Enzyme-Linked Immunosorbent Assay by Image Analysis Using a Charge-Coupled Device Array Detector

F. García Sánchez, A. Navas Díaz, and J. Lovillo
Departamento de Química Analítica, Facultad de Ciencias, Universidad de Málaga, 29071-Málaga, Spain

Received September 14, 1995

This paper describes a fluorescence enzyme-linked immunosorbent assay (ELISA) for the quantification of (-)-2-(2,4-dichlorophen-oxy)propionic methyl ester (dichlorprop methyl ester). Antibodies for dichlorprop methyl ester were produced by immunizing rabbits with a conjugate of dichlorprop methyl ester with bovine serum albumin. Data acquisition on microtiter wells is performed by a spectrofluorometer through a fiber optic and by a charge-coupled device camera. A correlation was obtained between the image analysis data on ELISA and the data acquired by the spectrofluorometer. The results demonstrate that the fluorescence image analysis performed by the charge-coupled device detector is applicable to ELISA, and the analysis time, sensitivity, and precision of the ELISA procedure are compared to conventional fluorescence ELISA performed by the spectrofluorometer. The ELISA procedure was selective for structurally similar compounds or usually found in formulation pesticides. Concentrations for 50% displacement curves were dichlorprop, 83.59 μg/ml, and 2,4-D, 388.23 μg/ml; triclopyr, ioxynil, bentazone, and MCPA had no response.

Pesticide residue monitoring has attracted enormous attention in the past few years as public concern has focused on the environmental impact of widespread pesticide residues, accompanied by a need for rapid and simple testing procedures.

Analysis for pesticide residues is generally carried out with two distinct purposes, one of which is the screening analysis, in which the analysis is focused to detect components in a sample. In this case, dynamic techniques, such as chromatography (LC and GC), are used. The other type of analysis is related to the detection and quantification at ng/ml levels of a specific pesticide in a sample. In this case, an immunoassay gives the adequate specifications to certify existence or not of a single pesticide.

The specimen preparation is generally not less time consuming in immunoassays compared to chromatography. The real advantage is batch processing in immunoassays.

Immunological reactions based on the specific combination of antigens and antibodies have been used for quantitative and semiquantitative assays of pesticides, including triazine (1), 2,4-D (2), alachlor (3), molinate (4), paraquat (5), isoproturon (6), and dichlorprop (7, 8) among others.

Assay sensitivities have also been greatly enhanced by methods that use easily detected enzyme-linked immunosorbent assay (ELISA) procedures. Another factor influencing sensitivity in ELISA is the extent of detectability of the substrate products. Although colored products of enzyme reactions are the most commonly used indicator systems in conventional ELISA, fluorescent end products of enzyme reactions are detectable at lower concentrations. In fact, the sensitivity of enzyme immunoassays which utilize fluorogenic substrates has been demonstrated to be higher than those of assays which employ the same enzyme with a chromogenic substrate (9, 10). The fluorimunoassays are generally blank limited which means that the sensitivity achieved ultimately depends on the background level within the measurement and their ratio to the specific signal. Thus, the fluorescent properties of fluorogenic substrate play the most important role in defining the performance characteristics of assay designs. The fluorogenic substrate used needs to have a high quantum yield, and in order to achieve a good resolution from the background noise, the fluorescent wavelengths need to differ from the background; thus, the

1 Abbreviations used: ELISA, enzyme-linked immunosorbent assay; CCD, charged-coupled device; MDC, minimum detectable concentration; RDL, reliable detection limit.
excitation should be over 300 nm, emission over 400 nm, and the Stokes shifts as long as possible to avoid interference from scattering.

ELISA procedures use solid supports such as a microtiter plate, and two-dimensional information is required. The local chemical information is acquired by scanning, where several detectors are moved in y direction over the plate; however, lineal scanners are time consuming when multidimensional data sets are required and it is better to acquire two-dimensional information by direct imaging. Recent instrument developments in image detectors have stimulated the usage of charge-coupled device (CCD) detectors with associated software for chemical analysis, the creation of data being possible from the obtained images by image analysis, permitting the extraction of quantitative and qualitative information.

The advantages of CCDs as imaging detectors have been described (11) and are eliminated of mechanical components, fast data acquisition, and the ability to use sophisticated image-processing algorithms for quantitation, sample detectability, and dynamic signal range, and it is not necessary to resort to the use of intense excitation sources. Other advantages of the CCD as a detector in immunoassays is the possibility of using a smaller volume of the sample. This is possible because in image analysis (a bidimensional technique) the area occupied by the fluorophore proceeds from the signal, instead of a tridimensional sample such as a cuvette.

The most contemporary microplate reader has eight photodiodes mounted below the sample wells that measures the transmitted light and read a maximum of eight wells simultaneously. A CCD can read a microplate of 96 wells at one time, and it is only necessary to put the camera at an adequate distance and to use approximation lenses with the purpose of improving the sensitivity of the measure.

In image analysis the capture of the image is achieved in 1/25 s; with conventional readers the time employed in the measuring of the 96 wells is approximately 1 min.

The image analysis is particularly useful for chemical analysis on solids and other nonhomogeneous media. Their usage is undergoing great expansion in different areas such as electrophoretic gels (12, 13), thin-layer chromatographic plates (14, 15), cancer detection (16), and chemiluminescence ELISA (17) among others (18). Although CCD applications on microtiter plates have been suggested, as far as we know, no assays have appeared evaluating image analysis data of fluorescent ELISA on microtiter plates.

The aim of this study was to compare and evaluate ELISA data obtained from fluorescent intensities measured with a spectrofluorometer and with a charge-coupled device camera and to describe a fluorescent enzyme-linked immunosorbent assay for the selective quantification of dichlorprop methyl ester.

EXPERIMENTAL

Reagents. Dichlorprop [(±)-2-(2,4-dichlorphen-ox)-propanoic acid], triclopyr [3,5,6-trichloro-2-pyridinyl]oxy acetic acid], MCPA [(4-chloro-o-tolyl]oxy acetic acid], and bentazone [3-isopropyl-(1H)-2,1,3-benzothiadiazin-4(3H)-one 2,2-dioxide] were provided by Dr. Ehrensteiner Laboratories (Augsburg, Germany). 2,4,5-T [2,4,5-trichlorophenoxy acetic acid], goat anti-rabbit-IgG peroxidase conjugates, and homovanillic acid (4-hydroxy-3-metoxo-phenylacetic acid) were from Sigma. Isoxynil (4-hydroxy-3,5-diiodobenzonitrile) and dichlorprop methyl ester were obtained from Riedel de Haën (Seelze, Germany). All other chemicals used were from Merck R.A.

Stock standards of dichlorprop methyl ester (4.2 × 10⁻³ m) were prepared in methanol and stored in the dark at 4°C. Buffer solutions were prepared from phosphate (pH 7.5, 0.1 m), carbonate (pH 9.6, 50 mm), and Tris-HCL (pH 8.5, 0.1 m).

Rabbit anti-dichlorprop was produced by previously immunizing a rabbit with a conjugate of dichlorprop and bovine serum albumin following a previously described procedure (7). The excess hapten was separated from the conjugate by dialysis. Effective conjugation was confirmed by inspection of the uv spectra. The number of amino groups substituted by the hapten in each carrier molecule was determined by 2,4,6-trinitrobenzenesulfonic acid titration.

Instrumentation. Fluorescent readings were accomplished in a Perkin-Elmer plate reader accessory, supplied with polystyrene well plates. A bifurcated high-grade fused silica fiber optic (Oriel, Stratford, CT, Ref. 77565) with a high transmission range of 240-2200 nm was used to transfer the excitation and emission energies between the well plate and the spectrometer. An IBM-PC computer was used for on-line data acquisition at an integration time of 10 s. Data were collected and processed by FLDM software (Perkin-Elmer).

Fluorescent images of the microtiter plate were captured with a Sony SSC-M370CE video camera with a 12-mm F/1.2 lens. Digitization of the video images was transferred in binary format to a host computer which controls the working of the system through Visilog version 4.0 software. The wells of the microtiter plate were excited with a 6-W Mineralight uv lamp Model UVGL-58 placed in front of the microtiter plate at 45°. Images obtained were stored in digital format in the computer and they were able to be both displayed and manipulated.
Image analysis. The charge-coupled devices are composed of a framework of semiconductors that transform incident ray light into electric charges. The photosensitive sensors are generally arranged into square or rectangular matrices which include several hundred rows and columns in an area of less than one square centimeter. CCD cameras have a very high sensitivity, detecting up to 80% of the incident photons.

The first step of image analysis is the digitization of the video images, which consists of the transformation of an image into a matrix, where each element corresponds to a picture element, called “pixel.” For a monochrome image each pixel is characterized by its grey level value coded by an integer number. According to the digitizer and the available mass storage memory, each grey level is coded on several bits per pixel, allowing 256 grey levels values.

Raw digitized images generally require some preliminary processing for enhancing the contrast of grey level values, reducing the noise or simplifying the data for further processing. The way of reducing random noise is to cumulate and take the average value of images of the same scene. This method is time consuming. Noise is more commonly reduced by local filtering. With linear filters, the grey level of each pixel is replaced with the linear combination of its neighbors. Median filtering may be more appropriate; in this case the neighbors of a given pixel are sorted according to their grey level. The pixel to be processed is replaced by the median grey level.

ELISA procedure. Microtiter plates were coated by adding 200 μl of human K-globulin-dichlorprop (HGG-dichlorprop) dissolved in 50 mM carbonate buffer (10 μg/ml of antigen coating) to each well and incubated for 36 h at 4°C. The plates were emptied and washed three times with washing solution (0.1 M phosphate buffer, pH 7.5, supplemented with 0.1% Tween 20). Unoccupied sites on the polystyrene well surface were blocked by adding 200 μl of 0.2% (w/v) gelatin solution in phosphate buffer and incubated for 20 min at 4°C. The plates were emptied and washed as described above. Diluted antisemum (1:3000) in phosphate buffer, pH 7.5, supplemented with 0.05% Tween 20 was preincubated for 1 h with dichlorprop methyl ester at concentrations covering the range 0.1–10,000 ng/ml; 200 μl of these preincubated mixtures was transferred to the wells of the microtiter plate and incubated for 15 min at 4°C. One column of the plate received no dichlorprop and no antisemum to determine nonspecific binding of the secondary antibody-labeled horseradish peroxidase enzyme in the following step. Another column received no dichlorprop to determine the maximum fluorescent reading. The plates were washed as before. Goat anti-rabbit–horseradish peroxidase conjugate diluted (1:500) was added (200 μl/well) to the plates. The plates were incubated for 1 h at 4°C, emptied, and washed.

Substrate. Sixty microliters of a 20 mg/ml solution of homovanillic acid, 60 μl of 0.1% hydrogen peroxide solution, and 100 μl of 0.1 M Tris buffer, pH 8.5, were added to the plate. Fluorescence was allowed to develop for 2 h and was measured as a 315-nm wavelength excitation and a 425-nm wavelength emission (19). Readings were corrected for nonspecific binding of the antibody-labeled enzyme.

Fluorescent values of standards (F) were divided by the maximum fluorescent value (F0) representing those wells in which binding of antibody to the coating conjugate was not challenged with free dichlorprop methyl ester in solution. The F/F0 values were plotted against the logarithm of dichlorprop methyl ester concentration to construct a standard curve.

Apple extraction procedure. A 250-g amount of apples was chopped in a food chopper and 15 g was transferred into a blender cup and blended with 50 ml of acetone containing 0.3 ml of orthophosphoric acid at high speed for 3 min. The homogenate was filtered through a fritted-glass Büchner funnel (coarse porosity) under reduced pressure and the filter cake washed with 5 ml of acetone. The filtrate was transferred into a 50-ml volumetric flask and diluted in volume with acetone. An aliquot of 1 ml of the extract in acetone was diluted with methanol to a final volume of 5 ml. This solution was used for analysis.

RESULTS AND DISCUSSION

Standard curves. To construct a calibration curve aliquots of dichlorprop methyl ester covering the range 0.1–10,000 ng/ml were preincubated with antisemur (1:3000) for 1 h at 4°C and then incubated for 15 min with previously coated HGG-dichlorprop, as described in the ELISA procedure. A plot of fluorescent readings against log concentration of the standard dichlorprop methyl ester (Fig. 1) displays a typical calibration graph fitted to an IC50 four-parameter logistic model. A dynamic range covering standard concentration of dichlorprop methyl ester between 0.3 and 10,000 ng/ml is deduced.

The minimum detectable concentration (MDC) is defined for a four-parameter logistic model (20) as “the lowest concentration which results in an expected response less than the one-sided 1 level (I = 0.05) lower confidence limit at zero concentration.” The MDC calculation is illustrated graphically in the inset to Fig. 1, and its value was 0.3 ng/ml and was calculated by substituting the average response minus the relative standard deviation (σp–1) of three samples containing 0.1 ng/ml in each in the calibration equation. The reliable detection limit (RDL) (19) is also shown graphically in
determined and the cross-reactivity (CR) of a compound was determined as (22)

\[
\%CR = \frac{\text{Apparent dose of dichlorprop methyl ester}}{\text{Actual dose of structurally related compound}} \times 100.
\]

Table 1 gives formulas and cross-reactivity percentages for the components studied. From these results it can be deduced that bentazone, MCPA, ioxynil, and triclopyr do not affect the analytical method of determining dichlorprop methyl ester. At 50% displacement the inset to Fig. 1 and can be defined as “the lowest concentration that has a high probability of producing a response that is greater than the estimated one-sided \( t \) level \((t = 0.05)\) upper confidence limit at zero.” To calculate RDL the average response was substituted in the equation of the upper confidence limit and the obtained value was 0.65 ng/ml. The precision of the method was determined by measuring the fluorescent intensity of 10 separate samples, each containing 50 ng/ml of dichlorprop methyl ester, and gave a relative standard deviation of 10.78% \((P = 0.05)\).

Specificity of the assay. To characterize the specificity of the assay several pesticides with structures very similar to that of the dichlorprop methyl ester (2,4,5-T, triclopyr, dichlorprop) or usually found in formulations (21) (ioxynil, bentazone, MCPA) were tested.

The cross-reactivity was calculated by using the 50% displacement method; the concentration of compound required to produce 75% displacement was only determined in each case. Displacement curves were prepared by incubation of various doses of cross-reactant with a constant amount of antibody, followed by separation of bound and unbound fractions, and quantitation of the cross-reactant at each dose. After fitting the curves by the four-parameter logistic model the doses of cross-reactant that give 50 or 75% displacement were

**TABLE 1**

Cross-Reactivity of the Dichlorprop Methyl Ester and Related Compounds

<table>
<thead>
<tr>
<th>Formula</th>
<th>Compound</th>
<th>( %RC_{75%}^a )</th>
<th>( %RC_{50%}^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image.png" alt="Image" /></td>
<td>Dichlorprop methyl ester</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><img src="image.png" alt="Image" /></td>
<td>Dichlorprop</td>
<td>1.03</td>
<td>0.27</td>
</tr>
<tr>
<td><img src="image.png" alt="Image" /></td>
<td>MCPA</td>
<td>0.024</td>
<td>&gt;10^6</td>
</tr>
<tr>
<td><img src="image.png" alt="Image" /></td>
<td>2,4,5-T</td>
<td>0.053</td>
<td>0.06</td>
</tr>
<tr>
<td><img src="image.png" alt="Image" /></td>
<td>Triclopyr</td>
<td>0.007</td>
<td>&gt;10^6</td>
</tr>
<tr>
<td><img src="image.png" alt="Image" /></td>
<td>Bentazone</td>
<td>&gt;10^6</td>
<td>&gt;10^6</td>
</tr>
<tr>
<td><img src="image.png" alt="Image" /></td>
<td>Ioxynil</td>
<td>&gt;10^6</td>
<td>&gt;10^6</td>
</tr>
</tbody>
</table>

\( ^a \) Cross-reactivity estimated at 75% displacement of signal.

\( ^b \) Cross-reactivity estimated at 50% displacement of signal.
TABLE 2
Recovery of Dichlorprop Methyl Ester from Apple Samples

<table>
<thead>
<tr>
<th>Fortification (Conc, ng/ml of sample)</th>
<th>Found (Conc, ng/ml of apple)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>0.833</td>
</tr>
<tr>
<td></td>
<td>503.77 ± 7.47a</td>
</tr>
<tr>
<td>50</td>
<td>0.083</td>
</tr>
<tr>
<td></td>
<td>48.35 ± 4.78</td>
</tr>
<tr>
<td>1</td>
<td>1.67 × 10⁻³</td>
</tr>
<tr>
<td></td>
<td>1.035 ± 0.129</td>
</tr>
</tbody>
</table>

* Standard deviation for eight determinations.

The recoveries were 103.5, 96.7, and 100.8%, respectively. The results obtained are given in Table 2.

Applications. Extracts of apple samples were spiked at three concentrations: 1, 50, and 500 ng/ml. The recoveries were 103.5, 96.7, and 100.8%, respectively. The results obtained are given in Table 2.

Image analysis. Standard curves were constructed from solutions identical to that described above. We obtained the profiles of the intensities from each row of the microtiter plate. In these profiles 12 peaks appeared whose heights represent the fluorescent intensity of each well. Intensity values of standards (I) were divided by the maximum intensity value (I₀), that represents the well that did not contain dichlorprop methyl ester in solution. The I/I₀ values were plotted against the logarithm of dichlorprop methyl ester concentration (Fig. 2). A dynamic range covering standard concentration of dichlorprop methyl ester between 0.15 and 10,000 ng/ml is deduced. The MDC and RDL were 0.15 and 0.22 ng/ml, respectively. The precision of the method was determined by measuring the intensity of 10 separate samples, each containing 50 ng/ml of dichlorprop methyl ester, and gave a relative standard deviation of 8.5% (P = 0.05).

A comparison of the results of image analysis performed by using the CCD detector and those obtained with the spectrofluorimeter in terms of speed, MCD, RDL, and precision reveals that all the parameters of the ELISA procedure are improved with respect to conventional fluorescent ELISA performed by the spectrofluorimeter. The correlation between the ELISA performed by the spectrofluorimeter and by the CCD was 0.9997 (r), the slope of the regression line was 1.0025, and the intercept was 0.4821.

CONCLUSIONS

The results obtained in this work demonstrate the validity of image analysis as a detection system in fluorescent immunoassay for pesticides performed on microplates. This detection system presents several advantages in comparison with other systems, such as photodiode array. (i) It is not necessary to resort to intense excitation sources; (ii) smaller sample volumes (100 μl) can be employed, and the sensibility of the method is not affected; (iii) the images captured by the camera can be stored in a digital format and be operated on in future procedures; on the other hand, these images can be processed through mathematical algorithms specialized to reduce the background noise, to eliminate irrelevant parts, and to improve the quality of the same; (iv) with image analysis, the time used in the data acquisition is inferior and 96 wells can be read...
at one time. The sensibility and the precision of the method are similar to those obtained with other microplate readers.

The difference of cost between an ELISA using conventional detectors or image analysis resides in the price of the detector. At present cameras are very efficient with inferior prices to the conventional plate readers.

ACKNOWLEDGMENT

This work was supported by a grant (BIO94–0548) from the Spanish Comisión Interministerial de Ciencia y Tecnología (CICYT).

REFERENCES