Monoclonal Antibodies for the Enzyme Immunoassay of the Mycotoxin Cyclopiazonic Acid

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Murine monoclonal antibodies (MAB) were raised from hybridomas obtained by fusions of splenocytes from Balb/c mice immunized intraperitoneally with cyclopiazonic acid-keyhole limpet hemocyanin (CPA-KLH) conjugates, with different myeloma lines. Hybridomas secreting antibodies against CPA were detected by enzyme immunoassay, purified by limiting dilution cloning, and adapted to a batch fermentation process for the large-scale production of MAB. From 15 independent hybridomas obtained, 1 was used further. MAK S14-2-B6 (IgG_1) is extremely selective for cyclopiazonic acid (the only cross-reactant detected so far is cyclopiazonic acid imine, which displays a cross-reaction of 20%) and can be used in an antibody-immobilized enzyme immunoassay format measuring from 7 to 300 ng per assay of the mycotoxin. The intra-assay coefficient of variation is 2.5%, and inter-assay variability is 4.4% (within-day) and 8.1% (between-day). Crude extracts from fungal strains can be analyzed for CPA, thus allowing an efficient screening of fungal isolates for toxin production. The assay can be used to analyze white-mold fermented cheese for CPA.

Cyclopiazonic acid (CPA, 1), a metabolite synthesized from tryptophan and acetate by several fungi of the genera *Aspergillus* (Gallagher et al., 1978) and *Penicillium* (Holzapfel, 1968), produces necroses in spleen, liver, pancreas, and kidney tissue and, when administered intraperitoneally, is acutely toxic to rats, while the oral toxicity is much lower (Purchase, 1971). On a molecular basis, CPA was shown to strongly inhibit the Ca^{2+}-ATPase of sarcoplasmic reticulum (Seidler et al., 1989). This may explain the tremorgenic effects of high doses of CPA.

Interest in monitoring CPA stems from the occurrence of CPA-producing fungal species belonging to the genera *Aspergillus* and *Penicillium* on stored grain and seeds, cereal products, nuts, and dried foods (Gallagher et al., 1978; Trucksess et al., 1987; Lansden and Davidson, 1983; Ross et al., 1991). CPA is also produced commonly by *P. camemberti* (Geisen et al., 1990, and references cited therein) used as starter culture in the production of white-mold fermented cheeses (Le Bars, 1979), and the presence of CPA in these cheeses has been reported (Still et al., 1978; Le Bars, 1979).

Recently, we have shown that high-affinity polyclonal antibodies against CPA can be raised when rabbits are immunized with CPA-protein conjugates prepared by Mannich condensation of the toxin to the immunogenic carrier (Hahnau and Weiler, 1991). These sera proved to be highly specific and, in unprocessed fungal extracts, selectively detected CPA without apparent cross-reactions by other metabolites. While this assay considerably facilitates CPA analysis, the limited availability of antisera precludes widespread use of CPA immunoassays, e.g., in food screening, in the monitoring of cheese products for CPA content, as well as in selection programs for toxin-free industrial starter strains. To this end, we have produced MAB against CPA which match the previously reported antisera in terms of specificity and allow us to quantitate nanogram amounts of the toxin in fungal extracts and cheese products without the necessity of cumbersome sample cleanup procedures.

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MATERIALS AND METHODS

Materials. Cyclopiazonic acid and moniliformin were purchased from Sigma Chemical Co.; tenuazonic acid, fumitremorgin B, paxillin, and penitrem A were kind gifts of Dr. Fink-Gremmels, Utrecht. Ergocristin base and dihydroergocristin methanesulfonate were gifts from TAD Pharmazeutische Werke, Cuxhaven, Germany. Microtitration plates (96-well, flat-bottom type, ELISA grade) were from Greiner, Nürtingen, Germany (catalog no. 655081).

Fungal Material. Reference strains were those described earlier (Hahnau and Weiler, 1991). White-mold and blue-mold cheeses were obtained from local markets and used to isolate fungal strains. Blue-mold cheese fungal isolates and white-mold cheese fungal isolates were grown as described (Hahnau and Weiler, 1991). These strains were designated B-codes and W-codes, respectively.

Synthesis of CPA-Protein Conjugates. CPA was linked to alkaline phosphatase using the formaldehyde condensation technique (Hahnau and Weiler, 1991). CPA conjugation to keyhole limpet hemocyanin (KLH) was performed as follows: CPA (5.0 mg, 14.9 μmol) was dissolved in 1.0 mL of 0.05 M $NaHCO_3$ in 50% (by vol) aqueous methanol. KLH (10.42 mg) was dissolved in 0.2 mL of H_2O, and 0.4 mL of 3 M sodium acetate solution was added. To this solution was added dropwise 0.2 mL of an aqueous solution of formaldehyde (37% by wt) with stirring over 1 min at room temperature. After 10 min, the solution of CPA was added (dropwise over 5 min) while stirring was continued. The reaction mixture was then stirred for 16 h at room temperature, followed by dialysis against water at 4 °C (3 days, 3 × 10 L of water/day). The product was lyophilized, redissolved to a protein concentration of 1 mg/mL in phosphate-buffered saline (6 mM K_2HPO_4, 2 mM NaH_2PO_4, 150 mM NaCl, pH 7.4; PBS), and stored aliquotted at −18 °C until use.

Immunization Protocol. Balb/c mice (10–12 weeks old) were given intraperitoneal injections of 0.1 mg of CPA-bovine serum albumin conjugate (Hahnau and Weiler, 1991), dissolved in PBS and emulsified with an equal volume of complete Freund's adjuvant at days 0, 7, and 14, followed by three injections of the same dose of immunogen, but with use of incomplete Freund's adjuvant, at days 30, 37, and 44. One month later, three weekly injections of 0.01 mg of the new CPA-KLH conjugate in complete Freund's adjuvant were given intraperitoneally, followed by three intraperitoneal boost injections of 0.01 mg of the conjugate dissolved in PBS. Splenocytes were removed from the immunized animals one day after the final boost injection.

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Cell Fusion and Hybridoma Culture. Splenocytes from immunized animals were fused with myeloma cells, line P3-X63-Ag.8.653 (Kearney et al., 1979) or PAI (Stocker et al., 1982), using poly(ethylene glycol) and subjected to HAT selection (Galfre and Milstein, 1981). The production of antibodies directed against CPA was monitored by enzyme immunoassay as described below. Cells from positive wells were subjected to recloning by limiting dilution, and this step was repeated three more times, until a stable and homogeneously positive response was obtained for all subclones. MAB preparations were the culture fluids from 1–2-L batch cultures grown to stationary phase. The antibody subclasses were determined by double-diffusion analysis (Ouchterlony, 1958).

Enzyme Immunoassay. Flat-bottom polystyrene microtitration plates were precoated overnight at 4 °C with 0.2 mL per well of a rabbit anti-mouse immunoglobulin (RAMIG) solution (0.025 mg/mL of 50 mM $NaHCO_3$, pH 9.6). This antibody was raised in rabbits by immunizing with mouse immunoglobulin (Sigma, catalog no. 15381) using standard procedures (Weiler, 1986) and purified by the rivanol–ammonium sulfate technique (Hurn and Chantler, 1980). After coating, plates were emptied and rinsed twice with deionized water. Then the wells were loaded with 0.2 mL of the MAB [cell culture fluid suitably diluted with TBS buffer (50 mM tris(hydroxymethyl)aminomethane, 150 mM NaCl, 1 mM $MgCl_2$, pH 7.8)]. After overnight incubation at 4 °C, the plates were washed two times with deionized water and were then blocked with TBSG buffer [TBS buffer containing 0.1% (by wt) gelatin] for 1 h at 37 °C. After the blocking solution was decanted, plates were ready to use.

For the assay, 50 μL of TBSG buffer was delivered into each well followed by 100 μL of CPA standard or sample solution [suitably diluted in TBS buffer; for the determination of maximum tracer binding, B_0, buffer alone was delivered, for the determination of unspecific binding, UB, an excess (5 nmol/well) of CPA was used]. The plates were mixed, covered, and incubated at 37 °C for 30 min. Then 50 μL of the CPA–alkaline phosphatase conjugate suitably diluted in TBSG buffer (representing 49 pkat of enzyme activity) was added to each well. After mixing, the plate was covered again and incubated at 37 °C for 2.5 h. Plates were then washed two times with deionized water, and substrate (0.2 mL of a solution of 1 mg/mL of *p*-nitrophenyl phosphate in 1 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8) was added. The reaction was allowed to proceed for 1 h at 37 °C and was then stopped by the addition of 50 μL of 5 N KOH. Absorbances were read differentially at 405–620 nm in an SLT EAR 400 microplate reader.

Extraction and Sample Preparation. Fungal mycelia and agar samples were extracted as described (Hahnau and Weiler, 1991). Cheese samples were extracted as follows: Per gram of product, 2 mL of methanol was added and the sample shaken at 4 °C for 16 h. After centrifugation, the clear supernatant was separated and diluted 10-fold with TBS buffer and aliquots of 0.1 mL were analyzed by enzyme immunoassay.

Immunohistograms were used to analyze for potential cross-reactants present in the biological extracts. These were obtained as follows: Methanolic extracts from agar surface cultures of fungi were filtered through 0.45-μm Spartan filters (Schleicher & Schuell, Dassel, Germany). Aliquots of 0.1 mL of these extracts were subjected to HPLC using the procedure of Urano et al. (1992) with slight modifications as follows. Separations were carried out on a Biophase Octyl, 5-μm narrow-bore column (Bioanalytical Systems, Inc.), 200 mm × 2 mm i.d. [solvent A, methanol/water (85:15 by vol); solvent B, 4 mM $ZnSO_4$ in methanol; solvent program, 2 min solvent A, then within 10 min from A to 100% solvent B (linear gradient), followed by 10-min elution with 100% B and return to 100% A within 2 min; flow rate, 1 mL/min]. Elution was monitored at 279 nm. Fractions of 0.5 mL were collected and supplemented with 0.1 mL of TBS buffer. Then the methanol was evaporated. Finally, the samples were made up to 1.0 mL by the addition of further TBS buffer. Aliquots (0.1 mL) of these samples were subjected to immunoassay.

In each case, triplicate analyses were performed.

Table I. Characteristic Parameters for the Competitive Enzyme Immunoassay of Cyclopiazonic Acid

parameter	value
measuring range, ng of CPA per assay	7–300
midrange (B/B_0 = 50%), ng per assay	50
unspecific binding, %	0
intra-assay variability,[a] CV (n = 4)	2.5
inter-assay variability,[a] % CV	
within day (n = 4)	4.4
between days (n = 10)	8.1

[a] Averaged for all standards throughout measuring range.

RESULTS AND DISCUSSION

Generation and Properties of Monoclonal Antibodies against CPA. While conjugates of CPA and BSA were found to be immunogenic in rabbits (Hahnau and Weiler, 1991), none of the Balb/c mice immunized with this type of conjugate developed measurable titers of anti-CPA antibodies. Therefore, we switched to KLH, a protein strongly immunogenic in mice, as a carrier protein during the course of the immunization. At the stage of splenocyte removal, anti-CPA antibodies were detected in the serum of the animals. From six independent fusions, a total of 15 primary hybridomas screening anti-CPA antibodies were selected, and the cell lines were purified by repeated recloning. The MAB from three of the cell lines (S14-6-C2, S14-2-B6, and S14-8-A6) were characterized in more detail, but the results for only one of the antibodies, secreted by the hybridoma line S14-2-B6 (fusion partner P3-X63-Ag.8.653), are detailed here. The cell line secretes an IgG_1 antibody as determined by Ouchterlony double-diffusion analysis. Binding of CPA to all MAB was relatively pH-independent in the range from 6 to 8 but sharply declined at pH >8.0 and at acidic pH below 5. Therefore, pH 7.8 was chosen for all further tests. The use of rabbit anti-mouse immunoglobulin polyclonal antibody-precoated polystyrene plates rather than untreated plates to accommodate the MAB has several advantages recognized earlier (Weiler, 1986): (i) it reduces within-plate variability due to differences in absorbing capacity on the test plates and (ii) it eliminates the necessity to use highly enriched MAB to achieve high coating efficiencies, which is especially useful when screening. The amounts of specific MAB and of CPA–alkaline phosphatase used were adjusted so that both were present in limiting amounts, giving, under the standard assay conditions detailed under Materials and Methods, [ΔOD(405–620 nm), B_0] – [ΔOD(405–620 nm), UB] = 0.6. This was achieved at a final tracer dilution of 1:5000 (49 pkat of enzyme activity per well) and with hybridoma culture fluid diluted between 20- and 40-fold during MAB coating.

Assay Sensitivity and General Characteristics. The timing of assay steps was based on detailed CPA and tracer binding experiments (not shown). Kinetics followed usual time courses. The determination of the general assay characteristics was achieved using established routines, and the values obtained are given in Table I. The measuring range was defined as that part of the standard curve which gave linearity in the logit–log plot (cf. Figure 1, inset). As compared to the serum-based assay (Hahnau and Weiler, 1991), the MAB-based technique displays a narrower useful measuring range and thus a better precision. This is also evident from the assay variability data. The MAB-based assay is, however, considerably less sensitive than the serum-based technique. In the case of CPA analysis, this is a definite advantage because the use of the serum-based immunoassay required relatively

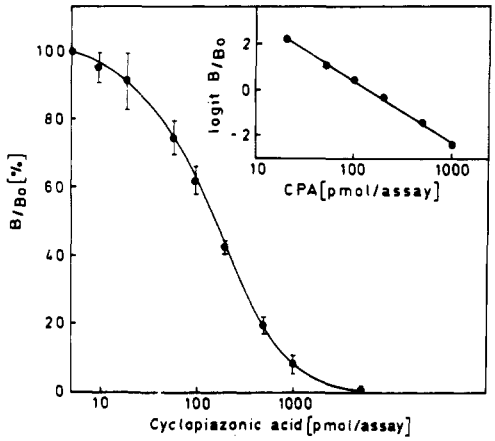

Figure 1. Standard curve for the competitive enzyme immunoassay of cyclopiazonic acid based on MAB S14-2-B6. The curve shown is the mean ± sd of 10 individual standard curves run on differents days. The insert shows a logit–log plot of the data to visualize the useful assay range.

high dilutions of samples, thus introducing serial dilution error. As given under Materials and Methods, convenient, one-step dilutions giving much less error are sufficient for the MAB-based technique.

Assay Specificity. To obtain some general information about the structural requirements for the binding of CPA to the monoclonal antibodies, a range of structurally related molecules was assayed for cross-reactions. The structures of CPA (1) and of these compounds (2–11) are shown in Figure 2. The MAB proved highly specific. Except for CPA-imine (2), which has a cross-reactivity of 20%, none of the compounds tested (3–11) gave any detectable cross-reaction up to the highest concentration assayed, which means that any potential cross-reaction would be much lower than 0.0008% (on a molar basis, compared to CPA = 100%). CPA-imine is spontaneously formed from CPA in the presence of aqueous ammonia. The imine has been isolated from fungal mycelium but is not considered a true metabolite, but rather a product of reaction of CPA with the cellular pool of free ammonia (Holzapfel et al., 1970). The techniques described here do not lead to the formation of CPA-imine during processing, which would lead to a loss in immunoassayable CPA and thus underestimation. However, it has to be noted that some HPLC techniques and also TLC methods use solvent systems containing ammonia [for a survey of TLC systems for mycotoxins, see Betina (1985) and Goto et al. (1987)]. We found that under these chromatographic conditions CPA may be converted quantitatively to the imine, precluding immunological analysis (not shown). It should also be noted that the cross-reactivity of CPA-imine with the serum 527 P (Hahnau and Weiler, 1991) is zero. The non-cross-reacting metabolites tested in this study include the biosynthetic precursor of CPA, tryptophan (5), as well as a widespread, simple, tryptophan metabolite, indoleacetic acid (6), the isoleucine analogue of CPA, tenuazonic acid (Stickings and Townsend, 1961) (3), a range of other indole-derived mycotoxins, such as paxillin (7), fumitremorgin B (4), and penitrem A (8), and the ergot alkaloids ergocristin (9) and dihydroergocristin (10). The loosely related mycotoxin moniliformin (11) was not cross-reactive either, indicating that simple diketones are not recognized by the antibody. KHL and BSA, the two carriers used for immunization, also had no effect on the binding of CPA to the MAB.

To further substantiate the selectivity of the assay, a typical CPA producer, *P. camemberti* SP 438, grown as an agar surface culture, was extracted (together with the

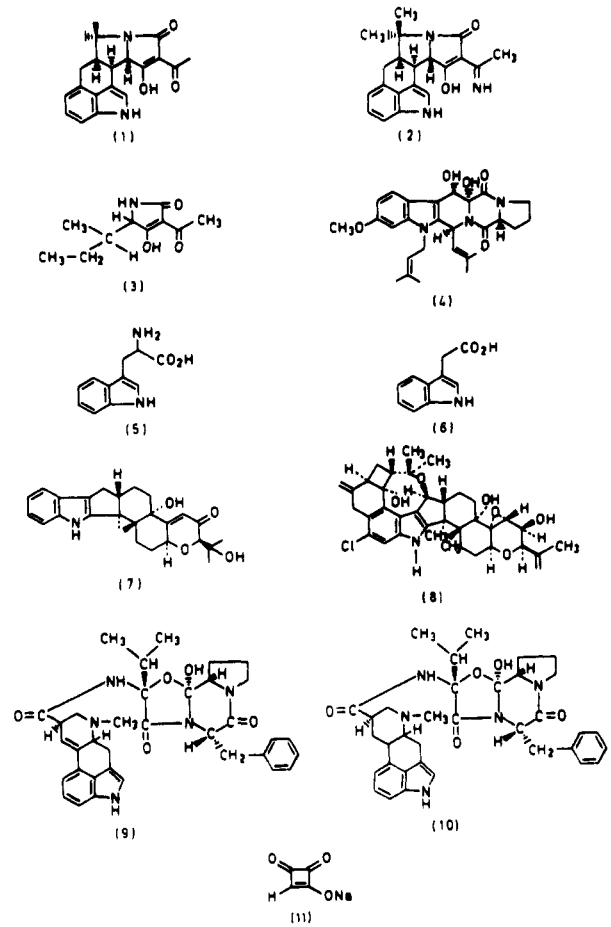

Figure 2. Structures of CPA (1) and related compounds used to analyze cross-reactivity. The names for all compounds are given in the text.

agar) as described and the extract subjected to HPLC. The distribution of immunoreactivity was analyzed by enzyme immunoassay. The result is shown in Figure 3. To obtain this figure, the immunological background recorded for a blank run had to be subtracted on a fraction-to-fraction basis, because it was observed that the increasing amounts of $ZnSO_4$ from the gradient led to a steady rise in immunological background which was due to interference of $ZnSO_4$ with the assay. It can be seen that the only immunoreactive zone detected in these extracts is located at the position of CPA. Recovery of CPA from spiked extracts after HPLC was found to be quantitative within the limits of experimental error.

CPA Production by Fungal Agar Surface Cultures. It was one of the aims of the present study to devise a rapid assay to be used in screening fungal strains for CPA nonproducers. First, the recovery of CPA added to agar in defined amounts before pouring the plates was analyzed (Table II). Approximately 80% of the added CPA was recovered by our extraction procedure. A range of fungal reference strains (Hahnau and Weiler, 1991), among them known CPA producers as well as nonproducers, showed CPA production for all of the reported producers but none for the reported nonproducers (not shown). This finding was substantiated by the analysis of fungal isolates obtained from commercial white- and blue-mold fermented cheeses (Table III). All tested white-mold cheese isolates were CPA producers, while none of the blue-mold cheese isolates gave indications for the presence of CPA. The small positive values obtained in these cases reflect the cumulative analytical background of the method. Thus,

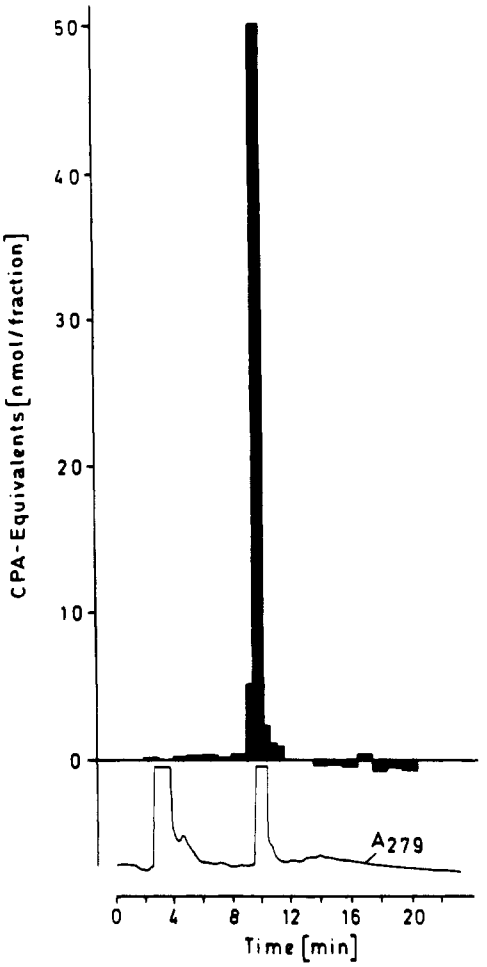

Figure 3. Distribution of immunoreactive material in fractions of an extract from *P. camemberti* Sp 438 after separation by HPLC. For this experiment, 2 g of mycelium together with underlying agar was extracted with 5 mL of extractant as detailed under Materials and Methods. After processing, an aliquot of 0.1 mL, representing 10% of the original extract, was subjected to HPLC.

Table II. Recovery of Cyclopiazonic Acid from Agar

CPA added, μg/g of agar	CPA recovered,[a] μg/g of agar	recovery, %
100	87.5 ± 21.3	88
80	62.6 ± 19.5	78
40	30.0 ± 8.3	75
20	16.0 ± 2.5	80

[a] Mean ± sd from a triplicate analysis.

Table III. Production of Cyclopiazonic Acid in Agar Surface Cultures of White- (W) and Blue-Mold (B) Cheese Fungal Isolates

strain	CPA,[a] μg/g	strain	CPA,[a] μg/g
W6	15.9	B1	1.3
W9	19.1	B2	0.8
W12	23.1	B3	0.1
W13	34.3	B4	0.2
W14	20.4	B5	0.4
W15	19.6	B6	0.2
W18	18.0	B7	0.4
W23	11.9	B8	1.1

[a] Mycelium plus agar underneath mycelium was extracted using the standardized technique of Hahnau and Weiler (1991).

the method described here provides a sensitive and specific means of analyzing fungal isolates for CPA.

CPA in White-Mold Fermented Cheeses. A preliminary survey was made for the level of CPA in cheese, basically to check performance of the assay for this matrix. Samples were obtained from local sources, kept refrigerated, and analyzed immediately. As Table IV shows, CPA was not detected in all samples analyzed, albeit the fungal isolates from these cheeses all produced CPA on agar. A correlatin between the CPA production on agar on the data in Table IV was not obvious (not shown). This is not surprising, because CPA is produced during the growth of the mycelium, and thus CPA levels observed in the cheese might be influenced by recent mycelial growth. Consistent with this is the observation that CPA levels in the cheese rise upon storage at elevated temperatures (Still et al., 1978, and own unpublished observations) and the finding that an older mycelium may contain significantly more CPA than young ones, resulting in clear CPA gradients on agar plates with growing mycelium (Hahnau and Weiler, 1991). Within the cheese, the highest level of CPA was found to be associated with the outer mycelial rind, while much lower levels could be extracted from the central parts, in agreement with earlier papers (Le Bars, 1979). Also, the levels of CPA in cheeses as recorded immunologically are in good agreement with the data of Le Bars (1979), who has used a TLC method and mass spectrometry to detect CPA. These levels are very unlikely to produce a potential health hazard given the doses eventually ingested by consumers as contrasted with the oral toxicity of the compound (Purchase, 1971). Nevertheless, the availability of toxin-free starter cultures (Geisen et al., 1990) would be an advantage for the producing industry. The assay reported here should facilitate the selection of the appropriate fungal strains.

Table IV. Cyclopiazonic Acid in White-Mold Cheese Extracts

cheese sample[a]	CPA, μg/g of cheese	cheese sample[a]	CPA, μg/g of cheese
K1	4.5	K17	7.9
K3	≈2.0[b]	K18	7.1
K6	nd[c]	K19	2.9
K8	nd	K20	6.1
K9	nd	K23	≈2.0
K11	≈2.0	K24	≈2.0
K12	≈2.0	K25	4.9
K13	2.7	K27	7.1
K14	6.8	K28	5.5

[a] Code numbers do not correspond to numbers in Table III. [b] Limit of detection. [c] Not detected.

ABBREVIATIONS USED

B_0, binding of tracer to antibody in the absence of unlabeled antigen; CPA, cyclopiazonic acid; HPLC, high-pressure liquid chromatography; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; TLC, thin-layer chromatography; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; UB, unspecific binding.

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