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Quantification of the Urea Herbicide, Diuron, in Water by Enzyme Immunoassay

N. Lee,[1] J. H. Skerritt,[2] M. Thomas,[3] W. Korth,[3] K. H. Bowmer,[3] K. A. Larkin,[4] B. S. Ferguson[4]

[1]CSIRO Division of Plant Industry, PO Box 7, North Ryde, NSW 2113, Australia
[2]CSIRO Division of Plant Industry, GPO Box 1600, Canberra, ACT 2601, Australia
[3]CSIRO Division of Water Resources, PMB 3, Griffith, NSW 2680, Australia
[4]ImmunoSystems Inc, a subsidiary of Millipore Corporation, 4 Washington Ave, Scarborough, Maine 04074, USA

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Urea herbicides are widely applied to soil for total vegetation control, or for pre- or post-emergence treatment in crops. Uses include pre-plant treatment of fields in cotton, sugar cane, and vegetables; removal of annual weeds in citrus orchards; and for treatment of irrigation canal, road, and railroad rights of way. Several of the compounds are highly persistent in the environment; this can lead to damage to crops if contaminated water is used for irrigation and increase the possibility of unacceptable residues in drinking water. Diuron and related urea herbicides have rather simple chemical structures, being characterised by a substituted aryl moiety on one of the nitrogen atoms of urea, while two methyl groups are attached to the other urea nitrogen. Diuron has been one of the major urea herbicides in use since the 1950s. After simazine, it is the second most persistent herbicide in common agricultural practice (Hassall 1990). Its persistence in soil is due to a combination of three properties - chemical stability, low aqueous solubility (El-Dib and Aly 1976) and strong adsorption to soil (Kozak and Weber 1983; Alva and Singh 1990). Urea herbicides can be difficult to detect instrumentally at low part-per-billion levels. HPLC of water extracts following concentration can typically detect down to only 0.5-1 ppb in water. While more sensitive gas-liquid chromatography (GC) methods have been developed, the low volatility of these compounds can lead to decomposition by use of high column temperatures before separation (Bowmer and Adeney 1978; Peterson and Batley 1991). A number of ELISAs for phenylurea herbicides, differing in the sensitivity and specificity for diuron, have been developed. These used either rabbit antisera (Newsome and Collins 1990, Liegeois et al. 1992; Schneider et al. 1994) or mouse monoclonal antibodies (Karu et al. 1994a). With the exception of analyses of groundwater samples using the monoclonal antibody assay (Karu et al. 1994b) these antibodies have only had limited application to water. We describe here an immunoassay for diuron based on use of sheep polyclonal antibodies to chlortoluron (Aherne 1991), and report the specificity and sensitivity properties of this assay and its application to detection of diuron in surface and sub-surface water.

MATERIALS AND METHODS

Two types of water samples were studied:

1. Surface water samples collected from November 1991 to April 1992 from the Griffith area of SW New South Wales, Australia. They comprised water from canals draining an irrigated agricultural system consisting of rice, citrus, vegetable and vines. Water samples were buffered by addition of 10 mL/L 5 M sodium phosphate (pH 6.9 upon dilution) and stored at 4° C in amber glass bottles until analysis (up to 2 weeks). Buffering was not performed if samples were frozen or analysed immediately.

Correspondence to: J. H. Skerritt

2. Subsurface drainage water collected in May 1992 from a series of 49 tile drains below irrigated crops (in which urea herbicides and other agrochemicals were used) within the Murrumbidgee Irrigation Areas of NSW, Australia. A second set of samples was obtained from the drains in September 1992. All samples were stored unbuffered at -20° until analysis.

Subsurface water samples were analysed by gas chromatography - mass spectrometry (GC/MS) on a Varian 3300 chromatograph (Varian, Walnut Creek, CA, USA) with a model 5970 Mass Selective Detector (Hewlett Packard, Palo Alto, CA, USA; operated in the single ion monitoring mode), using a 5% phenylmethylsilicone column (25 m long, 0.2 mm diameter, Hewlett Packard). For these analyses, 500-mL water samples were extracted using three extractions with 50-mL dichloromethane in a separatory funnel; the extracts were combined and concentrated to 0.5 mL using a Turbo Vap. One μL was directly injected using a splitless injection (splitter off 1 min) and an injector temperature of 200° C. The column temperature was ramped from 50° C (isocratic 1 min) to 160° C at 20° C/min; the temperature was held at 160° for 4 min and then ramped at 5° C/min to a final temperature of 250° C. Identification and detection of diuron was based on a breakdown product and not on the parent compound. Diuron was quantified using fenchlorphos as an internal standard only when the absence of linuron in the same standard was confirmed by HPLC. This was necessary because both diuron and linuron thermally degrade in the injection port of the gas chromatograph to form an identical breakdown product. Samples analysed by HPLC used a Waters system (Milford, MA, USA) with a Spectrofocus UV scanning detector (Spectrophysics, San Jose, CA, USA). Fifty-mL water samples were loaded onto octadecylsilyl Sep-Paks (Millipore) which had been preconditioned using 3-mL acetonitrile then 10-mL water. Diuron was eluted using 1 mL of 80% acetonitrile, followed by 1-mL water. Aliquots of the eluate (150 μL) were analysed using a octadecylsilyl column (Alltech ODS-2, 250 x 4.6 mm, Deerfield, IL, USA), which was eluted at 1.5 mL/min isocratically for 15 min using 38 % acetonitrile in water; detection was at 215 and 245 nm. Under these conditions diuron eluted at 9-10 min. A step gradient to 50 % acetonitrile was used to wash the column for 5 min between sample applications.

The hapten used for both antibody generation in sheep and peroxidase conjugation was a chlortoluron derivative, 1-(carboxymethyl)-3-(3-chloro-4-methylphenyl)-1-methylurea, as described by Aherne (1991). The hapten was attached to both ovalbumin and horseradish peroxidase using the N-hydroxysuccinimide active ester reaction described by Gadow et al. (1984) and Aherne (1991). A 1/10,000 dilution of antisera in 50 mM sodium carbonate, pH 9.6 (250 μL/well) was pre-immobilised onto polystyrene microwell strips (Maxisorp, Nunc, Roskilde, Denmark). The assay was performed by addition of either 100 μL/well urea herbicide standard or water samples and 100 μL/well pesticide-enzyme conjugate (57 ng/mL), incubating 1 hr at room temperature, then washing wells 3 times with purified water. Antibody binding was revealed by addition of 120 μL/well color developer (3,3',5,5"-tetramethylbenzidine- hydrogen peroxide in acetate buffer, pH 5.5, Hill et al 1991) and incubating 30 min. After stopping color development by addition of 40 μL/well 1.25 M sulfuric acid, color was then read at 450 nm using a microplate reader. Color developed is inversely related to the concentration of urea herbicide in the original sample. After initial screening, water samples that produced more than 80 % inhibition were diluted 1/10 with purified water and reanalysed.

Standards were prepared by dilution from a 1 mg/mL solution of diuron (ChemService, West Chester, PA, USA) in methanol stored at 4° C or -20° C, and diluted in purified water using borosilicate glass tubes. Standard curves were prepared using 7 - 9 concentrations of diuron in the range 0.01 - 10 ppb, either in purified water (for analysis of purified water spiked with diuron) or field water (for analysis of field samples spiked with diuron standard and the analysis of diuron residues in irrigation drainage water).

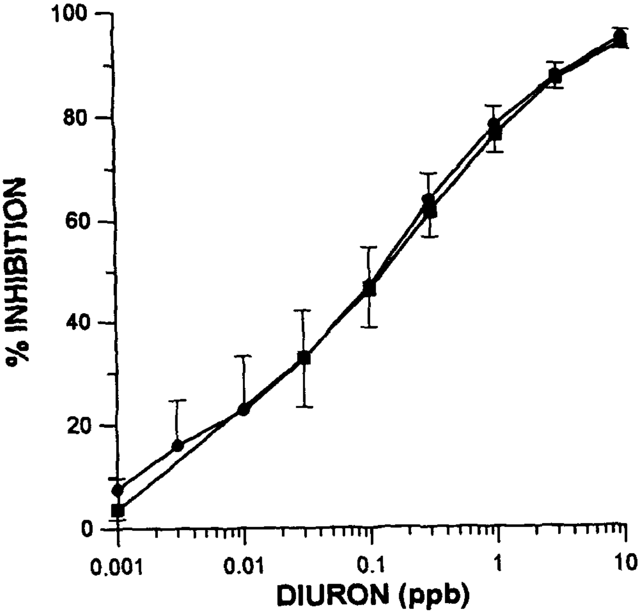

Figure 1. Standard curves for diuron prepared in field surface water (30 NTU) (■) and in purified water (●). Data shown are means+/-SD for 16 assays.

RESULTS AND DISCUSSION

The mid-point of the standard curve for diuron was between 0.1 and 0.2 ppb in purified water (50 % inhibition mean 0.12 ± 0.06 ppb; data from 1-2 assays on each of 12 days) (Fig. 1). The limit of detection (85 % Bo, where Bo is defined as the color developed in the absence of diuron in the test sample) of the assay in pure water was about 0.01 ppb, and the range of quantitation of the assay was 0.05 - 1 ppb. A ten-fold difference in diuron concentration gave a 31% difference in absorbance. The assay was therefore sufficiently sensitive for monitoring adherence to the European Community Drinking water guidelines (0.1 ppb tolerance), and for screening compliance with the Australian guidelines (ANZECC, 1992). The latter guidelines specify the following maximum permitted concentrations of diuron for: irrigation water - 2 ppb, potable water - 40 ppb, aquatic ecosystems - 16 ppb.

The sensitivity of this assay for diuron is about twice that of Schneider et al. (1994); it was also about ten times more sensitive than the monoclonal antibody assay of Karu et al. (1994a,b). Although spacer arm and substitution position heterology is often required to obtain assays of part-per-billion sensitivity for herbicide haptens (Harrison et al. 1990), the immunogen conjugate used in our assay for antibody generation and the peroxidase conjugate were derivatised at the same position (on the urea nitrogen distal to the aromatic ring), but our assay used a shorter spacer arm (carboxymethyl) than the assays described above. We have developed other highly sensitive assays for agrochemicals, (eg. chlorpyrifos (Hill et al. 1994) and diazinon (Ferguson et al. 1993)) without use of such heterology, although in other situations heterology was essential (e.g., bioresmethrin, Hill et al. 1993). Therefore, it appears that general rules for hapten design may be useful for research planning but there remains a degree of uncertainty in the response of the immune system.

Karu et al. (1994a) prepared haptens based on diuron and developed mouse monoclonal antibodies. Highest sensitivity was obtained using the diuron analog of the chlortoluron hapten used in the current work (i.e., differing only by substitution of a methyl group for a chloro- at the 3- position of the aromatic ring). However, in order to obtain adequate sensitivity for free diuron, screening conjugates based on the same hapten could not be used. Instead, best results were obtained using haptens conjugated by a 4-carbon spacer arm linked to the proximal rather than distal nitrogen. Newsome and Collins (1990) used a similar hapten based on a diuron derivative [N'-(3,4-dichlorophenyl)-N-methyl-N-butyrylurea], but did not obtain an antibody response. However, sensitive immunoassays were obtained using the analogous monuron and fenuron haptens.

The current assay detected urea herbicides with 3 and/or 4- substituted phenyl- groups, providing they were not larger than four atoms (Table I), with most sensitive detection if both substituents were present. The relative cross-reactions based on the mid-points of the standard curve (50 % Bo) and the limits of detection (90 % Bo) were not identical;

Table 1. Specificity of urea herbicide assay

Compound	Cross-reaction		Substituents		
	50 % Bo	90 Bo	N-phenyl 3-	4-	N- (urea N)
<u>3,4- substituted</u>					
diuron	100	100	Cl	Cl	methyl, methyl
neburon	125	25	Cl	Cl	methyl, propyl
chlortoluron	65	25	methyl	Cl	methyl, methyl
linuron	40	25	Cl	Cl	methyl, methoxy
chlorbromuron	35	20	Br	Cl	methyl, methoxy
metoxuron	1	1	methoxy	Cl	methyl, methyl
<u>4- substituted on ring</u>					
monuron	9	7	Cl	H	methyl, methoxy
monolinuron	6	18	Cl	H	methyl, methoxy
metobromuron	6	12	Br	H	methyl, methoxy
isoproturon	0.2	0.1	isopropyl	H	methyl, methyl
chloroxuron	<0.001	-	p-Cl-phenol	H	methyl, methyl
<u>3- substituted on ring</u>					
fluometuron	2	1	H	CF_3	methyl, methyl
<u>no ring substiuents</u>					
fenuron	0.2	0.05	H	H	H, H
siduron	0.08	0.08	H	H	H,2-methylcyclohexyl
<u>other related herbicides</u>					
methabenzthiazuron	-	-			
tebuthiuron	-	-			
chloranocryl	35	34	(3',4'-dichloro-2-methylacrylanilide)		
chloromethiuron	-	-	(3-(4-chloro-*o*-tolyl)-1,1-dimethylthiourea)		
propanil	1	1	(3,4-dichloropropionanilide)		
propyzamide	0.08	-	(3,5-dichloro-N-1,1-dimethylpropynyl) benzamide		

cross reaction = 50 % Bo concentration, relative to diuron (100 %); - = < 0.0005 %

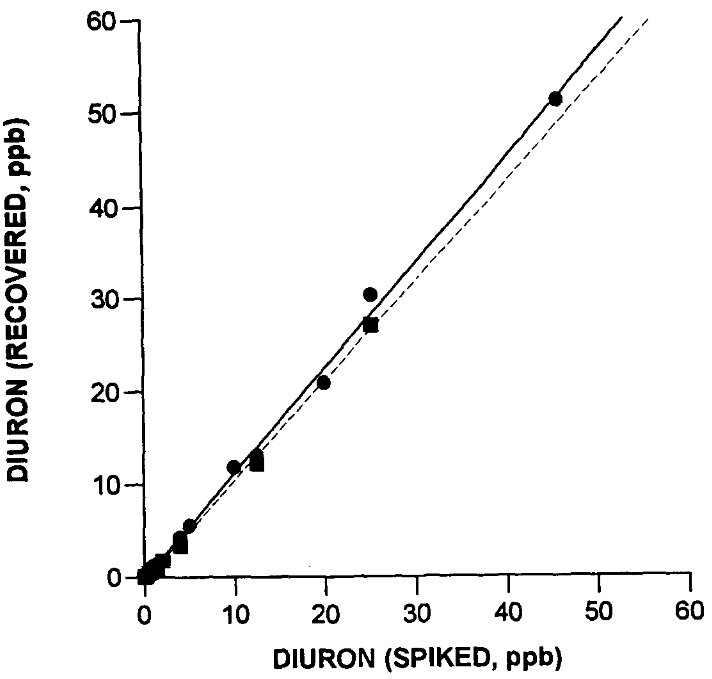

Figure 2. Spike and recovery of diuron in surface water - analysis by GC/MS (■, solid lines) and immunoassay (●, dashed lines).

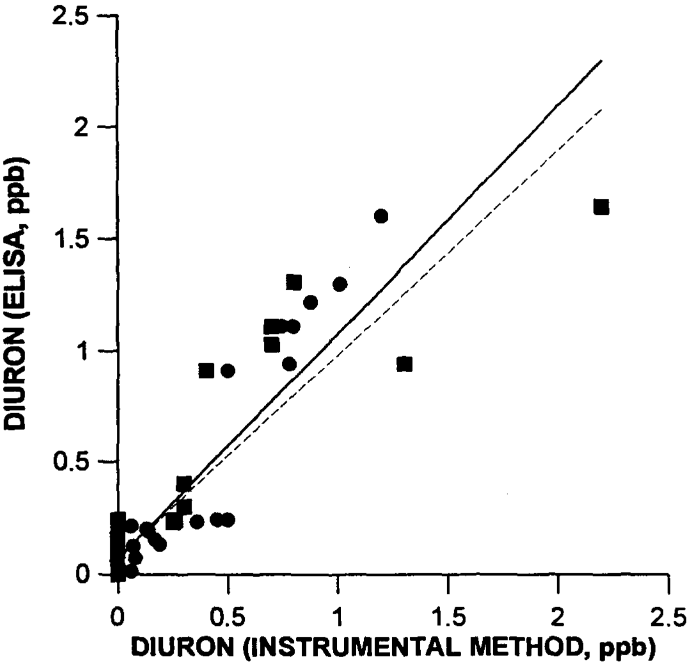

Figure 3. Relationship between diuron determined by immunoassay and GC/MS (●, dashed lines) or HPLC (■, solid lines) in surface water.

non-parallel inhibition curves are possibly due to interaction with several populations of antibodies within the polyclonal antiserum used. Interestingly, since the assay sensitively detected diuron the antibody did not discriminate between the methyl- and chloro-groups at position 4- on the aromatic ring. Urea herbicides with large groups on the urea nitrogen or with groups other than phenyl- were either detected weakly or not at all. The discontinued herbicide, chloranocryl (3',4'-dichloro-2-methylacrylanilide) is not a urea, but is similar in spatial orientation of functional groups to diuron. Other herbicides - atrazine, alachlor, 2,4-D, chlorsulfuron and simazine, MCPA or paraquat (Aherne 1991) that could occur in surface or sub-surface water did not cross-react. Several diuron metabolites (Hassall 1990) may be found in water from fields treated some time before with the parent herbicide. 3-(3,4-Dichlorophenyl)-1-methyl urea, which has some phytotoxicity, exhibited 6% cross-reaction (at 50 % Bo), while 3-(3,4-dichlorophenyl) urea (low phytotoxicity) exhibited 0.3% cross-reaction and dichloroaniline (not herbicidally active) was not detected at 1 ppm.

Other antibodies that have been described in the literature also cross-react with a range of urea herbicides, but the relative degrees of cross-reaction differ. Karu et al. (1994a) produced a set of monoclonal antibodies to the same hapten. In general, the cross-reaction patterns of each of the monoclonal antibodies reflected the polyclonal antiserum studied here: diuron > linuron > monuron > fenuron. The major difference between the current assay and the four antisera reported by Schneider et al (1994) is that the latter antisera generally cross-reacted to a greater extent with mono-substituted ureas such as monuron and monolinuron. This difference was expected, as the hapten used by these workers was a derivative of monuron rather than chlortoluron. Newsome and Collins (1990) prepared a panel of antisera to fenuron, monuron and a ring-derivatized (4-succinamidophenyl) hapten, and in each case obtained antibodies that cross-reacted extensively with other urea herbicides. In contrast, antisera raised to isoproturon haptens are rather specific for the parent compound. This has been observed for both sheep polyclonal antibodies to a hapten based on coupling through one of the methyl substituents on the urea (analogous to the chlortoluron hapten in this study; Aherne 1991) and for mouse monoclonal antibodies prepared using an isopropylaniline derivative (Liegeois et al. 1992).

Routinely, seven diuron concentrations were used in standard curve preparation: 10, 3, 1, 0.3, 0.1, 0.03 and 0.01 ppb. The relative error (deduced concentration ± standard deviation / nominal standard concentration x 100) for fifteen standard curves run on 8 different days were 1.3, 2.5, 4.5, 7.6, 29.6, 45.2 and 54.5 %, respectively. The increased relative error at low analyte concentrations for hapten immunoassay has been discussed previously by Harrison et al. (1989). However, the lowest two concentrations in the diuron immunoassay fall below the usual regulatory action levels. Thus, the high relative error below 0.1 ppb is not of concern. The coefficient of variation measured by comparing five standard curves run on the same day showed the same trend as for assays run on different days, ranging from 0.9 % for 10 ppb to 22 % for 0.01 ppb. The precision of sample analyses was examined by spiking surface water samples at 11 concentrations (range 0.1 - 46 ppb) of diuron. Analysis of samples on three different days gave coefficients of variation (CV) on the mean recoveries ranging from 1.4 to 23.6 %. The average CV calculated from these samples was 12 %, indicating that precision was similar to GC/MS (Korth, unpublished).

In an attempt to keep the assay as simple as possible, unfiltered field water samples were added directly to the microwells, even though some of these had visible turbidity (up to 100 nephelometric turbidity units (NTU)). With a surface drainage water sample (30 NTU), determined by GC/MS to contain <0.05 ppb diuron, a slight shift in the 50% inhibition value for diuron was noted compared with standards prepared in pure water (Fig. 1); since this accounted for under a 20 % shift in assay sensitivity, it is of minor practical concern. We can also not exclude the possibility of trace levels of other urea herbicides being present. A range of ions that are potentially found in surface and subsurface water did not affect the assay: Na^+ (up to 1 M), K^+ (up to 5 mM), Mg^{++} (up to 5 mM), Ca^{++} (up to 5 mM), Cl^- (up to 1 M), HCO_3^- (up to 1 M), SO_4^{--} (up to 5 mM) and NO_3^- (up to 5 mM).

Several diuron spikes (0.8 - 45.7 ppb) into surface water were analysed by both immunoassay and GC/MS (Fig. 2). There were good correlations between the level of the spike and that recovered: GC/MS recovery was (107 %, $n = 11$, $r = 0.99$), ELISA recovery (113 %, $n = 18$, $r = 0.99$). A set of surface water samples collected from supply channels and major drains in the Murrumbidgee Irrigation area of Southern NSW, Australia in November-December 1992 were analysed by both GC/MS and ELISA, with the following results: Diuron (ELISA, ppb) = 0.86 x Diuron (GC/MS, ppb) + 0.01; $r = 0.84$, $n = 14$.

For analysis of subsurface water samples, an initial set of 49 samples were collected from tile drains under orchards in May 1992, and analysed by GC/MS, HPLC and ELISA. Out of 29 samples negative (i.e. < 0.05 ppb diuron) by GC/MS and HPLC, 26 samples were negative by ELISA and 2 which were negative by HPLC and ELISA were positive by GC/MS. One sample was negative by GC/MS but positive both by HPLC and ELISA. Some of the samples negative by GC/MS contained triazines, molinate, chlorpyrifos or bromacil, but these samples were also negative by immunoassay. A second set of subsurface water samples, obtained in September 1992, were analysed by GC/MS and ELISA only. This enabled us to both further study the relationship between data obtained using the instrumental methods and ELISA, and to demonstrate the advantage of the high throughput of the ELISA in performing dissipation studies. For the two sets of samples, the regressions between data obtained using the different methods were: Diuron (ELISA) = 1.01 (GC/MS) + 0.06, $n = 64$, $r = 0.92$; Diuron (ELISA) = 0.91 (HPLC) + 0.07, $n = 49$, $r = 0.91$ (Fig. 3).

This urea herbicide ELISA has shown to be highly sensitive for monitoring of diuron in two water matrices. While the assay detects several urea herbicides, it did not detect its breakdown products. In general, the ELISA tended to give slightly lower results than the instrumental methods with surface water, but slightly higher with sub-surface water. The former water matrix was low in dissolved salts but high in particulate matter. The latter matrix is high in salt, and although we did not note interference with the immunoassay, the matrix may have had slight effects on sensitivity.

Water samples can be analysed directly without need for extraction, clean-up and concentration of the sample. The good correlations between instrumental data and ELISA also suggested the assay can be a useful tool for detection of diuron in water. Applications include regular monitoring of surface water samples for the presence of diuron before they are recycled, especially for use on crops that would be susceptible to diuron. In addition, extensive studies on the dissipation of diuron, for example through soil into subsurface water are possible.

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