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Homogeneous fluoroimmunoassay of a pyrethroid metabolite in urine

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Abstract

Pyrethroids are widely used in agriculture as insecticides. In this study, we describe a simple one-step homogeneous fluoroimmunoassay for the glycine conjugate of phenoxybenzoic acid (PBAG), a putative pyrethroid metabolite that may be used as a biomarker of exposure to pyrethroids. Quenching fluoroimmunoassay (QFIA) is based on the competition of labeled and non-labeled pesticide for binding with antibodies and the resulting calibration curve is based on the relationship between analyte concentration and fluorescence quenching of labeled pesticide by specific antibodies. We developed a QFIA for PBAG in aqueous solution using fluorescein-labeled PBAG and polyclonal antibodies. The estimated IC_{50} (analyte concentration giving 50% inhibition of quenching) for PBAG was 4.5 nM. The detection limit (DL) was 0.9 nM. The dynamic range of the calibration curve was 2–50 nM. The average analytical recovery obtained by applying the method to urine samples (400- or 1000-fold urine dilution) was 85–111%. This demonstrates the QFIA to be a very simple and rapid detection method for PBAG; no washing steps and no enzyme conjugates were required.

Keywords: Homogeneous fluoroimmunoassay; Fluorescence quenching; Pyrethroid metabolite; Polyclonal antibodies; Urine

1. Introduction

Pyrethroids have been one of the most heavily used insecticide classes in the world [1,2]. These neurotoxins act on the axons in the peripheral and central nervous systems by interacting with sodium channels in mammals and insects and cause a series of neurotoxic effects, such as hyperactivity, tremor, ataxia, convulsion and possible paralysis [3–5]. Careful use of pyrethroids in management of both medical and agricultural pests offers many advantages in terms of cost, human safety, and environmental safety. However, production personnel such as farm workers are exposed to pyrethroids during manufacture and application. Although, these compounds are claimed to be safe for humans [6], after exposure, reversible symptoms of poisoning, such as headaches, dizziness, nausea, irritation of the skin and nose and paraesthesia have been reported [7,8]. It was supposed (based on studies of chronic exposure effects [9]) that fenvalerate

Abbreviations: Ab, polyclonal antibody (antibodies) against PBAG; DL, detection limit; DMF, dimethylformamide; ELISA, enzyme-linked immunosorbent assay; F-PBAG, PBAG labeled with fluorescein; IC_{50}, analyte concentration giving 50% inhibition (quenching); PBAG, glycine conjugate of phenoxybenzoic acid, *N*-(3-phenoxybenzoyl)glycine; QFIA, quenching fluoroimmunoassay; r.u., relative units; Tris, tris(hydroxymethyl)aminomethane

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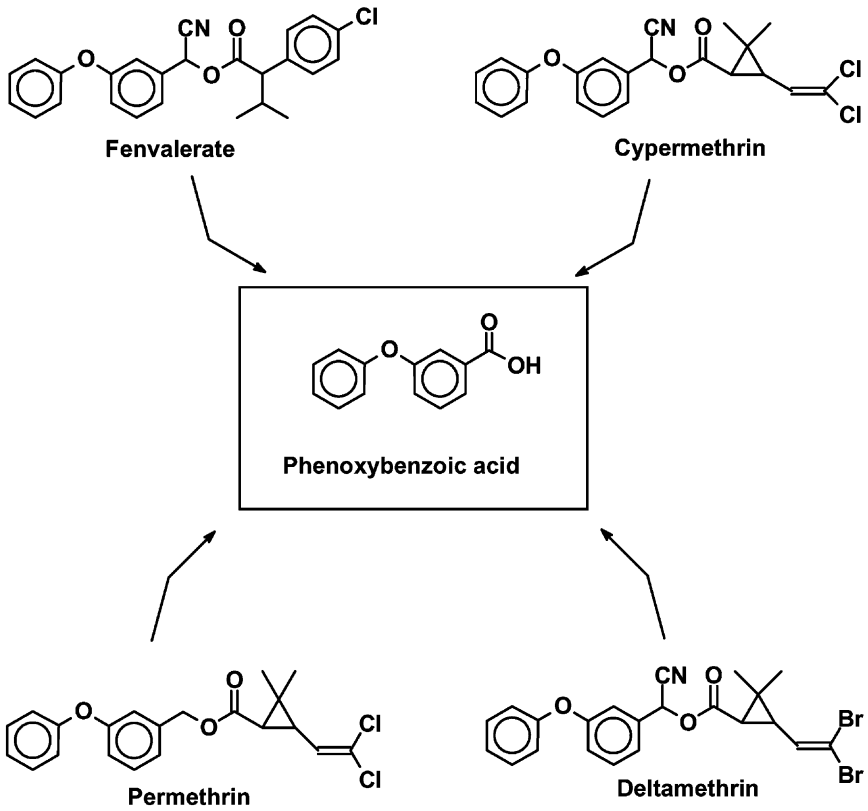


Fig. 1. The structures of some pyrethroids and the major metabolite, 3-phenoxybenzoic acid.

may cause lymph node and splenic damage as well as carcinogenesis. Suppressive effect of high doses of pyrethroids on the immune system was reported [10]. Therefore, it is desirable to develop sensitive and efficient analytical methods for both toxicological and epidemiological monitoring with the goal of optimizing effective use while minimizing exposure.

Studies of pyrethroid metabolism in mammals have revealed that pyrethroids are metabolized rapidly by hydrolytic cleavage of the ester linkage, preceded or followed by oxidation and conjugation [11–15]. The major metabolite is 3-phenoxybenzoic acid (Fig. 1). This metabolite can be conjugated to glucuronic acid and glycine, and then excreted renally [2,16–19]. Although, no study has been reported about the conjugates of pyrethroid metabolites in humans, it has been well established that conjugation with one of a variety of amino acids (such as glycine) is an important biotransformation pathway for xenobiotics containing a carboxylic acid group [20,21].

Analytical methods for detection of pyrethroid metabolites in urine or blood are based on multi-step sample preparation including solid-phase and liquid–liquid extractions and derivatization, followed by instrumental detection using high-pressure liquid chromatography (HPLC) [18] or gas chromatography with mass spectrometry (GC–MS) [22–28]. Although, these methods are very sensitive (detection limit (DL) for pyrethroid metabolites is as low as 0.3–0.5 μg/l in urine [23–26], and 5 μg/l in blood plasma [25]), they are time-consuming and expensive and not suitable for a routine analysis.

Availability of an immunoassay method involving the use of specific antibodies could be a suitable alternative to the chromatographic methods reported for pyrethroid detection. Immunoassay techniques

are widely used in clinical diagnostics, environmental monitoring, food quality, and agriculture. Immunoassays are highly sensitive and selective analytical tools to determine trace chemicals, such as pesticide residues and their metabolites as key urinary biomarkers of exposure [29]. Several immunoassays have been developed for determining pesticide metabolites, including triazines [30,31] and parathion metabolites [32]. Recently, a heterogeneous enzyme-linked immunosorbent assay (ELISA) has been developed for the pyrethroid esfenvalerate (with an $IC_{50} = 30\,\mu g/l$ and a detection limit $= 3\,\mu g/l$ [33]) and for the glycine conjugate of the pyrethroid metabolite 3-phenoxybenzoic acid, PBAG (with an $IC_{50} = 0.4\,\mu g/l$ and quantitation limit $= 1.0\,\mu g/l$ [34]). However, these heterogeneous assays require several washing steps and the use of enzyme conjugates that may be sensitive to matrix effects.

A previous study of the immunochemical reaction between fluorescein-labeled hapten and specific anti-hapten antibodies showed that the formation of the immunocomplex causes the fluorescence quenching of the label [35]. This quenching may be caused by the change of the fluorescein microenvironment to a more hydrophobic one (fluorescein becomes located more closely to the hydrophobic active site of the antibody). The addition of a defined amount of a non-labeled hapten (for example from the standard or sample), which competes with a labeled hapten for binding sites of antibodies, leads to a partial restoration of the initial fluorescence intensity. Thus, quenching fluoroimmunoassay (QFIA) is based on the competition of labeled and non-labeled haptens for binding with antibodies, and the resulting calibration curve is based on the relationship between pesticide concentration and fluorescence quenching by specific antibodies. One can construct a calibration curve using resulting 'steady-state' (close to equilibrium) quenched fluorescence intensity [36,38] or initial quenching rate [37]. Similar quenching effect was observed for binding of coproporphyrin (the analyte which itself is a fluorescent label) to specific anti-coproporphyrin antibodies, and initial quenching rate was used to develop a stopped-flow fluoroimmunoassay for coproporphyrin detection in human urine [39]. Previous QFIAs were performed, in general, in aqueous buffer solution. In some cases, sample matrices were tested, for example stopped-flow QFIAs were demonstrated in juice [37] and urine [39] matrices. A QFIA has also been developed that can be used for routine determination of gentamicin in serum [40,41]. However, stopped-flow assays, being fast and sensitive, required the measurement of the initial reaction rate within the first second and special equipment. QFIA was developed for pesticides propazine [36] and 2,4-dichlorophenoxyacetic acid [37,38] labeled with fluorescein.

In the present study, we describe a simple and rapid one-step homogeneous QFIA for the glycine conjugate of phenoxybenzoic acid (PBAG) that might be used as a biomarker of human exposure to pyrethroids. This is the first example of a steady-state QFIA in presence of such a complex sample matrix as human urine; the interference of urine matrix is discussed. The proposed assay does not require any special equipment; a simple cheap fluorometer that can measure the intensity of fluorescein fluorescence is suitable.

2. Experimental

2.1. Instrumentation

Fluorescence measurements in standard 1 cm cuvettes were performed using a Fluoromax II spectrofluorometer, Jobin Yvon - Spex, USA, at room temperature, at an excitation wavelength of 490 nm and excitation/emission slits at 5 nm each. Fluorescence measurements in 96-well plates ("Costar" black polystyrene non-treated plates) were performed using a Fluostar plate reader (Phenix Research Products, USA) at room temperature, at an excitation of 485 and emission of 538 nm. A Shimadzu UV-2101 PC UV–VIS scanning spectrophotometer was used for absorbance measurements. HPLC analysis was performed using a Capcell Pak C-18 column (UG 120, S-5; Shiseido Co. Ltd., Tokyo, Japan) with a mobile phase of MeOH:H_2O (75:25) and UV detection at 254 nm.

2.2. Reagents

Esfenvalerate and permethrin were obtained from Riedel-de-Haen (Germany). 3-Phenoxybenzoic acid and 3-phenoxybenzyl alcohol were from Aldrich Chemical Co. (USA). Syntheses of *N*-(3-phenoxybenzoyl)glycine (PBAG) and *N*-[(*S*)-4-chloro-α-

(1-methylethyl)benzene-acetyl]glycine were described in [34]. Other chemicals were purchased from Aldrich Chemical Co. (USA) and Fisher Scientific (USA).

Polyclonal antibodies against PBAG (Ab #19) were obtained as described before [34]. Briefly, three New Zealand white rabbits were immunized intradermally with 100 mg of the immunogen (conjugate of *N*-(3-phenoxybenzoyl)-4-amino-L-phenylalanine with fetuin). Two rabbits (#18 and #20) died prior to final bleed. Final serum of the rabbit #19 was used as the specific Ab, and serum collected before immunization from the same rabbit was used as the control Ab. Antibodies were purified by precipitation with half-saturated aqueous ammonium sulfate solution. Following dialysis against 50 mM Tris buffer (pH 8.0), the dialysate was stored as aliquots at −20°C. The antibody concentration was estimated spectrophotometrically at 280 nm, assuming that the absorbance of a 1 mg/ml antibody solution was 1.38 and its molecular weight was 140 kD.

Urine samples were collected from four individuals of different age and gender at different time (mornings, days, evenings) and stored frozen at −20°C or stabilized by chloroform and stored at +4°C.

2.3. Synthesis of F-PBAG

The fluorescein-labeled hapten was synthesized by the *N*-hydroxysuccinimide ester method. *N*-(3-phenoxybenzoyl)glycine (26 mg, 0.1 mmol) was dissolved in 1.0 ml of dry DMF with equimolar *N*-hydroxysuccinimide and a 10% molar excess of dicyclohexylcarbodiimide. After 4 h of stirring at room temperature, the precipitated dicyclohexylurea was removed by centrifugation. The DMF supernatant was added to 35 mg of fluoresceinamine isomer I (0.1 mmol) in 2 ml of methylene chloride, and stirred at 15°C for 8 h. The mixture was then treated with 2 ml of water. The organic phase was then immediately flash-chromatographed on 15 g of silica gel (CH_2Cl_2:EtOAc) to give 48 mg (85%) of the product as a pale yellow solid (after removing the solvent), which was one spot by TLC: $R_f = 0.38$ (EtOAc). The spot was visualized by strong quenching of plate fluorescence at 254 nm and strong fluorescence at 280 nm. This product was verified by ELISA inhibition test [34] and HPLC with a purity of 95%, and characterized by electrospray ion source mass spectrometry.

2.4. Procedure

Stock solutions of PBAG and F-PBAG were prepared in MeOH. All working solutions were prepared in Tris buffer (50 mM, pH 8.0). To prepare calibration standards, the standards were mixed with the working solution of F-PBAG. Each mixture contained the same F-PBAG concentration (20 nM) and different PBAG concentrations in the range 0–50 μM, which was achieved by serial dilutions of the initial PBAG + F-PBAG mixture with working F-PBAG solution, or alternatively 2–5 μl aliquots of a methanolic standard PBAG solution was added to a working F-PBAG solution. The methanol concentration was kept below 0.5% and did not affect fluorescence or antibody activity. Samples were mixed with the working solution of F-PBAG so as to retain the same final F-PBAG concentration as used for calibration standards (20 nM of F-PBAG).

Fluorescence intensity in 1 cm glass cuvette was measured (at excitation 490 nm, emission 512 nm) after mixing of standard/sample PBAG + F-PBAG solution (1 ml) with Ab solution (1 ml; optimized Ab concentration was 150 nM) and incubation of the resulting mixture at room temperature for 20–60 min. Final optimized concentrations in a cuvette were [F-PBAG] = 10 nM and [Ab] = 75 nM. All measurements, except mentioned in Section 3.8 and in Fig. 7B, were performed in a cuvette. Measurements in a 96-well plate (see Section 3.8 and Fig. 7B) were performed at the same concentrations and mixing order, at 10-fold less volume (final volume in a well was 200 μl).

A calibration curve was constructed as plot of fluorescence intensity of the label versus PBAG concentration (concentrations are given per cuvette). Analyte concentration of the unknown sample was calculated by extrapolation from the calibration curve. The IC_{50} was determined from the calibration curve as the analyte concentration giving 50% of the maximal quenching. DL was determined as the analyte concentration corresponding to the fluorescence intensity value at zero analyte concentration plus three times the standard deviation of the response at zero analyte concentration ($n = 11$). Quantification limit was

determined as the analyte concentration corresponding to the fluorescence intensity value at zero analyte concentration plus 10 times the standard deviation of the response at zero analyte concentration ($n = 11$).

3. Results and discussion

The calibration curve was constructed using the 'steady-state' (close to equilibrium) quenched fluorescence intensity. We expressed the quenched fluorescence in the following form: quenched fluorescence, $I_0 - I$, as a percentage of initial fluorescence I_0:

$$\text{fluorescence intensity (\%)} = \frac{I_0 - I}{I_0} \times 100,$$

where I is the fluorescence intensity of the mixture F-PBAG + Ab, and I_0 the fluorescence intensity of F-PBAG in absence of Ab, which was normally the same as fluorescence intensity of F-PBAG+Ab+excess of PBAG (Fig. 2). From Fig. 2, we see that the main change caused by the presence of Ab is the quenching of F-PBAG fluorescence. There was also a slight shift of the emission maximum from 512 to 513–514 nm. Since the quenching efficiency depends on both F-PBAG and Ab concentrations, careful optimization of these parameters was critical.

3.1. Influence of F-PBAG concentration

The concentration of fluorescein-labeled analyte should be as low as possible (depending on the equipment) in order to reach the lowest DL in a competitive immunoassay. At the same time, it should be markedly higher than background fluorescence. The background fluorescence intensity for 50 mM Tris buffer (pH 8.0) using the Fluoromax fluorometer was about 0.5 relative units (r.u.) at the chosen excitation and emission wavelengths. The blank signal obtained for antibodies in the absence of F-PBAG was about 0.7 r.u. In this study, the fluorescence of the fluorescein label that can be accurately detected

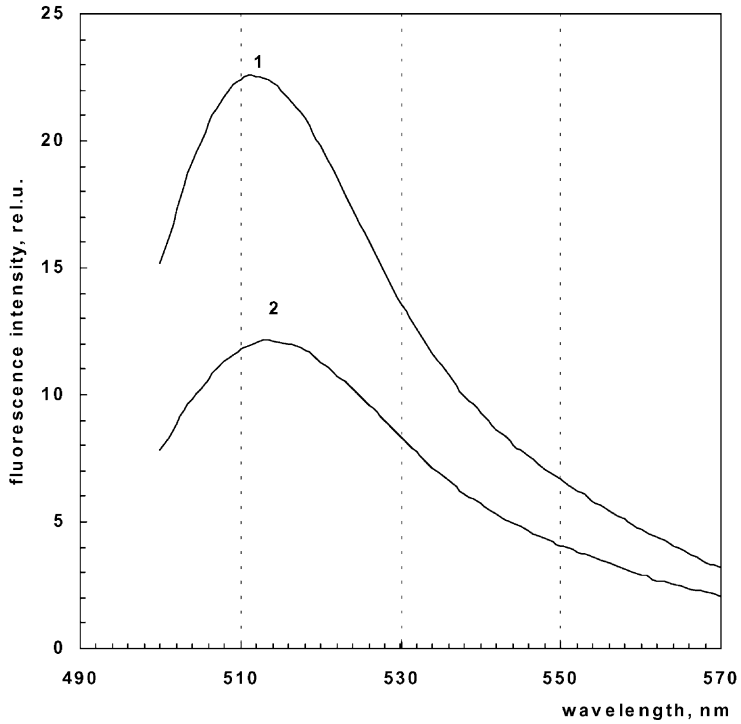


Fig. 2. Fluorescence emission spectra of F-PBAG (1) and F-PBAG + Ab (2). [F-PBAG] = 10 nM, [Ab #19] = 75 nM, excitation wavelength 490 nm, in 50 mM Tris, pH 8.0.

was chosen to be around 15 r.u. (after quenching), which corresponded to 10 nM of F-PBAG in the cuvette.

The working F-PBAG concentration in a 96-well plate (10 nM) was chosen similarly for optimized excitation and emission wavelength for the reader used (emission used was 538 nm instead of closer to maximum of fluorescein fluorescence 515 nm because of much larger background/signal fluorescence ratio at 515 relative to 538 nm).

3.2. Influence of Ab concentration

The Ab concentration has an effect on the sensitivity and background signal of the assay. A low concentration of Ab results in an insufficient level of Ab to F-PBAG binding to create a detectable signal. A high concentration of Ab results in a high background signal and prevents the detection of analyte binding to the Ab. An optimized Ab concentration is one at which there is 60–80% of bound F-PBAG (Fig. 3). Therefore, the optimal Ab concentration used in this study was 75 nM (both for cuvette and 96-well format).

3.3. Kinetics estimation

The immunochemical reaction between fluorescein-labeled hapten and specific antibodies is characterized by a very high initial rate of fluorescence quenching.

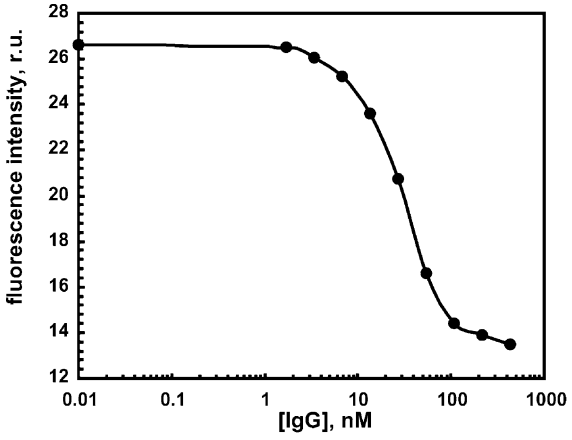


Fig. 3. Antibody dilution curve obtained for Ab #19 in 50 mM Tris, pH 8.0, [F-PBAG] = 10 nM. Optimal [Ab] = 75 nM (at about 80% maximal quenching).

The kinetic behavior of the system was estimated by fast manual mixing of F-PBAG and Ab solutions (1:1 v/v) and immediate recording of the resulting kinetic curve (Fig. 4A). The data show that a 20–40 min incubation gives quenched fluorescence close to equilibrium. Longer incubation of the mixture at room temperature leads to Ab inactivation and decreasing of the quenching efficiency (Fig. 4B). Short incubation leads to larger errors in quenching determination due to the bigger difference between incubation times for each separate sample. Thus, the optimal incubation time was about 30 min, but increasing the incubation time up to 24 h (at +4°C) was also possible (Fig. 4B).

3.4. Calibration graph and detection limit

The calibration curve obtained for PBAG in buffer solution (50 mM Tris, pH 8.0) at incubation time 0.5 h is presented in Fig. 5 (black circles). The DL calculated from this graph was 0.9 nM, which corresponds to 0.25 μg/l. The quantification limit was 2 nM (0.54 μg/l). The IC_{50} was found to be 4.5 nM (1.2 μg/l).

3.5. Effect of urine matrix

In order to study the potential effect of the urine matrix on the calibration curve, calibration standards were run in the absence and presence of 0.1–5% urine. As expected, since the method is homogeneous (without any washing steps) and rather sensitive, the presence of urine led to a significant contribution to F-PBAG fluorescence even at low urine concentration (0.1%). This led to an overestimation of the analyte concentration of 10-fold and more.

We found a very simple way to correct the calibration curve in the presence of the urine matrix. The correction procedure involved subtraction of the urine background fluorescence (which was measured for the same dilution of the urine sample alone, without F-PBAG and Ab) from the fluorescence of F-PBAG + PBAG + Ab mixture in the presence of urine. After such correction, the calibration curve in presence of urine (0.1%) became suitable for analysis (Fig. 5).

Calibration curves at increased urine percentages are shown in Fig. 6 (studies on one urine sample). Following correction, increasing urine concentration up to 5% still allowed rough estimation of PBAG at its

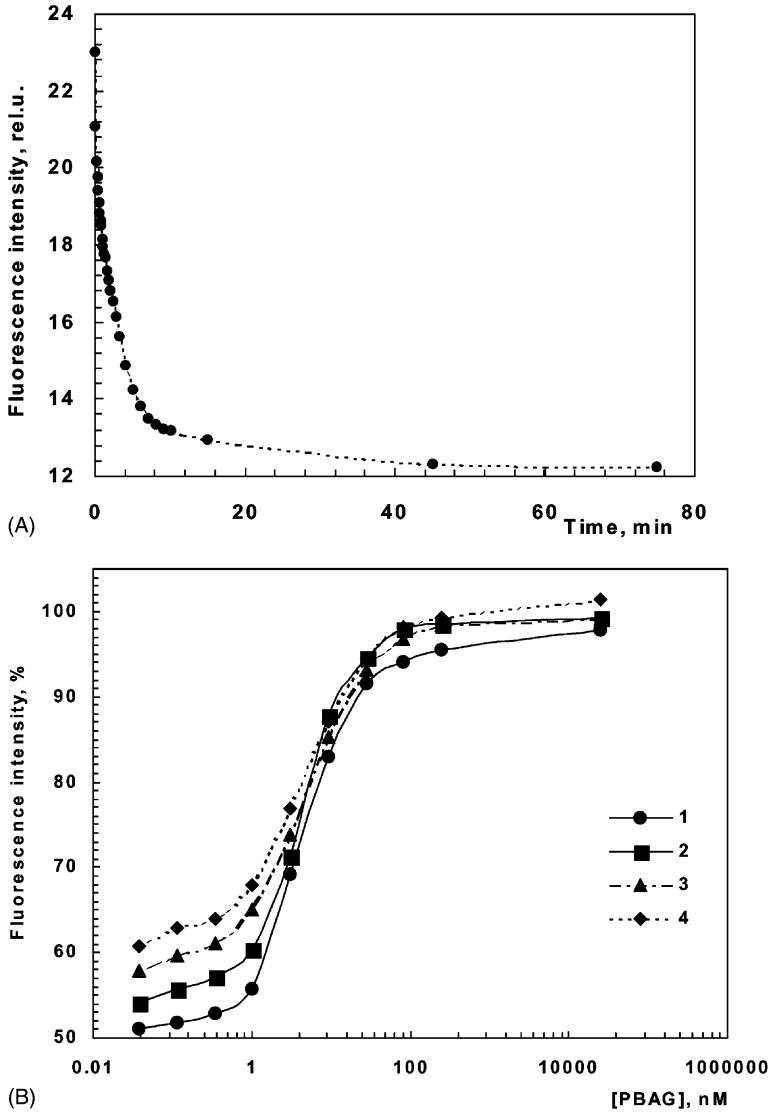


Fig. 4. Time effect on quenching: (A) kinetics of F-PBAG fluorescence quenching after mixing with Ab ([F-PBAG] = 10 nM, [Ab] = 75 nM, in 50 mM Tris, pH 8.0, room temperature); (B) calibration curve for PBAG at different incubation times: (1) 20 min (room temperature), (2) 1 h (room temperature), (3) 2 h (room temperature), (4) 24 h (+4°C).

high concentrations. Urine at 20% gave an incorrect calibration even after correction, although it did not markedly change the pH of the solution.

3.6. Recovery study

We have studied the PBAG recovery using four different urine concentrations: 0.1, 0.25, 0.5, and 5%. Twelve urine samples were spiked with different amounts of PBAG to give the final concentrations in the cuvette: 1, 2.5, 10, 40, and 100 nM. Untreated urine was used in each analysis to measure background signal. The best recoveries were obtained at 0.1 (see Table 1) and 0.25% urine samples with 2.5 and 10 nM concentration of PBAG in the cuvette, which is above the quantification limit and close to

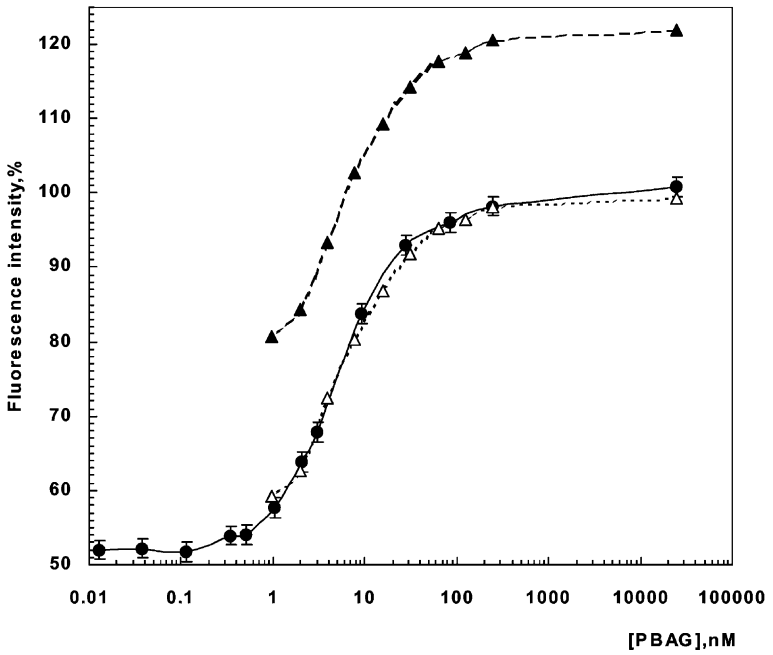


Fig. 5. Effect of urine matrix on calibration graph (incubation time 30 min): (●) calibration curve in the absence of urine, triplicate; (▲) calibration curve in the presence of 0.1% urine, not corrected; (△) corrected calibration curve in the presence of 0.1% urine.

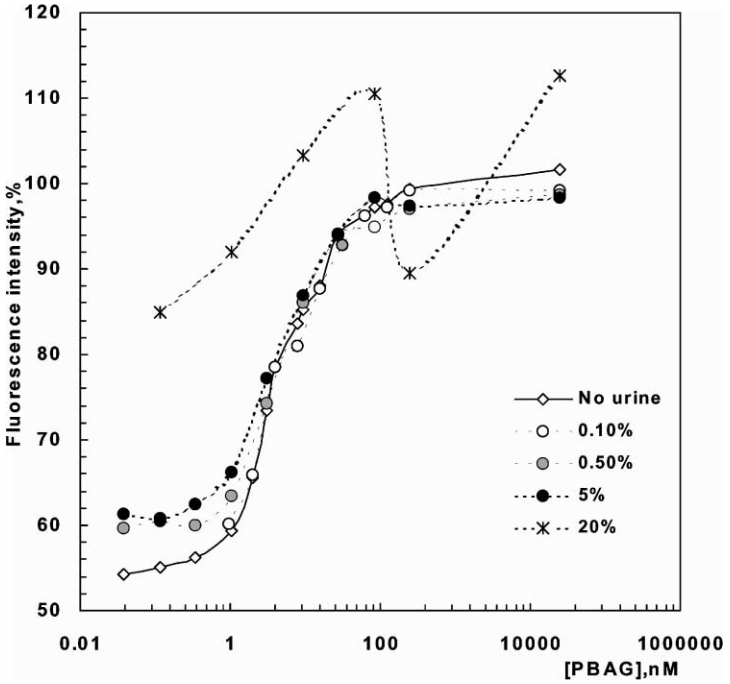


Fig. 6. Calibration graph (corrected) in the presence of different urine concentrations (incubation time 45 min).

Table 1
Analytical recoveries of PBAG added to urine (0.1% urine) at highly variable background fluorescence

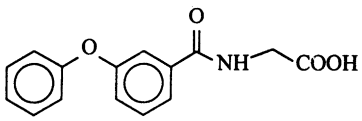
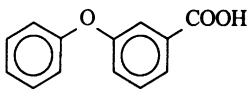
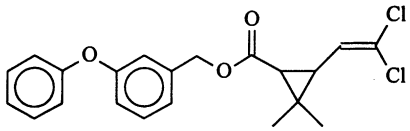
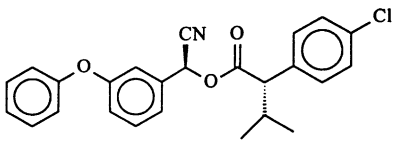
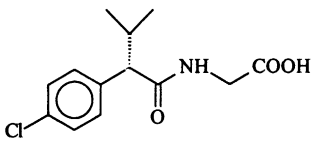
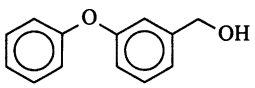
Sample	Spiked to [PBAG] in urine/[PBAG] in cuvette										Urine background fluorescence (%)[a]
	1 μM/1 nM		2.5 μM/2.5 nM		10 μM/10 nM		40 μM/40 nM		100 μM/100 nM		
	Found (nM)	Recovery (%)	Found (nM)	Recovery (%)	Found (nM)	Recovery (%)	Found (nM)	Recovery (%)	Found (nM)	Recovery (%)	
Water	0.96	96	2.65	106	10.1	101	38	95	101	110	0
Urine-1	1.18	118	2.68	107.2	12.5	125	48	120	125	117.5	19
Urine-2	0.68	68	2.36	94.4	9	90	41	102.5	90	80	3
Urine-3	0.65	65	2.84	113.6	12	120	36	90	120	80	1.8
Urine-4	0.9	90	2.2	88	9.5	95	34	85	95	85	20
Urine-5	1.35	135	2.8	112	12.5	125	42	105	125	120	1.7
Urine-6	1.25	125	2.5	100	14	140	34	85	140	200	9
Urine-7	1.3	130	2.26	90.4	11.5	115	28	70	115	400	29
Urine-8	1.05	105	2.77	110.8	11	110	38	95	110	200	5
Urine-9	0.95	95	2.72	108.8	11.5	115	45	112.5	115	150	4.5
Urine-10	0.8	80	2.6	104	10	100	29	72.5	100	250	4.8
Urine-11	1.4	140	2.7	108	9.5	95	39	97.5	95	110	63
Urine-12	1.1	110	2.83	113.2	10.5	105	45	112.5	105	145	3

[a] Percentage of F-PBAG fluorescence (10 nM).

Table 2
Average PBAG recovery from urine at different dilutions

Urine dilution (concentration)	Spiked concentration of PBAG		Average recovery ± S.D.	Number of urine samples
	In urine (μM)	In cuvette (nM)		
1:1000 (0.1%)	1	1	105 ± 26	12
	2.5	2.5	104 ± 9	12
	10	10	111 ± 15	12
	40	40	96 ± 16	12
	100	100	161 ± 92	12
1:400 (0.25%)	1	2.5	93 ± 16	12
	4	10	91 ± 15	12
	16	40	85 ± 23	12
1:200 (0.5%)	0.2	1	103 ± 83	6
	2	10	93 ± 41	9
1:20 (5%)	0.05	2.5	150 ± 200	6
	0.2	10	27 ± 28	4
	2	100	6 ± 5	4

Table 3
Cross-reactivities of PBAG-related compounds

Cross-reagent	Structure	IC_{50} (nM)	CR (%)	
			This work (QFIA)	[34]
N-(3-phenoxybenzoyl)glycine (PBAG)		4.5	100	100
3-Phenoxybenzoic acid		1300	0.35	0.02
Permethrin		>25000	<0.02	NA
Esfenvalerate		325	1.4	0.02
N-[(*S*)-4-chloro-α-(1-methylethyl) benzene-acetyl]glycine		>25000	<0.02	<0.01
3-Phenoxybenzyl alcohol		72	6.2	0.01

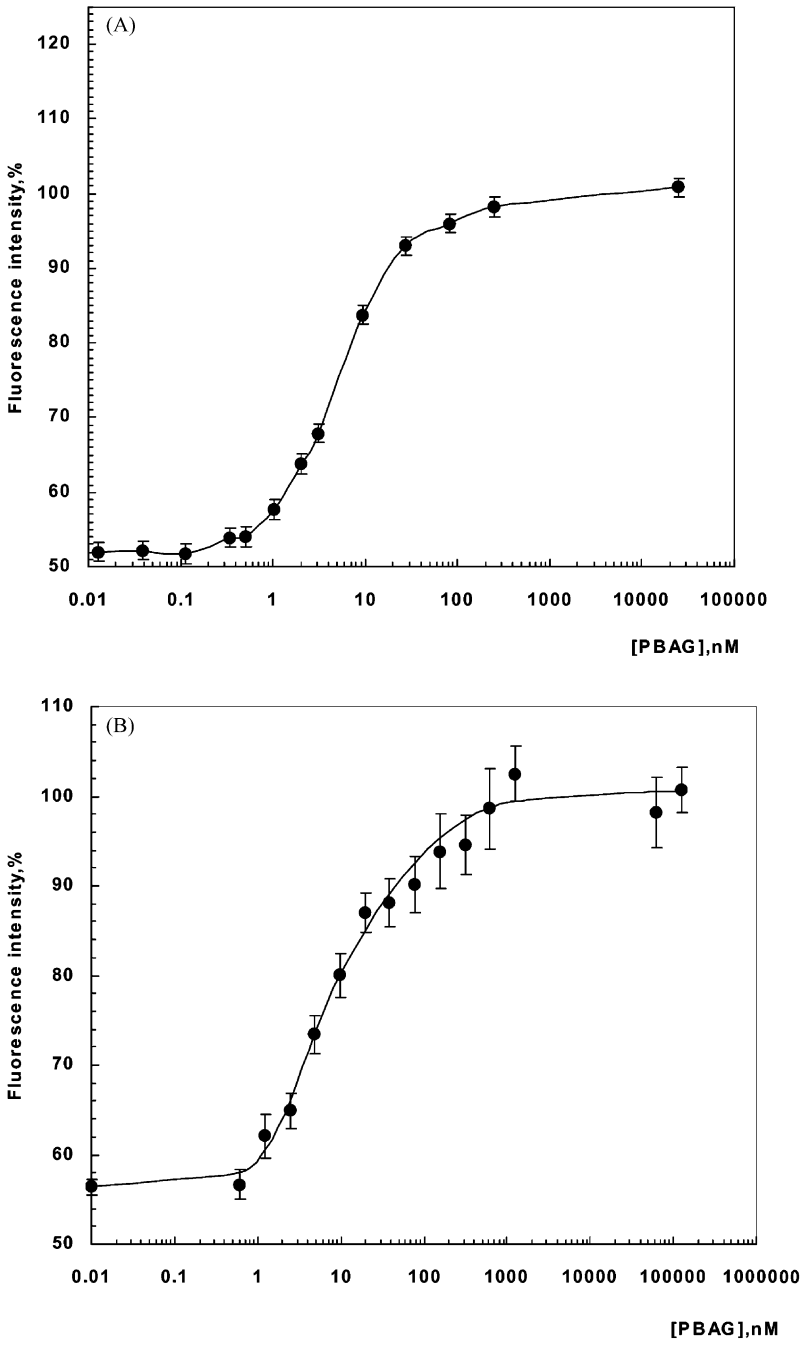


Fig. 7. Calibration curve for PBAG using: (A) the cuvette format (triplicates); (B) the 96-well plate format (quadruplicates). [F-PBAG] = 10 nM, [Ab] = 75 nM, incubation time 20 min.

Table 4
Cuvette/plate formats for QFIA of PBAG

Instrument	Format	Sensitivity (minimal F-PBAG concentration[a])	IC_{50}		DL		Notes
Fluorometer (Fluoromax-2)	1 cm cuvette	10 nM	4.5 nM	9 pmol per cuvette	0.9 nM	1.8 pmol per cuvette	
Plate attachment to a fluorometer (Fluoromax-2–Micromax)	96-well plate	2 μM	NA	NA	NA	NA	Sensitivity may be improved (background signal decreased) by using a cut-on long pass filter
Fluorescent plate reader (Fluostar)	96-well plate	10 nM	8 nM	1.6 pmol per well	1.5 nM	0.3 pmol per well	Background may be decreased by using longer wavelength emission (not in maximum)

[a] Minimal F-PBAG concentration to be correctly determined, at least two times higher signal compared to the background signal.

IC_{50}. Reasonable recoveries were obtained for the lowest PBAG concentration of 1 nM that is close to the DL. At the higher urine concentrations of 0.5 and 5%, which give higher background fluorescence, reasonable recovery could be obtained only for some urine samples with lower background. At 0.5% urine (which gave background fluorescence in the range 10–200%), recoveries varied from 0 to 180% when spiked to 0.2 μM in urine, or 1 nM in a cuvette (six urine samples tested), and from 10 to 150% when spiked to 2 μM in urine, or 10 nM in a cuvette (10 urine samples tested). Lower recoveries were obtained for the urine sample with higher background, especially if the background was 100% or more. Thus, urine exhibiting a 200% background gave 10% recovery, two urine samples with 94 and 117% background gave 60 and 80% recoveries, and the other seven urine samples with backgrounds of 10–35% gave recoveries of 90–150% (spiked to 2 μM in urine, or 10 nM in a cuvette, at 0.5% urine). No correlation between background fluorescence and recovery was observed at background values of less than 50%. At a high urine concentration of 5% (four samples tested, background up to 980%), no reasonable recoveries were obtained.

The effect of urine dilution on recovery and precision was estimated by averaging the single recovery experiments for different urine samples; the results are given in Table 2. Low urine concentrations can be used for detection of 1–10 nM PBAG (spiking urine to 1–10 μM correspondingly). Higher urine concentrations lead to a decrease in precision and an increase in the recovery error.

3.7. Cross-reactivity study

The cross-reactivity for similar to PBAG compounds was evaluated by using the following equation: percent cross-reactivity (CR) = 100 × (IC_{50} of the cross-reagent/IC_{50} of PBAG). Table 3 shows the results obtained for each cross-reactant tested. Cross-reactivities previously determined [34] using a heterogeneous ELISA method are also given. Although, all cross-reactivities determined by QFIA were slightly higher than those determined with the heterogeneous ELISA, the cross-reactivity pattern was the same. No cross-reactivity was detected with 3-phenoxybenzoic acid, permethrin, esfenvalerate, and *N*-[(*S*)-4-chloro-α-(1-methylethyl)benzene-acetyl]glycine, and only slight cross-reactivity (6%) with 3-phenoxybenzyl alcohol.

3.8. Throughput

Since, we have used equipment that only allow measurements to be conducted sequentially, the time required to measure each sample was about 5 s, and the practical sample throughput, including the incubation time and changeover in the system, was about 25 samples/h. However, this value can be dramatically increased if using a fluorescence plate reader and/or automated pipetting. Taking into account that no washing steps are needed — simply mixing, incubating 20 min, and measuring — hundreds of samples could be measured per 1 h.

The assay was optimized on a standard fluorometer (Fluoromax) in a 1 cm glass cuvette. Using the same conditions, we compared the assay in a 96-well format using a Fluostar fluorescent reader (Fig. 7 and Table 4). Such a format facilitates complete sample automation and allows a far higher sample throughput and lower reactant volumes. However, some loss in precision may occur (compare error bars for calibration curves in a cuvette and in a 96-well plate, Fig. 7).

4. Conclusions

A simple and rapid one-step homogeneous QFIA for the pyrethroid metabolite, glycine conjugate of 3-phenoxybenzoic acid, is described. This is the first demonstration of using a steady-state QFIA in presence of a human urine sample matrix. The proposed assay does not require any special equipment; a simple, cheap fluorometer suitable for measurement of the intensity of fluorescein fluorescence can be used. QFIA exhibited a high selectivity for the target PBAG analyte; no or little cross-reactivity was measured to the parent pyrethroids and other related metabolites. Although, the sensitivity of the assay is not high in urine, the assay is very simple and rapid, and would be adaptable to automation and direct sample analysis.

The urine matrix studied drastically increases the DL; however, at 400–1000-fold dilution, QFIA can be used for determination of more than 1 μM (0.27 μg/ml) of PBAG in human urine. This value

is close to the concentration of pyrethroid metabolites detected in urine of highly exposed pest control operators, 0.277 μg/ml [24,25]. QFIA may be used as a rapid screening method to separate the samples that can be further analyzed by more sensitive instrumental or ELISA methods. For example, gas chromatography–tandem mass spectrometry detects less than 0.3 ng/ml of pyrethroid metabolites in urine after solid-phase, liquid–liquid extraction steps and derivatization step [42], and ELISA allows detection of 5 ng/ml of PBAG in urine, which can be improved up to 1 ng/ml when using a solid-phase extraction step [34]. Since the DL of QFIA for PBAG in buffer solution is 0.9 nM (0.25 ng/ml), a similar solid-phase extraction step can be used during urine sample preparation to decrease the DL for QFIA in urine if needed and may decrease background fluorescence further improving detectability. Since PBAG is charged under basic conditions, many interfering materials can be removed by simple partition or an ion-exchange solid-phase system.

Alternatively, the high background of the urine matrix could be avoided by using other fluorescent labels. Human urine and other complex matrices are known to have high and variable levels of interfering materials which either quench or fluoresce. Higher sensitivity in urine also can be obtained by using alternate fluorescent labels that have optical characteristics different from those of interfering materials in the urine. More sophisticated reading systems, such as time-resolved fluorescence that also take advantage of the unique optical properties of reporters may also improve sensitivity. Labels with longer excitation and emission wavelengths and larger Stokes' shift, and phosphorescent long-lived labels are of special interest. Such developments currently are underway in this and other laboratories.

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