Selective Recognition of the Herbicide Atrazine by Noncovalent Molecularly Imprinted Polymers

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Molecular imprinting was applied to the synthesis of methacrylic acid−ethylene glycol dimethacrylate copolymers specific for the herbicide atrazine. In toluene, these imprints bound atrazine with dissociation constant (K_D) values as low as 10^{-6} M, and could be used for the development of a ligand binding assay for the detection of atrazine. The selectivity profile of the imprints for triazines structurally related to atrazine was comparable to those of antibodies. The substituent of carbon C1 is the most important feature determining the level of cross-reactivity, and the binding affinity decreased in the order Cl > OCH_3 > OH > SCH_3 (atrazine, the imprint species, has Cl at this position). No cross-reaction of structurally unrelated herbicides and other compounds was detected. Rebinding experiments in phosphate buffer containing 0.15% Tween 20 gave similar results. The polymers could be packed into HPLC columns and used for the separation of triazine derivatives. The potential uses of imprinted polymers for the detection of pollutants in environmental analysis and as sorbents for the specific removal of toxic compounds in waste water treatment are discussed.

Keywords: *Ligand binding assays; antibody binding site mimics; chromatography; immunoassay-style techniques*

INTRODUCTION

Intensive agriculture, associated with the use of a large number of pesticides, has increasingly caused problems due to the contamination of ground and drinking water. European Union directives stipulate a maximum limit for pesticide concentrations of 0.1 μg/L for each single substance and 0.5 μg/L for the sum of all pesticides in the sample (EEC Drinking Water Directive, 1980). There is a need for monitoring very large numbers of samples and, consequently, inexpensive and rapid analytical techniques are required. Several methods based on biorecognition, in most instances antibody−antigen interaction, as the primary reaction are available (Van Emon and Lopez-Avila, 1992). The ever increasing number of contaminants subjected to monitoring demands a fast and inexpensive strategy for assay development. Molecular imprinting (Mosbach, 1994; Wulff, 1993; Shea, 1994) offers a means for the preparation of synthetic polymers that are highly selective for a predetermined substance. Such polymers may become a valuable complement to antibodies for use in pesticide determinations.

Molecularly imprinted polymers (MIPs) have already been used in a broad range of applications from structural studies of ligand−receptor interactions to selective binding-matrices in detection, separation, and purification (Mosbach, 1994; Andersson et al., 1995a). Imprint synthesis entails polymerization around an imprint species with monomers that are selected for their ability to form specific and definable interactions with the imprint molecule. "Cavities" are formed in the polymer matrix whose size and shape are complementary to that of the imprint molecule. Interactions between complementary functionalities present in the imprint molecule and the monomer(s) prior to the initiation of polymerization are conserved in the product polymer. Subsequent removal of the imprint species exposes "memory" sites around which the spatial arrangement of the monomer residues is dependent on the chemical structure of the imprint molecule. These recognition sites enable the polymer to rebind the imprint species selectively from a mixture of closely related compounds, in many instances with binding affinities approaching those demonstrated by antigen−antibody systems (Vlatakis et al., 1993; Andersson et al., 1995b). Furthermore, selectivity profiles that are comparable to those of antibodies are often observed. Thus, the imprinted polymers can be regarded as antibody binding site mimics that efficiently mimic the ability of biological antibodies to bind an antigen specifically.

Although atrazine is not considered to be a highly toxic herbicide, residue analysis of this substance is a significant parameter in environmental testing programs because of its persistence and widespread application. Various formats of immunoassays, based on the use of poly- or monoclonal antibodies, have been applied to atrazine detection (Dunbar et al., 1990; Giersch, 1993; Schneider and Hammock, 1992; Weil et al., 1991; Wüst and Hock, 1992). Another type of assay is based on the inhibition by atrazine of photosynthetic electron transport in purified spinach thylakoids (Brewster and Lightfield, 1993). Furthermore, GC (Lee and Chau, 1993; Sirons at al., 1973) and HPLC (Vickrey et al., 1980; Vermeulen et al., 1982) techniques offer sensitive and accurate methods for the determination of atrazine and other triazine pesticides.

Here we report on the imprinting of atrazine and demonstrate the ability of the resultant polymers to bind atrazine selectively. For a highly preliminary account on atrazine imprinting, see also Andersson et al. (1995b). The potential applications of such MIPs in chromato-

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graphic separations of triazine herbicides and in immunoassay-style analytical methods for the determination of atrazine are discussed.

MATERIALS AND METHODS

Polymer Preparations. Imprint molecule (0.56 mmol; 121 mg of atrazine; 45 mg of triazine), methacrylic acid (MAA, 3.4 mmol), and ethylene glycol dimethacrylate (EGDMA, 17 mmol) were weighed into borosilicate glass test tubes and mixed with 5 mL of dried methylene chloride. The polymerization mixture was cooled on ice, purged with nitrogen for 5 min, and sonicated thoroughly. Initiator (2,2′-azobis(2,4-dimethylvaleronitrile), 0.24 mmol) was added and the tubes were placed under a UV light source (366 nm) at 4 °C for 16 h. The bulk polymers were ground in a mechanical mortar (Retsch, Haan, Germany) and wet sieved through a 25-μm sieve (Retsch, Haan, Germany). Fines were removed by repeated sedimentation from acetonitrile. The imprint molecules were extracted by washing with large volumes of methanol:acetic acid (9:1; v/v) and methanol. Finally, the particles were dried under reduced pressure and stored in a desiccator. Safety precautions during the preparation of the polymerization mixture and the grinding and the extraction of the polymer. These steps were performed in a hood because they involve handling of the possible carcinogen methylene chloride and/or the toxic compounds methacrylic acid, ethylene glycol dimethacrylate, and 2,2′-azobis(2,4-dimethylvaleronitrile).

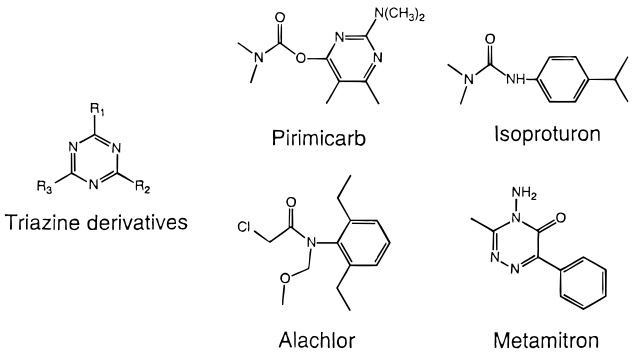
High-Performance Liquid Chromatography (HPLC). Polymer particles were slurried in chloroform and packed into stainless steel columns (200 × 4.6 mm i.d.) with acetone at 300 bar by an air-driven fluid pump (Haskel Engineering Supply, Burbank, CA; Andersson and Mosbach, 1990). The columns were washed on line with methanol:acetic acid (9:1; v/v) and equilibrated with 4% acetic acid in acetonitrile. Then, 20 μL of 0.1 mM samples, dissolved in the eluent, were injected onto the column and eluted isocratically at a flow rate of 1 mL/min. Acetone was employed as a void marker, and detection was at 254 nm. Plate number, (N), retention factor (k'), and separation factor (α), were calculated using standard chromtographic theory (Snyder and Kirkland, 1974). The normalized retention index is defined as $K = (k'_{s,imp}/k'_{s,non\text{-}imp})/(k'_{im,imp}/k'_{im,non\text{-}imp})$, where k'_s is the retention factor for substrate and k'_{im} is the retention factor for the imprint molecule on imprinted (imp) and nonimprinted reference (non-imp) polymers, respectively.

Frontal Chromatography. Varying concentrations of atrazine ($S_0 = 0.056-0.46$ mM) were injected onto a 50 × 2.5 mm i.d. column packed with imprinted polymer in a total volume of 5 mL. The eluent was 2.5% acetic acid in acetonitrile and the flow rate was 0.2 mL/min. The results were plotted as follows: $1/\{[S_0](V - V_0)\}$ versus $1/[S_0]$, where V and V_0 are the elution volume of atrazine and the void marker acetone, respectively (Kempe and Mosbach, 1991; Fassina and Chaiken, 1989). The number of accessible binding sites (L_t) was calculated from the intercept on the ordinate ($1/L_t$), and the dissociation constant (K_D) was calculated from the intercept on the abscissa ($-1/K_D$).

Radioligand Binding Assays. Anti-atrazine MIP (1 mg), 12.5 nCi of [*ring*-U-^{14}C]atrazine (specific activity 58 mCi/mmol), and various amounts of atrazine or other herbicides ($1-10^6$ ng/mL) were mixed in 1 mL of toluene:acetonitrile (19:1). The suspension was incubated at room temperature for 2 h. After centrifugation (10000g, 5 min), the amount of radiolabeled atrazine in the supernatant was measured by liquid scintillation counting. The relative cross-reactivities were estimated after log−logit transformation (Price and Newman, 1991). The binding experiments performed with aqueous solvents were incubated in 10 mM phosphate buffer (pH 7.0):acetonitrile (19:1) containing 0.15% Tween 20.

RESULTS AND DISCUSSION

Chromatographic Experiments. The molecular recognition abilities of the MIP preparations were assessed by HPLC, as has been described previously for other imprinted polymers (O'Shannessy et al., 1989; Andersson and Mosbach, 1990). For each compound analyzed on a particular column, the retention factor k' is correlated to the relative binding strength of that compound to the stationary phase, in our case the MIP. Further, a series of K values for compounds structurally similar to the imprint molecule provides an estimate of the level of selectivity of the MIP. The retention index, K, is defined as the normalized ratio of k' recorded on the imprinted polymer to k' on the nonimprinted reference polymer. By definition, $K = 1$ for the imprint compound. The presence of selective imprints in the polymer yields K values lower than unity, and low K values imply a high selectivity of the imprints.



Compound	R_1	R_2	R_3
Atrazine	Cl	$NHCH_2CH_3$	$NHCH(CH_3)_2$
Terbutylazine	Cl	$NHCH_2CH_3$	$NHC(CH_3)_3$
Simazine	Cl	$NHCH_2CH_3$	$NHCH_2CH_3$
Propazine	Cl	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$
De isopropyl atrazine	Cl	$NHCH_2CH_3$	NH_2
De ethyl de isopropyl atrazine	Cl	NH_2	NH_2
Prometryn	SCH_3	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$
Terbutryn	SCH_3	$NHCH_2CH_3$	$NHCH_2CH_3$
Atraton	OCH_3	$NHCH_2CH_3$	$NHCH(CH_3)_2$
Terbumeton	OCH_3	$NHCH_2CH_3$	$NHC(CH_3)_3$
Hydroxyatrazine	OH	$NHCH_2CH_3$	$NHCH(CH_3)_2$
s-Triazine	H	H	H

Figure 1. Structures of the herbicides.

Atrazine and *s*-triazine (Figure 1) were imprinted in copolymers composed of the acidic and hydrogen bonding methacrylic acid as the functional monomer and the cross-linking monomer ethylene glycol dimethacrylate. Furthermore, a nonimprinted reference polymer, with the same composition except that the imprint compound was omitted, was made under identical conditions. On both columns with imprinted polymer, stronger retardations of the imprint species were recorded than on the reference column (Table 1). The retardation factor (k') for atrazine increased 14 times. The affinity of atrazine for the atrazine MIP and the number of accessible binding sites under the conditions used during chromatography were determined by frontal chromatography by a previously described method (Kempe and Mosbach, 1991). The apparent dissociation constant, K_D, was 0.8

Table 1. Chromatographic Data Obtained by HPLC Analyses of Imprinted Polymers

	anti-atrazine MIP		*anti*-triazine MIP		nonimprinted polymer
compound	k'	K	k'	K	k'
atrazine	6.00	1.00	0.67	0.49	0.42
terbutylazine	6.00	1.00	0.67	0.49	0.42
propazine	6.00	0.88	0.67	0.42	0.48
simazine	4.55	0.76	0.67	0.49	0.42
deisopropylatrazine	4.50	0.35	1.20	0.41	0.90
deethyldeisopropylatrazine	4.95	0.22	1.93	0.37	1.57
prometryn	1.50	0.25	0.60	0.43	0.42
terbutryn	1.30	0.16	0.40	0.21	0.58
terbumeton	1.30	0.20	0.40	0.27	0.45
atraton	1.63	0.20	0.40	0.21	0.58
hydroxyatrazine	0.65	0.08	0.20	0.10	0.58
s-triazine	0.01	0.01	0.23	1.00	0.07
metamitron	0.45	0.15	0.47	0.68	0.21
isoproturon	0.15	0.08	0.27	0.63	0.13
alachlor	0.15	0.15	0.12	0.52	0.07
pirimicarb	0.15	0.15	0.12	0.52	0.07

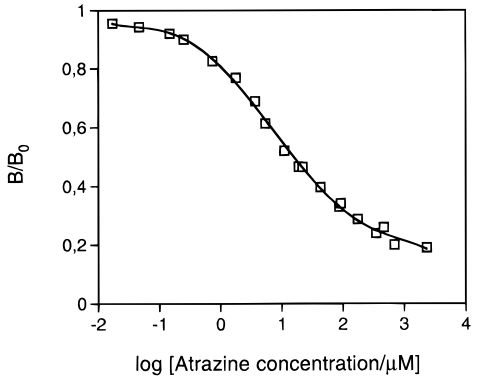


Figure 2. Dose−response curve for atrazine determination using the atrazine imprinted polymer. The assay was performed using toluene:acetonitrile (19:1) as the solvent and [^{14}C]-atrazine as the tracer. Each point represents the average of three independent measurements, with a relative standard deviation of ≤5%. B/B_0 is the ratio of the amount of tracer bound in the presence of displacing ligand, B, to the amount bound in the absence of displacing ligand, B_0.

mM, and the number of sites, L_t, associated with this affinity was 7.7 μmol/g of dry polymer.

The substituent R_1 on carbon C1 (Figure 1) is the most important feature determining the retention of the triazine herbicides (Table 1). The derivatives with the same R_1 as the imprint species, namely −Cl, were all strongly retained on the atrazine MIP column. Two of these compounds (propazine and terbutylazine) coeluted with atrazine, whereas the other compounds eluted just slightly prior to atrazine. These compounds were well separated from derivatives in which R_1 is a different functionality, such as −OH, −OCH_3, and −SCH_3. The alkyl side groups R_2 and R_3 were almost identical for all compounds and had little relevance to the extent of retardation. Non-triazine herbicides were not significantly retained on the column. Analysis of the K values, rather than the retardation factors, gives a clearer picture of the selectivity of the MIP, because retardation due to nonspecific interaction with the polymer varies significantly between the compounds. Under the conditions used (4% acetic acid in acetonitrile), the nonspecific retardation is stronger for more polar compounds, such as the dealkylatrazines, than for less polar compounds, such as atrazine, as recorded on the nonimprinted reference polymer. This result is because of the presence of carboxylic acid groups on the polymer surface, which can be involved in hydrogen bonding with polar molecules. The K values were in all instances <1, except for the structurally very similar terbutylazine (Table 1), demonstrating that the MIP efficiently recognized atrazine compared with other triazine derivatives. Low K values are recorded for the dealkylatrazines, which lack one or both alkyl groups. Hence, they are only weakly recognized by the atrazine MIP. Because of their strong nonspecific retention, consideration of retention factors only would miss this observation.

The *s*-triazine polymer demonstrated a significantly lower but still detecable specific binding of the imprint species, in this case *s*-triazine. Analysis of K values rather than retention factors was necessary because, in contrast to the atrazine MIP, the imprint species was not the last to elute (Table 1). Instead, a relatively weak retention was observed on both the imprinted and the reference column. The fact that high K values are obtained also for structurally unrelated compounds, such as metamitron (Table 1), is further evidence for the weak selectivity shown by the *s*-triazine polymer system. This weak selectivity is due to *s*-triazine being a small molecule with few structural features that can serve as points of identification for recognition and binding.

Radioactive Ligand Binding Assays. The binding of radiolabeled atrazine to the anti-atrazine polymer in the absence and presence of varying concentrations of competing ligands was analyzed under conditions where the number of polymeric binding sites was limited. The experimental design is analogous to the conceptually identical competitive immunoassays, where labeled and unlabeled analyte molecules compete for binding to a restricted number of antibody binding sites (Price and Newman, 1991). The incubation conditions of the assay were established by applying protocols described previously for MIP ligand binding assays (MIA; Vlatakis et al., 1993; Andersson et al., 1995b). Ligand binding was investigated in several solvents. The binding strength increased with decreased polarity of the solvent used. A mixture of 5% acetonitrile in toluene was a useful incubation solvent. This solvent combined high binding affinity for atrazine with low nonspecific binding, evident as weak binding to the nonimprinted reference polymer. The binding of radiolabeled atrazine with increasing concentration of imprinted and nonimprinted polymer was investigated. One milligram of polymer/per milliliter was required to bind ∼50% of the added radioligand, and this polymer concentration was used in subsequent experiments. At this polymer concentration, the nonimprinted reference polymer bound 10% of

Table 2. Cross-Reactivity of Triazine Derivatives and Other Herbicides for Binding of Labeled Atrazine to Anti-atrazine MIP and Anti-atrazine Antibodies

compound	MIP IC_{50} (mM)	cross-reactivity (%)			
		MIP	Mab[a]	Mab[b]	Pab[c]
atrazine	0.016	100	100	100	100
terbutylazine	0.036	44	20	26	1.2
propazine	0.090	18	147	136	116
simazine	0.13	12	32	4	1.1
deisopropylatrazine	0.03	53		8	
deethyldeisopropyl-atrazine	0.27	5.9		0.0	
prometryn	0.95	1.6	17	<0.1	10.1
terbutryn	1.0	1.6	19	0.3	<0.1
terbumetone	0.22	7.4			
atratone	0.23	7.0		0.0	
hydroxyatrazine	0.50	3.2	2		<0.1
s-triazine	56	≪0.1			
metamitron	25	<0.1			
isoproturon	95	≪0.1			
alachlor	>100	≪0.1			
pirimicarb	>100	≪0.1			

[a] Data for the monoclonal antibody clone AM7B2.1 in Schneider and Hammock (1992). [b] Data for clone K4E7 in Giersch (1993). [c] Data for the polyclonal antibody serum S84 in Wüst and Hock (1992).

the added radioligand. The binding reaction was rapid, equilibrium was reached within 1 h, and 90% of the binding occured within 30 min. Because of binding site heterogeneity of the MIP, the calibration graph for atrazine determination is flat and extends over four orders of magnitude. The detection limit of the assay is 0.25 μM (55 μg/L) (the concentration of atrazine that gives a B/B_0 value of 0.9) and an IC_{50} value (the concentration of atrazine that displaces 50% of the binding of the radiolabelled ligand) of 16 μM (3.4 mg/L). Biological antibody-based assay systems show a better sensitivity, with IC_{50} values typically around 0.2 μg/L (Giersch, 1993; Schneider and Hammock, 1992; Weil et al., 1991; Wüst and Hock, 1992). In these instances, the assays were performed in the ELISA format, where sensitive enzyme tracers such as peroxidase coupled to atrazine were used for recording the binding reaction. In our case, there is the potential to improve the assay, because we used a radiolabeled tracer, obtained commercially, with only low specific activity (see Materials and Methods). Ongoing work with the objective of improving the binding capacity of the MIPs includes the use of new cross-linking agents, such as tri- and tetramethacrylylerythritol (Kempe and Mosbach, 1995), and the preparation of beaded polymer particles.

The specificity of the atrazine MIP was determined via the cross-reactivity of several triazine derivatives and some other pesticide compounds (Table 2). Structurally unrelated pesticides and *s*-triazine did not interfere with the binding of radiolabeled atrazine even at concentrations up to 1 mg/mL. In contrast, several of the triazine herbicides showed significant cross-reactivity. This result was expected, however, because the triazine herbicides have very similar structures and even antibodies have difficulty distinguishing between them (Table 2). Close analysis of the binding data demonstrates that the level of cross-reactivity is roughly proportional to the structural resemblance to the imprint species. The single most important factor to affect the cross-reactivity is the substituent on carbon C1 (R_1 in Figure 1). The binding affinity is strongest for atrazine, somewhat lower for other triazines where R_1 is chlorine, and then decreases in the rank order $-Cl > -OCH_3 > -OH > -SCH_3$. The identities of the alkyl groups R_2 and R_3 are less important because structural variations of these cause only marginal effects on the level of cross-reactivity. It must be emphasized, however, that the imprint species was the compound best recognized by the MIP, a finding that contrasts with the specificities observed for antibody clones (compare atrazine and propazine, Table 2). Preparation of biological antibodies against a low molecular weight compound, such as atrazine, necessitates its conjugation to a carrier protein before immunization of the animal. Such derivatization may alter the properties of the antigen presented to the immune system, and the resultant antibodies may be directed to a somewhat different structure than desired. For atrazine, the conjugation is usually carried out by derivatization of the R_2 position with a caproic acid spacer, which then is used to link the atrazine molecule to a surface amino group of the protein. The resultant antibodies would be expected to have difficulty in favoring ethyl (as in atrazine) over larger alkyl groups (as in propazine) at this position during the binding event.

In aqueous buffers, very strong nonspecific adsorption of the hydrophobic atrazine to both the imprinted and nonimprinted polymers was observed. Addition of surfactants to the incubation mixtures reduced the nonspecific binding. Several surfactants were tested, and addition of 0.15% Tween 20 to 10 mM sodium phosphate (pH 7) reduced the nonspecific adsorption to an acceptable low level. Under these conditions, 52, 21, and 12% cross-reactivity were recorded for atratone, prometryn, and hydroxyatrazine, respectively. These values were significantly higher than those obtained in the toluene-based assay. Likewise, higher IC_{50} values were obtained: 1.5 mM for atrazine; 2.9 mM for atratone; 7.0 mM for prometryn; and 13.0 mM for hydroxyatrazine. Although binding affinity and selectivity decreased, these results demonstrate that MIP recognition is possible in aqueous solutions.

Conclusions. This work is a pilot study on the synthesis and use of imprinted polymers with predetermined selectivity for environmental pollutants, such as atrazine. Such MIPs may be applied in the development of ligand binding assays, were imprinted polymers substitute for the antibodies used in conventional immunoassay. However, the very high sensitivity characteristic for many antibody-based assays has not yet been achieved. Advantages of the MIP-based approach include the high mechanical, thermal, and solvent stability of the polymers used. As shown in this report, it is possible to prepare MIPs exhibiting atrazine selectivity comparable to that of antibodies, at least if the binding reaction is performed in toluene. In this context, there is a desire to perform immunoassays in anhydrous organic solvents (Russell et al., 1989, Wetall, 1991), especially in situations where the analyte is hydrophobic and sample workup necessitates a solvent extraction step. The triazine pesticides are examples of such analytes with poor water solubility, and attempts have been made to perform the antibody–antigen binding reaction in organic solvents (Stöcklein et al., 1990). Hence, for the detection of atrazine, the use of imprinted polymers is a potentially interesting alternative to antibodies.

Another application within the environmental analysis area may be the use of atrazine MIPs as a solid-phase extraction sorbent for preconcentration of samples

before determination of residue concentrations using traditional methods. Another potential use of imprinted polymers is as sorbents for the removal of specific toxic compounds in water treatment. The MIPs may be distributed at the site of contamination where they would adsorb atrazine, or any other biohazardous compound against which a MIP is available. When the binding capacity of the sorbent is saturated, the sorbents may be regenerated and reused. The contaminants can then be disarmed by incineration or deposition or be recycled. In the case of atrazine, biological methods for the decontamination of water and soil are being investigated (Leeson et al., 1993). These processes involve ozonization of the pesticide followed by biomineralization that is catalyzed by microorganisms. The results obtained indicate low efficiency in field environments, at least by the strains tested thusfar. In this context, the use of imprinted polymers should be considered as a potential alternative to the technologies presently studied in environmental clean-up applications.

ADDED IN PROOF

It has come to our attention that, during the past few months, two related studies appeared on a similar topic: Muldoon, M. T.; Stanker, L. H. *J. Agric. Food Chem.* **1995**, *43*, 1424−1427. Matsui, J.; Doblhoff-Dier, O.; Takeuchi, T. *Chem. Lett.* **1995**, 489.

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