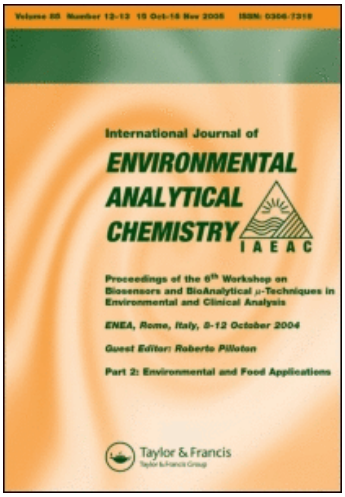

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Kinetic Determination of 2,4-Dichlorophenoxyacetic Acid by Stopped-Flow Fluorescence Polarization Immunoassay

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KINETIC DETERMINATION OF 2,4-DICHLOROPHENOXYACETIC ACID BY STOPPED-FLOW FLUORESCENCE POLARIZATION IMMUNOASSAY

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A kinetic metodology was applied to the determination of 2,4-dichlorophenoxyacetic acid (2,4-D) by using fluorescence polarization immunoassay (FPIA). The analytical parameter used was the initial rate of the antigen-antibody reaction which was obtained from the kinetic curve degree of polarization-time and measured in only 1 s. The stopped-flow mixing technique was used for this purpose which allows the application of the method to routine analysis. The dynamic range of the calibration graph is 10–1000 ng ml^{-1} and the detection limit is 4 ng ml^{-1}, lower than that afforded by the conventional FPIA. The relative standard deviation was close to 4%. The method was directly applied to the analysis of river water, grape juice and white wine and the analytical recoveries ranged from 92 to 110 %.

Keywords: 2,4-Dichlorophenoxyacetic acid; kinetic method; fluorescence polarization immunoassay; stopped-flow; environmental and food samples

INTRODUCTION

Chlorinated phenoxy acids are an important class of herbicides widely used in agriculture. They are of particular concern because of their potential toxicity to animals and humans. Among these compounds, 2,4-dichlorophenoxyacetic acid (2,4-D) is widely used for broadleaf weed control in cereal and can appear in

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environmental and food samples. As this compound is a potential cancer-causing agent [1], its determination is required when a risk of contamination of these samples exists. Thus, the availability of a simple, fast and selective method for screening or monitoring purposes is desirable.

Gas chromatography has been the most used technique for the determination of 2,4-D [2–4], although the corresponding methods require the previous extraction and derivatization of the analyte because of its low volatility and thermic unstability, which limit their use for routine screening of 2,4-D in a large number of samples. Alternative methods involving capillary electrophoresis [5,6], supported liquid membrane technique [7] and several sensors [8,9] and immunosensors [10–12] have been reported. Generally, they show good sensitivity but are complicated and time consuming. Although chromatographic methods still dominate the field of pesticide analysis, advances in immunoassay technology have allowed the development of very selective and sensitive methods for the determination of these compounds. Thus, several radio- and enzyme-immunoassay methods [13–19] have been reported for 2,4-D determination. In order to avoid radioactivity, the latter methods are usually preferred although most of them involve multiple addition and wash steps and need special instrumentation and non-stable immunoreagents.

With regard to homogeneous immunoassay, where the separation step is not required, fluorescence polarization immunoassay (FPIA) has been widely applied in clinical analysis to the determination of drugs and other small molecules [20]. The suitable features of this technique justify its application to other areas such as envirommental analysis. Thus, it has been applied to the determination of 2,4-D [21,22]. As any homogeneous immunoassay, the main limitation of FPIA when it is applied to the analysis of real samples is the relatively high detection limits obtained as a result of the background signal, which is caused partly by scattered ligth and partly by the sample matrix. However, an alternative approach to avoid or minimize this effect, which gives rise to a static signal, is to use the initial rate of the immunochemical reaction as analytical parameter instead of the signal obtained when the reaction reaches or is close to the equilibrium. Because the competitive antigen-antibody reactions are usually very fast, kinetic data can be obtained by using stopped-flow mixing technique (SF), which allows the measurements to be performed shortly after mixing the reagents and, in addition, to automatize this step of the analytical process. SF-FPIA has shown its usefulness in clinical analysis for the determination of therapeutic and abuse drugs [23,24], yielding better detection limits than conventional FPIA. This paper shows the first application of this technique to environmental and food analysis by developing a method for the determination of 2,4-D in river water, grape juice and white wine samples.

EXPERIMENTAL

Reagents

Monoclonal anti-2,4-D antibodies (lot E2/G2) were obtained in the Veterinary Research Institute (Brno, Czech Republic) as described elsewhere [14]. The IgG fraction from ascitic fluids was precipitated by half saturated aqueous ammonium sulphate solution, dialysed against phosphate saline buffer and lyophilized. The concentration of IgG was calculated by UV measurements based on the absorbance value obtained for 1 mg ml^{-1}, which is 1.3. The preparation of the fluorescein labeled 2,4-D (tracer) was made by conjugation of the pesticide with fluoresceinthiocarbamyl ethylenediamine as described earlier [25]. The concentration of tracer was determined by using $\varepsilon_{492} = 8.78 \times 10^4$ M^{-1} cm^{-1} for the fluorescein. 2,4-D and the compounds used in the cross-reactivity study were obtained from Sigma or Aldrich. The stock solution of each pesticide (1 mg ml^{-1}) was prepared in methanol. Distilled water (Millipore pure 18.2 Mom) was used to prepare more diluted solutions. Diluted antibody and tracer solutions were made in 0.1 M phosphate buffer (pH 7.4) containing 0.1 % sodium azide and 0.1% bovine γ-globalin (TDx dilution buffer, Abbott Diagnostics).

Apparatus

An SLM-Aminco (Urbana IL, USA) Model 8100 photon-counting spectrofluorimeter, equipped with a 450 W xenon arc source, two R928 photomultiplier tubes and three polarizers (Glan-Thompson calcite prism type) was used. In order to obtain polarization data, one of the polarizers was placed horizontally in the excitation lightpath and the other two in the corresponding emission channels of the T-format configuration of the instrument, placing horizontally the polarizer of the left channel (channel A) and vertically that of the right channel (channel B). The excitation wavelength was set at 490 nm with the excitation monochromator. The emission wavelength was selected by placing a Schott OG-550 filter on each of the two emission channels. The integration and resolution time selected was 0.3 and 0.5 s, respectively. The instrument was fitted with an SLM-Aminco Milliflow stopped-flow reactor which was furnished with an observation cell of 0.2 cm path length. This module was controlled by the associated electronics, the computer and a pneumatic syringe drive system. The solutions in the stopped-flow module were kept at a constant temperature of 30° C by circulating water from a thermostated tank.

Procedure

Of the two drive syringes of the stopped-flow module, one was filled with 1.1 ml of a solution containing the tracer (1 nM) and 2,4-D standard or sample solution at a final concentration between 0.9 and 90 ng ml^{-1}. The other syringe was filled with a solution containing the antibody (10 μg ml^{-1}). In each run, 0.04 ml of each solution was mixed at a flow rate of 20 ml s^{-1} in the mixing chamber. The variation of the fluorescence intensity with time in each emission arm of the T-format configuration of the instrument was monitored by placing the two polarizers perpendicular to each other. The kinetic curves thus obtained were processed by the microcomputer to record the variation of the degree of polarization (P) with time. The initial rate (V_p = dP/dt) of the immunochemical reaction was determined in 1 s by running a program for application of the reaction-rate method. Each sample was assayed in triplicated.

Determination of 2,4-D in environmental and food samples

River water, grape juice and white wine samples were spiked with appropriate amounts of 2,4-D. Pretreatment was not required when these model samples were used. Each determination was carried out by using 0.1 ml of sample, which was treated as described above.

RESULTS AND DISCUSSION

Kinetic study of the 2,4-D immunochemical system

In order to study the usefulness of SF-FPIA in environmental and food analysis and its potential advantages, compared with conventional FPIA, the kinetic behaviour of the 2,4-D immunochemical system was investigated by using the initial rate of the antigen-antibody reaction, V_p, for this purpose. This kinetic parameter was obtained from the expression of the degree of polarization, $P = [(A/B) - 1]/[(A/B) + 1]$, where A and B are the fluorescence intensities measured with the emission polarizer parallel (from channel A) and perpendicular (from channel B), respectively, to the excitation polarizer, and by using a spectrofluorimeter in a T-format configuration, which allowed the corresponding kinetic curves to be obtained simultaneously. By processing both curves by the microcomputer, the variation of P with time was easily obtained and, from it, the value of V_p. The high initial rate of this system required the use of SF, so that the analyte and the tracer were placed in one syringe and the antibody in the other.

As the main purpose of this study was to check the potential effect of the sample matrix on the initial rate, several samples were assayed (white wine, grape juice and river water) previous to carry out the optimization of the system. Figure 1 shows the kinetic curves obtained for this system in the absence and presence of a white wine sample, which was diluted eleven times. As can be seen, the initial rate can be measured in only 1 s and it is not affected by the presence of the sample, unlike the equilibrium signal, which decreased about a 10%. Similar results were obtained for the grape juice and river water samples, although the effect of the latter on the equilibrium signal was less marked than that obtained for the other two samples.

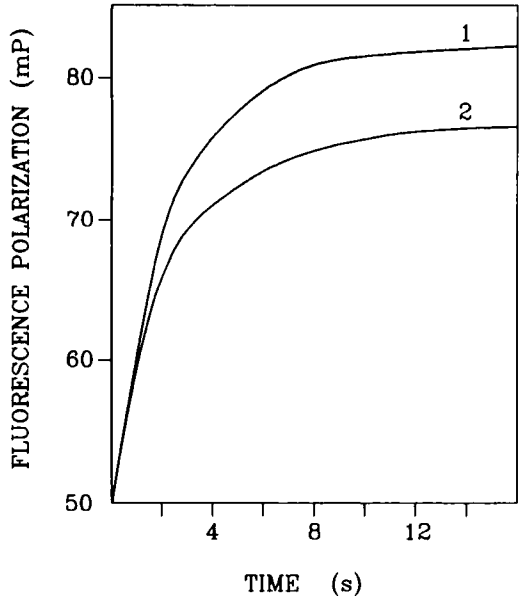

FIGURE 1 Variation of the degree of polarization with time for 10 ng ml^{-1} 2,4-D in the absence (1) and presence (2) of white wine (10% v/v). [tracer] = 1 nM, [antibody] = 10 $\mu g\ ml^{-1}$

Optimization of variables

The system was optimized by altering each variable in turn while keeping all others constant. All reported concentrations are initial concentrations in the syringes (twice the actual concentrations in the reaction mixture at time zero after mixing). Each kinetic results was the average of three measurements.

Two of the most important variables in a competitive immunoassay are the concentration of tracer and antibody. Taking into account that both variables are

interdependent and that the detection limit obtained in an immunoassay of this type decreases as the tracer concentration decreases, a sufficiently low concentration of this reagent was chosen (1 nM), which gave an adequate initial rate with a low noise in the absence of 2,4-D, in order to study the effect of adding decreasing amounts of antiserum on the initial rate of the immunochemical reaction. The results obtained were compared with those obtained by using the values of P in the equilibrium (Figure 2). As can be seen, both dilution curves are very similar as both P and V_p decrease as the antiserum dilution increases because binding of the tracer to the antibody disminishes. According to these results, a 200-fold dilution of the antibody, equivalent to 10 μg ml^{-1} of IgG was chosen.

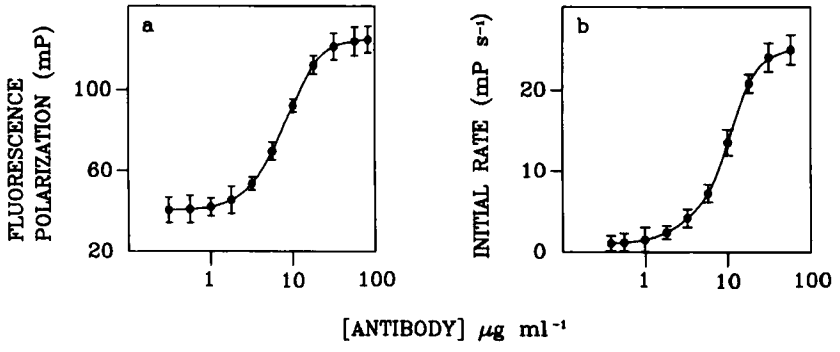

FIGURE 2 Antibody dilution curves obtained with 1 nM tracer by using equilibrium (a) and kinetic (b) measurements

The study of the effect of the temperature in the range 20–40° C showed that the initial rate of this system was constant between 25 and 35° C, but decreased at higher values. As one of the samples below analysed (white wine) contains ethanol, the potential effect of the concentration of this alcohol on the system was also studied, finding that the initial rate was independent of this variable in the range 0.1–40% ethanol.

Features of the proposed method

The kinetic curves obtained at different 2,4-D concentrations were processed by using the initial-rate method. In order to study the effect of the sample matrix on the calibration graph, this was obtained in the absence and presence (10% v/v) of white wine. Figure 3 shows both calibration graphs, where can be seen that the effect of the matrix is practically negligible. Similar results were obtained when

grape juice and river water were assayed. The dynamic range of the calibration graph, referred to the white wine sample, is 10–1000 ng ml^{-1}, the final concentrations in the syringes being 0.9–90 ng ml^{-1}. The detection limit, calculated according to IUPAC recommendations [26] and in the presence also of the white wine sample, was 4 ng ml^{-1}.

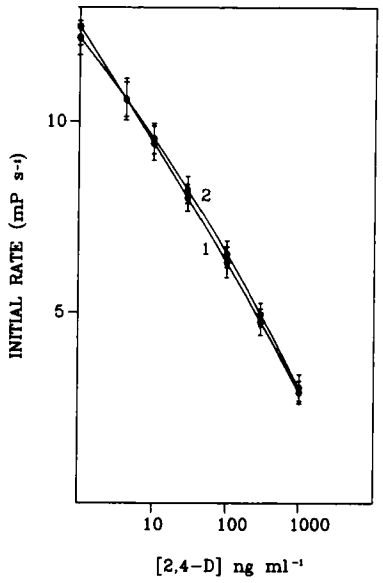

FIGURE 3 Calibration graphs obtained for 2,4-D in the absence (1) and presence (2) of white wine (10% v/v). [tracer] = 1 nM, [antibody] = 10 μg ml^{-1}

The precision of the method was also studied in the presence of the white wine diluted sample above indicated, by adding two different amounts of 2,4-D to the samples, in order to obtain concentrations of 20 and 100 ng ml^{-1}. The relative standard deviations obtained (n = 10) were 4.2 and 3.4%, respectively.

Several structurally-related substances [2-methyl-4-chlorophenoxyacetic acid,2,4,5-trichlorophenoxyacetic acid and 2-(2,4-dichlorophenoxy)propionic acid] were assayed to study the cross-reactivity of the method. The percent cross-reactivity was calculated from: %CR=([F]/[A])×100, where [F] is the 2,4-D concentration found from the calibration graph and [A] the interferent concentration added. The method was very selective at low interferent concentrations (1–100 ng ml^{-1}), but the cross-reactivity ranged from 10 to 30% at higher interferent concentrations (1–100 μg ml^{-1}), which is similar to that found

for this antibody by ELISA method [14]. Also, the potential effect of other common herbicides such as atrazine, simazine and carbaryl was studied, but all them were tolerated in a 100-fold excess relative to the analyte.

Other salient feature of this method is its high throughput, taking into account that the time needed to measure each initial-rate value is only 1 s and that the samples assayed do not require any pretreatment. Thus, hundreds of samples could, theoretically, be analysed during 1 h. However, the practical throughput is about 60 samples h^{-1}, including the time taken to perform three replicates analyses and changeover in the system.

Applications

The method was applied to the direct analysis of three samples, namely river water, grape juice and white wine. A sample volume of 0.1 ml was used in each analysis. None of these samples gave an analytical signal. Table I lists the analytical recoveries obtained by adding three different amounts of 2,4-D to each sample; these recoveries ranged from 92 to 110 %, with a mean of 103 %. The analysis of red wine samples was also assayed, but the sample matrix caused a significant effect on the initial rate of the system, which precluded the direct application of the method to the analysis of these samples.

TABLE I Analytical recoveries of 2,4-D

Sample	*Added ng ml^{-1}*	*Found* ng ml^{-1}*	*Recovery (%)*
River water	125	134 ± 5	107
	250	255 ± 10	102
	500	530 ± 20	106
Grape juice	125	119 ± 6	95
	250	260 ± 20	104
	500	460 ± 20	92
White wine	125	137 ± 5	110
	250	263 ± 8	105
	500	540 ± 20	108

* Average of three determinations ± standard deviation

CONCLUSIONS

The interest in the determination of 2,4-D is evident taking into account the numerous methods described for this pesticide in the last few years. Among

these methods, those based on the use of immunochemical reactions, involving enzymatic reagents, play a leading role owing to their high selectivity and low detection limits, which reach values of few pg ml^{-1}. However, the practical utility of some of these methods has not been shown as data from the analysis of real samples were not reported [10,18,19]. As known, in some instances, substances from the sample matrix may bind nonspecifically to the antibody in such a way as to prevent analyte binding, which will affect to the detection limit of the immunoassay. Also, the incubation and separation steps required in most of these immunoassay methods are time-consuming and make the automation difficult. On the contrary, the SF-FPIA method reported here reduces reactant manipulations and shows a high sample throughput, as the measurement step takes only 1 s, so that it is a useful alternative to the automatic rutine determination of 2,4-D.

The conventional FPIA method reported for 2,4-D [21,22] showed a linear range of 1–200 μg ml^{-1}, so that its lower concentration corresponds to the upper concentration of the calibration graph of the SF-FPIA method. With regard to the detection limit, the value obtained by conventional FPIA was 100 ng ml^{-1}, which is 25-times higher than that obtained by SF-FPIA. The improvement attained by the kinetic methodology could be ascribed to the fact that the dynamic measurement obtained at the beginning of the reaction between the tracer and the antibody avoids or minimizes the background signal and the potential interferences from the sample matrix. Finally, while the conventional FPIA method was only applied to the analysis of tap water samples, which have a very simple matrix, the SF-FPIA method has shown its utility for the direct analysis of samples which have a more complex matrix.

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References

[1] S.K. Hoar, A. Blair, F.F. Holmes, C.D. Boysen, R.J. Hoover and J.F. Fraumeni, *J. Am. Med. Assoc.* **256**, 1141–1147 (1986).

[2] S. Butz, Th. Heberer and H.-J. Stan, *J. Chromatogr. A*, **677**, 63–74 (1994).

[3] H.B. Lee, T.E. Peart, J.M. Carron and H. Tse, *J. Assoc. Off. Anal. Chem.*, **74**, 835–842 (1991).

[4] R.E. Cline, G.D. Todd, D.L. Ashley, J.Grainger, J.M. McCraw, C.C. Alley and R.H. Hill, *J. Chromatogr. Sci.*, **28**, 167–172 (1990).

[5] Y. Mechref and Z. El Rassi, *Anal. Chem.*, **68**, 1771–1777 (1996).

[6] M. Jung and W.C. Brumley, *J. Chromatogr. A*, **717**, 299–308 (1995).

[7] M. Knutsson, G. Nilve, L. Mathiasson and J.-A. Jonsson, *J. Agric. Food Chem.*, **40,** 2413–2417 (1992).
[8] A.N. Reshetilov, O.V. Fedoseeva, T.P. Eliseeva, Yu.V. Sergeev, E.P. Medyantseva and G.K. Budnikov, *J. Anal. Chem.*, **50,** 414–417 (1995).
[9] E.P. Medyantseva, M.G. Vertlib, G.K. Budnikov, S.S. Babkina and S.A. Eremin, *J. Anal. Chem.*, **50,** 719–722 (1995).
[10] T. Kalab and P. Skladal, *Anal. Chim. Acta,* **304,** 361–368 (1995).
[11] M.F. Yulaev, R.A. Sitdykov, N.M. Dmitrieva, B.B. Dzantiev, A.V. Zherdev and K.A. Askarov, *J. Anal. Chem.*, **50,** 194–197 (1995).
[12] S.M. Khomutov, A.V. Zherdev, B.B. Dzantiev and A. N. Reshetilov, *Anal. Lett.*, **27,** 2983–2995 (1994).
[13] J.C. Hall, R.J.A. Deschamps and K.K. Krieg, *J. Agric. Food Chem.*, **37,** 981–984 (1989).
[14] M. Franek, V. Kolar, M. Granatova and Z. Nevorankova, *J. Agric. Food Chem.*, **42,** 1369–1371 (1994).
[15] G. Matuszczyk, D. Knopp and R. Niessner, *Fresenius J. Anal. Chem.*, **354,** 41–47 (1996).
[16] C. Wittmann, F.F. Bier, S.A. Eremin and R.D. Schmid, *J. Agric. Food Chem.*, **44,** 343–350 (1996).
[17] B.B. Dzantiev, A.V. Zherdev, M.F. Yulaev, R.A. Sitdikov, N.M. Dmitrieva and I.Yu. Moreva, *Biosens. Bioelectron.*, **11,** 179–185 (1996).
[18] F.F. Bier, E. Ehrentreich-Forster, C.g. Bauer and F.W. Scheller, *Fresenius J. Anal. Chem.*, **354,** 861–865 (1996).
[19] A. Dzgoev, M. Mecklenburg, P.-O. Larsson and B. Danielsson, *Anal. Chem.*, **68,** 3364–3369 (1996).
[20] M.C. Gutiérrez, A. Gómez-Hens and D. Pérez-Bendito, *Talanta,* **36,** 1187–1201, 1989.
[21] Yu. V. Lukin, I.M. Dokuchaev, I.M. Polyak and S.A. Eremin, *Anal. Lett.*, **27,** 2973–2982 (1994).
[22] S.A. Eremin, in "*Immunoanalysis of Agrochemicals: Emerging Technologies*" ACS Symposium Series No. 586. (J.O. Nelson, A.E. Karu and R. Wong, eds., American Chemical Society, Washington DC, 1995), p. 223.
[23] A. Gaikwad, A. Gómez-Hens and D. Pérez-Bendito, *Anal. Chim. Acta,* **280,** 129–135 (1993).
[24] D. Pérez-Bendito, A. Gómez-Hens and A. Gaikwad, *Clin. Chem.*, **40,** 1489–1493 (1994).
[25] S.A. Eremin, I.M. Lunskaya and A.M. Egorov, *Bioorganicheskaya Khimica (in Russsian)* **19,** 836–843 (1993).
[26] G.L. Long and J.D. Winefordner, *Anal. Chem.*, **55,** 712A-724A (1983).