

Development of a simple enzyme-linked immunosorbent assay for quantitative estimation of aflatoxin B₁ albumin adduct in humans

Aflatoxin B₁ (AFB₁), a hepatotoxicant and hepatocarcinogenic secondary metabolite of *Aspergillus flavus* and *Aspergillus parasiticus*, is a frequent contaminant in several foods (e.g. groundnut, maize, chillies, etc.)¹. Human exposure to AFB₁ occurs through dietary intake of aflatoxin-contaminated food². AFB₁ heightens the risk of liver cirrhosis and hepatocellular carcinoma (HCC)³, particularly in individuals affected with hepatitis B virus (HBV) due to synergistic interaction between AFB₁ and HBV in the causation of HCC⁴. Identification of aflatoxin-exposed individuals would facilitate introduction of preventive interventions to reduce the risk of liver disorders and HCC³. Various measures that reduce fungal infestation and toxin contamination in foods are being enforced to prevent human exposure to aflatoxins². Some of them involve assessment of aflatoxin exposure in humans to identify high-risk communities or individuals to facilitate HCC preventive interventions³.

Based on the knowledge of AFB₁ metabolism in humans, biomarkers in the blood, urine, faeces and tissue have been identified that have been used as a proxy to monitor AFB₁ exposure in humans⁵. The AFB₁-8-9-epoxide lysine (AFB₁-lys) adduct found in human serum albumin (HSA) is a widely used AFB₁ biomarker as it reflects actual dose resulting from aflatoxin exposure over a period of 2–3 months^{6,7}. Various techniques based on mass spectrometry, high-performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA) have been used to monitor AFB₁-lys^{7,8}. In the present study, we have produced high titred polyclonal antibodies against synthetic AFB₁-lys and developed a simple indirect competitive (IC)-ELISA for quantitative estimation of AFB₁-lys adducts in HSA.

Unless specified, all the reagents used in the study were procured from Sigma-Aldrich, USA. Three AFB₁-8,9-epoxide conjugates, AFB₁-lys, AFB₁-ovalbumin (AFB₁-ova) and AFB₁-bovine serum albumin (AFB₁-BSA), were synthesized as described before^{9,10}, except that biphasic reactions were incubated overnight (~14 h). AFB₁-lys and AFB₁-ova were used as reference standard and

coating conjugate respectively, in IC-ELISA; whereas AFB₁-BSA was used as an immunogen to produce antiserum in a New Zealand White inbred rabbit. Immunization via intramuscular injections was performed using 250 µg AFB₁-BSA in 250 µl of 0.1 M phosphate buffer, pH 7.2, emulsified with an equal volume of Freund's complete adjuvant. Four subsequent injections were given at weekly intervals using AFB₁-BSA emulsified in equal volumes of Freund's incomplete adjuvant. The rabbit was bled for polyclonal antiserum a week after the last injection for four weeks at weekly intervals. Subsequently, a booster immunization was given with AFB₁-BSA emulsified in incomplete Freund's adjuvant. After two weeks of rest, the animal was bled for polyclonal antiserum at weekly intervals for eight weeks.

The titre of each bleed of antiserum was determined by IC-ELISA performed in 96-well microtitre plates (Nunc® MaxiSorp, Sigma-Aldrich)^{11,12}. Prior to utilizing this procedure, optimum concentrations of the AFB₁-lys and AFB₁-ova; antiserum and alkaline phosphatase (ALP)-labelled anti-rabbit IgG conjugate were determined. Wells of the ELISA plates were coated with 150 µl of 10 ng/ml AFB₁-ova in 0.2 M carbonate coating buffer, pH 9.6, and incubated overnight at 4°C. The wells were replaced with 0.2% BSA in PBS containing 0.05% Tween-20 (PBST) to block free sites of the well. In each subsequent step,

plates were incubated at 37°C for 1 h followed by three washes with PBST. Hundred microlitres of AFB₁-lys standards ranging from 7.8 to 2000 pg/ml PBST were added in duplicate wells and they were supplemented with 50 µl of 1:40,000 (v/v) AFB₁-lys antiserum in PBST containing 1.5% BSA (PBST-BSA). Sample wells constituted the same, except, instead of the standard, 20 µl of 1:5 (v/v) hydrolysed albumin (described below) was added. This was followed by addition of 150 µl of ALP-labelled anti-rabbit IgG at 1:2000 in PBST-BSA. In the final step, 1 mg/ml para-nitrophenyl phosphate in 10% diethanolamine buffer, pH 9.8, was added and the plates were incubated at 37°C for 1–3 h. Optical densities (OD) were read in an ELISA plate reader (Multiskan Plus, Labsystems) fitted with a 405 nm filter. Recordings were taken till OD values of the control well reached 1.5 ± 2.0 OD. The toxin concentration was calculated using SigmaPlot version 2.01, and the formula $(A \times D \times E) / G$ pg/mg albumin, where *A* is the concentration of AFB₁-lys (pg/ml), *D* the dilution factor (ml), *E* the extraction solvent volume (ml) and *G* the sample weight (mg).

The procedures used in the present study have contributed to the production of high titred and specific antiserum against AFB₁-lys, and high yields (55.5 µg/µl) of synthetic AFB₁ adduct. Titre of the antiserum prior to booster

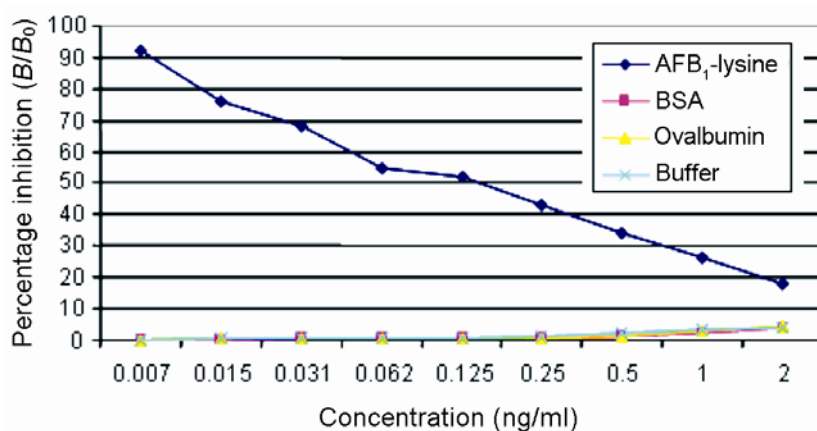


Figure 1. Evaluation of polyclonal antibodies (1:40,000 v/v) against AFB₁-lys, BSA and ovalbumin in IC-ELISA.

Table 1. Percentage recovery of AFB₁-lys from spiked human serum albumin by IC-ELISA

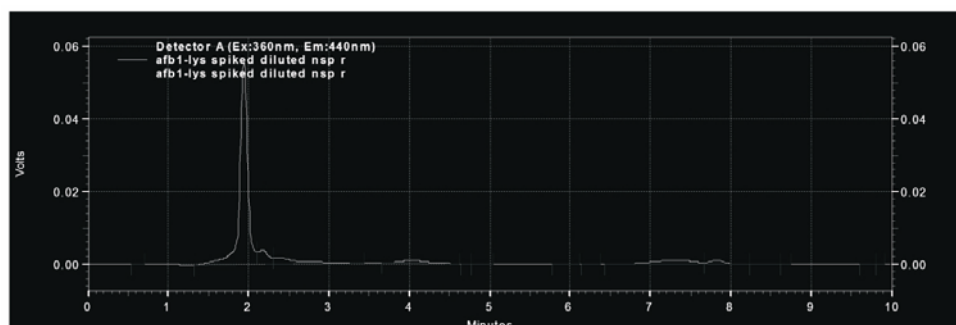
Sample no.	AFB ₁ -lys spiked in HSA (pg)	Estimated by IC-ELISA (pg/mg)		Recovery* (%)		
		Exp. 1	Exp. 2	Exp. 1	Exp. 2	SD (±)
1	2000	1677	1832	83.8	91.6	5.5
2	1000	1049	979	104.9	97.9	4.9
3	500	556	483	111.2	96.6	10.3
4	200	189	212	94.5	106	8.1
5	50	51.38	52.85	102.7	105.7	2.1

*(Concentration estimated by IC-ELISA/amount of AFB₁-lys spiked) × 100.

Table 2. Quantitative estimation of AFB₁-lys in human serum albumin by HPLC and in HPLC fraction by IC-ELISA

Sample no.	Concentration of AFB ₁ -lys (pg/mg)		Recovery of AFB ₁ -lys in HPLC fraction by IC-ELISA (%)
	HPLC	IC-ELISA	
1 ^a	72	69.53	96.5 (± 1.7)
1 ^b	72	67.2	93 (± 3.4)
2 ^b	109.08	103	94.2 (± 4.3)
3 ^b	101	75.82	75.0 (± 17.8)
4 ^c	0 ^d	— ^d	—

^aHydrolysed sample purified with Sep-Pak cartridge. ^bHydrolysed sample purified by ethanol precipitation. ^cNegative control. ^dNo peak in HPLC and OD equivalent to negative control in ELISA.

**Figure 2.** Reversed phase HPLC of proteinase-K hydrolysed human serum albumin spiked with AFB₁-lys.

immunization was between 1:2000 to 1:10,000; and titre of the antiserum post-booster injection was 1:40,000 to 1:75,000, suggesting positive effect of longer resting time and booster dose on improving the antiserum titre. The optimum antiserum dilution for sensitive detection of AFB₁-lys was determined by 50% displacement values of B/B_0 , where B is the OD of the AFB₁-lys, and B_0 the OD of the negative control (Figure 1). This has identified 1:40,000 (v/v) as the optimum antiserum dilution for the detection of up to 5 pg AFB₁/mg HSA.

To determine the detection limit of IC-ELISA, 2 mg/ml HSA in PBS was spiked with 50–2000 pg/ml of AFB₁-lys and then hydrolysed with 0.67 mg of pro-

teinase K (Amresco, Ohio) in 0.8 ml PBS at 37°C for ~17 h (ref. 13). Undigested proteins were removed using Sep-Pak cartridges (WAT051910, Waters Ltd, UK)¹³ or by precipitation with cold ethanol. This revealed $96 \pm 11\%$ recovery of the AFB₁-lys in IC-ELISA (Table 1). Comparison of efficacy of albumin hydrolysis using cold ethanol precipitation procedure and Sep-Pak cartridges showed no significant differences, indicating that low-cost ethanol precipitation approach is effective (Table 1).

Results of IC-ELISA were compared with HPLC using reversed-phase C18 column (Shimadzu Liquid Chromatography-LC-10AT VP) with a particle size 5 µm diameter linked to a fluorescence

detector (Shimadzu RF-10 AXL). Solvent flow rate was 1 ml/min with mobile phase water : acetonitrile : methanol (70 : 17 : 17). Fluorescence detection parameters were set to excitation wavelength at 360 nm and emission wavelength at 440 nm. For each HPLC run, 20 µl of the hydrolysed HSA was injected into the column and fractions were collected at 1 min interval. Two peaks were obtained, one major peak at 2.0 min corresponding to AFB₁-lys and one minor peak at 2.4 min (Figure 2), suggesting that AFB₁-lys had a relative retention time of 2 min. Each HPLC fraction was precipitated in a vacuum evaporator, reconstituted with 250 µl of PBS and analysed in IC-ELISA. Results of IC-ELISA were

Table 3. Estimation of AFB₁-lys in human serum albumin by IC-ELISA

AFB ₁ -lys adduct concentration (pg/mg albumin) ^a	Number of samples
– ^b	232
≤ 5	1
6–25	13
26–50	3
51–75	1
Total	250

^aAlbumin purified from serum fraction was hydrolysed with proteinase-K, purified with ethanol and tested in IC-ELISA. ^bOD equivalent to negative control.

similar to HPLC results (SD ± 1.7 to 17.8; Table 2) and there was no difference in the samples prepared by Sep-Pak cartridge or ethanol precipitation.

IC-ELISA was validated by testing 250 blood samples that include 85 HBV positive samples from unidentified subjects from the Apollo Health City, Hyderabad, and 165 blood samples collected from ICRISAT campus, Hyderabad. Serum was separated by centrifugation at 5000 rpm for 10 min and it was heat-treated at 56°C for 45 min to inactivate any infectious HIV. Albumin fraction was extracted from 500 µl serum as detailed in Chapot and Wild¹⁴, and its concentration was estimated by the Bradford method¹⁵. Two milligram albumin was hydrolysed with proteinase-K, precipitated with ethanol and tested in IC-ELISA, as described above. AFB₁-lys at a concentration between 2.5 and 75 pg/mg albumin was detected in 12 samples (Table 3). All the samples that were positive to AFB₁-lys were from HBV-positive subjects, indicating a potential risk of HCC in 4.8% of the subjects tested in the present study. This validation confirms the suitability of IC-ELISA, which is simple, cost-effective and enables high-throughput analysis.

An earlier study in India using immunoperoxidase test detected AFB₁ deposits in 58% of 32 human liver biopsy samples from HCC cases, 15 of which were positive to HBV¹⁶. However, in the same study ELISA assay for AFB₁ biomarker was negative¹⁶. We speculate that negative results in ELISA could be linked to the time of assessment, as AFB₁-lys biomarker is detectable for up

to 2 months from first exposure to AFB₁. Nonetheless, earlier¹⁶ and current studies have demonstrated significant association of AFB₁ toxicity with HCC cases in India, and emphasize the need for wider surveillance to determine the AFB₁-exposed populations in the country. After thorough validation, IC-ELISA has the potential to serve as a tool for epidemiological studies to identify vulnerable groups and implement appropriate interventions to minimize aflatoxin contamination in diets of communities at high risk of AFB₁ exposure^{17,18}. Vulnerable individuals can be subjected to further specific tests to assess HCC risk and implement remedial treatments¹⁹.

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