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Articles

Monoclonal Antibody-Based ELISAs for Part-per-Billion Determination of Polycyclic Aromatic Hydrocarbons: Effects of Haptens and Formats on Sensitivity and Specificity

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As a first step toward developing sensitive enzyme-linked immunosorbent assays (ELISAs) for multianalyte detection of polycyclic aromatic hydrocarbons (PAHs), haptens with different lengths of carboxylic acid spacers at various positions were derived from naphthalene, fluorene, anthracene, phenanthrene, pyrene, fluoranthene, chrysene, and benzo[*a*]pyrene (BaP). These haptens were coupled with bovine serum albumin (BSA) to form competitor conjugates. All of these haptens were recognized to different extents by monoclonal antibodies (MAbs) 4D5 and 10C10 originally derived by Gomes and Santella (*Chem. Res. Toxicol.* 1990, *3,* 307−310). The most sensitive indirect ELISAs were obtained by coating wells with the least competitive conjugates. Direct ELISAs using horseradish peroxidase conjugates of pyrene and BaP were less sensitive. The MAbs bound BaP with spacers at either C1 or C6. The cross-reactivity profiles of the eight PAHs were different with each PAH−BSA conjugate used as coating antigen. The ELISA results for BaP closely correlated with those by gas chromatography (GC), but the detection limit of the ELISA was ∼150-fold more sensitive than that of GC, with 2−600 nM spike recoveries of 80−127% from human urine and canal and tap water.

Polycyclic aromatic hydrocarbons (PAHs) are environmental contaminants produced mainly by incomplete combustion of organic matter. PAHs in smoke, soot, and dust particles in the air are carried into water, soil, and crops to contaminate the environment. Many PAHs are carcinogens in animals and are suspected carcinogens in humans.[1] The U.S. Environmental Protection Agency (EPA) has listed 16 PAHs as priority pollutants in wastewater and 24 PAHs in soils, sediments, hazardous solid wastes, and groundwater.[2] Benzo[*a*]pyrene (BaP) is one of the most potent carcinogens of the PAH family, BaP content correlates well with total PAH content in contaminated environmental samples, and BaP adducts of protein and DNA are biomarkers of exposure to PAHs.[3]

Determination of PAHs in environmental matrixes typically involves several steps. These include extraction of the sample by either liquid−liquid or solid-phase extraction, concentration and cleanup of the extracts followed by analysis with high-performance liquid chromatography (HPLC), gas chromatography (GC) or GC/mass spectrometry (GC/MS).[4] To avoid these complex procedures, immunochemical analyses employing either polyclonal or monoclonal antibodies have been developed for PAHs.[5−9] Commercially available immunoassay kits for PAHs, total petroleum hydrocarbons (TPHs), and carcinogenic PAHs in various matrixes are marketed by Strategic Diagnostics, Inc. (Newark, DE). U.S. EPA has compiled a series of immunochemical methods for environmental analysis. Method 4035 detects several PAHs to different degrees and measures their cumulative responses to

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(1) International Agency for Research on Cancer Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans. *Polynuclear Aromatic Compounds, Part 3*; International Agency for Research on Cancer: Lyon, France; 1984; Vol. 34.

(2) Patnaik, P. In *Handbook of Environmental Analysis*; Patnaik, P., Ed.; CRC Press: Boca Raton, FL, 1997; pp 165−169.

(3) International Agency for Research on Cancer Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans. *Polynuclear Aromatic Compounds, Part 1*; International Agency for Research on Cancer: Lyon, France; 1983; Vol. 32.

(4) Osborne, M. R.; Crosby, N. T. In *Benzopyrenes*; Cambridge University Press: New York, 1987; pp 251−300.

(5) Gomes, M.; Santella, R. M. *Chem. Res. Toxicol.* **1990**, *3*, 307−310.

(6) Knopp, D.; Vaananen, V.; Zuhlke, J.; Niessner, R. *Immunochemical Technology for Environmental Applications*; ACS Symposium Series 657; American Chemical Society: Washington, DC, 1997; pp 71−76.

(7) McDonald, P. P.; Almond, R. E.; Mapes, J. P.; Friedman, S. *JAOAC Int.* **1994**, *77*, 466−472.

(8) Roda, A.; Bacigalupo, M. A.; Ius, A.; Minutello, A. *Environ. Technol.* **1991**, *12*, 1027−1035.

(9) Roda, A.; Pistillo, A.; Jus, A.; Armanino, C.; Braldini, M. *Anal. Chim. Acta* **1994**, *298*, 53−64.

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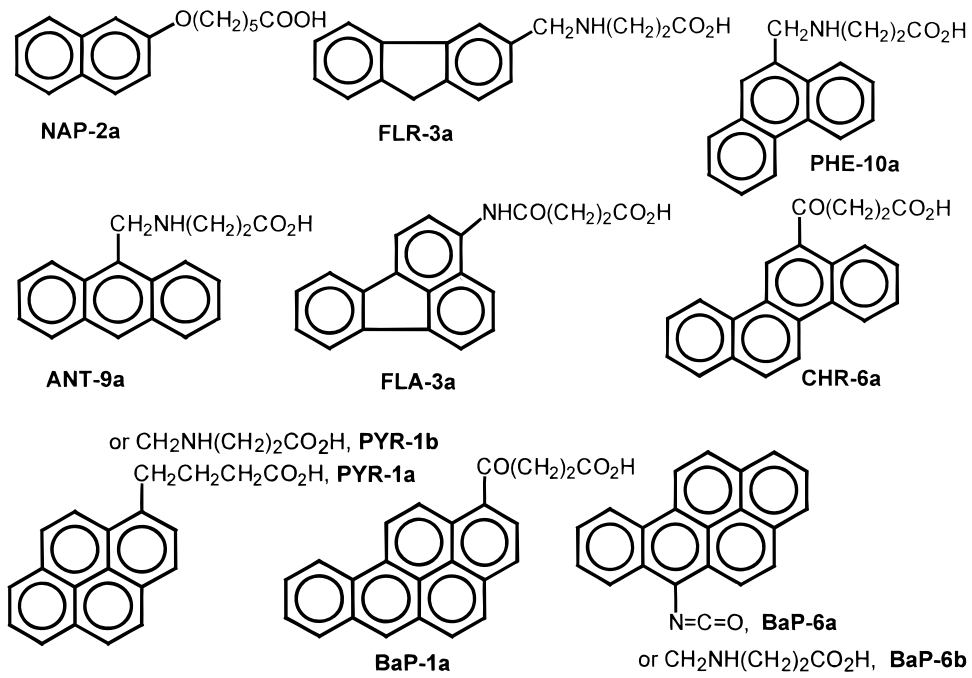

Figure 1. Structures of haptens used in the preparation of antigens.

determine the total PAH in the sample.[10] These assays provide an easy and fast alternative for PAH screening.

Antibodies are the primary agents that determine immunoassay performance, and properties of the antibody are largely determined by the immunogen.[11] The similar shape, hydrophobicity, lack of hydrogen-bonding atoms, and other characteristics of PAHs make it difficult to derive antibodies that are highly selective for individual PAHs. Cross-reactivity is expected and will have to be exploited in PAH immunoanalysis of environmental samples. Monoclonal antibodies (MAb), 4D5 and 10C10, originally derived and characterized by Gomes and Santella,[5] cross-react with several PAHs of major concern as environmental pollutants. In this paper, we describe several new PAH haptens we prepared for a systematic study of cross-reactivity by these MAbs. In addition, we recently cloned and expressed recombinant Fab antibodies (rFabs) with similar cross-reactivity from these two MAbs.[12] A model based on the rFab sequences indicates that the PAH binding sites accommodate a variety of PAHs.[13] The experiments we report here were done to maximize the differences in PAH cross-reactivity of these two antibodies in the direct and indirect formats of enzyme-linked immunosorbent assays (ELISAs). The half-maximal inhibition (I_{50}) of the PAHs and haptens was measured with MAbs 10C10 and 4D5. Suitable conjugates were further examined to optimize the assays. In addition, the most sensitive assay was tested for the analysis of BaP spiked in tap and canal water and human urine and was compared with GC analysis. The ELISA development approach is generally applicable to developing immunoassays for other small molecules of environmental significance.

EXPERIMENTAL SECTION

Reagents and Instruments. All PAH standards and derivatives were obtained from Aldrich (Milwaukee, WI). PAH stock solutions were prepared in methanol or DMSO in sealed glass vials. Immunochemicals, enzyme substrates, and buffer capsules were obtained from Sigma (St. Louis, MO). The ELISA was carried out in 96-well polystyrene plates (MaxiSorp F96, Catalog No. 439454, Nalge Nunc International, Denmark). The optical density (OD) was measured using a Vmax microplate reader (Molecular Devices Corp., Sunnyvale, CA). Hybridoma cell lines 4D5 and 10C10, kindly provided by R. M. Santella (Columbia University, New York), were grown, and IgG was purified as previously described.[14] Precoated TLC plates were silica gel F254 and were purchased from E. Merck (Darmstadt, Germany). NMR spectra were recorded with a Nicolet NT 300-MHz instrument. Mass spectra were obtained with an AEI-MS 30 or VG Trio 2 (VG BioTech) spectrometer with a direct insertion probe and fast atom bombardment (FAB) ionization.

Safety Considerations. Syntheses were done in chemical fume hoods with charcoal filters. PAH-impermeable nitrile gloves were used. All byproducts and wastes from the syntheses and primary solutions of PAH analytes and haptens from assays were disposed of as hazardous materials.

Synthesis of Haptens. All haptens except 4-(1-pyrene)butyric acid (PYR-1a), which was a commercial product from Aldrich (Milwaukee, WI), were synthesized from PAH derivatives and characterized by NMR and MS (Figure 1, Table 1).

6-(2-Naphthoxy)hexanoic Acid (NAP-2a). NAP-2a was prepared by reaction of 2-naphthol with ethyl bromohexanoate

(10) US EPA method 4035. In *SW-846 Test Methods for Evaluating Solid Waste Physical/Chemical Methods*, 3rd ed.; US EPA, Office of Solid Waste: Washington, DC, 1996; Chapter 4.

(11) Tijssen, P. In *Laboratory Techniques in Biochemistry and Molecular Biology*; Burdon, R. H., Knippenberg, P. H., Eds.; Elsevier Verlag: Amsterdam; 1985; Vol. 15, pp 128−135.

(12) Li, K.; Bell, C.; Chen, R.; Zhao, B.; Karu A.; Li, Q. X. 215th ACS national meeting. Dallas, TX, March 29−April 2, 1998; Abstr. AGRO-62.

(13) Roberts, V. A.; Pellequer, J.-L.; Bell, C. W.; Karu, A. E.; Li, K.; Li, Q. X. *81st Canadian Soc. for Chem. Conf., Exhib.* Whistler BC, Canada, June 1998.

(14) Karu, A. E.; Goodrow, M. H.; Schmidt, D. J.; Hammock, B. D.; Bigelow, M. W. *J. Agric. Food Chem.* **1994**, *42*, 301−309.

Table 1. Chemical Characterization of Haptens by ^{1}H NMR and MS

chemical	spacer arm	^{1}H NMR (300 MHz)[a]	MS *m*/*z* (rel int, %)
NAP-2a	$O(CH_2)_5COOH$	4.15 (t, 2H), 2.36 (t, 2H), 1.88 (m, 2H), 1.74 (m, 2H), 1.55 (m, 2H)	258 (M^+, 12), 144 (100), 115 (30)
FLR-3a	$CH_2NHCH_2CH_2COOH$	3.70 (s, 2H), 2.75 (t, 2H), 2.30 (t, 2H)	268 (MH^+, 34)[b]
PHE−10a	$CH_2NHCH_2CH_2COOH$	3.58 (s, 2H), 2.53 (t, 2H), 2.12 (t, 2H)	279 (M^+, 38), 218 (8), 206 (32), 191 (100), 178 (12)
ANT-9a	$CH_2NHCH_2CH_2COOH$	4.25 (s, 2H), 2.89 (t, 2H), 2.35 (t, 2H)	279 (M^+, 66), 218 (10), 206 (18), 191 (100), 178 (35)
FLA-3a	$NHCOCH_2CH_2COOH$	3.20 (t, 2H), 2.80 (t, 2H)	317(M^+, 13), 299 (25), 217 (100), 189 (25)
CHR-6a	$COCH_2CH_2COOH$	3.60 (t, 2H), 3.00 (t, 2H)	328 (M^+, 40), 255 (100), 226 (71), 113 (46)
PYR-1a	$CH_2CH_2CH_2COOH$	3.42 (t, 2H), 2.52 (t, 2H), 2.24−2.18 (m, 2H)	288 (M^+, 36), 239 (100), 215 (55), 135 (65)
PYR-1b	$CH_2NHCH_2CH_2COOH$	3.58 (s, 2H), 2.99 (t, 2H), 2.45 (t, 2H)	303 (M^+, 37), 242 (10), 230 (21), 215 (100), 202 (25)
BaP-1a	$COCH_2CH_2COOH$	3.60 (t, 2H), 3.02 (t, 2H)	352(M^+, 38), 279 (60), 251 (30), 250 (26), 149 (55), 125 (75)
BaP-6b	$CH_2NHCH_2CH_2COOH$	3.75 (s, 2H), 2.80 (t, 2H), 2.30 (t, 2H)	353 (M^+, 20), 292 (8), 280 (12), 265 (100), 252 (18)

[a] Only signals from spacer arm are listed. [b] Obtained using FAB mode.

followed by hydrolysis.[15] Briefly, 2-naphthol (1.0 mmol) and ethyl 6-bromohexanoate (1.0 mmol) were dissolved in acetone (50 mL). Potassium iodide (40 mg) were added to the acetone solution. The heterogeneous mixture was refluxed for 24 h. After filtering off the salt and evaporating acetone, the crude residue was purified with silica flash chromatography (methylene chloride−ethyl acetate−hexane 1:1:8). The purified ethyl 6-(2-naphthoxy)hexanoate (200 mg) was hydrolyzed in ethanol under the catalysis of lithium hydroxide (2 mL, 1 N). Upon addition of 4 N HCl, the product precipitated out as a white solid.

***N*-(3-Fluorenemethyl)-3-alanine (FLR-3a), *N*-(10-Phenanthrenemethyl)-3-alanine (PHE-10a), *N*-(9-Anthracenemethyl)-3-alanine (ANT-9a), *N*-(1-Pyrenemethyl)-3-alanine (PYR-1b), and *N*-(6-Benzo[*a*]pyrenemethyl)-3-alanine (BaP-6b).** A PAH aldehyde (1.0 mmol) and *β*-alanine (1.0 mmol) were dissolved in a mixture of methanol (10 mL) and triethylamine (1 mL). The reaction mixture was refluxed for 2 h. After cooling to room temperature, the reaction product was reduced with an excess amount of sodium borohydride (100 mg). The solution was adjusted to pH 2 using 2 N HCl to precipitate the product, which was then recrystallized with a mixture of methanol and water (2:1). BaP-6-carboxaldehyde (BaP-6-CHO) was used as an intermediate for the synthesis of BaP-6b and was prepared by reaction of BaP and *N*-methylformanilide in the presence of phosphoryl chloride according to the procedures described by Dewhurst and Kitchen.[16]

***N*-(3-Fluoranthene)succinamic Acid (FLA-3a).** 3-Aminofluoranthene (0.5 mmol) and succinic anhydride (0.6 mmol) were dissolved in pyridine (1 mL), and the mixture was stirred at room temperature for 24 h. After all starting materials were consumed as indicated by TLC (ethyl acetate−hexane 1:3), water (20 mL) was added and the precipitate was collected. The product was purified on silica gel column using ethyl acetate−hexane (1:5) as eluant.

(1-Benzo[*a*]pyrene)succinic acid (BaP-1a) and (6-chrysene)succinic acid (CHR-6a) were prepared by reaction of succinic anhydride with BaP and chrysene, respectively, using aluminum chloride as catalyst following the procedures of Buu-Hoi and Lavit.[17]

6-Benzo[*a*]pyreneisocyanate (BaP-6a) was synthesized as described by Griffin et al.[18] and was generously provided by T. Vo-Dinh.

Hapten−Protein Conjugates. A carboxylic hapten (0.20 mmol), *N*-hydroxysuccinimide (0.20 mmol), and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (0.22 mmol) were dissolved in dimethylformamide (1 mL). The mixture was stirred at room temperature for 3.5 h and centrifuged to remove the precipitate. The active ester in the supernate (0.25 mL) was added very slowly to a solution of bovine serum albumin (BSA) (50 mg) in 15 mL of 0.05 M borate buffer, pH 9. After it was stirred gently overnight at 4 °C, the solution was dialyzed against 0.02 M phosphate-buffered saline, pH 7.5 (PBS), over 24 h, with six changes of 4 L each. To prepare horseradish peroxidase (HRP) conjugates, 130 *μ*L of active ester solution was added slowly to a HRP solution (0.67 mg/mL 0.13 M $NaHCO_3$). The solution was stirred gently overnight at 4 °C, and the conjugate was then dialyzed against 4 L of 0.13 M $NaHCO_3$ at 4 °C for 3 days with buffer changes twice a day. BaP-6a−BSA was prepared by covalently coupling BaP-6a with BSA.[19]

Enzyme-Linked Immunosorbent Assays. *Direct Competitive ELISA.* Microplate wells were coated with purified MAb 10C10 (100 *μ*L/well, 0.2 *μ*g/mL 0.05 M carbonate−bicarbonate buffer, pH 9.6). The coating solution was evaporated to dryness at 37 °C overnight. The plate was washed four times with PBS containing 0.05% Tween 20 (PBST) and blocked with 0.3% BSA in PBST (PBST−BSA) (200 *μ*L/well) at 37 °C for 1 h. Alternatively, the plate was precoated overnight at 37 °C with affinity-purified goat anti-mouse IgG (Boehringer Mannheim, 1 mg/mL, 1:2000 in PBST−BSA) and subsequently incubated with 0.2 *μ*g/mL purified MAb 10C10 (100 *μ*L/well) at 37 °C for 1 h. The coated wells were washed three times with PBST. Samples containing different amounts of analyte and 25 ng of PYR-1a−HRP competitor in PBST−BSA were added to coated microwells (100 *μ*L/well) and incubated at 37 °C for 1 h. After another washing, 100 *μ*L of *o*-phenylenediamine (OPD) solution (1.0 mg/mL in 0.05 M citrate−phosphate with 0.03% sodium perborate, pH 5.0) was added in each well. After 15−30 min at room temperature, the

(15) Chiu, Y. W.; Carlson, R. E.; Marcus, K. L.; Karu, A. E. *Anal. Chem.* **1995**, *67*, 3829−3839.
(16) Dewhurst, F.; Kitchen, D. A. *J. Chem. Soc., Perkin Trans. 1* **1972**, 710−712.
(17) Buu-Hoi, N. P.; Lavit, D. *Tetrahedron* **1960**, *8*, 1−6.
(18) Griffin, G. D.; Ambrose, K. R.; Thomason, R. N.; Murchison, C. N.; McManis, M.; St. Wecker, P. G. R.; Vo-Dinh, T. Production and characterization of antibodies to benzo[*a*]pyrene. *Proceedings of 10th International Symposium on Polycyclic Aromatic Hydrocarbons*, October 21−23, 1985.
(19) Vo-Dinh, T.; Tromberg, B. J.; Griffin, K. R.; Ambrose, M. J.; Sepaniak, M. J.; Gardenhire, E. M. *Appl. Spectrosc.* **1987**, *41*, 735−738.

reaction was stopped with 2 M sulfuric acid (50 μL/well). The OD at 490 nm was then read on a Vmax microplate reader, and the data were fitted using Softmax software (Molecular Devices, Sunnyvale, CA).

Indirect Competitive ELISA. Microplates were coated with PAH hapten−BSA conjugate (1−5 ng/100 μL per well in 0.05 M carbonate−bicarbonate buffer, pH 9.6) by drying at 37 °C overnight and blocked with PBST−BSA as described above. Hybridoma culture supernate (1:500−1:2000 in PBST−BSA) was mixed with different amounts of analyte, the solution was added to the plate (100 μL/well) and incubated at 37 °C for 1 h. After washing, the plate was incubated for 1 h at 37 °C with HRP−goat anti-mouse IgG (1:12000 in PBST−BSA, 100 μL/well), the plate was washed, and the procedure for the color development was as described above. Where indicated, the signal was amplified with biotinylated goat anti-mouse IgG and avidin−HRP conjugate. After the incubation with the mixture of PAH antibody and analyte, the plate was washed, incubated with biotinylated goat anti-mouse IgG (1:12000 in PBST-BSA, 100 μL/well) at 37 °C for 1 h, followed by another wash and incubation with avidin−HRP (1:12000 in PBST−BSA, 100 μL/well) at 37 °C for 1 h. The rest of the procedures were the same as described above. This protocol is referred to as labeled avidin−biotin ELISA (LAB-ELISA).

GC Analysis. Water samples from the Ala Wai canal (Honolulu, HI) were spiked with BaP at concentrations of 20, 40, 100, 200, and 400 nM (i.e., 5−100 ppb). A 400-mL aliquot of the 20 nM spiked sample and 200 mL of the 40−400 nM spiked samples were extracted with 3 × 100 mL of ethyl acetate, the extracts were combined and dried over anhydrous sodium sulfate, concentrated by rotary evaporation, and reconstituted in ethyl acetate to 2.0 (20 nM spiked sample) or 5.0 mL (40−400 nM spiked sample). Analysis was done on a Hewlett-Packard (HP) 5890 GC equipped with a DB-5 capillary column (J & W, 30 m × 0.25 mm, 0.25-μm film thickness) and flame ionization detector. The injector and detector temperatures were 275 and 300 °C, respectively. The oven temperatures were increased from 65 to 140 °C at a rate of 25 °C/min and then to 290 °C at a rate of 10 °C/min. The temperature was maintained at 290 °C for 15 min. Peak areas recorded with a HP 3396A integrator were quantified relative to an external standard of BaP.

RESULTS AND DISCUSSION

Selection and Synthesis of Haptens. The MAbs 10C10 and 4D5 used in this study were originally derived by Gomes and Santella using a 6-amino-BaP−BSA (a BaP 6-isocyanate hapten) as immunogen.[5] These antibodies recognized BaP and cross-reacted with a number of BaP metabolites as well as several other PAHs.[5] As a possible means of expanding the usefulness of these MAbs, we synthesized and characterized the new PAH haptens in Figure 1 and examined the performance of the MAbs with them in direct and indirect ELISAs. These 11 haptens represent the major two- to five-ring PAHs with aqueous solubilities (0.002−30 μg/mL) and vapor pressures (4.92×10^{-2}−6.9×10^{-7} Torr at 20 °C) that are most amenable to immunoassay.[20] BaP and chrysene are carcinogenic PAHs found in urban air.[21] These and fluoranthene are mutagenic in the Ames test.[20] Naphthalene, phenanthrene, fluorene, and fluoranthene as well as others are found in cigarette smoke and exhaust gases.[21] All are present in different amounts in oils, fuels, lubricants, cooked foods, and a wide variety of biological and environmental matrixes.[22,23] Metabolites of chrysene, pyrene, and BaP form protein and DNA adducts that are biomarkers of PAH exposure.[24,25]

The FLR-3a, PHE-10a, ANT-9a, PYR-1b, and BaP-6b haptens were prepared by reacting PAH aldehyde intermediates with β-alanine. FLA-3a was synthesized by reaction of 3-aminofluoranthene with succinic anhydride. Friedel−Crafts acylation of chrysene and BaP with succinic anhydride gave CHR-6a and BaP-1a, respectively. BaP-6a differs in linkage position and spacer from BaP-1a. BaP-6b differs from BaP-6a only in the spacer. These carboxylic haptens were conjugated to carrier protein or enzyme by the active ester method.[26] The MAbs bound both the haptens and PAH−BSA conjugates with very different relative affinities, and this affected the sensitivity and selectivity of direct and indirect ELISAs for various PAHs.

Enzymes and Assay Formats. While OPD was used as a substrate for goat anti-mouse IgG−HRP, *p*-nitrophenyl phosphate (NPP) was used for alkaline phosphatase (AP). Indirect ELISAs using BaP-6a−BSA or PYR-1a−BSA as coating antigen were used to compare these enzymes and substrates. There was no significant difference in I_{50} or limit of detection (LOD; I_{20}) for BaP regardless of whether commercial goat anti-mouse IgG−HRP or −AP conjugate was used (I_{50} = 852 and 1008 nM, respectively with BaP-6a−BSA coating; both at 128 nM with PYR-1a−BSA coating). Goat anti-mouse IgG−HRP conjugate and the substrate OPD were used for the subsequent work because they gave much faster color development.

In the direct ELISA, the I_{50} for BaP was 2952 nM when affinity-purified MAb 10C10 IgG (20 ng/well) was directly coated on the plate and the enzyme tracer PYR-1a−HRP was used at 25 ng/well. When MAb 10C10 was captured from culture supernate on wells coated with 20 ng/well of affinity-purified goat anti-mouse IgG, the I_{50} for BaP decreased to 2015 nM. Wells could not be coated directly with unpurified hybridoma culture supernate because it contains a large excess of serum proteins.

The direct ELISA could be run in the shortest time among the three ELISA formats, but it was less sensitive than the indirect ELISA. Conditions optimized for indirect ELISA detection by goat anti-mouse IgG−HRP (each well coated with 2.7 ng of BaP-6a−BSA, and a 1:500 dilution of MAb 10C10 culture supernate) gave an I_{50} of 980 nM for BaP. The sensitivity was improved 3−4-fold (I_{50} = 330 nM BaP) when conditions were optimized for detection

(20) Sims, R. C.; Overcash, M. R. Fate of polynuclear aromatic compounds in soil-plant systems. In *Residue Reviews*; Gunther, F. A., Gunther, J. D. Eds.; Springer-Verlag: New York, 1983; Vol. 88, pp 2−68.

(21) Pucknat, A. W. *Health impacts of polynuclear aromatic hydrocarbons*; Noyes Data Corp.: Park Ridge, NJ, 1981; p 271.

(22) Neff, J. M. *Polycyclic Aromatic Hydrocarbons in the Aquatic Environment*; Applied Science Publishers Ltd.: London, 1979.

(23) Lee, M. L.; Novotny, M. V.; Bartle, K. D. *Analytical chemistry of polycyclic aromatic compounds*; Academic Press: New York, 1981; p 462.

(24) *Biomarkers and risk assessment: concepts and principles*; World Health Organization: Geneva, Switzerland, 1993.

(25) Schnell, F. C. Protein adduct-forming chemicals and molecular dosimetry: potential for environmental and occupational biomarkers. In *Reviews in Environmental Toxicology*; Hodgson, E. Ed.; Toxicology Communications, Inc.: Raleigh, NC, 1993; Vol. 5, pp 51−60.

(26) Klibanov, A. L.; Slinkin, M. A.; Torchlin, V. P. *Appl. Biochem. Biotechnol.* **1989**, *22*, 45−58.

Table 2. Sensitivity of Indirect LAB-ELISA for BaP and Relative Binding of MAb 10C10 to PAH−BSA Conjugates

PAH−BSA conjugate	recognition index (I_{50}, ppb)[a]	I_{50} (ppb) for BaP with indicated coating conjugate[b]
PYR-1a−BSA	0.55 (51.6)	11.7 (46.8 nM)
PYR-1b−BSA	0.61 (47.0)	28.7
FLA-3a−BSA	0.70 (40.5)	47.2
CHR-6a−BSA	0.75 (38.0)	17.9
ANT-9a−BSA	0.95 (29.9)	14.3
BaP-6a−BSA	1.0 (28.5)	25.5
BaP-6b−BSA	1.2 (23.7)	35.0
BaP-1a−BSA	1.9 (14.9)	14.5
PHE-10a−BSA	2.1 (13.4)	23.4
FLR-3a−BSA	9.2 (3.1)	24.7
NAP-2a−BSA	95 (0.3)	134.0 (536 nM)

[a] Recognition index, I_{50} of BaP-6a−BSA/I_{50} of a conjugate. I_{50} values in parentheses were obtained by indirect LAB-ELISAs of the corresponding PAH−BSA conjugate in the left column using MAb 10C10 (1:6000) on plates coated with BaP-6a−BSA (2.0 ng/100 μL per well). Data were means of four wall replicates. [b] Indirect LAB-ELISAs of BaP using MAb 10C10 on plates coated with the corresponding hapten−BSA in the left column. Data were means of four well replicates.

using biotinylated goat anti-mouse IgG and avidin−HRP (LAB-ELISA: each well coated with 1.0 ng of BaP-6a−BSA, and a 1:2000 dilution of MAb 10C10 culture supernate). The LAB-ELISA obviously consumed less reagents. We subsequently found that one incubation step could be eliminated by adding the biotinylated goat anti-mouse IgG along with the MAb in the incubation with the analyte. The assay performance was the same, but the time required for the LAB-ELISA was significantly shortened. Therefore, we used the indirect LAB-ELISA for all subsequent work.

Selection of Coating Antigens. The sensitivity and specificity of competitive ELISAs depend on relative affinities of antibody for the competitor haptens and the analytes. The competitor conjugates may vary in hapten structure and density. The most sensitive assays are usually those in which the antibody has a lower affinity for the competing hapten (i.e., haptenated conjugate) than it does for the analyte.[27] Two indirect competitive LAB-ELISA experiments were done to identify which haptenated conjugates improved the sensitivity and gave adequate binding for signal detection. First, to compare the relative binding of MAb 10C10 to the PAH−BSA conjugates, each hapten−BSA conjugate was used as the inhibitor in solution on plates coated with BaP-6a−BSA. The conjugates that were recognized best by MAb 10C10 had the least MAb bound to the coating and thus gave the lowest I_{50} value. The conjugates that were weakly recognized by the MAb left more MAb bound to the coating and gave a higher I_{50} value. These results were expressed as a "recognition index" (RI) and calculated as ratio of the I_{50} value of BaP-6a−BSA to that of the test conjugate, i.e., RI = I_{50} of BaP-6a−BSA/I_{50} of a conjugate (Table 2). Thus, conjugates to which MAb 10C10 bound more strongly than to BaP-6a−BSA had a RI greater than 1. Using such a conjugate as coating antigen may reduce assay sensitivity. In addition, screening the PAH conjugate competitors in solution suggested that MAb 10C10 may be able to bind protein adducts formed by metabolites of pyrene, chrysene, BaP, and other PAHs for exposure monitoring without hydrolysis of the adducts, which is often required for instrumental analysis.

In the second experiment, an indirect competitive LAB-ELISA for BaP was done on plates coated with a PAH−BSA conjugate. The I_{50} values for BaP indicated the relative sensitivity. The results of both experiments, shown in Table 2, led to several conclusions. First, all of the listed two-, three-, four-, and five-ring PAH hapten−BSA conjugates in solution competitively bound MAb 10C10 in the presence of immobilized BaP-6a−BSA, underscoring the broad cross-reactivity of this MAb. Second, MAb 10C10, which was raised against BaP-6a−BSA, bound BaP haptens with the spacer arm at either C1 or C6 on the ring. Third, differences of only 2-fold or less in sensitivity for BaP were observed using different linkers between the hapten and BSA (PYR-1a and -1b, BaP-6a and -6b), supporting the notion that aromatic ring binding was the predominant factor in recognition. Fourth, the RI was an approximate predictor of the I_{50} for BaP, although only the largest and smallest RI and BaP I_{50} values correlated well. When the weakest cross-reactive conjugate, PYR-1a−BSA, was used as coating antigen, it gave the most sensitive ELISA for BaP. Conversely, the NAP-2a−BSA conjugate, which was most strongly bound, gave an assay for BaP that was roughly 10-fold less sensitive. Presumably, recognition of the PAH−BSA conjugates was affected primarily by the MAb's affinity for the hapten and the hapten density on the BSA. For example, FLR-3A conjugate had a recognition index ~17-fold higher than PYR-1a, but the I_{50} for BaP was only 2.1-fold higher when FLR-3A, rather than PYR-1a, was the coating antigen. Although hapten densities on the conjugates were not determined in this study, they were presumably similar because of similar chemical properties of the PAH haptens and parallel synthesis of these conjugates using the same mole ratio of hapten to BSA under the same condition. However, hapten densities must be considered when they vary significantly.

Because PYR-1a−BSA gave the lowest I_{50} for BaP, it was used as coating antigen in all subsequent LAB-ELISAs. The assay was optimized to use 0.5 ng/100 μL per well of PYR-1a−BSA and 1:6000 dilution of MAb 10C10 culture supernate. Standard curves fitted well with the four-parameter logistic model over the entire range or with a semilog model over the linear portion (percent inhibition, % $I = 29.04 + 20.28 \log X$, $r = 0.999$) (Figure 2). The average I_{50} for BaP was 10.8 nM (2.7 ppb), with a LOD defined as the I_{20}, of ~0.4 nM (0.1 ppb). The linear range of the curve was about 0.4−1000 nM of BaP. The assay is able to measure BaP accurately when its concentrations are above 0.4 nM in samples. The assay sensitivity was improved ~4-fold relative to that reported by Gomes and Santella and ~89-fold compared to that obtained in our initial assays using BaP-6a coating conjugate following the procedure of Gomes and Santella.[5]

Cross-Reactivity. Because of the planarity and similar shapes and chemical properties of PAHs, antibodies such as 4D5 and 10C10 with broad cross-reactivity are likely to occur as a rule rather than an exception. To test whether assay conditions and reagents could be manipulated to obtain consistent and interpretable reaction patterns with different PAHs, indirect competitive LAB-ELISAs were performed to obtain dose−response curves for eight PAHs on plates coated with each of six PAH−BSA conjugates that gave classical sigmoid dose responses. The I_{50} values

(27) Carlson, R. E. Hapten versus competitor design strategies for immunoassay development. In *Immunoanalysis of Agrochemicals: Emerging Technologies*; Nelson, J. O., Karu, A. E., Wong, R. B. Eds.; ACS Symposium Series 586; American Chemical Society: Washington, DC, 1995; pp 140−152.

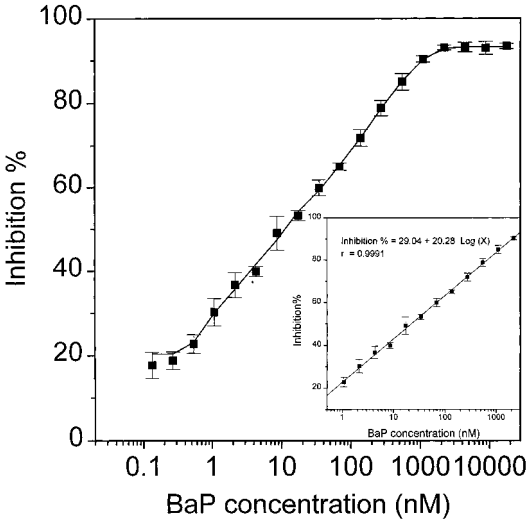

Figure 2. Standard curve of inhibition by BaP using MAb 10C10 in the indirect LAB-ELISA. The plate was coated with 0.5 ng/100 μL per well PYR-1a−BSA, and MAb 10C10 was diluted 1:6000. Error bars, ± one standard deviation. Inhibition percent, $100(A_0 - A)/A_0$, where A_0 is the absorbance with no BaP present and A is the absorbance with BaP present. The data were means of four replicates.

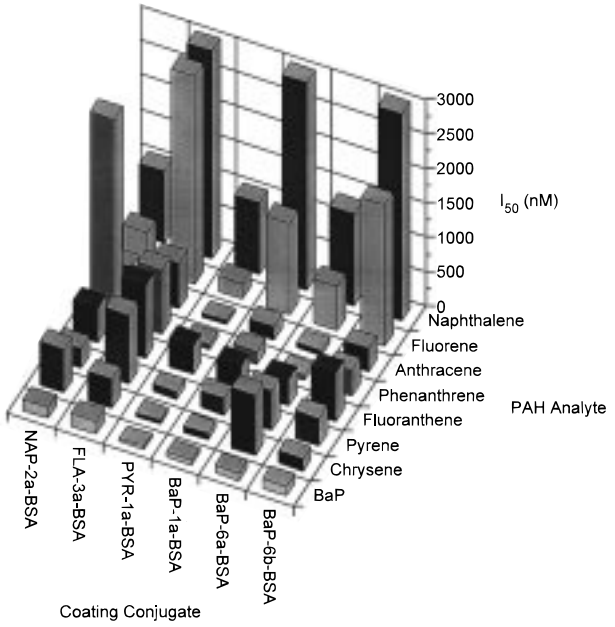

Figure 3. Half-maximal inhibition (I_{50}) as a measure of relative binding of MAb10C10 to eight PAHs in indirect LAB-ELISAs with six different competitor conjugates (coating antigens). The shortest bars are the most sensitive responses. The plate was coated with 2.0 ng/100 μL per well PAH−BSA except NAP-2a−BSA (2.0 pg/100 μL per well), and MAb 10C10 was diluted 1:6000. Overall standard deviations averaged 10.8% of mean values. The numerical data for this figure are available from the authors upon request. The data were means of four replicate experiments.

were determined as a measure of the assay sensitivity. The results, shown in Figure 3, illustrate the differences in cross-reactivity pattern for each coating antigen. The shorter the bar, the more sensitive was the detection. The I_{50} values varied from 42 nM to 305 μM for different PAHs with two to five fused rings. BaP was the most sensitively detected PAH regardless of the coating conjugate, probably because MAb 10C10 was originally evoked by BaP-6a−BSA. The graph also shows that the most sensitive

Table 3. Cross-Reactivity of Parent Analytes and Haptens[a]

analyte	I_{50} (nM)	hapten	I_{50} (nM)	I_{50} ratio[b]
naphthalene	370	NAP-2a	120	3.1
anthracene	46	ANT-9a	742	0.2
fluoranthene	336	FLA-3a	36	9.3
pyrene	34	PYR-1a	18	1.9
		PYR-1b	2	17
chrysene	20	CHR-6a	58	0.3
benzo[*a*]pyrene	11	BaP-6b	14	0.8
		BaP-6-CHO	29	0.4
		BaP-1a	99	0.1

[a] Indirect LAB-ELISAs using MAb 10C10 culture supernate diluted 1:6000 and the coating antigen PYR-1a−BSA at 0.5 ng/100 μL per well. Data were means of four well replicates. [b] I_{50} ratio, (I_{50} of a PAH/I_{50} of the corresponding hapten).

assays for the largest number of different PAHs were obtained with the PYR-1a−BSA conjugate that was most weakly recognized in the experiment of Table 2. The least sensitive assays were obtained for the two- and three-ring PAHs using the FLA-3a− and NAP-2a−BSA conjugates as competitors. However, some of the largest differences between PAHs were evident from the less sensitive assays. New antibodies with greater sensitivity for the two- and three-ring PAHs should provide information that could be used to identify PAHs in conjunction with the results obtained using MAb 10C10.

Similar indirect LAB-ELISAs were done to compare recognition of the haptens and the parent analytes (Table 3). The results show enhanced recognition by MAb 10C10 of NAP-2a, FLA-3a, PYR-1a and PYR-1b (I_{50} ratio >1), suggesting some binding interaction with the spacers on these haptens. By contrast, haptens ANT-9a, CHR-6a, BaP-6b, BaP-6-aldehyde, and BaP-1a were not recognized as well as the corresponding parent compounds (I_{50} ratio <1), indicating that the spacers on these haptens reduced the affinity of MAb 10C10 binding. However, the haptens ANT-9a, PYR-1b, and BaP-6b had the same β-alanine spacer, which suggests that the observed affinity differences arise from different fitting of the PAH moiety in the antibody's binding pocket.

The cross-reactivity data (Figure 3, Table 3) demonstrate the well-established principle that changing the competing hapten is a way to modulate the sensitivity and cross-reactivity pattern of a particular antibody in an ELISA.[27−30] Another way to acquire the cross-reactivity data is to use antibodies with differing cross-reactivities.[31−35] PAH detection appears to be a good model system to explore the use of both methods. Selection of an appropriate

(28) Van Weemen, B. K.; Schuurs, A. H. W. M. Sensitivity and specificity of hapten enzyme-immunoassays. In *First International Symposium on Immunoenzymatic Techniques*; Feldmann, M., et al., Eds.; North-Holland Publishing Co.: Amsterdam, 1976; Vol. 2, pp 125−133.

(29) Szurdoki, F.; Bekheit, H. K. M.; Marco, M.-P.; Goodrow, M. H.; Hammock, B. D. Important Factors in Hapten Design and Enzyme-Linked Immunosorbent Assay Development. In *New Frontiers in Agrochemical Immunoassay*; Kurtz, D. A., Skerritt, J. H., Stanker, L. Eds.; AOAC International: Arlington, VA, 1995; pp 39−63.

(30) Goodrow, M. H.; Sanborn, J. R.; Stoutamire, D. W.; Gee, S. J.; Hammock, B. D. Strategies for Immunoassay Hapten Design. In *Immunoanalysis of Agrochemicals: Emerging Technologies*; Nelson, J., Karu, A. E., Wong, R., Eds.; ACS Symposium Series 586. American Chemical Society: Washington, DC, 1995; pp 119−139.

(31) Muldoon, M. T.; Fries, G. F.; Nelson, J. O. *J. Agric. Food Chem.* **1993**, *41*, 322−328.

Table 4. Comparison of PAH Cross-Reactivity of MAbs 10C10 and 4D5[a]

	MAb 10C10		MAb 4D5	
analyte	I_{50} (nM)	CR (%)	I_{50} (nM)	CR (%)
benzo[*a*]pyrene	11 ± 4	100	11 ± 5	100
chrysene	20 ± 4	55	24 ± 4	46
pyrene	34 ± 6	32	21 ± 5	52
phenanthrene	39 ± 5	28	64 ± 8	17
anthracene	46 ± 5	24	71 ± 9	16
fluorene	132 ± 17	8	144 ± 21	8
fluoranthene	336 ± 43	3	313 ± 48	4
naphthalene	370 ± 52	3	292 ± 37	4

[a] Indirect LAB-ELISAs with culture supernate diluted 1:6000 for MAb 10C10 and 1:4000 for MAb 4D5. PYR-1a−BSA (0.5 ng/100 μL per well) was used as coating antigen. The I_{50} values were means ± standard deviations for 4 plates and eight wells per data point per plate.

Table 5. Variations among ELISA Runs for the Analysis of BaP[a]

	CV (%)	
BaP conc (nM)	intraassay	interassay
0	6.5	6.9
2.28	6.5	4.7
9.13	8.6	9.0
36.5	6.5	12.9
146	12.5	25.9
584	14.0	27.8

[a] Indirect LAB-ELISAs using MAb 10C10 culture supernate diluted 1:6000 and the coating antigen PYR-1a−BSA at 0.5 ng/100 μL per well. Data were mean values from four plates and eight replicate wells for each concentration.

combination of antibody−antigen is an essential step to develop a sensitive ELISA. After an antibody is developed, the assay sensitivity and specificity are primarily governed by the relative affinity of antibody−antigen and antibody−analyte. A relatively low affinity of 10C10 to PYR-1a−BSA (I_{50} = 52 ppb) was demonstrated to afford a sensitive assay for PAHs (Table 2, Figure 3). A linker substituent of PAH haptens and the linker and BSA moieties of PAH−BSAs influenced antibody recognition as shown by I_{50} changes (Tables 2 and 3). Such changes probably resulted from factors such as linker type and length of linker, position on the hapten, and hapten density on the conjugates. Interestingly, among the PAHs and their corresponding haptens and hapten−BSA conjugates, little I_{50} changes of pyrene, PYR-1a, and PYR-1a−BSA (34 nM, 18 nM, and 52 ppb, respectively) indicated negligible contribution of the linker and BAS moieties on 10C10 recognition. I_{50} values of PAH conjugates are more direct and reliable criteria than those of free haptens or parent PAHs to guide the selection of coating antigen for assay development. In addition, relative inhibitions of analytes and the corresponding haptens and hapten conjugates should be considered as illustrated in Tables 2 and 3.

Comparison of MAbs 10C10 and 4D5. In addition to MAb 10C10, a second MAb, 4D5, developed by Gomes and Santella from BaP-6a−BSA immunogen, was examined. MAbs 4D5 and 10C10 differ by 23 amino acids but have similar equilibrium dissociation constants for BaP (C. W. Bell and A. E. Karu, in preparation). To determine whether the sequence differences affected selectivity of these two MAbs, indirect competition LAB-ELISAs were done with PYR-1a−BSA as coating antigen, and the I_{50} values for various PAHs were compared (Table 4). Both antibodies showed similar patterns of recognition. Analysis of variance using one-way ANOVA ($\alpha = 0.05$) revealed no significant difference between I_{50} values obtained with 10C10 and 4D5 except for pyrene, phenanthrene, and anthracene. The differences in percent cross-reactivity are too small to be of practical use for these three. Thus, MAbs 10C10 and 4D5 have essentially the same PAH recognition patterns, despite the amino acid sequence differences, and only MAb 10C10 was further investigated.

Effect of Organic Solvents. The effects of methanol, acetone, and DMSO on the indirect competitive LAB-ELISA were examined. Samples containing up to 2% (v/v) acetone or DMSO or up to 5% methanol could be used with no significant change in OD readings compared with controls in PBS (data not shown). This solvent tolerance is sufficient for analyses of solvent extracts of many environmental samples.

Assay Variation. Reproducibility and precision are important criteria of an immunoassay. Standard curves for the indirect competitive LAB-ELISA of BaP from four consecutive days were compared and variations were calculated (Figure 2). Detection was linear with the log of BaP concentration from 0.4 to 1000 nM. The variations in replicates from well to well (intraassay) and plate to plate (interassay) were measured (Table 5).[36] Intraassay variations were generally lower than interassay variations. Variations in the standards were greatest at the lowest BaP concentrations. This nonlinearity of variance with concentration is common in competitive ELISA.[37−39] However, the CVs for samples increased with increasing BaP concentrations (Table 5). Factors that contribute variations include quality of the hapten, coating, plate wells and multichannel pipettor, edge effects due to evaporation, uneven temperature during incubation, and day-to-day variations in the preparation of reagents. A standard curve should be created every time an assay is performed to reduce the variations.

Analysis of Spiked Samples. PBS buffer, tap water, Ala Wai canal water, and human urine from a nonsmoker were spiked with BaP to 2−600 nM (i.e., 0.5−150 ppb), and BaP concentrations were determined by the LAB-ELISA using MAb 10C10 culture supernate. The assay was shortened by adding both the MAb (1:3000) and biotinylated goat anti-mouse IgG (1:6000) to the analyte mixtures, combining two incubation steps into one. Recoveries of the BaP spikes ranged from 81 to 127% in all samples (Table 6).

(32) Cheung, P. Y. K.; Kauvar, L. M.; Engqvist-Goldstein, A. E.; Ambler, S. M.; Karu, A. E.; Ramos, L. S. *Anal. Chim. Acta* **1993**, *282*, 181−192.

(33) Jones, G.; Wortberg, M.; Hammock, B. D.; Rocke, D. M. *Anal. Chim. Acta* **1996**, *336*, 175−183.

(34) Wortberg, M.; Kreissig, S. B.; Jones, G.; Rocke, D. M.; Hammock, B. D. *Anal. Chim. Acta* **1995**, *304*, 339−352.

(35) Karu, A. E.; Lin, T. H.; Breiman, L.; Muldoon, M.; Hsu, J. *Food Agric. Immunol.* **1994**, *6*, 371−384.

(36) Bookbinder, M. J.; Panosian, K. J. *Clin. Chem.* **1986**, *32*, 1734−1737.

(37) Wellington, D. Statistical Aspects of Enzyme Immunoassay. In *Enzyme Immunoassay*; Maggio, E. T., Ed.; CRC Press: Boca Raton, FL, 1980; pp 249−273.

(38) Canellas, P. F.; Karu, A. E. *J. Immunol. Methods* **1981**, *47*, 375−385.

(39) Karu, A. E.; Perman, M.; McClatchie, I. R. T.; Speed, T. P.; Richman, S. J. AutoElisa: A data management system for regulatory and diagnostic immunoassays, using parallel fitting for data evaluation. In *New Frontiers in Agrochemical Immunoassay*; Kurtz, D. A., Skerritt, J. H., Stanker, L., Eds.; AOAC International: Arlington, VA, 1995; pp 261−282.

Table 6. Recoveries of BaP in Spiked Samples[a]

spiked level (nM)	recovery determined by LAB-ELISA (% ± SD [b])			
	buffer	tap water	canal water	urine
2	84 ± 8	96 ± 12	94 ± 10	118 ± 17
4	96 ± 7	86 ± 9	96 ± 9	113 ± 11
20	105 ± 7	97 ± 5	86 ± 7	85 ± 10
40	83 ± 9	81 ± 9	92 ± 5	89 ± 10
200	85 ± 7	84 ± 8	106 ± 5	127 ± 19
400	98 ± 5	85 ± 8	100 ± 7	127 ± 9
600	117 ± 15	87 ± 9	80 ± 9	118 ± 13
av	95	88	93	111

[a] BaP in spiked samples was directly analyzed by LAB-ELISA without sample preparation. MAb 10C10 culture supernate was diluted 1:6000 and the coating antigen was PYR-1a−BSA at 0.5 ng/100 μL per well. [b] Values were means ± standard deviations for four replicates.

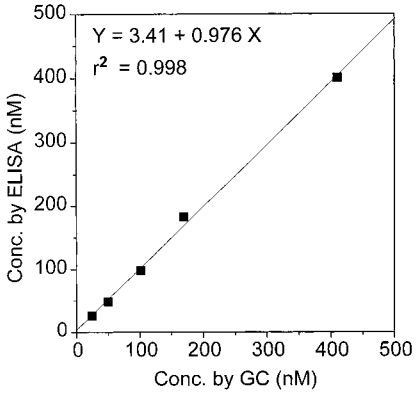

Figure 4. Correlation of BaP concentrations measured by GC and those directly by ELISA using MAb10C10 for fortified canal water samples (100 nM of BaP = 25 ppb). The plate was coated with 0.5 ng/100 μL per well PYR-1a−BSA, and MAb 10C10 was diluted 1:6000. The data were means of four replicate experiments.

Canal water samples fortified with 20−400 nM BaP (i.e., 5−100 ppb) were split for ELISA and GC determination. The portions for ELISA were analyzed with no concentration or cleanup. The portions for GC analysis were extracted with ethyl acetate and concentrated. The concentrations determined by ELISA (26−400 nM) agreed well with those by GC (24−411 nM) (Figure 4). The least-squares fit had a slope of 0.976 and a coefficient of determination (r^2) of 0.998. The ELISA detected BaP as low as ∼10 pg/well. The GC method can measure ∼1.5 ng/injection. Therefore, the ELISA was ∼150-fold more sensitive than GC for these analyses.

CONCLUSION

The new approach of hapten screening used in this study should be applicable to new immunoassay development and optimization for other small molecules of environmental significance. The LAB-ELISA was optimized by cross-screening PAH−BSA conjugates as competitors in solution and as coating antigens immobilized on the ELISA wells. A recognition index was defined to compare relative affinities of the antibodies to the PAH conjugates and roughly to predict assay sensitivities with a given conjugate as coating antigen. PYR-1a−BSA, which was identified as the least competitive inhibitor among 11 PAH conjugates, gave the most sensitive assay for BaP and other PAHs when it was used as coating antigen. The indirect LAB-ELISA format was very sensitive because the biotinylated second antibody could accommodate multiple copies of the avidin−HRP probe. The resulting amplification allowed us to reduce the concentrations of coating conjugate and MAb.

Although absolute immunospecificity for a single analyte is desirable, it may be impractical to achieve for highly similar molecules such as the two- to five-ring PAHs. The present work explored the potential of using antibodies that cross-react and changing the sensitivity and cross-reactivity by using different competing haptens. MAbs 4D5 and 10C10 might be used to complement the reaction patterns of other PAH antibodies as components of more complex multianalyte PAH assays and sensors with the new PAH haptens described here. The reactivity profiles with the new haptens suggest additional combinations of antibodies and conjugates (for example, antibodies with strong preference for naphthalene, fluorene, or fluoranthene) that would be useful together with MAbs 4D5 and 10C10.

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