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DEVELOPMENT OF A RAPID AND SENSITIVE RADIOIMMUNOASSAY FOR MEASUREMENT OF AFLATOXIN B1 IN BIOLOGICAL SAMPLES

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ABSTRACT

Aflatoxin B1 (AFB) is a well known hepatocarcinogen in several animal species and probably a causative agent in human hepatocellular carcinoma. Humans are exposed to AFB by ingesting contaminated food. Aflatoxin contamination encountered in human foods is usually at low levels which is difficult to measure by chromatographic methods. Therefore in the present study we have developed an immunoassay for AFB detection which is specific, sensitive and reliable. This method is applicable to a variety of biological samples such as food products, serum, milk, urine, etc. The antibody produced against AFB-bovine serum albumin is highly specific with a low cross reactivity towards structurally-related aflatoxins. Other characteristics of this method including assay validation, reproducibility, recovery and statistical validations are discussed. We suggest the use of this technique as a routine method for screening food products designated for human consumption.
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INTRODUCTION

Aflatoxins are a group of mycotoxins produced as secondary metabolites by *Aspergillus flavus*. The fungus grows rapidly on a variety of foodstuffs under favorable conditions of moisture and temperature.[1] Among four naturally occurring aflatoxins, aflatoxin B1 (AFB) is the most potent hepatotoxic and hepatocarcinogenic agent in several animal species including man. The main route of human exposure to AFB is through ingestion of contaminated food.[1]

Several procedures have been developed for the measurement of aflatoxin levels in biological and industrial samples. These procedures are thin layer chromatography (TLC), high performance liquid chromatography (HPLC), radioimmunoassay (RIA) and enzyme-linked immunosorbantassay (ELISA).[3-6] In the developed countries, immunological techniques with relatively high specificity and sensitivity are being used for screening commercial and biological samples for AFB.

Due to the limits on animal feed with AFB contaminated grain of <300 ppb, regulatory agencies have an imperative need for a screening method which can quickly and accurately identify contaminated feed in order to prevent it from reaching the market. Our country is not however an exception, since apart from large quantities of pistachio nuts exported annually, we import large quantities of corn, wheat, etc. which are usually imported via southern ports with a favorable climate for the growth of aflatoxin-producing fungi.

To our knowledge, presently TLC and gas chromatography (GC) are the methods routinely used at the authorized institutes in Iran. In the present study, an attempt has been made to develop a rapid, sensitive and specific immunoassay for measuring the toxin in various clinical and commercial samples. Further as to our information, this is the first immunoassay reported for AFB in this country.

MATERIALS AND METHODS

Charcoal, dextran T-70, gelatin, complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), AFB1, AFB1 -1 ortho-carboxymethyloxime-BSA (AFB1-1O-CMO-BSA), and structurally-related aflatoxins such as AFB2, AFG2, AFM1, AFM2, AFP1, AFQ1 were obtained from Sigma Chemical Co., St. Louis, MO., U.S.A. Generally labeled ^{3}[H] AFB1 was purchased from American Radiolabeled Chemical Co., St. Louis, MO., 63146U.S.A. SEP-PAK cartridges (classic) were obtained from Milipore Coorporation, Milford, Massachusetts 01757. All other solvents and reagents were of analytical grade products of E. Merck, Germany.

Immunization

Two New Zealand white rabbits were injected with 500 µg each of immunogen (AFB-BSA) dissolved in 1.0 mL of physiologic saline and emulsified in 1.0 mL of CFA. The resulting emulsion was administered to 40 places on the back of the animal following the procedure of Vaitukaitis et al.[7] This was followed by a booster injection 30 days later, with 250 µg of immunogen for each animal emulsified in 1.0 mL of saline and 1.0 mL of incomplete Freund's adjuvant (IFA) intramuscularly into each thigh and shoulder. This was continued for6 months while the animals were injected every 15 days and bled seven days after each injection. Blood samples were obtained from the marginal ear vein and the serum was separated. Each bleeding was checked for titer, specificity and sensitivity. The sample was supplemented with a proper preservative and stored at -20°C until use.

Titer and dose-response assay

Each bleeding was checked for the titer and a standard curve was constructed following the procedure of Abraham et al.[8] Briefly; different dilutions of antibodies were prepared in RIA buffer (10 mmol PBS, pH= 7.4 containing 0.1% gelatin and 0.1% sodium azide) and dispensed 100 µL each to duplicate tubes. ^{3}[H] AFB was diluted in the same buffer and added to every tube (100 µL, approx. 10,000 cpm). These were then incubated overnight and the reaction was terminated by adding 200 µL of dextran-coated charcoal (prepared by adding 0.625g Norit A and 0.0625g dextran T-70 in 100 mL of RIA buffer) incubated for 10 min, centrifuged at 2000 g for 10 min. at 4°C and the supernatant was dispensed toa scintillation vial containing 5mL of scintillation cocktail (containing 0.5g popop and 0.25g of ppo in 2.5 liter of toluene) and counted in an LKB β-emitter spectrometer. Similarly for dose-response curve, various concentrations of aflatoxin standard from an ethanolic stock solution were prepared in RIA buffer and added along with antibody and ^{3}[H]-aflatoxin to each tube. Other procedures were reperformed as described above.

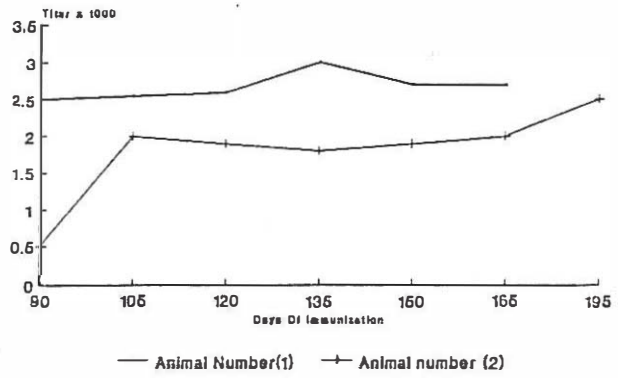

Fig. 1. Immunization schedule of antibody raised against aflatoxin B1-1 O-CMO BSA.

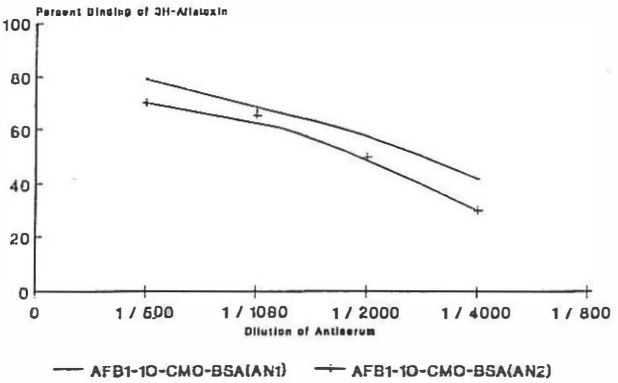

Fig. 2. Titration curve of antibody raised against aflatoxin B1-1O-CMO-BSA.

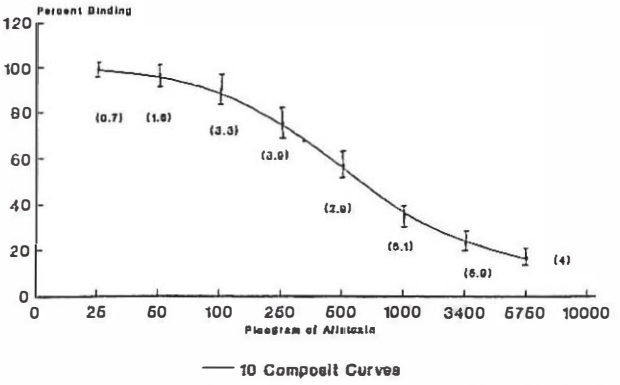

Fig. 3. Standard plot of anti-aflatoxin B1- 1O-CMO-BSA by RIA.

Cross Reaction

Possible structurally related aflatoxins such as AFB2, AFG2, AFM1, AFM2, AFP1 and AFQ1 were checked in parallel with AFB up to 200 ng of concentration.

Recovery

Human serum, urine and cow's milk were treated with dextran-coated charcoal and supplemented with exogenous aflatoxin in different ranges. The serum and milk samples

Table 1. Cross reactivities of antibody raised against AFB1-1 O-CMO-BSA with the structurally related compounds.

SN	Structurally-Related Aflatoxins	% Cross Reactivities AN(1)		% Cross Reactivities AN(2)	
		*	**	+	++
1	AFB1	100.0	100.0	100.0	100.0
2	AFB2	2.8	6.0	5.0	10.0
3	AFM1	3.75	12.5	2.0	15.0
4	AFG1	5.0	18.7	10.0	18.0
5	AFG2	1.0	5.0	1.0	2.0
6	AFQ	---	5.4	---	5.0
7	AFP	---	5.2	---	5.0

*,+ Bleeding After 45 Days.
**,++ Bleeding After 200 Days

Table II. Effect of time and solvent on the percentage of extraction

	Solvent				
Time* (min).	Acetone	Chloroform	Hexane	Ether	Ethanol
15	30 %	32.5 %	1 %	17 %	50 %
30	33 %	29 %	2 %	29 %	90 %
45	31 %	31 %	1.9 %	19.5 %	79 %
65	28 %	28 %	---	20 %	85 %

*shaking time

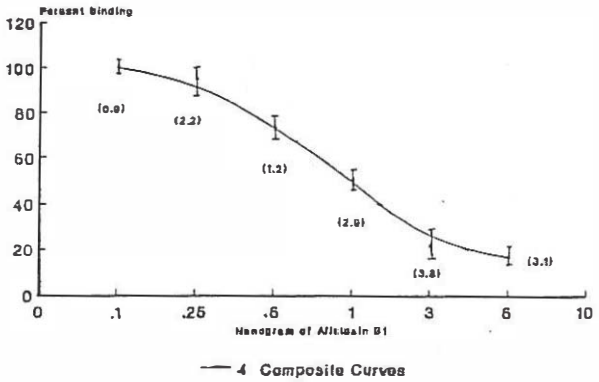

Fig. 4. Standard plot of anti-aflatoxin B1-1 O-CMO-BSA by RIA.

were then treated with about 2 ml ethanol and shaken for 30 min. at room temperature. The resulting suspension was then centrifuged at 11000g for 30 min. and the ethanolic phase was separated. These were then used for the assay directly. Urine samples were assayed directly without any pretreatment. To check for the best recovery conditions we also examined a number of common solvents such as chloroform, acetone, hexane and ether. Various shaking time periods (i.e.: 15, 30, 45, and 65 minutes) were tried in this relation.

Assay Validation

Inter-and intraassay validations were performed by recovering three different concentrations of sample standard following a similar procedure reported elsewhere[9] by repeating each concentration from the recovery step six times. The assay was then perfomed six times in six replicates.

Test of parallelism

A spiked sample of stripped urine, milk and serum was serially diluted and the assay was performed in parallel with the standard dose response as described.

Table III. Analytical recoveries of AFB1 added to charcoal-treated serum, urine and milk.

Conc.	AFB1 added pg/100 µL	SERUM Mean ± SE	CV%	URINE Mean ± SE	CV%	MILK Mean ± SE	CV%
Low	50	100 ± 27	1.26	94.2 ± 2.37	11.5	112.0 ± 2.5	9.13
	300	----------	------	----------	------	101.1 ± 1.4	6.92
Medium	1000	97 ± 4.07	4.19	----------	------	102.6 ± .23	5.58
	900	----------	------	101 ± 6.71	6.6	----------	-----
High	1500	92 ± 6.77	7.36	----------	------	98.5 ± 1.24	6.34
Very High	3000	----------	------	94.0 ± 0.7	0.7	----------	-----

Conc.= Concentration
Each concentration assayed 25 times

Table IV. Inter- and intra-assay variation of aflatoxin B1 added to charcoal-stripped milk.

Inter-assay	n	Mean ± SE Pool A ng/mL	CV%	Mean ± SE Pool B ng/mL	CV%	Mean ± SE Pool C ng/mL	CV%
1	6*	0.36 ± 0.04	11.2	1.0 ± .02	6.4	1.52 ± .08	5.6
2	6	0.28 ± 0.01	6.1	1.0 ± .03	8.1	1.44 ± .08	5.1
3	6	0.31 ± 0.03	11.7	1.0 ± .03	5.8	1.46 ± .17	11.7
4	6	0.29 ± 0.04	14.8	1.0 ± .06	5.9	1.42 ± .11	8.2
5	6	0.30 ± 0.03	9.5	1.1 ± .03	5.2	1.57 ± .08	5.3
6	6	0.31 ± 0.01	3.9	1.1 ± .04	5.3	1.57 ± .08	5.3
Intra-assay	6	0.30 ± .02	9.0	1.0 ± .05	4.76	1.49 ± .06	4.40

n= number of times assay carried out.
*= result of 6 replicates.

C18 column chromatography

In order to observe the effect of partial purification and concentration of the urine samples, we performed a C18 column chromatography for six samples following the procedure of Stubblefield et al.[10] Briefly, a C18 cartridge equipped with an adaptor was attached to a 30 mL syringe as sample reservoir. The cartridge was connected to a peristaltic pump and pretreated cartridge with 5mL methanol, followed by 5mL H_2O. A 10 mL clearified urine was added and the sample was drawn through the column at a flow rate of 20mL/min. The column was rinsed with 3mL water followed by 5mL acetonitrile-H_2O (15 + 85) at a flow rate of 1 drop/s. The cartridge was washed with 5mL H_2O (fast run) and allowed to run dry. Finally the AFB was eluted with 1.5 mL MeOH-H_2O (7 + 3).

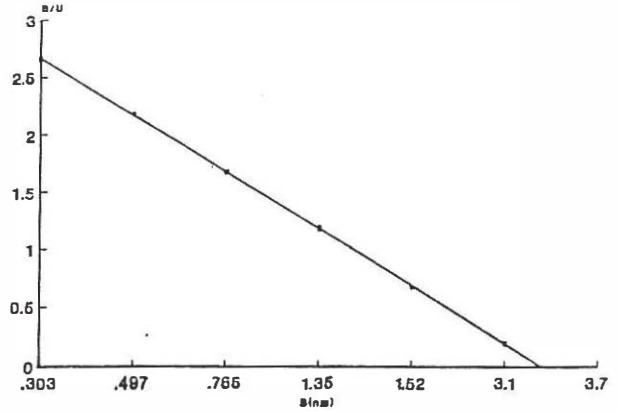

Fig. 5. Scatchard plot of anti-aflatoxin-B1-1O-CMO-BSA *by RIA.

RESULTS

Fig. 1 shows the immunization schedule for anti-aflatoxin B1-1O-CMO-BSA raised in two rabbits. Both animals reached approximately a titer of 1/2500 after six months of immunization (Fig. 2). Percent cross reaction of antibody with the structurally-related aflatoxins was calculated and shown in Table 1. The results presented denote that the antibody obtained is highly specific during the first four months of immunization but the cross reaction increases as time elapses (data not shown here). Results of 10 and 4 composite standard curves have been presented in Figs. 3 and 4. These results indicate that antibody raised in animal number 1 is of more sensitivity. In Figs. 5 and 6 the calculated affinity constant of antibodies are shown. These results show that antibody raised in animal number one

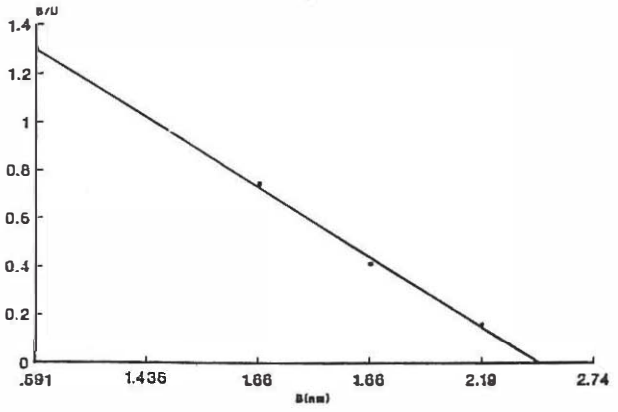

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Table IV. Inter- and intra-assay variation of aflatoxin B1 added to charcoal-stripped milk.

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n= number of times assay carried out.
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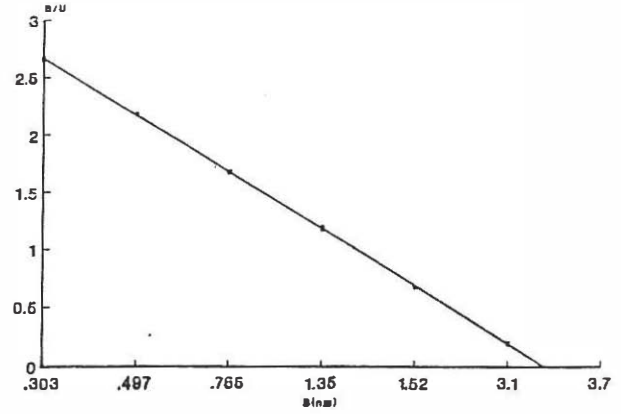

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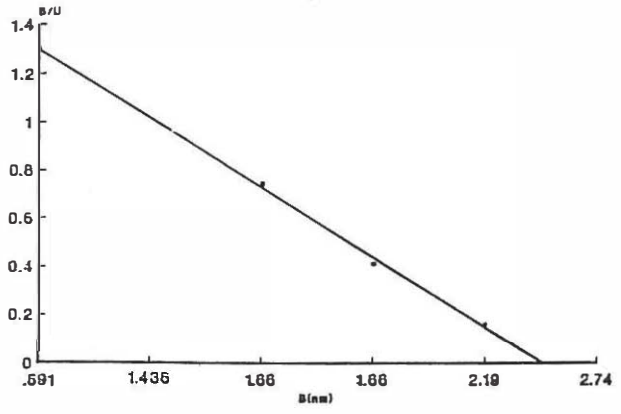

Fig. 6. Scatchard plot of anti-aflatoxin-1O-CMO-BSA* by RIA.

Table V. Inter - and intra-assay variation of aflatoxin B1 added to the charchoal-stripped serum.

Inter-assay	n	Mean ± SE Pool A ng/mL	CV%	Mean ± SE Pool B ng/mL	CV%	Mean ± SE Pool C ng/mL	CV%
1	6*	0.05 ± 0.00	0.2	0.88 ± 0.01	4.32	1.31 ± 0.4	8.26
2	6	0.05 ± 0.00	2.1	0.94 ± 0.05	1.4	1.24 ± 0.2	5.02
3	6	0.05 ± 0.00	0.3	0.85 ± 0.01	5.41	1.31 ± 0.4	8.67
4	6	0.05 ± 0.00	8.2	0.89 ± 0.02	5.97	1.21 ± 0.3	6.10
5	6	----------	-----	0.91 ± 0.01	3.98	1.29 ± 0.4	8.50
6	6	----------	-----	0.87± 0.01	4.73	1.29 ± 0.8	5.90
Intra-assay	6	0.05 ± 0.00	0.0	0.89 ± 0.03	3.55	1.27 ± .04	3.20

n= number of times assay carried out.
*= number of replicates.

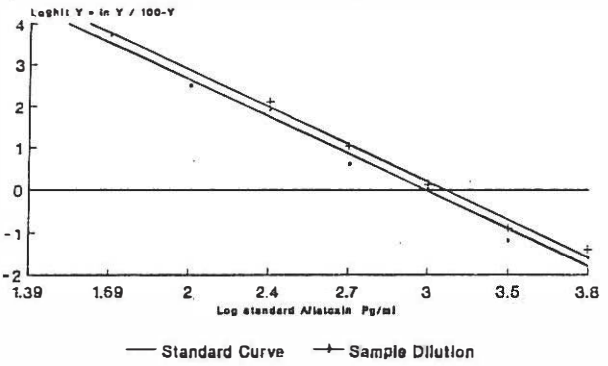

Fig. 7. Logit-log transformation of the dose response curve and sample dilution of milk by RIA (test of parallelism).

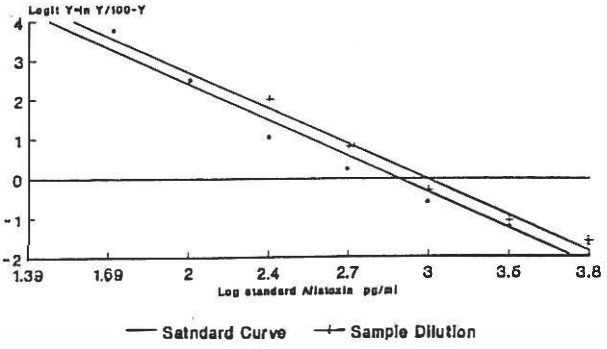

Fig. 8. Logit-log transformation of the dose reponse curve and sample dilution of serum by RIA (test of parallelism).

possesses a higher affinity, ie. 1.2×10 L/Mol. The data presented in Table II show thata one-step ethanol extraction among other solvents withoutany further treatment resulted in about 90-100% recovery after 30 minutes of shaking. The recovery data presented in Table III show that the assay is well validated in three ranges of low, medium and high concentration whereby between 92-102% recovery is obtained in different samples (urine, serum and milk). The precision profile of each assay system for three different samplesare listed in TablesIV, V and VIformilk, serumand urine respectively, each sample performed in six replicates and each assay repeated six times. Coefficient of variation (CV), standard error of mean (SE) and standard deviation (SD) were all within the acceptable ranges. In Figs. 7,8 and 9 the results of parallelism test are shown. The slopes of standard curve and sample dilutions in all three cases are in agreement. Finally, in Table VII we have presented data related to various clinical, normal and commercial samples. The results indicate the absence of aflatoxin in the urine and serum of normal males living in Tehran when using 200 µL of urine for analysis without any further treatment. However, when the same samples (10 mL) were passed through a C18 sep-pak column and concentrated for almost 20 times all samples under investigation showed AFB contamination between 80-100 pg/mL. We also detected 250 pg/mL of AFB in one out of 50 different milk samples obtained from various parts of the country. Similarly, we could detect that two samples (prediagnosed as liver cancer patients from northern regions of the country) out of 19 samples obtained from patients suffering from various cancers contained about250pg/mLaflatoxin. These results have been repeated for three times, every time with nearly similar results.

DISCUSSION

In the developed countries, chromatographic methods are being replaced by immunoassays for the detection of

Table VI. Inter- and intra-assay variation of aflatoxin B1 added to the charcoal-stripped urine.

Inter-assay	n	Mean ± SE Pool A ng/mL	CV%	Mean ± SE Pool B ng/mL	CV%	Mean ± SE Pool C ng/mL	CV%
1	6*	0.049 ± 0.00	2.0	0.83 ± 0.01	4.1	3.05 ± 0.03	2.74
2	6	0.046 ± 0.00	3.1	0.86 ± 0.02	8.02	3.01 ± 0.01	1.34
3	6	0.47 ± 0.00	3.3	0.83 ± 0.01	4.84	2.94 ± 0.02	1.67
4	6	0.049 ± 0.00	3.6	0.84 ± 0.02	5.81	3.10 ± 0.04	3.53
5	6	----------	-----	0.84 ± 0.01	4.62	3.21 ± 0.06	5.22
6	6	----------	-----	----------	-----	3.38 ± 0.03	2.47
Intra-assay	6	0.047 ± 0.00	3.1	0.84 ± 0.01	1.37	3.11 ± 0.15	5.08

n= number of times assay carried out.
*= number of replicates.

mycotoxins.[11-14] The presence of AFB has been encountered in several food and feed commodities imported to Iran (personal communication). In case of man, due to the mechanical elimination and processing of the contaminated food, only trace amounts of aflatoxin residues are left in the grains and their derived products. This minor quantity of AFB is practically not detectable by TLC, while the measurement of basal levels of AFB by immunological techniques possesses diagnostic value.

AFB like several other genotoxic agents is known to be mutagenic and carcinogenic *in vitro* and *in vivo* systems irrespective of the dose.[15-17] The measurement of AFB in human serum, urine and milk could therefore be of immense importance for monitoring the relationship between human exposure and the environmental contamination of the toxin.[10-16] The work presented here introduces a radioimmunoassay which is specific, simple, sensitive and reliable with a high throughput for the measurement of low levels of AFB1 in various samples. From the immunological point of view this technique has other advantages over similar methods reported elsewhere.[12,13] Use of one step ethanol extraction without any column chromatography has shown acceptable precision in routine operation. We also found that this type of extraction will result in an excellent recovery (i.e. 95-110%). Presence of AFB in human serum and urine is reported by many workers. In most of these studies the antibody used cross reacts with a number of structurally related aflatoxins.[14] Therefore, the results obtained can not be expressed as AFB content only. In these studies the column chromatography not only serves as a purification step but also results in considerable concentration of the sample. Here we have prepared a very specific antiserum with a high affinity and very low cross-reactivity with other aflatoxins. This will

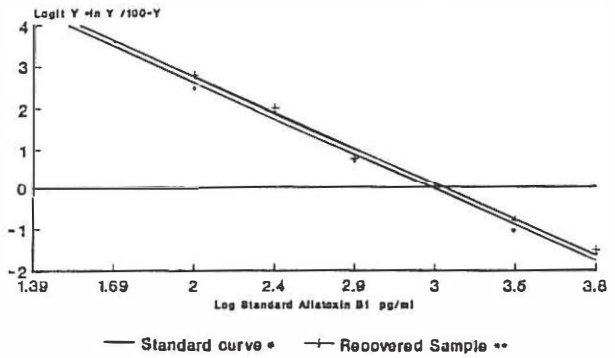

Fig. 9. Logit-log transformation of the dose response curve and sample dilution of urine by RIA (test of parallelism).

overlook the need for time-consuming extraction and purification chromatography for the purpose of purification and clean up. However, if this is required in the case of very low exposure our results indicate that the use of a C18 column to concentrate 10 mL urine will improve the assay sensitivity. To this end we found no significant difference using either a C18 column for one mL urine or assaying the sample directly without any pretreatment (data not shown). The low limit of AFB detection in this assay is 25 pg/tube i.e. using 0.2 mL urine, serum or milk extract (equivalent to 0.2 mL of sample) or 125 pg/mL. The standard curve is well validated between 25 pg up to 10ng. The inter-and intra-assay variations are well in the acceptable range (Tables IV-VI). Since this method is fully validated under various circumstances, we therefore suggest that it may replace current methods of AFB detection in this country.

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