



ELSEVIER

Journal of Chromatography A, 697 (1995) 131–136

JOURNAL OF
CHROMATOGRAPHY A

Development and optimisation of an immunoaffinity-based solid-phase extraction for chlortoluron

S.J. Shahtaheri, M.F. Katmeh, P. Kwasowski, D. Stevenson*

Robens Institute, University of Surrey, Guildford, Surrey GU2 5XH, UK

Abstract

The determination of trace organics such as pesticides in biological and environmental samples require rapid, easy-to-use, reliable sample preparation procedures. Solid-phase extraction using silica or bonded silicas has proven useful for broad-range screening. We have used antisera to chlortoluron immobilised onto silica as a “tailor-made” solid-phase extraction system. The chlortoluron can be selectively retained and eluted using a simple phosphate buffered saline/ethanol mixture. Preliminary studies have demonstrated that the immuno column has a high volume breakthrough (at least 1 l of water) and can retain up to 500 ng of chlortoluron. Quantitative recovery from tap water, river water, drinking water, plasma and urine is achieved along with an HPLC trace free from co-eluting compounds at the chlortoluron retention time.

1. Introduction

The measurement of trace levels of organic chemicals such as drugs, metabolites, pesticides and other pollutants in biological and environmental samples is a challenging and exacting task. Growing concern about the risk to human health posed by toxic chemicals has led to an increase in the need for simple, reliable analytical methods. Although there are now many elegant separations on highly sophisticated instrumentation the rate-limiting step (and that most prone to errors) in most assays is during sample extraction and trace enrichment [1].

Many analytical methods use liquid–liquid extraction or solid-phase extraction techniques to perform sample clean-up [2]. Liquid–liquid methods cannot easily be automated and use large volumes of solvents that present environ-

mental concerns. Solid-phase extraction methods using silica or bonded silicas have proven useful in simplifying sample preparation. Trace enrichment and clean-up can be achieved in a single step and only low volumes of solvents are used.

The use of commercially available low cost vacuum manifolds allows many samples to be processed simultaneously. Furthermore, complete automation of procedures based on solid-phase extraction is now possible using commercially available instrumentation. A wide range of phases from many suppliers based on silicas are available including reversed-phase, normal-phase, ion-exchange and mixed-mode phases. Although useful for broad-range screening, these phases do not actually have good selectivity and there is continued interest in the development of alternative sample preparation procedures. Recently there has been growing interest in the use of antibody mediated extractions since in principle highly selective analyte–antibody interactions

* Corresponding author.

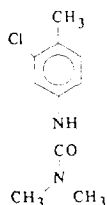


Fig. 1. Structure of chlortoluron.

are possible [3–7]. Immunoassays have long been used in clinical chemistry/biochemistry laboratories but more recently these techniques have become popular for monitoring toxic chemicals (such as pesticides) in the environment [8]. The antisera developed for such immuno methods provides the key reagent for antibody columns (also termed immunoaffinity columns).

This paper describes how we have successfully used antibodies to the herbicide chlortoluron (see Fig. 1) to develop a simple solid-phase extraction procedure for chlortoluron. The aims of the work were to develop a simple protocol for the retention and elution (in a small volume) of chlortoluron from an immunoaffinity column and to carry out a preliminary evaluation of the capability with respect to enrichment and clean-up.

2. Experimental

2.1. Chemicals

Chlortoluron reference standard was obtained from Greyhound, Birkenhead, UK. Methanol, ethanol, hydrochloric acid, disodium 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, 0.2 g of potassium dihydrogenorthophosphate and 2.9 g disodium hydrogenorthophosphate to 1 l of distilled water.

2.2. Chromatographic conditions

The pump was a Beckman 110B (Beckman Instruments, High Wycombe, UK), operated at 1.0 ml/min. Detection was by UV at 244 nm using a Pye LC-UV spectrophotometer (Unicam, Cambridge, UK). The mobile phase consisted of methanol-water (70:30). Injection volumes of up to 100 μ l were delivered using a WISP 710A (Waters Associates, Northwich, UK). The column was a Bondacelone 10 C₁₈ (Phenomenex, Macclesfield, UK).

2.3. Immuno extraction

The immuno-extraction column was prepared by adding chlortoluron antisera to aldehyde activated porous silica (Clifmar Associates, Guildford, UK). The chlortoluron antisera had been raised in sheep and was used unpurified. Unbound aldehyde groups were deactivated using glycine. Columns contained approx. 1 g of solid phase and approx. 200 μ l of antisera. The basic protocol for the immuno extraction was: (a) wash the column with 0.3% hydrochloric acid; (b) wash the column with 10 ml phosphate-buffered saline (PBS) at pH 7.4; (c) load the sample typically 1 ml (this fraction is labelled break-through in tables); (d) wash with 5 \times 1 ml PBS (these fractions are labelled W in tables); (e) elute analyte with 2 \times 1 ml PBS-ethanol (50:50) (these fractions are labelled E in tables).

3. Results and discussion

Initial results showed that chlortoluron was retained by the antibody column using a simple extraction procedure shown earlier. When applying 1 ml of 1 μ g/ml chlortoluron to the column quantitative elution of analyte was achieved in five fractions of PBS, pH 8-ethanol (50:50), it was not possible to elute in one 1-ml fraction. It should be noted that recovery values are only approximate as no internal standard is used to allow for small volume changes.

Experiments using PBS-ethanol at different pH values have shown that pH could be used to

Table 1
Recovery of chlortoluron (1 ml of 1 $\mu\text{g}/\text{ml}$) in immuno-column fractions using different pH values to elute

Eluent pH	Fractions												Total
	BT	W1	W2	W3	W4	W5	E1	E2	E3	E4	E5	E6	
2	0	0	0	5	16	7	0	74	0	0	0	0	102
3	0	0	0	0	0	0	0	120	0	0	0	0	120
4	0	0	0	0	0	0	0	62	18	0	0	0	80
5	0	0	0	0	0	0	0	25	23	9	5	0	62
6	0	0	0	0	0	0	0	83	21	7	2	0	113
8	0	0	0	0	0	0	0	2	53	22	12	7	96

optimise the elution of chlortoluron (see Table 1) from the columns. At low pH it was possible to elute the chlortoluron in one $\times 1$ ml fraction. Further experiments using 1 ml of chlortoluron over the range 0.1–0.5 $\mu\text{g}/\text{ml}$ gave quantitative recovery in one 1-ml fraction even after retaining the chlortoluron through up to five wash steps. This suggested that the column has a capacity to retain up to 500 ng of chlortoluron. Optimum elution was achieved using 1 ml of pH 2 PBS-ethanol (50:50).

The effect of sample pH on chlortoluron retention in the immuno column was evaluated by monitoring the pH of all samples (distilled water, tap water, river water, plasma and urine). The pH varied over the range 5.7 to 8.3 and had no effect on the retention and elution profile of chlortoluron.

In order to evaluate the water volume breakthrough of the immuno-extraction cartridge 1-ml samples of 0.5 $\mu\text{g}/\text{ml}$ chlortoluron were diluted into different volumes and added to the column. The results are shown in Table 2 demonstrating that up to at least 1000 ml of sample could be applied, without loss of recovery. In all cases the chlortoluron eluted in one 1-ml fraction. This allowed accurate measurement as low as at least

0.5 $\mu\text{g}/\text{l}$, though this is dependent on the detection system rather than the extraction procedure. A limit of detection as low as 0.1 $\mu\text{g}/\text{l}$ was possible using $3 \times$ baseline noise. It clearly demonstrated that the breakthrough of chlortoluron was due to the capacity of the column for chlortoluron, not due to the volume of water it was contained in. Trace enrichment from a large volume is thus possible. These experiments have shown that the columns we describe could retain up to 500 ng of chlortoluron. This is clearly very compatible with current chromatography detection systems.

In order to demonstrate that the retention was due to the immobilised antibody the elution profile of the antibody column was compared with that of activated silica and an immobilised non-immune antibody from the same sheep before it was injected with chlortoluron. The results are shown in Table 3 and clearly demonstrate that the chlortoluron is hardly retained by the activated silica but is retained and then selectively eluted by the chlortoluron antibody column.

Preliminary experiments to look at the effect of different matrices (drinking water, tap water, river water, urine and plasma) have also been

Table 2
Recovery of chlortoluron using different sample volumes (500 μg applied)

Volume (ml)	1	10	20	30	40	50	70	100	150	300	500	1000
Recovery (%)	116	127	105	102	89	105	115	118	102	89	103	119

Table 3
Recovery of chlortoluron from activated silica, a non-immune antibody column and a chlortoluron antibody column

Column	Fractions													
	BT	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	E1	E2	E3
Activated silica	0	0	0	5	26	29	18	9	5	3	2	0	0	0
Non-immune antibody	0	0	0	2	23	29	22	11	7	3	1	0	0	0
Chlortoluron antibody	0	0	0	0	0	0	0	0	0	0	0	0	91	0

carried out. The results showed that quantitative recovery (> 91%) in one fraction was obtained from all matrices. The highly efficient clean-up that was obtained for all matrices is shown in Fig. 2 for urine and plasma. Very large interfering peaks eluted in wash fraction 1, getting less with fraction 2 and 3. By wash fraction 4 and 5 and the elution fractions no interference was seen at the chlortoluron retention time. For plasma the E2 fraction shows that unspiked plasma contained no interfering peak at the chlortoluron retention time. It should also be noted that all the experiments described in this paper have been carried out on the same column after appropriate regeneration, demonstrating the reusability of the immuno columns.

The reproducibility that might be achieved using this immuno-extraction procedure was evaluated by analysis of six 1-ml samples of distilled water spiked with 0.5 $\mu\text{g/ml}$. These results showed a within-day relative standard deviation (R.S.D.) of $\pm 6.9\%$. The results in Table 2 using the same mass of chlortoluron but in different volumes of water gave a mean recovery of 108% with an R.S.D. of $\pm 10.9\%$.

The work described here demonstrates the feasibility of using antibody-antigen interactions for trace enrichment and clean-up prior to chromatography. Although primarily aimed at isolation from the matrix antibodies can show cross-reactivity to structurally similar compounds, though in many cases they are separated by the chromatographic technique. Preliminary experiments with the chlortoluron immunoaffinity column showed that there was some cross-reactivity

with linuron, chlorbromuron and chloroxuron but very little with isoproturon and methoxuron. Cross-reactivity could however be seen as an advantage when there was the need to isolate a class of compounds (such as phenylureas) which could then be separated by instrumental chromatography. Further experiments to investigate this interesting possibility are being carried out.

4. Conclusions

This work demonstrates that antibodies to chlortoluron can be successfully immobilised onto activated silica, without loss of immune response. The immuno columns can selectively extract chlortoluron from a variety of matrices, and give a clean sample for HPLC using a simple protocol involving PBS and ethanol at different pH values. Using water as an example it is possible to preconcentrate chlortoluron from up to 1000 ml of sample. The capacity of immuno-affinity columns for mass of chlortoluron is limited (approx. 500 ng) but this is compatible with chromatographic detection limits, and it is low values that potentially cause problems not such high concentrations. As the procedures are based on solid-phase extraction, both on and off-line automation should be possible. Immuno-based solid-phase extraction offers a new sample preparation approach using "tailor-made" columns either alone or in combination with other clean-up steps. Now a very simple protocol has been developed this ought to be evaluated for a

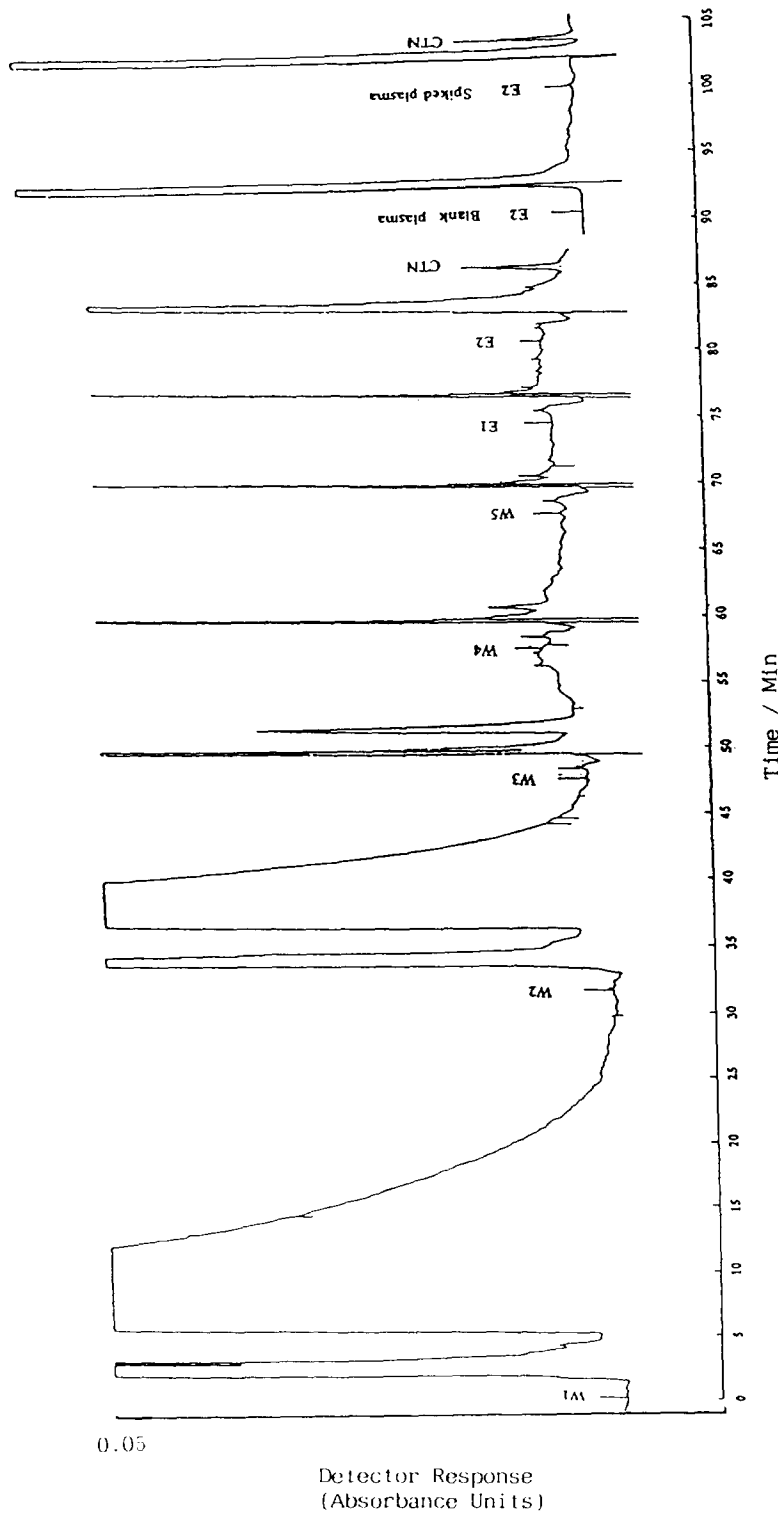


Fig. 2. Chlortoluron (CTN) immuno extraction from urine and plasma. HPLC conditions as in Experimental section.

wider range of analytes for which antisera is available, e.g. drugs and other pesticides.

Acknowledgements

This programme of work has received financial support from the Health and Safety Executive (to support M.F.K.) and the Iranian Ministry of Health (to support S.J.S.).

References

- [1] U.A.Th. Brinkman, *J. Chromatogr. A*, 665 (1994) 217.
- [2] D. Barceló, *Analyst*, 116 (1991) 681.
- [3] C. van de Water and N. Haagsma, *J. Chromatogr.*, 411 (1987) 415.
- [4] A. Farjam, A.E. Bragman, H. Lingeman and U.A.Th. Brinkman, *Analyst*, 116 (1991) 891.
- [5] A. Farjam, A.E. Bragman, A. Soldaat, P. Timmerman, H. Lingeman, G.J. de Jong, R.W. Frei and U.A.Th. Brinkman, *Chromatographia*, 31 (1991) 469.
- [6] M. Sharman, S. MacDonald and J. Gilbert, *J. Chromatogr.*, 603 (1992) 285.
- [7] G.S. Rule, A.V. Mordehai and J. Henion, *Anal. Chem.*, 66 (1994) 230.
- [8] G.W. Aherne, *Anal. Proc.*, 24 (1987) 140.