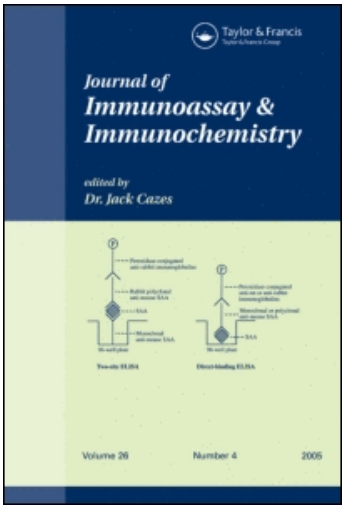

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DEVELOPMENT OF A DIRECT AND INDIRECT CHEMILUMINESCENCE IMMUNOASSAY FOR THE DETECTION OF AN ORGANOPHOSPHORUS COMPOUND

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(KEY WORDS: Organophosphorus Compound, Monoclonal Antibodies, Chemiluminescence Immunoassay, Detection System, Camera Luminometer)

ABSTRACT

The following study describes the development of two imunoassays for the semiquantitative determination of the organophosphorus compound methyl phosphonic acid, *p*-aminophenyl 1,2,2,-trimethyl-propyl diester (MATP). These assays are a direct competitive, labelled-hapten, enzyme immunoassay and a competitive enzyme immunoassay with indirectly labelled antibody, both with luminescence determination of peroxidase. A camera luminometer was used to determine final signal strength. A constant light reaction was reached using luminol (5-amino-2,3-dihydro-1,4-phalazinedione), and by adding coumaric acid as an enhancer. A five minute interval was selected as exposure time. This phototechnical system makes a semiquantitative detection of MATP possible. The detection limits for both assays were at 10^{-6}mol/l MATP. With the help of a microtiter luminometer (reader) the detection limit was reduced to 5 x 10^{-8} mol/l MATP, by measuring the real intensity of the chemiluminescence signal.

INTRODUCTION

Chemiluminescent reactions provide the basis of numerous assays for substances present in low concentrations (1). The range at which sensitive tests, such as ELISA, can detect antigens could be expanded with the development of this new method of detection, based on chemiluminescent reactions (2). The luminescence immunoassay (LIA) is one of these new procedures. Even though LIA is a more sensitive technique than the other widely used methods of detection, it has not yet been frequently applied (3, 4, 5).

For the assay of organophosphorus compounds direct as well as indirect competitive chemiluminescence assay techniques are suitable because organophosphates are haptens with only one epitope where antibodies can be bound.

In view of the increasing production, application in plant production, and potential use in chemical warfare, the importance and necessity for better methods of detection for organophosphates has become apparent. Various immunological methods have already been successfully employed in the detection of organophosphates (6, 7, 8, 9). Specifically for MATP, which is used in this study as a model substance for an organophosphorus compound, specific and sensitive methods of detection have been developed using polyclonal (10) and monoclonal antibodies (MAb; (11)).

These immunological methods as well as other possible methods of detection such as thin-layer chromatography, spectrometry and gas chromatography, are all highly specific, sensitive and precise. Their disadvantage, however, is that they are time consuming and require expensive equipment and specialized staff.

The purpose of this study was to find a cheap, practical, and easy to perform technique to detect an organophosphorus compound, that at the same time remains specific and sensitive without requiring overly expensive equipment and large staff.

MATERIALS AND METHODS

Reagents, Chemicals, and Materials

Methyl phosphonic acid, *p*-aminophenyl 1,2,2-trimethyl-propyl diester (MATP) was synthesized by TNO, Rijswijk, Netherlands. The MAb F71D7 against MATP was produced in our institute (11). The following reagents were also used: Glutaraldehyde, coumaric acid, 5-amino-2,3,-dihydro-1,4,-phalazinedione (luminol), and bovine serum albumin (BSA; Sigma Chemie GmbH, Deisenhofen, FRG); horseradish peroxidase (HRP; Boehringer, Mannheim, FRG); goat anti mouse IgG-HRP (Medac, Hamburg, FRG). All other chemicals were obtained from Merck, Darmstadt, FRG. Polyvinylchloride microtiter plates were obtained from Dynatech (Billinghurst, Sussex, UK), Polaroid film type 612, 20,000 ASA 1440 from Sigma Chemie GmbH (Deisenhofen, FRG), camera luminometer from Dynatech (Billinghurst, Sussex, UK), and microtiter luminometer (MTP-Reader; Hamamatsu Photonics, Herrsching, FRG).

Development of a Direct Competitive, Labelled-hapten, Enzyme Immunoassay

In this direct competitive assay an antigen and a peroxidase labelled antigen compete for a defined number of specific binding sites of the antibody which is bound to the microtiter plates (Fig.1). The luminescence signal is inversely proportional to the unlabelled antigen concentration (12). The solid phase was coated with the MAb F71D7 which could be classified as an IgG1 antibody.

To produce MATP-HRP, we used the procedure described by Erhard et al. (11). The assay was performed with the MAb concentrations and conjugate dilutions that were determined as optimal in preliminary tests. Since luminol and most of the other reagents required for the test are light sensitive, the substances were immediately used after their preparation.

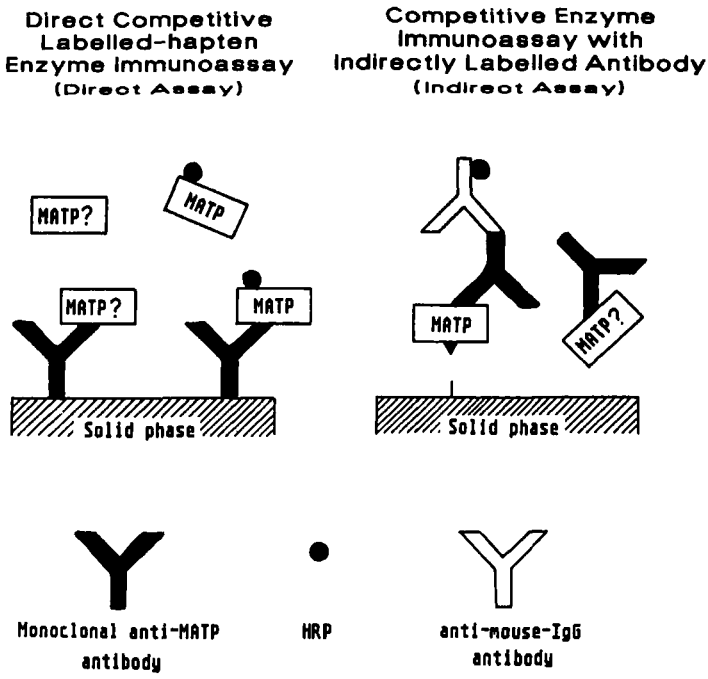

FIGURE 1. Schematic representation of the direct competitive, labelled-hapten, enzyme immunoassay and the competitive enzyme immunoassay with indirectly labelled antibody, both with luminescence determination of peroxidase.

The microtiter plates were coated with 2.5 µg MAb F71D7 per ml in 0.1 mol/l carbonate buffer (pH 9.6, 200 µl/well) for 1 h at 37 C. Then 50 µl of MATP-HRP (1:35,000 in PBS-Tween) and 50 µl of MATP in different dilutions were added to each well and incubated for 1 h. MATP was used in a log10 and a log2 dilution. After adding the substrate (1.25 mmol/l luminol, 30 mmol/l coumaric acid, 2.7 mmol/l H_2O_2 in 100 mmol/l tris-acetate buffer, pH 8.6; (13)), the emitted light was measured for 5 min. Between each step the plates were washed three times with saline-Tween.

Development of a Competitive Enzyme Immunoassay with Indirectly Labelled Antibody

The indirect assay consists of a two phase reaction (Fig.1). In the first phase the antigen which is to be analyzed is preincubated with a definite amount of the specific MAb F71D7. Those antibodies which are not bound to the free antigen bind to the immobilized antigen, which is bound in a known concentration to the microtiter plates. For detection an peroxidase labelled antibody (anti mouse IgG) is added. The enzyme reaction in this competitive assay is inversely proportional to the antigen concentration (12).

For coating the microtiter plates have to be activated by glutaraldehyde (0.2% in phosphate buffer, pH 5.0, 200 µl/well) for 4 h at room temperature. After washing the plates three times with phosphate buffer (pH 5.0), the microtiter plates were coated with 15 µg MATP per ml phosphate buffer pH 8.0 (200 µl/well). The plates were incubated for 3 h at 37 C and then overnight at 4 C. The free binding sites were blocked with 0.1 mol/l ethanolamine in phosphate buffer pH 8.0 (200 µl/well). The probes were preincubated for 1 h at 37 C with MAb F71D7, 5 µg per ml PBS-Tween (50 µl), and MATP in a log10 dilution (50 µl), starting with 10^{-3} mol/l MATP or in the case of the log2 dilution starting with 10^{-5} mol/l MATP. We pipetted 100 µl of the preincubated probes into the wells. To have a control, in one column no antigen was pipetted. The probes were incubated for 1 h at 37 C. Goat anti mouse IgG-HRP (1:500, 50 µl/well; 1 h at 37 C) was used as conjugate. Between each incubation stage the plates were washed three times with NaCl-Tween. The last step was the addition of the substrate solution (200 µl/well; see direct assay). Then the light emission was measured over a period of 5 min.

Quantification, Correlation, and Validation

To check the validity of the direct competitive, labelled-hapten, enzyme immunoassay the luminescence signal was detected quantitatively with a microtiter luminometer (MTP-Reader). The percent of inhibition in the competitive luminescence determination was compared with the inhibition of the direct competitive ELISA (11). The technique of the assays and the concentrations of the probes were the same in all tests.

Detection of the Antigen in Biological Fluids

MATP was diluted log2 in goat serum, cow milk, and drinking water starting with 10^{-4} mol/l MATP. The direct competitive, labelled-hapten, enzyme immunoassay was performed as described above.

RESULTS

Direct Competitive, Labelled-hapten, Enzyme Immunoassay

To find the optimal relationship between plate coating (MAb F71D7) and MATP-HRP concentration, we chose a MATP-HRP concentration at which a clear difference in the light intensity was visible between the neighboring dilutions, and at which the MATP-HRP concentration was as low as possible.

In the direct assay the MATP probes were added in a log10 dilution (Fig.2) starting with a concentration of 10^{-3} mol/l MATP in column 8.

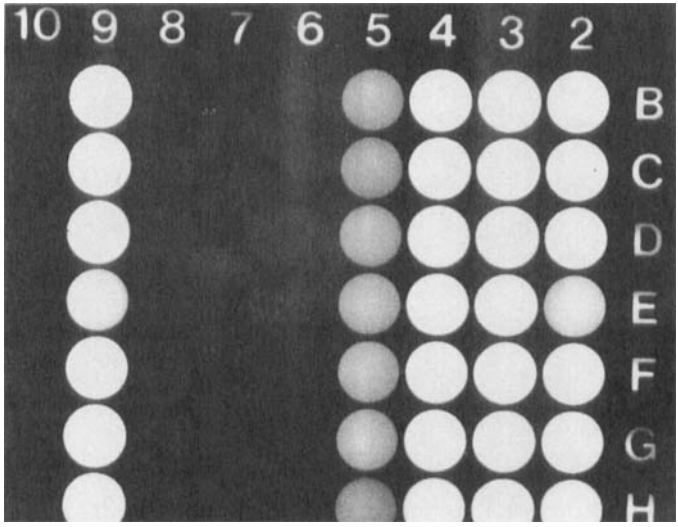

FIGURE 2. Performance of the direct competitive, labelled-hapten, enzyme immunoassay with luminescence determination of peroxidase (n=7). The MATP probes were added in a log10 dilution starting in column 8 with a concentration of 10^{-3} mol/l MATP to column 2 (10^{-9} mol/l MATP). Column 10 was the negative control (PBS-Tween) and column 9 was the positive control (PBS-Tween and MATP-HRP).

Column 4 (10^{-7} mol/l MATP) did not show any difference to the positive control which did not contain any free antigen. The limit of detection in this test was at a concentration of 10^{-6} mol/MATP (column 5).

To check the validity of our detection system we made instead of a log10 dilution of our antigen a log2 dilution (Fig. 3). The first dilution used was 5×10^{-6} mol/l MATP (column 8). The limit of detection was now at a MATP concentration of 1.25×10^{-6} mol/l (column 5). Therefore, up to this concentration it is possible to differentiate between MATP and the standard value.

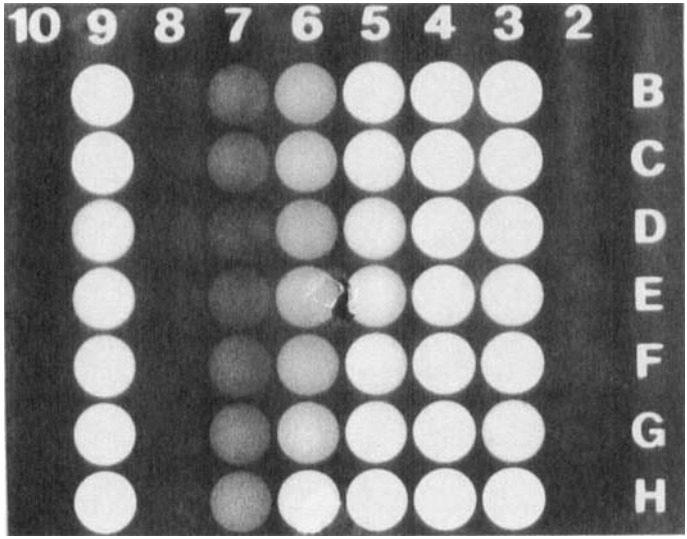

FIGURE 3. Performance of the direct competitive, labelled-hapten, enzyme immunoassay with luminescence determination of peroxidase using a log2 dilution of MATP (column 8 to 3; n=7). The first dilution used was 5×10^{-6} mol/l MATP (column 8). Column 10 and 2 were the negative control (PBS-Tween) and column 9 was the positive control (PBS-Tween and MATP-HRP).

Competitive Enzyme Immunoassay with Indirectly Labelled Antibody

The limit of detection of MATP was first determined in a log10 dilution (starting with 10^{-3} mol/l MATP) as described in direct assay. It is possible to clearly distinguish the light intensity between the positive control and MATP at a concentration of 10^{-6} mol/l MATP. In addition, a further indirect assay with a MATP log2 dilution (starting with 10^{-5} mol/l MATP) was performed for checking the validity. The limit of detection was the same achieved in direct assay (10^{-6} mol/l MATP; results not shown).

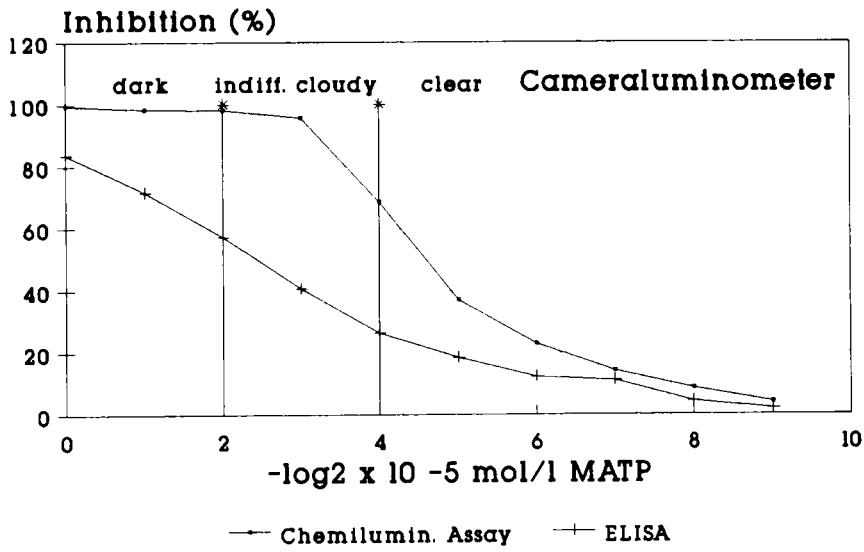

FIGURE 4. Inhibition of the antibody binding (MAb F71D7) to peroxidase labelled MATP by different quantities of MATP using direct competitive, labelled-hapten, enzyme immunoassay with microtiter luminometer (reader) and direct competitive ELISA as a reference detection system. The correlation (r) was 0.99 in the linear part of the curves. In comparison the different light signals (dark, indifferent cloudy, clear) of the camera luminometer and their area of detection are shown on the top of the figure.

Quantification, Correlation and Validation

The luminescence signal was quantitatively determined in the direct competitive, labelled-hapten, enzyme immunoassay using a microtiter luminometer. The not inhibited probe, with an MATP-HRP concentration of 1:35,000, achieved over a period of 5 min a constant signal intensity of 15,000 cpm. The percent of inhibition depending on the concentration of MATP is shown in figure 4. The chemiluminescence signal was compared with the exposure intensity using the cameraluminometer.

TABLE 1

Validation and statistical testing of the direct competitive, labelled-hapten, enzyme immunoassay using a camera luminometer (log_2 dilution).

Assay	circles on the polaroid film	MATP (mol/l)	probes
Intraassay	black	$\geq 5.00 \times 10^{-6}$	(7/7)
	indifferent cloudy (heavy)	2.50×10^{-6}	(7/7)
	indifferent cloudy (light)	1.25×10^{-6}	(6/7)
	clear (white)	$\leq 6.25 \times 10^{-6}$	(7/7)
Interassay	black	$\geq 5.00 \times 10^{-6}$	(42/42)
	indifferent cloudy (heavy)	2.50×10^{-6}	(39/42)
	indifferent cloudy (light)	1.25×10^{-6}	(40/42)
	clear (white)	$\leq 6.25 \times 10^{-6}$	(42/42)

The direct competitive ELISA (11) was used as a reference method. The correlation (r) of the direct chemiluninesence assay and the direct ELISA was 0.99 in the linear part of the measured curves (Fig. 4).

The statistical testing of the direct competitive, labelled-hapten, enzyme immunoassay was performed by analysing a significant numbers of samples known to contain certain concentrations of analyte. The results of the intra- and the interassay are shown in table 1. All samples (100 %) with a concentration of 5×10^{-6} mol/l MATP or higher got a black circle on the polaroid film. Using a concentration of 2.5×10^{-6} mol/l MATP, 93 % of the circles were indifferent cloudy (either heavy). When testing the concentration of 1.25×10^{-6} mol/l MATP, 95 % of the circles were indifferent cloudy (either light). Higher concentrations of MATP resulted in a maximal intensity of chemiluminescence without inhibition and clear circles (100 %).

Detection of MATP in Biological Fluids Using Camera Luminometer

The limit of detection for MATP in goat serum, cow milk, and drinking water was uniformly at 10^{-6} mol/l and coincides therefore with the values achieved in direct and indirect assay MATP was diluted in saline-Tween.

DISCUSSION

Pesticides have a widespread range of application as herbicides, insecticides, rodenticides or also as fungicides in agriculture, gardening and forestry. This broad spectrum of application increases the risk of man and animals accidentally poisoning themselves through careless handling or ignorance of the toxicity of the pesticide. Man can endanger or hurt himself either directly through the production and distribution of pesticides or indirectly through pesticide residues (14, 15). Organophosphates, also known as phosphoric acid esters or organophosphorus compounds, are easily disintegrated but have a high acute toxicity, which means that even a single low dose can cause noticeable damage to the body.

For the detection of MATP, which we used as a model substance, competitive test principles are descripted (10, 11, 16). In this study we attempted to establish a sensitive, specific and easy to perform detection system for an organophosphate that can be used under field conditions.

The disadvantages of bioluminescent substrates are their costs and their instability in solutions. Since the development of a field test was the goal, a chemiluminescent substrate was chosen. Good results could be achieved using aminobutylethylisoluminol (ABEI), a derivative of isoluminol (17, 18). When ABEI was used in the test systems developed in this study the results were not useable. However, the substrate described by Sankolli et

al. (13) performed well. This substrate uses luminol as the luminogenic substance and coumaric acid as an enhancer. A suitable enhancer to prolong the light signal is indispensable to achieve an optimal luminescence signal on the polaroid film. The sensitivity of this test method, using a camera luminometer, depends on how sensitive the polaroid film is. The polaroid film (type 612, 20,000 ASA) used in these experiments is sensitive enough to detect light emissions in the analytically relevant range of the chemiluminescent reaction (19, 20).

Direct competitive, labelled-hapten, enzyme immunoassay is superior to competitive enzyme immunoassay with indirect labelled antibody in that it is easier and quicker to perform. Since only one antibody is used instead of two, such as in indirect assay, one incubation step and washing step can be spared. However, since indirect assay has been described as the more sensitive test (2, 21), we decided to additionally perform an assay using the indirect assay principle. In this study we got the same detection limit in both systems.

The specificity of the antibody used and its ability to bind to the microtiter plate is a contributory determinant for the specificity and sensitivity of the test system. Erhard et al. (11) described the antibody when developing an immunoassay for MATP. In contrast to the immunoassay the camera luminometer is easy to handle, does not need an electrical source, and requires very little space (22).

Since it is easy to interpret the light spots, even unversed observers can quickly evaluate the results. The test system's limit of sensitivity, determined by the difference in light intensity between the row of maximum light intensity and the samples row, is distinct and clear in all performed and established test systems. The limit of detectability (10^{-6} mol/l MATP) was the lowest dilution at which there still was a distinct difference

in light intensity to the column with maximum light intensity (positive control). To check the validity of the direct competitive assay the luminescence signal was detected quantitativly with a microtiter luminometer. With this reader the detection limit could be reduced to 5 x 10^{-8} mol/l MATP.

The chemiluminescence test system described in this paper is a true alternative to ELISA systems. The field test developed does not detect low concentrations as well as the ELISA test (10^{-7} mol/l MATP), but it does not require the apparatus necessary for ELISA. In the ELISA test after enzyme inhibition a exactly defined time must be held after which the signal has to be measured. This results in only one single measurement, whereas the light signal in the chemiluminescence immunoassay is stable and measured over a longer period (several minutes) of time. Due to the constant light signal it is possible, without haste, to pipette the substrate into a microtiter plate. The time measured can be varied between a few seconds and several minutes. It is also possible to repeat measurements. Extending the exposure time often results in higher sensitivity.

To sum up, it can be said that the test developed in this study does not require electrically and can be used as a field test. Even though the tests results are semiquantitative, they are more than adequate to determine if MATP is present in very low concentrations.

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