Phenobarbital Molecularly Imprinted Polymer Selectively Binds Phenobarbital

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Molecularly imprinted polymer (MIP) was prepared against phenobarbital using methacrylic acid as the functional monomer and ethylene glycol dimethacrylate as the cross linking monomer. We analyzed the recognition properties of the phenobarbital MIP. In some organic solvents, imprinted polymer showed selective binding to phenobarbital. Two dissociation constants of binding were calculated by Scatchard plot analyses; K_d values were 1.8, 121.7 μM, and the number of binding sites was 8.3, 92.3 μmol/g MIP in toluene–heptane–acetic acid (25 : 75 : 1, v/v), respectively. The relationship between the binding affinity to phenobarbital MIP and the polarity of the solvent system, as well as the structure of the template molecule is also discussed.

Key words molecularly imprinted polymer (MIP); molecular imprinting; phenobarbital; molecular recognition; antibody mimic

The concentrations of drugs and hormones in body fluids with a narrow therapeutic index and variable rates of elimination are generally measured by immunoassays.[1] Specific monoclonal antibodies, first produced by Köhler and Milstein,[2] constitute a powerful immunochemical technology with which to monitor therapeutic drugs. It is necessary, however, to handle antibodies carefully in order to prevent loss of activity.

Molecular recognition mechanisms have been intensively studied. One approach has been the synthesis of a three dimensional 'host' structure, such as crown ether, which selectively binds 'guest' molecules. To create a specific host, Mosbach[3] introduced a specific imprint molecule to coordinate the assembly of synthetic functional and cross linking monomers around a template molecule. After polymerization, the imprint molecule is removed from the polymer by extraction to leave individual sites with complementary binding points within the polymer which are complementary to both shapes and chemical functions. A macroporous structure allows the imprint molecule to diffuse into and out of the polymer matrix.[3–5] This new technique is now being evaluated in areas such as chiral separation, antibody mimics, enzyme mimics and substrate-selective sensors.[3] The advantage of the method is the simple, rapid and inexpensive preparation of the molecularly imprinted polymers (MIPs) and their stability.

In this study, we prepared a phenobarbital MIP against the antiepileptic drug phenobarbital using methacrylic acid as the functional monomer and ethyleneglycol dimethacrylate as the cross linking monomer, and demonstrated the recognition properties of the phenobarbital MIP.

MATERIALS AND METHODS

Materials Phenobarbital, hexobarbital, metharbital, amobarbital, carbamazepine, ethotoin, nitrazepam were of Japan Pharmacopeia grade. Methacrylic acid (MAA), ethylene glycol dimethacrylate (EDMA), 2,2′-azobis (2-methylpropionitrile) (AIBN), theophylline, nifedipine, disopyramide, acetonitrile, chloroform were purchased from Wako Pure Chemical Industry, Osaka, Japan.

Polymer Preparation A phenobarbital MIP was constructed by the method of Mosbach *et al.*[4] Functional monomer MAA (1.8 g [20.9 mmol]) was mixed with phenobarbital (0.94 g [4.0 mmol]), EDMA (18.7 g [94.3 mmol]), and AIBN (0.24 g) in chloroform (50 ml) in a round bottomed flask (200 ml). The mixture was degassed under vacuum in a sonicating water-bath and sparged with nitrogen for 5 min before polymerization under ultraviolet-illumination at 4 °C for 16 h. The bulk polymer was ground into particles in a mechanical mortar, and the fine particles were removed by repeated sedimentation from acetonitrile. The particles were extracted by extensive shaking with a large amount of methanol–acetic acid (9 : 1, v/v) until phenobarbital was no longer detected spectrophotometrically in the extract solvent. Three or four extractions were usually needed to accomplish this. Repeated times shaking was required to complete extraction with methanol–acetic acid (9 : 1, v/v). The particles extracted were then washed with a large amount of methanol and water, respectively, and then dried under vacuum.

Binding Assay The phenobarbital MIP was shaken for an appropriate period at room temperature in 2 ml of assay solution containing a test compound. After centrifugation for 5 min at $800 \times g$, the supernatant (S1) was collected. The pellet was washed with the same incubation buffer, centrifuged, then the supernatant (S1′) was collected and combined with S1. The pellet was extracted with methanol–acetic acid (9 : 1, v/v) by vortex mixing, and the supernatant (S2) was recovered after the centrifugation. The solvent of S1 + S1′ and S2 was evaporated, respectively, and the residues were redissolved in 2 ml of 1 M NaOH when phenobarbital or in 2 ml of methanol when other compounds were tested. The absorbance (λ_{max}) of each sample was measured and determined for each concentration.

RESULTS

Polymer Preparations Ultraviolet light can initiate the

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polymerization of phenobarbital MIP at 4 °C, because polymers constructed at lower temperatures exhibit higher recognition due to weak noncovalent interactions, such as the hydrogen bonding believed to be essential for imprint formation and subsequent recognition.[4] The molar ratio of methacrylic acid to phenobarbital was 5.0.

Time Course of the Phenobarbital Binding to the Phenobarbital MIP We investigated ligand binding during several intervals (0, 15, 30 min, 1, 2, 3 and 6 h). Binding was assayed in 2 ml of toluene–heptane–acetic acid (75:25:1, v/v), using 10 mg of phenobarbital MIP and 10 μg of phenobarbital. The polymer particles were centrifuged ($800 \times g$, 5 min), and the phenobarbital concentrations were determined spectrophotometrically in the supernatant. The ratio of the binding to MIP markedly increased for 2 h, then became saturated at 3 h (data not shown). Accordingly, the reaction was gently shaken for 3 h at room temperature in subsequent experiments.

The Binding Capacity and Recovery of Phenobarbital from Phenobarbital MIP We investigated the binding capacity and the recovery of phenobarbital from phenobarbital MIP. The assay proceeded in 2 ml of toluene–heptane–acetic acid (75:25:1, v/v), using from 10 to 50 mg of phenobarbital MIP and 10 μg of phenobarbital in a 3 h incubation at room temperature with gentle shaking. As shown in Table 1, the phenobarbital bound to phenobarbital MIP was quantitatively recovered from MIP, and the recovery was 98.8—103.2%. The amount of phenobarbital bound to phenobarbital MIP determined from the phenobarbital concentration in the supernatant of the incubation mixture was apparently the same as that of the phenobarbital recovered from the MIP. Therefore, the ratio of the binding of phenobarbital to phenobarbital MIP was determined from the ratio of phenobarbital recovered from the MIP or the ratio of the unbound phenobarbital (remaining in the supernatant of the reaction mixture).

Binding Selectivity of Phenobarbital MIP against Several Compounds The assay was performed in 2 ml of toluene–heptane–acetic acid (75:25:1, v/v), using 10 mg of phenobarbital MIP and 10 μg of each compound for 3 h at room temperature (about 20 to 25 °C) with gentle shaking. The binding capacity of phenobarbital, hexobarbital, metharbital, amobarbital, carbamazepine, theophylline, nifedipine, ethotoin, nitrazepam and disopyramide was tested to the phenobarbital MIP. The MIP selectively bound phenobarbital in this solvent system (Table 2). About 47.7% of the phenobarbital bound to the polymer. Phenobarbital, however, did not selectively bind in toluene–heptane (75:25, v/v) (Table 2), suggesting that acetic acid was essential for the selective binding of phenobarbital to phenobarbital MIP. Only the structurally closely related compounds hexobarbital and amobarbital significantly cross-reacted.

The Binding of Phenobarbital and Composition of the Assay Solvent The assay was performed in 2 ml of solvent, using 10 mg of phenobarbital MIP and 10 μg of phenobarbital for 3 h at room temperature with gentle shaking. As shown in Fig. 1, the binding rate of phenobarbital was increased as the ratio of heptane was increased in the assay solvent. Phenobarbital MIP did not bind to phenobarbital in methanol or ethanol, which are protonic solvent. Moreover, in other aprotonic solvents, such as acetonitrile ($\varepsilon = 37.5$, 20 °C), the MIP also did not bind to phenobarbital. On the other hand, phenobarbital bound to the MIP only in toluene ($\varepsilon = 2.379$)–heptane ($\varepsilon = 1.924$). The binding ratio of phenobarbital to MIP tended to be linear with the ratio of heptane in the solvent.

Scatchard Plot Analysis Scatchard plot analyses were performed[6] to examine the relationship between the

Table 1. Recovery of Phenobarbital from Imprinted Polymers

Polymer (mg)	Supernatant (%)	Pellet extract (%)	Recovery (%)[a]
10	63.4	37.8	103.2
20	38.5	60.9	99.0
50	23.4	75.7	98.8

a) Recovery (%) = [pellet extract (%)/(100 − supernatant (%))] × 100.

Table 2. Reactivity of Barbiturates and Other Compounds for Binding to Phenobarbital Imprinted Polymers and Effect of Acetic Acid

Compound	Reactivity (%)	
	Reaction solvents (v/v)	
	Toluene–heptane (75:25)	Toluene–heptane–acetic acid (75:25:1)
Phenobarbital	64.4	47.7
Hexobarbital	N.D.	49.1
Metharbital	N.D.	<1
Amobarbital	N.D.	13.4
Carbamazepine	58.7	<1
Theophylline	73.2	<1
Nifedipine	14.3	<1
Ethotoin	<1	<1
Nitrazepam	31.6	<1
Disopyramide	96.0	<1

a) N.D.: not determined.

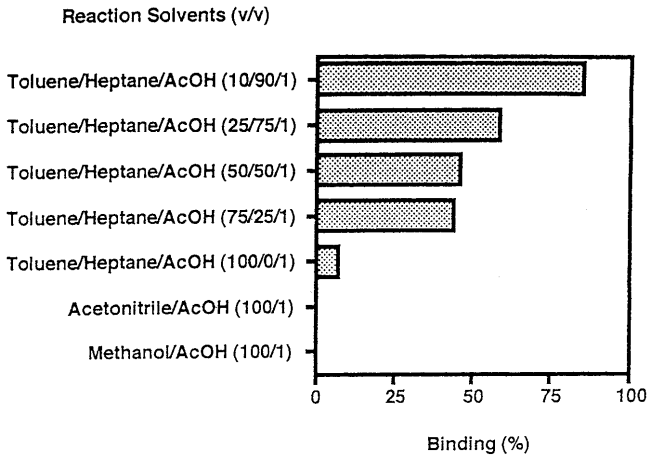

Fig. 1. Effects of Reaction Solvents against Phenobarbital Molecularly Imprinted Polymer

Phenobarbital MIP (10 mg) and 2 ml of phenobarbital dissolved in each solvent (5 μg/ml) were mixed and incubated for 3 h. Phenobarbital binding was assayed spectrophotometorically. The dielectric constants of acetonitrile, toluene, heptane and acetic acid were 37.5, 2.28, 1.92, and 6.15, respectively (20 °C; except for toluene, 25 °C).

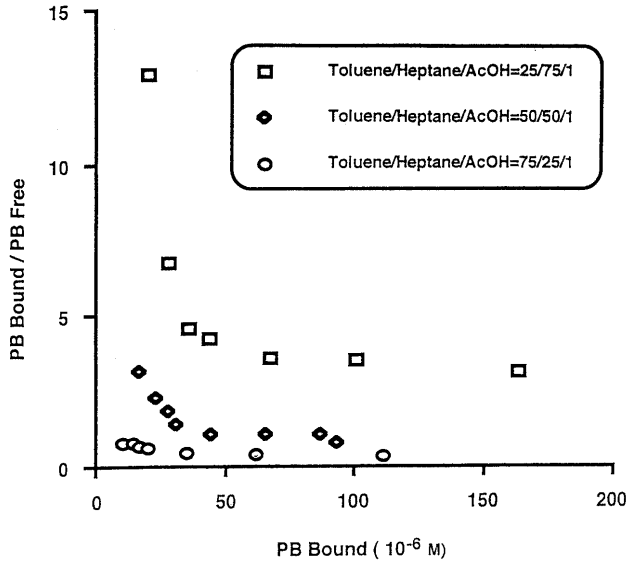

Fig. 2. Scatchard Plot Analysis of Phenobarbital Binding to Phenobarbital MIP

Phenobarbital MIP (10 mg) was incubated with 2 ml of phenobarbital dissolved in various solvents (concentrations varied from 5 to 200 μg/ml). Free and bound phenobarbital values were calculated and then analyzed by Scatchard plots.

Table 3. Dissociation Constant (K_d) and Population of Sites (S)

Reaction solvent					
Toluene–heptane–acetic acid (v/v)	$\varepsilon^{a)}$	$K_{d1}{}^{b)}$	$S_1{}^{c)}$	K_{d2}	S_2
25 : 75 : 1	2.082	1.8	8.3	121.7	92.3
50 : 50 : 1	2.191	8.7	8.6	166.7	41.6
75 : 25 : 1	2.307	50.7	9.9	640.7	63.7

a) Geometric average, see legend of Fig. 1. *b*) μM. *c*) μmol/g polymer.

binding and the reaction solvent. The reaction solvents used to 10 mg of phenobarbital MIP were 2 ml of toluene–heptane–acetic acid (25 : 75 : 1, v/v), toluene–heptane–acetic acid (50 : 50 : 1, v/v), and toluene–heptane–acetic acid (75 : 25 : 1, v/v) at room temperature. The plots were nonlinear (Fig. 2), due to the heterogeneous population of sites with various affinities for phenobarbital, and were approximated best by models with two apparent K_d values for high- and low-affinity binding sites. The apparent K_d values for phenobarbital binding to phenobarbital MIP were 1.8 and 121.7 μM in toluene–heptane–acetic acid (25 : 75 : 1, v/v), associated with site populations of 8.3 and 92.3 μmol/g, respectively (Table 3). In toluene–heptane–acetic acid (75 : 25 : 1, v/v), however, the K_d values were 50.7 and 640.7 μM for high- and low-affinity binding sites, associated with site populations of 9.9 and 63.7 μmol/g, respectively.

DISCUSSION

Phenobarbital MIP was prepared by non-aqueous bulk polymerization method, and methacrylic acid was used as the functional monomer to prepare it. In some organic solvents, imprinted polymer showed selective binding to phenobarbital. Two dissociation constants of binding were calculated by Scatchard plot analyses; K_d values were 1.8, 121.7 μM, and the number of binding sites was 8.3, 92.3 μmol/g MIP in toluene–heptane–acetic acid (25 : 75 : 1, v/v), respectively. The carboxylic acid function of the functional monomers was expected to form a strong hydrogen bond with the carboxylic acid function of the print molecules.[4] Therefore, the binding force between phenobarbital and phenobarbital MIP might be an interaction between carboxylic acid of the MIP binding site and heterogeneous atoms, such as nitrogen of the phenobarbital skeletal structure. Only the structurally closely related compounds hexobarbital and amobarbital in tested compounds showed significant cross-reactivity, whereas the cross-reactivity of metharbital was minimal.

Vlatakis *et al.* demonstrated that a theophylline MIP bound selectively to theophylline in acetonitrile–acetic acid (99 : 1, v/v).[4] However, our phenobarbital MIP did not bind to phenobarbital in acetonitrile–acetic acid (99 : 1, v/v) but bound in a toluene–heptane mixture. The apparent K_d values for binding of phenobarbital were one order lower than that of theophylline MIP or diazepam MIP as reviewed by Vlatakis *et al.*[4] We believe that the difference of K_d values between phenobarbital MIP in this report and others may be due to the differences in chemical properties of printed molecules; it is important for the binding that the number and of the functional groups or atoms present on the print molecule are able to interact with carboxylic acid of the phenobarbital MIP.

The highest affinity binding was found in an aqueous buffer, as nonspecific binding to a nonimprinted reference polymer (data not shown). In the organic mixture of toluene–heptane (75 : 25, v/v), the phenobarbital MIP bound phenobarbital with high affinity, but the cross-reactivity was also higher, and was recognized as nonspecific binding (Table 2). However, the binding was most specific in toluene–heptane–acetic acid (10 : 90 : 1, v/v) (Table 2). The inclusion of acetic acid in the solvent mixture may increase the formation of the hydrogen-bonding interactions between phenobarbital and phenobarbital MIP and produce optimal specific interactions as described by Andersson *et al.*[5] Structurally similar compounds, such as amobarbital, cross-reacted weakly in an immunoassay with a specific monoclonal antibody. It is possible that the binding-site of phenobarbital is the N site, since methacrylic acid as the functional monomer induces the creation of anionic binding sites.[5] Hosoya and co-workers reported that non-covalently MIP phases prepared by a two step swelling technique was very useful for chiral resolution by HPLC.[7]

In conclusion, this study demonstrated that synthetic polymers can be used as receptor-binding-site mimics and that phenobarbital MIP construction is a simple, rapid, and inexpensive preparation and the product is stable. Molecular recognition technology using chemical preparations should be further developed.

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