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Competitive Enzyme-linked Immunosorbent Assay for the Determination of the Phenylurea Herbicide Chlortoluron in Water and Biological Fluids

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A competitive ELISA method suitable for the monitoring of the herbicide chlortoluron [*N*-(3-chloro-4-methylphenyl)-*N'*-dimethylurea] in different types of water and biological fluids was developed. The production of the immunogen utilized in this work was achieved by covalently coupling bovine thyroglobulin with the synthesized hapten (*N'*-3-chloro-4-methylphenyl-*N*-carboxypropyl urea) using the *N*-hydroxysuccinimide active ester method. The chlortoluron antibody, raised in sheep after immunization with the immunogen, showed no cross-reactivity with a large range of pesticides, although some cross-reactivity was displayed with various phenylurea herbicides (*i.e.*, chlorbromuron, isoproturon and metoxuron). The limit of detection of the chlortoluron ELISA method was 0.015 μg l^{-1}, well below the legal European limit for individual pesticides in drinking water (the EC maximum admissible concentration, 0.1 μg l^{-1}). In addition, reproducible and quantitative recovery of chlortoluron from water, obtained from various sources, and biological fluids was possible without any sample preparation. The ELISA technique for chlortoluron developed and described here proved to be rapid, sensitive and specific, fulfilling the needs of present legislation relating to the use and levels of pesticides in the environment.

Keywords: *Chlortoluron; antiserum production; hapten; urea herbicide; maximum admissible concentration*

Introduction

Pesticide residue analysis has been a fundamental part of safe and effective product development, but there is now additional pressure to provide more information about the fate of pesticides in the environment. The presence of pesticide residues in water, soil and food is an escalating problem that has aroused public concern over potential health hazards. In 1985, the EEC issued a Drinking Water Directive, No. 80/778/EEC,[1] setting standards for the quality of water for human consumption, irrespective of the source. In this Directive, pesticides are considered as a single group, with a maximum admissible concentration (MAC) set at 0.1 μg l^{-1} for any individual substance in the group, and 0.5 μg l^{-1} for total pesticides and related products. As a result, frequent monitoring for the presence of pesticides in food, drinking water, *etc.*, is required in order to meet the needs of present legislation relating to the use and levels of pesticides.

Chlortoluron (Fig. 1) is used extensively in the control of the four types of wild oats and black grass in winter barley. Currently, the presence of urea herbicides in water supplies is an emerging problem of concern to the water industry. Indeed, results available from the UK Department of the Environment in 1988 illustrate that chlortoluron as well as other commonly used herbicides (*i.e.*, isoproturon, triazine and phenoxyalkanoic acids) were present at levels exceeding the MAC.[2] The traditional and approved techniques for measuring chlortoluron, *i.e.*, chromatographic methods, involve extensive sample preparation using solvent or solid-phase extraction procedures, which adds to assay costs and reduces the number of samples that can be handled at one time.[3–6] Thus, the use of chromatographic techniques for pesticide residue analysis is not entirely suited to the task of rapid and frequent monitoring of a large numbers of samples.

Immunochemical techniques based on the use of specific antibodies provide a convenient, sensitive, cost-effective option for the purpose of monitoring pesticides in different matrices. A number of reviews on immunochemical analysis of pesticides have been published,[7–11] which illustrated the advantages of immunological methods for pesticide analysis as supplemental methods for screening and confirmatory assays. Different forms of ELISA, which are now widely available, have been described and one of the most common enzyme systems is horseradish peroxidase with a colorimetric end-point.[12]

An enhanced chemiluminescent immunoassay for chlortoluron published recently[11] showed the chemical synthesis of the chlortoluron immunogen and the procedure for raising the chlortoluron antiserum in sheep. This method was essential for the development of a portable photographic detection system using a camera luminometer. However, costly reagents and equipment are required for the detection of light quantitatively. This paper describes a direct competitive ELISA method for detecting chlortoluron in various matrices, offering a highly sensitive and inexpensive method of analysis which complements the existing techniques. Furthermore, a visual qualitative measurement can be achieved. It also facilitates the simple and rapid performance of assays in the laboratory as a means of screening water samples for the presence of chlortoluron.

Experimental

Materials and Equipment

Unless stated otherwise, all chemicals were of AnalaR grade from Merck (Poole, Dorset, UK). Barbital solution (0.07

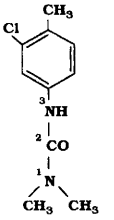

Fig. 1 Chlortoluron chemical structure.

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mol l^{-1}), pH 9.6 (14.4 g l^{-1} sodium barbitone), was used for coating the antibodies onto the solid support. Phosphate-buffered saline (PBS), pH 7.4 [0.15 mol l^{-1}, containing 0.05% v/v of Tween 20 (PBST)] (Sigma, Poole, Dorset, UK), was used as the washing buffer for the assay. Horseradish peroxidase (HRPO) was purchased from Biozyme (Blaenavon, Gwent, UK). A stock standard solution (1 mg ml^{-1}) of chlortoluron in methanol was prepared from stock powder (Greyhound Chemicals, Birkenhead, Merseyside, UK) and stored at 4 °C. A ^{14}C chlortoluron label, [phenyl-(u)-^{14}C]-chlortoluron: 637.5 MBq $mmol^{-1}$, was supplied by Ciba-Geigy (Cambridge, UK). Dextran-coated charcoal was purchased from Pharmacia (Milton Keynes, Buckinghamshire, UK). Nunc-Immuno microtitre plates, Maxisorp F96 grade 1, were supplied by Gibco Europe (Uxbridge, Middlesex, UK).

N-*(3-Chloro-4-methylphenyl)*-N-*methyl*-N-*carboxypropyl Urea (Fig.* 2, III)

The first stage of the synthesis, involving the formation of 3-chloro-4-methylphenyl isocyanate (**II**), was carried out with triphosgene [bis(trichloromethyl)carbonate] as a convenient and less hazardous replacement for the monomer. Triphosgene (0.85 g, 2.865 mmol) was weighed into a two-neck flat-bottomed flash (150 ml) fitted with a reflux condenser and an addition funnel mounted on a suitable hot-plate stirrer. Dry toluene (6 ml) was added and the solution stirred using a magnetic stirrer. A solution of 3-chloro-4-methylamine (**I**) (1.14 g, 8.4 mmol) in dry toluene (7 ml) was added dropwise and the funnel was washed out with dry toluene (5 ml). The mixture was refluxed gently and all solids slowly dissolved with the evolution of HCl gas ($CaCl_2$ guard tube). Heating was continued for 2 h and the flask and contents were left overnight at room temperature.[11,13]

For the second stage, the intermediate isocyanate solution (**II**) was evaporated to remove toluene and the viscous residue was added dropwise to a stirred solution of *N*-methylaminobutyric acid hydrochloride (1.3 g, 8.5 mmol) and sodium hydroxide (0.74 g, 18.5 mmol) in water (10 ml) in a 50 ml conical flask. The solution was stirred for 2 h at room temperature at which time the pH was 11. A fine precipitate was formed and a small amount of this was filtered off and washed with water (7 ml). The filtrate was acidified with 2 mol l^{-1} HCl to pH 3 and the resulting precipitate was filtered on a Whatman No. 50 filter-paper (Büchner funnel) and washed with water until the filtrate was at pH 5. The product was dried *in vacuo* over $CaCl_2$ to 725 mg. Examination by thin-layer chromatography [ethyl acetate–hexane (4 + 1)] showed one major spot only. Fig. 2 illustrates the presumed chemical reactions for synthesizing the hapten (**III**).

Antiserum Production

The chlortoluron antiserum was raised in two mature Suffolk sheep after immunization with an immunogen consisting of *N'*-3-chloro-4-methylphenyl-*N*-methyl-*N*-carboxypropyl urea (**III**) conjugated to bovine thyroglobulin. The conjugation method was *via* the *N*-hydroxysuccinimide active ester, which links carboxyl groups on the hapten to amino residues on the carrier protein.[11,13] The immunization procedure was similar to that reported earlier.[11,13] A radioimmunoassay (RIA) procedure using a ^{14}C chlortoluron radiolabel and dextran-coated charcoal was utilized to screen the antiserum for the presence of specific antibodies. The chosen bleed was subsequently partially purified using diethylaminoethyl (DEAE) cellulose ion exchanger as previously described.[11,12] The purified antiserum was stored at 4 °C with 0.1% thimerosal solution added as preservative.

Preparation of Peroxidase–Chlortoluron Conjugate

The chlortoluron hapten was also conjugated to HRPO by the *N*-hydroxysuccinimide active ester technique. Various molar ratios of HRPO : hapten were used. Subsequently, the conjugates were dialysed for 48 h against PBS buffer of pH 7.4 and the enzyme solutions were then stored at 4 °C after the addition of 0.1% thimerosal solution.[11]

Direct Competitive Chlortoluron ELISA Final Protocol

A microtitre plate was coated with 200 µl per well (0.17 µg ml^{-1}) of partially purified chlortoluron antibody diluted in 0.07 mol l^{-1} sodium barbitone buffer of pH 9.6 (1 + 7999 dilution). The plate was incubated overnight at 4 °C in a moist chamber and washed three times with PBST on the next day. Standard solutions (100 µl per well) of chlortoluron in the range 0–100 µg l^{-1}, made up in tap water, were applied to the plate followed by the addition of 100 µl per well of HRPO-labelled chlortoluron at a 1 + 15 999 dilution (0.13 µg ml^{-1} peroxidase), diluted in PBS buffer containing 2.5% normal sheep serum, to bring the total volume to 200 µl per well. The plate was incubated in a shaker incubator (Enfer, Derby, UK) for 1 h at 37 °C. After incubation, the plate was washed four times with tap water and 200 µl per well of TMB chromagen-substrate[7] were placed in all the wells. Finally, after a further 30 min incubation in the shaker, the reaction was stopped by the addition of 1 mol l^{-1} HCl (50 µl per well) and the colour was read using a Multiskan plate reader (Labsystem, Basingstoke, Hampshire, UK) at 450 nm. Calibration graphs were evaluated off-line using a four-parameter logistic plot (RiaCalc, Wallac, Milton Keynes, Buckinghamshire, UK).

Results

A positive immune response was observed in both sheep immunized with the chlortoluron immunogen. Although sheep 1 had a higher concentration of antibody during the four booster doses, sheep 2 reacted very positively to the fifth booster injection, producing a high titre antiserum. Thus, the third bleed after five boosts (*i.e.*, 91 weeks following the initial immunization) was chosen from sheep 2 for immunoglobulin purification and ultimately to establish the assay procedure. The results of the immune response are shown in Fig. 3. Titre is the dilution of the antiserum which binds 50% of the immunoreactive radiolabel.

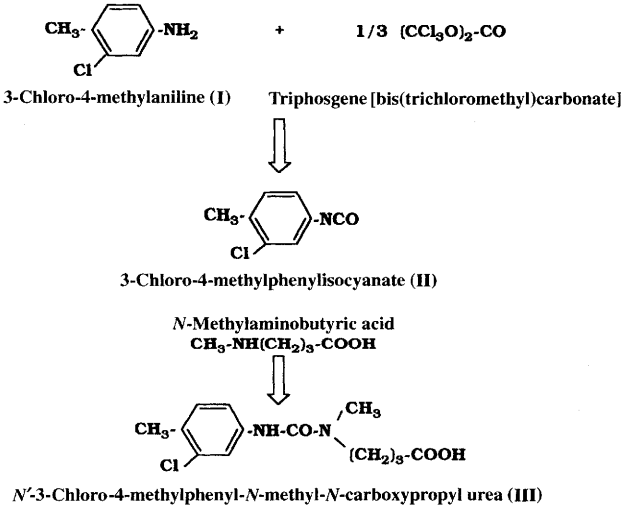

Fig. 2 Chemical synthesis of the chlortoluron hapten.

Optimization of Assay Conditions

The development and performance of an immunoassay is dependent on the availability of two key reagents: the antiserum with the desired specificity and avidity towards the analyte and an immunoreactive labelled form of that analyte.[14]

Coating antibody

Four different dilutions of purified chlortoluron antibody solution (1 + 999, 1 + 1999, 1 + 3999 and 1 + 7999), made up with sodium barbitone buffer of pH 9.6, were applied to a microtitre plate containing appropriate dilutions of standard. Chlortoluron enzyme label at a 1 + 999 dilution with a 1 : 20 molar ratio of 'peroxidase : hapten' was then added. Fig. 4 shows that the 1 + 7999 dilution (0.17 μg l^{-1}) of the chlortoluron coating antibody yielded the steepest calibration graph with a high B_0 reading (the binding measured in the absence of chlortoluron).

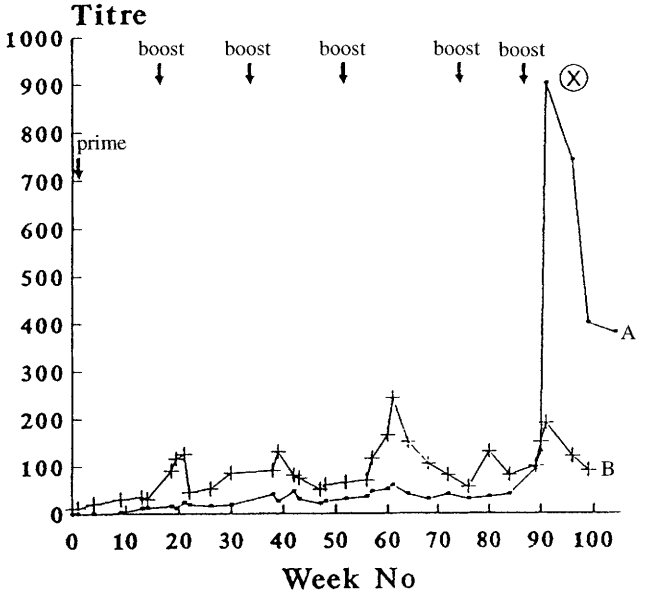

Fig. 3 Immunization chart of chlortoluron in two sheep (sheep 1, A, and sheep 2, B). The antiserum was collected from sheep 2 after 91 weeks of the primary injection. ⊗: Fifth boost: third bleed (V : C).

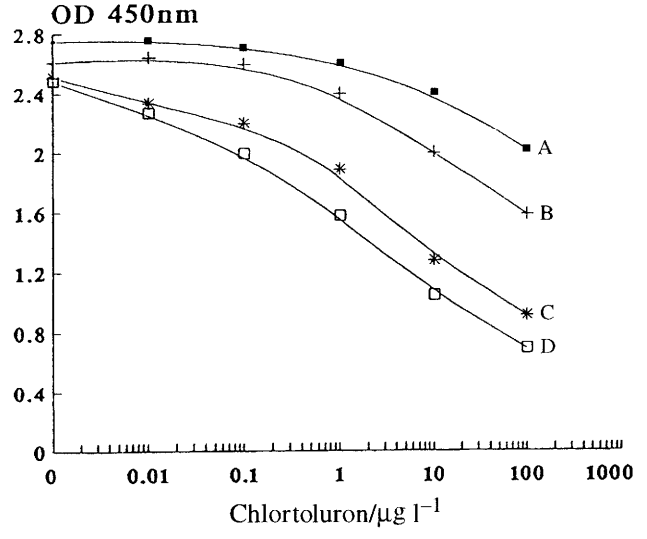

Fig. 4 Optimization of the chlortoluron coating antibody dilutions for the calibration graph (A, 1 + 999; B, 1 + 1999; C, 1 + 3999; and D, 1 + 7999). HRPO label was used at a dilution of 1 + 999 with a 1 : 20 molar ratio.

Enzyme label

Several dilutions of chlortoluron enzyme label (1 + 999, 1 + 1999, 1 + 3999, 1 + 7999 and 1 + 15 999) from each conjugate prepared previously (1 : 5, 1 : 10, 1 : 20 and 1 : 40 'peroxidase : hapten') were used in the assay for calibration graphs constructed on one plate coated with the optimum dilution of the coating antibody (1 + 7999). The addition of a 1 + 15 999 dilution (0.13 μg ml^{-1} peroxidase) of the chlortoluron enzyme label (1 : 20 molar ratio) to the chlortoluron assay produced a calibration graph with the steepest slope (Fig. 5).

The effects of temperature, incubation time and different types of microtitre plates on the interaction between antibody and antigen were investigated. As a result, the irradiated polystyrene Immuno-2-microtitre plates incubated overnight at 4 °C (16 h) showed high absorbance readings accompanied by a low relative standard deviation. Also, the incubation of the related label and unlabelled chlortoluron, for 1 h while the plate was shaken at 37 °C, produced a calibration graph with high absorbance and high sensitivity (*i.e.*, steepest slope). Consequently, the conditions optimized above were utilized for further assay validation.

Assay Validation

Cross-reactivity

The specificity of the chlortoluron antiserum was assessed by deriving cross-reactivity curves for different types of herbicide compounds. A series of calibration graphs were constructed whereby one consisted of decreasing concentrations of chlortoluron (0–100 μg l^{-1}, six standards) whilst the others were composed of greater concentrations of compounds (0–10 000 μg l^{-1}, six standards) which might possibly cross-react. The optimized concentrations of chlortoluron label and antiserum were added to the plates.

Percentage cross-reactivity was calculated from the masses of chlortoluron and cross-reactants required to reduce the binding at zero standard concentration by 50%. Table 1 demonstrates the degree of cross-reactivity of the chlortoluron antiserum with various pesticides. As expected, structurally unrelated compounds, *e.g.*, triazines and phenoxyacetic acids, did not show any significant cross-reactivity, but some

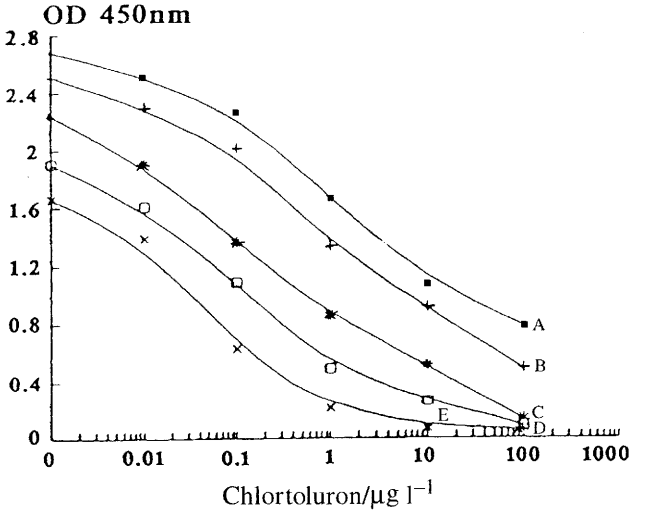

Fig. 5 Optimization of the chlortoluron–HRPO label for the calibration graph (A, 1 + 999; B, 1 + 1999; C, 1 + 3999; D, 1 + 7999; E, 1 + 15 999). Molar ratio: 1 : 20. The coating antibody dilution was 1 + 7999.

structurally related phenylurea herbicides were recognized by the antibodies.

Intra-assay variation

The variation between chlortoluron calibration graphs (0–100 μg l^{-1}, eight standards) obtained on one microtitre plate was determined using the final protocol procedure. The intra-assay variation ranged from 2.7 to 6.5% across the calibration graph compared with the inter-assay variation (variation between six plates using six calibration graphs), which ranged from 6.9 to 12.4%, as shown in Table 2. The assay limit of detection was 0.015 μg l^{-1}, defined as the concentration equivalent to a three standard deviation fall from binding at B_0, well below the MAC (0.1 μg l^{-1}) for a single pesticide in drinking water.

Recovery of chlortoluron from different water samples

The accuracy of measurement can be assessed by determining the recovery of analyte in authentic samples fortified with a known amount of analyte. Water samples obtained from various sources (river water, lake water and tap water) were fortified with different concentrations of chlortoluron stock solution (1 μg l^{-1}) and then measured using the ELISA. Table 3 shows that the recovery of chlortoluron from different water matrices ranged between 78 and 127%.

Recovery of chlortoluron from biological fluids

Six chlortoluron standard solutions in the range 0–100 μg l^{-1}, made up in normal human plasma and urine and stored at 4 °C, were used to assess the intra- and inter-assay variation over a period of 10 d. In addition, plasma and urine samples, spiked with different concentrations of chlortoluron standard (0.1, 0.5 and 1 μg l^{-1}) and stored at 4 °C, were measured with each assay calibration graph. Tables 4 and 5 demonstrate the intra- and inter-assay variation of chlortoluron in plasma and urine, respectively, across the calibration graphs displaying also the percentage recovery of chlortoluron from plasma and urine samples.

Discussion

The ELISA technique developed and described here for the measurement of chlortoluron proved to be sensitive and specific, fulfilling the needs of present legislation relating to the use and levels of pesticides in the environment. Chlortoluron is commonly used as a herbicide which effectively controls a wide range of grasses. So far, no simple and sensitive methods are available for detecting this phenylurea herbicide at low concentrations (MAC 0.1 μg l^{-1}). The assay was based on a direct competition between the enzyme-labelled and unlabelled form of chlortoluron, offering a speedy system for monitoring the presence of pesticides. Also, sample pre-treatment was not necessary and only small sample volumes were required for each assay, resulting in a highly beneficial technique for environmental analysis.

Table 1 Specificity of the chlortoluron antiserum towards a selection of pesticides

Compound	Cross-reaction (%)
Chlortoluron	100
Chlorbromuron	71
Isoproturon	47
Metoxuron	8.8
Metsulfuron	3.45
Chlorsulfuron	1.3
Metamitron	<0.001
2,4-D	<0.001
(4-Chloro-2-methylphenoxy)acetic acid	<0.001
Atrazine	<0.001
Simazine	<0.001
Mecoprop	<0.001
Propyzamide	<0.001
Paraquat dichloride	<0.001
Terbutryn	<0.001
(4-Chloro-2-methylphenoxy)butyric acid	<0.001

Table 3 Percentage recovery of chlortoluron from various water samples previously showing no immunoreactivity

Chlortoluron added/μg l^{-1}	Amount determined/μg l^{-1}	RSD (%) (n = 6)	Recovery (%)
Tap water (*day* 1):			
0.2	0.162	4.3	81
0.8	0.691	5.7	86
Tap water (*day* 2):			
0.2	0.209	11.2	105
0.8	0.703	8.2	88
Tap water (*day* 3):			
0.2	0.156	7.8	78
0.8	0.810	6.7	101
River I:			
0.2	0.223	9.3	112
0.8	1.012	6.4	127
River II:			
0.2	0.194	7.9	97
0.8	0.878	5.3	110
Lake I:			
0.2	0.207	4.8	104
0.8	0.874	5.1	109
Lake II:			
0.2	0.223	6.5	112
0.8	0.821	8.4	103
Lake III:			
0.2	0.177	4.4	89
0.8	0.787	7.3	98

Table 2 Precision of the chlortoluron ELISA within- and between-assays (mean of six calibration graphs)

Chlortoluron standard/ μg l^{-1}	Within-assay variation		Between-assay variation	
	Mean B/B_0 (%) (± s)	RSD (%)	Mean B/B_0 (%) (± s)	RSD (%)
100	5 (± 0.20)	5	7 (± 0.80)	11.4
10	6 (± 0.16)	2.7	9 (± 1.09)	12.1
1	11 (± 0.70)	6.4	12 (± 0.96)	8
0.5	15 (± 0.80)	5.3	16 (± 1.10)	6.9
0.1	19 (± 1.04)	5.5	21 (± 1.80)	8.6
0.05	41 (± 1.60)	3.9	47 (± 5.10)	10.8
0.01	63 (± 4.10)	6.5	66 (± 8.20)	12.4
0	100 (± 4.90)	4.9	100 (± 9.30)	9.3

The antiserum was characterized for specificity towards closely related and unrelated pesticides.[15] Although the cross-reactivity of all urea herbicides has not been determined, the antibody displayed significant percentage cross-reaction with some phenylurea herbicides (*i.e.*, chlorbromuron, isoproturon and metoxuron). This lack of specificity towards related phenylureas could be used to an advantage. In contrast to many other applications of immunoassays where absolute specificity for a single compound is required, the antibody can be utilized for screening purposes for phenylurea herbicides in general. Such use of immunoassays using non-specific antisera has previously been exploited in forensic toxicology, *e.g.*, where a negative result excludes a range of compounds from further investigation while a positive result indicates the class of compound present and gives an approximate estimate of the amount, and thus simplifies the choice of method for confirming the immunoassay result.[16] Consequently, samples contaminated with pesticides could be screened using the chlortoluron antibody for the presence of phenylurea herbicides.

The assay exhibited a limit of detection of 0.015 μg l^{-1}, well below the EC MAC for a single pesticide in drinking water. Furthermore, reproducible and quantitative recovery of chlortoluron was achieved from various spiked samples (*i.e.*, different water sources, biological fluids) without extensive sample preparation, *e.g.*, solvent or solid-phase extraction procedures, as required by the GC and HPLC methods. The present assay is therefore accurate, adaptable and suitable for applications such as screening programmes and also offers a convenient diagnostic aid in the case of accidental poisoning and provides an opportunity to carry out occupational monitoring (*i.e.*, in workers).

Although the potential use of immunoassays for pesticide residue analysis has not yet been fully realized, this assay may complement, not replace, the traditional methodology. The type of assay used will depend on the concentration and matrix of the analyte and the specific analytical requirements. In conclusion, the work described here has demonstrated the analytical versatility of antibodies and thus exemplified how further work will ensure an important place for immunoassays in environmental monitoring of pesticides as well as other potentially toxic compounds.

Table 4 Intra- and inter-assay variation of chlortoluron calibration graphs in plasma and the recovery of chlortoluron from spiked plasma samples

	Within-assay variation		Between-assay variation	
Chlortoluron standard/ μg l^{-1}	Mean B/B_0 (%) (± *s*)	RSD (%) (*n* = 6)	Mean B/B_0 (%) (± *s*)	RSD (%) (*n* = 6)
100	4 (± 0.17)	4.3	6 (± 0.5)	8.3
10	8 (± 0.54)	6.8	10 (± 1.1)	11
1	13 (± 0.72)	5.5	20 (± 2.5)	12.5
0.1	20 (± 0.98)	4.9	28 (± 4.1)	14.6
0.01	59 (± 2.60)	4.4	64 (± 7.3)	11.4
0	100 (± 5.10)	5.1	100 (± 8.9)	8.9
Chlortoluron added/μg l^{-1}	Amount determined, mean ± *s*/μg l^{-1}		RSD (%) (*n* = 5)	Recovery (%)
Plasma:				
0.1	0.113 ± 0.01		8.8	113
0.5	0.537 ± 0.06		11.2	107
1	0.945 ± 0.08		8.5	95

Table 5 Intra- and inter-assay variation of chlortoluron calibration graphs in urine and the recovery of chlortoluron from spiked plasma samples

	Within-assay variation		Between-assay variation	
Chlortoluron standard/ μg l^{-1}	Mean B/B_0 (%) (± *s*)	RSD (%) (*n* = 6)	Mean B/B_0 (%) (± *s*)	RSD (%) (*n* = 6)
100	5 (± 0.13)	2.6	4 (± 0.42)	10.5
10	11 (± 0.60)	5.5	12 (± 1.60)	13.3
1	15 (± 1.00)	6.6	18 (± 2.60)	14.4
0.1	24 (± 1.10)	4.6	30 (± 3.90)	13.0
0.01	66 (± 3.30)	5.0	62 (± 5.80)	9.4
0	100 (± 4.90)	4.9	100 (± 11.10)	11.1
Chlortoluron added/μg l^{-1}	Amount determined, mean ± *s*/μg l^{-1}		RSD (%) (*n* = 5)	Recovery (%)
Plasma:				
0.1	0.870 ± 0.097		11.1	87
0.5	0.521 ± 0.050		9.6	104
1	0.934 ± 0.100		10.7	93

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