DIPSTICK IMMUNOASSAY FORMAT FOR TERBUTHYLAZINE ANALYSIS IN WATER SAMPLES

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ABSTRACT:

In this paper are reported some results on development of a new dipstick immunoassay format for terbuthylazine determinations in environmental water samples.This dipstick assay was applied on surface and ground water samples and results were correlated with those from gas chromatography (GC/MS) and ELISA. Our results confirmed that at this developmental stage the dipstick method reported can be very useful as qualitative/semiquantitative “field test” for identifying “positive” samples, reducing the number of samples to be reanalysed in laboratory. Further improvements are possible in order to optimise the whole system on the strictly analytical aspects.

Keywords: terbuthylazine; dipstick immunoassay; environmental monitoring.

INTRODUCTION

Recently, immunoassays have seen a great development in the field of organic micropollutants analytical determination [7] and specifically in the s-triazines analyses [2; 3; 6; 8; 9], but these methods are generally restricted to laboratories.
In this paper a new dipstick assay for terbuthylazine, using a monoclonal antibody (P6A7) showing a high specificity for the analyte, was developed. Dipstick assays, widely used as a diagnostic tool to detect toxins [4], hormones or drugs [1], allow a rapid determination of the analytes. Dipstick assays, following the ELISA procedural schemes, are based on a membrane, adhered to a plastic strip, as the antibody coating support [5]. The competitive reaction between the pesticide and the enzyme-tracer for binding the membrane-coated specific monoclonal antibody (mAb) involves a change in colour intensity which can be measured as remission values at 657 nm, by a portable reflectometer. Terbuthylazine dipstick assay reported is suitable as a qualitative or semi-quantitative methods to select and identify ”in field” all “positive” water samples and to obtain preliminary significant information on the concentration levels. Dipstick assays are rapid, easy-to-perform and inexpensive and could be advantageous in respect to ELISA or GC/MS for “in field” environmental analysis. Further improvements of analytical parameters such as precision, accuracy and detection limits are required.

The evaluation of the analytical performance of dipstick assay, in comparison with other analytical methods such as GC/MS and ELISA, is also reported.

MATERIALS AND METHODS

Murine hybridoma cell line secreting monoclonal antibodies P6A7 (terbuthylazine specific) as well as the s-triazine derivative [4-chloro-6(isopropylamino)1,3,5-triazine-2-(6-amino) caproic (atrazine-caproic acid)] were kindly provided by Prof. B. Hock and Dr T. Giersch. Dipstick assay procedure can be decribed as two different step:

antibodies coating and dipsticks preparation

The strips for dipstick assay were prepared as follows.
Polystyrol sheets (cut into strips of 0.7 x 7 cm) were soaked in acetone, washed in 40 mM phosphate-buffer saline (sodium chloride, 0.150 mM) [PBS] and incubated with the monoclonal antibody P6A7 (0.2 g/mL in Carbonate buffer 50 mM at +4°C for 24 hours. After being washed twice with PBS-washing buffer containing 0.05% Tween 20, the polystyrol strips were blocked with 2% Casein in distillated water solution for 30 minutes. The test strips were washed three times, dried for 1 hour at room temperature and were ready for use in the assay.
All the incubation steps were carried out at room temperature under constant shaking. Finally the test strips were ready for use

in the assay and can be stored at +4°C for at least 6 months without loss of efficiency.

assay protocol

The test strips were incubated in 2 mL glass test tubes with a mixture of 200 ?L of enzyme tracer 4-chloro-6(isopropylamino)1,3,5-triazine-2-(6-amino) Horse Radish Peroxidase conjugate prepared according to the literature method [3] and 800 ?L of standard solution (or sample). The reaction was allowed to proceed 45 minutes and the strips are washed three times with PBS/tween 20. The strips were incubated for 20 min in 800 ?L substrate buffer prepared as following described: 400 ?L of TMB (3,3',5,5'-tetramethylbenzidin) stock solution (6 mg/mL in DMSO) + 1 mL of Dioctyl Sulpho Succinate solution (8 mg/mL in ethanol) + 3.6 mL of 0.1 M sodium acetate solution pH 5.5 + 100 ?L of 1% hydrogen peroxide. All incubations are carried out at room temperature under constant shaking and, as a general principle, 30 strips can be analysed in the same experiment, including a sufficient number of replicates both for standard solutions and samples.
The absorption of the coloured product was measured with a portable reflectometer RQflex reflectometer (Merck, Darmstadt, Germany) at 657 nm. RQflex reflectometer was calibrated using the barcode "testroutine", which is strip included to the device. This barcode permits the output of absorption values in percentage measured in the transmission mode at 657 nm.

The remission values, relative to the standard solutions (concentration in a range from 0.01 to 100 ?g/L), were fitted to a four-parameter logistic function using a commercial software package (ORIGIN^{TM}).
The remission values R were normalized as % B/Bo, according to the following equation :

$$B/Bo\ [\%] = (R - Rxs / \ Ro - Rxs) \times 100$$

R = Remission at 657 nm
Ro = Remission at concentration 0 of the hapten
Rxs = Remission at an excess of the hapten

and elaborated with the same software package:
The IC 50 (the amount of hapten required to occupy 50% of mAb binding sites) was 4.5 ?g/L.
Surface and ground water samples were collected in an agricultural area in Northern Italy (Veneto). Regarding dipstick assay for TBA analysis all samples were also analysed by GC/MS (with a terbuthylazine detection limit of 0.01 ?g/L) and by ELISA performed with the same monoclonal antibody terbuthylazine specific (P6A7).

RESULTS AND DISCUSSION

Polystirol, as plastic support for mAb immobilization was chosed for terbuthylazine dipstick assay development, due to the best

colour precipitation obtained. Dipstick assay standard curve for terbuthylazine is shown in the Figure. The least detectable dose (LDD) was calculated by t-student test at 95 % confidence interval and is about 1.2 ?g/L for the analyte.

Several calibration curves were analysed in order to evaluate their reproducibility. A relatively high coefficients of variation suggested the necessity to include an internal calibration curve for each set of analyses in the analytical protocol. Because the colour developed on the membrane is relatively unstable, timing of instrumental readings should be considered critical and it is advisable to analyse no more than 30 test strips in a single set of analyses, to avoid loss of analytical consistance of the data. Therefore, the best compromise is based on the simultaneous analysis of 5 samples (three replicates each) and 5 different standard solutions concentrations (three replicates each) in order to obtain significant quantitative results.

On the basis of this analytical protocol terbuthylazine was determined in 25 real water samples with TBA dipstick assays. Four samples resulted TBA positive at 1.2-3.8 ?g/L concentration level (with a detection limit of 1.2 ?g/L). ELISA, using monoclonal antibody P6A7 anti-TBA specific (with a detection limit of 0.3 ?g/L), confirmed the four positive samples and evidenced 5 more TBA positive samples at 0.6-0.7 ?g/L concentration level. GC/MS analyses evidenced the presence (even at trace level) of TBA in 23 out of 25 samples (with a detection limit of 0.01 ?g/L), negative in ELISA because the TBA concentrations were under the ELISA detection limit.

The differences between results from GC/MS and immunoassays for TBA determination could be justified with the P6A7 mAb cross-reactivity to other TBA degradation product.

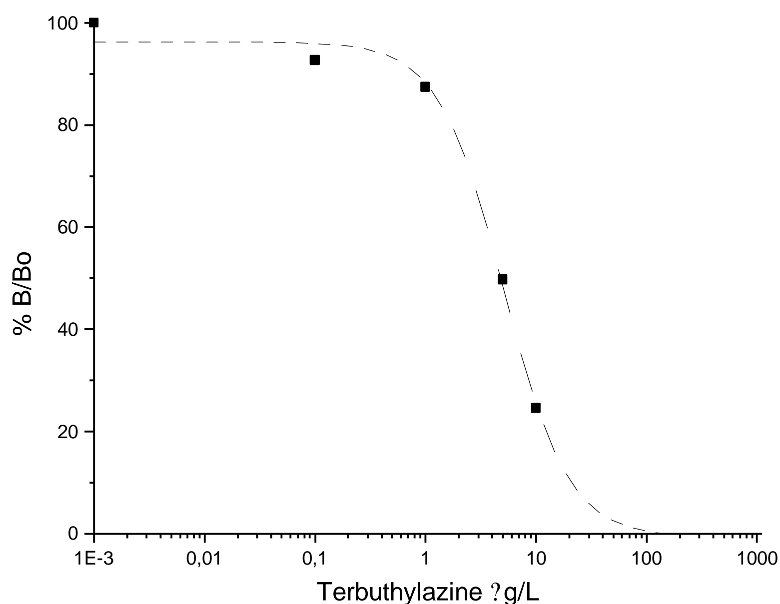

Figure : Representative terbuthylazine calibration curve obtained with P6A7 dipstick assay. The two-fold standard deviation (? 2? representing 95% confidence interval) is indicated as error bars.

CONCLUSION

The proposed dipstick assay seems to be a fast and reliable “field test” for a preliminary qualitative/semiquantitative screening of environmental water samples. The real samples can be analysed without any preliminary enrichment or clean-up treatment, the

method is relatively rapid, easy to perform and quite inexpensive. All positive samples can be successively analysed in the laboratory according to an analytical standard method, in order to confirm the presence of the analytes and obtain officially acceptable quantitative data.

On the strictly analytical point of view, at this stege of development, the main existing problems seem to be a relatively poor precision, probably due to problems in the homogeneity of the monoclonal antibody coating on the polystyrol transparents, and the instability of the coloured TMB charge-transfer complex. The practical analytical protocol proposed in this paper permits to minimize these problems as shown by the reasonably good agreement among results from GC/MS, ELISA and dipstick assays.

Dipstick assay detection limits are suitable for environmental monitoring purposes (runoff water, surface water etc.), but it is still too high for drinking water analysis according to the EU guidelines for drinking water.

Further improvements are needed in order to optimise the system both from the screening “field test” point of view (reducing the number of sample replicates and standard solutions to be processed) and the strictly analytical aspects (increasing precision and accuracy of the method and lowering the detection limits.

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