IMMUNO-SELECTIVE EXTRACTION FOR MONITORING HERBICIDE 2,4-D PRIOR TO HPLC

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Introduction

Chlorophenoxyacetic acids are extensively used in agriculture for weeds control, making the atmosphere and life ecosystems to be polluted and mostly are found in the different environmental matrices (1). Trace-level analysis of such chemical components in the environmental matrices includes different processes i.e. *a*) sampling, *b*) sample preparation, and *c*) determination. From these stages, sample preparation is usually the most time-consuming and error prone process when an environmental and biological matrices is going to be determined (2-5). Although liquid-liquid extraction and solid phase extraction using silica bonded phases still are commonly used for environmental pollutants (6,7), the lack of selective solid phase for enrichment of low concentration of pesticides in the environmental waters is a serious drawback of the SPE technique. Therefore, the most popular silica phases such as C18 and C8 sorbents cannot provide the selectivity necessary for the trace pesticide residue pre-treatment. Thus, the major problem associated with the silica cartridges is the poor selectivity in the applied sorbents. To overcome this problem, immuno-extraction methods have attracted increasing recognition and considerable acceptance in the field of environmental analysis (8-10). Although immunoassays have long been used in pathology and biochemistry laboratories, these techniques have become popular for monitoring environmental and biological pollutants. It is now possible to produce antibodies against some pollutants such as pesticides (8,9). In this approach, antibodies are raised against small molecules (already linked to the carrier proteins to make it immunogen) and are applied as tailor made tools for trapping analytes of interest from the complex environmental and biological samples. This study explains how the immuno-sorbent can selectively retain and extract the 2,4-D (Figure 1) from the large volume of environmental sample followed by an efficient desorption in a very low volume of modified organic eluent, developing a simple protocol of solid-phase immuno-extraction procedure for the compound of interest.

Material and Methods

Reagents

2-methyl,4-chlorophenoxyacetic acid (99%) (2,4-D) as standard, was obtained from Greyhound, Birkenhead, UK. Methanol, ethanol, acetonitrile, n-hexane, acetic acid, hydrochloric acid (all HPLC grade), deionized water, and standard buffered solution at three pH values (4.00±0.02, 7.00±0.02, and 9.00±0.02) were purchased from BDH-Merck, Poole, UK. Hydrogenorthophosphate, potassium dihydrogenorthophosphate, potassium chloride and sodium chloride were analytical-reagent grade from BDH-Merck, Poole, UK. Phosphate-buffered saline (PBS) pH 7.4 was prepared by adding 8.0 g sodium chloride, 0.2 g of potassium chloride, and 0.2 g of potassium dihydrogenorthophosphate to 1 liter of distilled water.

Apparatus

A 10-place vacuum manifold (made in our laboratory) was used for elution of the Bond Elute silica cartridges. The pH values of the solution were measured with a Metrohm 744 digital pH meter (Metrohm, Switzerland). The amounts of reagents were measured, using a Satorius CP225D balance (Satorius, Germany) for milligram quantities or less. Quantitative liquid transfers were performed with Gilson Pipettman (Gilson Medical Electronics, Villiers-leBil, France). Vortex Genie from Scientific Industries, INC. (Bohemia NY, USA) was used for mixing solutions in the test tubes. A reversed-phase HPLC system (Knauer Company, Germany) was used for the measurements performance, consisting of a K-1001 series high-pressure pump; the analytical column was a bondclone 10 C18 (30 cm × 3.9 mm) (Phenomenex, Macclesfield, UK). The detector was a Pye LC-UV spectrophotometer (Unicham, Cambridge, UK), combined with LaserJet 1200 series printer for recording the chromatogram, using a 1456-1 Chromogate Data System Version 2.55. Because the reagents

used in this study were HPLC-grade, there was no need to filter them. However, the analytical column in HPLC system was equipped with a filter on the top. An on-line degasser attached to the solvent delivery system degassed solvents and mobile phase used in HPLC analysis.

Immuno-extraction

The immuno-extraction column was prepared by covalently binding 2,4-D antisera to aldehyde activated porous silica (Colifmar Associates, Guildford, UK). The 2,4-D antisera had been raised in sheep and were used unpurified. Unbound aldehyde groups were deactivated using glycine. Columns contained approximately 1 g of solid phase and approximately 200 µl of antisera. The basic protocol for immuno-extraction was: (a) wash the immuno-column (pre-clean up) with 25 ml 0.3% hydrochloric acid; (b) wash (activation) the immuno-column with 20 ml phosphate buffered saline (PBS) at pH 7.4; (c) load the sample typically 1 ml (this fraction is labeled BT, breakthrough in the following table; (d) wash (clean up) the immuno-column with (5-7) × 1 ml PBS (these fractions are labeled W in the tables); (e) elute (extraction) analyte with 2 × 1 ml PBS-ethanol (50:50) at pH 2 (these fractions are labeled E in table). The extracts were then analyzed by HPLC-UV.

Chromatographic conditions

The pump was operated at 1.0 ml/min, detection was by UV at 280 nm, the mobile phase consisted of methanol/water, 75:25 (v/v) containing 0.01 M acetic acid, flow rate, 1 ml/min injection volume was 100 µl, the analytical column was C18 (30 cm×3.9 mm i.d.), and the ambient temperature was used for the chromatographic system. In this study, peak height was used as detector response and extraction recoveries were calculated by comparison of the peak height in the chromatogram of extracts with those in the chromatogram of standard solutions prepared in the same solvent as following:

$$\text{Recovery (\%)} = \text{peak area (sample)/peak area (standard)} \times 100$$

Results and Discussion

The simple protocol described earlier was successfully used to retain and then disrobe 2,4-D from the immuno-extraction column. The pH of the elution solvent was crucial in obtaining quantitative recovery in a single 1 ml fraction. At a pH above two, 2,4-D began to elute in early wash fractions, whereas at pH 2 or below 100% recovery was obtained in the second 1 ml elution fraction using ethanol/PBS (50:50) at pH 1.5. The effect of the sample pH, entering the immuno-column was also investigated. Sample within the pH rang 2 to 11 were run through the column and breakthrough, washing and elution fractions were collected. The results showed that maximum recovery was obtained at a sample pH 6 and that at a pH below 4 or above 9, recovery dropped to below 50%.

In order to establish that the retention of 2,4-D was based on antibody-antigen interaction the 2,4-D column was compared with an activated silica column and a column bonded with non-immune antibody from the same sheep before it had been injected with 2,4-D. The results shown in Table 1, clearly demonstrate that retention is due primarily to the 2,4-D antibody.

Solutions with different concentrations of 2,4-D were run through the column to establish the retention capacity of the column in terms of mass of analyte. Using the immuno-column containing 1 g of dry solid phase and 200 µl of raw antibody the maximum retention capacity was 200 ng. In order to evaluate the potential for trace enrichment, the breakthrough volume capacity of the immuno-column was assessed. Spiked sample containing 100 ng of 2,4-D in water volumes of 1 to 1000 ml were passed through the immuno-column, washed and eluted as described in the experimental section. The results indicated that up to1000 ml of sample could be run through the immuno-column with the 2,4-D still eluted in a one ml fraction. No 2,4-D was eluted in any of the breakthrough, wash or elution fractions other than the E2 fraction. These results clearly demonstrate that the breakthrough of 2,4-D was due to the mass of 2,4-D rather than the volume of sample. The variation in recovery is due to analytical variation as no internal standard is used to correct for the possibility of volume changes, etc. The mean recovery was 95% with a relative standard deviation of 10%. The effect of sample flow rate on recovery was investigated since the possibility of trace enrichment from large

volumes is even more interest if this can be achieved in a relatively short time. The higher flow rate was obtained using reduced pressure at the column outlet. A flow rate of up to 5 ml/min could be used without adversely affecting recovery. More experiments were performed on drinking water to validate the present method. The spiked water samples of 50 ml of 2,4-D were used for extraction followed by HPLC-UV determination. Linear standard curve (for extracted samples) over the range 0.1-2.0 µg/ml were obtained each day (n=6) with correlation coefficient of 0.997 or greater. The extraction procedure was reliable and reproducible from day-to-day and within-day. The coefficient of variation (%CV) of 13.50, 9.99, and 5.38 were obtained for 0.10, 1.0, and 2.0 µg/ml respectively for day-to-day reproducibility and 7.40, 9.99, and 5.80, at the same concentrations, respectively for within-day reproducibility, showing suitable accuracy and precision (see Table 2). Specimen chromatograms have been shown in Figure 2. The detection limit of the method (signal/noise: 3:1), using a 50 ml sample volume is 100 ng/l as well as reproducible and quantitative recoveries, ranging from 94% to 105% were possible. Further experiments using 1 liter of sample at 5 ml/min showed that a concentration as low as 5 ng/l could be detected if necessary using the current procedure.

There was some cross-reactivity with other similarly related compounds, between 13-84% (MCPB>2,4,5-T>MCPA), in which, getting zero for both 2,4,5-T and MCPA as well as getting significantly less for MCPB (about 18%). However, this co-immuno-extraction sometimes could be considered as an advantage when there is a need to isolate a class of compounds, which can be separated by instrumental chromatography. It should be noted that such cross-reactivity usually arises by chance and has not been deliberately designed into the production of the antibody.

The work described here has shown that antibodies to 2,4-D can be immobilized on silica without losing their ability to bind 2,4-D. The immuno-column may extract 2,4-D from a variety of matrices and can pre-concentrate 1000 times from drinking water. Very low limits of detection (100 ng/ml using 50 ml water sample) can be obtained for HPLC-UV following a simple extraction-desorption step with no further sample preparation. The columns evaluated in this work typically contained 200 µl of antiserum and had a capacity for 200 ng of 2,4-D. If columns were to be used in monitoring programmes at lower concentrations, fewer antibodies could be used. The immuno-column can be re-used up to at least 50 times with drinking water samples and this approach warrants further investigation in automated systems and with different matrices.

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References
1. Devon J., Sutherland G., Stearman K., and Wells M. J. M. *J. Agric. Food Chem.* 2002; 51: 14.
2. Pool S. K., Dean T. A., Oudsema J. W., and Poole C. F. *Analytica Chimica Acta* 1990; 236: 3.
3. McDowall R. D. *J. Chromatogr.* 1989; 492: 3.
4. McDowall R. D. *J. Pharm, Biomed. Anal.* 1989; 7: 1087.
5. Wells M. J. M., and Yu l. Z. *J. Chromatogr.* 2000 *A*, 885: 237.
6. Biesaga M., Janknowska A., and Pyrzynska K. *Microchimica Acta* 2005; 150: 317.
7. Yu L. Z., and Wells M. J. *J. Chromatogr.* 2007 *A*, 1143(1-2): 16.
8. Shahtaheri S. J., Katmeh M. F., Kwasowski P., and Stevenson D. *J. Chromatogr. A*, 1995; 697: 131.
9. Shahtaheri S. J., Kwasowski P., and Stevenson D. *Chromatographia* 1998; 47: 453.
10. Chung J. C., Emon J. M., Van Durnford J., Thomas K. *Talanta* 2005; 67: 658.

Table 1. Recovery of 2,4-D from activated silica, a non-immune antibody column and an 2,4-D immuno-column, 1 ml of 100 ng/ml of sample was applied. BT is the breakthrough fraction. Ws are wash fractions, and Es are the elution solvent fractions.

Columns	Fractions										
	BT	W_1	W_2	W_3	W_4	W_5	W_6	W_7	E_1	E_2	E_3
Act. silica	0	0	31	45	20	11	0	0	0	0	0
Non-imm. ant.	0	0	0	39	34	25	0	0	0	0	0
2,4-D ant.	0	0	0	0	0	0	0	0	0	96	0

Table 2. Day-to-day (D-day) and within day (W-day) reproducibility of 2,4-D spiked in drinking water, sample volume: 50 ml, N=6

Statistical data	Concentrations added (µg/L)					
	0.10		1.0		2	
	D-day	W-day	D-day	W-day	D-day	W-day
Mean	0.09	0.10	1.05	1.05	1.95	2.0
SD	0.01	0.007	0.11	0.11	0.11	0.12
%CV	13.5	7.40	9.99	9.99	5.38	5.80

Figure 1. Structure of 2,4-D

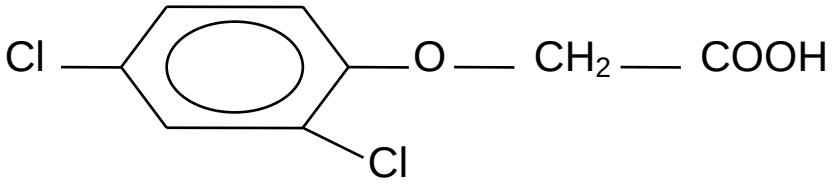

Figure 2. Chromatograms showing 2,4-D immuno-extraction from 50 ml drinking water, extracted in 1 ml eluent. HPLC conditions as in experimental section