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Solid-Phase Radioimmunoassay of Ochratoxin A in Serum

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A solid-phase radioimmunoassay (RIA) for the determination of ochratoxin A in serum was developed. [^{14}C]Ochratoxin A, with a specific activity of 130 Ci/mol, was used as tracer. Antibody was obtained by repeated injection of ochratoxin A–bovine serum albumin conjugate in rabbits. A rapid sample cleanup was achieved on a Sep-Pak C_{18} cartridge. Immobilization of antibody on different solid phases was tested. RIA with antibody coupled to protein A–Sepharose CL-4B allowed a detection of ochratoxin A in serum as low as 0.4 ng/mL. Recoveries in the 0.4–20 ng/mL range were 83.4–87.5% with standard deviations of 0.9–2.1%.

INTRODUCTION

The ochratoxins are a group of isocoumarin-containing toxic secondary metabolites produced by a number of fungal species of the *Aspergillus* and *Penicillium* genera. Ochratoxin A, the most toxic of this mycotoxin series, has been found in a number of agricultural commodities. Because of its association with mold-induced porcine nephropathy (Krogh, 1978) and with endemic Balkan nephropathy (Krogh et al., 1977), a fatal human kidney disease, several assay procedures of ochratoxin A in cereals have been developed (Lee and Chu, 1984; Rousseau et al., 1985).

One of the main problems in the analysis of cereals is the sampling of the suspected commodities. In very large parcels the moldiness mostly appears in so called "hot spots", which are isolated contamination zones. Since homogenization of the whole parcel is not possible, samples must be taken under well-defined conditions. Statistical analyses have shown that 5 kg of fine cereals to 25 kg of nuts are required to have a reasonable chance of encountering contaminated parts of a heterogeneous parcel (Dickens and Whitaker, 1982).

To overcome this problem, analysis of the toxin in biological samples (serum, urine, kidney) of slaughter animals, especially pigs, fed with the suspected cereals was suggested. Consequently the presence of ochratoxin A in serum and kidneys of pigs has been reported after detection by means of high-performance liquid chromatography (HPLC) (Bauer et al., 1984) and thin-layer chromatography (TLC) (Sandor, 1984). These results indicate that a broad survey of biological samples of slaughter animals is necessary. Ochratoxin A determinations by TLC and HPLC often require an extensive cleanup and consequently are time consuming. Immunoassay procedures have several advantages: they are very sensitive and specific and allow rapid determination of a large number of samples. Hence, an RIA for the detection of ochratoxin A in serum was developed in our laboratory.

As solid-phase systems offer several advantages, the binding of rabbit antibodies against ochratoxin A on various coated glass beads and on protein A–Sepharose CL-4B was thoroughly compared. Derivatized glass beads are often used for immobilization of proteins and enzymes (White and Kennedy, 1980; Slegers et al., 1984).

The characteristic biological property of protein A is its ability to interact with IgG molecules. Since the interaction of protein A and IgG does not involve the antigen-binding portion of the immunoglobulin molecule, it is possible to develop a competitive RIA with protein A–Sepharose CL-4B as solid phase, which to our knowledge, has not yet been employed.

EXPERIMENTAL SECTION

Materials. Ochratoxin A was obtained from Janssen Chimica (Beerse, Belgium) and RIALUMA (cumene) from Lumac/3M (Schaesberg, The Netherlands). Ochratoxin B was supplied by Dr. E. Creppy (Institut de Biologie Moléculaire et Cellulaire du CNRS, Strasbourg, France). Certified serum samples (one negative and three positives) of pigs were supplied by Dr. B. Hald (Royal Veterinary and Agricultural University, Copenhagen, Danmark). Protein A–Sepharose CL-4B and Sephadex G-25 columns were obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Controlled-pore glass beads of 500-Å pore size (80–100 mesh) were purchased from Pierce Chemical Co. (Rockford, IL), and Sep-Pak C_{18} cartridges were from Waters Associates (Millford, MA). Silanes were a gift of Union Carbide (Brussels, Belgium).

Apparatus. Scintillation counting was performed with a Packard Tri-Carb Model 3390 liquid scintillation spectrometer.

Synthesis of [^{14}C]Ochratoxin A. [^{14}C]Ochratoxin A was synthesized by methods previously described (Rousseau et al., 1984). Its specific acitvity was 130 Ci/mol.

Production of Ochratoxin A Antiserum. Synthesis of ochratoxin A–bovine serum albumin conjugate was

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Table I. Antibody Immobilization on Different Solid Phases

carrier	silane	spacer arm	active end gp	coupling type	% B[a]	% NSB[b]
CPG	A 1100[d]	glutaraldehyde	aldehyde	Schiff base	53.2	18.7
CPG[c]	A 1100	succinate	acid chloride	amide	55.3	4.1
CPG[c]	A 1100	succinate	*N*-hydroxysuccinimide	amide	51.5	0.4
CPG[c]	A 1100	thiophosgene	isothiocyanate	thiourea	55.1	3.3
CPG	A 1100	arylamine–thiophosgene	isothiocyanate	thiourea	57.2	15.1
CPG	A 1100	arylamine	diazonium	azo	59.8	37.4
CPG	A 1120	glutaraldehyde	aldehyde	Schiff base	56.5	23.2
CPG[c]		cyanogen bromide	cyanogen bromide	carbamate	56.9	3.3
CPG[c]		carbonyldiimidazole	carbonyldiimidazole	carbamate	53.5	0.3
CPG	A 187	glutaraldehyde	aldehyde	Schiff base	32.5	2.4
CPG	A 187	cyanogen bromide	carbamate	42.1	2.6	
CPG	A 187	carbonyldiimidazole	carbonyldiimidazole	carbamate	43.0	3.1
sepharose CL-4B[c]		CNBr–protein A	protein A		56.5	0.5

[a] % B = % bound of the zero standard. [b] NSB = nonspecific binding. [c] Coated beads for which a standard curve was established. [d] A 1100, $(C_2H_5O)_3SiCH_2CH_2CH_2NH_2$; A 1120, $(CH_3O)_3SiCH_2CH_2CH_2NHCH_2CH_2NH_2$; A 187, $(CH_3O)_3SiCH_2CH_2CH_2OCH_2$  .

performed by the method of Chu et al. (1976). Ochratoxin A antiserum was produced in rabbits by an immunization and bleeding schedule previously described (Rousseau et al., 1985).

Sample Preparation. A Sep-Pak C_{18} cartridge was wet with 2.5 mL of methanol and flushed with 7.5 mL of a 2% acetic acid solution. A 0.5-mL portion of serum, the pH of which was adjusted to ca. 4.5 with 1 M acetic acid, was applied onto the cartridge and slowly pumped through by means of a syringe. Impurities were removed with 10 mL of a 2% acetic acid solution. Ochratoxin A was eluted with 7.5 mL of methanol. The eluate was evaporated to dryness and dissolved in 100 μL of 0.1 M sodium phosphate buffer (NaPB), pH 7.4.

Antibody Immobilization on Various Coated Glass Beads. For the immobilization of antibodies on various coated glass beads, binding of impurities must be minimized. Hence, purification of antibodies was achieved by affinity chromatography. A 1.5-g sample of freeze-dried protein A–Sepharose CL-4B was swollen in distilled water. The swollen gel, approximately 5 mL, was equilibrated with phosphate-buffered saline (PBS), pH 7.2, in a column (150 × 9 mm). One milliliter of antiserum was applied onto the column. Impurities were eluted with PBS until absorbance at 280 nm was negligible. IgG was eluted in 10–15 mL of 1 M acetic acid. The eluate was immediately neutralized with 0.5 M Tris, desalted on a small Sephadex G-25 column, and lyophilized. The purity of the IgG was about 100% as controlled by electrophoresis and immunoelectrophoresis.

Controlled-pore glass beads with 500-Å pore size were ultrasonicated in 1 M HCl, washed with water, and hydrated by boiling in 5% HNO_3 for 1 h. After the beads were rinsed with water, derivatization was carried out under generally applied methods (Weetall and Hersh, 1970; Weetall and Filbert, 1974; Weetall, 1976; Regnier and Noel, 1976; Scouten, 1981).

The binding of IgG antibodies was performed by incubating 15 mg of beads with 20 μL of a 1:40 dilution of purified antiserum for 1 h at room temperature. The beads were then washed twice with 0.5 mL of 0.1 M NaPB, pH 7.0. Unreacted groups were blocked by shaking the beads with 100 μL of 2 M glycine for 15 min. Washing with NaPB was repeated.

Immobilization of Antibody on Protein A–Sepharose CL-4B. Eight milligrams of freeze-dried protein A–Sepharose CL-4B was swollen and equilibrated with 0.1 M NaPB, pH 7.0. The NaPB was removed, and the gel was incubated with 10 μL of a 1:75 dilution of unpurified antiserum for 1 h at 37 °C. Excess IgG and impurities were removed by washing twice with 0.5 mL of NaPB. The immobilized antibody was stable for weeks when stored at 4 °C.

Screening of the Antibodies Immobilized on Different Solid Phases. One hundred microliters of [^{14}C]-ochratoxin A tracer solution (ca. 8000 cpm) and 100 μL of 0.1 M NaPB, pH 7.0, were added to 15 mg of antibody-loaded glass beads. The mixture was shaken for 4 h at room temperature. The supernatant was collected, and the beads were washed twice with 0.5 mL of NaPB. The supernatants were pooled, radioactivity was measured, and the percent bound of the zero standard was calculated. To determine nonspecific binding, the procedure was repeated with unloaded glass beads.

The screening of protein A–Sepharose CL-4B with 8 mg of IgG-loaded protein A–Sepharose CL-4B was performed in the same conditions as described for the glass beads. Centrifugation for 30 s at 5000 rpm (710*g*) was needed before the supernatants were collected.

Radioactivity Measurements. Radioactivity was measured after mixing each sample with 8 mL of RIA-LUMA. Dpm calculations were made with [^{14}C]toluene as the internal standard. The counting efficiency was 85%.

RIA. A 100-μL portion of standard solutions of ochratoxin A or purified unknown sample and 100 μL of [^{14}C]ochratoxin A (ca. 8000 cpm) in 0.1 M NaPB, pH 7.4, were added to 8.0 mg of antibody-loaded protein A–Sepharose CL-4B, and the mixture was shaken for 4 h at 37 °C. After centrifugation for 30 s at 5000 rpm (710*g*), the supernatant was collected. The gel was washed twice with 0.5 mL of NaPB and centrifuged again, and the supernatants were pooled. Radioactivity was measured and ochratoxin A concentration calculated by a curve of percent bound vs. log concentration of standards.

RESULTS AND DISCUSSION

Comparison of Antibody Immobilization on Different Solid Phases. Results of the antibody immobilization on different solid phases are shown in Table I. A standard curve was determined for the best five results of the antibody immobilization on glass beads and for protein A–Sepharose CL-4B. Figure 1 shows that an RIA with protein A–Sepharose CL-4B as the solid phase has the highest sensitivity. A linear relationship between percent bound and log concentration exists in the 0.2–10 ng/assay range. Good within-day reproducibility of the standard curve as well as good day-to-day reproducibility were obtained. Standard deviations in the 0.2–10 ng/assay range were, respectively, within 1.8% and within 3.5%.

When a standard curve was established by means of spiked serum samples, purified with the Sep-Pak C_{18} column cleanup, no interferences could be detected.

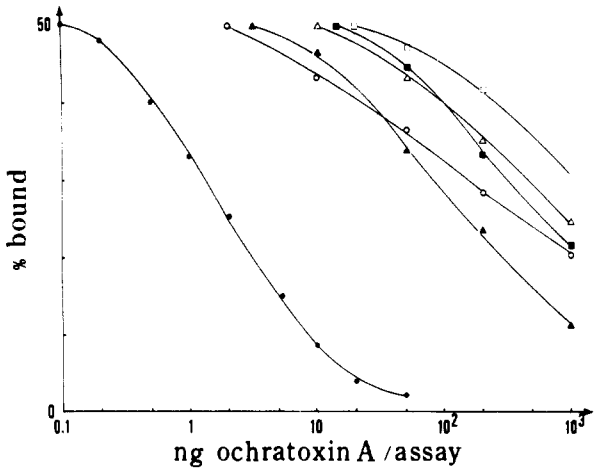

Figure 1. Standard curves for the determination of ochratoxin A with antibody immobilized on different solid phases: protein A–Sepharose CL-4B (●); CPG–cyanogen bromide–cyanogen bromide (▲); CPG–A 1100–succinate–*N*-hydroxysuccinimide (○); CPG–A 1100–succinate–acid chloride (△); CPG–carbonyldiimidazole–carbonyldiimidazole (■); CPG–A 1100–thiophosgene–isothiocyanate (□).

Hence antibody binding on protein A–Sepharose CL-4B was the method of choice.

Optimization of the RIA Procedure. The different parameters of the binding of IgG antibodies on protein A–Sepharose CL-4B as well as the interaction of antigen with immobilized IgG antibodies were tested in order to determine the optimal reaction conditions. NaPB and PBS were investigated as incubation buffer. NaPB showed the best results. Different NaPB concentrations, pH values, and incubation times and temperatures were tested. Optimal binding of IgG antibodies on protein A–Sepharose C-4B was obtained in 0.1 M NaPB, pH 7.0, and after 1 h incubation at 37 °C (Figure 2a–c). Optimal interaction of antigen and immobilized IgG antibodies was obtained after 4-h incubation at 37 °C in 0.1 M NaPB, pH 7.4 (Figure 2d–f). RIA was then performed in these optimized conditions as described in the Experimental Section.

Table II. Recovery (%) ± One Standard Deviation of Ochratoxin A from Serum after Sample Cleanup[a]

toxin added, ng/mL	reproducibility	
	within-day	day-to-day
0.4	83.4 ± 2.1	83.8 ± 2.6
1	84.9 ± 1.9	85.2 ± 2.6
5	85.6 ± 1.9	85.6 ± 2.4
10	87.3 ± 1.1	85.5 ± 2.1
20	87.4 ± 1.0	87.8 ± 1.8

[a] Data are means of four to six analyses.

Cross-Reactions. The cross-reactivity of the antibody with different ochratoxin A analogues was tested by competition experiments of [^{14}C]ochratoxin A with ochratoxin A, ochratoxin B, ochratoxin α, β-L-phenylalanine, and bovine serum albumin over a wide concentration range. The concentrations that give 50% displacement of [^{14}C]-ochratoxin A were found to be 3 ng for ochratoxin A, 10^5 ng for ochratoxin B, and even more for all other analogues, which indicates that the immobilized IgG is very specific to ochratoxin A but not to its analogues (Figure 3).

Recovery Studies. To test the efficiency of the Sep-Pak C_{18} column cleanup, known amounts of ochratoxin A were added to serum (0.4–20 ng/mL) and the purified extracts were subjected to RIA. Within-day and day-to-day reproducibility were also tested and were found to be very good even at a concentration of 0.4 ng/mL (Table II).

Three serum samples of ochratoxin A contaminated pigs were also subjected to Sep-Pak C_{18} column cleanup and RIA. Concentrations of 200–350 ng/mL of ochratoxin A were found. These results were confirmed by means of HPLC analysis (Schweighardt et al., 1980) (Table III).

CONCLUSION

In this study an optimized radioimmunoassay was developed and validated for the measurement of ochratoxin A in serum. As the method allows a low detection limit

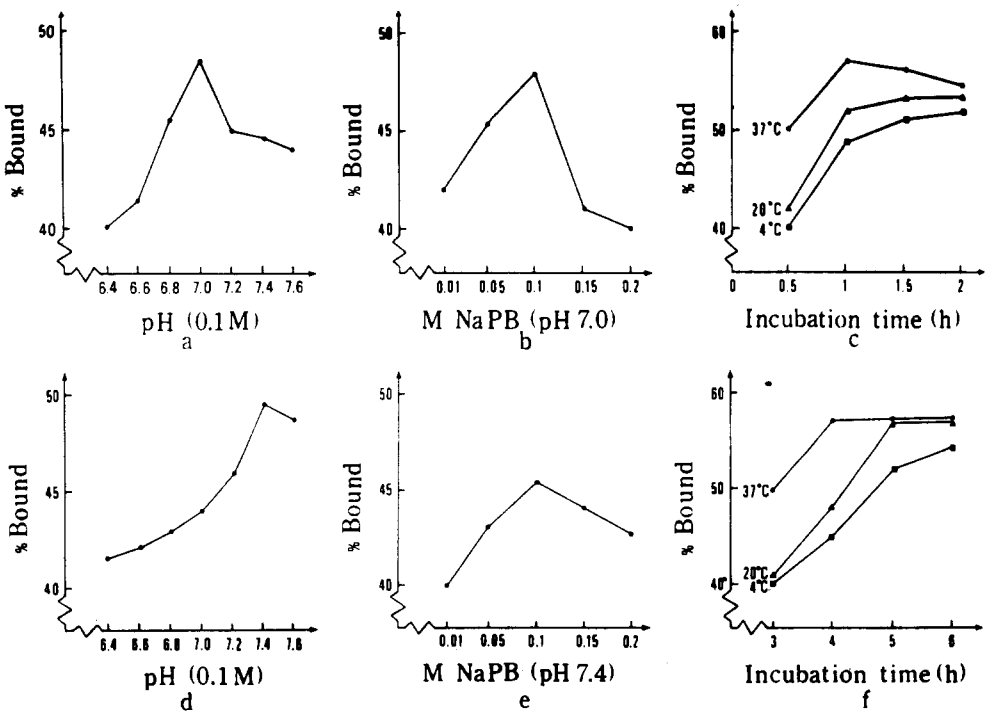

Figure 2. Optimization of the IgG antibody immobilization on protein A–Sepharose CL-4B: (a) influence of pH of 0.1 M NaPB; (b) influence of NaPB concentration at pH 7.0; (c) influence of incubation times and temperatures. Optimization of the interaction of antigen and immobilized IgG antibodies: (d) influence of pH of 0.1 M NaPB; (e) influence of NaPB concentration at pH 7.4; (f) influence of incubation times and temperatures.

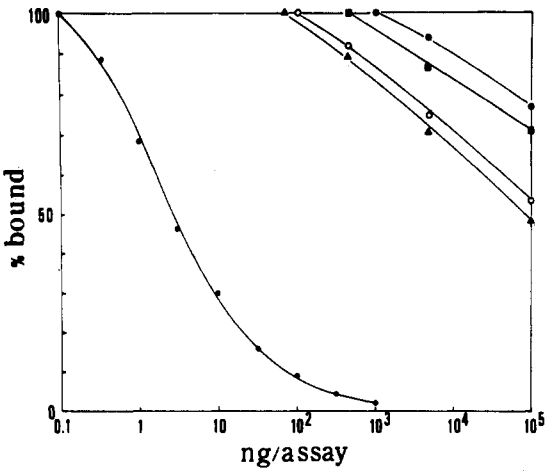

Figure 3. Effect of structural analogues of ochratoxin A on the binding of [^{14}C]ochratoxin A with IgG: ochratoxin A (●); ochratoxin B (▲); bovine serum albumin (○); β-L-phenylalanine (■); ochratoxin α (*). The extent of binding of [^{14}C]ochratoxin A with IgG in the absence of unlabeled toxin was considered as 100% of binding.

Table III. Comparison between RIA and HPLC Analysis of Porcine Serum Samples[b]

	RIA, ng/mL	HPLC, ng/mL
neg sample	<0.4[a]	<5[a]
pos sample 1	350.4 ± 6.4	363.1 ± 9.3
pos sample 2	199.6 ± 3.7	209.4 ● 5.5
pos sample 3	274.7 ± 5.3	282.7 ± 7.4

[a] Detection limit. [b] Data are means of three to five analyses.

(0.4 ng/mL) and as the entire procedure can be done in 1 day, the method can be useful for broad surveys of biological samples of slaughter animals.

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In Vitro Metabolism of T-2 Toxin by Rat Liver Microsomes

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Rat liver microsomes biotransform T-2 toxin in vitro to a variety of metabolites including HT-2, neosolaniol, 4-deacetylneosolaniol, T-2 triol, 3′-OH T-2, and 3′-OH HT-2, in addition to two unidentified compounds. Metabolism resulting from cleavage of the ester linkages of the parent T-2 toxin was more extensive than hydroxylation of the 3′-carbon side chain by mixed-function oxidases. Treatment of rats with phenobarbital resulted in enhanced oxidative hydroxylation of T-2 toxin at the 3′-carbon position, and addition of paraoxon to the microsomal preparation inhibited the hydrolysis of the C3′-oxidized product.

INTRODUCTION

T-2 toxin (4β,15-diacetoxy-8α-[(3-methylbutyryl)oxy]-3α-hydroxy-12,13-epoxytrichothec-9-ene) is a trichothecene mycotoxin produced by species of *Fusarium* (Bamburg and Strong, 1971; Pathre and Mirocha, 1977). Although detected only sporadically in nature compared with other trichothecene mycotoxins such as deoxynivalenol, T-2 has been implicated in several serious cases of human and animal toxicoses (Hsu et al., 1972; Joffe, 1971; Bamburg et al., 1971).

The distribution and metabolism of tritium-labeled T-2 toxin was investigated after oral administration in chickens (Yoshizawa et al., 1980a), mice (Matsumoto et al., 1978), and a lactating cow (Yoshizawa et al., 1981) and after intravascular administration in swine (Corley et al., 1985). In all species, T-2 was rapidly biotransformed to a variety of metabolites. Minor metabolites in the cow and chicken were initially identified as simple hydrolysis products including HT-2, 4-deacetylneosolaniol, and neosolaniol; however, the major metabolites remained unidentified.

Yoshizawa et al. (1982) characterized two of the main metabolites present in bovine urine, initially labeled TC1 and TC3, as 3′-OH T-2 and 3′-OH HT-2, respectively. Recently a third metabolite, TC6, was tentatively identified as 3′-OH-7-OH HT-2 (Pawlosky and Mirocha, 1984).

The compound HT-2 was reported as the only in vitro metabolite in human and bovine liver homogenates (Ellison and Kotsonis, 1974) and laboratory animals (Ohta et al., 1977). In addition to HT-2, 4-deacetylneosolaniol,

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