Highly Selective Antibody-Mediated Extraction of Isoproturon From Complex Matrices

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Key Words

Column liquid chromatography
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Summary

For the analysis of pesticides such as isoproturon in complex matrices the rate determining step is sample preparation. A novel solid-phase extraction system for isoproturon based on antibody mediated extraction has been developed. The isoproturon can be retained while the immuno-extraction column is washed with phosphate buffered saline and eluted using phosphate buffered saline/ethanol at low pH. The column can preconcentrate isoproturon from up to at least 1000 mL water and elute in 1 mL. Quantitative recovery of isoproturon from water, plasma and urine can be obtained and the proposed water assay can detect as low as 50 ng L^{-1} with a 50 mL sample, or 5 ng L^{-1} with a 1 L sample.

Introduction

Sample preparation is currently recognised as the most important step in many analytical procedures particularly those involving the determination of trace concentrations of organics in complex matrices. The most commonly used sample preparation methods for aqueous samples are liquid-liquid extraction and solid-phase extraction, though a wide variety of procedures are used either alone or in combinations [1, 2]. Liquid-liquid extraction is not easily automatable and can use large volumes of solvents that require expensive disposal methods. The popularity of solid-phase extraction methods is thus growing as these use less solvent and are easily automated. With solid-phase extraction it is also possible to achieve trace enrichment and clean-up in one step.

A wide range of solid phases is available and these use various different separation mechanisms to isolate and then release analytes. In general the phases are suitable for the extraction of many analytes under the same conditions but they do not offer high selectivity for a single analyte or a closely related group of analytes. Recently there has been growing interest in the use of immobilised antibodies as selective solid-phase extraction columns and procedures have been developed for several drugs and other trace organics [3–8].

Immuno-extraction offers several potential advantages, including the use of low volumes of environmentally friendly extraction solvents based on aqueous buffers. As the extraction is based on antibody-antigen interactions analyte retention should be highly selective. The main limitation is the need for antisera, but for many drugs and now pesticides antisera are available. One other major advantage of using an immuno-extraction (or indeed a conventional solid-phase extraction) followed by chromatography is that it allows the possibility of confirming the identity of an analyte by spectroscopic techniques such as mass-spectrometry.

We have recently shown that it is possible to use antisera covalently bound onto silica as a highly selective solid-phase extraction and enrichment method [9]. Most of the current descriptions of immuno-extraction use soft gels and cannot elute in a small volume, which may indicate a large contribution from non-specific binding. As antisera are more easily obtained for large molecular weight compounds there are relatively few examples in the literature of immuno-extraction of small molecular weight compounds such as drugs and pesticides.

The aims of the work described here were to demonstrate the successful immobilisation of antibodies to the herbicide isoproturon, the development of a protocol that was clearly based on antibody-antigen interactions and desorption in a low volume of eluent. Isoproturon was chosen as a model compound for immuno-extraction as we had a large quantity of good quality an-

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tiserum available from previous work on the development of an ELISA [10].

Isoproturon is a phenylurea used to control broad leaf weeds and grasses. There has recently been some concern over levels found in drinking water. Current chromatographic methods typically involve derivatisation prior to GLC [11] or HPLC with UV detection [12–14] for which extensive sample preparation is needed. Alternatively immunoassays for determining isoproturon in environmental samples have been described [10, 15].

This paper describes the development and optimisation of an antibody mediated extraction of isoproturon. Application to drinking water and preliminary work on biological samples is described.

Experimental

Chemicals

Isoproturon reference standard was obtained from Greyhound, (Birkenhead, UK). Methanol, ethanol, hydrochloric acid, glacial acetic acid, disodium hydrogen orthophosphate, potassium dihydrogen orthophosphate, potassium chloride and sodium chloride were analytical reagent grade from BDH-Merck, (Poole, UK). Phosphate-buffered saline (PBS), pH 7.2–7.4, was prepared as previously described [9].

Chromatographic Conditions

The pump was a Beckman 110B (Beckman Instruments, High Wycombe, UK), operated at 1.0 mL min^{-1}. Detection was by UV at 244 nm, (AUFS=0.02), using a Pye LC-UV spectrophotometer (Unicam, Cambridge, UK). The mobile phase was methanol-water (70–30 v/v). Injection volumes of up to 200 µL were made with an autoinjecter (WISP 710A, Waters Associates, Northwich, UK).The column was a Bondaclone 10 C-18 (Phenomenex, Macclesfield, UK).

Immuno-Extraction

The immuno-extraction column was prepared by covalently binding antiserum (raised against isoproturon hapten, details in reference [10]) to aldehyde activated porous silica (Clifmar Associates, Guildford, UK). The antiserum was used unpurified. Unbound aldehyde groups were deactivated using glycine. Columns contained approximately 1 g of dry solid phase and 200 µL of antiserum. The basic protocol for the immuno-extractions described was : (a) wash the column with 0.3 % hydrochloric acid; (b) wash the column with 10 mL of PBS at pH 7.4; (c) load the sample, typically 1–50 mL (this fraction is labelled BT, breakthrough in the following tables); (d) wash with 5 × 1 mL PBS (these fractions are labelled W in the tables); (e) elute the analyte with 2 × 1 mL of PBS-ethanol (50–50) at pH 2, (these fractions are labelled E in the tables).

Results and Discussion

The simple protocol described earlier was successfully used to retain and then desorb isoproturon 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 3 isoproturon began to elute in early wash fractions, whereas at pH 3 or below 100 % recovery was obtained in the second 1 mL elution fraction using ethanol-PBS (50–50) at pH 2. The effect of the pH of the sample entering the immuno-column was also investigated. Samples over the pH range 2 to 11 were run through the column and breakthrough, washing and elution fractions were collected as described earlier. The results showed that maximum recovery was obtained at a sample pH of 7, and that at a pH below 4 or above 9, recovery dropped to below 50 %.

In order to establish that the retention of isoproturon was based on antibody-antigen interactions the isoproturon 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 isoproturon. The results, shown in Table I, clearly demonstrate that the retention is due primarily to the isoproturon antibody.

Different concentrations of isoproturon (all loaded in 1 mL of sample) were run through the column to establish the retention capacity of the column in terms of mass of analyte. Using the immuno-column described earlier containing 1 g of dry solid phase and 200 µL of raw antibody the maximum retention capacity was 200 ng. The breakthrough volume capacity of the immuno-column was assessed in order to evaluate the potential for trace enrichment. Spiked samples containing 100 ng of isoproturon 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, shown in Table II, indicated that up to 1000 mL of sample could be run through the immuno-column with the isoproturon still eluted in a one mL fraction. No isoproturon eluted in any of the breakthrough, wash or elution fractions other than the E2 fraction. These results clearly demonstrate that the breakthrough of isoproturon was due to the mass of isoproturon 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 shown in Table II is 95 % with a relative standard deviation of 10 %. The capacity of the column not the affinity of the antibody is the limiting factor.

Following this important finding the effect of sample flow rate on recovery was investigated since the possibility of trace enrichment from large volumes is even more exciting if this can be achieved in a relatively short time. The higher flow rates were obtained using reduced pressure at the column outlet. A flow rate of up to 5 mL

Table I. Recovery of isoproturon from activated silica, a non-immune antibody column and an isoproturon antibody column, 1 mL of 100 ng mL^{-1} of sample was applied. BT is the breakthrough fraction, W are wash fractions, and E are the elution solvent fractions.

Column	Fractions													
	BT	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	E1	E2	E3
Activated silica	0	0	23	42	23	12	0	0	0	0	0	0	0	0
Non-immune antibody	0	0	0	42	35	15	0	0	0	0	0	0	0	0
Chlortoluron antibody	0	0	0	0	0	0	0	0	0	0	0	0	93	0

Table II. Recovery of isoproturon using different sample volumes (100 ng applied)

Volume (mL)	1	10	20	30	40	50	70	100	150	300	500	1000
Recovery (%)	100	100	85	85	77	92	85	108	100	100	108	100

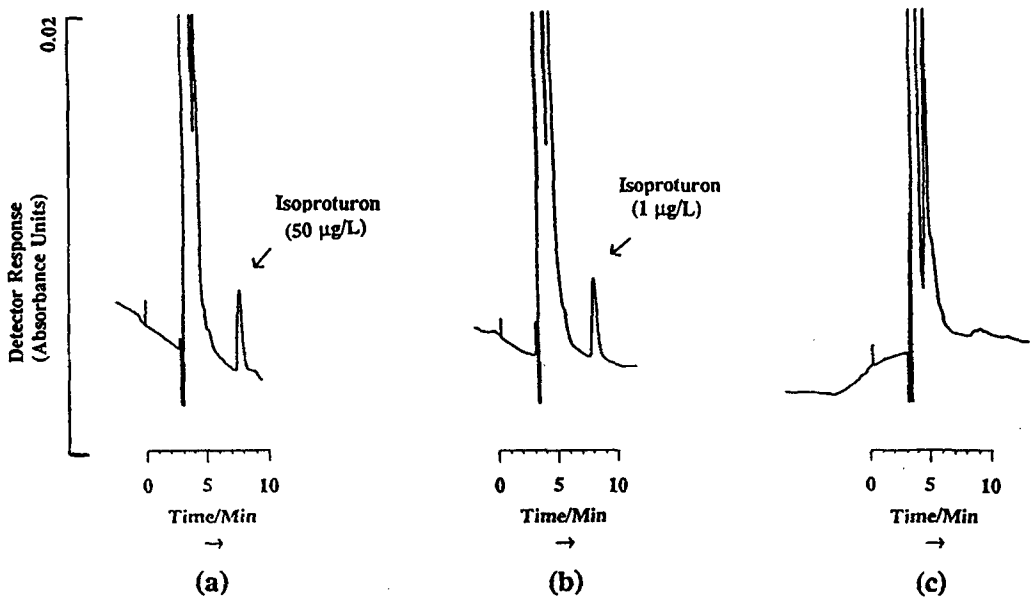

Figure 1
Specimen chromatograms showing isoproturon immuno-extraction from 50 mL drinking water, (a) non extracted standard, (b) water spiked with 1 µg L^{-1} isoproturon, 50 mL sample taken through immuno-extraction column and eluted in 1 mL (c) extracted water blank (also 50 mL taken through immuno-extraction column and eluted in 1 mL. HPLC conditions as in Experimental section.

min^{-1} could be used without adversely affecting recovery.

Further experiments were carried out on drinking water. A preliminary validation of the possible use of the immuno-column for measuring isoproturon in drinking water was carried out using spiked samples and standards. Samples of 50 mL were used for extraction and HPLC. Linear standard curves (immuno-extracted) over the range 0.1–2.0 µg L^{-1} were obtained each day ($n = 6$) with a correlation coefficient of 0.996 or greater. The day-to-day relative standard deviation of the method was investigated by spiking tap water with isoproturon. The day-to-day relative standard deviation ($n = 6$) was 22, 12, 7, 11, 10 and 9.4 % respectively at a concentration of 0.1, 0.2, 0.6, 1.0, 1.4, and 2.0 µg L^{-1}. It should be noted that the immuno-column extract (E2) is injected with no further sample preparation, i.e. no evaporation

to dryness, etc. and that no internal standard has been added. Specimen chromatograms are shown in Figure 1. The limit of detection possible using a 50 mL sample volume is 100 ng L^{-1} using twice baseline noise. Further experiments using 1 L of sample at 5 mL min^{-1} showed that a concentration as low as 5 ng L^{-1} could be detected if necessary using the current procedure.

Although primarily aimed at showing extraction and enrichment of isoproturon the immuno-column has been tested for cross-reactivity against other pesticides. There was 100 % cross-reactivity with chloroxuron, some with chlorbromuron (59 % in the E2 fraction), linuron (48 % in E2), and methabenthiazuron (37 % in E 2), very little with chlortoluron (17 % in E2), and none with metoxuron, atrazine, 2,4-D, 2,4,5-T, MCPA and MCPB. The remainder of the herbicides eluted in the wash fractions. As stated in a previous publication [9] cross-reactivity could be seen as an advantage when there is the need to isolate a class of compounds (e.g. phenylureas) which can be separated by instrumental chromatography. The possibility of using antibody cross-reactivity to advantage has also been shown for triazines and phenylureas [16,17]. It should be noted that such cross-reactivity usually arises by chance and has not been deliberately designed into the production of the antibody.

Conclusions

The work described here has shown that antibodies to isoproturon can be immobilised on silica without losing their ability to bind isoproturon. The immuno-column can extract isoproturon from a variety of matrices and can pre-concentrate 1000 times from drinking water. Very low limits of detection (100 ng mL^{-1} using a 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 aniserum and had a capacity for 200 ng of isoproturon. If columns were to be used in monitoring programmes at lower concentrations less antibody could be used. The immuno-column can be reused 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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