ELISA of dichlorprop by digital image analysis

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Received 5 Apr 2011; Accepted 5 Jun 2011; Available Online 5 Jun 2011

Abstract

An image based detection of enhanced chemiluminescence enzyme-linked immunosorbent assay (CL-ELISA) has been developed for the quantification of dichlorprop with human γ-Globuline (HGG). Data acquisition on microtiter wells is performed using an economical charge coupled device (CCD) camera for capturing images. The standard curve was produced for 0.1-5000 mg/L of dichlorprop. The minimum detectable concentration was 4.5 mg/L and the relative standard deviation was 0.22% (n =10) for a 10 mg/L sample concentration.

Keywords: Chemiluminescence; Dichlorprop; ELISA; Digital Image

1. Introduction

Among the immunoassay methods, enzyme-linked-immunosorbent technique (ELISA), is one of most versatile analytical tools for bio-sensing, which employs a label enzyme and a substrate to produce an amplified signal for antigen, haptons or antibody quantitation. This technique has been well established and considered as the technology of choice for a wide variety of applications in diagnostics [1], research [2,3], food testing [4], process quality [5,6] assurance [7], quality control [8-10], environmental testing [11-14], and analysis of herbicides [15].

Herbicides are viewed with particular concern due to their increased uses. Dichlorprop, a herbicide of chlorophenoxy compounds, used to kill annual and perennial broadleaf weeds, is a component of many common weed-killers. There has been concern that chlorophenoxy herbicides including dichlorprop may cause cancer. This class of compounds has been ranked by IARC as possible carcinogen to humans [16]. Separation methods are the mainly used to quantify this compound by HPLC [17-21], CE and HPLC [22] or GC-MS [23]. By indirect methods, combined with chemiluminescence detection (CL-ELISA), the obtained product is excited, reverts to ground state with emission of light and has been used to quantify dichlorprop methyl ester, a similar compound of this family [24], or other pesticides [25, 26].

The purpose of this work is to couple to chemiluminescence signal a charge coupled device camera (CCD) as image formation detector. CCD camera is connected to the computer towards a frame-grabber card, which is capable of digitizing the video signal delivered by the camera. Images can then be treated by advanced software to reduce the noise, eliminating irrelevant parts, improving quality and to quantify intensity. The image based detection methods have been used in different applications: gel electrophoresis [27-29], thin layer chromatography [30-31], immunoassay on well plate [32-34] or DNA sequencing blots [35-38] among others, being especially useful in low [39-40] and high throughput screening assays [40-42].

In this paper, commercial image analysis software is combined with data processing to reduce thermal noise and to measure a significant chemiluminescent signal. The assay imaging system was applied to detect light emitted by a CL-ELISA developed for the quantification of dichlorprop.
2. Experimental

2.1. Chemicals

Dichlorprop [(±)-2-(2,4- dichlorophenoxy) propanoic acid, DPP] was provided by Dr. Ehrenstoffer Labs. (Augsburg, Germany). N,N’-dicyclohexylcarbodiimide (DCC), N-hydroxy succinimide (NHS), Bovine serum albumin (BSA), 2,4,6-trinitrobenzene sulfonic acid (TNBS), archilamida, bis-acrilamide, Freund’s adjuvants, p-iodophenol, human-γ-globulin (HGG), and luminol (5-amino-2,3-dihydrophthalazine) were purchased from Sigma-Aldrich Química S. A. (Spain). All other chemicals used were from Merck.

2.2. Production of polyclonal antibody. BSA-DPP conjugate

DPP was coupled to BSA as carrier protein, using the activated Flekker procedure [43]. Briefly, equimolar quantities of NHS (220 mg), DPP (470 mg) and DCC (390 mg) were dissolved in dioxane (25 mL) and left for 18 h at room temperature. After that it was filtered and the solution obtained was dried, under vacuum conditions, yielding DPP-NHS-ester, a white powder checked by $^1$H-NMR (supporting information). Then, DPP-NHS-ester (28 mg) was dissolved in 1.5 mL of dioxane and mixed with BSA (100 mg), previously dissolved in 3 mL of borax/HCl buffer solution (pH 9), stirred and left to react for 1 h at room temperature. Then, the product was dialyzed in cellophane membrane for 36 h against deionized water at 4 °C, where the excess of hapten was separated, and lyophilized overnight at -40 °C under 50 mTorr. Effective conjugation was confirmed by inspection of the UV spectra. The number of amine groups substituted by the hapten in each carrier molecules was determined by TNBS titration, according to Habeed procedure [44] and by electrophoresis. Rabbit anti-DPP was produced by subcutaneous injection of BSA-DPP in New Zealand White rabbits, as previously described [45].

2.3. ELISA procedure

Each well of microtiter plates (FluoroNunc MaxiSorp purchased from Nunc) was coated with 200 μL (15 μg/mL) of DPP-HGG conjugate, previously synthesised according to the Flekker procedure [43], dissolved in carbonate buffer (50 mM, pH 9.6) and incubated for 48 h at 4 °C. The plates were emptied and washed with washing solution (0.1 M sodium phosphate buffer, pH 7.5, supplemented with 0.1% Tween 20). Following the incubation and washing procedure, diluted antiserum (1:1000) in the phosphate buffer (7.5), supplemented with 0.05% Tween 20 was pre-incubated for 24 h with DPP 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 DPP and no antiserum to determine non-specific binding of the secondary antibody-labelled DPP-HGG in the following step. Another column received no DPP to determine the maximum CL reading. The plates were washed as before. HGG 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

Aliquots of 0.01 M of luminol (82.94 μL), 0.1 M of hydrogen peroxide (165.88 μL), 0.001 M of p-iodophenol (1077 μL) and 0.1 M Tris-HCl buffer (197.65 μL, pH 8.5) were mixed and used as substrate to promote the CL assay. Aliquots of DPP solution were incorporated for the quantitative analysis. The reaction begins when 200 μL of CL substrate is transferred to each well.

2.5. Image analysis

CL images of the microtiter plate (Figure 1) were acquired in the dark, with a National Electronics, Inc. model T-390 C monochrome video CCD camera [12 mm F/1.2 lens, 0.01 lux] and digitised with an Imagraph IC-PCI frame grabber using a custom program developed in C++ (Microsoft Visual C++ 6.0). Frame grabber is a method for providing short-term storage in the computer memory, which stores one or more images and can be accessed rapidly, usually at video rates [46]. Digital images were processed with MATLAB v. 5.1 to obtain quantitative results. The overall imaging method used can be split into several steps:

1. Image acquisition process on the microtiter well by using integration of 256 images to reduce thermal noise and to obtain a significant CL signal. Longer integration times (6 min approximately) are essential at the lowest light levels since it is necessary to obtain statistically valid results. Such integration times can be achieved by averaging or summing up successive images as they are produced. We acquire a pack of 256 integer images 2 min after the beginning of the CL reaction and we go on acquiring such images until the chemiluminescence signal practically disappears (about 30 min.). Due to the non-constant light emitted by the CL reaction, a rigorous control of the time interval between the beginning of the CL reaction and the image acquisition is necessary.
Figure 1. Digital images obtained and processed using the hardware and software used.
2. Pre-processing of digital images to correct some defects and to improve their quantitative characteristics (enhancing the contrast of grey level values, reducing the noise). Processing of digital images involves procedures that are expressed in algorithmic form.

3. Image segmentation in which the image is divided into different regions, e.g. well and background. It is the process of transforming a grey level image into a binary image, where the background is black (value 0) and objects of interest are white (value 1 or 255, normally a value different from zero is chosen).

4. Feature extraction for each detected object of the image. Quantitative data such as maximum intensity, area and volume of each well in the microtiter well, are provided for the detected wells on the image.

The maximum intensity is the measurement of the grey level of the pixel with the most brightness in each well. The area (in square pixels) of a well is defined as the number of pixels contained within their boundary [46]. The area values are very similar for each well. The volume is the integration of the light intensity of all pixels in each well. The volume of each well is a measurement of the light detected from each well [47]. This parameter has been used for standard curve representation.

Figure 1A shows the digital image obtained before processing, integrating 15 images per second for 6 min (5400 images). In Figure 1B, we can see the normalized image by adjusting the grey levels to pixel levels, as indicated in the inserted scale (0 black, 1 white). Figure 1C shows the same image but containing a circular mesh, thus measuring only the CL signal within the mesh. Figure 1D shows how the program written in MATLAB detects the different circles corresponding to the wells, indicating the geometric parameters of the different wells: metrics, eccentricity and area. Finally, Figure 1E represents an image in false colour of the data contained in Figure 1B with the purpose of best visualization of the intensity difference.

3. Results and Discussion

3.1. Optimization of chemical parameters

The experimental conditions were optimized by means of the univariate approach, to obtain the best CL signal to be detected by the CCD. Different concentrations of coating agent were tested (5, 10, 15 and \(\mu\)g/mL) with different dilutions of antiserum (1:200, 1:400, 1:1000 and 1:3000). Previously, each well was incubated overnight at 4 \(^\circ\)C with 0.1, 10 and 1000 mg/mL of DPP, and the emission was recorded for 6 min after the addition of CL substrate as described in the ELISA procedure. The best CL signal was obtained when 15 \(\mu\)g/mL of coating agent and a primary antibody (Ab) (dilution of 1:1000) were mixed. Previously the kinetics of CL emission was studied for 30 min, and observed that in the first 4 min the best CL signal is obtained and it is constant after 20 min, and hence 6 min was selected for recording the assay. Light emission from CL oxidation of luminol catalyzed by BSA-DPP is enhanced by the addition of \(p\)
iodophenol. Figure 2 shows the CL intensity-time curves for the different levels of DPP added. Because antigen-antibody binding is characterized by weak intermolecular bonds, changes in pH could affect this interaction. CL signal shows the high dependence on the pH, reaching a maximum at pH 8-8.7 for the assay [48]; a pH of 8.5 was chosen as optimum. In the proposed method, the effect of Tris–HCl concentration was studied in the range 0.01-0.2 M. The maximum CL inhibition with DPP was observed at a Tris–HCl concentration of 0.1 M; therefore, Tris–HCl buffer (0.1M and pH 8.5) was selected and used for subsequent studies.

3.2. Calibration curves
To construct standard curve, aliquots of DPP covering 0.1-5000 mg/L were pre-incubated with antiserum (1:1000) overnight at 4°C, under the optimum conditions previously mentioned. The CCD recorded the image after 2 min of addition of CL substrate and for 6 min. The calibration curve obtained (Figure 3) by representation of log [DPP] vs. V/V₀, where V represents the integrated CL signal recorded from each well and V₀ is the recorded blank signal. The calibration curve fitted to a IC₅₀ four parameter logistic (γ = (a / (1 + bxᶜ)) + d) [49]:

\[
V/V₀ = (0.9379 / (1 + 0.2135 [DPP]^{0.3011})) + 0.6700 \quad (χ² = 1.24×10^{-3})
\]

where a (0.3799) is the highest value obtained for V/V₀; [DPP] is concentration in µg/mL; c is the slope, and d (0.6700) is the minimal signal or background signal. Minimal detectable concentration (MDC) is defined for a four parameter logistic model as the lowest concentration giving a response significantly lower than the response for a concentration equal to zero. It was calculated by substituting the average response (V/V₀) minus the relative standard deviation of three samples containing 10 µg/mL of dichlorprop (minimum assayed concentration giving a signal different from the maximum signal). The precision of the method obtained as RSD was 0.22% (n=10 and 50 µg/mL).

4. Conclusions
This image-based detection of CL-ELISA for dichlorprop determination offers good analytical characteristics: 1) the long CL signal (nearly 30 min) due to the use of HRP as a catalyst; 2) the intense light emission due to the use of p-iodophenol as enhancer, and 3) signal acquisition through a circular mesh adjusted to the border of microtiter wells obtained by software implementation, reducing the contribution of light from the neighboring wells.
References

Supporting Information

1. -Product between N-hydroxysuccinimide and Dichlorprop.

\[ \text{Product structure} \]

\(^1\text{H-NMR (DCCl}_3\): \( \delta \) 1.84 (d, \( J = 6.9 \) Hz, 3 H CH\(_3\)), 4.9 (q, \( J = 6.9 \) Hz, 1 H CH), 6.94 (d, \( J = 8.8 \) Hz, 1 H aromatic), 7.2 (q, \( J = 6.3 \) Hz, 1 H aromatic), 7.36 (d, \( J = 2.4 \) Hz, 1 H aromatic).