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Characterization of the selectivity of a phenytoin imprinted polymer

Tímea Pap [a,*], George Horvai [b]

[a] *Research Group of Technical Analytical Chemistry, Hungarian Academy of Sciences, Budapest University of Technology and Economics, Szt. Gellért tér 4, H-1111 Budapest, Hungary*
[b] *Department of Chemical Information Technology, Budapest University of Technology and Economics, Szt. Gellért tér 4, H-1111 Budapest, Hungary*

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Abstract

The selectivity of analytical methods based on molecularly imprinted polymers (MIPs) is due to the preferential adsorption of the analyte(s) as compared to other substances (interferences). This paper shows the theoretical and practical difficulties, which have to be considered and solved when real samples need to be analysed in a wide range of analyte and interferant concentrations. It is shown that the estimation of interference effects requires either many measurements or a realistic model of the adsorption equilibrium in mixed solutions of the analyte and the interferences. Examples are shown for positive (cooperative) interference effects, for better experimental design and interpretation of binary isotherm measurements and for establishing the chemical model of interference from selectivity measurements. The usual MIP model consisting of a cavity, which closely fits the shape of the template from all sides, appears unsuitable for this MIP, and it is replaced with a more realistic, more open model. The applicability of the results to using non-imprinted polymers as selective sorbents and to screening drug candidates is also shown.

Keywords: Molecularly imprinting; Selectivity; Adsorption; Binding studies; Stationary phases, LC; Phenytoin

1. Introduction

Molecularly imprinted polymers (MIPs) have recently gained much popularity [1]. They may serve as chromatographic and solid-phase extraction sorbents, as sensor materials, as artificial antibodies, as artificial enzymes, etc. [1–4]. Preparation of MIPs is technically quite simple but their exact chemical characterization – particularly the elucidation of the structure of binding sites – is difficult since they are usually insoluble amorphous solid materials, not well amenable for most spectroscopic and surface analysis methods.

The main feature of MIPs is their capability to selectively bind the substance used for imprinting, i.e. the template. This selectivity or specificity manifests itself in useful ways, e.g.:

– Higher chromatographic retention for the template than for other substances of the sample.
– A sensor signal influenced mainly by the template and only little by other components of a sample.
– (Nearly) exclusive and exhaustive binding of the template from complex sample matrices.

It is not enough, however, to obtain sufficient selectivity in one particular sample. As it is normal with any analytical method, a variety of samples need to be considered with the analyte concentration and the matrix composition, respectively, varying in a wide range. Thus, one needs to be able to estimate the effects of interfering substances in such a range. This problem has rarely been discussed with respect to MIPs.

In this paper, we show that MIP selectivity can be a complex matter. A recently developed MIP for the antiepileptic drug phenytoin [5] seems to be a case particularly suited to show some of the problems that may arise. At the same time, the data to be presented here allow to better understand how selectivity is created in MIPs.

2. Theory

Non-covalent MIPs are usually prepared by radical polymerization of suitable monomers in the presence of the

* Corresponding author. Tel.: +36-1-4631480; fax: +36-1-4631480.
E-mail address: pap-t@mail.bme.hu (T. Pap).

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template. The monomers and the template should have some functional groups capable of interacting with each other by reasonably strong secondary forces like ionic, hydrogen bonding, π–π, etc. The monomers and the template are thought to form such complexes during polymerization. After leaching out the template from the polymer by a suitable solvent, the binding functional groups of the polymer network remain in their binding positions. Thus, they are expected – and often found – to rebind only the template or molecules having similar functional groups to those of the template in the right geometrical positions.

2.1. Quantitation of selectivity in simple binding models

The above rudimentary description of the imprinting process leaves some questions open concerning the binding sites, such as:

- Are all imprinted binding sites identical (homogeneous) or are there different imprinted sites on the MIP?
- Are there only imprinted sites on the MIP or are there also other binding sites there (e.g. sites that would also be found on the NIP)?
- Are the binding sites binding the template (or any other compound) with a strict 1:1 stoichiometry? (Note that a negative answer to this question may involve various alternative mechanisms, e.g. two or more sites being close and interacting, or two or more template and other molecules being bound as a cluster.)
- Is the chemical and geometrical structure of the binding sites independent from the sample matrix? (Note that a negative answer may again mean various alternatives, e.g. swelling or shrinking of the MIP, creation of new or modified binding sites, etc.)

The most simple MIP would thus have homogeneous, well-separated binding sites, characterized by strict 1:1 stoichiometry towards any adsorbable compound and free of matrix effects. These requirements practically coincide with the Langmuir model of adsorption [6]. In this model, there is a single, well-defined free energy change (and thus single equilibrium constant) associated with the adsorption of the template from a given solvent. Other adsorbable compounds (which may be considered interferants in analytical problems) also have their respective binding energies/equilibrium constants. The adsorption isotherm for any adsorbable substance can be described – in the absence of other adsorbable substances – by the Langmuir isotherm, which is essentially reformulation of the law of mass action together with the mass balance. With this model, binding equilibria can also be quantitatively characterized in mixed solutions of two or more adsorbable substances: one has to write only for all the simultaneous equilibria, the law of mass action and consider the mass balances. A simple consequence of these equations is that adsorbable substances compete for the binding sites. The Langmuir model can be quite straightforwardly extended to two or three well-defined but different types of sites if all are acting independently with 1:1 stoichiometry. These are the bi-Langmuir, etc. models [6]. A great advance of Langmuirian systems is that the equilibrium constants measured for individual adsorbable substances may be used directly to calculate the isotherms of their mixtures.

Despite all the convenient features of the Langmuir model one needs to see clearly that:

- A good fit to the Langmuir equation does not mean that the physical system really fulfils the model assumptions; the bi- and tri-Langmuir cases are notorious for such problems.
- The Langmuir model does not say anything about the detailed chemical and spatial structure of the sites.

2.2. Quantitation of selectivity in complex binding models

Many early studies of non-covalent MIPs used the Langmuir models. This was based on reasonably good fits of these models to the adsorption isotherms of the respective templates [1,7–11]. More recent studies have cast doubts on the general usability of the Langmuir models. On the one hand, better fits were obtained to the isotherm with the Freundlich model [12] and other models which assume many different sites with a wide range of binding energies [13,14], still with 1:1 stoichiometries. On the other hand, several studies showed evidence that binding may occur with other stoichiometries than 1:1, e.g. due to binding of template clusters or binding of a template molecule to an already adsorbed other template molecule (cooperative binding) [15–18]. Based on these results, it is also easy to imagine MIPs with a variety of sites, where some sites are Langmuirian (and thus competitively occupied), while others are not observing 1:1 stoichiometry and therefore show cooperative binding.

These complex models do not seem to allow the quantitation of simultaneous binding equilibria of two or more substances from data measured in separate solutions of these substances (as was the case with the Langmuir models described earlier). One should also note that these models were obtained from measurements with the template alone. In the simultaneous presence of template and interferant(s), one may—by extension—expect non one-to-one stoichiometries of binding with mixed occupation by template and interferant(s). Thus, one can expect cooperation between the template and the interferant(s). In this paper, we show first experiments where this seems to be the case.

In the non-Langmuirian cases of simultaneous adsorption of two or more substances, we do not have any proven formula available to quantitatively describe the adsorbed concentration (q_i, q_j, etc.) of each substance as a function of the equilibrium concentrations of all these substances in solution (c_i, c_j, etc.) and the concentrations of available sites, as it was the case for Langmuir adsorption. The existence of

such complex systems has apparently not yet been reported. In this paper, we show experimental results proving their existence and hinting to problems to be expected when using some MIPs in real samples.

2.3. Shape selectivity

As mentioned earlier, adsorption measurements made in separate solutions of the template and the interferants, respectively, cannot be used in the case of non-Langmuirian MIPs for predicting interferences in mixed solutions. Such measurements can still be useful to compare in a qualitative manner the interactions of these compounds with the binding sites. In this paper, we compare the binding of phenytoin and some chemically similar molecules to the phenytoin MIP and the corresponding blank polymer (NIP). The observed selectivities are used to study the widely held model of molecular imprinting which calls for the imprinting of cavities which sterically fit quite closely the shape of the template [19,20]. This spatial fit has been thought to contribute in some way to selective binding – e.g. van der Waals forces [20] and size exclusion – whereas the main binding forces are due to interactions between functional groups, charges, dipoles and π–π bonds.

Our data and also common sense seem to exclude the model of stiff binding cavities in solid (as opposed to swollen gel type [21]) MIPs. It is difficult to see how molecules of molecular weight of around 300 can get into almost closed cavities. The exact fit would also completely prevent binding of molecules larger than the template – by steric exclusion – but this is not the case. On the other hand, the selectivity for smaller molecules, which carry the main binding functionalities of the template in the right positions, should be as large as for the template if binding occurred only by interactions between functional groups. We shall show that this is not the case, either. With our data at hand, we shall suggest a more open model, which still uses the steric exclusion effect but not in the shape of a closed cavity.

Before going to these experimental data, we need to mention that they were obtained on a MIP and a NIP column, respectively, by measuring the individual chromatographic retention data of the substances studied. The relationship between the chromatographic peak's retention and shape on the one hand and the isotherm on the other is quite complex [6]. Since we shall use only retention data and these will also used only for qualitative conclusions, we do not discuss the matter in detail. In the chromatographic assessments of MIPs, there are generally two different types of selectivity considered. One is the analytical selectivity (α), i.e. the discrimination between the template and other substances by the MIP. The analytical selectivity is widely used in case of characterizing chiral MIPs, since the effects of non-specific binding cancel out when enantiomers are compared [19,20]. The other is the so-called imprinting factor (IF), which might also be termed as selectivity due to the imprinting. The IF is the ratio between the respective retention factors of a substance (this may be the template or any other compound) on a MIP column and the NIP column:

$$\mathrm{IF} = k'_{\mathrm{MIP}}/k'_{\mathrm{NIP}}$$

The IF has been generally used to show that imprinting has indeed increased the binding of the template. It is important to do this because the NIP may itself also bind the template. The NIP has the same functional groups available for template binding as the MIP, albeit possibly not in the right positions for multiple binding. We shall use in this study the IF values to compare the effect of imprinting on the rebinding of different compounds. We shall see that even in cases when a substance (X) is more strongly bound by the MIP than its template (T), i.e. $k'_{\mathrm{MIP,X}} > k'_{\mathrm{MIP,T}}$ (or $\alpha_{\mathrm{X,T}} = (k'_{\mathrm{MIP,X}}/k'_{\mathrm{MIP,T}}) > 1$), the IF for the template is higher than for the other substance, i.e. $\mathrm{IF}_{\mathrm{MIP,X}} < \mathrm{IF}_{\mathrm{MIP,T}}$.

3. Experimental

3.1. Materials

Atrazine and ametryn were generously provided by Novartis (Basel, Switzerland). Phenobarbital and barbital were of Pharmacopoeia Hungarica VII grade. Barbituric acid was purchased from Reanal (Budapest, Hungary). Phenytoin (5,5-diphenylhydantoin), hydroxy-phenytoin (5-phenyl-5-(*p*-hydroxy)phenylhydantoin), methyl-phenytoin (5-(*p*-methylphenyl)-5-phenylhydantoin), dimethylhydantoin (5,5-dimethylhydantoin), diphenylmethane, hydantoin and *S*-(−)-1,1-diphenyl-1,2-propanediol were purchased from Sigma–Aldrich (St. Louis, MO, USA). For structures see Fig. 1. All solvents used were HPLC grade, methanol was purchased from Carlo Erba (Milan, Italy), acetonitrile was a product of Romil (Loughborough, UK). Milli-Q RG ultrapure water (Millipore, Billerica, MA, USA) was used to prepare eluent and calibration solutions. $NaH_2PO_4{\cdot}H_2O$ ("puriss" grade) from Fluka was used for preparation of buffer solution. The Eppendorf-type test tubes (1.5 ml) were from Spektrum 3D (Budapest, Hungary).

3.2. Molecularly imprinted polymer preparation

The MIP and NIP were prepared by Bereczki and co-workers as previously described elsewhere [5]. The composition of the polymerization mixture was as follows. MIP: 2 mmol phenytoin (504.5 mg) as template, 8 mmol methacrylamide (680.8 mg) as functional monomer, 40 mmol EDMA (7.6 ml) as cross-linker, 8.32 ml acetonitrile and 2.88 ml tetrahydrofuran (THF) as porogen and 100 mg AIBN as initiator. The NIP was prepared with the same composition but without the template phenytoin. The grounded and sieved polymer particles (25–36 μm) were packed into LC columns for elution chromatographic measurements or weighed into Eppendorf tubes for batch

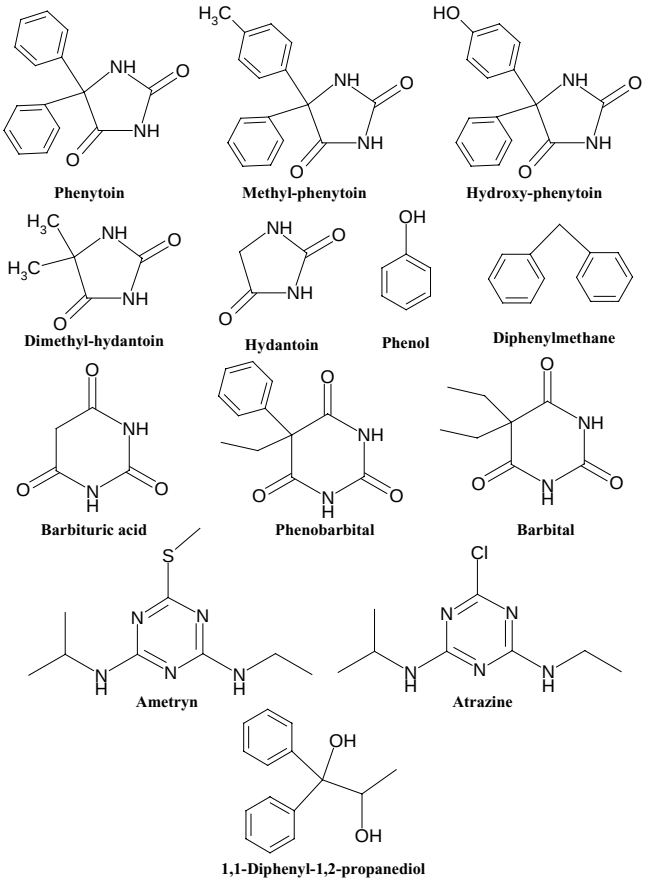

Fig. 1. Structures of the compounds tested in elution chromatography.

adsorption measurements. Before the batch adsorption experiments, the imprinted polymer was thoroughly washed. The template bleeding was determined to be below the quantitation limit of the batch equilibrium measurements.

3.3. Batch adsorption measurements

A 50.0 mg amount of air-dry MIP or NIP in an Eppendorf tube was equilibrated at room temperature ($25 \pm 1\,^{\circ}C$) with mixtures of phenytoin and atrazine prepared at different concentrations in acetonitrile. Every experimental point was measured in duplicate or triplicate. The supernatant in each tube was replaced with fresh solution repeatedly, following equilibration periods of at least 3 h each time, until the polymer was in equilibrium with the solution (the supernatant concentration did not change and was equal to the concentration of the fresh solution). Then, the content of the tube was centrifuged and the supernatant was carefully removed. The mass of the solution remaining in the tube (among the polymer particles and in pores) was determined by weighing the tube with its contents and subtracting the known mass of the vial and the dry polymer. From the known concentration, mass and density of the solution phase the number of moles of the solutes in the solution phase can be computed. The total amount of phenytoin and atrazine remaining in the tube (in solution or adsorbed) was removed for subsequent determination by a two-step washing protocol. In the first step, the washing solvent was acetonitrile with 2% methanol ($4 \times 600\,\mu l$), and in the second step, pure acetonitrile was used ($4 \times 600\,\mu l$). These mild conditions were chosen to avoid structural changes of the polymer during the wash. The wash fractions were collected, dried under nitrogen stream in a TurboVap LV evaporator (Zymark, Hopkinton, MA, USA), re-dissolved in 200 μl mobile phase and injected (10 μl) into a Perkin-Elmer Series 200 chromatograph. The mobile phase (30% methanol–30% acetonitrile–40% phosphate buffer (0.01 M), pH 4.8) was delivered (flow rate 400 μl/min) at room temperature to a Purospher RP-18 e.c. (5 μm, 3 mm × 125 mm, Merck) column and the concentration of the samples were determined using TurboChrom software from the UV absorbance of peaks measured at 240 nm. If the second fraction contained more than 2% of the total washed amount, a further wash cycle was applied. The amounts found in the wash fractions were added and the number of moles that remained on the solution phase (see earlier) was subtracted to give the number of moles adsorbed on the polymer.

3.4. Elution chromatography

To determine the imprinting factors in acetonitrile eluent (flow rate 1 ml/min, room temperature), 20 μl of 1 mM solutions of acetone (void marker, t_0) and either phenytoin or its structural analogues or other compounds (listed in Table 2) were injected onto two 125 mm × 4 mm columns, one packed with imprinted polymer (MIP) and the other packed with non-imprinted polymer (NIP). Experimental or estimated $\log P_{ow}$ values (P_{ow} = octanol–water partition coefficient) [22,23] and pK_a values of the compounds studied are shown also in Table 2. The measurements were done with a Perkin-Elmer Series 200 chromatograph; the peaks were detected by UV-Vis detection at 240 nm. The capacity factor was calculated as $k' = (t_R - t_0)/t_0$. The imprinting factor of each substance was calculated as the ratio of the respective capacity factors on the imprinted and non-imprinted polymer filled columns ($IF = k'_{MIP}/k'_{NIP}$).

4. Results and discussion

4.1. Simultaneous adsorption measured by the batch method

The conventional batch adsorption measurement method is not quite suitable for the three-dimensional mapping ($q_i = f(c_i, c_j)$) required in simultaneous adsorption studies. In the conventional method, a solution of the two compounds at known concentration is contacted with the sorbent and after an equilibration time period the decreased value of each concentration is measured. Thus, a designed experiment based on the equilibrium concentrations – which need to be

plotted in the isotherm – is not possible, since the equilibrium concentrations are not known before the experiment. We have modified the conventional batch adsorption method in such a way that the equilibrium solution concentration of each compound is fixed in advance. This allowed to determine directly how the change of the interferant concentration changes analyte adsorption and vice versa. With the conventional batch method, these results could have been only obtained from much more experimental points and only by using badly defined interpolations.

We have chosen atrazine (as the second compound besides phenytoin) for the simultaneous binding experiments for several reasons. The elution chromatographic study (Table 2) shows that the retention of atrazine from acetonitrile eluent is rather low on the MIP and it is virtually identical on the MIP and the NIP. This allowed the conclusion that the binding of atrazine occurred on surface sites that were present to the same extent both on the MIP and the NIP. Thus, atrazine could be regarded as a prototypic weakly interfering matrix species. We also knew from the previous study [8] that although the NIP adsorbed phenytoin less than the MIP, the adsorption of phenytoin was still substantially higher on the NIP than the adsorption of atrazine. From these observations, it has been concluded that in a simultaneous batch adsorption experiment, atrazine would not influence the adsorption of phenytoin (by competition) except perhaps at large atrazine excess. On the other hand, phenytoin – at not too high concentrations – would not influence the adsorption of atrazine, because phenytoin would preferentially adsorb to the most strongly binding imprinted sites, whereas atrazine could equally bind to many other sites.

Based on these expectations, the simultaneous batch binding experiments were designed in such a way that phenytoin concentration would be kept at a constant low level (10^{-5} M) whereas atrazine concentration would be increased from the same low level (10^{-5} M) to a 100-fold excess (10^{-3} M) over phenytoin. The number of concentration steps had to be kept small because the experiments are laborious. The results are shown in Table 1.

The data (Table 1) confirm first of all the chromatographic findings, i.e. the low and nearly equal adsorption of atrazine on both the NIP and the MIP, the preference for phenytoin compared to atrazine on both polymers, and finally the greater adsorption of phenytoin on the MIP than on the NIP.

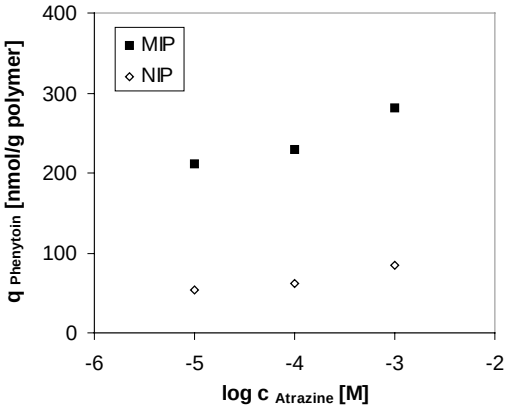

Fig. 2. Dependence of phenytoin adsorption (q) on the equilibrium atrazine concentration (c) for the MIP and the NIP, respectively. The equilibrium concentration of phenytoin in the solution is constant (10^{-5} M).

Besides these findings, a positive interaction of the two substances can be observed both on the NIP and the MIP (Fig. 2): an increase in atrazine concentration in solution and the concomitant increase of adsorbed atrazine concentration enhance the adsorption of phenytoin (while the equilibrium phenytoin concentration in the solution is kept constant). Phenytoin shows a similar enhancing effect on the adsorption of atrazine on the NIP (Table 1): the addition of phenytoin at 10^{-5} M solution concentration level increases the adsorption of atrazine in its 10^{-5} M solution from 5.2 to 9.1 nmol/g. This observed mutual enhancement of adsorption between atrazine and phenytoin may be due to atrazine and phenytoin molecules being co-adsorbed next to each other or on top of each other. All this occurs at low surface coverage, since we know from earlier work [8] that phenytoin adsorption can be more than 10 000 nmol/g NIP (at 10^{-2} M solution concentration). Thus, the observed positive interactions are not due to overcrowding of the surface.

Table 1
Adsorption equilibrium concentrations in solution (c) and on the MIP or NIP (q) with the relative standard deviations of the measurements (n was typically 3)

c (M)		q (nmol/g polymer) (R.S.D., %)			
Phenytoin	Atrazine	Phenytoin on NIP	Phenytoin on MIP	Atrazine on NIP	Atrazine on MIP
10^{-5}	–	61[a]	207[a]	–	–
–	10^{-5}	–	–	5.2 (11)	4.5 (18)
10^{-5}	10^{-5}	54 (4.8)	211 (2.1)	9.1 (4.9)	–
10^{-5}	10^{-4}	62 (3.2)	230 (0.6)	116 (28)	79 (4.7)
10^{-5}	10^{-3}	84 (13)	281 (1.3)	2050 (21)	2350 (29)
–	10^{-3}	–	–	1280[b]	1380[b]

[a] Frontal chromatographic data, the R.S.D. of the original breakthrough volumes was 12 and 6.3%, respectively.
[b] Interpolated data from atrazine adsorption isotherm (conventional batch adsorption measurement).

Table 2
Elution chromatographic data, $\log P_{ow}$ and pK_a values of phenytoin and the other test compounds (mobile phase: acetonitrile)

Test compound	$\log P_{ow}$	pK_a	MIP		NIP		IF
			t_R	k'_{MIP}	t_R	k'_{NIP}	
Acetone			1.48		1.50		
Phenytoin	2.47	8.33	11.15	6.52	3.43	1.29	5.04
Methyl-phenytoin	2.71[a]		8.22	4.55	3.29	1.20	3.78
Hydroxy-phenytoin	1.7		18.72	11.63	5.93	2.96	3.93
Hydantoin	−1.69	9.12	3.77	1.54	3.01	1.01	1.52
Dimethyl-hydantoin	−0.48	9.17	3.09	1.09	2.53	0.69	1.57
Diphenylmethane	4.14		1.70	0.15	1.70	0.13	1.08
Phenobarbital	1.47	7.3	4.33	1.92	3.18	1.13	1.71
Barbital	0.65	8.14	3.55	1.39	2.89	0.93	1.49
Barbituric acid	−1.47	4.04	5.96	3.03	3.96	1.65	1.83
Atrazine	2.61	1.7	2.04	0.38	1.98	0.32	1.16
Ametryn	2.98	4.1	1.98	0.34	1.95	0.30	1.11
Phenol	1.46		2.06	0.39	2.05	0.37	1.06
S-(−)-1,1-Diphenyl-1,2-propanediol	2.52[a]		1.96	0.32	1.98	0.33	0.99

[a] Estimated using KowWin program, see Section 4.2.

4.2. Chromatographic versus imprinting selectivities

Contrary to the batch adsorption measurements, the elution chromatographic method allows collection of many data in reasonable time. We used a NIP and a MIP filled chromatographic column, respectively, and measured the retention time (at peak maximum) for various, separately injected compounds (Fig. 1). The injected sample concentrations were always 10^{-3} M. The ratio of the respective k' values for the same compound on the MIP and the NIP is the imprinting factor. The results are shown in Table 2. The octanol–water partition coefficients ($\log P_{ow}$, from the literature or estimated) and the pK_a values are also shown. For the estimation of octanol–water partition coefficients, the KowWin program uses an atom/fragment contribution method [22,23]. Most compounds in the table are chemically related to the template phenytoin (see Fig. 1) and some of them are also drugs with similar (sedative) action (the barbiturates). There are shown also results for atrazine and ametryn. These are structurally unrelated compounds, but they also have functional groups capable of forming H-bonds – which is essential to have retention on this acrylamide type polymer – and their hydrophobicities are similar to phenytoin.

In the following discussion, we attribute the observed differences between different compounds in their k' and IF = k'_{MIP}/k'_{NIP} values, respectively, to linear thermodynamic effects. This is an approximation, since k' values depend also on isotherm non-linearity and kinetic effects.

As a thermodynamic characteristic, k' depends on the following interactions: solvent–solute, solute–solute, solvent–solute (as adsorbate) – different binding sites on the surface, solvent–surface interactions, and on the surface density of each type of binding site.

In the IF value, the solvent–solute interactions and the solute–solute interactions are cancelled out because they are independent of the polymer. The solvent–solute (as adsorbate) non-selective binding site interactions are also expected to cancel out, as these sites should exist on the NIP and MIP surface equally. This explains the marked differences between the IF selectivity pattern and the chromatographic selectivity patterns (k') of each column (Table 2). The IF values reflect differences in the site structure (nature and concentration of sites) of the NIP and the MIP, which is, of course, an expected result of imprinting. Here, we assume that other differences between the two polymers (e.g. specific surface area, pore size distribution, etc.) are negligible. The IF selectivity pattern is useful for understanding the imprinting effect but it should not be confused with the MIP's chromatographic selectivity pattern which is of higher practical relevance. For example, hydroxy-phenytoin is more retained than phenytoin on both columns, but the IF of phenytoin is higher than that of hydroxy-phenytoin.

One should note, however, that the NIP itself shows a remarkable retention for all hydantoin and barbituric compounds, which means that methacrylamide-based polymers can be used as solid phases in separations of these groups of compounds even without imprinting.

4.3. Molecular structure and imprinting selectivity

The IF of the imprinted template phenytoin is the highest among all compounds investigated. In particular, the IF of phenytoin is greater than either those of its smaller "fragments" or those of its derivatives with bulkier side groups (hydroxy-phenytoin and methyl-phenytoin). The molecular structure of phenytoin may be decomposed into two fragments, which are quite different in their expected binding behaviour: the heterocyclic hydantoin ring and the diphenylmethane group. The diphenylmethane fraction is quite bulky and its geometrical position with respect to the phenytoin ring is stiff (the geometry of the phenytoin molecule is like a three-bladed screw). The hydantoin ring has four functional groups capable of hydrogen bonding and it is very likely that it is bound to the polymer's amide

groups with at least one pair of H-bonds, i.e. with a two point, oriented binding. The most selective and strongest bonds could be possibly formed with two pairs of H-bonds. Due to the important role of these H-bonds in the binding of the hydantoin ring, the adsorption is expected to be quite strictly oriented.

We have investigated these two fragments of phenytoin as separate entities and the experiments have shown a profound difference between the binding and IF of them. Diphenylmethane was very little bound to either the NIP or the MIP (and its IF is also close to 1), much less than the other main fragment, hydantoin. This suggests that the interactions between the diphenyl part of phenytoin and either polymer are negligible in acetonitrile. Hydantoin's retention on the NIP is significant and similar to phenytoin. This indicates that the binding of phenytoin to both polymers occurs by the hydantoin ring or part of this ring. (Similar conclusion can be drawn from a quite similar system [24].) The difference between the retention of phenytoin and hydantoin on the NIP can be a result of the different solvation effects and the somewhat different capability of forming H-bonds. On the other hand, the IF of hydantoin (1.52) is significantly higher than 1, but much less than the IF of phenytoin (5.04). This shows the importance of the diphenyl part in the imprinting effect. These two observations together allow to assume that the role of the diphenyl group in this imprinting is mainly steric. This assumption is confirmed by noting that methyl-phenytoin (and also hydroxy-phenytoin) has lower IF than phenytoin, apparently due to their bulkier diphenyl derivative side groups.

All these conclusions are apparently in agreement with the accepted model of the imprinting effect, which assumes the correct steric positioning of binding functional group(s) within a cavity, which closely fits the shape of the template. A contradiction becomes immediately apparent, however. Since the binding of phenytoin occurs only at the hydantoin ring, the need to fit the diphenyl group into a fitting cavity should decrease the IF of phenytoin, as compared to hydantoin, for entropic reasons. We have, however, observed the opposite.

This contradiction leads us to think, that the prime role of imprinting with respect to non-binding but bulky side groups is not to provide a well-fitting cavity (with possible van der Waals interactions between the cavity wall and the bulky side group [20]) but to prevent the formation (during the polymerization process) of obstacles in the way of access and oriented binding to the functional groups of the polymer.

During the preparation of the MIP, the template molecules are thought to bind to the still changing polymer network. This binding may coordinate two or more functional groups of the polymer in a favourable position so that the binding strength between the resulting MIP surface site and the template is stronger than the binding with the less well-positioned NIP functional groups. It is important to remember here, that the template–polymer binding is often due to directed forces like H-bonds. Therefore, not only the distances between the functional groups of the polymer need to fit to the template but also their spatial orientation is important. The presence of better and/or more binding functionalities on the MIP (as compared to the NIP) is reflected here by the IF of hydantoin (1.52). These MIP sites arise apparently due to the coordinating role of phenytoin's hydantoin ring in the imprinting process.

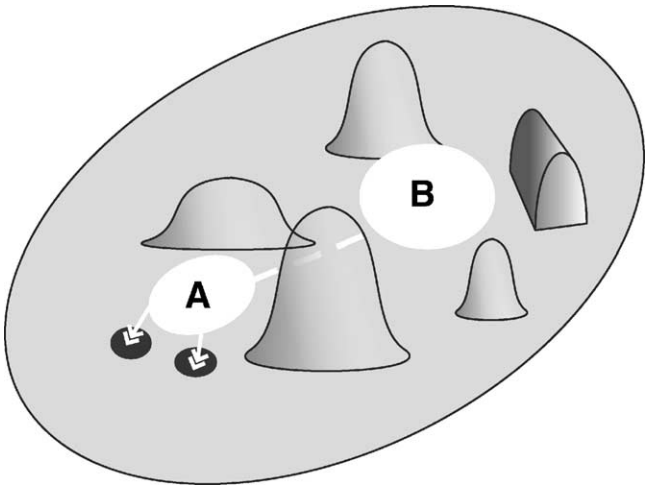

Fig. 3. Schematic model of the MIP surface (grey) with the adsorbed template (white). The white arrows symbolize the specific binding groups (e.g. H-bonding function groups). The white dashed line symbolizes that part of the template molecule which connects the specifically bound part (A) and the bulky, non-bound part (B). The length and flexibility of this connection may both be important in imprinting. Note that on the NIP, the space needed for substructutre (B) may be occupied by polymer (i.e. a "grey hill") and thus the template cannot bind from the best direction and thus its binding will be weaker or impossible.

The role of phenytoin during imprinting is, however, more than this. The phenytoin template, which is bound to the developing polymer network through its hydantoin ring, prevents also the formation of polymer where the phenyl groups are. This makes the obstacle-free rebinding of phenytoin in the correct position possible (Fig. 3). Thus, in the case of phenytoin, the imprinting has two effects: it increases the strength and/or number of hydantoin binding sites and it also makes these sites accessible for phenytoin to bind from the right direction. Had we used hydantoin instead of phenytoin for imprinting, the functional groups may have been imprinted just as well but polymeric material may have protruded in the vicinity of hydantoin in those areas which the diphenyl group of phenytoin would occupy. Thus, phenytoin might not access most of these sites. While we have not done this experiment, the lower IF of the bulkier methyl-phenytoin compared to phenytoin shows the validity of this argument.

A very recent study [20] has produced many fine data with respect to bulky side group effects in imprinting. The enantioselectivity of the MIPs in that study was also reduced when the side group was either increased or decreased compared to the template. Although the conclusions of the authors are different from ours, this need not mean a contradiction, since they studied a system where only a single,

weakly orientation-dependent binding functionality was involved. This is certainly a case where our arguments about strict orientation would not necessarily be applicable.

A side result of our work (Table 2) has been that the IF of barbituric acid and barbiturates is significantly higher than the IF of other bioactive heterocycles like atrazine and ametryn. Barbiturates are both chemically and pharmacologically close to phenytoin, thus the IF selectivity – at least in this case – seems to be a useful indicator of chemical and pharmacological similarity.

5. Conclusion

In this paper, we have addressed the selectivity problem of MIPs. For practical usefulness, the selectivity of an analytical method needs to be determined over a fairly large range of the analyte and interferant(s) concentrations. In the case of MIPs, this means that the adsorbed amount of the analyte (per gram of MIP) needs to be determined or estimated. In the case of a single interferant, this means the determination or estimation of a response surface (i.e. adsorbed amount) over a (usually rectangular) domain of the analyte concentration–interferant concentration plane. If this surface can be well described by a model equation, one needs only to determine the constants of the model from a few measurements. (In some models like Langmuir models, these constants may even be determined from the individual isotherms.) Otherwise, one has to map the surface at many measurement points. If we want the measurement points to be arranged in a simple (e.g. rectangular) pattern, the usual batch method is not suitable. In this paper, we have shown a modified batch method suitable for measurements in predefined points.

We have also shown in a particular case, that competition, as a source of interference is not the only possibility. Atrazine and phenytoin appear to interact cooperatively on the NIP and the MIP. Such cooperativity is not very surprising because the adsorption of both the template and the interferant occurs by hydrogen bonds. Since both compounds can participate in several hydrogen bonds, they can interact with the sorbent and with each other simultaneously. Thus, the binding of each compound may be increased by the presence of the other.

We have been careful not to exclude in the foregoing discussion the possibility of cooperative, competitive and indifferent interaction occurring at the same time. One can easily imagine a MIP with a variety of sites, some of which are occupied by two substances in competition, others in cooperation and still others independently, i.e. only by one or the other substance. Our experimental data only show that by increasing the equilibrium solution concentration of a substance B, one can observe an increase in the adsorbed equilibrium concentration of another substance A, while the equilibrium solution concentration of A is unchanged. This does not prove any binding mechanism, neither does it prove that there is only one binding mechanism present, but it clearly contradicts that A and B would only participate in competition or that they would only be adsorbed on independent sites. The word cooperativity should also not be interpreted mechanistically: our data do not exclude, e.g. the – for other reasons unlikely – possibility that addition of atrazine reduces the solubility of phenytoin in solution and thereby increases its adsorption.

In chromatographic separations, if the template and interferant peaks become separated close to the top of the column, the problems with simultaneous adsorption disappear. Yet in those cases where the MIP column is used to realize difficult separations, the peaks traverse a substantial part of the column unseparated and thus one has to consider simultaneous adsorption in wide concentration ranges.

Retention selectivity data may also prove to be very useful to understand the mechanism of adsorption and the mechanism of the imprinting process. We have shown the substantial difference in the usefulness of imprinting factors and chromatographic selectivities (separation factor, α). The IF values of a set of fragments and derivatives of the template have revealed that the template need not and actually should not be surrounded by a polymer cage. The steric role and effect of imprinting is probably much less restrictive: the template molecule becomes adsorbed on the still growing polymer in a well-defined position and thereby it prevents polymer growth at parts of the adsorbing surface. Fig. 3 shows schematically that imprinting may occur on a rough but essentially plane surface. The efficiency of imprinting may depend on many factors, e.g. the orientation sensitivity of the non-covalent bonds, the distance of the space-filling part of the template from its binding centre(s) and on the flexibility of the connecting covalent bonds between the binding centre(s) and the space-filling part.

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