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Evaluation of a Pentachlorophenol Immunoassay for Environmental Water Samples

Jeanette M. Van Emon[1] and Robert W. Gerlach[2]

[1]Exposure Assessment Research Division, United States Environmental Protection Agency, Environmental Monitoring Systems Laboratory, P.O. Box 93478, Las Vegas, Nevada 89193-3478, USA and [2]Lockheed Engineering & Sciences Company, 1050 East Flamingo Road, Las Vegas, Nevada 89119, USA

Immunoassays are analytical methods based on specific antibodies directed to a particular target analyte. The binding between antibody and analyte can be used for detection and quantitation. Immunoassay can provide a sensitive, specific, simple to perform, and cost-effective analysis for many compounds of environmental concern (Van Emon et al. 1989). Methods can be designed for rapid, field-portable, semi-quantitative analysis or as standard quantitative laboratory procedures. Immunoassays can be used as screening methods to identify samples that must be further characterized by classical analytical methods. Screening can substantially reduce the costs incurred for hazardous waste site monitoring. Immunoassays may not consistently provide useful information when used for environmental samples containing an unknown compound or complex mixture, and may not be as accurate or precise as conventional analytical procedures. However, to analyze a large number of samples for one or two target analytes or to screen samples so that rapid decisions can be made in the field, immunoassay could be the method of choice.

The potential of immunoassay for environmental analysis has not been fully realized. Extensive validation and performance evaluations are necessary for full acceptance of immunoassay methods by analytical chemists. Specific immunoassays that have been developed for a particular environmental application need to be evaluated to ensure that the intended performance characteristics are met (Van Emon 1990).

Pentachlorophenol (PCP) is used in agriculture and as a wood preservative. Although the compound is clearly beneficial, there are risks associated with its use. Numerous waste sites contain high levels of PCP. To follow remedial actions at these sites, rapid and cost-effective monitoring methods are needed.

Westinghouse Bio-Analytic Systems (WBAS), Rockville, Maryland, developed an immunoassay appropriate for screening drinking-water, surface-water, and groundwater samples for PCP. The method is a 96-well microplate immunoassay designed to accommodate a high sample load as might be encountered in a laboratory setting. This immunoassay was submitted to the U.S. Environmental Protection Agency (EPA), Environmental Monitoring Systems Laboratory-Las Vegas (EMSL-LV) for evaluation as a rapid, cost-effective screening method to monitor and characterize sites of EPA concern.

Send reprint requests to J. M. Van Emon at the above address.

MATERIALS AND METHODS

Most environmental contaminants are small molecules that are not capable of stimulating antibody production. However, when covalently attached to carriers, such as proteins, these small molecules (i.e., haptens) may elicit the production of antibodies. In many cases the antibodies will bind to the hapten itself without the carrier being present. Table 1 shows the generalized steps in immunoassay development using the WBAS PCP immunoassay as an example.

Table 1. Steps of PCP immunoassay development

Hapten Designs	2,3,5,6-Tetrachlorophenol
	2,6-Dichlorophenol
Conjugation Procedure for Antigens	Mixed Anhydride[a]
Carrier Proteins	Bovine Serum Albumin
	Thyroglobulin
Antibody Production	Monoclonal (rat)
Cross-Reactivity Determination	See Table 2
Immunoassay Type	Competitive Inhibition
Immunoassay Format	96-Well Plate
End Use	High Sample Capacity
	Quantitative Analysis

[a]From Erlanger, 1980

A solid-phase, competitive, enzyme-linked immunoassay was used for quantitation. The immunoassay was based on a rat monoclonal antibody selective for PCP, developed in response to a tetrachlorophenol hapten conjugated to bovine serum albumin. A dichlorophenol hapten conjugated to thyroglobulin (DCP-THY) was used as the coating antigen. The assay required 2 days for completion, but had a high sample capacity. On the first day of analysis, 96-well polystyrene microtiter plates were sensitized with 100 µL of DCP-THY in 7-mM phosphate buffer (pH 7.4) solution containing 0.14 M NaCl (phosphate buffered saline, PBS). The plates were covered with adhesive sealers and were stored overnight at 4°C. On the second day of analysis, the plates were washed with PBS containing 0.05 percent Tween 20 (PBS-Tween) before samples and standards (50 µL) were added into triplicate wells. A 1:1,000 dilution of the rat monoclonal anti-PCP antibody (50 µL) was next added into the wells, and the plate incubated at room temperature for 1 hour. The plate was washed before the addition of a secondary antibody (goat anti-rat) labelled with alkaline phosphatase. After a 1-hour incubation, the plate was again washed and the enzyme substrate (p-nitrophenyl phosphate) was added. Color development of the yellow end-product (p-nitrophenol) was monitored at 405 nm. Final absorbance readings were determined within 40 minutes of substrate addition. Quantitation was achieved by constructing a standard curve of absorbance vs. log concentration of PCP standards.

Cross-reactivity of the assay toward selected compounds structurally related to PCP is shown in Table 2. The greatest cross-reactivity was observed with certain tetra- and trichlorophenols. No cross-reactivity was observed with a variety of mono- and dichlorophenols.

Table 2. Cross-reactivity of selected compounds in the WBAS PCP immunoassay

Compound	Molar IC_{50}[a]	Cross-reactivity[b]
2,3,5,6-Tetrachlorophenol	5.3 (± 0.6) x 10^{-6}	42
2,4,6-Trichlorophenol	1.8 (± 0.3) x 10^{-6}	12
2,3,6-Trichlorophenol	2.5 (± 0.1) x 10^{-5}	8.8
2,6-Dichlorophenol	1.2 (± 0.1) x 10^{-4}	1.8
Tetrachlorohydroquinone	2.8 (± 0.1) x 10^{-4}	0.8
2,3,4-Trichlorophenol	4.5 (± 0.3) x 10^{-4}	0.5
2,3,5-Trichlorophenol	4.3 (± 0.3) x 10^{-4}	0.5
Pentachloroaniline	NI	0
Pentachlorobenzene	NI	0
2,3-Dinitrotoluene	NI	0
2,4-Dinitrotoluene	NI	0
2,4,5-Trichloronitrobenzene	NI	0

[a]Molar concentration of compound that inhibits 50 percent antibody binding in immunoassays.
[b][IC_{50} PCP/IC_{50} compound] x 100
NI = Not inhibitory; 1.0 x 10^{-3} M

The immunoassay analysis was compared to gas chromatography (GC) results obtained using the procedure described in EPA Method 604 (Longbottom and Lichtenberg 1982) with minor modifications: (1) preliminary extractions of the samples showed no interferences, so the basic pH wash was omitted and (2) the phenols were analyzed underivatized. Several water extracts contained phenols other than PCP; however, quantitation was performed for only the PCP component.

Sample preparations consisted of one of three methods: a solid-phase extraction protocol developed by WBAS (*Anonymous 1991, Protocols used in the EPA Evaluation of the Westinghouse Bio-Analytic Systems Pentachlorophenol Immunoassays. Van Emon JM and RW Gerlach (eds) U.S. EPA, EMSL-LV, in preparation*) that produced samples in 100 percent 2-propanol, an extraction procedure from EPA Method 604 (Longbottom and Lichtenberg 1982) that also produced samples in 100 percent 2-propanol, or no extraction step (direct immunoassay analysis). For the direct immunoassay, concentrated (X10) PBS-Tween was added to each sample to obtain a buffer concentration equal to that of PBS-Tween, and 100 percent 2-propanol was added until the sample was 25 percent 2-propanol. The solid-phase and EPA Method 604 extracts were each diluted 1:4 with PBS-Tween. Additional dilutions for all sample extracts were made with PBS-Tween containing 25 percent 2-propanol. Several dilutions of each sample were analyzed by immunoassay to determine the optimum dilution for the final immunoassay analysis.

The immunoassay was tested on three types of environmental water samples; drinking water, ground water, and surface water. Four different sources of each type were used, and splits of each sample were sent to each of the two laboratories (WBAS and EMSL-LV). The laboratories spiked aliquots of each sample at two different

levels of PCP, ranging from a low level of 15 ng/mL to a high level of 2,800 ng/mL. Spiking solutions were prepared from low-level Phenols-Water Pollution Quality Control Samples obtained from EMSL-Cincinnati. Each laboratory analyzed an aliquot of the spiked samples with immunoassay. Another aliquot of spiked sample was processed with the solid-phase extraction. Subsamples of this extract were exchanged between laboratories and were also analyzed by immunoassay. In addition, EMSL-LV analyzed their original solid-phase extract with the GC procedure given in EPA Method 604. Another extract was processed by EMSL-LV using the extraction procedure in EPA Method 604 and was analyzed by EMSL-LV with immunoassay and GC, and a split of this extract was analyzed by WBAS with immunoassay.

Every immunoassay determination was the average of results from three wells, and each immunoassay or GC sample determination was carried out in triplicate. Blank samples were split in parallel with the factors of water type, sample source, extraction laboratory, extraction procedure, detection laboratory, and detection type. The only factor where the blank samples were not split in conjunction with different procedure variations was spike level.

RESULTS AND DISCUSSION

The median imprecision, based on triplicate determinations, was 10 percent relative standard deviation (RSD) for the immunoassays run at WBAS (n = 95) and 12 percent for the immunoassays run at EMSL-LV (n = 95), which compares favorably to the 10 percent RSD found for the GC analysis (n = 47). Single operator precision for the GC calibration is previously reported as 10 percent (Longbottom and Lichtenberg 1982).

The average of each triplicate determination was used as a single value for each sample in the following discussion. Excluding the results of sample splits from one original blank sample, which appeared to have become contaminated, there was a false positive rate of 9 percent. The false negative rate was 0 percent. Accuracy was assessed in terms of percent recovery and is summarized in Table 3 for each sample type for both immunoassay and GC detection. Accuracy was best for drinking-water samples and occasionally variable for selected surface-water and ground-water samples. The high mean bias and variability for ground-water samples A and D are due primarily to a few high values not qualifying as statistical outliers.

Table 3. Means and standard deviations for immunoassay and gas chromatography results in units of percent recovery

Sample Type	Surface Water				Drinking Water				Ground Water			
	A	B	C	D	A	B	C	D	A	B	C	D
IA Mean	80	99	96	117	118	86	98	118	152	102	121	147
IA SD	21	30	28	99	36	31	12	29	91	36	37	107
GC Mean	97	95	76	79	132	101	94	94	118	98	97	83
GC SD	27	13	22	9	44	12	15	16	17	15	11	16

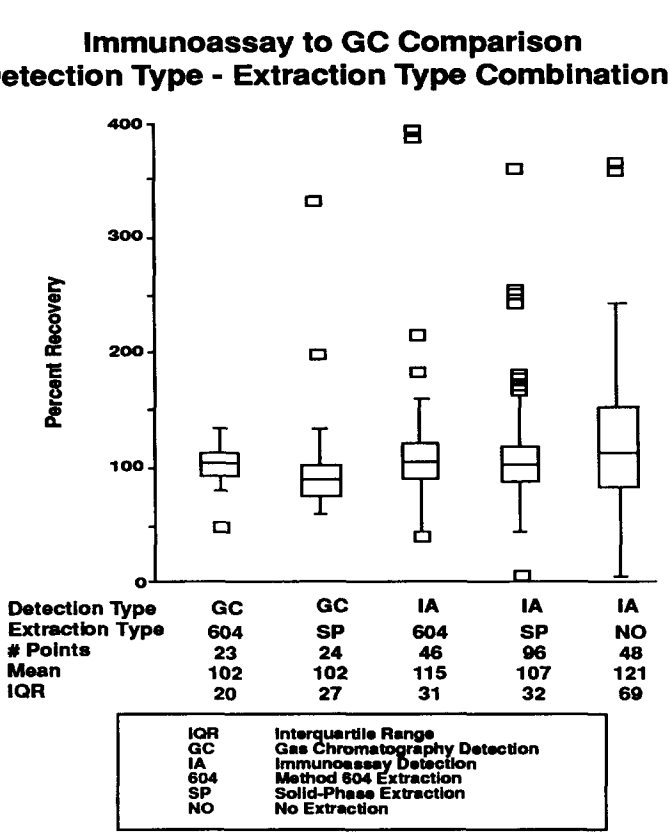

Figure 1. Box plot comparison of immunoassay and GC analysis by detection type and extraction type.

The experimental design allowed us to study the effects of the various controlled factors with analysis of variance (ANOVA) methods. Several ANOVAs were performed by using various subsets of the data (Van Emon and Gerlach 1990).

However, the only statistically significant result ($\alpha = 0.05$) was the identification of a laboratory bias when the direct immunoassay method was used; the EMSL-LV recoveries were lower than recoveries from WBAS. There were no practical differences found between (1) immunoassay and GC detection of samples extracted by the EPA Method 604 protocol, (2) immunoassay and GC detection of samples processed using the WBAS solid-phase extraction, (3) detection laboratory for samples analyzed by immunoassay following the WBAS solid-phase or Method 604 extraction protocols, and (4) precision of the direct immunoassay at the two laboratories.

A summary of results organized by extraction and detection methods is shown using box plots (Chambers et al. 1983) in Figure 1. These plots show that immunoassay results using either extraction technique had a larger variability than the GC results. The variability of the direct immunoassay results was twice as large as the variability

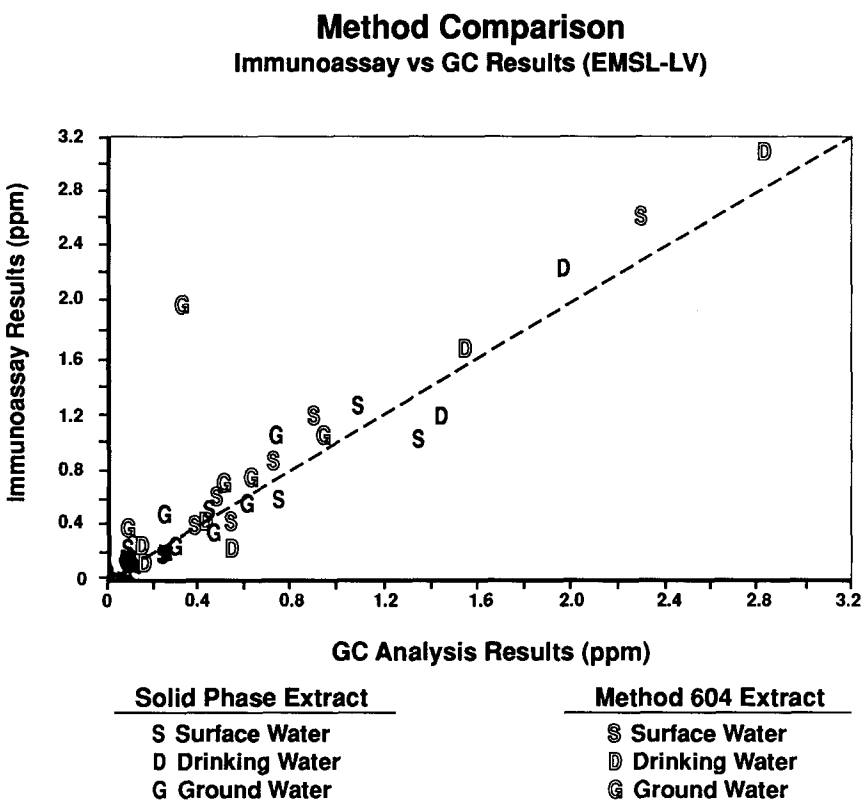

Figure 2. Comparison between EMSL-LV immunoassay and GC results.

for the immunoassay results when extractions were used. Further study and an improved analysis protocol are needed in order to reduce the variability associated with the direct immunoassay method. We note that the number of outlying points (shown individually in Figure 1) is roughly related to the number of points in each extraction type/detection type category.

A graphical comparison of immunoassay to GC results (both analyzed at EMSL-LV) is shown in Figure 2. This plot shows good agreement between these two methods for all combinations of samples and extraction methods. A Spearman rank correlation coefficient of 0.92 (n = 47, 95 percent confidence interval of 0.86 to 0.96) summarizes the comparability seen in Figure 2. Though the data in Figure 2 suggest a slight shift toward higher immunoassay results compared to GC values, the difference was not statistically significant at the 95 percent confidence level on the basis of a paired t-test (t = 1.41, n = 47). In this study, an immunoassay response within a factor of two of the GC value was considered to be an acceptable value in terms of practical differences related to field screening. Only 4 of 44 comparisons (9 percent) had mean recoveries differing by a factor of 2 or more; immunoassay results were higher than GC results for all 4 comparisons.

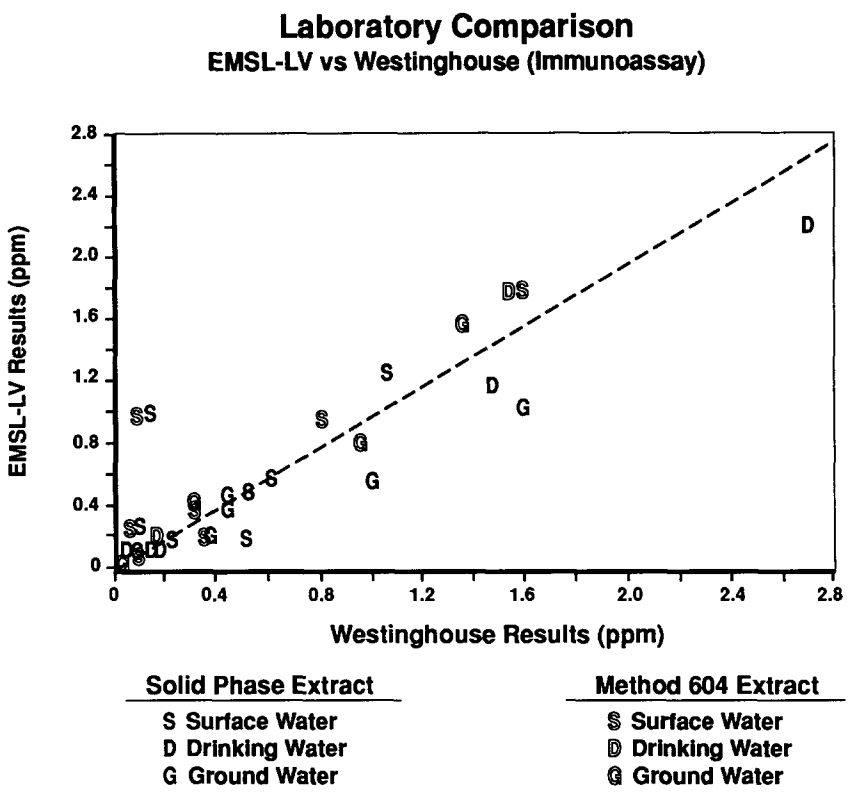

Figure 3. Comparison of immnoassay results between the EMSL-LV and WBAS.

Figure 3 compares the immunoassay results from EMSL-LV to immunoassay results from WBAS. Slightly more variability is evident than was seen in the immunoassay to GC plot. It is possible that interlaboratory effects added to the variability observed in Figure 2. Nevertheless, the comparability between laboratories is good.

A comparison of performance parameters for the WBAS immunoassay and the GC method is given in Table 4. The GC analysis is more accurate, but more time consuming and expensive. This immunoassay evaluation demonstrated many features desired in a field screening method. It is sensitive to the target compound and somewhat sensitive to highly related compounds. It is fast, has a high sample capacity, and has less than a 10 percent false positive rate. Of great importance for screening, it is conservative with respect to generating false negatives; none were found in this study. These characteristics combine to make immunoassay an attractive, low-cost alternative to more expensive traditional laboratory analysis methods.

Table 4. PCP Water analysis comparison of WBAS plate immunoassay to EPA method 604

Performance Parameters	WBAS Immunoassay	EPA GC Method 604
Detection Limit (ppb)	30 - 40	1 - 15
Linear Range (ppb)	30 - 400	1 - 200
Precision	20 - 30%	10 - 20%
Accuracy	±40 - 50%	±30%
Analysis Time based on Sample Load	2.5 hours/ 40 samples	0.5 hour/ 1 sample
Extraction Required	NO	YES
Cost/Sample	$2.50	$100 - $300
Key Interferents	2,3,5,6-tetra-chlorophenol	polyaromatic hydrocarbons
Matrix Spike Recovery	60 - 160%	70 - 120%
Rapid On-site Analysis Capability	YES	NO
Total Analysis Time	5 hours/ 40 samples	4.5 hours/ 1 sample

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