Development of a simple enzyme-linked immunosorbent assay for quantitative estimation of aflatoxin B_1 albumin adduct in humans

Aflatoxin B1 (AFB_1), 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 AFB_1 occurs through dietary intake of aflatoxin-contaminated food[2]. AFB_1 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 AFB_1 and HBV in the causation of HCC[4]. Identification of aflatoxin-exposed individuals would facilitate introduction of preventive interventions to reduce the risk of liver disorders and HCC[3]. Various measures that reduce fungal infestation and toxin contamination in foods are being enforced to prevent human exposure to aflatoxins[2]. Some of them involve assessment of aflatoxin exposure in humans to identify high-risk communities or individuals to facilitate HCC preventive interventions[3].

Based on the knowledge of AFB_1 metabolism in humans, biomarkers in the blood, urine, faeces and tissue have been identified that have been used as a proxy to monitor AFB_1 exposure in humans[5]. The AFB_1-8-9-epoxide lysine (AFB_1-lys) adduct found in human serum albumin (HSA) is a widely used AFB_1 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 AFB_1-lys[7,8]. In the present study, we have produced high titred polyclonal antibodies against synthetic AFB_1-lys and developed a simple indirect competitive (IC)-ELISA for quantitative estimation of AFB_1-lys adducts in HSA.

Unless specified, all the reagents used in the study were procured from Sigma–Aldrich, USA. Three AFB_1-8,9-epoxide conjugates, AFB_1-lys, AFB_1-ovalbumin (AFB_1-ova) and AFB1-bovine serum albumin (AFB_1-BSA), were synthesized as described before[9,10], except that biphasic reactions were incubated overnight (~ 14 h). AFB_1-lys and AFB_1-ova were used as reference standard and coating conjugate respectively, in IC-ELISA; whereas AFB_1-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 AFB_1-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 AFB_1-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 AFB_1-BSA emulsified in incomplete Freund's adjuvant. After two weeks of rest, the animal was bleed 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 AFB_1-lys and AFB_1-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 AFB_1-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 AFB_1-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) AFB_1-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 AFB_1-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 AFB_1-lys, and high yields (55.5 µg/µl) of synthetic AFB_1 adduct. Titre of the antiserum prior to booster

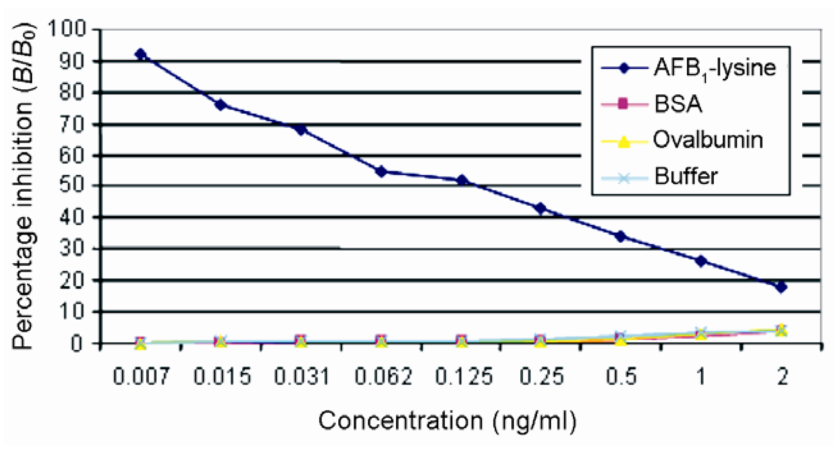

Figure 1. Evaluation of polyclonal antibodies (1 : 40,000 v/v) against AFB_1-lys, BSA and ovalbumin in IC-ELISA.

Table 1. Percentage recovery of AFB_1-lys from spiked human serum albumin by IC-ELISA

Sample no.	AFB_1-lys spiked in HSA (pg)	Estimated by IC-ELISA (pg/mg) Exp. 1	Estimated by IC-ELISA (pg/mg) Exp. 2	Recovery* (%) Exp. 1	Recovery* (%) Exp. 2	Recovery* (%) 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 AFB_1-lys spiked) × 100.

Table 2. Quantitative estimation of AFB_1-lys in human serum albumin by HPLC and in HPLC fraction by IC-ELISA

Sample no.	Concentration of AFB_1-lys (pg/mg) HPLC	Concentration of AFB_1-lys (pg/mg) IC-ELISA	Recovery of AFB_1-lys in HPLC fraction by 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]	–

[a]Hydrolysed sample purified with Sep-Pak cartridge. [b]Hydrolysed sample purified by ethanol precipitation. [c]Negative control. [d]No peak in HPLC and OD equivalent to negative control in ELISA.

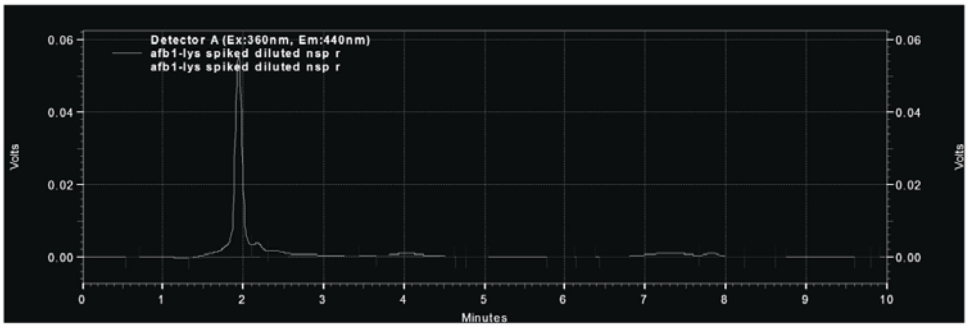

Figure 2. Reversed phase HPLC of proteinase-K hydrolysed human serum albumin spiked with AFB_1-lys.

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 AFB_1-lys was determined by 50% displacement values of B/B_0, where B is the OD of the AFB_1-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 AFB_1/mg HSA.

To determine the detection limit of IC-ELISA, 2 mg/ml HSA in PBS was spiked with 50–2000 pg/ml of AFB_1-lys and then hydrolysed with 0.67 mg of proteinase 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 ± 11% recovery of the AFB_1-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 AFB_1-lys and one minor peak at 2.4 min (Figure 2), suggesting that AFB_1-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 AFB_1-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

[a]Albumin purified from serum fraction was hydrolysed with proteinase-K, purified with ethanol and tested in IC-ELISA. [b]OD 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 Wild[14], and its concentration was estimated by the Bradford method[15]. Two milligram albumin was hydrolysed with proteinase-K, precipitated with ethanol and tested in IC-ELISA, as described above. AFB_1-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 AFB_1-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 AFB_1 deposits in 58% of 32 human liver biopsy samples from HCC cases, 15 of which were positive to HBV[16]. However, in the same study ELISA assay for AFB_1 biomarker was negative[16]. We speculate that negative results in ELISA could be linked to the time of assessment, as AFB_1-lys biomarker is detectable for up to 2 months from first exposure to AFB_1. Nonetheless, earlier[16] and current studies have demonstrated significant association of AFB_1 toxicity with HCC cases in India, and emphasize the need for wider surveillance to determine the AFB_1-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 AFB_1 exposure[17,18]. Vulnerable individuals can be subjected to further specific tests to assess HCC risk and implement remedial treatments[19].

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