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Determination of carbofuran in water by homogeneous immunoassay using selectively conjugate mastoparan and terbium/dipicolinic acid fluorescent complex

Maria Angela Bacigalupo *, Giacomo Meroni, Renato Longhi

Istituto di Chimica del Riconoscimento Molecolare, CNR, via M. Bianco 9, Milan 20131, Italy

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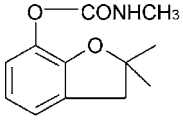
Abstract

Homogeneous immunoassay (LITRFIA) for carbofuran (CF) determination was performed using liposomes and mastoparan (Mast) conjugate as cytolitic agent. Mast was conjugated to the 5-(2-2-dimethyl-2,3-dihydro-benzofuran-7-yloxy)-pentanoic acid (CPCF) both randomly and selectively to a single (V^1- or K^4-) amino-group. The conjugated compounds have been tested for the cytolytic activity on liposomes trapping Tb/citrate complex. Dipicolinic acid (DPA) was used as fluorescent chelating agent. The CPCF–V^1–Mast derivative (retaining almost the same lytic activity as Mast) was used in the immunoassay in competition with standard CF. Liposome lysis was proportional to the standard concentrations in a dynamic range between 10 pg and 10 ng. Assay has been performed for tap water analysis and for 10 real samples taken from an agricultural area to the south of Milan. Recovery in samples spiked with two different CF concentrations was between 92.5 and 105%.

Keywords: Carbofuran; Mastoparan; Liposomes; Lantanide fluorescence; Dipicolinic acid

1. Introduction

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl-methylcarbamate) is a pesticide worldwide used to control soil and leaves-feeding insects and nematodes.



Carbofuran (CF)

CF toxicity is very high in human and birds, as it is a potent inhibitor of cholinesterase, by binding to the serine residues [1]. It is widely used and can be found as pollutant in the air, water, soil, and foods. Determination of pesticide residues in water from field application is generally restricted to area around to the site of application. However, contamination may be more extensive [2] and reach aquifer confining systems, that requires large-scale monitoring programs; so an increasing interest exists in making analytical methods more practical, less time-consuming and with simple laboratory equipment. For the reason given above and because of the great potential health hazards, many assays [3,4] have already been developed to evaluate CF concentrations in water and foods, either by gas-chromatography [5,6], electro-chromatography [7–10], HPLC [11–14] or immunoassay [15–17]. The last one is 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 reduction and many samples can be processed simultaneously. However, even if immunoassays are rather widely used in clinical chemistry they have not yet gained a similar diffusion in environmental and food analysis.

In this paper we reported a homogeneous time-resolved fluoroimmunoassay (LITRFIA) in which we raised several advantages: we combine sensitivity of immunological methods with reduction of background signal by using time-resolved fluorescence and we overcome the disadvantages of the separation of bound and unbound labelled immunoreagent by using liposomes. We used Mast (a 14-amino acid polypeptide from wasp venom) selectively conjugated to the hapten as a cytolytic agent. Furthermore, we used as marker dipicolinic acid (DPA) and lipo-

* Corresponding author. Fax: +39 02 28901239.
E-mail address: mariangela.bacigalupo@icrm.cnr.it (M.A. Bacigalupo).

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somes trapping Tb/citrate complex that are simple and cheap reagents [18].

2. Experimental

2.1. Materials

All of the common chemicals, including bovine serum albumin (BSA), goat anti-rabbit IgG, standard CF and the related cross-reacting compounds: 2,3-dihydro-2,2-dimethyl-7-benzofuranol; bendiocarb; carbaryl; methiocarb; propoxur and aldicarb were obtained from Sigma–Aldrich (Milan, Italy). The rabbit anti-carbofuran polyclonal antibody (obtained using a carbofuran derivative conjugated to KLH as immunogen) was purchased from Europa Bioproducts (Cambridge, UK); 9-fluorenylmethoxycarbonil (Fmoc)-amino acid derivatives; *N*-α-Fmoc-*N*-ε-1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene) ethyl-L-lysine (Fmoc-Lys(Dde)-OH); Rink-amide MBHA resin; 2-chlorotrityl chloride resin; Novasyn TRG® resin; 1-hydroxybenzotriazole (HOBt); 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were from NovaBiochem (Läufelfingen, Switzerland); surface water specimens were collected from Olona River and two wells and five irrigation ditches in the agricultural area to the south of Milan. The water samples were collected in glass vials, filtered and stored at 4 °C until use.

2.2. Apparatus

A single-photon-counting time-resolved fluorometer (1232 DELFIA Fluorometer; Wallac, Turku, Finland) was used to measure fluorescence. MALDI-TOF MS measurements were performed with a Voyager-De pro Applied Biosystems (Foster City, CA, USA). NMR spectrum was obtained using a Gemini 300 spectrometer, Varian Medical Systems (Cernusco, Milan, Italy).

2.3. Syntheses

2.3.1. Liposomes trapping the Tb/citrate complex

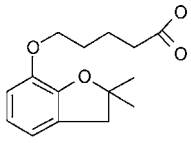
Unilamellar vesicles were prepared using a detergent dialysis technique [19] with the following modifications. Phosphatidylcholine from fresh egg yolk (25 mg) and phosphatidylethanolamine (2.5 mg) dissolved in 550 μL chloroform and 125 μL methanol were mixed with 4.3 mg cholesterol dissolved in 600 μL chloroform. The mixture was first dried to a thin film under a nitrogen stream, and then further dried under reduced pressure for 4 h to remove any organic solvent residues. The phospholipid/cholesterol film was again dissolved in 3.8 mL of 0.2 M octylglucoside solution, leading to a lipid-detergent solution in which 12 mg of sodium citrate and 1.5 mg $TbCl_3$ were dissolved. The solution was dialysed against 0.05 M Tris–HCl, pH 7.5, at 4 °C; in order to remove the Tb^{3+} ions from the outside wall of liposomes, the dialysate was gently shaken with 1 mL of 0.01 M EDTA for 5 min and then chromatographed on a column of Sepharose CL-4B, eluting with the same buffer, to remove the EDTA. The liposomes were stored at 4 °C under nitrogen and diluted 1:10 with Tris–HCl buffer before use.

2.3.2. Mastoparan

The Mast carboxyamidated peptide was assembled by means of a Biosystem 433A peptide synthesiser using the stepwise solid phase Fmoc method [20,21] on 0.5 g of Novasyn TRG® resin (100 μmol). The sequence was:

VDWKKIGQHILSVL-NH_2
Mastoparan (Mast)

2.3.3. Hapten



5-(2-2-dimethyl-2,3-dihydro-benzofuran-7-yloxy)-pentanoic acid (CPCF)

CPCF was obtained from 5-(2-2-dimethyl-2,3-dihydro-benzofuran-7-yloxy)-pentanoic acid ethyl ester intermediate, as described below.

2,3-Dihydro-2,2-dimethyl-7-benzofuranol (6.1 mmol) was dissolved in 10 mL of absolute ethanol containing 6.9 mmol of sodium. Ethyl 5-bromovalerate (6.9 mmol) was added and mixture was heated at reflux for 2 h under nitrogen. Purification by column chromatography (2 cm × 30 cm) on silica gel (230–400 mesh) eluted with 10% methanol in chloroform produced 1.2 g of 5-(2-2-dimethyl-2,3-dihydro-benzofuran-7-yloxy)-pentanoic acid ethyl ester. This compound was unequivocal on TLC plates (silica gel F_{254}) with an Rf = 0.89 (methanol:chloroform 1:9). The compound was added to 50 mL of methanol and 10 mL 2 M NaOH and the solution was heated at reflux for 1 h. Solution was acidified to rise pH 5 with acetic acid, diluted to 200 mL with water and than extracted with ethyl acetate. The solvent was removed to yield 980 mg of the product chromatographically unequivocal on TLC; Rf = 0.64 (methanol:chloroform 1:9); ^{1}H NMR (acetone-d_6) δ: 1.44 (6H, s), 1.53–1.80 (4H, m), 2.36 (2H, t), 3.02 (2H, s), 4.04 (2H, t), 6.73–6.85 (3H, m).

2.3.4. Randomly conjugated Mast–CPCF

Randomly conjugate peptide was obtained from CPCF (15 μmol) dissolved in 400 μL anhydrous dioxane, and cooled to 4 °C before the addition of 5 μL tri-*n*-butylamine. After 10 min, 2 μL isobutylchloroformate was added, and the solution was left for 20 min at 4 °C before being added to Mast (2.5 μmol) dissolved in 2.5 mL 50% dioxane, in aqueous 0.9% NaCl. The pH was adjusted to 8.5 using 1N NaOH, and the reaction mixture left overnight at 4 °C before being eluted on Sephadex G-10 with 0.05 M Tris–HCl buffer pH 7.5, and stored frozen at −20 °C. The CPCF molar residues per mol of Mast were evaluated by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry.

2.3.5. Selectively conjugated compounds

Selectively conjugated compounds (Mast–V^1–CPCF and Mast–K^4–CPCF) were performed manually: coupling reaction was carried with the HBTU/HOBt activation procedure [22]. Mast–K^4–CPCF was assembled using Fmoc-

Lys(Dde)-OH as protecting groups, as described in Ref. [23].

2.3.6. Activity evaluation

The lytic activity of each derivative was measured as follows: 100 μL of 10-fold diluted liposome suspension in 0.05 M Tris–HCl buffer, pH 7.5, containing 0.9% NaCl, were incubated in polystyrene microwells with 500 ng of standard Mast in 10 μL Tris–HCl buffer or with 10 μL Mast derivative solutions, corresponding to the 500 ng of peptide. After 10 min, 20 μL of 15×10^{-3} M DPA in buffer solution were added and fluorescence was measured using a single-photon-counting time-resolved fluorometer at 545 nm; the delay time was 500 μs and the counting time 1400 μs.

2.3.7. Antibody titre evaluation

The antibody was assayed by incubation with Mast derivatives: 100 μL of buffer or 100 μL of serial dilutions of antiserum in buffer (1:500, 1:1000 and 1:5000) were applied to the wells with 0.5 μg of each Mast derivative. After 10 min, 10 μL of liposome dilution was added and the microwells incubated at 30 °C for 15 min; 20 μL of 15×10^{-3} M DPA in buffer solution were then added and fluorescence was measured. Dilution giving about 90% reduction of fluorescent signal was chosen to use in immunoassay.

2.3.8. LITRFIA

Homogeneous immunoassay was performed with the CF standard dissolved in tap water; 100 μL of serial dilutions of standard between 10 pg and 10 ng were transferred in duplicate into polystyrene microwells. A dilution of specific antibody (50 μL in buffer 0.05 M Tris–HCl, pH 7.5, containing 0.9% NaCl at the working titre of 1:1000) 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 river and ditch samples were assayed with 100 μL of sample applied in duplicate instead of the standard. For recovery evaluation, the samples were spiked by adding 2 and 50 μg/L of CF standard. Mast–V^1–CPCF (10 μL, corresponding to 0.5 μg of peptide) was pipetted into wells and after 5 min incubation, 20 μL of diluted Tb/citrate liposomes were added. After 10 min, fluorescence was determined as described in Section 2.3.6.

Influences of the solvents on the fluorescence signal were evaluated by adding to the standards methanol or dimethylformamide (DMF) at different concentrations between 0.5 and 10% v/v.

In order to evaluate the ionic strength influence on assay, standard curve was performed with the standard dilutions in NaCl from 0 to 500 mM, buffered at pH 7.5 with 0.05 M Tris–HCl.

3. Results and discussion

3.1. Liposome lysis and fluorescence

CPCF molar incorporation for randomly conjugate Mast–CPCF has been assayed by MALDI mass spectrometry. The graph shows two peaks corresponding to Mast conjugated with one (MW = 1882.02) or two (MW = 2150.13) molecules of CPCF. The lysis and inhibition have been average values. The lower % inhibition could be ascribed to the presence of a small quantity of unconjugated Mast.

The three derivatives Mast–CPCF, Mast–V^1–CPCF and Mast–K^4–CPCF were assayed for liposomes lytic activity; fluorescence values were compared with those of liposomes incubated in the same way with a solution of Brij 5% in water. Lysis was reported as percent fluorescence values expressed as counts per second (arbitrary units) over the value of the completely lysed liposomes, after subtraction of blank according to the formula:

$$\%\ \text{lysis} = \frac{100 \times (\text{cps of sample} - \text{cps of intact liposomes})}{\text{cps after detergent lysis} - \text{cps of intact liposomes}}$$

The percent of lysis for each compound has been reported in Fig. 1.

Inhibition of lysis by different dilutions of specific antibody has been shown in Table 1. Both randomly coupled Mast–CPCF (% lysis = 75 ± 3.8) and Mast–K^4–CPCF (% lysis = 74 ± 4.2) are suitable for use in competitive immunoassay. Mast–V^1–CPCF was chosen because of its higher lytic activity that increases assay sensitivity.

Liposomes entrapping Tb/citrate complex are easy to prepare and have a good stability (at least six mounts under nitrogen). Nevertheless, the fluorescence intensity of the Tb^{3+} ions (inside the liposomes) on its own is very low, but is enhanced 10^4 times after interaction with DPA [24]. That is why the $\{Tb\ (DPA)_3\}^{3-}$ complex is useful for application as fluorescent marker in a time-resolved immunoassays. Because fluorescence intensity of

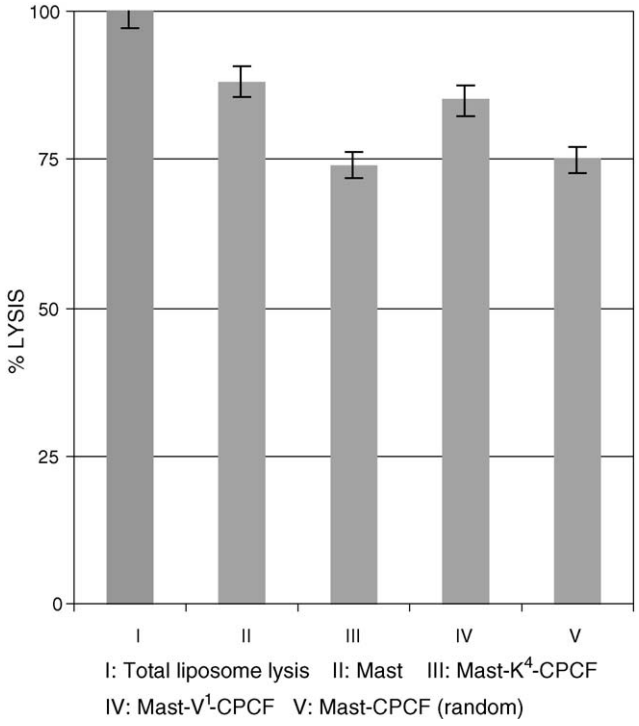


Fig. 1. % Lysis of liposomes with different Mast conjugated compounds. The total lysis obtained with 0.5% Brij 58 in water is set at 100% (bars correspond to the standard deviations).

Table 1
Inhibition of liposome lysis by specific anti-CF antibody

Antibody titre	Compounds (0.5 μg/well)	% Inhibition of liposome lysis
1:5000	Mast–CF (random)	50 ± 3.5
1:1000		75 ± 5.0
1:500		85 ± 2.2
1:5000	Mast–V[1]–CPCF	74 ± 2.2
1:1000		87 ± 1.8
1:500		95 ± 4.1
1:5000	Mast–K[4]–CPCF	75 ± 3.6
1:1000		88 ± 4.4
1:500		99 ± 4.0

Values are referred to the fluorescence of liposomes lysed by the same concentration of each compounds (mean of three determinations in triplicate; blanks have been subtracted).

Tb^{3+}/DPA complex depends from pH, the assay must be carried in buffered solution at neutral pH above the second pK of the acid [25].

3.2. Calibration graph

Fluorescence values for each concentration of standard are reported as % F/F_0 where F was the mean of cps for each standard and F_0 was the mean of cps for zero CF concentration (Fig. 2). Liposome lysis was proportional to the standard concentrations in a dynamic range between 10 pg and 10 ng. Blank value calculated as the number of cps of intact liposomes (4112 ± 440 cps) was subtracted.

Assay performances are not significantly affected by a low percent of methanol or DMF: F_0 and midpoint dose (I_{50}) values in different assay conditions are reported in Table 2. On the other hand, a solvent concentration higher than 10%, results in a loss of liposome stability. Since the ionic strength can affect antibody binding, a higher salt concentration resulted in lower F_0 fluorescence and higher I_{50} value. Thus, to minimize this systematic error, the presence of 0.9% NaCl in running buffer appeared to be important.

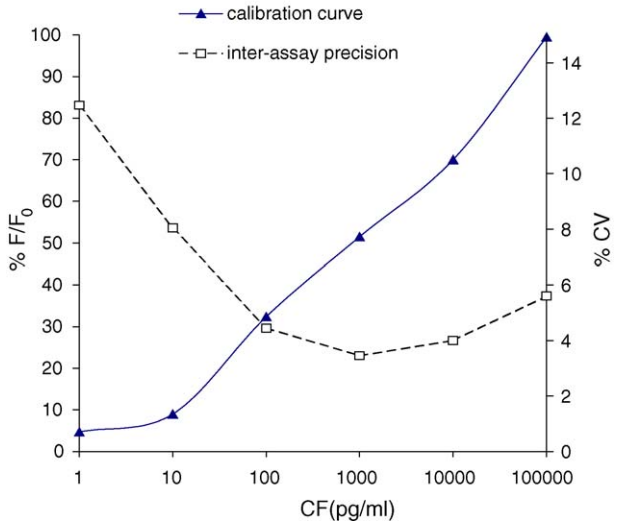


Fig. 2. LITRFIA standard curve and inter-assay precision profile for carbofuran in water (mean values of 10 determinations).

3.3. Antibody specificity

Specificity of the polyclonal antibody was evaluated by assaying the cross-reactions of CF-related compounds (Table 3), calculated at 50% of fluorescent signal reduction against the 100% of CF.

Cross-reaction values were in agreement with those obtained by enzyme immunoassay (unpublished data) and competitive heterogeneous time-resolved immunoassay.

3.4. Accuracy

Carbofuran concentrations between 206 ± 23 and 372 ± 10 pg mL^{-1} were found in four of real assayed samples. Accuracy was evaluated by six water samples in which carbofuran was not detectable: these samples were spiked with standard CF at two different concentrations (2 and 50 μg L^{-1}): the values obtained by LITRFIA method reported in Table 4 shown a good recovery for all assayed samples.

Table 2
Influence of solvent and ionic strength on assay performance: total fluorescent signals at zero standard concentration and I_{50} values for different assay conditions are reported (mean of three determinations in duplicate)

		F_0 (cps)	I_{50} (ng/ml)
Methanol (%v/v) (in Tris–HCl 0.05 M pH 7.5 + NaCl 0.15 M)	0	8300 ± 500	0.90
	1	8830 ± 530	0.88
	2	8040 ± 890	0.88
	5	9100 ± 300	0.98
	10	13050 ± 1010	n.d.
DMF (%v/v) (in Tris–HCl 0.05 M pH 7.5 + NaCl 0.15 M)	1	8800 ± 720	0.85
	2	8450 ± 200	0.89
	5	9500 ± 800	0.95
	10	12850 ± 640	n.d.
NaCl (mM) (in Tris–HCl 0.05 M pH 7.5)	0	12080 ± 780	0.78
	100	9025 ± 450	0.90
	200	8860 ± 530	0.94
	500	7815 ± 1200	1.45

Table 3
Cross-reactions of carbofuran related compounds with anti-carbofuran polyclonal antibody calculated at 50% of fluorescence signal reduction

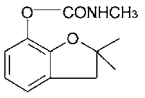
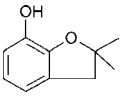
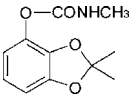
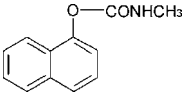
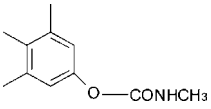
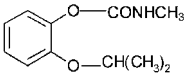
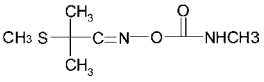
		%CR LITRFIA
	Carbofuran	100
	2,3-Dihydro-2,2-dimethyl-7-benzofuranol	120
	Bendiocarb	0.2
	Carbaryl	18
	Methiocarb	<0.01
	Propoxur	22
	Aldicarb	<0.01

Table 4
CF values in six water samples (spiked with 2 and 50 μg/L of standard) assayed by LITRFIA (mean of 10 different assays run in a week; each sample was assayed in duplicate)

Samples	LITRFIA (100 μL added) mean of 10 determinations run in duplicate		
	Expected (ng)	Found (ng)	% Recovery
River	0.2	0.19 ± 0.02	95
	5.0	4.85 ± 0.4	97
Well no. 1	0.2	0.19 ± 0.02	97.5
	5.0	5.10 ± 0.5	102
Well no. 2	0.2	0.21 ± 0.03	105
	5.0	4.90 ± 0.4	98
Ditch no. 2	0.2	0.18 ± 0.02	92.5
	5.0	4.90 ± 0.3	98
Ditch no. 4	0.2	0.2 ± 0.05	100
	5.0	5.20 ± 0.5	104
Ditch no. 5	0.2	0.19 ± 0.04	95
	5.0	4.95 ± 0.3	99

3.5. Sensitivity

The detection limit was determined by calculating the minimum amount of CF that could be significantly distinguished from zero (mean binding at zero dose at three times the S.D.). This value, calculated from three curves prepared in duplicate was 10 pg/well. Consequently, its high degree of sensitivity also makes it useful for determining CF levels in drinking water within the European Community limits of 0.1 μg/L. Assay sensitivity was good as shown by a high slope of calibration curve.

4. Conclusions

Mast can be easily conjugated to the various haptens because of the presence of reactive amino groups. To improve the sensitivity of assay, we have synthesized Mast derivatives with CPCF

bound on a single amino group of peptide and we have chosen Mast–V^1–CPCF conjugate as the more suitable, because its higher lytic activity and good recognition by antibody. The described method should provide a useful alternative to conventional chromatographic methods: in fact many samples can be analysed simultaneously in a short incubation time; terbium chelates label emission characteristics (narrow and strong emission bands around 600 nm and an exceptionally long decay time) allow the elimination of the high background of the common fluorescent labels. Furthermore, DPA give a good fluorescence signal and is a cheap commercial product. Sensitivity of described method is helpful in CF determination, as this pesticide is usually present in very high dilution in aqueous samples. Moreover, because of its widespread use, solubility in water and persistent chemical nature, CF is monitored in many agricultural countries and many samples have to be assayed in ground and surface water. Consequently, a homogeneous method performed by simple laboratory equipment such as described above, can co-operate in order to facilitate automation.

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