An indirect format enzyme-linked immuno-sorbent assay (ELISA) on graphite rods (Ø 0.8 mm × 20 mm) and, for comparison, on microtiter plates has been developed against terbuthylazine. For this purpose, a series of 2-aminoalkyl-4-chloro-6-terbuthyl-s-triazine-2,6-diamine ELISA haptens with alkyl spacer lengths of 2, 4, 6, and 8 CH₂ groups has been synthesized. The graphite rods or the microtiter plates were covered by a polymerized glutaraldehyde network, and the ELISA hapten has been coupled by imino coupling to the free aldehyde groups of that network. ε-Aminocaproic acid has been used as an agent to block unspecific binding sites. The ELISA was run in a competitive mode, where the immobilized ELISA hapten and the solute terbuthylazine as a target analyte compete for the solute antibody.

Key words: Indirect Format ELISA, Graphite, Triazines

Introduction

Today enzyme-linked immuno-sorbent assays (ELISAs) are commonly applied to trace level analyses in aqueous systems. Competitive systems are by far dominating for the determination of low molecular weight hapten. In most cases the antibody is immobilized on a suitable surface. In this normal format assay the analyte and a structurally related enzyme-labeled compound compete in the sample solution for the antibody binding sites and thus the enzyme may get into contact with sample solution components which may affect the enzyme. Therefore, indirect formats using immobilized hapten are gaining interest. In most such cases a hapten-protein conjugate is immobilized. However, the synthesis of tracer or coating hapten-protein conjugates often lacks reproducible preparation of these conjugates with respect to coupling densities (Kaur et al., 2004; Wortberg et al., 1995). Therefore, direct immobilization of the hapten should be favoured.

Some papers report direct immobilizations of hapten to surfaces like silanized optical fibre (Fry and Bobbitt, 2001), gamma-irradiated polystyrene (Hofstetter et al., 1997), or hydroxyl succinimide activated polymer, Affiprep10 (Silvaieh et al., 2002). In Geckeler and Müller (1993) possible polymer materials are discussed. However, only a few papers report competitive ELISAs with direct immobilization of hapten on surfaces. Bier et al. (1994) immobilized an atrazine derivative on a SiO₂–TiO₂-waveguide with glutaraldehyde after silanization. In Piehler et al. (1996) glass-type surfaces modified by several hydrophilic polymers of different functional properties to immobilize aminocaproic acid atrazine are used, and in Trau et al. (1997) 2,4-D (2,4-dichlorophenoxyacetic acid) was immobilized to the surface of quartz capillaries utilizing poly-l-lysine. A glutaraldehyde network was used for immobilizing several atrazine derivatives on polystyrene microtiter plates (MTP) (Holthues et al., 2001, 2005). A thiolated pyrene derivative was immobilized on a gold surface in an amperometric immunosensor for the determination of benzo[a]pyrene (Liu et al., 2000).

In particular, surfaces other than MTP are of interest with regard to applications in the field of biosensors. In the present communication the development of an indirect ELISA for terbuthylazine, a common herbicide, on graphite rods is reported. The assays for two different antibodies and
4 different coating haptens are compared with assays of the same format in MTP.

**Material and Methods**

**Antibodies**

Two different monoclonal antibodies have been used here. The lyophilized antibodies (2 mg each) were dissolved in 200 µl sterile phosphate buffered saline (PBS) buffer (80 mmol l⁻¹, pH 7.6). The monoclonal antibodies P6A7 and 4A54 were a kind gift from T. Giersch and B. Hock (Technical University of Munich, Weihenstephan, Germany), and are described in detail elsewhere (Giersch *et al.*, 1993). P6A7 is highly specific for the determination of terbuthylazine (Giersch *et al.*, 1993), 4A54 is highly sensitive to a series of s-triazines (Winklmair *et al.*, 1997), including terbuthylazine, since it was prepared through a multi-hapten immunogen consisting of 10 different haptens linked to one carrier protein.

**Apparatus**

Adjustable pipettes [Finpette 40–200 µl and Finpette 200–1000 µl (Labsystems, Helsinki, Finland), Eppendorf pipette 0.5–10 µl, and Eppendorf Multipette (Eppendorf, Hamburg, Germany)] were used to dispense the liquids. Data evaluation has been performed by Kaleidagraph 3.5 (Synergy Software, Reading, PA, USA). ELISAs were performed in 96-well flat-bottom polystyrene microtiter plates (MTPs, Maxisorb No. 442404, Nunc, Wiesbaden, Germany). A Dynatech MR5000 (Dynatech Laboratories, Virginia, USA) ELISA reader was used. The software Biolinx 2.21 (Dynatech Laboratories) was used to control the reader. Pencil rods (graphite rods) were a kind gift from Staedler (Nuremberg, Germany).

**Chemicals**

Organic reagents for synthesis were purchased from Sigma (Steinheim, Germany) and Merck (Darmstadt, Germany). Terbuthylazine was obtained from Fluka-Riedel de Häen (Seelze, Germany). Silica gel 60 (0.063–0.1 mm) and aluminium-backed silica-gel 60 F254 thin-layer chromatography (TLC) plates were from Merck. Tween 20 was obtained from Serva (Heidelberg, Germany). Horseradish peroxidase (HRP) (lyophilized, 250 U/mg) was purchased from Boehringer (Mannheim, Germany). Goat anti-mouse antibody (IgG), goat anti-mouse IgG-peroxidase conjugate and bovine serum albumin (BSA) were obtained from Sigma. Glutaraldehyde (GA) (25% in water) was purchased from Baker (Griesheim, Germany). All other chemicals were of analytical grade.

**Experimental**

**General**

ELISA was carried out in the competitive indirect mode:

1.) A suitable ELISA hapten was immobilized on a carrier surface (MTP or graphite pencil rods, 0.8 mm Ø, 20 mm length).

2.) The target analyte and the ELISA hapten competed in a solution to which a mouse antibody against the analyte was added.

3.) After a washing step the bound amount of mouse antibody was determined by a peroxidase (POD) labeled goat anti-mouse antibody (GaM-POD).

4.) The amount of bound anti-antibody was determined by the peroxidase induced reaction and the photometric detection of the reaction product at 450 nm.

All these steps have been thoroughly optimized, with all details given by Fouad (2003).

**ELISA haptens**

Since in a competitive ELISA the relative affinity of the analyte and of the ELISA hapten towards the antibody strongly affects the sensitivity of the ELISA, several bridge homologous ELISA haptens have been synthesized starting from cyan-

![Fig. 1. Structure of the synthesized haptens 4-chloro-2-ethyl-6-terbuthyl-s-triazine-2,6-diamine (C2), 2-butyl-4-chloro-6-terbuthyl-s-triazine-2,6-diamine (C4), 4-chloro-2-hexyl-6-terbuthyl-s-triazine-2,6-diamine (C6), and 4-chloro-2-octyl-6-terbuthyl-s-triazine-2,6-diamine (C8).](image-url)
uric chloride through dichloroterbuthylazine to yield the alkyl-series 2-aminoalkyl-4-chloro-6-terbuthyl-s-triazine-2,6-diamine, following methods described elsewhere (Holthues et al., 2005). The purity of all compounds was confirmed by TLC and proton nuclear magnetic resonance spectroscopy ($^1$H NMR), respectively. The structure of the haptens is shown in Fig. 1.

2,4-Dichloro-6-terbuthylamino-s-triazine (dichloroterbuthylazine)

To a well stirred solution of cyanuric chloride (0.1 mol in 80 ml acetone) at 0°C, 0.1 mol of terbuthylamine was added drop-wise, so that the temperature did not exceed 5°C. Then 50 ml of a 2 N Na$_2$CO$_3$-solution were added and the temperature was kept between 0–5°C. The reaction mixture was then filtered off, and the solid product was dried in a desiccator. Re-crystallization three times from petroleum ether (40/65) gave white crystals, yield 65%, m.p. 130°C. – $^1$H NMR (CDCl$_3$): δ = 1.7 (s, 9H, 3CH$_3$), 1.3 (s, 9H, 3CH$_3$), 1.4 (m, 4H, CH$_2$ and NH$_2$), 2.6 (t, 2H, CH$_2$), 3.0 (br, 2H, CH$_2$), 3.4 (m, 4H, CH$_2$ and NH$_2$), 7.7 (s, 1H, NH), 7.8–7.9 (t, 1H, NH); after adding D$_2$O, bands for NH$_2$ and NH groups disappeared.

2-Butyl-4-chloro-6-terbuthyl-s-triazine-2,6-diamine (C4)

2-Butyl-4-chloro-6-terbuthyl-s-triazine-2,6-diamine was synthesized starting with di-chloro-terbuthylazine and 1,4-diaminobutane. A white solid was obtained, m.p. 106°C. – $^1$H NMR (DMSO): δ = 1.5 (s, 9H, 3CH$_3$), 1.7–1.9 (br, 4H, 2CH$_2$), 2.8–2.9 (br, 2H, CH$_2$), 3.3–3.5 (m, 4H, CH$_2$ and NH$_2$), 7.7 (s, 1H, NH), 7.8–7.9 (t, 1H, NH); after adding D$_2$O, bands for NH$_2$ and NH groups disappeared.

4-Chloro-2-hexyl-6-terbuthyl-s-triazine-2,6-diamine (C6)

4-Chloro-2-hexyl-6-terbuthyl-s-triazine-2,6-diamine was synthesized starting with di-chloro-terbuthylazine and 1,6-diaminohexane. A white solid was obtained, m.p. 89°C. – $^1$H NMR (CDCl$_3$): δ = 1.7 (s, 9H, 3CH$_3$), 1.3 (s, 9H, 3CH$_3$), 1.4 (m, 4H, CH$_2$ and NH$_2$), 2.9 (br, 2H, CH$_2$), 3.3 (t, 2H, CH$_2$), 3.4 (m, 4H, CH$_2$ and NH$_2$), 7.5 (s, 1H, NH), 7.8–7.9 (t, 1H, NH); after adding D$_2$O, bands for NH$_2$ and NH groups disappeared.

4-Chloro-2-octyl-6-terbuthyl-s-triazine-2,6-diamine (C8)

4-Chloro-2-octyl-6-terbuthyl-s-triazine-2,6-diamine was synthesized starting with di-chloro-terbuthylazine and 1,8-diaminooctane. A white solid was obtained, m.p. 76°C. – $^1$H NMR (DMSO): δ = 1.3 (s, 9H, 3CH$_3$), 1.4–1.7 (br, 12H, 6CH$_2$), 2.5–2.6 (t, 2H, CH$_2$), 3.0–3.4 (m, 4H, CH$_2$ and NH$_2$), 7.5 (s, 1H, NH), 7.8–7.9 (t, 1H, NH); after adding D$_2$O, bands for NH$_2$ and NH groups disappeared.

Assay procedure

ELISA haptens were immobilized on MTP and graphite rods using a GA network. The assay on MTPs was carried out as described previously (Holthues et al., 2001). Each well of the MTP was treated with 200 µl GA solution (6.25% v/v in 0.05 M carbonate buffer). Incubation was for 2 h at room
temperature. Graphite rods have been incubated for 4 h in a 400 µl reaction vessel, each with 200 µl GA solution, as described with the MTPs.

After washing with distilled water coating with the ELISA haptons (C2 to C8, respectively) was performed through imino coupling of the ELISA haptons to the free aldehyde groups of the GA polymer network using 200 µl coating hapten solutions prepared in PBS buffer at pH 8. Reaction was over night at room temperature.

A second coating with 250 µl ε-aminocaproic acid, 0.5 m in PBS buffer at pH 7.5, has been applied in order to block unspecific antibody or anti-antibody binding sites yet available on the GA polymer network, or even on the polystyrene or graphite carrier.

For the competition step 100 µl of a standard terbutylazine solution in PBS buffer (pH 7.5) mixed with 100 µl antibody solution in PBS buffer (pH 7.5) have been incubated for 2 h at room temperature. After a washing step 200 µl of the secondary anti-antibody GaM-POD dilution in PBS buffer (pH 7.5) + BSA (0.06% w/w) buffer were added and then incubation was for 2 h at room temperature. The final enzyme reaction was in 200 µl of the substrate solution and was stopped after 30 min by 50 µl 2 N H2SO4. The detection was by absorption at 450 nm in an ELISA reader.

Pre-incubation of the analyte and the antibody prior to incubation with the ELISA hapten for a certain time has been checked for the P6A7 antibody. For this purpose, a mixture of 100 µl of the terbutylazine standards with 100 µl of the antibody solution was allowed to pre-incubate for 1 h. The subsequent competition step with the immobilized ELISA hapten on MTP or graphite rods was for 1 h.

**Data evaluation and error considerations**

The ELISA reader results have been evaluated in the usual way and normalized plots of % B/B_0 are presented:

\[
% \frac{B}{B_0} = \frac{(A_{ex} - A)}{(A_{ex} - A_{0})},
\]

where \(A_{ex}\) and \(A_{0}\) are the absorptions of the considered analyte sample at excess of the analyte and in absence of the analyte, respectively. The so-called four-parameter curve was used to fit the sigmoidal calibration curves (Rodbard, 1974):

\[
Y = \frac{(a-d)}{(1 + (x/c)^b)} + d.
\]

Here \(Y\) is the dose response absorption, \(a\) is the response at zero dose, \(c\) is the midpoint of test, most often called IC50 value, \(d\) is the response at infinite dose of the analyte, \(x\) is the dose of the analyte, and \(b\) is the slope of the sigmoidal curve at the IC50 value.

\(R^2\) as a measure for the fit quality was in all cases larger than 0.992, mostly between 0.995 and 0.997.

All assay signals in this communication are means of four replicates in MTPs and three replicates with graphite rods. The errors reported here may be compared to the IUPAC guidelines (Krotzky and Zeeh, 1995), which recommend for ELISAs that the coefficient of variation of the IC50 value and of the limit of detection should not exceed 15% and 25%, respectively. The reproducibility of the IC50 value at the same day and under different conditions should not exceed 12.5%, and for different days not 25%.

**Results**

Effect of the antibody dilution and of the concentration of the ELISA hapten coating solution

Since the sensitivity of a competitive ELISA strongly depends on each step, careful optimization of all the reaction partners is essential. In a first step the effect of the hapten coating concentration as well as the antibody dilution were checked by measuring \(A_{0}\) and \(A_{ex}\). The relative difference of these two values should be high to ensure low errors in the calibration curve. As an example Figs. 2 and 3 show the effects of different antibody dilutions for the C6 hapten on MTP.
For the antibody 4A54 a lower dilution factor is required than for the antibody P6A7 which applies for both surfaces. Typically the antibody dilution for P6A7 was 1:20000 to 1:30000 for MTP (data not shown) and 1:15000 for graphite, for 4A54 1:5000 for MTP (data not shown) and 1:600 to 1:1000 for graphite (data not shown). The optimized hapten concentrations were 0.2 nmol/l to 0.4 nmol/l for MTP for both antibodies. In case of the graphite surface the optimized hapten concentration was 1 nmol/l for all haptens, with the antibody 4A54. In addition in the case of C6, the hapten concentration dependence was found to be very small; hapten concentrations of 0.2 nmol/l and 1 nmol/l gave the same relative difference between $A_0$ and $A_{ex}$.

**Effect of the ELISA hapten spacer length on the IC50 value**

The spacer length of the ELISA hapten may influence the IC50 values and therefore the different haptens were tested in an ELISA using the optimized conditions for both surfaces. Fig. 4 shows the calibration curves for the ELISA on MTP for the two considered antibodies 4A54 and P6A7. In both cases the IC50 values were lowest for the C2 hapten. For C2 and P6A7 the IC50 value was 10.8 µg/l, for 4A54 0.17µg/l being clearly lower than for C4, C6 and C8. For graphite as surface the results for both antibodies are summarized in Table I. For all haptens a concentration of 1 nmol/l was used. Here C2 also showed the lowest IC50 value compared to the other spacer lengths, being 0.43 µg/l for 4A54 and 23.0 µg/l for P6A7. In the case of P6A7 the IC50 value increased with increasing spacer length of the hapten. The coefficients of variation of the IC50 values which were obtained on different days were within the IUPAC guidelines.
Comparing the two antibodies 4A54 showed for all haptens and surfaces lower IC50 values than P6A7. Comparing the two surfaces, graphite based assays resulted in slightly higher IC50 values than MTP based.

**Effect of pre-incubation**

Pre-incubation was tested to improve the IC50 value. In all cases C6 was used as hapten and P6A7 as antibody. The antibody dilution was 1:30000 for MTP and 1:10000 for graphite as a surface. A hapten coating concentration of 0.2 nmol/l was applied in all cases.

For MTP the IC50 value could be improved slightly from 58 µg/l in the standard assay to 25 µg/l when using pre-incubation. However, in the case of graphite as surface pre-incubation remarkably improved the IC50 value from 52 µg/l to 8.5 µg/l.

**Discussion**

ELISAs using the indirect format with immobilization of the hapten have to be carefully optimized in regard to the hapten coating concentration and the antibody dilution, because both will influence the IC50 value. As the immobilized hapten and the free hapten, the analyte, compete for the antibody binding sites, the lower the amount of bound hapten is the less analyte can be detected. However, extremely low hapten coating concentrations result in absorbance values in the assay, which are too low to clearly discriminate between the different analyte concentrations.

High antibody dilutions are favoured, because of low values of $A_{0}$, but the $A_{ex}$ value should not decrease below 0.1.

For the two antibodies 4A54 and P6A7 the optimized dilutions are different due to their binding constants. P6A7 is highly specific for the determination of terbuthylazine (Giersch et al., 1993). An affinity constant of $4 \cdot 10^8$ l/mol can be deduced from the results reported by Piehler et al. (1997). 4A54 is highly sensitive to a series of s-triazines with a reported affinity constant of $(4.3 \pm 0.9) \cdot 10^{10}$ l/mol for terbuthylazine (Winklmair et al., 1997). Since the affinity constant of P6A7 is lower than that of 4A54, P6A7 requires lower dilution factors which holds for assays on MTP as well as on graphite. With regard to the hapten coating concentrations graphite required an up to fivefold higher concentration and as a consequence the antibody dilution was lower than on MTP for 4A54 and P6A7, respectively. Applying the optimized hapten coating concentrations and antibody dilutions the IC50 values for both surfaces were comparable, showing that graphite is a suitable surface for an ELISA with directly immobilized haptens. Terbuthylazine derivatives having different spacer lengths were used as coating haptens and the IC50 values obtained in the ELISA were compared. For both antibodies and both surfaces the assay employing C2 gave the lowest IC50 value. For P6A7 and graphite as surface the IC50 values increased from C2 via C4, C6 to C8. Since Holthues et al. (2005) have shown, that different spacer length haptens should only be compared with respect to their IC50 values, if working in the affinity limit, this finding should not be overstressed. To improve the IC50 value pre-incubation techniques are discussed in literature (Zettner and Duly, 1974; Weller, 1992). Using this sequential incubation of antibody and hapten (Holthues, 1998) could decrease the IC50 value by a factor of two, compared to an ELISA without pre-incubation, for a competitive ELISA with immobilization of atrazine derivatives to MTP. In the present report pre-incubation was checked for the antibody P6A7 and C6 as coating hapten and the IC50 value decreased by a factor of two in the case of MTP, but for graphite the IC50 value decreased markedly by nearly a factor of six.

**Conclusion**

Graphite rods as surface could be successfully applied in a competitive ELISA for terbuthylazine using direct immobilization of the hapten. After optimization of the assay parameters the IC50 values were comparable with the values obtained in ELISAs on MTP. Using the pre-incubation technique the IC50 value could be improved in particular remarkably on graphite rods. These results using graphite will open up the use of this material in the field of immunosensors with immobilized haptens.

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