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Short communication

Class-specific immunoaffinity monolith for efficient on-line clean-up of pyrethroids followed by high-performance liquid chromatography analysis☆

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ABSTRACT

A class-specific monolithic immunoaffinity column was developed for on-line clean-up of pyrethroid insecticides in complex samples. Deltamethrin was oxidized with ozone to generate the hapten of (RS)-α-cyano-3-phenoxybenzyl (RS)-cis,trans-2,2-dimethyl-3-formyl-cyclopropane carboxylate. Class-specific antibodies against pyrethroids were produced using the conjugate of above hapten with bovine serum albumin as the immunogen. Poly(ethylene dimethacrylate-glycidyl methacrylate) monolith was synthesized in a 50 mm × 4.6 mm i.d. stainless steel cartridge with two auxiliary pipette tips. The polymerization method was proved to be economic and reproducible. Antibodies against pyrethroids were covalently immobilized onto the monolithic support via Schiff base reaction. With a column-switching valve system, the immunoaffinity monolith (IAM) could be readily adapted to the reversed-phase high-performance liquid chromatography (HPLC) system. Under the optimum loading, washing and eluting conditions, the IAM specifically retained deltamethrin, flumethrin, flucythrinate and cis/trans permethrin, which were further baseline separated by C18 column using acetonitrile–water (83:17, V/V) as the mobile phase at a flow rate of 1.0 mL/min. The established system provides a highly efficient approach for high-throughput on-line clean-up of pyrethroid in various samples.

1. Introduction

In the field of synthetic insecticides analysis, detection of synthetic pyrethroids is becoming particularly important due to the dramatic increase of their use as relatively safer alternatives to the highly toxic organochlorine and organophosphate insecticides [1,2]. Animal studies have demonstrated that pyrethroid exposure may affect neurological development [3], suppress the immune system [4], and disrupt the endocrine systems [5–7]. It is highly demanded to develop reliable and high-throughput detection methods not only to guarantee the pesticides have sufficiently decomposed before the grain is processed for consumption but also to evaluate the accumulated human exposure to pyrethroid from different sources.

Analysis of pyrethroids has been difficult because of the numerous analogues and isomers in the family and the relatively low application level. Immunoassays have been developed for monitoring of these compounds with the merits of rapidity, sensitivity and relatively low cost [8–13]. They are especially useful to check if the residue levels in food are below the legally set maximum residue limits. However, immunoassay can neither differentiate analogues or isomers nor provide their accurate concentration, which is disadvantageous for effective control of the use of these compounds.

Chromatographic techniques such as gas chromatography (GC) [14–21] or high-performance liquid chromatography (HPLC) [22–28] combined with mass spectrometry (MS) may give reproducible and reliable detection results for individual compounds, but they usually require multi-step sample pretreatment including extraction, clean-up, and even derivatization for GC/MS when dealing with samples with complex matrices.

Immunoaffinity chromatography has been recognized as a powerful technique to selectively isolate and concentrate minor components of interest from complex mixtures [29]. However, few reports are available now on the immunoaffinity extraction methods for clean-up and preconcentration of synthetic pyrethroids. In our previous work [30], we have developed a very efficient monolithic immunoaffinity column for the selective on-line extraction and preconcentration of bisphenol A in environmental water samples. To extend the approach to a more general platform with multi-targeted analytes such as pyrethroids, there are two major challenges. First, class-specific antibodies need to be prepared. Second, the commonly used mobile phase for pyrethroids analysis on HPLC is composed of acetonitrile and water, which is not compatible with immunoaffinity column. So, a more com-

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plicated column-switching system needs to be designed. In this study, we successfully addressed the above two issues in a more environmental-friendly way and established a simple on-line clean-up method for HPLC quantification of pyrethroids. Besides, the preparation procedure of the monolithic column was also substantially improved.

2. Experimental

2.1. Chemicals and reagents

Flumethrin, permethrin (cis/trans mix isomer), flucythrinate, glycidyl methacrylate (GMA, 97% pure), ethylene dimethacrylate (EDMA, 98% pure), bovine serum albumin (BSA), ovalbumin (OVA), sodium cyanoborohydride (94% pure) and azobisisobutyronitrile (AIBN, 98% pure) were all purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Deltamethrin was kindly provided by College of Agriculture and Biotechnology, China Agricultural University (Beijing, China). 1-Dodecanol (DoOH, 98% pure), cyclohexanol (CyOH, 98% pure) and periodic acid were all obtained from Beijing Chemical Co. (Beijing, China). All other chemicals were of the highest purity commercially available and were used without further purification.

2.2. Instrumentation

A 2PB00C peristaltic pump from Beijing Satellite Factory (Beijing, China) was used to deliver the solvents (or antibody solutions) through the monolith column during the preparation. A Waters Lambda-Max 481 LC Spectrophotometer from Waters (Milford, MA, USA) was used to monitor the on-line UV absorption signals of proteins flowing out of the column. HPLC analysis was performed on an Agilent 1100 HPLC with an ultraviolet detector (UVD). The immunoaffinity monolith (IAM) was connected to a C18 column (Dikma Technologies Diamonsil, 5 μm, 150 mm × 4.6 mm) using a Rheodyne 7000 six-port valve (Cotati, CA, USA) as the switching device. The flow rates of the mobile phases were 0.5 mL/min for the IAM and 1.0 mL/min for the C18 analysis. The detection wavelength was set at 220 nm.

2.3. Preparation of class-specific antibodies against pyrethroids

The hapten was generated by oxidization of deltamethrin with ozone according to the method by Mak [10] with some improvements. The reaction scheme was illustrated in Fig. 1. In brief, ozone gas from an ozone generator was passed through a solution containing 505 mg (1 mmol) of deltamethrin in 5 mL of CH_2Cl_2 and 1 mL of methanol with the reactor flask placed in a dry ice/acetone cooling bath until the solution turned golden yellow. Then the reaction progress was monitored by thin-layer chromatography (TLC) (silica gel, eluent petroleum ether:ethyl acetate = 15:1) until the reactants were completely consumed. To the obtained solution, 1 mL of dimethyl sulfide was added at room temperature to reduce the activated intermediate produced in the first step and the solution gradually turned clear and colorless. When the reaction was completed according to the silica-TLC (ether:ethyl acetate = 4:1) results, the resulting mixture product was purified by column chromatography on silica gel (eluted with petroleum ether:ethyl acetate = 4:1). The collected fractions containing the product were dried with rotary evaporator to yield 289 mg (83%) of the hapten. The structure of the obtained hapten was confirmed with H NMR, as shown in the supplementary material (Fig. S1).

The obtained hapten was used to react with BSA to prepare the complete antigen (PRD-BSA). To 5 mL of 0.01 mol/L phosphate-buffered saline (PBS, pH 7.4) containing 60 mg of BSA, 20 mg/mL hapten in dimethyl sulfoxide (DMSO) and 5 mol/L sodium cyanoborohydride were added dropwise in turn with gently magnetic stirring. The reaction was allowed to proceed for 24 h at 4 °C. The resulting solution was dialyzed against 0.01 mol/L, pH 7.4 PBS with 6 changes over 48 h at 4 °C. Then it was further dialyzed against pure water for 48 h at the same temperature with six changes. The solution was then lyophilized and the white crystal obtained (about 40 mg) was stored at −20 °C. The conjugation ratio of hapten/BSA was measured using a Coomassie Brilliant Blue spectrophotometric method [31].

Three New Zealand white rabbits in good health weighing 2–3 kg were immunized with the above PRD-BSA conjugate according to the regular methods [32]. The blood cells were removed by centrifuging to obtain the antiserum, followed by purification using a Protein G affinity column (Pharmacia) according to the manufacturer's instructions. The collected IgGs were immediately used for immobilization or stored at −20 °C until further use.

2.4. Synthesis of the poly(EDMA-GMA) monoliths with auxiliary end-tips

The preparation method of the poly(ethylene dimethacrylate-glycidyl methacrylate) (EDMA-GMA) monolith was further improved on the basis of our previous work [30]. In brief, two pipette tips were respectively attached to the two ends of the empty stainless steel cartridges (50 mm × 4.6 mm i.d.) to facilitate the polymerization process. A picture of the device is shown in the supplementary material (Fig. S2). First, the bottom outlet of the device was thermally sealed and the two joint parts between the tip and the cartridge were wrapped with polytetrafluoroethylene (PTFE) film to prevent possible leakage. GMA, EDMA, CyOH and DoOH at a volume ratio of 12:8:21:9 were previously mixed thoroughly in a clean and dry flask, to which AIBN was added as the initiator at a ratio of 4.7 mg/mL of the mixture. The solution was ultra-sonicated for 3 min and then purged with nitrogen for 3 min before slowly transferred into the cartridge through the top inlet with an injector. Then the top inlet was also thermally sealed and the whole device was vertically put into a water bath. The temperature of the water bath was increased from room temperature to 80 °C in half an hour and then held at 80 °C for 24 h. After the polymerization was completed, the cartridges were taken out and the auxiliary tips were all removed with the outside polymer. The monolithic column was attached to the peristaltic pump and 100 mL of acetonitrile was pumped through the column

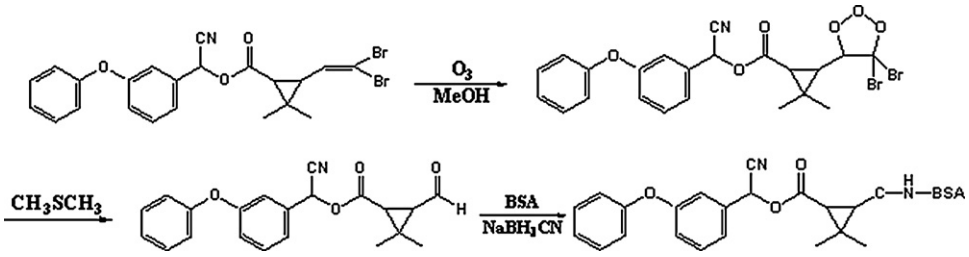

Fig. 1. Synthesis of the hapten and the hapten/BSA conjugate.

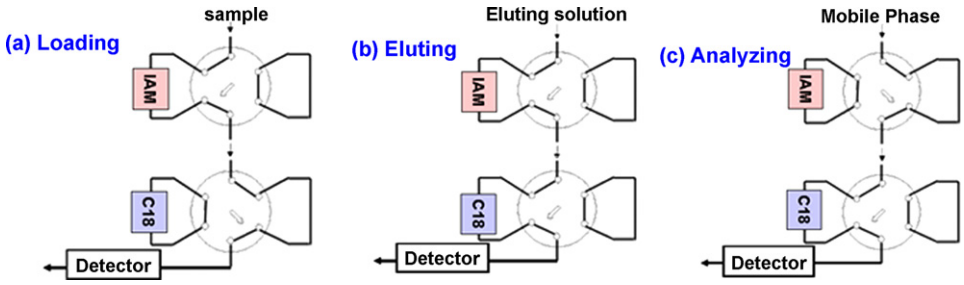

Fig. 2. Column-switching configuration of the IAM-HPLC-UVD system.

at a flow rate of 0.5 mL/min to remove the unreacted monomers and porogenic diluents.

The purified IgGs obtained in Section 2.3 were covalently immobilized onto the monolith via the Schiff base reaction according to our previous work [30]. A control column was prepared with an irrespective protein (OVA) as the substitute of the anti-pyrethroid antibody.

2.5. Optimization of the operating conditions and characterization of the binding performance of the obtained IAM

The column-switching configuration of the IAM-HPLC-UVD system was illustrated in Fig. 2. The loading, washing and eluting conditions were all optimized using deltamethrin as the reference compound. The bound pyrethroids on the IAM were eluted with methanol in water at different volume ratios [33].

To assess the binding selectivity and recovery of the IAM, four pyrethroids were tested (Fig. 3). The HPLC analytical separation conditions including the mobile phase composition and the flow rates were all optimized. The nonspecific adsorption of the support was estimated by performing the same experiments as above using the control columns.

3. Results and discussion

3.1. Preparation of class-specific antibodies against pyrethroids

As shown in Fig. 3, the tested pyrethroid compounds all have a 3-phenoxybenzyl moiety, which is important to improve their relative stability in the environment. Deltamethrin and flumethrin have very similar structures and the only difference between them is the halogen atoms on the vinyl group. Permethrin has the same acid structure as above two compounds but lacks an α-cyano group at the phenoxybenzyl alcohol. Flucythrinate has the same α-cyano-3-phenoxybenzyl structure as deltamethrin and flumethrin, but the acid portion is altered to include a phenyl ring. In these molecules, there is no obvious functional group for direct conjugation to the carrier protein. Since the specificity of the antibodies is determined by the site and chemistry of the conjugation [34], we chose to break the vinyl group in deltamethrin and preserve the common structure of the phenoxybenzyl moiety and the cyclopropane ring.

Ozone was used to oxidize the vinyl side chain since it yielded the least side products compared to other oxidants such as potassium permanganate [11]. For reduction of the activated intermediate produced in the first step, we used dimethyl sulfide instead of zinc powder [10] so that no filtration step was needed after the reaction. Besides, we added methanol to the reaction system to eliminate the toxic product (Br_2O). The hapten (RS)-α-cyano-3-phenoxybenzyl (RS)-cis,trans-2,2-dimethyl-3-formyl-cyclopropane carboxylate was used to conjugate to BSA to produce the immunogen. The conjugation ratio of hapten/BSA was observed to be 22:1.

With the above conjugate, the resulted antibodies differentially recognize a range of synthetic pyrethroids containing the structure of 3-phenoxybenzyl carboxylate and the cyclopropane ring, as confirmed by enzyme-linked immunosorbent assay (data not shown).

3.2. Improvement of the polymerization device for the monolith preparation

In our previous work, the monolith column was prepared by directly immersing the empty cartridges with two ends open in the polymerization solutions [30]. With a programmatic temperature increase during the polymerization, the columns were quite reproducible. But an obvious drawback of the method was the volume of the solution outside the cartridges was very large. Approximately 20 mL of the pre-polymerization solution was needed to prepare one monolith column. Besides, the polymer outside the cartridge needs to be removed laboriously. In this study, we tried to improve this by placing two auxiliary pipette tips on both ends of the cartridge. With this new device, only 1.5–2.0 mL of the pre-polymerization solution is needed for each column. Thus the cost was greatly reduced and the subsequent treatment procedure was

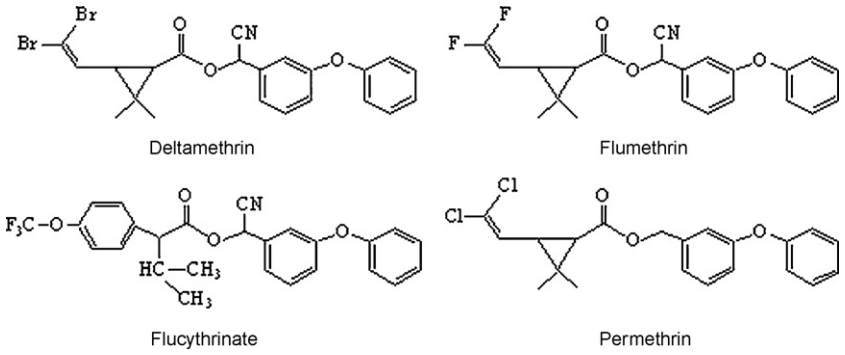

Fig. 3. Chemical structures of the tested pyrethroids.

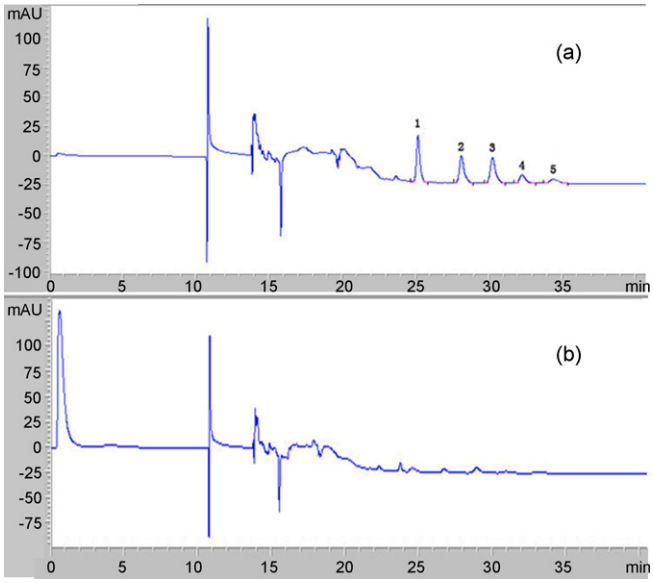

Fig. 4. Comparison of the binding–desorption of the pyrethroids on (a) IAM and (b) control column. Peaks 1–5: flucythrinate, deltamethrin, trans-permethrin, cis-permethrin, flumethrin.

also notably simplified. The amount of immobilized antibody on the monolith was determined by comparing the antibody concentration in the circulating solution before and after the coupling reaction, and was found to be 0.20 ± 0.02 μmol IgG per gram of the dry polymer monolith ($n = 3$).

3.3. Binding performance of the obtained IAM

The effects of pH and ionic strength on the binding ability of the IAM to pyrethroids were systematically investigated using deltamethrin as the reference compound. The maximum binding ability for deltamethrin was observed both in 0.01 mol/L PBS (pH 5.5) and in pure water as the application buffer. The recoveries of the bound deltamethrin on the IAM were found to be $101 \pm 2\%$ ($n = 4$) when the volume ratio of methanol/water in the eluting solutions was in the range from 70 to 90%. A lower recovery of $89 \pm 2\%$ ($n = 3$) was observed for eluting solution containing 60% of methanol in water. Therefore 70% methanol in water was chosen as the eluting solution in the following experiments. After elution, the IAM was regenerated with 0.01 mol/L PBS (pH 7.4).

The chromatograms of the mixture standard solution of the four tested pyrethroids on the IAM and corresponding control column were compared in Fig. 4. As can be seen, the IAM showed specific binding property to deltamethrin, flumethrin, flucythrinate and cis/trans permethrin, which were further baseline separated using acetonitrile–water (83:17, V/V) as the mobile phase at a flow rate of 1.0 mL/min.

To further confirm the specificity of the immobilized antibodies, three structurally related compounds including 2-amino-4-chlorophenol, 2-methoxybenzoic acid and 3-phenoxybenzoic acid (metabolite of many pyrethroids in human body) were also tested and their retention on the IAM was observed to be negligible.

The detection limits of the whole method were determined using the mixture standard solution of the pyrethroids. The linear calibration range was found to be 0.05–10 μg/mL for all the four tested compounds with an injection volume of 20 μL. The detection limits for a loading volume of 1 mL were found to be 0.4, 0.6, 0.4, 1.0 and 1.0 ng/mL for flucythrinate, deltamethrin, trans-permethrin, cis-permethrin and flumethrin, respectively.

The day-to-day reusability of the IAM was examined using the same column and the same sample in four consecutive days and the RSDs of the peak area of the tested pyrethroids were observed to be flucythrinate (±3.0%), deltamethrin (±3.2%), trans-permethrin (±2.8%), cis-permethrin (±3.7%) and flumethrin (±4.1%), respectively, indicating an acceptable repeatability of the obtained column for practical use. The stability of the IAM after relatively long-term storage was also examined. The IAM stored at 4 °C in 0.01 mol/L PBS (pH 7.4) containing 0.02% NaN_3 for 30 days showed similar binding capacity as the original column. But the IAM preserved for 30 days at the same temperature without addition of NaN_3 showed 70% loss of the binding capacity. This indicates that the addition of antibacterial agents is a key factor to preserve the binding ability of the immobilized antibodies.

Conventional clean-up procedure for analyzing pyrethroids normally involves multiple off-line operation steps [26,27] before being analyzed by HPLC. The employment of on-line IAM greatly simplified the pretreatment procedure and reduced the sample lost or contamination risks. The presented method can be readily used to detect pyrethroid contamination in excess of the regulatory limit. Higher sensitivity may be achieved by increase the loading volume or using other detectors based on fluorescence or mass spectrometry.

4. Conclusion

An efficient monolithic immunoaffinity column was developed for on-line clean-up of pyrethroid insecticides. Class-specific antibodies against pyrethroids were produced using the conjugate of (RS)-α-cyano-3-phenoxybenzyl (RS)-cis,trans-2,2-dimethyl-3-formyl-cyclopropane carboxylate with BSA as the immunogen. The modified polymerization method for poly(EDMA-GMA) monolith was proved to be economic and reproducible. Under the optimum loading, washing and eluting conditions, the IAM specifically retained deltamethrin, flumethrin, flucythrinate and cis/trans permethrin, which were further baseline separated by C18 column using acetonitrile–water (83:17, V/V) as the mobile phase at a flow rate of 1.0 mL/min. The established system would be widely applicable for developing on-line clean-up approach for other compounds of interest in various samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2009.06.007.

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