DEVELOPMENT OF CHEMILUMINESCENT FLOW INJECTION IMMUNOASSAY (FIIA) FOR DDT ORGANOCHLORINE PESTICIDES

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The chemiluminescent flow injection immunoassays were developed for the detection of organochlorine pesticides belonging to DDT family. The immunosensor was based on the principle of heterogeneous indirect competitive immunoassay. Anti-DDT monoclonal antibodies LIB-DDT5-25 and LIB-DDT5-52 with different specificity to DDT family compounds were labelled with horseradish peroxidase. DDT antigen was immobilised on commercially available nylon membrane support. The interaction between immobilised antigen and monoclonal antibody-enzyme conjugate was directly detected using the reaction of enhanced chemiluminescence. The limits of detection were 1 and 3 nM for LIB-DDT5-25-HRP and LIB-DDT5-52-HRP respectively. Optimal membrane support regeneration was achieved using 0.2 M Glycine-HCl buffer, pH 1.9, and under these regeneration conditions immunosensor was reusable for 40 consecutive assay cycles without significant loss of performance. The immunosensor was able to run a whole assay in 20 min.

Introduction

1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane (DDT) was patented in 1939 as the first synthetic multipurpose insecticide with low nontarget toxicity. DDT proved economical and versatile for use in both agricultural and public health applications and more than 1 million tons of this chlorinated compound have been used worldwide. Great stability of DDT and its main metabolites 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE) and 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane (DDD) to physical, chemical and biological degradation has resulted in the accumulation of their residues in the adipose tissues of animals and man, as well as in the environment [1]. Some toxic effects of DDT residues have been reported [2, 3] and these effects lead to the need of methods able to determine DDT residues in environmental samples.

Immunochemical techniques are considered as a good alternative to the more classical chromatographic methods, since immunoassays are fast, sensitive, selective and economic. The ability of producing antibodies against any organic molecule and the development of new technologies for the production of monoclonal antibodies (Mabs) have stimulated the development of immunochemical techniques applied to environmental pollutants such as insecticides [4].

Flow-injection immunoassays (FIIA) are recently used for environmental analysis when continuous monitoring and high sample throughput are required [5]. Immunosensors are devices that use immunochemical principles to carry out large-scale analysis in rapid way. Flow-injection heterogeneous immunosensors combine the sensitivity and specificity of immunoassays with the precision and ability to be automated of flow technique. However, immunosensors need to exhibit reproducibility and repeatability as good as possible, must be able to work with non-pretreated samples and has to be regenerable to be cost-effective.

The aim of this work was the development of flow injection immunoassays for the determination of DDT residues. For the immunoassay development, previously selected anti-DDT monoclonal antibodies with different specificity to DDT family compounds were labelled with horseradish peroxidase. DDT hapten was immobilised on membrane support (HyBond), which then was placed in thin-layer flow-through cell. Assay procedure was based on the indirect competitive format. Reaction of enhanced chemiluminescence was used for detection of the peroxidase label. The sensor optimisation regarding its sensitivity and dynamic range is described. Other analytical aspects such as cross-reactivity, reusability and operation stability were also studied.

Materials and Methods

Chemicals

Analytical standards of *p,p′*-DDT, *o,p′*-DDT, *p,p′*-DDD, *o,p′*-DDD, *p,p′*-DDE, and *o,p′*-DDE were from Riedel–de Haen (Seelze, Germany). Stock solutions (1 mg/ml) were prepared in dry DMF and stored at 4°C. Working standard solutions were prepared daily from stock solutions in glass tubes and used within 30 min to avoid pesticide loss through adhesion to glass surfaces.

Mouse anti-DDT monoclonal antibodies LIB-DDT5-25 and LIB-DDT5-52 obtained from the synthetic hapten 4-{4-[1-(4-Chlorophenyl)-2,2,2-trichloroethyl]phenyl}-butanoic acid (DDT5) and conjugate DDT5 with ovalbumin (OVA-DDT5) were previously prepared and characterised by ELISA [6].

Bovine serum albumin (BSA), casein, horseradish peroxidase (HRP), luminol, and *p*-iodophenol (PIP) were pur-

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chased from Sigma (St. Lous, MO, USA). All other reagents of analytical grade were supplied by Reakhim (Moscow, Russia). All solutions were prepared with water purified with a Milli-Q system (Millipore).

Preparation of HRP-monoclonal antibody conjugates

Conjugates of monoclonal antibody and horseradish peroxidase were prepared by the periodate method of Wilson and Nakane [7]. 4 mg of HRP was dissolved in 1 ml of H_2O. 0.2 ml of fresh 0.1 M $NaIO_4$ was added to the peroxidase solution. Obtained mixture was stirred for 20 min in darkness. Mixture was dialyzed against 0.001 M Na-acetate buffer, pH 4.4, overnight at 4°C. 1 ml of monoclonal antibody suspension under 50% $(NH_4)_2SO_4$ with concentration 1 mg/ml was centrifuged and the precipitate was dissolved in 300 μl of 0.01 M K-phosphate buffer, pH 7.4, with 0.15 M NaCl (PBS). This monoclonal antibody solution was dialyzed against 0.2 M Na-carbonate buffer, pH 9.5, at 4^0C overnight. This monoclonal antibody solution was dialyzed against 0.2 M Na-carbonate buffer, pH 9.5, at 4°C overnight. pH value of horseradish peroxidase solution after the dialysis was brought to 9.5 with 0.2 M Na-carbonate buffer and after that monoclonal antibody solution was added immediately to achieve an HRP/monoclonal antibody molecular ratio 2:1. Mixture was stirred for 2 h at RT. Then 0.1 ml of fresh 0.1 mM $NaBH_4$ solution was added. Mixture was incubated for 30 min at RT. Obtained solution was dialyzed against PBS at 4°C overnight. Obtained conjugates were stored in 50% glycerol solution at −20 °C.

Membrane support

Nylon membranes with a pore size 0.45 μm (Hybond-N+ nylon transparency, RPN2020B) were obtained from Amersham (UK).

Preparation of membrane support

Prior to immobilisation, membrane was activated by *p*-azidobenzoic acid. Membranes were immersed in the activation mixture (50 ml of DMF, 0.4 g of N-hydroxysuccinimide ester of p-azidobenzoic acid, 0.71 g of NN′-dicyclohexylcarbodiimide) and shake for 5 days without access of light. After that membranes were washed with DMF, 3 times for 20 min in shaker, until the white precipitate (dicyclohexylurine) was eliminated. Then membranes were dried between layers of filtration paper during 2–3 days, changing the paper time to time. Activated membranes are kept at RT in dark place (during 6 months). To perform indirect assays, antigen (OVA-DDT5) was immobilised on activated membrane support. The place of sampling was allocated by stencil (test tube, diameter 7 mm) on the piece of activated membrane. Membrane was carefully placed on the surface of water. Antibodies (90 μl in TBS or PBS) were dropped on membrane and membrane were incubated for 10–15 min. Membrane was carefully taken down and dried at RT during 15 min. Then membrane covered with the lid of the polystyrene plate was irradiated by UV light from the distance of 10 cm during 3 min. Membrane was washed for 10 min with TBST or PBST (in shaker) and then 10 min with TBS or PBS. Free binding sites were blocked by TBS with 0.5% of casein and 0.5% of BSA during 1 h at RT in shaker. Casein was solved on heating (80 °C) and mixing. The solution of casein obtained was kept at −20 °C. Membrane was dried and stored at 4 °C before use.

System design

Flow manifold consists of Tecator FIA 5020 instrument and flow luminometer Lumiscan. The flow-through thin-layer cell containing membrane support was placed directly into the measuring compartment of the luminometer [9].

Assay protocol

The scheme of the assay protocol, based on the principles of competitive indirect enzyme immunoassay, was the following. First, a fixed amount (1.8 ml) of enzyme conjugate solution was mixed with the appropriate amount (0.4 ml) of standard solution, and the mixture was injected through the immobilised antigen membrane cell at 0.5 ml/min flow rate. After washing with PBST (1 ml) in order to remove all unbound reagents, substrate mixture (1 mM luminol, 0.5 mM *p*-iodophenol, 1 mM H_2O_2 in 0.2 M borate buffer, pH 8.6) (1 ml) was injected and the luminescent intensity was registered. Finally, a fixed amount (1 ml) of desorbent solution was injected, followed by another washing step with PBST for complete immunosensor regeneration. The total assay time was around 20 min per cycle.

Results and Discussion

Sensor optimisation and characterisation

Chemical and hydrodynamic parameters were optimised for maximal sensitivity, sensor stability and speed of analysis. The most suitable monoclonal antibodies LIB-DDT5-25 and LIB-DDT5-52 were chosen previously [6] and conjugates of these monoclonal antibodies with horseradish peroxidase were synthesised, while sample volume, flow rate, desorbent solution, antigen density on membrane support and enzyme-monoclonal antibody conjugates concentrations were tested in the flow-injection system.

The OVA-DDT5 conjugate (molar ratio 1:11) was immobilised on nylon membrane in concentrations ranging from 5 to 40 μg/ml. The optimum antigen density on the membrane support was achieved when the solution of OVA-DDT5 with concentration 20 μg/ml was used in immobilisation procedure. Higher antigen density (40 μg/ml) diminished assay sensitivity and lower antigen density (10 and 5 μg/ml) led to considerable loss of activity.

HRP-monoclonal antibody conjugate dilutions between 1:500 and 1:4000 were tested, and dilutions 1:2000 for LIB-DDT5-25-HRP and 1:1000 for LIB-DDT5-52-HRP were chosen as optimum.

Different sample volumes were assayed ranging between 150 and 500 μl. The best results were found for both LIB-DDT5-25-HRP and LIB-DDT5-52-HRP with sample volume 400 μl at flow rate 0.5 ml/min. Higher flow rate and lower sample volumes diminished assay sensitivity, while lower flow rates and higher sample volumes increased assay time without improving sensitivity.

Disruption of the antigen-antibody complex, i. e., immunosorbent regeneration, is an essential step to render an immunosensor reusable [8]. For this purpose, different dissociating reagents for membrane regeneration, such as high salt concentration (5 M NaCl), methanol:water mixture (50:50 v/v), and buffers with low pH values (0.2 M Glycine/HCl) were tested. To assess optimum conditions for desorption from the antigen-MAB-HRP complex, membrane was treated using 1–3 cycles (500 μl each) of the desorbent solutions. The effectiveness of these solutions is shown in Table 1. Desoprtion effectiveness was calculated according to the following equation: Desorption effectiveness $= (1 - A/A_0) \times 100$, where A_0 represents the luminescent signal obtained before applying any desorption step and A represents the signal obtained after each desorption cycle. The best dissociating reagent was a low pH buffer (0.2 M Glycine/HCl, pH 1.9) applied for a short time (2 cycles), since it allowed the fast and complete dissociation of the antigen–Mab–HRP complex for both LIB-DDT5-52-HRP and LIB-DDT5-25-HRP.

In these conditions, competitive calibration curves were made with DDT standards at concentrations ranging from 0 to 10000 nM. The normalised signals expressed as $100 \times (B/B_0)$ (where B and B_0 are the chemiluminescent signals obtained with DDT standards and blank sample respectively) were plotted vs. DDT concentration, and the experimental points were fitted to a four-parameter logistic equation. Figure 1 shows calibration curves obtained from three measurements with I_{50} 22 ± 2 and 58 ± 8 nM for LIB-DDT5-25 and LIB-DDT5-52 respectively.

Apart from I_{50}, the limit of detection was calculated from competitive curves as the analyte concentration for which the normalised signal was 90%. Limits of detection were 1 nM in the immunoassay with LIB-DDT5-25-HRP and 3 nM with LIB-DDT5-52-HRP. Dynamic ranges, defined by the analyte concentrations that inhibited maximum signal by 20% and 80%, were comprised between 5–100 and 10–400 nM for LIB-DDT5-25 and LIB-DDT5-52 respectively.

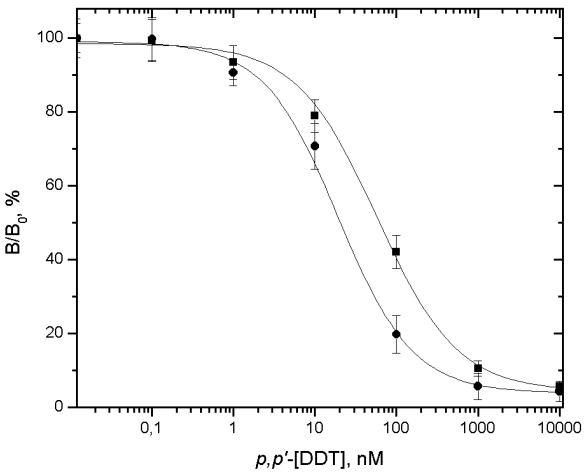

Fig. 1. Calibration curves for the DDT flow-injection immunoassays, using both LIB-DDT5-25-HRP (●) and LIB-DDT5-52-HRP (■) conjugates. Each point represents the mean ± SD of three measurements.

Immunoassays developed were sensitive enough to be applied to drinking water samples without preconcentration.

Both sensitivities and dynamic ranges obtained with the developed flow-through immunosensor were comparable to those obtained in indirect competitive ELISA with enzyme-monoclonal antibody conjugates, but were less sensitive than those described for DDT ELISAs based on the use of secondary HRP-labeled antibodies [6].

Sensor reusability

Reusability of immunosurfaces is the main problem in sensor development. To estimate operation stability of the immunosensor, repeated analyses of blanks and standards with DDT concentration near I_{50} were carried out. The sensor was considered useful while the normalised B/B_0 signal remained constant, and B_0 did not diminish more than 15% of the initial value.

In a continuous work, B/B_0 remained constant for 30–40 assay cycles. Along these assays, B_0 diminished to 85% of the initial value. More than 40 assay cycles resulted in rapid decrease of the B_0 value and in significant variations of B/B_0. Therefore, the operation stability of the immunosensor was established as 30–40 assay cycles. No physical damage or loss of hydrodynamic properties of

Table 1

Desorption effectiveness (%) of different desorbents

Mab-HRP conjugate	Cycle	Desorbent solution			
		0.2 M Gly, pH 1.9	0.2 M Gly, pH 3.0	MeOH:H_2O (1:1)	5 M NaCl
LIB-DDT5-25-HRP	1	90	65	5	20
	2	98	80	10	30
	3	100	85	15	35
LIB-DDT5-52-HRP	1	85	50	5	20
	2	98	65	8	25
	3	100	80	10	28

Table 2

Cross-reactivity of DDT-related compounds

Compound	Enzyme-Monoclonal antibody conjugate			
	LIB-DDT5-25-HRP		LIB-DDT5-52-HRP	
	I_{50}, nM	Cross-reactivity, %	I_{50}, nM	Cross-reactivity, %
p,p'-DDT	22	100	57	100
o,p'-DDT	34	65	48	120
p,p'-DDD	10	215	1154	5
o,p'-DDD	28	78	1442	4
p,p'-DDE	24	92	1442	4
o,p'-DDE	71	31	2885	2

the support was observed. The same batch of membrane support with immobilised OVA-DDT5, stored at 4°C for 2 months, was used with no detectable loss of activity.

Cross-reactivity

For interference studies, competitive curves were performed with DDT-related compounds, and their corresponding I_{50} values were determined. Cross-reactivity was then calculated as the percentage ration I_{50} for DDT/ I_{50} for related compound, both I_{50} expressed in nM units. As shown in Table 2, cross-reactivity values with conjugate LIB-DDT5-52-HRP were low except o,p'-DDT. In the case of conjugate LIB-DDT5-25-HRP cross-reactivities were high for almost all DDT-related compounds. Therefore immunoassays developed can be applied for class-specific (conjugate LIB-DDT5-25-HRP) and DDT-specific (conjugate LIB-DDT5-52-HRP) analysis of real samples.

These results are also very similar to those obtained previously in DDT ELISAs [6].

Conclusions

The chemiluminescent flow-injection immunoassays developed have shown to be rapid and sensitive enough to be applied to class- and DDT-specific determination of DDT residues in drinking water without preconcentration. The reusability of the immunosensor, a very important practical requirement of immunosensors, has been successfully accomplished, so that 40 analyses can be performed with the same membrane support.

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