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Development of a Radioimmunoassay Procedure for Aflatoxin B_1 Measurement

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A radioimmunoassaay (RIA) procedure to measure aflatoxin B_1 (AfB_1) in agricultural commodities was developed. AfB_1 oxime derivative was synthesized, characterized, and used for preparation of ^{125}I-labeled AfB_1. Antiaflatoxin B_1 serum was raised in-house using AfB_1−bovine serum albumin conjugate as immunogen. The assay system was optimized in the range of 0.2−5 ng/mL, using a liquid phase (PEG) as well as a solid phase (coated polystyrene beads) separation system. Inter-assay and intra-assay variations, recovery, and parallelism studies validated the assay. AfB_1 analysis was carried out in nearly 130 samples of different agricultural commodities. The correlation coefficient was determined using commercial ELISA and in-house-developed RIA methods.

KEYWORDS: Immunoassays; RIA; aflatoxin

INTRODUCTION

Aflatoxins are a group of toxic secondary metabolites produced by *Aspergillus* fungi, which contaminate agricultural products. Aflatoxin B_1 (AfB_1) is one of the most potent carcinogens classified as Class I by the IARC and hence a potential hazard to human and animal health. Stringent regulations are being imposed on the presence of AfB_1 in agricultural commodities. Being specific, sensitive, and user-friendly in nature, immunoassays have become popular for AfB_1 analysis (*1*−*3*). There are reports regarding the variability associated with different enzyme immunoassays for AfB_1 analysis (*4*). The matrix effect and other interferences are generally observed in enzyme-based assays. On the other hand, radiometric measurements are seldom affected by sample matrix. Hence, as the first step a radioimmunoassay (RIA) for AfB_1 was developed to serve as a basic method to develop an enzyme-based cutoff assay at a later stage. The RIA could be carried out at a central laboratory to which the samples are sent from many users to obtain accurate values, whereas the enzyme assay could be used in the users' laboratories for screening. We describe here the development of an RIA procedure for $AfB_1$and its application for sample analysis. The RIA was standardized both for liquid phase separation using polyethylene glycol (PEG) and for solid phase separation using beads coated with antibodies.

MATERIALS AND METHODS

Aflatoxins B_1, B_2, G_1, and G_2, histamine, Chloramine-T, bovine serum albumin (BSA), carboxymethyl hydroxylamine, isobutylchloroformate, and aflatoxin B_1−BSA conjugate were purchased from Sigma Chemical Co., St. Louis, MO. Freund's adjuvant was from Difco Laboratories. PEG MW 6000 was from Loba Chemicals India. Other reagents used were of analytical grade.

Carrier-free ^{125}I as sodium iodide (specific activity = 15−17 mCi/g, radioactive concentration = 100−110 mCi/ml) was from Dupont NEN Products, Boston, MA.

An NaI (Tl) well-type scintillation counter from Electronic Corporation of India Ltd. was used for all radioactivity measurements. UV−vis spectrophotometer V-530 was from M/S. Jasco. ^{1}H NMR spectra was recorded on a 300 MHz Varian VXR 300 S spectrometer. $CDCl_3$ was used as solvent and tetramethylsilane as internal reference.

Preparation of Radioiodinated AfB_1. The AfB_1 oxime derivative was synthesized according to a procedure described by Chu et al. (*5*). ^{125}I-Labeled AfB_1 was prepared by conjugating AfB_1 oxime with [^{125}I]-histamine by using a mixed anhydride method as reported elsewhere (*6*). In brief, histamine (220 ng in 10 μL of 0.5M phosphate buffer) was labeled with 2 mCi (74 MBq) of Na^{125}I using Chloramine-T (50 μg in 10 μL) as the oxidant at pH 8 for 60 s. The reaction was stopped by the addition of sodium metabisulfite (300 μg in 10 μL). AfB_1 oxime (2 mg in 50 μL of dioxin) was activated with isobutylchloroformate in the presence of tributylamine at 10 °C for 30 min; 35 μg of this activated AfB_1 was conjugated with [^{125}I]histamine.

Histamine labeling yield was determined by paper electrophoresis. The extent of incorporation of radioiodine in AfB_1 was calculated from the distribution of the activity in the different phases obtained during purification. The radiochemical purity was estimated by paper electrophoresis and TLC.

Preparation and Characterization of Anti-aflatoxin Serum. AfB_1−BSA (8−10 mol of aflatoxin per mole of BSA) was used as the immunogen. AfB_1−BSA (3 mg/mL solution in saline) was emulsified with 2 volumes of Freund's complete adjuvant for immunization. Primary injection of 0.5 mg/0.5 mL/rabbit by intradermal route was followed by subsequent boosters at 250 μg/0.5 mL/rabbit concentration, also intradermally at monthly intervals for 6 months. The sixth booster was given after a rest period of 3 months, whereas the seventh and

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eighth boosters were given after a rest period of 4 months. Serum samples from each rabbit after 10 days of each booster were tested for their suitability for RIA. The best of the crops was chosen for further optimization of the assay procedure.

The affinity constant was determined by setting up standard curves in the range of 0.2−10 ng/mL for incubation at 25 °C. The specificity of the AfB_1 antiserum was determined in terms of cross-reactivity with related toxins (*7*).

Assay Optimization and Evaluation. The optimal concentration of the reagents and assay reaction parameters such as time, temperature, and pH of the reaction were chosen so that standard curves with desirable slope and minimum errors at different measured doses resulted. The assay was optimized with both liquid phase and solid phase separation systems. In liquid phase separation, γ-globulins were precipitated using PEG wherein 1 mL of 24% PEG was added to a reaction mixture of 0.5 mL, preceded by the addition of 0.1 mL of the carrier, 2% bovine γ-globulin. To achieve solid phase separation, polystyrene beads coated with 1:20000-fold diluted antibody were used. The standards were prepared in bulk over the range of 0.2−10 ng/mL, lyophilized and stored at 4 °C. Assay performance was evaluated for imprecision in terms of %CV in dose both for "within assay" and "between assays". Studies were also carried out to estimate the recovery of added standard and exhibition of parallelism on sample dilution. Contaminated agricultural commodities, which showed high levels of aflatoxins, were used for carrying out parallelism studies. Two such samples from three varieties of grains were used for these parallelism studies.

Sample Analysis. Samples from different agricultural commodities were analyzed using the RIA system. Indian tropical climatic conditions are conducive for fungal contamination. Hence, sample analysis was carried out in agricultural commodities that were stored under proper conditions (normal samples) as well as those infected with fungus (both by inoculation of *Aspergillus flavus* and by addition of known amounts of AfB_1)**.** Five kinds of agricultural commodities, namely, groundnuts, maize, soybean, wheat and rice, were used for analyses. In all, 35 samples each of normal, inoculated, and spiked samples of the above grains were analyzed. Additionally, 25 samples of a chewable tobacco product (containing betel nut, tobacco, lime, and cathechu—locally called "Gutka") sold in the market and used widely by certain strata of society were also analyzed.

The samples were extracted with chloroform. The chloroform extracts were evaporated and dissolved in 10% methanol in buffer and assayed at different dilutions. The values obtained were compared with the instrumental analysis of aflatoxin using an aflatoxicometer, which detects aflatoxin adsorbed on a Florisil minicolumn by fluorometric measurement as reported by Velasco (*8*).

Performances of liquid phase (PEG) and solid phase separation systems were evaluated by sample analysis. Assays were set up under identical conditions of reaction and reagents such as standards, tracer, and buffer system, varying only the separation system. Values of the same samples analyzed by both the PEG and solid phase separation methods were correlated. The developed RIA system was also compared with a commercially available ELISA kit by means of correlation of the sample values obtained on analyses by both methods. An ELISA kit from Neogen Corp., "Veratox aflatoxin quantitative test kit", was used for these studies.

RESULTS AND DISCUSSION

Preparation of ^{125}I-Labeled Histamine—AfB_1Oxime. The yield of radioiodination to obtain [^{125}I]histamine was found to be ∼60%. However, ∼20% of the radioiodine was observed to be present in the organic phase containing the purified [^{125}I]histamine−AfB_1 oxime conjugate. The radiochemical purity of the tracer was 98% as determined by TLC. The specific activity of the tracer was ∼20 MBq/nM (1.8 μCi/ng). The tracer was aliquoted as 100 kBq/vial and lyophilized. The tracer was found to be stable for at least 75 days when stored at 4 °C.

Anti-AfB_1 Serum Production. Eighth booster antiserum was used for standardization of the assay system at 1:50000 dilution.

Table 1. Cross-Reactivity with Related Toxins

aflatoxin	% cross-reactivity	aflatoxin	% cross-reactivity
B_1	100	G_1	11
B_2	18.5	G_2	not detectable

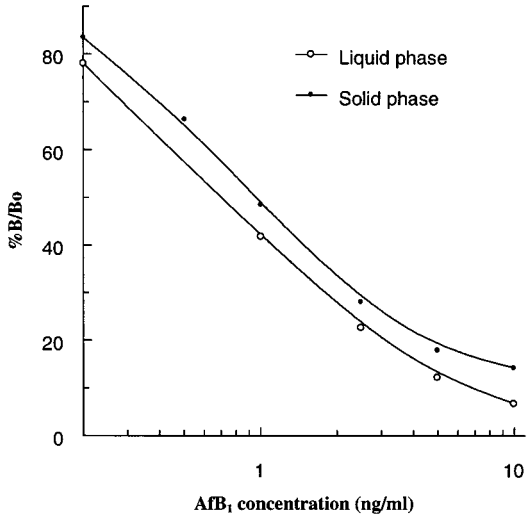

Figure 1. Comparison of solid phase and liquid phase AfB_1 RIA.

The affinity constant determined was 2.33×10^9 L/M at 25 °C. The cross-reactivity of the antiserum with other aflatoxins is shown in **Table 1**. It is seen that there is some cross-reaction with aflatoxins B_2 and G_1. Generally, these B_2 and G_1 aflatoxins are seldom present in amounts comparable to that of B_1 in products with stringent regulations. Because the presence of AfB_1 is of greater concern and because such levels of cross-reaction would not introduce intolerable errors in the estimate, this antiserum was deemed to be suitable for the assay.

Optimization of the Assay System. The optimized assay was carried out using 100 μL of tracer containing ∼10−12 pg of AfB_1 and 1 kBq (1000 disintegrations per second, equivalent to 0.027 μCi) of ^{125}I-labeled AfB_1 tracer and either 100 μL of 1:50000 diluted antibody for the liquid phase system or a coated bead for the solid phase system. Phosphate buffer (0.05 M) containing 0.2% BSA and 0.05% sodium azide was used as assay buffer. One hundred microliters of standard or sample was used per tube. AfB_1 calibrators in the range of 0.2−10 ng/mL were used for setting up standard curves**.** The assay was carried out for 3 h at room temperature. **Figure 1** shows the comparison of the standard curves for AfB_1 using the two different separation systems, the coated beads solid phase and the PEG liquid phase. The two standard curves are parallel, and a small decrease in the sensitivity is observed in the solid phase separation system when compared with the PEG separation system. However, both systems would be suitable for screening commodities for AfB_1 levels.

Validation of the Assay. The sensitivity of the assay defined as the smallest amount that is significantly different from the zero standard at a 95% confidence limit was found to be 45 pg/mL. This sensitivity was adequate for routine assays. The results of the recovery studies are given in **Table 2**. The recovery values obtained ranged between 92 and 107%, which is within the acceptable limits of ±10%. The results indicate that there is no interference from sample matrix on the assay performance.

Table 3 lists the aflatoxin values obtained in the parallelism studies using serially diluted samples of agricultural commodi-

Table 2. Recovery Studies with the Aflatoxin B_1 Radioimmunoassay[a]

sample value (ng)	standard added (ng)	observed value (ng)	expected value (ng)	% recovery
3.0	0.5	3.5	3.5	100
0.32	0.5	0.8	0.82	97.5
1.28	2.5	3.5	3.8	92
1.35	5.0	4.3	6.35	99.2
1.35	2.5	3.9	3.85	101
1.9	0.2	2.25	2.1	107

[a] Assays based on liquid phase separation were used for these studies.

Table 3. Parallelism Studies in Samples[a]

type of sample	sample dilution	estimated AfB_1 content (ng/g)
rice sample 1	1:100	92
	1:500	86
rice sample 2	1:100	4.8
	1:500	4.6
wheat sample 1	1:100	6
	1:500	6.4
wheat sample 2	1:100	14.8
	1:500	15.2
soybean sample 1	1:10	220
	1:500	220
soybean sample 2	1:10	40
	1:500	53

[a] Assays based on liquid phase separation were used for these studies.

Table 4. Sample Analysis of Normal Agricultural Commodities (Values in Nanograms per Gram)[a]

rice	wheat	maize	ground nuts	soybean
0.9	0.6	3.5	12	5.5
<0.2	0.3	2.5	2	1.3
<0.2	<0.2	48	720	2.8
<0.2	<0.2	3.7	800	40
<0.2	<0.2	80	12	5
<0.2	<0.2	25	680	1.2
<0.2	<0.2	80	>5000	<0.2

[a] Assays based on liquid phase separation were used for these studies.

ties. It is seen that the sample values obtained are close to the estimated values and the response is linear with dilution, indicating the suitability of the assay for analysis.

Reproducibility was evaluated by determining inter-assay and intra-assay variations by construction of precision profiles. The assays had <10% error throughout the assay range between 0.2 and 10.0 ng/mL. The results indicate that the assay could be used for the measurement of AfB_1 as low as 0.2 ng/mL with >90% precision for both solid phase and liquid phase separation assays.

Sample Analysis. Table 4 gives the results of analysis of naturally occurring samples of five different varieties of grains. Aflatoxin could be measured in detectable concentrations in many samples by the developed RIA. When the sample values were low, the aflatoxicometer did not detect aflatoxin as its sensitivity is far below that of immunoassays. When the sample values were >100 ng/g, the aflatoxicometer indicated the presence of the toxin. Although the comparisons of the sample values indicated in this table are not between two similar methods, they were made because the aflatoxicometer has been the conventional method of detection of aflatoxins (total). Grossly, the correlation is seen between the two methods in samples with high levels of toxins.

Table 5. Sample Analysis by ELISA and Liquid Phase Radioimmunoassay

RIA (ppb)	ELISA (ppb)	RIA (ppb)	ELISA (ppb)
4.7	5.3	4.3	4.9
25.5	31.1	<0.5[a]	~0
14.5	12	<0.5[a]	~0
10.5	10.5		

[a] Values obtained were below the detection limit of 0.5 ppb.

All 35 samples spiked with aflatoxin registered increased levels of aflatoxins. This indicated that the extraction procedure was efficient in transporting aflatoxins added to the grains. All 35 samples inoculated with the fungus showed very high levels of aflatoxin, as expected. The average levels of AfB_1 in these samples were ~20 μg/g in ground nuts, 0.25 μg/g in maize, 0.1 μg/g in rice, 0.09 μg/g in wheat, and 0.02 μg/g in soybeans.

The chewable tobacco samples were analyzed to study if aflatoxin levels were high in these, because a high incidence of oral cancers is noted in the users of this product. The presence of aflatoxins would add to the risk that already exists in such products containing tobacco. It was observed that of 25 samples, nearly one-third (8 samples) exhibited >20 ng/g levels of aflatoxin. These studies were undertaken at the behest of a group studying oral cancers.

The intra-assay variations at three different levels (10.48 $\pm$ 0.7, 79.4 $\pm$ 3.9, and 148.8 $\pm$ 5.9 ng/g) were found to be <7%, which is an acceptable limit. Samples with aflatoxin concentrations varying from 10 to 150 ng/g were analyzed in five different assays. The inter-assay variations are higher (7−14% CV) yet within tolerable limits, considering that such assays will be mainly for screening purposes when the detectable limit rather than the precision would be the main criterion.

To compare the two RIA systems developed and to judge the suitability of the solid phase user-friendly system, a few samples were analyzed by both liquid phase and solid phase RIA. The two sets of values correlated well, with a correlation coefficient of 0.99. **Table 5** gives the sample values obtained using the commercial ELISA kit and the in-house-developed RIA kit. The correlation coefficient obtained was 0.98. Hence, it could be seen that the assays developed are comparable to the commercially available ELISA system.

Conclusion. A radioimmunoassay procedure for aflatoxin B_1 was developed. The optimized assay was thoroughly validated for intra- and inter-assay precision, recovery, and parallelism. The suitability of the assay for analysis of samples had been demonstrated by analyzing several samples. The sample analysis was compared with a commercially available ELISA kit, and the correlation was found to be good.

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LITERATURE CITED

(1) Pestka, J. J. Enhanced surveillance of foodborne mycotoxins by immunochemical assay. *J. Assoc. Off. Anal. Chem.* **1988**, *71*, 1075−1081.

(2) Ram, B. P.; Hart, L. P. Enzyme linked immunosorbent assay of aflatoxin B_1 in naturally contaminated corn and cottonseed. *J. Assoc. Off. Anal. Chem.* **1986**, *69*, 904−907.

(3) Samarajeeva, U.; Wei, C. L.; Huang, T. S.; Marshal, M. R. Application of radioimmunoassay in food industry. *CRC Crit. Rev. Food Sci. Nutr*. **1991**, *29*, 403−434.
(4) Whitaker, T. Variability associated with analytical methods used to measure aflatoxin in agricultural commodities. *J. Assoc. Off. Anal.Chem.* **1996**, *79*, 476−485.
(5) Chu, F. S.; Ueno, I. Production of antibody against aflatoxin B_1. *Appl. Environ. Microbiol*. **1977**, *33*, 1125−1128.
(6) Korde, A.; Banerjee, S.; Pillai, M. R. A.; Venkatesh, M. Preparation and evaluation of ^{125}I-aflatoxin B_1. *J. Radioanal. Nucl. Chem.* **2001**, *250*, 231−237.
(7) Chard, T. *An Introduction to Radioimmunoassay and Related Techniques*; Work, T. S., Work, E., Eds.; Elsevier Publications: New York, 1990; pp 42−70.
(8) Velasco, J. Fluorometric measurement of aflatoxin adsorbed on Florisil in minicolumns. *J. Assoc. Off. Anal. Chem*. **1975**, *58*, 757−763.

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