Enhanced chemiluminescence kinetic ELISA of dichlorprop methyl ester

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Abstract

A competitive enzyme-linked immunosorbent assay (ELISA) for dichlorprop methyl ester (DME) with an anti-rabbit secondary antibody conjugated to peroxidase was developed in polystyrene plates. The plate was developed with a luminol–H₂O₂-horseradish peroxidase-p-iodophenol mixture by using a fibre optic to collect the chemiluminescent emission. The standard curve was produced for 0.05 to 10000 ng ml⁻¹ DME. The minimum detectable concentration was 0.11 ng ml⁻¹ and the relative standard deviation was 7.26% for a 50 ng ml⁻¹ sample of (n = 10). The ELISA procedure was selective with respect to structurally similar compounds usually found in formulations of pesticides. This method was applied to DME added to wheat grain extracts with recoveries between 97.1 and 103.6% for 5–500 ng ml⁻¹.

Keywords: Immunoassay; Kinetic methods; Chemiluminescence; Luminol; Horseradish peroxidase

1. Introduction

An immunoassay is an analytical technique which is capable of specifically detecting very small quantities of a particular compound. In addition, a large number of samples can be detected simultaneously within a relatively short period of time (h). Among the immunoassay methods, very useful is the enzyme-linked immunosorbent assay (ELISA), which is as sensitive as radioimmunoassay. Chemiluminescence (CL) has had a considerable impact on biomedical analysis, on medicine and as clinical diagnosis [1]. It is the phenomenon observed when the excited product of a chemical process reverts to its ground state with emission of light. Many investigators have explored the use of CL in order to increase the sensitivity of immunoassays [2].

Luminol and its derivatives are widely used in CL immunoassays, but microperoxidase or horseradish peroxidase (HRP)-catalyzed reactions result in the short-lived production of light of low intensity. The introduction of enhancers of chemiluminescence, such as: 6-hydroxybenzothiazole derivatives [3], phenol derivatives [4–6], 2-naphthol derivatives [7], aromatic amines [5,6,8,9] and aryloboric acids [10], produce two kinds of positive influence on the system: the chemiluminescence produced by peroxidase–luminol–H₂O₂ systems is increased by a factor greater than 1000 in some cases, whereas the blank signal is decreased.

Because CL intensity varies with time as reactants arc consumed, it is necessary to initiate CL reactions in a controlled manner to achieve repeatable results.

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The most common method is to rapidly mix the chemiluminescent reactants and then measure the emission. Some instruments do not permit the initiation of the luminescent reaction inside the luminometer. When the intensity–time curve is analyzed by measuring the intensity at a fixed time after mixing, it is necessary to rigorously control the time interval between initiating the reaction and obtaining the data. The maximum intensity may be subject to poor precision due to changes in reaction rate when the concentration of horseradish peroxidase varies. In these cases, the precision of the method can be improved by measuring its rate of intensity change over a wide time interval after mixing.

These considerations led us to develop an enhanced chemiluminescent competitive ELISA for the screening and determination of dichlorprop methyl ester (DME). In this work, we employed a secondary antibody labelled with horseradish peroxidase, and used p-iodophenol as the enhancer. This assay is highly sensitive and specific.

2. Experimental

2.1. Apparatus

Readings of chemiluminescence were accomplished in a Perkin-Elmer plate reader accessory, supplied with polystyrene well plates. A 185-cm glass single fibre-optic (Ref. 77527, Oriel, Stratford, CT) with a light transmission range of 390–1500 nm was used to transfer the light between the well plate and the spectrometer. Instrumental parameters were controlled by a fluorescence Data Manager (FLDM) software (Perkin-Elmer) with the light source switched off. The spectrometer was set in the phosphorescence mode with a 0.00 ms delay time and a 120 ms gate time; the slit width of the emission monochromator was set at 20 nm with \( \lambda_{cm} = 425 \) nm and the photomultiplier voltage set manually to 900 V.

2.2. Reagents and solutions

Dichlorprop [(\( \pm \))-(2,4-dichlorophenoxy)propanoic acid], triclopyr [3,5,6-trichloro-2-pyridinyl]oxyacetic acid], MCPA [(4-chloro-o-tolyloxyacetic acid], and bentazone [3-isopropyl-(1\( H \))\-2,1,3-benzothiadiazin-4(3\( H \))\-one 2,2-dioxide] were provided by Dr. Ehrenstoffer Labs. (Augsburg, Germany). 2,4,5-T [2,4,5-trichlorophenoxy acetic acid], goat anti-rabbit-IgG peroxidase conjugates, p-iodophenol and luminol (5-amino-2,3-dihydrophthalazine dione) were from Sigma (St. Louis, MO). Ioxynil (4-hydroxy-3,5-diodobenzonitrile) and dichlorprop methyl ester were obtained from Riedel-de-Haën (Seelze, Germany). All other chemicals used were Merck R.A.

Stock standards of dichlorprop methyl ester (4.2 \( \times 10^{-3} \) M) were prepared in methanol and stored in the dark at 4\( ^{\circ} \)C. Luminol (0.01 M) was prepared by dissolving 0.0913 g of 97% luminol in a small amount of NaOH solution; the final volume was made up to 50 ml with Tris–HCl buffer (0.1 M), pH 8.5. Hydrogen peroxide (0.1 M) was prepared by diluting 2.83 ml of hydrogen peroxide (6%, w/v) from Panreac (Montplet and Esteban, Barcelona, Spain) in 50 ml of bidistilled water.

Rabbit anti-dichlorprop was produced by previously immunizing a rabbit with a conjugate of dichlorprop and bovine serum albumin following the procedure previously described [11]. The excess of 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 molecules was determined by 2,4,6-trinitrobenzenesulfonic acid titration.

2.3. ELISA procedure

Microtiter plates were coated by adding to each well 200 \( \mu l \) of \( \gamma \)-globulin–dichlorprop dissolved in 50 mM carbonate buffer at pH 9.6 (10 \( \mu g \) ml\(^{-1} \) antigen coating) and incubated for 36 h at 4\( ^{\circ} \)C. The plates were emptied and washed three times with washing solution (0.1 M sodium phosphate buffer, pH 7.5, supplemented with 0.1% Tween 20). Unoccupied sites on the polystyrene well surface were blocked by adding 200 \( \mu l \) of 0.2% (w/v) gelatin solution in the phosphate buffer and incubated for 20 min at 4\( ^{\circ} \)C. The plates were emptied and washed as described above. Diluted antiserum (1:3000) in the phosphate buffer, pH 7.5, supplemented with 0.05% Tween 20 was pre-incubated for 1 h with a dichlorprop methyl ester standard.
Aliquots of the pre-incubated mixture were transferred to the wells of the microtiter plate (200 µl per well) and incubated for 15 min at 4°C. One column of the plate received no dichlorprop and no antiserum to determine nonspecific binding of the secondary antibody-labelled horseradish peroxidase in the following step. Another column received no dichlorprop to determine the maximum chemiluminescence reading. The plates were washed as before. Goat anti-rabbit HRP conjugate diluted 1:500 was added (200 µl per well) to the plates. The plates were incubated for 1 h at 4°C, emptied and washed.

2.4. Chemiluminescence assay

A 40-µl aliquot of 0.01 M luminol, 40 µl of 0.1 M hydrogen peroxide, 1640 µl of 1 × 10⁻³ M p-iodophenol and 2280 µl of 0.1 M Tris buffer, pH 8.5, were mixed [12]. The optical fibre was situated over the well, and 200 ml of the anterior mixture were added. Chemiluminescence intensity was monitored vs. time for each well. The slope between 150 and 600 s was calculated.

Readings were corrected for nonspecific binding of the antibody labelled enzyme.

Slope values of standards (tg) were divided by the maximum slope value (tg₀) representing those wells in which binding of antibody to the coating conjugate was not challenged with free dichlorprop methyl ester in solution. The tg/tg₀ values were plotted against the logarithm of dichlorprop methyl ester concentration to construct a standard curve.

2.5. Extraction procedure for wheat grain

Portions of 10 g of representative wheat grain samples were washed with 20 ml of acetone in order to eliminate any impurities present. The pesticides were extracted by adding 20 ml of acetone and sonicating for 5 min. The solution was filtered through a glass frit (coarse porosity) under reduced pressure and the filter cake was washed with acetone. The filtrate was evaporated to dryness in a rotary evaporator. The residue was dissolved in methanol (5 ml). This solution, after spiking with DME, was used for analysis.

3. Results and discussion

3.1. Standard curves

To construct a standard curve aliquots of dichlorprop methyl ester covering the range 0.05–10000 ng ml⁻¹ were pre-incubated with antiserum (1:3000) for 1 h at 4°C and then incubated for 15 min with previously coated γ globulin–dichlorprop, as described in the ELISA procedure. Light emission from chemiluminescent oxidation of luminol catalyzed by HRP conjugates in the immunometric assay for DME-specific antibody is enhanced by the addition of the p-iodophenol. These enhanced reactions improve discrimination between DME concentrations. Fig. 1 displays chemiluminescent intensity–time curves for a series of DME doses. These curves were smoothed by employing a binomial filter. The smooth factor was 3 and there were 13 points.

In this method, HRP is conjugated to a secondary antibody and its concentration on the wells is inversely proportional to DME concentration. The shape of the intensity–time curve depends on the peroxidase concentration. The decay of light emission being more pronounced at high peroxidase concentrations. The curves showed a similar behavior...
between 150 and 600 s. The slope were calculated in
this interval as \( t_g = \frac{(C_{150} - C_{600})}{\Delta t} \) where \( C_{150} \) and \( C_{600} \) are chemiluminescent intensities at 150
and 600 s, respectively.

Plot of decay slope values against log concentra-
tion of the standard dichlorprop methyl ester (Fig. 2)
displays a typical calibration graph fitted to an IC\(_{50}\)
four-parameter logistic. The dynamic range covering
standard concentration is \( 0.1-10^4 \) ng ml\(^{-1}\) dichlor-
prop methyl ester.

Table 1
Cross-reactivity of 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>Cl(\text{O})(\text{O} \text{CH}_3)</td>
<td>Dichlorprop methyl ester</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Cl(\text{O} \text{CH}_3)</td>
<td>Dichlorprop</td>
<td>0.156</td>
<td>0.072</td>
</tr>
<tr>
<td>Cl(\text{O} \text{COOH})</td>
<td>MCPA</td>
<td>0.021</td>
<td>0.0020</td>
</tr>
<tr>
<td>Cl(\text{N} \text{COOH})</td>
<td>2,4,5-T</td>
<td>0.055</td>
<td>0.0078</td>
</tr>
<tr>
<td>Cl(\text{O} \text{COOH})</td>
<td>Triclopyr</td>
<td>0.015</td>
<td>&gt;10 (^3)</td>
</tr>
<tr>
<td>Cl(\text{N} \text{SO}_2\text{NCH}_3)</td>
<td>Bentazone</td>
<td>&gt;10 (^6)</td>
<td>&gt;10 (^4)</td>
</tr>
<tr>
<td>Cl(\text{N} \text{CN})</td>
<td>Ioxynil</td>
<td>&gt;10 (^6)</td>
<td>&gt;10 (^4)</td>
</tr>
</tbody>
</table>

\(^a\) Cross-reactivity estimated at 75% displacement of signal.
\(^b\) Cross-reactivity estimated at 50% displacement of signal.
The minimum detectable concentration (MDC) is defined in the four-parameter logistic model [13] as "the lowest concentration which results in an expected response less than the estimated one-sided \( \alpha \)-level \((\alpha = 0.05)\) lower confidence limit at zero concentration". The MDC calculation is illustrated graphically in Fig. 2; its value is 0.11 ng mℓ⁻¹. The reliable detection limit (RDL) [13] is also shown graphically in Fig. 2, and can be defined as follows: "the lowest concentration that has a high probability of producing a response that is greater than the estimated one-sided \( \alpha \)-level \((\alpha = 0.05)\) upper confidence limit at zero". The RDL was 0.20 ng mℓ⁻¹.

The precision of the method was studied in two regions of the standard curve. One is the zone that coincides with the minimum slope of the curve, the other is the zone of maximum slope.

We prepared two series of 10 samples, containing 0.5 ng mℓ⁻¹ and 50 ng/ml DME, respectively. These samples were analysed according to the described procedure. The chemiluminescence intensity was monitored against the time with the purpose of calculating the slopes of each one of the samples. The relative standard deviation (R.S.D.) referred to concentration was 20.6% at concentration level of 0.5 ng ml⁻¹, and of 7.23% at 50 ng ml⁻¹ of DME.

### 3.2. Selectivity of the assay

To characterize the selectivity of the assay several pesticides having structures closely similar to that of dichlorprop methyl ester (i.e., 2,4,5-trichlorophenoxy acetic acid, triclopyr, dichlorprop) or those usually found in formulations [14] (ioxynil, bentazone, MCPA) were tested.

The cross-reactivity was calculated by using the 50% displacement method; also the concentration of compound required to produce 75% displacement was also determined in each case. Displacement curves were prepared by incubation of various doses of cross-reactant, followed by separation of bound and unbound fractions, and quantitation of the bound cross-reactant at each dose. After fitting the curves by the four parameter logistic model the doses of cross-reactant that gave 50% or 75% of bound cross-reactant were determined and the cross-reactivity (CR) of a compound was determined given in [15].

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

### Table 2

<table>
<thead>
<tr>
<th>Fortification Conc. of sample (ng ml⁻¹)</th>
<th>Equivalent conc. in grain (µg g⁻¹)</th>
<th>Found Conc. of sample (ng ml⁻¹)</th>
<th>Equivalent conc. in grain (µg g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>12.5</td>
<td>518 ± 20 a</td>
<td>12.9 ± 0.49 a</td>
</tr>
<tr>
<td>50</td>
<td>1.25</td>
<td>48.6 ± 3.6</td>
<td>1.21 ± 0.09</td>
</tr>
<tr>
<td>5</td>
<td>0.125</td>
<td>5.1 ± 0.3</td>
<td>0.128 ± 0.008</td>
</tr>
</tbody>
</table>

a Standard deviation for five determinations.
Table 1 gives formulae and %CR for the compounds studied. From these results it can be deduced that bentazone, MCPA, ioxynil, and triclopyr do not affect the analytical method to determine dichlorprop methyl ester. At 50% displacement dichlorprop, and 2,4,5-T display affinity for the antiserum, with IC50 of 65.6 µg ml⁻¹ and 604 µg ml⁻¹, respectively. At 75% displacement dichlorprop, 2,4,5-<i>T</i>, MCPA, and triclopyr display affinity for the antiserum at concentrations above 3.9, 11.1, 29.4, and 39.7 µg ml⁻¹, respectively.

3.3. Application

Extracts of wheat grains were spiked at three concentration levels 5, 50, and 500 ng ml⁻¹, the recoveries were 102, 97.1, and 103.6%, respectively. The results obtained are given in Table 2.

4. Conclusion

Enhanced chemiluminescence detection in ELISA methods gives good detection limits and precision. The drawback of chemiluminescence detection arising from the transient nature of the analytical signal has been solved by applying kinetic measurements.

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References