

Chart 1. Tracings of μPorasil column chromatograms of aflatoxin standards and serum extracts. Three typical resolutions of samples are shown. Applied aflatoxin standard concentrations were 20 ng of AFB_1 and AFB_2 and 1 ng of AFG_1 and AFG_2.

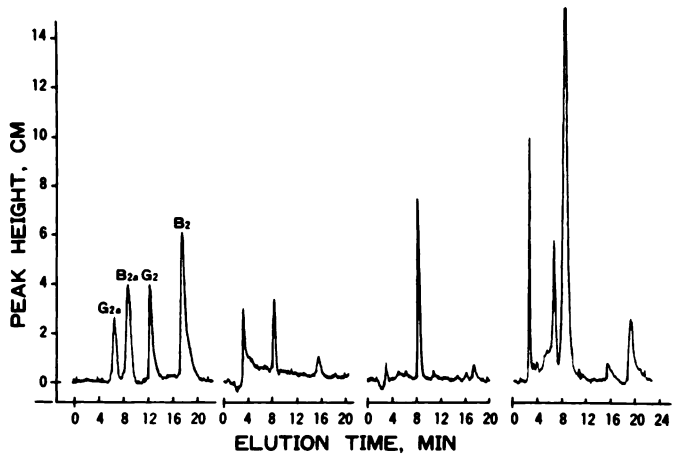

Chart 2. Tracings of C_{18}-μBondapak column chromatograms of aflatoxin standards and serum extracts. Three typical resolutions of samples collected by μPorasil column chromatography are shown. The AFB_1 values of these 3 samples obtained by a Waters Data Module were 93, 271, and 1375 pg/ml serum (from *left*). Those obtained by RIA were 105, 195, and 1169 pg/ml serum. The amount of each aflatoxin standard applied was 500 pg.

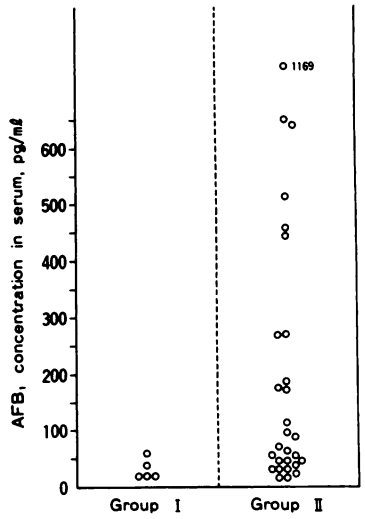

Chart 3. AFB_1 in Japanese serum samples. Group I consisted of 20 healthy men in Kobe. Fasting blood samples were collected. Group II samples were collected from 2 groups; 30 healthy men in Kobe, and 50 healthy men in Osaka. The serum samples were collected 2 to 3 hr after lunch.

DISCUSSION

Recent HPLC and RIA studies have demonstrated the presence of AFB_1 in human serum (6, 14, 16). Nelson *et al.* (6) detected AFB_1 levels of 2.52 to 4.68 ng/ml in serum samples from hospital control patients and patients with Reye's syndrome. They found that the lowest detectable level of AFB_1 was about 20 pg by RIA and approximately 200 pg by HPLC. Siraj *et al.* (14) examining liver, urine, and blood samples from normal subjects and patients with Reye's syndrome reported AFB_1 levels of 2 to 12 ng/ml serum by HPLC. The levels of AFB_1 reported in these studies were higher than those of the present

Table 1

AFB_1 in Japanese serum samples

	No. of samples	No. AFB_1-positive	Age (yr)	AFB_1 (pg/ml)
Group I	20 (Kobe)	5 (40)[a]	20–54 (28 ± 7.8)[b]	20–56 (33.6 ± 14.6)[b]
Group II	30 (Kobe)	12 (40)	20–27 (23.6 ± 2.4)	20–640 (122.7 ± 167.8)
	50 (Osaka)	17 (34)	23–62 (38.8 ± 11.1)	20–1169 (285.5 ± 303.3)
Group II total	80	29 (36)	20–62 (34.2 ± 11.8)	20–1169 (218.1 ± 268.3)

[a] Numbers in parentheses, percentage.
[b] Numbers in parentheses, mean ± S.D.

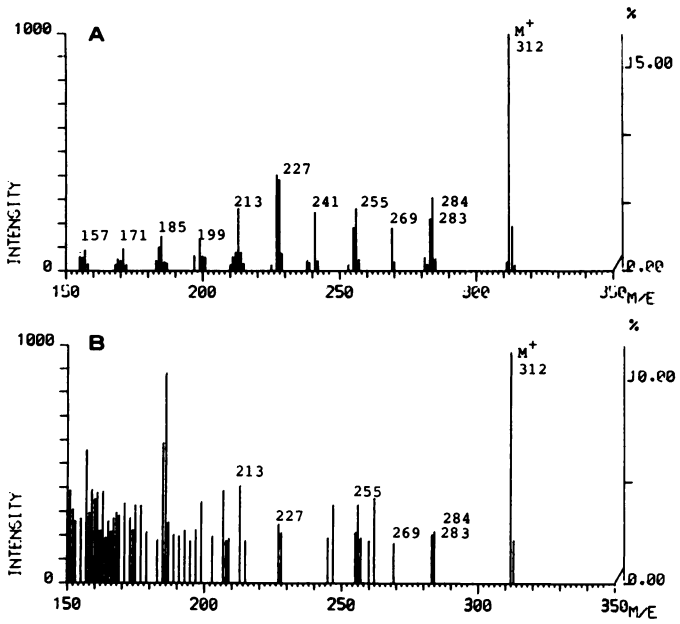

Chart 4. Mass spectrum of AFB_1 in serum samples. The extracted solutions from serum samples (total volume, >3200 ml) were separated by HPLC. Peaks containing AFB_1 were collected, pooled, evaporated to dryness, and separated 5 times by HPLC changing the concentration of isopropyl alcohol in elution buffer. The obtained material was subjected to mass spectral analysis. The concentration of AFB_1 standard (*A*) subjected to the analysis was 1 μg. AFB_1 in the sample (*B*), measured by RIA, was more than 50 ng.

studies. This discrepancy may be due to the fact that we used RIA and the 2 HPLC techniques for analyzing AFB_1 in serum samples. The separation using the μPorasil column was necessary for the determination of AFB_1 because it permitted the exclusion of other components which might have interfered with the RIA. The subsequent use of a C_{18}-μBondapak column after the conversion of AFB_1 to the water adduct AFB_2a, therefore, provided a very sensitive method for determining AFB_1. The loss of standard and samples was large; however, the Waters Data Module with the Shimadzu RF 530 fluorescence monitor could detect AFB_2a in amounts less than 80 pg; this limit of detection was found at Range 4 of the monitor.

Although the antibody was specific for AFB_1, AFB_2 and AFG_1 also showed some cross-reactivity at a concentration 10 times greater than that of AFB_1. In several samples analyzed by HPLC, these aflatoxins were not detected. The fractions before and after the AFB_1 fractions were checked to ensure specificity of the RIA; no cross-reactivity was seen.

Although mass spectral analysis confirmed the presence of AFB_1, each sample obtained by HPLC separation was too small to be subjected to mass spectral analysis. It required careful consideration to evaluate the presence of AFB_1. A significant positive correlation was found between the values obtained by RIA and those obtained by μBondapak column chromatography; thus, the best method for the detection of AFB_1 in serum samples may be first to separate the sample by μPorasil column chromatography and then to measure AFB_1 by RIA or by μBondapak column chromatography after the conversion of AFB_1 to AFB_2a.

The AFB_1 levels obtained in this study were very low except for 4 samples which contained more than 500 pg/ml. The source of ingested AFB_1 is unknown. The volunteers with high levels of AFB_1 consumed fried foods with soy sauce, coffee, and milk prior to the sampling. We had previously reported the presence of AFB_1 in certain types of foods (7, 15). We had detected AFB_1 in soy sauce, coffee, beer, and some kinds of processed foods. The amount of AFB_1 found in these materials was less than 10 ppb, the maximum permissible amount in Japan. The focus of this study was to evaluate the prevalence of aflatoxins in 2 large cities on the basis of the data obtained from serum samples. At present, it is not clear what levels of AFB_1 in serum are carcinogenic. Gross and Newberne (3) have shown that foods considered "aflatoxin free," in the range of 10 ppb, may contain toxic levels that are within the carcinogenic range when subjected to an appropriate induction period.

The study of environmental carcinogens is becoming of great importance as the number and concentration of contaminants in the environment increase (1).

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