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Original Article

Development and characterization of enzyme-linked immunosorbent assay for aflatoxin B_1 measurement in urine sample using penicillinase as label

Malihe Paknejad[1], Mohammad Javad Rasaee[2], Javad Mohammadnejad[2], Mehdi Pouramir[3], Masoume Rajabibazl[2] and Mohammad Kakhki[4]

[1]Department of Biochemistry, School of Medicine, Medical Sciences/University of Tehran, Tehran, Iran
[2]Department of Clinical Biochemistry, School of Medical Sciences, Tarbiat Modarres University, Tehran, Iran
[3]Department of Clinical Biochemistry, School of Medical Sciences, Babol University of Medical sciences, Babol, Iran
[4]Pasteur Institute of Iran, Tehran, Iran

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ABSTRACT — A simple, sensitive, rapid and specific enzyme linked immunosorbent assay (ELISA) for quantitative measurement of aflatoxin B_1 (AFB_1) in urine samples was developed in this study. Polyclonal antibodies were raised against a C_1-carboxymethyl oxime (CMO) derivative of AFB_1 conjugated bovine serum albumin (BSA). AFB_1-C_8-penicillinase (AFB_1-C_8-P) and AFB_1-C_1-carboxymethyl oxime-penicillinase (AFB_1-CMO-P) were prepared and used as tracer molecule. A heterologous combination of antibody and enzyme conjugates (AFB_1-C_1-CMO-BSA and AFB_1-C_8-P) proved to work better with respect to specificity and sensitivity. Ig purified antibody (4 μg/well) was coated onto the pre-coated (BSA) wells of microtiter plate. The assay procedure was completed within 3 hr and the sensitivity was calculated to be from 200 pg/ml. The standard curve was linear up to 10 ng/ml so was able to detect high concentration of AFB_1 in sample. Affinities were calculated for homologous and heterologous system in which the heterologous system showed better affinities (1.9×10^8 M^{-1}). The antibody prepared in this study showed minimal cross-reaction with structurally related molecules being affected by homology and heterology of the assay system that is the site of conjugation of carrier protein for antibody production using the hapten BSA conjugate and the site of enzyme conjugated on the hapten molecule used as tracer as well as direct and indirect coating of antibody on the surface of microtiter plat. The results reported here indicated that the heterologous combination of antibody and enzyme conjugate performs better in assay qualities in general. More than 90% recovery of AFB_1 added to stripped urine samples were observed in this type of assay. Inter and intra-assay percent of coefficient of variations for ten successive assays were found to be 10.2 and 6.9% respectively. Logit -log transformation of standard curve and sample dilution with urine sample containing no AFB_1 in a serial manner exhibited parallel line with the slope of −1.03 and −1.03 respectively. A correlation of 0.90 was found between the ELISA reported in this study and radioimmunoassay (RIA) of AFB_1 in urine samples.

Key words: ELISA, Penicillinase, Aflatoxin B_1, Urine, Heterology

INTRODUCTION

Aflatoxins are a group of secondary metabolites produced mainly by the fungi *Aspergillus flavous, Aspergillus parasiticuse and Aspergillus nomius* (Weidenbörner., 2001). These metabolites were shown to be highly toxic and found to contaminate food and feed stuff widely and implicated in hepato-cellular carcinoma and classified as a carcinogenic substance of group1 by the International Agency for Research on Cancer (IARC) (Park *et al*., 2002). The hepato-carcinogenicity of aflatoxin B_1 (AFB_1) in experimental animals indicates a possible relationship between exposure to AFB_1 and hepatocellular carcinoma in human (Renzulli *et al*., 2004; Guerra *et al*., 2005). Regulatory measures have been adapted in many countries and an imperative need for fast and easy screen-

Correspondence: M.J. Rasaee (E-mail: Rasaee_m@modares.ac.ir)

ing methods exists (Mably *et al*., 2005; Murugavel *et al*., 2007). Studies show a positive correlation between aflatoxin consumption and its urinary excretion (Stubblefield *et al*., 1991; Johnson *et al*., 2008). Different techniques have been adapted to monitor human exposure to AFB_1 such as thin layer chromatography (TLC), gas chromatography (GC) and high pressure liquid chromatography (HPLC) in various biological samples. (Kamkar *et al*., 2008; Stroka *et al*., 2000; Zöllner and Mayer, 2006). Immunological procedures have been adapted during recent years, because of their specificity, sensitivity, ease of performance and speed. Therefore, techniques such as radioimmunoassay, enzyme immunoassay, fluroimmunoassays, antibody affinity columns and strip liposome immunoassay are reported for the accurate measurement of AFB_1 or its adducts (AFB_1-DNA or AFB_1-albumin) in various biological and non-biological samples (Shim *et al*., 2007; Chen *et al*., 2005; Arduini *et al*., 2007; Bacigalupo *et al*., 1994). Few enzyme linked immunosorbent assay (ELISA) procedures using horseradish peroxidase (HRP) and alkalin phosphatase as label have been reported or AFB_1 (Kolosova *et al*., 2006; Nayak *et al*., 2001). In these assays, the quality of enzyme label determines the sensitivity and specificity. Penicillinase as enzyme label is used in the immunoassay of many small molecules (haptens) in recent years (Malakaneh *et al*., 2001; Omidfar *et al*., 2002; Kumari and Dhir, 2003). The quality of antibody is another important factor in immunological procedures. Two common derivatives of AFB_1 are used (C_1 and C_8) for carrier protein conjugation; the antibodies rose using showed different specificities toward the structurally similar molecules (Basu *et al*., 2006). But Enzyme Immuno Assay (EIA) also depends on another very important factor, namely homology and heterology of tracer and antibody used in assay which also play important rule on quality (specificity as well as sensitivity) of the assay (Kumari and Dhir, 2003; Basu *et al*., 2006; Shrivastav *et al.,* 2005).

In this paper, we report the development of a simple, sensitive and specific ELISA using the enzyme penicillinase as label to measure AFB_1 in various biological samples specially urine. To best of our knowledge, this is the first ELISA reported for AFB_1 using penicillinase as the label enzyme.

MATERIALS AND METHODS

Compounds

AFB_1, penicillinase (EC: 3.5.2.6), bovine serum albumin (BSA), penicillin V, Ferund's complete and incomplete Ferunds adjuvant's (FCA & FIA), aminooxy acetic acid, pyridine, N-hydroxy succinimide, Diethyl Amino Ethyl Cellulose (DEAE) cellulose, dimethyl formamide (DMF), 1,ethyl-3-(3-dimethyl carbodiimid) (EDC), dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Company, St. Louis, MO, USA. C_{18} Sephadex columns were obtained from Walter Associates, Inc. (Millipore Corp., MA, USA). Structurally related aflatoxins (AFB_2, AFG_1, AFG_2, AFQ, AFP_1 and AFM_1) were purchased from Fluka (Switzerland).

Sample collection and preparation

Urine samples were collected from normal individuals residing in Tehran and taking their normal diet (grains, rice and milk). In order to concentrate and partially purify the samples, two strategies were selected. In one procedure 10 ml of urine sample was collected, centrifuged and passed through a C_{18} sephadex mini column following the procedure explained by Chu *et al.* (1981) with a minor modification as follow. A C_{18} sep pak column was activated with 5 ml of methanol and washed with 5 ml of a 7% solution of methanol in water. Ten milliliter urine sample containing 7% methanol was passed through the column with a speed of one ml/min; the column was then washed with 5 ml of a 7% methanol in water and eluted with 80% methanol in water. The residue was allowed to dry at 37°C over night and reconstituted to 1 ml with a 10 mmol PBS buffer pH 7.2 containing 0.1% gelatin and 0.01% sodium azid (here after called as assay buffer). In another sets of experiment, 10 ml urine samples were treated with 2 ml of chloroform, vortexes and shaked for 30 min at room temperature. One milliliter of solvent phase was then removed, evaporated and reconstituted with 500 µl of PBS buffer. The efficiency of the above procedures was examined by adding a fix concentration of radioactive AFB_1 to urine samples and calculating the percent recovery after chloroform extraction and column chromatography. Urine sample prepared for recovery experiments were obtained by charcoal treatment of normal urine which was then added with the required amount of AFB1 from stock ethanolic solution.

Antibody preparation

Two male rabbits were immunized with AFB_1-C_1-carboxymethyl oxime (CMO)-BSA as immunogen following the low dose multi-intradermal method of Vaitukaitis (1986). Blood samples were obtained after two months and antibody titer was calculated by radioimmunoassay (RIA). High titer antibodies obtained during one year of immunization were characterized (affinity and specificity) following the method of Abraham (1975) purified following the procedure of Andrew and Titus (2001) and stored

as explained by Shrivastav (2005).

Tracer preparation

AFB_1 was either brominated or chlorinated at C_7-C_8 double bound following a modified procedure of Sizaret and Malaveille (1983) briefly as follow. A 200 µg/ml of ethanolic solution of AFB_1 was dried under nitrogen gas and was dissolved in minimum amount of DMSO (200 µl). One drop of Br_2 dissolved in 1 ml of DMSO was added to AFB_1 solution slowly until a pale yellow color appeared. Alternatively, AFB_1 was dissolved in 200 µl of dichloroethan and chlorine gas was passed through the solution. A yellow color development indicated complete chlorination of AFB_1 molecule at C_7-C_8 position. Penicillinase type I (800 µg) was dissolved in 250 µl of PBS (10 mM, pH 7.2) and was added slowly to either of brominated or chlorinated AFB_1 solution while stirring. The mixture was allowed to be stirred at 4°C for another 2 hr. The product was then chromatographed on a G-25 Sephadex. AFB_1-C_1-CMO was prepared following the method of Chu *et al.* (1977) with a minor modification as follow. AFB_1 (530 µg) and carboxymethylamin hydrochloride (300 µg) were dissolved in 400 µl of water/pyridine/methanol (1:1:4) and refluxed (2.5 hr at 58°C). The product was evaporated, dissolved in 200 µl of DMF, added with 4.7 mg of EDC dissolved in 50 µl of distilled water. The final product was incubated at room temperature while stirring for another 4 hr. At the end of incubation time, the product was added slowly to a 1 mg penicillinase (equivalent to 140 µg protein) solution in 500 µl of distilled water while being stirred. The product was incubated at 4°C for 16 hr and chromatographed on a Sephadex G-25 column.

ELISA of AFB_1

Different concentrations (2, 4, 8, 16, and 100 µg/well) of purified antibody was coated onto the wells of microtiter plat, or alternatively BSA (0.1%) was first coated (at 37°C, overnight), washed and the antibody was coated in the similar manner as above (indirect coating) and blocked with 0.3% solution of gelatin in PBS (10 mM, pH 7.2). The wells were washed with a 10 mM PBS containing 0.05% tween 20, added with different dilutions (1:200, 1:400, 1:800 and 1:1,600) of tracer molecule (AFB_1-C_1-CMO-P and AFB_1-C_8-P), incubated for 1 hr, washed and added with a 100 µl freshly prepared substrate solution (280 mg/l solution of penicillin V in a 0.2 M PBS buffer of pH 7.2). Wells were incubated for another 1 hr at 37°C and added with 150 µl of starch iodine solution reagent, the preparation of which is explained elsewhere (Rassaie *et al*., 1992) incubated for 10 min and the optical density (OD) was measured at 600 nm using a Multiscan ELISA reader. In order to construct a standard curve, concentrations of AFB_1 from 20 pg/ml up to 100 ng/ml were prepared from a stock ethanolic solution in assay buffer. These were added into a 4 µg/well antibody coated (direct and indirect) wells of ELISA plate, incubated for 1 hr at 37°C, added with an optimum dilutions of tracer prepared in assay buffer and incubated for another 1 hr. Finally the wells were washed and added with 100 µl of substrate solution. Rests of experiments were performed as explained in above section.

Cross reactivity

Concentrations of aflatoxin B_2, G_1, G_2, Q, P_1 and M_1 were prepared in above manner in doses from 400 pg/ml up to 400 ng/ml. The standard assay was performed along with concentrations of AFB_1 and above possible cross-reacting aflatoxins as explained in the above section. Results were calculated following the method of Abraham (1975).

RESULTS

Fig. 1 shows a typical checkerboard titration assay for anti-AFB_1-C_1-BSA antibody and AFB_1-C_8-Penicillinase (AFB_1-C_8-P) used as tracer in which a dilution of 4 µg/well of antibody indirectly coated (IC) onto the wells of microtiter plate and 1:500 dilution of enzyme conjugate were show to work properly, when using Br_2 derivative for penicillinase conjugation. While when chlorination product was used for tracer preparation minimum antibody and tracer concentration required for detectable reaction was found to be 4 µg/well and 1:400 dilution of enzyme conjugate respectively (the results are not shown). Hence all our further experiments were performed with AFB_1 brominated derivative. Fig. 2 shows a heterologous (anti-AFB-C_1-CMO-BSA and AFB_1-C_8-P) standard curve constructed in buffer; here results of ten experiments are reported. The sensitivity of the standard curve was found to be from 200 pg/ml covering up to 100 ng/ml in a linear manner. It is to be denoted that the antibody for this experiment was coated indirectly. Affinities of antibody toward AFB_1 was calculated by Scatchard analysis and was found to be $Ka = 1.9 \times 10^8\ M^{-1}$. A typical curve is shown in Fig. 3. Table 1 summarizes our results for antibody and tracer characteristics when the homologous and heterologous as well as coating conditions were used. The results indicated that although in case of homologous combinations, the titer of tracers were generally high but the sensitivity and affinity in term of cold AFB_1 decreases notably. However in general the heterologous system is

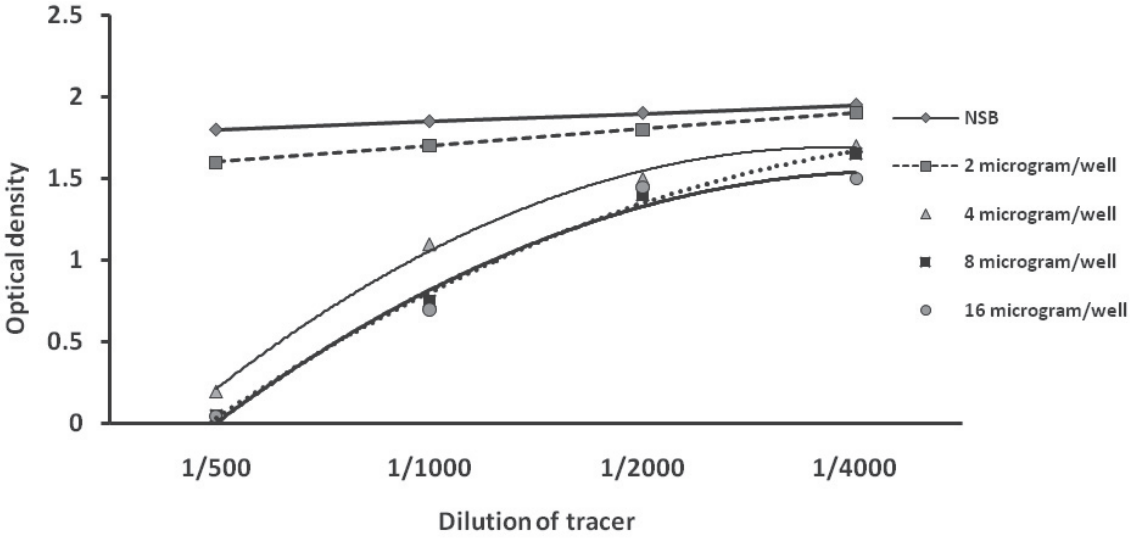

Fig. 1. Checkerboard titration for anti-AFB_1-C_1-BSA antibody and AFB_1-C_8-P. All assays were performed in duplicate. NSB = Non Specific Binding

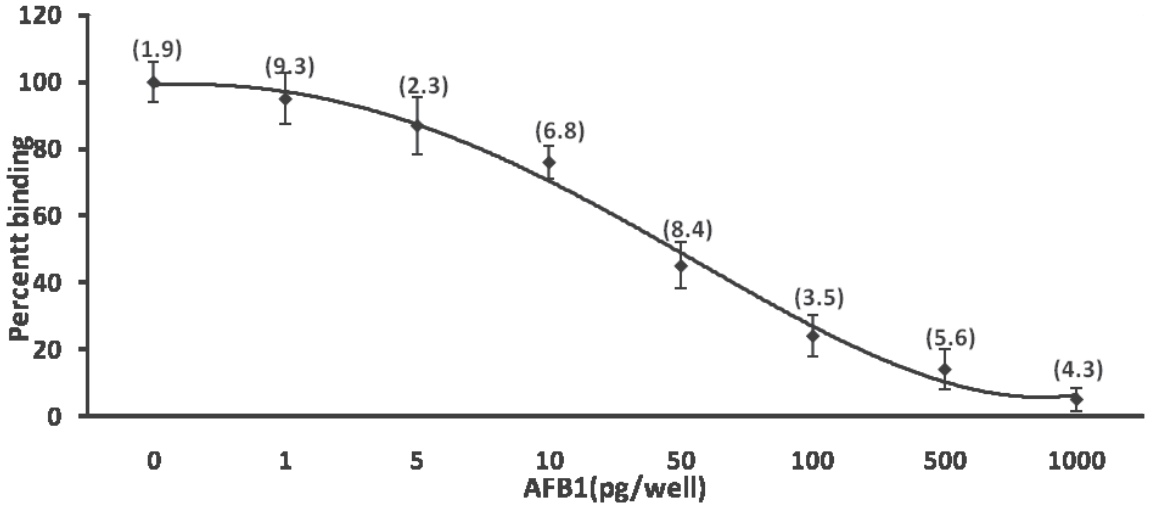

Fig. 2. Standard curve of anti-AFB-C_1-CMO-BSA and AFB_1-C_8-P by ELISA. Results of ten experiments. All assays were performed in duplicate. Bars indicate S.D. while values in parenthesis show the percent coefficient of variation (% CV).

more sensitive and the antibody exhibits better affinities. Table 2 shows the results for specificity of anti-aflatoxin antibodies by RIA and ELISA (homologous and heterologous) and when the antibody was coated directly and indirectly. Here the normal trend of increased specificity for homologous combination was observed while the indirect coating also affected positively in terms of specificity. However the lowest cross-reaction was observed

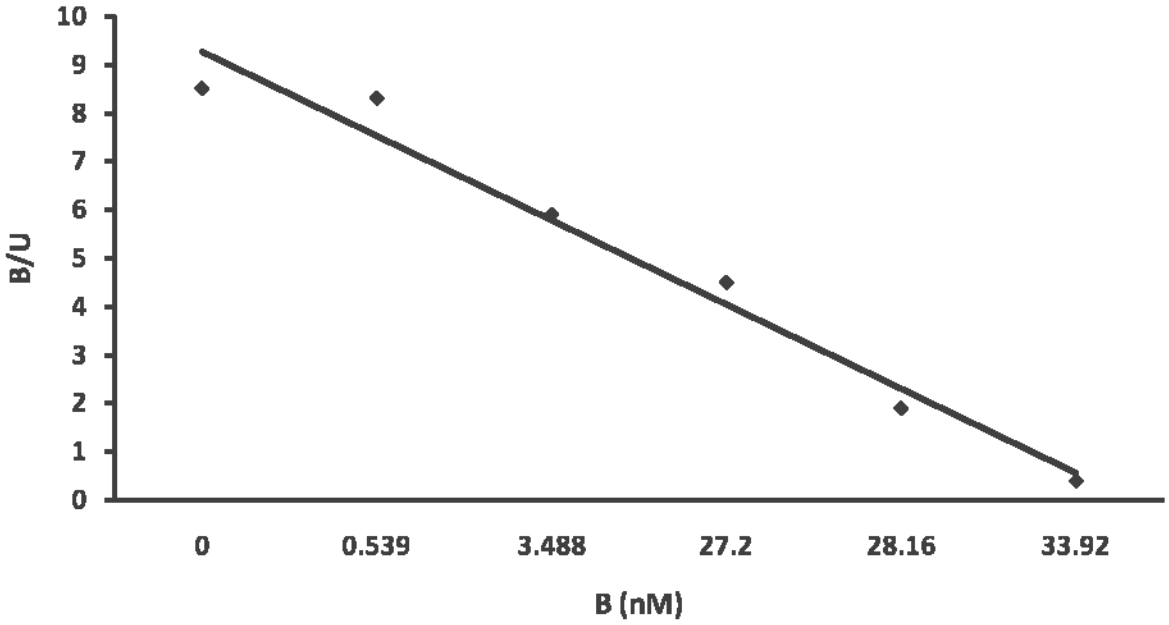

Fig. 3. Afinity analysis of anti-AFB-C_1-CMO-BSA using AFB_1-C_8-P as tracer by ELISA. All assays were performed in duplicate. (Ka was calculated as 1.9×10^8 M^{-1})

Table 1. Characteristics of titer, sensitivity and affinity of antibody (AFB_1-C_1-CMO-BSA) and enzyme conjugate.

SN		*AFB_1-C_8-CMO-P		AFB_1-C_1-CMO-P	
	Character	IC	DC	IC	DC
1	Ab titer	4 µg/well	4 µg/well	4 µg/well	4 µg/well
2	Tracer titer	1:400	1:500	1:600	1:1,200
3	Sensivity	200 pg/ml	20 pg/ml	200 pg/ml	2,000 pg/ml
4	Affinity	2×10^8 l/mol	1.9×10^8 l/mol	3×10^6 l/mol	4×10^6 l/mol

*AFB_1-$C_{8,9}$ Br_2 derivative of AFB_1 was used to prepare tracer (AFB_1-C_8-CMO-P). IC: Indirect Coating, DC: Direct Coating
SN = Serial Number

in heterologous system with indirect antibody coating. The results of recovering AFB_1 added to charcoal stripped urine samples are shown in Table 3. These are calculated in three ranges (10, 50 and 100 pg/50 µl of sample). Each assay was repeated six times and percent recovery was calculated to be from 90 to 98. The results for inter and intra-assay variation were calculated for heterologous assay of indirect coating which are shown in Table 4. The assays were carried out in 4 replicates and each assay was repeated for at least 5 times. The CVs here varied from 1.8 up to 10.2%. Fig. 4 exhibits the logit-log transformation of standard curve and AFB_1 containing sample diluted serially in which the slope of standard curve and sample dilution was calculated to be −1.03 and −1.03 respectively. Finally, a correlation coefficient between aflatoxin measured by ELISA and RIA both developed in our laboratories (data for RIA not shown) were calculated to be 0.90. Fig. 5 presents our results for 20 samples measured in this report.

Table 2. Percent cross-reaction of anti-AFB_1-C_1-CMO-BSA in RIA and ELISA.

SN	Molecules	% Cross reaction				
		ELISA				RIA
		AFB_1-C_8-CMO-P		AFB_1-C_1-CMO-P		
		IC	DC	IC	DC	
1	AFB_1	100	100	100	100	100
2	AFB_2	14	94	10	133	49
3	AFG_1	66	119	35	100	4
4	AFG_2	58	221	32	53	< 0.05
5	AFM_1	4	119	< 0.05	9.4	< 0.05
6	AFM_2	16	103	< 0.05	32	3.7
7	AFQ_1	110	75	50	47	< 0.05
8	Cortisol	ND	< 0.05	ND	ND	ND
9	Progesterone	ND	2.3	ND	ND	ND

SN = Serial Number
DC = Direct antibody coating
IC = Indirect antibody coating
ND = Not detected

Table 3. Recovery of added AFB_1 from urine samples stripped-off endogenous AFB_1.

SN	Added AFB_1 (pg/50µl)	N	Concentration observed pg/50 ul	% Recovery	% CV
1	10	6	9.8	98	17.5
2	50	6	47	94	6.7
3	100	6	90.3	90.3	3.2

SN = Serial Number
N = Number of assays performed

DISCUSSION

Epidemiological studies implicate aflatoxins as of importance in the induction of liver cancer in man. In this study we have developed a simple, sensitive and high through-put enzyme immunoassay for AFB_1 measurement in urine samples. Two AFB_1 enzyme conjugate were prepared, one using C_8-C_9 double bound in which halogenation performed using chlorine and bromine. However bromine derivative gave raise to a better enzyme conjugation product. Another derivative of carbon number one (C1) was prepared and conjugated to enzyme. Using these tracers along with the antibody raised for a C1 derivative of AFB_1 it was observed that the homologous assay (AFB_1-C_1-CMO-P and AFB_1-C_1-CMO-BSA) was highly insensitive and the slope of standard curve although changing the assay performance criteria (such as coating conditions, pH, temperature, etc the results of which are not shown here) could not improve the results. However a hetrologous combination of antibody and tracer (AFB_1-C_1-CMO-BSA and AFB_1-C_8-P) improved the assay quality (sensitivity, slope of standard curve etc). Rassaie *et al*. (1992) found a similar effect in the immunoassay of testosterone derivatives in which the homologous combination

Table 4. Inter- and intra-assay variation of AFB_1 assays.

Inter assay							
EN	N	10 pg/well		50 pg/well		100 pg/well	
		Mean ± S.D.	% CV	Mean ± S.D.	% CV	Mean ± S.D.	% CV
1	4	0.68 ± 0.068	10	1.11 ± 0.638	3.4	1.26 ± 0.05	4
2	4	0.70 ± 0.06	9.3	1.12 ± 0.023	2.1	1.21 ± 0.02	2.3
3	4	0.633 ± 0.06	10.2	1.083 ± 0.017	1.6	1.208 ± 0.036	3
4	4	0.609 ± 0.021	3.5	1.108 ± 0.015	1.4	1.62 ± 0.022	1.9
5	4	0.603 ± 0.052	8.6	1.068 ± 0.027	2.6	1.225 ± 0.022	1.8
Intra assay							
	4 × 5	0.64 ± 0.04	6.9	1.1 ± 0.02	2	1.22 ± 0.04	3

N = Number of replicates
EN = Number of experiments carried out

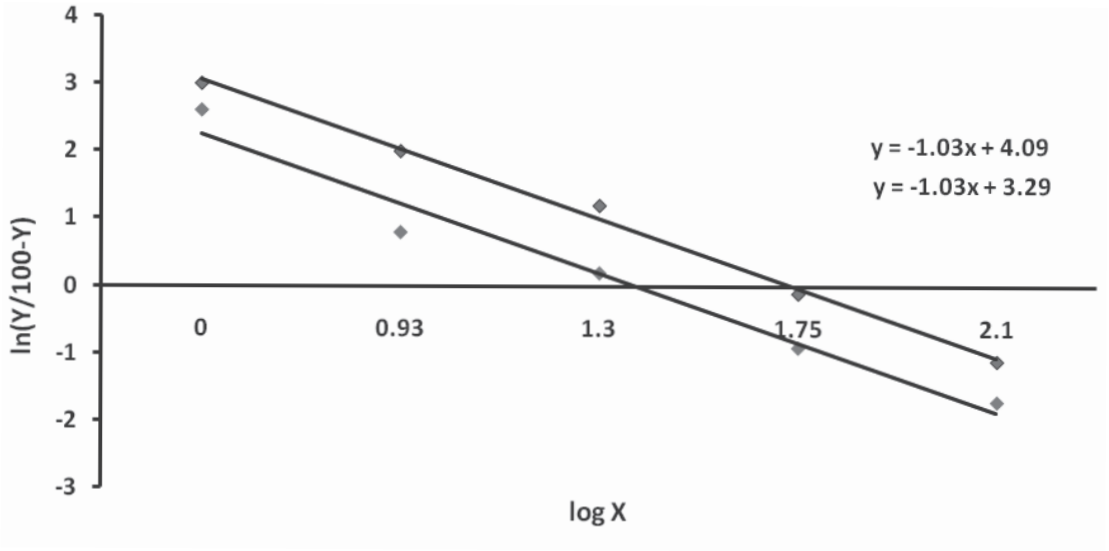

Fig. 4. Logit-log transformation of standard curve and AFB_1 containing sample dilution by ELISA (test of Parallelism). All assays were performed in duplicate

of derivatives used for antibody production and tracer formation ended up to a more specific assays with lower sensitivity. In this work the heterologous combination (derivatives used for antibody production and tracer formation) exhibited higher sensitivity while the assay experienced very low specificity (Rassaie *et al.*, 1992). Further, our initial experiments showed that with a heterologous assay some unknown factors cross reacted with the antibody. These were reported by others in previous studied (Sizaret and Malaveille, 1983) which was attribution to coating conditions in our experiments. In order to overcome this difficulty the antibody in our experiments was coated on to the wells of microtiter plate indirectly (i.e. a 0.1% concentration of BSA were first coated for a short

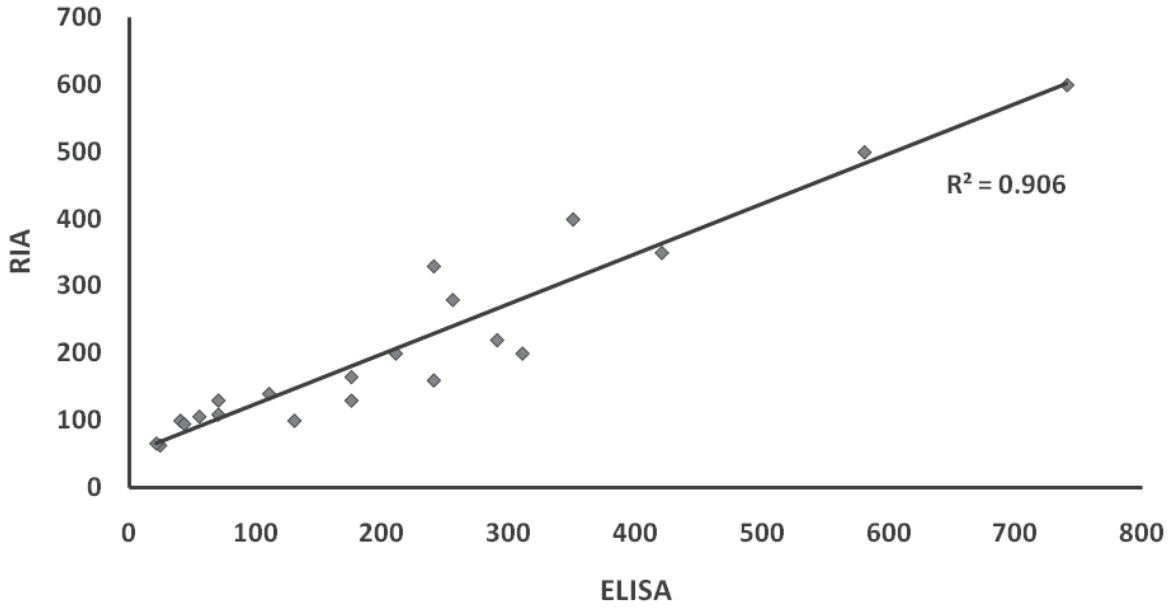

Fig. 5. Correlation Coefficient between aflatoxin measured by ELISA and RIA for 20 samples. (Coating: indirect, Buffer: PBS, Tracer: AFB_1-C_8-P). All assays were performed in duplicate. ($R^2 = 0.9$)

course of time and antibodies were than coated and dried on them). The results obtained here indicated higher specificity in both homologous and heterologous assays. However the affinities were higher in case of heterologous assay (Ka = 1.9×10^8 l/mol) as compare to homologous assays (Ka = 3×10^7 l/mol). This assay was further charactrized (recoveries, inter and intra assay variation, test of parallelism), the result of which were in acceptable ranges. Aflatoxin contents of urine sample were purified in our experiment by two method of Sep pack C_{18} column chromatogarphy and solvent extraction. Both methods proved to work in terms of percent recovery. These were confirmed by addaing radioactive AFB_1 to charcoal stripped sample to be in range of 95-98%.When 20 sample were purified in this way a correlation of 0.90 was observed between RIA and ELISA method of AFB_1 measurement in urine.

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