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Ultrasensitive Quantitative Determination of Paraquat: Application to River, Ground, and Drinking Water Analysis in an Agricultural Area

MARIA A. BACIGALUPO,*,† GIACOMO MERONI,† MARA MIRASOLI,‡ DANIELA PARISI,‡ AND RENATO LONGHI†

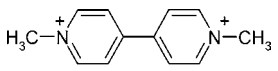
Istituto di Chimica del Riconoscimento Molecolare, CNR, Via M. Bianco 9, Milan 20131, Italy, and Dipartimento di Scienze Farmaceutiche, Università di Bologna, Via Belmeloro 6, Bologna 40126, Italy

The water specimens were collected from wells and irrigation ditches in the agricultural area to the south of Milan and from Olona River and Mantua Lake and analyzed for paraquat detection. The assay was performed using a specific polyclonal antibody raised in sheep and rabbit anti-sheep IgG conjugated with a chelating molecule 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid complexed with Eu^{3+} as a fluorescent marker. Bovine serum albumin conjugated with 5-(1′-methyl[4,4′]bipyridinyl-1-yl)pentanoic acid was used in solid phase preparation. The sensitivity achieved was 20 ng L^{-1}. The recovery in samples spiked with three different PQ concentrations was between 88 and 108%.

KEYWORDS: Paraquat; bipyridylium herbicides; immunoassay; europium chelate; time-resolved fluorescence

INTRODUCTION

The herbicide paraquat (PQ) is a quaternary nitrogen compound, active as a redox drug inhibiting reduction of NADP to NADPH during photosynthesis.



Paraquat

It is known to generate superoxide anions in mitochondria and cytosol of yeast and mammalian cells leading to the formation of several reactive oxygen species (*1*, *2*). It is used as a nonselective contact herbicide, in presowing and inter-row weed control of ploughed land, for stubble cleaning, and as a spray for weed control in maize, vineyards, and orchards. It is also applied for pasture renovation and in wedding noncrop areas. Because of its unavailability and its resistance to microbial degradation and breakdown by sunlight, PQ is long-lived. The bound residues transported in runoff with the sediment persist indefinitely. The reported half-life for PQ in soil ranges from 16 months (aerobic laboratory conditions) to 13 years (field study) (*3*).

Because of this widespread use, it often translates from foliage to foodstuffs (for instance to potato tubers before the harvest) (*4*) and to the environment. For protection of drinking waters, water quality criteria have been established for some pesticides, and PQ is included on a list of compounds to be monitored in pollution control. The European Union has established a maximum level of 0.1 μg L^{-1} (*5*), like other common pesticides. Bipyridylium herbicide analysis is not easy: in fact, they are cationic molecules and detection methods are either not sensitive (matrix effects were observed, and only 60 ng L^{-1} was raised in immunoassays) or time-consuming (as they need extensive sample pretreatment procedures). However, several analytical approaches such as derivative spectroscopy (*6*), high-performance liquid chromatography (HPLC) and liquid chromatography−mass spectrometry (*7*−*9*), various electrophoresis systems (*10*−*13*), and immunoassay (*14*−*16*) have been described for these compounds. In this paper, we reported a time-resolved fluoroimmunoassay (TR-FIA) in which we achieve higher sensitivity. In fact, we combine the sensitivity of immunological methods and the sensitivity of time-resolved fluorescent revelation system (*17*). Immunological methods are useful for pollutant determinations in aqueous samples, because very low concentrations can be detected without the need for previous laborious and time-consuming sample volume reductions. Furthermore, a time-resolved system is able to eliminate the interference of background fluorescence. TR-FIA is a very useful method to determine the concentration of widely used herbicides and pesticides, which have raised concerns to warrant large specific monitoring programs, in which a large number of samples must be assayed.

MATERIALS AND METHODS

Materials. All of the chemicals, including standard PQ and the related cross-reacting compounds, bovine serum albumin (BSA) and

* To whom correspondence should be addressed. Tel: ++39-02-28500023. Fax: ++39-02-28500036. E-mail: mariangela.bacigalupo@icrm.cnr.it.
† CNR, Milan.
‡ Università di Bologna.

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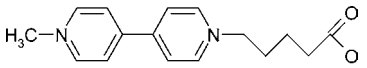
rabbit anti-sheep IgG, were obtained from Sigma-Aldrich (Milan, Italy); the sheep anti-PQ polyclonal antibody (obtained using PQ−ovalbumin conjugate as immunogen) was purchased from Maine Biotechnology Service (Portland, ME). The surface water specimens were collected from four wells and five irrigation ditches in the agricultural area to the south of Milan and from Olona River and Mantua Lake. The water samples were collected in glass vials and filtered to remove particulates. Particulates were discarded, and samples were stored at 4 °C in a refrigerator without any other preliminary treatment. All samples were assayed within a week.

Apparatus. A single photon-counting time-resolved fluorometer (1232 DELFIA Fluorometer; Wallac, Turku, Finland) was used to measure fluorescence; for matrix-assisted laser desorption/ionization (MALDI) time-of-flight measurements, the Voyager-DE pro Applied Biosystems (Foster City, CA) was used.

Synthesis. *4,7-Bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic Acid (BCPDA) and IgG−(BCPDA)$_n$.* The BCPDA and rabbit anti-sheep IgG−(BCPDA)$_n$ conjugate were synthesized as described in the literature (*18*). The value for n (≅36) was assessed as the absorbance of BCPDA at 325 nm; $\epsilon = 1.52 \times 10^4$ M^{-1} cm^{-1}.

Monoquat (MQ). The synthesis of MQ was performed from (4,4′)-bipyridine and iodomethane according to ref *19* dried and stored in a vacuum desiccator until used.

Hapten. CPMQ was obtained from the hydrolysis of 5-(1′-methyl-[4,4′]bipyridinyl-1-yl)pentanoic acid ethyl ester, synthesized using MQ and ethyl 5-bromovalerate as previously described (*19*).



CPMQ

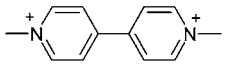
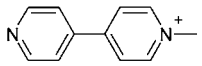
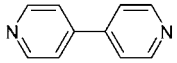
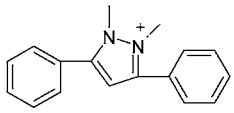
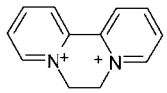
BSA−MQ. CPMQ (15 μmol) was dissolved in 400 μL of anhydrous dimethylformamide and cooled to 4 °C before the addition of 5 μL of tri-*n*-butylamine. After 10 min, 2 μL of isobutylchloroformate was added, and the solution was left for 20 min at 4 °C before being added to 8 mg of BSA dissolved in 2.5 mL of 50% dimethylformamide in aqueous 0.9% NaCl. The pH was adjusted to 8.5 using 1 N NaOH, and the reaction mixture was left overnight at 4 °C before being eluted on Sephadex G-50 with 0.05 M NH_4HCO_3 at room temperature, lyophilized, and stored at 4 °C.

Preparation of Solid Phase. Polystyrene microtiter wells were coated overnight at 27 °C with 200 μL of 0.1 M carbonate buffer, pH 9.0, containing 10 μg/mL of BSA−MQ conjugate. The microtiter wells were washed, and a second coat was made with 250 μL of 2% BSA solution in the same buffer for 4 h at 27 °C. The wells were washed five times with 0.05 M Tris-HCl buffer, pH 7.5, containing 0.9% NaCl and 0.05% NaN_3 and stored dry at 4 °C until use. The CPMQ incorporation, evaluated by MALDI MS, was 13 molar residues per mol of BSA.

Antibody Titer Evaluation. The antibody was assayed by incubation in wells coated with the BSA−MQ compound: 100 μL of serial dilutions of antiserum in buffer (1:250, 1:2500, and 1:25000) was applied to the coated wells, and the antibody bound to the solid phase was revealed by means of rabbit anti-sheep immunoglobulin G covalently bonded to BCPDA. After they were washed, 150 μL of dissociation solution (4mol L^{-1} urea, 1% sodium dodecyl sulfate, and 10^{-6} mol L^{-1} Eu^{3+}) was added to each well. Fluorescence was measured after 10 min, using the excitation wavelength of 345 nm. The delay time was 400 μs after excitation; the emitted light was read at 615 nm. The dilution giving 50% of fluorescence intensity in comparison with 100% of saturate solid phase fluorescence was chosen as the working titer.

TR-FIA. The same PQ standard dilutions and samples were assayed either by solid phase immunoassay (TR-FIA) and HPLC in order to compare the results. TR-FIA was performed with the PQ standard dissolved in tap water; 100 μL of serial dilutions of standard solution between 2 pg and 100 ng was transferred in duplicate into coated polystyrene microwells. A dilution of specific antibody (50 μL in buffer 0.05 M Tris-HCl, pH 7.5, containing 0.9% NaCl and 0.2% BSA at a working titer of 1:10000) was added to all of the wells other than that used for the blank evaluation, to which the same volume of buffer was added. The assay was performed directly in the water matrix by applying 100 μL of samples in duplicate instead of the standards. The wells were washed, and 150 μL of buffer containing an excess of rabbit anti-sheep IgG labeled with BCPDA was added and incubated for 30 min. After the wells were washed, 150 μL of dissociation solution was added to each well and fluorescence was measured as described in the antibody titer evaluation section. As the PQ concentration in the sample used for recovery evaluation was below the detection limit of assay, this sample was spiked by adding 0.25, 25, and 500 μg L^{-1} of the standard. The matrix effect was also assayed in 10 times concentrated samples in order to evaluate if the results might be affected by the high presence of salts or other organic compounds. Samples (50 mL) were partially lyophilized, and the volume was adjusted to 5 mL with distilled water; 100 μL of spiked and concentrated samples was assayed as described above.

Table 1. Percent Cross-Reactivity of PQ-Related Compounds with PQ Specific Antibody Calculated at 50% of Fluorescence Signal Reduction

Compounds	% CR
 Paraquat *1,1′-Dimethyl-[4,4′]bipyridinyl*	100
 Monoquat *1-methyl-[4,4′]bipyridinyl*	2
 *[4,4′]-Dipyridinyl*	0.4
 Difenzoquat *1,2-Dimethyl-3,5-diphenyl-1H-pyrazole*	< 0.01
 Diquat *9,10- Dihydro-8a,10a-diaza-phenanthrene*	< 0.01

RESULTS AND DISCUSSION

Antibody Specificity. The specificity of the polyclonal antibody was evaluated by assaying the cross-reactions of PQ-related compounds (**Table 1**), calculated at 50% of fluorescent signal reduction against the 100% of PQ. Cross-reaction values were reported in **Table 1**. All structurally related compounds did not interfere in the assay even when present at 10−1000 times higher concentrations.

Assessment of Solid Phase. Hapten residues bound to the BSA, evaluated by MALDI MS, result 13 mol/mol of BSA (**Figure 1**). The assay sensitivity was improved by reducing the concentration of BSA−MQ onto the microwell surface. The coating microwell concentration was chosen consistently with

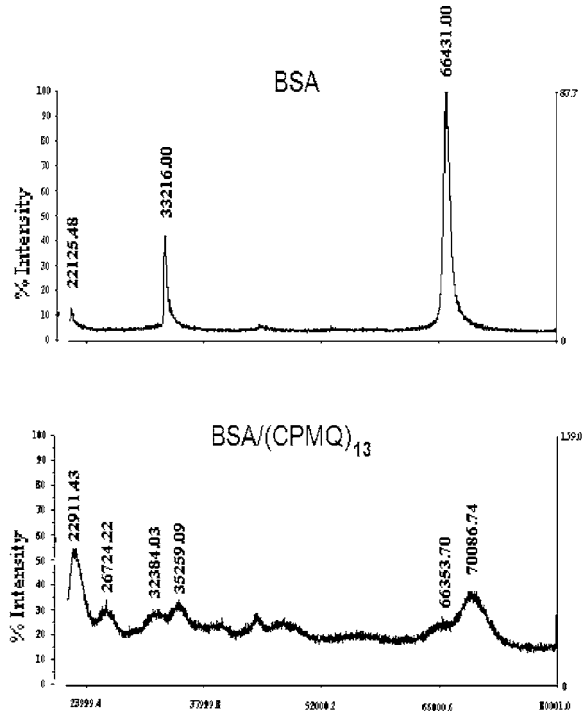


Figure 1. MALDI mass spectrometry for BSA–MQ conjugate in comparison with standard BSA (the peak corresponds to the molecular weight of BSA bound to 13 molecules of CPMQ).

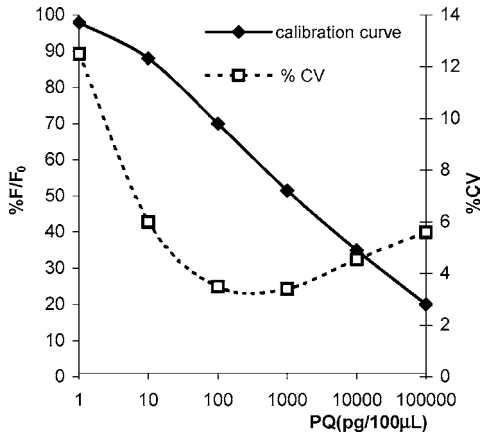


Figure 2. Dose–response curve and precision profile for TR-FIA of PQ. Each point represents the mean of 10 determinations in duplicate.

fluorescent signal intensity: 10 μg mL^{-1} of BSA−MQ was useful for our purposes and was used as coating dilution in the assay.

Calibration Graph. The antibody dilution giving 50% of fluorescence intensity in comparison with 100% of saturated solid phase fluorescence was chosen as the working titer. A dose−response curve is shown in **Figure 2**. The graph was obtained by averaging 10 individual curves normalized by reporting fluorescence values as % F/F_0 where F was the mean of cps for each standard and F_0 was the mean of cps for zero PQ concentration; the blank value (2540 ± 280 cps) was subtracted.

Assay Performances. *Recovery Evaluation.* The accuracy was evaluated by comparing PQ concentrations in seven water samples spiked with three different concentrations of standard PQ: the values obtained by TR-FIA show a good recovery for all sample dilutions (**Table 2**).

Table 2. PQ Values in Seven Water Samples Spiked with Low (0.25 μg L^{-1}), Medium (25 μg L^{-1}), and High (500 μg L^{-1}) Standard Concentrations Assayed by TR-FIA (Mean of 10 Different Assays; Each Sample Was Assayed in Duplicate)

spiked samples	expected (ng)	found (ng) ± %CV	% recovery
tap water	0.025	0.024 ± 0.0018	96.0
	2.50	2.55 ± 0.105	102.0
	50	48.8 ± 2.88	97.6
lake	0.025	0.027 ± 0.0020	108.0
	2.50	2.48 ± 0.190	99.2
	50	53.7 ± 4.830	107.5
lake shore	0.025	0.022 ± 0.0023	88.0
	2.50	2.62 ± 0.258	104.8
	50	52.8 ± 3.200	105.6
well no. 1	0.025	0.025 ± 0.0023	100.0
	2.50	2.48 ± 0.111	96.0
	50	53.3 ± 3.730	106.6
well no. 2	0.025	0.026 ± 0.0026	104.0
	2.50	2.44 ± 0.134	97.6
	50	49.0 ± 3.080	98.0
ditch no. 1	0.025	0.022 ± 0.0023	88.0
	2.50	2.22 ± 0.155	88.8
	50	48.8 ± 3.88	97.6
ditch no. 2	0.025	0.023 ± 0.0020	92.0
	2.50	2.60 ± 0.118	104.0
	50	51.0 ± 4.800	102.0

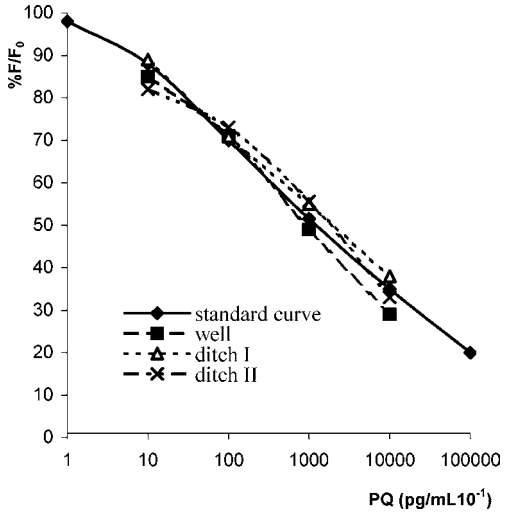


Figure 3. Parallelism of the assay expressed as logit-log function for water samples (two ditches and one well) at different dilutions.

Parallelism Test. To assess the matrix effect, three water samples with high PQ concentrations (100 μg L^{-1}) were diluted 10, 10^2, and 10^3 times with distilled water and results plotted in comparison with a typical standard curve show that the four curves were parallel (**Figure 3**). The same results were obtained from the assay of specimens concentrated 10 times.

Sensitivity. The assay sensitivity was good as shown by a high slope of the calibration curve. The detection limit of the method was determined by calculating the minimum amount of PQ that could be significantly distinguished from zero (mean binding at zero dose at three times the SD). This value, calculated from three curves prepared in duplicate with tap water, was 2 pg/well. Consequently, its high degree of sensitivity also makes it useful for determining PQ levels in drinking water within the European Community limits of 0.1 μg L^{-1}.

PQ concentrations were undetectable in all assayed specimens: The presence of PQ can be evaluated only for some of 10 times concentrated matrices. Concentrated samples from two ditches showed, respectively, 9 ± 1.1 and 4 ± 0.3 ng L^{-1};

samples from wells were not detectable. The only exception was the sample from the Olona River in which 10 ± 0.56 ng L^{-1} of the analyte has been directly documented thanks to the high sensitivity of the described method. In fact, HPLC carried out as described in ref *20* was unable to detect the analyte even in 10 times concentrated samples. Nevertheless, binding of PQ on glass vials has been demonstrated by an isotopic dilution method (*21*), so that the reported value should be inaccurate. To avoid systematic errors in measurements, the method of storage for high-diluted samples should be reconsidered.

Because of its widespread use in agriculture and solubility in water, PQ must be assayed in agricultural countries to verify seasonal variations according to the application periods. Conventional chromatographic methods meet with some difficulties in PQ determination, as PQ is a cationic compound. This assay should thus provide a useful alternative. Many samples can be analyzed simultaneously in a short incubation time, and the reagents used can be easily obtained. Furthermore, as the BCPDA chelate is fluorescent in aqueous solution, it's possible to get a direct fluorescence determination with a good sensitivity at a very high dilution in aqueous samples and it's suitable for a rapid, simple, and cheap screening.

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