Development of Enzyme-Linked Immunosorbent Assays for 4-Nitrophenol and Substituted 4-Nitrophenols[†]

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An enzyme-linked immunosorbent assay (ELISA) was developed for the determination of 4-nitrophenol and monosubstituted 4-nitrophenols such as 2-chloro-4-nitrophenol and 3-methyl-4-nitrophenol. An antiserum (Ab1812) was selected among eight antisera against three different corresponding immunogens. 4-(Nitrophenyl)acetyl OVA conjugate (C-OVA) as a coating antigen was selected among eight hapten–protein conjugates. Using an Ab1812 dilution of 1/1000, I_{50} values for 4-nitrophenol, 3-methyl-4-nitrophenol, 2-chloro-4-nitrophenol, and 2-amino-4-nitrophenol were 59, 64, 31, and 57 nM, respectively. There were no cross reactivities with other substituted phenols/nitrobenzenes, and 2- or 3-nitrophenol. The detection limit of this ELISA was 1000 times lower than that of a standard colorimetric method for the analysis of 4-nitrophenol. Under the optimized condition, this procedure can be used to determine the concentration of 4-nitrophenol and/or monosubstituted 4-nitrophenols at levels as low as 0.2 ppb in different water samples without extraction and cleanup.

INTRODUCTION

Nitrophenols are important intermediates in the synthesis of dyes, pesticides, and many other industrial chemicals and have a wide variety of other uses (Hartter, 1985). 4-Nitrophenol, for example, is used as an intermediate in the synthesis of insecticides such as ethyl and methyl parathion and as an indicator in the laboratory. In 1986, 59 million pounds of 4-nitrophenol were produced in the United States. Unfortunately, nitrophenols are also environmental contaminants in soil, water, and air (Glotfelty et al., 1987; Sethunathan et al., 1977; Shea et al., 1983). In addition, some organophosphate pesticides can be degraded to nitrophenols, such as 4-nitrophenol (Mulla and Mian, 1981a) and 3-methyl-4-nitrophenol (Takimoto et al., 1984), either in the environment or in organisms. Therefore, the potential exists to use these nitrophenols as biomarkers to assess exposure to and risks from these environmental toxicants. New detection methods are urgently required for the study of these chemicals since existing analytical methods for nitrophenols are either time-consuming and expensive (Cranmer, 1970; Volpe and Mallet, 1981) or insensitive (Katagi et al., 1989; Ramakrishna and Ramachandran, 1978; Zakrevsky and Mallet, 1977). Immunoassays have been used in biomedical sciences for many years (Luster et al., 1988; Voller et al., 1976) and introduced to agricultural applications successfully for the detection of a variety of pesticides, including organochlorine compounds, organophosphates, sulfonylureas, phenoxyaliphatic acids, diphenyl ethers, bipyridinium compounds, triazines, thiocarbamates, isoxazolidinone compounds, and other classes of compounds (Jung et al., 1989; Vanderlaan et al., 1988; Van Emon and Mumma, 1990). Although ELISA methods are generally more rapid and less expensive than competing methods such as GC and HPLC and are particularly suited to samples that can be analyzed without extensive workup, immunoassay procedures have received very limited use in the analysis of industrial chemicals and/or intermediates. This is unfortunate since biosensor technology allows immunoassays to provide continuous monitoring of waste-water streams. Because nitrophenols are water soluble and acidic, they can be extracted from solid samples, such as soil samples from waste sites, with aqueous base solution. They also can be easily concentrated by differential purification. While GC methods exist for nitrophenols, an ELISA method for their analysis could have significant advantages over existing methods.

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EXPERIMENTAL DESIGN AND METHODS

Chemicals. 3,4-Dichlorobenzyl bromide and 2,4-dichlorobenzyl chloride were purchased from Fairfield Chemical Co. 3-Hydroxy-6-nitrobenzaldehyde, 2-hydroxy-5-nitrobenzoic acid, 4-methyl-5-nitrobenzoic acid, 3-methyl-4-nitrobenzoic acid, 3,4-dichlorobenzaldehyde, 3,4-dichlorobenzoic acid, 2,4-dichlorobenzoic acid, 2,4-dichlorobenzaldehyde, 3,5-dichlorobenzaldehyde, 3,5-dichlorobenzoic acid, and 3-(2,4,5-trichlorophenoxy)propionic acid were purchased from Pfaltz & Bauer, Inc. 1,3-Dicyclohexylcarbodiimide (DCC) and proteins used in coupling were purchased from Sigma Chemical Co. Other chemical reagents were purchased from Aldrich Chemical Co. The chemicals and their purities were checked by ^1H NMR spectrophotometry (Varian EM-390 90 MHz, General Electric) and thin-layer chromatography after purchase.

Instruments. Enzyme-linked immunosorbent assays (ELISAs) were carried out in 96-well plates (Nunc, Roskilde, DK) and read with a V_{max} microplate reader (Molecular Devices, Menlo Park, CA) at dual wavelength 405–560 nm. Ultraviolet–visible (UV–vis) spectra were obtained on an HP 8452A diode array spectrophotometer (Hewlett-Packard).

Synthesis of Hapten–Protein Conjugates. The haptens listed in Table I were directly used to synthesize hapten–protein conjugates. Nucleophilic substitution, reductive alkylation, and active ester methods were used to couple carrier proteins with haptens containing active bromide, aldehyde, and carboxylic acid

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Table I. List of Haptens, Carrier Proteins, and Conjugates Synthesized[a]

chemicals used as hapten (hapten no.)	carrier proteins	conjugates
2-hydroxy-5-nitrobenzyl bromide (1)	BSA	1-BSA
	KLH	1-KLH
3-hydroxy-6-nitrobenzaldehyde (2)	BSA	2-BSA
	KLH	b
	OVA	2-OVA
2-hydroxy-5-nitrobenzoic acid (3)	OVA	3-OVA
4-methyl-3-nitrobenzoic acid (4)	OVA	4-OVA
3-methyl-4-nitrobenzoic acid (5)	OVA	5-OVA
2-methoxy-5-nitrobenzyl bromide (6)	OVA	6-OVA
2,4-dinitrobenzoic acid (A)	OVA	A-OVA
4-nitrophenylacetic acid (C)	OVA	C-OVA

[a] Conjugates 1-BSA, 1-KLH, and 2-BSA were used as immunogens. OVA conjugates were used as coating antigens. [b] No hapten–protein conjugate was obtained.

functional groups, respectively. All synthesized conjugates were exhaustively dialyzed against phosphate buffer (0.2 M, pH 7.4) at 4 °C. After dialysis, UV spectra were obtained to confirm the conjugation, and conjugates were stored at −80 °C for later use.

Nucleophilic Substitution. The active bromide hapten (50 μmol of compound 1 or 6), tri-*n*-butylamine (55 μmol), and a trace amount of 4-(dimethylamino)pyridine as catalyst were dissolved in 2 mL of dimethylformamide (DMF). Then, protein (50 mg, 1.1 μmol of OVA, 0.7 μmol of BSA, 0.07 μmol of KLH) dissolved in 3 mL of PBS buffer (0.2 M, pH 7.5) was added and stirred at room temperature for 2 h.

Reductive Alkylation. Compound **2** was conjugated to proteins according to the method of Gaur et al. (1981) with a slight modification. Protein (50 mg, OVA or BSA) dissolved in 4 mL of PBS buffer (pH 7.5, 0.2 M) was added to 50 μmol of compound **2** dissolved in 1 mL of methanol and stirred for 30 min at 37 °C. After 30 μmol of $NaBH_4$ dissolved in methanol was added, the reaction was stirred for another 30 min at 4 °C. Finally, 1 mL of 0.1 N HCl was added to destroy the excess of $NaBH_4$.

Active Ester Method. The haptens with a carboxylic acid functional group (compounds **3–5**) were coupled covalently to proteins by the active ester method according to the procedure of Bauminger and Wilchek (1980). Conjugation of compounds A and C to ovalbumin was reported by Eck et al. (1990).

Immunization of Rabbits. Rabbits 1803, 1805, and 1806 were immunized with 2-BSA, rabbits 1808, 1809, and 1810 with 1-KLH, and rabbits 1811 and 1812 with 1-BSA. The immunization was done according to the method of Li et al. (1991). NaN_3 was added to the isolated serum to a final concentration of 0.02%. The serum was stored at −80 °C.

Enzyme Immunoassay and Competitive Enzyme Immunoassay Procedure. Microtiter plates (96-well) were coated with coating antigens at 0.6–20 μg/mL (100 μL/well) in 0.1 M carbonate–bicarbonate buffer (pH 9.6). Plates were sealed with adhesive plate sealers and incubated at 4 °C overnight. The samples or standards (120 μL) were mixed with 120 μL of antiserum, at the appropriate dilution in phosphate-buffered saline solution containing 0.05% Tween 20 and 0.02% sodium azide (PBSTA) (pH 7.4), and were incubated overnight at room temperature. The following day, the plates were washed with PBSTA buffer. The samples or standards (for checkerboard titration, no analyte) mixed with antiserum were added to the wells (50 μL/well). After 3 h of incubation at room temperature, the plates were washed again, and 50 μL of goat anti-rabbit IgG conjugated to alkaline phosphatase diluted 1:2500 with PBSTA was added and incubated for 2 h at room temperature. After another wash, 100 μL of a 1 mg/mL solution of 4-nitrophenyl phosphate in 10% diethanolamine buffer was added and incubated for 20–60 min. The optical density was then read at dual wavelength (405–560 nm) on a V_{max} microplate reader. The inhibition curves were analyzed by an IBM personal computer using a four-parameter logistic curve-fitting procedure which calculated I_{50} values (molar inhibitor concentration giving 50% inhibition). Optimal concentrations of coating antigens and antisera were obtained from the results of checkerboard titrations.

RESULTS AND DISCUSSION

Hapten Selection and Conjugation. There are only two positions (ortho and meta to the hydroxyl group) on 4-nitrophenol available for spacer attachment that would not drastically affect its chemical properties. To minimize spacer recognition, compounds **1** and **2** with a short spacer (CH_2) were selected as immunizing haptens. Immunizing haptens with a spacer at different positions were used to obtain antibodies showing specificity to 2- or 3-substituted 4-nitrophenols. It is known that multiple combinations of antibodies and coating antigens may generate more sensitive and specific assays (Wie and Hammock, 1984). Therefore, other nitroaromatic chemicals (compounds **3–6**, A, and C) as well as compounds **1** and **2** were conjugated to the appropriate carrier proteins and used as coating antigens (Table I).

Screening of Antisera by Testing Their Titers. Screening the titers of eight antisera using eight coating antigens and two protein controls gave a total of 15 combinations of five antisera that showed reasonable titers as indicated in Table II. The titers of three groups of antisera (Ab1803, 1805, 1806; 1808, 1809, 1810; and 1811 and 1812) against corresponding immunogens indicated clearly that the chance of raising useful antibodies depended on the immunogens used and was found to vary among individual animals. Antisera (Ab1803, 1805, and 1806) against 2-BSA gave very low titers with most coating antigens except 1-BSA. BSA, however, was also the immunizing protein. This lack of response to most coating antigens may be due to the masking of a major determinant group, the nitro group, on the hapten of immunogen and/or coating antigens (Brimfield et al., 1985a; Hack et al., 1989; Vallejo et al., 1982). Seven useful combinations were obtained from Ab1808, 1809 and 1810 against 1-KLH, which had the same hapten as 1-BSA. The titer differentials between antibodies raised from 1-BSA and 1-KLH may be due to the fact that carrier proteins have different immunogenicity (Harlow and Lane, 1988). The importance of the hapten structure to the resulting antibody characteristics was partially shown by the higher titers for Ab1811 and Ab1812 (against 1-BSA) compared to Ab1803, 1805, and 1806 (against 2-BSA) in which the haptens differed only by spacer location. In spite of this apparently small structural modification, its effect on antibody characteristics is dramatic.

Screening of Antisera by Testing Their Recognition to Analytes. Compounds **18**, **21**, and **23** were selected further to screen antisera in competitive assays on those antibody/coating antigen combinations showing reasonable titers. The data in Table III show several trends. First, when the same coating antigen was used, the affinity of different antisera for an analyte varied. Antisera (Ab1808, 1809, and 1810) against 1-KLH were generally less sensitive for the analytes tested than those (Ab1811 and 1812) against 1-BSA. For example, I_{50} values for compound **18** using Ab1809, 1810, 1811, and 1812 with C-OVA as coating antigen were 17 400, 1200, 870, and 59 nM, respectively. Second, the relative affinity of an antiserum to an analyte varied depending on the coating antigen used. For example, I_{50} values for compound **18** using Ab1812 with 3-OVA, 4-OVA, 5-OVA, and C-OVA were 13 300, 139, 1300, and 59 nM, respectively. Third, the specificity and detection limit of a combination of antiserum and coating antigen varied among analytes. This was shown, in part, by Figure 3, and it will be discussed

Table II. Summary of Titration Results[a]

serum no.	coating antigens									
	1-BSA	2-OVA	3-OVA	4-OVA	5-OVA	6-OVA	A-OVA	C-OVA	OVA	BSA
1803	H	L	L	L	L	L	L	L	L	H
1805	H	L	L	L	L	L	L	L	L	H
1806	H	L	L	L	L	L	L	L	L	H
1808	L	L	*	L	L	L	L	L	L	L
1809	L	L	L	L	*	L	L	*	L	L
1810	L	L	*	*	*	L	L	*	L	L
1811	H	L	*	*	*	L	L	*	L	H
1812	H	L	*	*	*	L	L	*	L	H

[a] Sera 1803, 1805, and 1806 were against 2-BSA; 1808, 1809, and 1810 against 1-KLH; 1811 and 1812 against 1-BSA. L and H represent titers that were too low and too high, respectively. Recognition of the hapten and/or carrier protein, BSA, was responsible for the very high titers. *Represents those antigen/antibody combinations tested that were taken to the next screening stage.

Table III. Screening Results from Various Combinations of Antibodies and Coating Antigens Using Representative Analytes[a]

serum no.	coating antigen	4-nitrophenol (18)		3-methyl-4-nitrophenol (21)		2-amino-4-nitrophenol (23)	
		slope	I_{50}, nM	slope	I_{50}, nM	slope	I_{50}, nM
1808	3-OVA	1.034	4120	0.756	30200	0.736	4750
1809	5-OVA	1.045	14100	1.070	44600	0.846	14200
	C-OVA	1.275	17400	1.400	25700	1.320	14900
1810	3-OVA	1.089	1250	1.050	613	1.580	296
	4-OVA	0.826	568	0.677	225	1.240	2940
	5-OVA	0.994	25900	1.230	17800	0.868	140000
	C-OVA	0.975	1200	0.882	395	1.26	2070
1811	3-OVA	0.861	146	0.891	1230	1.120	282
	4-OVA	0.844	515	0.522	3000	0.771	571
	5-OVA	1.19	1040	1.130	5540	0.996	1910
	C-OVA	0.920	870	0.599	5960	0.856	414
1812	3-OVA	1.465	13300	1.280	5140	1.040	3090
	4-OVA	0.694	139	0.470	67	0.634	68
	5-OVA	0.582	1300	0.638	1980	0.415	342
	C-OVA	0.762	59	0.662	64	0.924	57

[a] The data are averages of two independent experiments each having four-well replicates.

in more detail in the following text. Fourth, the various specificities and affinities of antisera to different analytes were achieved by using different coating antigens. For example, the I_{50} values for compounds **18**, **21**, and **23** were 146, 1230, and 282 nM, respectively, when the combination of Ab1811 with **3**-OVA was used. For the combination of Ab1812 with **3**-OVA, I_{50} values for compounds **18**, **21**, and **23** are 13 300, 5140, and 3090 nM, respectively. The combination of Ab1812 with C-OVA gave almost identical detection limit with compounds **18** (I_{50} = 59 nM), **21** (I_{50} = 64 nM), and **23** (I_{50} = 57 nM). When the combination of Ab1812 and **4**-OVA was used, I_{50} values for compounds **18**, **21**, and **23** were 139, 67, and 68 nM, respectively. However, the assay of Ab1812 and **4**-OVA showed very slow color development after the substrate was added more than 8 h of incubation to get reasonable color). The various specificities of antisera may be due simply to their different affinities for different coating antigens and/or analytes. Those assays mentioned above offer more opportunities to obtain specific and sensitive assays for the target compound(s) and fully use the antibodies already generated. In this paper, only the combination of Ab1812 (1:1000 diluted in PBSTA in final concentration) with C-OVA (2.5 μg/mL) was selected for further study since this combination gave the lowest detection limit for the targets. Other combinations may yield more sensitive and specific assays for other targets.

Assay Optimization. *pH Effect.* Many immunoassays are equally sensitive over a wide range of pH values. However, as is common for potentially ionized species, the assay for 4-nitrophenol was pH sensitive. The affinity of Ab1812 to analytes (compounds **18** and **23**) and coating

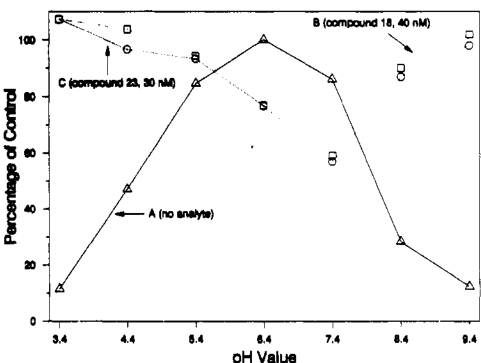

Figure 1. Effect of pH on antiserum 1812 and its interaction with coating antigen (C-OVA) and analytes (4-nitrophenol and 2-amino-4-nitrophenol). Assays were performed by using Ab1812 and C-OVA as coating antigen. Percentage of control for the points with analyte added was calculated according to the equation [the OD (with analyte)/the OD (no analyte) at the corresponding pH]. Percentage of control for noncompetitive assay (no analyte) was a relative value normalized to the percentage of the OD obtained at pH 7.4. (A) Noncompetitive assay (no analyte); (B) 40 nM 4-nitrophenol added; (C) 30 nM 2-amino-4-nitrophenol added. The data are means of four replicates with CV% from 1.0 to 5.6.

antigen (C-OVA) was very pH dependent as is shown in Figure 1. pH probably affects both the analytes and the antibodies. Both a maximal affinity of the antibodies to coating antigen (C-OVA) at pH 6.4 and that to the analytes at pH 7.4 suggest that a histidine residue may be involved in the binding site since the pK_a of histidine's imidazole NH^+ is about 6.0, and the pK_a values of 4-nitrophenol and 2-amino-4-nitrophenol are about 7.2 and

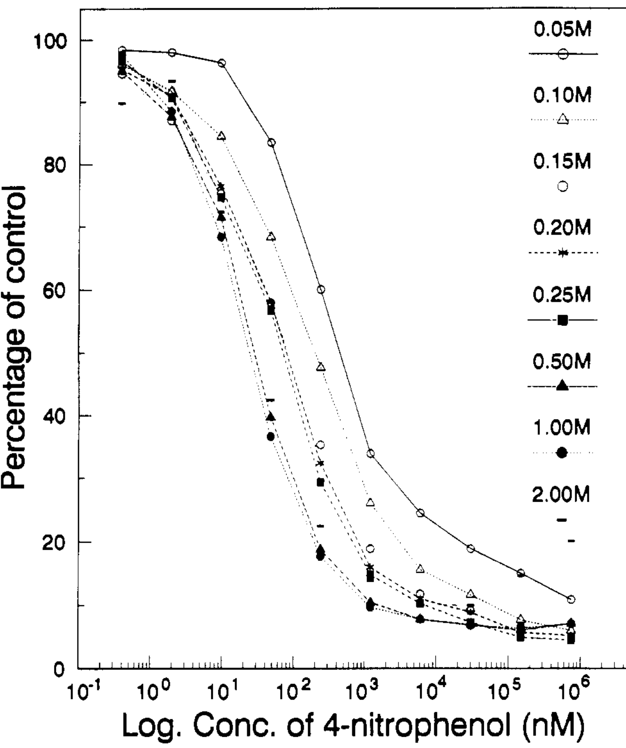

Figure 2. Effect of incubation buffer (PBSTA) concentration on the ELISA standard curve for 4-nitrophenol. The data are means of four replicates. Average CV% of standard curves in 0.05, 0.10, 0.15, 0.20, 0.25, 0.50, 1.00, and 2.00 M PBSTA are 2.5, 2.1, 2.2, 3.0, 2.3, 3.6, 2.6, and 5.9, respectively.

6.6 at 25 °C, respectively. A possible hydrogen binding exists between the nitrogen of histidine and the oxygen of the phenoxy. The pH values may also affect the ionization form and structure of the antibodies in both noncompetitive and competitive assays. The maximal affinity of the antibodies to the analytes at pH 7.4 was indicated by a maximal inhibition (minimal percentage of control) of compounds **18** and **23** at that pH and almost no inhibition at pH higher than 8.4 or lower than 5.4. Thus, it is necessary to adjust the pH of water samples to about neutrality to obtain the lowest limit of detection (LOD) and maximum accuracy in practice.

Effect of Incubation Buffer Concentration. 4-Nitrophenol was used as a representative compound to study the effect of buffer concentration and to optimize the assay. Even visually, the assay's LOD was significantly improved by an increase of incubation buffer concentration (Figure 2). When the buffer concentration was higher than 0.5 M, the I_{50} value was about half that at 0.2 M. This improvement may be due simply to dispersion and weakening of the nonspecific binding derived from heterogeneous antibodies. In other words, the higher ionic strength provides the same effect as purification of the antibody, by dampening the nonspecific antibody binding. However, after substrate was added, the color development was slowed significantly, having an OD about 0.5 in 30 min at 0.2 M compared with a similar OD requiring 1–2 h at 0.5–2.0 M. Thus, the buffer concentration of 0.2 M was used for further study. If a lower LOD is required and analysis time is not a limitation, a higher buffer concentration (0.5 M or higher) can be used to decrease the LOD, or a format can be used that allows high buffer strength for competitive binding and low buffer strength for color development. Reoptimization of the assay at the new condition may give desired results. Alternatively, one could use a reporter system resistant to ionic strength or use purified antibody.

Table IV. Specificity of Ab1812 toward 4-Nitrophenol and Di- and Monosubstituted 4-Nitrophenols[a]

(structure: OH, R_6, R_2, R_3, NO_2 — GROUP 1)

compd no.	R_2	R_3	R_6	I_{50}, nM	CR%[b]
18	H	H	H	59 ± 11	100.0
3	CO_2H	H	H	6740 ± 50	0.9
22	Cl	H	H	31 ± 9	190
23	NH_2	H	H	57 ± 13	104
31	NO_2	H	H	127 ± 16	48.1
60	$NHC(O)CH_3$	H	H	846[c]	7.0
21	H	CH_3	H	64 ± 14	92.0
49	CH_3	H	CH_3	1820 ± 500	3.3

[a] The data are averages of 4–13 independent experiments over 5 months, and each experiment has four-well replicates with average CV% from 2.1 to 7.7. [b] Percentage of cross reactivity (CR%) = (I_{50} of compound 18/I_{50} of other compound) × 100%. [c] Single experiment with four-well replicates.

Table V. Specificity of Ab1812 toward Substituted Nitrobenzenes[a]

(structure: R_1, R_2, R_3, NO_2 — GROUP 2)

compd no.	R_1	R_2	R_3	I_{50}, nM	CR%[f]
4	CO_2H	H	NO_2	>150 000	<0.05
5	CO_2H	H	CH_3	49 200 ± 110	0.12
33	NH_2	H	H	>150 000	<0.05
35	OCH_3	NH_2	H	>150 000	<0.05
36	H	H	H	>150 000	<0.05
37	$OP(S)(OEt)_2$	H	H	>150 000	<0.05
38	$OP(S)(OMe)_2$	H	H	>150 000	<0.05
62	$OP(O)(OEt)_2$	H	H	2830[b]	2.0
39	CH_2CO_2H	H	H	>150 000	<0.05
40	CO_2H	Cl	H	53 400 ± 3670	0.11
41	$OC(O)CH_3$	H	H	50 ± 17	118
56	$OC(O)OCH_3$	H	H	8.4[c]	702
42	Cl	H	H	>150 000	<0.05
43	SH	H	H	>150 000	<0.05
44	CH_3	H	H	>150 000	<0.05
45	CH_2OH	H	H	>150 000	<0.05
46	SCH_3	H	H	>150 000	<0.05
47	OCH_3	H	CH_3	>150 000	0.05
50	$OP(O)ClN(CH_2CH_2)_2O$	Cl	H	3 490 ± 500	1.69
52	SO_3K	H	H	23 000 ± 1620	0.26
53	α-D-G[d]	H	H	29 400 ± 7700	0.20
55	β-D-G[e]	H	H	68 200 ± 9590	0.09

[a] The data are means of three independent experiments, and each experiment has four-well replicates. [b] Single experiment with four-well replicates. [c] Average of two independent experiments. [d] 4-Nitrophenyl α-D-glucopyranoside. [e] 4-Nitrophenyl β-D-glucopyranoside. [f] CR% calculation is shown in Table IV.

Specificity of Ab1812. Excellent specificity of Ab1812 for four groups of related compounds was realized as shown in Tables IV–VI and Figure 3. Both the hydroxyl and nitro (Hack et al., 1989; Janda et al., 1988) groups and their para orientation are critical for antibody recognition as Ab1812 lacked recognition of mono- and disubstituted nitrobenzenes (Table V), mono- and disubstituted phenols, 2/3-nitrophenols, 4-nitrosophenol, and 4-nitropyridine *N*-oxide (Table VI) even at very high concentrations. Surprisingly, compounds **41**, **50**, **56**, and **62** gave I_{50} values of 50, 3490, 8.4, and 2830 nM, respectively. This may be due to their partial hydrolysis during competitive incu-

Table VI. Specificity of Ab1812 toward 2- or 3-Nitrophenol, Other Substituted Phenols, and 4-Nitropyridine *N*-Oxide[a]

GROUP 3

compd no.	R_2	R_3	R_4	I_{50}, nM	CR%[c]
19	NO_2	H	H	>150 000	<0.05
20	H	NO_2	H	>150 000	<0.05
61	NO_2	H	$NHC(O)CH_3$	61 300[b]	0.10
24	H	H	Cl	>150 000	<0.05
25	H	H	NO	11 600 ± 1850	0.51
26	H	Cl	Cl	>150 000	<0.05
27	H	H	NH_2	>150 000	<0.05
28	H	H	CH_3	>150 000	<0.05
29	H	CH_3	Cl	>150 000	<0.05
32	H	H	H	>150 000	<0.05
GROUP 4 (NO_2–pyridine N→O)				>150 000	<0.05

[a] The data are means of three independent experiments, and each experiment has four-well replicates. [b] Single experiment with four-well replicates. [c] CR% calculation is shown in Table IV.

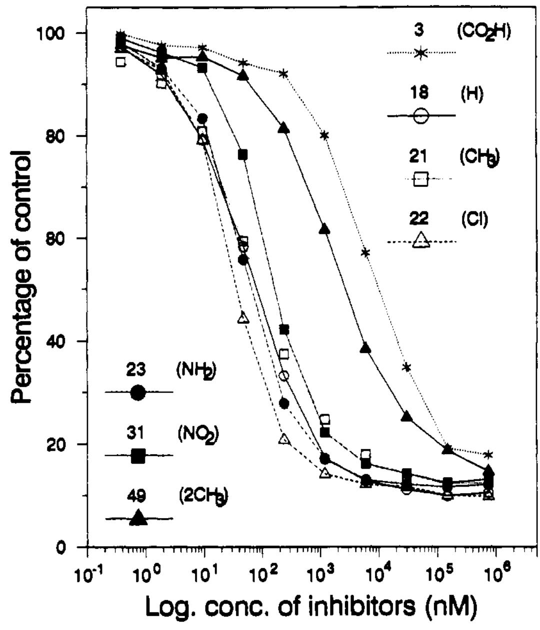

Figure 3. Standard curves of 4-nitrophenol and mono- and disubstituted 4-nitrophenols in PBSTA using the combination of Ab1812 and C-OVA as coating antigen. The structures of these compounds are shown in Table IV. The substituent on 4-nitrophenol is shown in parentheses. The data are averages of 4–13 independent experiments in 5 months, and each experiment has four-well replicates with average CV% from 2.1 to 7.7. Among experiments, CV% of the slopes of standard curves of compounds 3, 18, 21, 22, 23, 31, and 49 were 16.4, 9.5, 10.3, 10.4, 7.3, 3.2, and 2.3, respectively. I_{50} values and their standard deviations of different compounds are shown in Table IV.

bation and/or catalysis by the antibodies (Slobin, 1966), which is under study. Substitution of SH for OH (compound **43**) completely abolished the interaction with Ab1812. This observation was also found in the assay of paraoxon (Brimfield et al., 1985a) and soman (Brimfield et al., 1985b). We postulate that these effects are due to a reduction in hydrogen-bonding potential. Ab1812 showed excellent specificities among monosubstituted 4-nitrophenols. The I_{50} values of compounds **3, 18, 21, 22, 23, 31,** and **60** were 6740, 59, 64, 31, 57, 127, and 846 nM, respectively. Although different substituents on 4-nitrophenol had various contributions to the binding with Ab1812, there was no correlation obtained between the affinities and Hammett constants of the functional groups. The affinity of Ab1812 was also related to the steric structure of analytes; for example, compound **49** with a methyl at each side of 4-nitrophenol gave an I_{50} of 1820 nM, which is 28 times greater than that (64 nM) of 3-methyl-4-nitrophenol. This key-lock principle has been observed in many immunoassays (Huber and Hock, 1985; Nagao et al., 1989; Newsome and Collins, 1989; Van Emon et al., 1986).

If the specificity among monosubstituent 4-nitrophenols is required, the requisite resolution may be enhanced by using two types of antibodies. Consider a situation where the first antibody such as Ab1812 recognizes the unsubstituted face of monosubstituent 4-nitrophenols, while another antibody recognizes the other face as well as parts of the binding site of the first antibody (Haber, 1982). Such a two-antibody assay may be very useful in analyzing a required single analyte such as 4-nitrophenol, 2-chloro-4-nitrophenol, and 3-methyl-4-nitrophenol, which are common metabolites in the environment and/or organisms from methyl/ethyl parathion, dicapthon, and fenitrothion, respectively. While these insecticides are usually not tank-mixed or used simultaneously in the field, one could well encounter such a mixture of substituted 4-nitrophenols in an agricultural waste site.

Standard Curves and Interpretation of Standard Curves. An advantage of similar I_{50} values for these monosubstituted 4-nitrophenols is that this assay can be used to determine these compounds as a class. The standard curves for compounds **3, 18, 21, 22, 23, 31,** and **49** using Ab1812 and C-OVA had slopes of 0.883, 0.762, 0.662, 0.982, 0.924, 0.967, and 0.666, respectively, as well as different detection limits. As illustrated in Table III, different antigen/antibody combinations showed different LODs and sensitivities to analytes, so it may be possible to estimate statistically the concentration of each analyte in a mixture. Compounds **3, 22, 23,** and **31**, with substituents adjacent to the hydroxyl group, gave slopes steeper than those of compounds **18, 21,** and **49**. This seems to be related to the spacer location on the immunizing hapten (ortho to the hydroxyl group, Ab1812 against 1-BSA). However, no general rules for predicting the sensitivity (slope) and LOD (position) of standard curves of different analytes in different assays have been generated from the data in Table III. There is no doubt that both the slope and the LOD of a standard curve are related to the affinities of antibodies to the coating antigen and analyte and the ratio of both.

The independent binding of analytes to Ab1812 is illustrated in Figure 4. Curve A represents the average of four standard curves for compounds **18, 21, 22,** and **23**, respectively, while curve B represents the standard curve of a mixture of equimolar compounds **18, 21, 22,** and **23**. Identical curves A and B indicated that the four competitors (**18, 21, 22,** and **23**) acted on the same binding site of Ab1812. In other words, inhibition is cumulative for at least these four compounds. This provides a basis for analyzing these nitrophenols as a class, statistically estimating the concentration of each of them, and/or using these assays as a detection system following chromatographic separation.

Figure 5 shows a comparison between the detection limit of this assay and that of the colorimetric method for measuring 4-nitrophenol. The LOD of this ELISA method

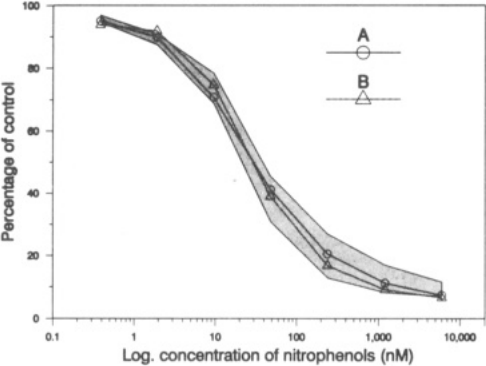

Figure 4. Standard curve (B) of a mixture of equimolar compounds **18, 21, 22**, and **23** and average curve (A) of the standard curves of compounds **18, 21, 22**, and **23** separately in PBSTA using the combination of Ab1812 with C-OVA as coating antigen. The shaded area is the average ± SD (n = 4) for curve A.

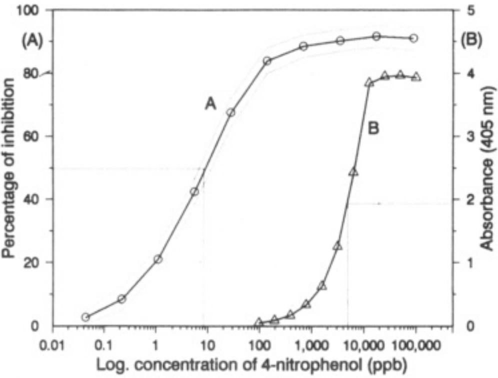

Figure 5. Comparison of ELISA (A) with colorimetry (B) for the determination of 4-nitrophenol. (A) is the average of 10 standard curves for 4-nitrophenol run independently in 6 months. The dotted lines are the average ± SD for curve A. Curve B was obtained by reading 200 μL of different concentrations of pure 4-nitrophenol dissolved in 10% diethanolamine buffer (pH 9.6) in microtiter plates at a wavelength of 405 nm.

is about 1000 times lower than that of colorimetry. One would expect an even larger difference in the LOD between these methods of analyzing real world samples since the ELISA method is specific for the target analyte(s) and thus increases the signal/noise ratio. Colorimetry, however, is nonspecific and will be subject to a relatively decreased signal/noise ratio. This assay illustrates that immunochemical methods can work very well for quite small molecules. (For example, the molecular weight of 4-nitrophenol is 139.)

An ideal ELISA procedure should satisfy the criteria of LOD (lowest I_{50}), sensitivity and accuracy (steepest slope), reproducibility, precision (least intra-interassay variation and least nonspecific interferences), wide linear window for quantitation (largest range of inhibition and moderate slope), and easy performance (least steps). Moderate slopes (0.66–0.98) for the different 4-nitrophenols as well as their broad range of inhibition afford a wide linear window for quantitating 4-nitrophenols within a class.

Matrix Effects. Since 4-nitrophenol and substituted 4-nitrophenols are metabolites of some organophosphorus pesticides as well as products of industry and laboratory wastes, these contaminants have been found in the environment (Mikami et al., 1985; Mulla et al., 1981b; Sethunathan et al., 1977; Weber, 1977), foods (Abbott et al., 1970), biological fluids (Elliott et al., 1960), and organisms (Anjum and Qadri, 1986; Garnas and Crosby, 1979; Kobayashi et al., 1985). A study of matrix effects on this assay is therefore crucial for its successful application to various samples. pH and ionic strength strongly

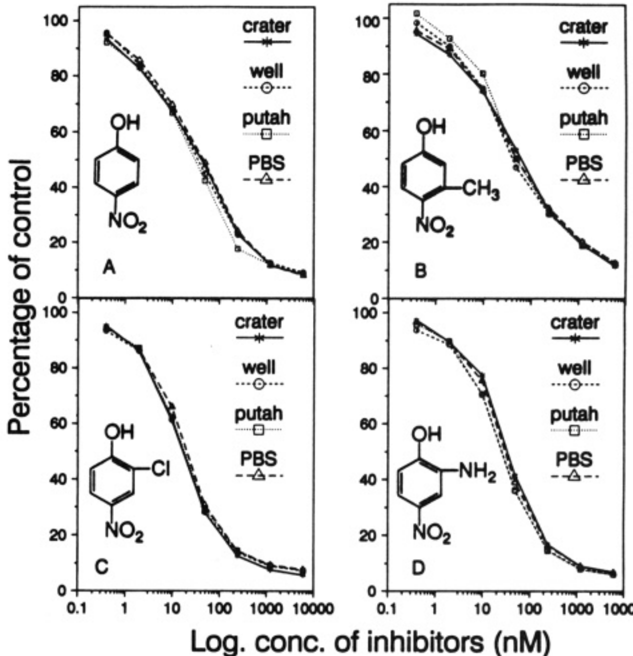

Figure 6. ELISA standard curves for compounds **18, 21, 22**, and **23** in the presence of 50% of different water samples in final concentration. The data supporting each point are means of four replicates with CV% ranging from 0.7 to 9.6.

affect the assay detectability as discussed in the previous paragraphs. Three types of water samples with free target analytes were used to test possible nonspecific interferences. A sample of well water from a well in the San Joaquin Valley of California represented groundwater. Crater Lake (Pristine) and UCD Putah Creek water represented clean and eutrophic surface water, respectively. PBSTA solution was used as the standard for comparison. When 50% of the test water sample was used in the final incubation solution, there was no significant effect on the standard curves for compounds **18** (Figure 6A), **21** (Figure 6B), **22** (Figure 6C), and **23** (Figure 6D). These data indicate that this assay can be used for the analysis of different 4-nitrophenols in environmental water samples and adapted to other samples.

CONCLUSION

A sensitive, specific, and reproducible ELISA method has been developed and optimized for the detection of 4-nitrophenol and monosubstituted 4-nitrophenols. The LOD is about 1000 times lower than that of colorimetric analysis of 4-nitrophenol. It has the advantages of low cost, rapidity, and ease of performance over conventional analytical methods such as GC, which requires extraction and/or derivatization of nitrophenols (Bengtsson, 1985; Kopecni et al., 1989). The linear ranges of inhibition for compounds **18, 21, 22, 23**, and **31** using Ab1812 with C-OVA is approximately 0.3–140, 0.3–150, 0.3–170, 0.3–150, and 1.8–220 ppb, respectively, and the I_{50} values for compounds **18, 21, 22, 23**, and **31** are 8, 10, 5, 9, and 23 ppb, respectively. There were no significant interferences for the analysis of different water samples tested. This assay demonstrates that immunoassay is a potential analytical tool for monitoring process streams even for quite small molecules such as 4-nitrophenol (molecular weight of 139), and it offers many advantages including low LOD, high specificity, good accuracy, low cost, ease of performance, and rapidity.

This assay can be used to determine a specific nitrophenol in a sample and/or the accumulated concentration

of several nitrophenols in a sample. Multiresidue analysis can be done by utilizing this assay (Ab1812 with C-OVA), and other combinations of the antibodies and coating antigens listed in Table III. These antibodies may be used as a small antibody library to be screened for the determination of other targets. This assay is now being explored as a tool in evaluating bioremediation of nitroaromatics, in analyzing 4-nitrophenol(s) as a biomarker(s) of human exposure to and risk from their precursors, and for the analysis of compounds such as parathion which can be hydrolyzed to 4-nitrophenol.

ABBREVIATIONS USED

Ab, antiserum/antibody; BSA, bovine serum albumin; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; GC, gas chromatography; IR, infrared spectroscopy; KLH, keyhole limpet hemocyanin; LOD, limit of detection; NMR, nuclear magnetic resonance spectroscopy; OD, optical density; OVA, ovalbumin; PBS, phosphate-buffered saline solution (pH 7.4); PBSTA, phosphate-buffered saline solution (pH 7.4) containing 0.05% Tween 20 and 0.02% sodium azide; UCD, University of California at Davis; CV%, percentage of coefficient variation; UV, ultraviolet-visible spectroscopy.

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