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Determination of carbofuran in surface water and biological tissue by sol–gel immunoaffinity extraction and on-line preconcentration/HPLC/UV analysis

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ABSTRACT

A selective and simple analytical method for the trace level determination of carbofuran in complex environmental and biological samples was developed based on immunoaffinity extraction (IAE) followed by on-line preconcentration and HPLC/UV analysis of the purified extract. The immunosorbent for IAE was prepared by sol–gel encapsulation of monoclonal anti-carbofuran antibodies, and was fully characterized for capacity, repeatability, binding strength, binding kinetics and cross-reactivity. Method performance was evaluated with two different types of difficult samples: dam water and methanolic extracts of epithelial cervical-uterine tissue. Linear behavior and quantitative recoveries were obtained from the analysis of samples spiked with carbofuran at 0.2–4 ng/mL (dam water, 50 mL samples) and 10–40 ng/mL (biological tissue extract, 2 mL samples). RSD ($n = 7$) and detection limits were, respectively, 10.1% (spike 0.40 ng/mL) and 0.13 ng/mL for dam water; 8.5% (spike 20 ng/mL) and 5 ng/mL for the biological tissue extract. The excellent sample purification achieved with the IAE column allows precise and accurate determination of carbofuran in complex matrices, even when using non-selective UV detection in the chromatographic analysis.

1. Introduction

The trace level determination of pesticides in complex environmental or biological matrices often requires long and laborious sample pretreatment prior to chromatographic analysis. Solid-phase extraction (SPE), in a variety of formats (cartridges, precolumns, fibers, disks) and operation modes, is actually the technique of choice for sample preconcentration and cleanup. Indeed, on-line coupling of SPE with high-performance liquid chromatography (SPE-HPLC) has been the basis for the development of highly sensitive methods, allowing determination of different pollutants at ppb (parts per billion) or sub-ppb levels in aqueous samples [1]. However, the reversed phase sorbents commonly used in these methods (C18 and polymeric phases) are non-selective; so, important amounts of other matrix components are co-extracted during the SPE of more complex samples, and may severely interfere in the determination of the compounds of interest [2]. Although selectivity is less of a problem when MS (mass spectrometry) is coupled to HPLC, high amounts of co-extracted material can affect the detector response or decrease the capacity of the SPE precolumn to quantitatively retain the analytes [3].

In the last decade, several papers have described the preparation of selective sorbents based on molecular recognition. Immunosorbents (IS) and molecularly imprinted polymers (MIP) have been successfully applied in environmental analysis for the extraction, preconcentration and cleanup of specific contaminants from water matrices, soil extracts and food extracts [3–6]. Immunosorbents are prepared by physical entrapment or chemical immobilization of an antibody in a porous solid support; as this antibody has been raised against the compound of interest, the selectivity and binding properties of the IS column derive from the strong affinity and specificity of antigen–antibody interactions. MIP are synthetic materials possessing specific cavities designed for a template molecule; the structure and functionalities of this molecule, as well as the reagents and solvent used for the synthesis, define the properties of the binding sites [6]. Incidentally, no MIP for pesticides of the N-methylcarbamate family has been yet reported, whereas, antibodies against various members of the family have already been produced and used for analytical purposes, mostly in immunoassays or immunosensors, but also in immunoaffinity extraction (IAE) columns [3,7–12].

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) is a systemic N-methylcarbamate pesticide, widely used in a variety of agricultural crops as insecticide nematicide and

Abbreviations: IAE, immunoaffinity extraction; IS, immunosorbent; SG-IAE, sol–gel immunoaffinity extraction; MIP, molecularly imprinted polymer; SPE, solid phase extraction; ELISA, enzyme-linked immunoassay; ACN, acetonitrile; MeOH, methanol; PBS, phosphate buffer saline solution; TEOS, tetraethoxysilane; Mab, monoclonal antibody; SD, standard deviation; MDL, method detection limit.

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acaricide. In Mexico, carbofuran is intensively applied for pest control in citric culture fields. Carbofuran is a potent cholinesterase inhibitor and, therefore, it is also highly toxic to human and wildlife (oral LD_{50} in rats is 8 mg/Kg) [13]. In recent years, an increasing number of cases of cervical-uterine anomalies, often turning to cancer, have been detected in young countrywomen from regions dedicated to citric culture in Mexico. Local clinicians suspect a direct contamination of the epithelial tissue with a toxic substance (in fact carbofuran) because of insufficient protection and deficient hygienic conditions. A regular screening of small cervical-uterine biopsies from exposed countrywomen with detected lesions would be necessary to determine if carbofuran is at the origin of the grave health problem in these regions. Moreover, monitoring of carbofuran in local water sources would also be necessary to evaluate the risks for the population and the extent of contamination.

Numerous publications have reported on the trace level determination of N-methylcarbamates, mainly in surface water and groundwater [14], but also in biological matrices such as fruits, vegetables and other foodstuffs [12]. However, studies for the detection of these pesticides or their metabolites in animal or human tissue are scarce [15–17]. In general, the preferred analytical technique has been HPLC because some N-methylcarbamates are thermally labile and not directly amenable to gas chromatography [12,18]. Indeed, analytical procedures reported in many works are variations of EPA method 531.1 (determination of N-methylcarbamates in aqueous samples), which is based on direct injection of the sample in a reversed phase column, followed by a two-step post-column reaction (hydrolysis and derivatization) and fluorescence detection [12,19,20]. Despite the high selectivity and sensitivity of the detection mode, application to biological extracts often requires laborious sample cleanup prior to chromatography to avoid severe interference from matrix components. Besides, the method requires sophisticated and relatively expensive equipment that is not commonly available in analytical laboratories of developing countries. The research group of Dr. Montoya [8–10], proposed an interesting alternative for the determination of carbofuran and other carbamates in water, fruits and vegetables, requiring practically no sample cleanup. This group produced monoclonal antibodies for the target pesticides and developed enzyme-linked immunoassays (ELISA), demonstrating that analytical results were comparable to those obtained with the HPLC-fluorescence method.

Although ELISAs are gaining reputation for the quantitative determination of pollutants in different matrices, most environmental and clinical laboratories still prefer the classical chromatographic techniques with robust detectors for routine analyses. Therefore, the aim of this work was to prepare an immunoaffinity extraction column by sol–gel encapsulation of a monoclonal anti-carbofuran antibody, and use it for the selective extraction of the pesticide from epithelial cervical-uterine extracts and surface water samples. In order to achieve a high sensitivity using a simple UV detector, the whole purified extract from the IAE column was re-concentrated and analyzed by on-line SPE-HPLC.

2. Experimental

2.1. Reagents and solutions

HPLC-grade acetonitrile (ACN) and methanol (MeOH) were from Prolabo and Fisher Scientific. Type 1 reagent water was obtained from a Nanopure deionizer (Barnstead Thermolyne). Pesticide and metabolite standards were from Chem Service (certified purity >99%), other common reagents were analytical grade from Baker. Stock solutions of carbofuran, four carbofuran metabolites and 2,4-dichlorophenol were prepared in ACN and stored at −20 °C Working standards at various concentrations were prepared from the stock solutions in MeOH–water 20:80 (v/v) or in phosphate buffer saline solution (PBS) and kept in refrigeration (4 °C) when not in use; these standards were frequently renewed. Unless otherwise indicated, PBS was 0.02 M NaH_2PO_4, 0.137 M NaCl and 0.0027 M KCl, adjusted to pH 7 with concentrated NaOH solution. PBS of higher concentration, required for some experiments, was prepared maintaining the same salt molar ratio and pH. Tetraethoxysilane (TEOS) from Fluka (99%) was the precursor for the preparation of sol–gel materials. Monoclonal antibody (Mab) LIB-BFNB67 raised against carbofuran, was purchased from I3BH – Universitat Politècnica de València, Spain. The original Mab suspension (in 50% saturated $(NH_4)_2SO_4$) was placed in a Centricon YM-30 tube (cutoff 30,000 Da, Amicon Bioseparations), and desalted by repeated washing with water and centrifugation; the retained portion was re-suspended in PBS, carefully transferred to a vial and kept at 4 °C until use. Concentration of the final Mab solution was 1 mg/mL.

2.2. Evaluation of the free Mab activity

The capacity of native Mab LIB-BFNB67 to form a complex with carbofuran was evaluated according to previously reported procedures with minimal modification [21–23]. Aliquots of 1 mg/mL Mab solution (50 μL, equiv. to 0.333 nmol) and 1 μg/mL carbofuran solution in PBS (70 or 140 μL, equiv. to 0.316 or 0.633 nmol) were diluted with 1 mL of PBS-ACN 98:2 (v/v) into a Centricon YM-30 tube and incubated for 15 min at room temperature. Then, the solution with unbound pesticide was separated from the carbofuran–Mab complex and the free Mab by centrifugation (2500 × *g*, 15 min); the retained portion was washed twice with 350 μL of the PBS-ACN mixture, centrifuging after each washing (same speed, 10 min). The flow-through fractions containing the unbound carbofuran were collected in the same beaker, brought to a volume of 2 mL, and analyzed by the developed on-line SPE-HPLC procedure described later (Section 2.8).

2.3. Sol–gel immunoaffinity extraction column (SG-IAE column)

The procedure used in previous works for the sol–gel entrapment of different polyclonal antibodies [22,23] was first assayed for the encapsulation of four anticarbofuran Mabs: LIB-BFNB67, LIB-BFNB62, LIB-BFNB52 and LIB-BFN21 (kindly provided for preliminary tests by Dr. A. Montoya from I3BH – Universitat Politècnica de València, Spain). From results obtained with the prepared immunosorbents, the Mab LIB-BFNB67 was chosen for further studies. However, these preliminary assays also showed that our sol–gel procedure was not adequate for the encapsulation of monoclonal antibodies as the maximum binding of carbofuran was 18 ng, which was too low compared to the capacity of previously prepared biomaterials.

Optimization of the most critical steps finally led to the following procedure: 2.5 mL of TEOS, 0.1 mL of 0.1 M HCl and 0.4 mL of water were chilled on ice for 30 min; the mixture was then submitted to continuous and gentle shaking (Burrell shaker, model 75) at ambient temperature until the sol became quite elastic, completely transparent and no TEOS odor was perceived (∼3.5–4 h). A 2-mL volume of concentrated PBS (0.1 M), immediately followed by a 500 μL aliquot of the Mab solution (500 μg of antibody) were added to the sol under vigorous stirring. Gelation occurred within 1 min. The formed hydrogel was separated from the walls of the recipient, completely covered with PBS and left at rest for 10 min; afterwards it was thoroughly crushed with a spatula and rinsed under vacuum with 25 mL each of the following: water, MeOH–water 50:50 (v/v), 5% glycerol in water and PBS. The drained particles were weighted and allowed to age and dry very slowly in refrigeration (4 °C) until loss of 50% weight. The obtained xerogel (3.6–3.9 g) was ground