Temperature sensitive dopamine-imprinted \((N,N\text{-methylene-bis-acrylamide})\) cross-linked polymer and its potential application to the selective extraction of adrenergic drugs from urine

Roongnapa Suedee\(^a,\,*\), Vatcharee Seechamnanturakita, Bhutorn Canyuka, Chitchamai Ovatlarnporn\(^a\), Gary P. Martin\(^b\)

\(^a\) Molecular Recognition Materials Research Unit, Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hatyai, Songkhla 90112, Thailand

\(^b\) Department of Pharmacy, Franklin-Williams Building, King’s College London, London SE1 9NH, UK

Received 8 November 2005; received in revised form 14 February 2006; accepted 14 February 2006

Available online 10 March 2006

Abstract

A temperature sensitive dopamine-imprinted polymer was prepared in 80% aqueous methanol solution by free-radical cross-linking co-polymerisation of methacrylic acid and acrylamide at 60 °C in the presence of \(N,N\text{-methylene-bis-acrylamide}\) as the cross-linker and dopamine hydrochloride as template molecule. The resulting molecularly imprinted polymer (MIP) formed temperature responsive materials, which could be used for the selective separation of appropriate dopamine and adrenergic compounds from a liquid matrix at ambient temperatures. The thermoresponsive MIP exhibited a swelling-deswelling transition in 80% aqueous methanol solution at about 35 °C. The capacity of the thermoresponsive MIP to recognise the template molecule when present in aqueous methanol solution changed with temperature, with the highest selectivity found at 35 °C. Additionally, binding parameters obtained from Scatchard analyses indicate that increasing temperature resulted in an increased affinity and binding capacity of specific binding sites, but had less effect on non-selective binding sites. Subsequently, the thermoresponsive MIP was tested for its application as a sorbent material, utilisable in the selective solid-phase extraction (SPE) of dopamine and other adrenergic compounds (epinephrine, isoproterenol, salbutamol and serotonin) from urine samples. It was shown that the compounds that were structurally related to dopamine could be removed by elution, while dopamine and serotonin, the analytes of interest, remained strongly adsorbed to the adsorbent during SPE applications. The thermoresponsive MIP displayed different efficiency in clean-up and enrichments using the SPE protocol at different temperatures.

© 2006 Elsevier B V. All rights reserved.

Keywords: Molecularly imprinted polymer; Polyacrylamide; Dopamine; Adrenergic drugs; Solid-phase extraction; Assay

1. Introduction

Natural receptors have evolved so as to achieve molecular recognition of ligands with high specificity and often efficiently bind complex molecules such as proteins. Many studies have investigated the design and the construction of synthetic receptors to mimic the selectivity of such natural receptors. In particular preparations of molecularly imprinted polymers (MIPs) have been investigated as a convenient and applicable means of creating three-dimensional networks with a cavity capable of memorizing the shape and functional group positions, complementary to the template molecule [1,2]. MIP receptors of this kind offer much potential in a number of application areas including analytical chemistry, separation science, sensor construction and drug design [3–5]. This is because of the potentially high selectivity and excellent stability of such polymers. For example, many kinds of MIP receptors have been prepared for the selective separation of some target compounds from liquid matrices, and these have been employed in clean-up procedures in highly sensitive analyses of such compounds in environmental and/or biological samples [6,7]. Generally, the preparation of MIP for organic compounds has been based on the hydrogen bonding interactions which occur between polymer and substrate in non-polar solvents and due to this it is much more

0021-9673/$ – see front matter © 2006 Elsevier B V. All rights reserved.
doi:10.1016/j.chroma.2006.02.033
difficult to prepare MIPs for polar compounds. Consequently, MIPs prepared for use in the sample preparation of biological and environmental samples do not usually allow the processing of samples in aqueous media.

Typically conventional molecular imprinting technology deals with highly cross-linked materials having relatively rigid structures, whereas natural receptors in contrast possess a more flexible and conformationally adaptable structure. The rigidity limits the number of binding sites available to the target molecule. Many studies have shown that lightly cross-linked polymer gels can undergo reversible swelling and shrinking under an external stimulus, which increases the number of binding interactions with the target molecule [8–10]. Cross-linked N-substituted polyacrylamides are among the most widely studied polymeric materials used for the molecular imprinting of biomolecules such as protein and DNA [11,12]. These polymers continue to receive much attention in the field of controlled drug delivery [13,14] because they can undergo a temperature-controlled volume phase transition in aqueous solution [15]. Combining the properties of a thermosensitive polymer with molecular imprinting techniques may provide a promising strategy for ensuring the system responds more rapidly to an external temperature change. In the present study, the co-polymerisation of acrylamide with a cross-linker and additional monomers, in the presence of a template, was used to synthesize imprinted polymers, which might exhibit reversible phase transition based phenomena at ambient temperature.

Dopamine was the compound of interest employed in this study, as the template molecule. It is a naturally occurring catecholamine, which can bind to adrenergic receptors and its hydrochloride salt is used in the treatment of acute congestive heart failure and renal failure [16]. The analytical detection of dopamine in urine has been reported to provide a valuable diagnosis of neuroblastoma in patients. Several methods have been described including a fluorescence-based method [17] and ion-exchange chromatography [18] both of which allow for precise measurement of the drug. Urine samples containing dopamine and its analogs (epinephrine and norepinephrine) have been analyzed by high-performance liquid chromatography (HPLC) with electrochemical detection after isolation of the compounds using ion exchange resins [19]. Integrated MIP and temperature-controllable mediated transitions could enable a novel selective extraction method to be developed.

The aim of the current study was to seek to prepare thermoresponsive imprinted polymers and compare their recognition ability to that obtained from structurally rigid polymers, prepared using ethylene glycol dimethacrylate (EDMA) as a cross-linking monomer. Dopamine (Fig. 1), a polar compound, which is not soluble in any organic solvents, was used as a template molecule. It was planned to prepare the polymers in an aqueous methanol solvent, with a view to strengthening any hydrogen bond interactions between dopamine and the chosen functional monomer. The temperature-dependence of the recognition property of the prepared thermoresponsive polymer and its application as an adsorption phase for the selective extraction of dopamine and other adrenergic compounds (Fig. 1) from the spiked human urine samples were also to be investigated.

2. Experimental

2.1. Materials

Ethylene glycol dimethacrylate (EDMA), N,N'-methylenebisacrylamide (MBAA), methacrylic acid (MAA) and acrylamide (ACM) were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). 2,2'-Azobis-(isobutyronitrile) (AIBN)
In this study, four molecularly imprinted polymers (MIP1, MIP2, MIP3 and MIP4) and corresponding non-imprinted polymers (NIP1, NIP2, NIP3 and NIP4) were prepared using a thermal method involving free radical polymerization, according to that reported previously [2]. For the preparation procedure of these polymers, the polymerizing compositions listed in Table 1 were dissolved in 25 ml of methanol/water (4:1, v/v) mixture. The solid phase extraction study was developed in off-line mode using a Supelco vacuum manifold (PA, USA) connected to a vacuum pump. High-performance liquid chromatography was carried out using an Agilent 1100 system consisting of a quaternary pump, an autosampler, a thermostated column compartment with a built-in-six-port switching valve and fluorescence detector (CA, USA).

Table 1

<table>
<thead>
<tr>
<th>Polymer composition</th>
<th>MIP1</th>
<th>NIP1</th>
<th>MIP2</th>
<th>NIP2</th>
<th>MIP3</th>
<th>NIP3</th>
<th>MIP4</th>
<th>NIP4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine HCl</td>
<td>0.62</td>
<td>–</td>
<td>0.62</td>
<td>–</td>
<td>0.62</td>
<td>–</td>
<td>0.62</td>
<td>–</td>
</tr>
<tr>
<td>MAA</td>
<td>1.12</td>
<td>1.12</td>
<td>0.56</td>
<td>0.56</td>
<td>–</td>
<td>0.56</td>
<td>5.04</td>
<td>5.04</td>
</tr>
<tr>
<td>EDMA</td>
<td>9.72</td>
<td>9.72</td>
<td>6.48</td>
<td>6.48</td>
<td>6.48</td>
<td>6.48</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MBAA</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1.10</td>
<td>1.10</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ARBN</td>
<td>0.71</td>
<td>0.71</td>
<td>0.71</td>
<td>0.71</td>
<td>0.71</td>
<td>0.71</td>
<td>0.71</td>
<td>0.71</td>
</tr>
</tbody>
</table>

The mean size as well as the size distribution of the prepared particles was determined at 25 °C using laser diffraction (Malvern Mastersizer, Worcester, UK) and water as the suspending medium. The mean of the triplicate measurements on the same batch was determined. The degree of swelling of the polymers was determined from the ratio between the volume of the swollen polymer and the volume of the dry polymer in each of four solvents: water, methanol, a methanol/water (4:1, v/v) mixture and phosphate buffer (pH 7.4), using calibrated measuring cylinder. A total of three replicates was used for such test. The determination of pore volume and specific surface area was carried out by nitrogen adsorption/desorption techniques using a Coulter SA3100 series surface area and pore analyzer (Coulter, USA), which enables pores between 0.3 and 200 nm to be measured. The samples were degassed at 120 °C and a 50-point pressure table was used. The surface area was determined from a Brunauer, Emmett and Teller (BET) plot whilst the average pore diameter and the cumulative pore volume were obtained using a Barrett, Johner and Halenda (BJH) model of the adsorption isotherm.

2.5. Binding experiments

The ability of the MIP prepared to selectively recognise the template molecule in comparison to the prepared NIPs was evaluated in four different solvents: water, methanol, a methanol/water mixture (4:1, v/v) and pH 7.4 phosphate buffer after equilibration of the polymers with a dopamine solution. In a typical binding assay, the powdered polymer (50 mg) was added to 5 ml of the solvent containing 5 μg ml⁻¹ of dopamine or 5 ml of the pure solvent (blank), and the suspension stirred for 24 h at room temperature (30 ± 1 °C). The polymer particles were then filtered off and the filtrate was analysed for dopamine using a fluorescence spectroscopy method. The quantity of drug in solution was determined by reference to a calibration curve. The amount of bound drug was obtained by subtracting the amount of free drug from the total amount of the drug added. The imprinting factor (α), which represented the effect of the imprinting pro-
cess, was the ratio of the amount of substrate bound by the MIP to that bound by the corresponding NIP.

The binding of dopamine to the thermoresponsive imprinted polymer (MIP4) and corresponding non-imprinted polymer (NIP4) was examined at six different temperatures (25, 30, 35, 40, 45 and 70 °C) using the binding assay protocol.

2.6. Determination binding characteristics of the thermoresponsive polymers

The binding characteristics of the thermoresponsive imprinted polymer (MIP4) as well as the control polymer were further examined at three different temperatures (25, 35 and 45 °C) using 50-μg samples of polymer with dopamine solutions ranging in concentration from 0.1 to 100 μg ml⁻¹, using methanol/water (4:1, v/v) mixture as medium. The amount bound (Q) was determined at each drug/polymer molar ratio (R). The binding parameters were determined from the equation, Bound/Free = (Bmax – R/Kd) where Kd is the equilibrium dissociation constant, and Bmax is the maximum number of binding sites which were obtained from the slope and intercept on x-axis of the straight line of the Scatchard plot, respectively. The association constant (Kd) value was obtained as the reciprocal of the Kd value. The mean dopamine binding constants calculated from triplicate independently derived results.

2.7. Selectivity evaluation

In order to verify selective recognition of the thermoresponsive MIP, the equilibrium binding analysis was examined using both a non-competitive and competitive ligand-binding assay.

2.7.1. Non-competitive ligand binding assay

Non-competitive ligand binding analysis of the polymers was determined by a saturation binding experiment using serotonin, salbutamol, isoproterenol, epinephrine and methyldopa as the related probes and histamine and ascorbic acid as non-related probes (Fig. 1). Particulate polymer (50 mg) was stirred into 5 ml methanol/water (4:1, v/v) solution containing 5 μg ml⁻¹ of each analyte of interest at room temperature. After 24 h, the filtrate was analysed for the amount of unbound analyte. The amount of each compound bound was calculated by subtraction of the concentration in the filtrate from the concentration in the original stock solution. The selectivity (%) was obtained by determining each specific the amount of compound sorbed per unit weight of MIP relative to the amount of dopamine sorbed.

2.7.2. Competitive ligand binding assay

In this experiment, the putative binding sites of dopamine on the polymer were identified by a displacement assay, using the same molecular probes as those used in the non-competitive ligand binding analysis. Particulate polymer (50 mg) was incubated with 5 ml of methanol/water (4:1, v/v) containing 5 μg ml⁻¹ of dopamine and a test probe within the concentration range 0.02–20 μg ml⁻¹, for 24 h at room temperature. The changes in fluorescence intensity of dopamine in solution were monitored at 320 nm following excitation at 279 nm. The binding of dopamine in the presence of substrates was calculated and reported as the % binding of dopamine to sites on the polymer. Each experiment was repeated three times.

2.8. Analysis method

Dopamine, serotonin, histamine or salbutamol in the samples was quantified using a fluorescence spectroscopic method. A sample (1 ml) containing dopamine was transferred to a 10.0 ml volumetric flask containing 2.0 ml of pH 3.6 acetate buffer solution, and diluted to volume with methanol. The fluorescence intensity of the solution was measured at 320 nm using an excitation wavelength of 279 nm (limit of quantitation (LOQ) = 7.3 ng ml⁻¹). The serotonin sample was determined by adding the sample (1 ml) to phosphate buffer pH 6.5 (2 ml), diluting with methanol (7 ml) and measuring the fluorescence intensity at wavelength of 335 nm with excitation wavelength at 300 nm (LOQ = 22.4 ng ml⁻¹). The assay of histamine in samples was carried out using 0.2% o-phthalaldehyde as a derivatizing agent. The sample (1 ml) containing histamine was transferred to 200 μl of 1 M sodium hydroxide contained in a 25.0 ml volumetric flask. Hydrochloric acid (100 μl, 3 M) and 50 μl of 0.2% o-phthalaldehyde was added and the solution diluted to volume with 4:1 (v/v) methanol/water mixture. The fluorescence intensity of the solution was measured at 430 nm using an excitation wavelength of 330 nm against a reagent blank, prepared using the same reagent concentrations but containing no histamine (LOQ = 9.0 ng ml⁻¹). For the assay of salbutamol-containing samples, the fluorescence intensity was measured at a wavelength of 309 nm with the excitation wavelength fixed at 218 nm (LOQ = 0.8 μg ml⁻¹).

Isoproterenol, epinephrine or methyldopa in the samples was assayed by UV spectroscopy using a wavelength of 279 nm (LOQs of isoproterenol, epinephrine and methyldopa were 12.0 ng ml⁻¹, 3.0 μg ml⁻¹ and 4.6 μg ml⁻¹, respectively).

The assay of ascorbic acid-containing samples was performed using a potentiometric titration method. A 2.0 ml sample was placed in a 150 ml vessel and 2 M potassium chloride (10 ml) added. The solution was diluted to 100.0 ml with distilled water before titration with a standard solution of 0.01 M sodium hydroxide. The pH values were recorded after the addition of each 0.1 ml titrant added. The plot of the titrant volume vs pH was made to determine the end-point (LOQ = 0.5 μg ml⁻¹).

2.9. Solid-phase extraction experiments

Twenty-five milligrams of the particulate polymer suspended in water was packed into a home-built SPE cartridge comprising a borosilicate glass tube (0.5 cm in internal diameter, 5 cm in length) with a double-walled water jacket for controlling temperature. Studies were conducted into the ability of thermoresponsive MIP to selectively extract dopamine and the other adrenergic compounds present in the mixture by evaluating the efficiency of SPE at three different extraction temperatures (25 °C, room temperature (30 °C) and 40 °C).

To determine the recovery of bound material from urine samples at three different temperatures, aqueous solution (1 ml)
including 5 μg ml⁻¹ of dopamine and 1.25 μg ml⁻¹ of each adrenergic compound presented as a mixture (serotonin, isoproterenol, epinephrine and salbutamol) was added to 4.5 ml of methanol and this solution was then diluted with human urine to 10 ml before filtering to remove any insoluble material. A sample (1 ml) of filtrate was loaded onto the SPE cartridge containing either MIP and the eluant collected for analysis. The column was then washed with 5 ml of 4:1 (v/v) methanol/water mixture and the analytes finally eluted with 5 ml of 1% acetic acid in methanol. Each experiment was run three times, using three different cartridges. The fractions eluted from each cartridge were collected separately and the amounts of recovered dopamine and other adrenergic compounds present in the mixture were quantified.

A reversed phase HPLC method was used for the quantitative analysis of dopamine and other adrenergic compounds after solid-phase extraction, using a method adapted from that reported by Wood and Hall [20]. Briefly, mobile phase A comprising 0.05% aqueous trifluoroacetic acid (THF)-methanol (97.5:2.5, v/v) and mobile phase B consisting of 0.05% aqueous TFA-methanol (40:60, v/v) were used for elution. An injection volume of 20 μl was employed and the analytical column was a Luna 5 μC18, 25 cm × 0.46 cm (Phenomenex, USA). A flow-rate of 1.0 ml min⁻¹ was used over 20-min with the following gradient: 0.00 min, 100% A; 1.00 min, 100% A; 16.00 min, 50% A and 50% B (linear gradient from 1 to 16 min); 16.05 min, 100% A to return column to initial condition by 20 min. The fluorescence detector used was set at λex 220 nm and λem 320 nm.

3. Results and discussion

3.1. Synthesis and characterisation of polymers

Putative thermoresponsive dopamine-imprinted polymers were synthesised using two functional monomers, MAA and ACM, such that the acid or amide group of the monomers might interact with the hydroxyl groups of the dopamine template, together with MBAA cross-linker. A mixture of methanol and water (4:1, v/v) was chosen as the porogen solvent since the dopamine (HCl) template is soluble only in polar solvent and it was the aim of this work to generate a MIP for use in an aqueous environment.

In the present work, the binding of dopamine to the thermosensitive imprinted polymer was compared to its binding to the structurally rigid polymers. The latter were prepared by using EDMA, a cross-linking monomer either singly or combined with MBAA monomer. Normally, EDMA generates an imprinted polymer that is compact, inert and highly stable with respect to rigidity of polymer structure. Molecularly imprinted polymers and corresponding non-imprinted polymers, consisting of MBAA and/or EDMA as cross-linker, were created following a common protocol for MIP synthesis using the compositions listed in Table 1. The physical characteristics of the polymers were examined and the data are summarised in Table 2. In general, the MBAA cross-linked polymers (MIP4, NIP4) were found to have both a large pore size and pore volume com-
pared with the more structurally rigid polymers (MIP1, MIP2, MIP3, NIP1, NIP2 and NIP3). Both NIP4 and MIP4 particles possessed micropores in the polymer network, and also exhibited specific swelling properties that were different to the structurally more rigid EDMA-cross-linked polymers. These observations show that the physical properties of the prepared polymers were markedly dependent upon the cross-linking monomer employed. It is apparent that the pore diameter and specific surface area of the MBAA cross-linked NIP (NIP4) were almost twice as large as those of the corresponding MIP (MIP4). Also, the pore volume of NIP4 was larger than that of MIP4. By contrast, pore diameter, pore volume and specific surface area of the NIPs and MIPs of the structurally more rigid EDMA cross-linked polymers were not significantly different. Indeed, either MIP4 or NIP4 was prepared with the same polymer component and the same polymerising conditions except the print molecule was present in the polymerising phase only when the MIP4 was synthesised. Hence, the smaller pore size of MIP4 in comparison to that of NIP4 must be related to the presence of dopamine during the preparation process. Probably, the dopamine template present in the lightly MBAA-cross-linked polymer gels causes a compactness of the size of the cavities, within gelling network, during the change of temperature from polymerising temperature (60°C) to extraction temperature (room temperature). This may have accounted for the specific surface area of MIP4 being lower than the specific surface area of the control.

3.2. Media effects on ligand-binding of MIPs

The influence of various binding media parameters (the type of the solvent, pH and ionic strength) was studied further so as to provide optimised parameters for binding in aqueous media, with a view to developing a thermoresponsive imprinted polymer adsorbent suitable for SPE analysis. Initially, the influence of the solvent on the recognition properties of MIP4 was studied and the binding of dopamine for this polymer was compared with that of the more structurally rigid polymers (MIP1, MIP2 and MIP3). Fig. 2 shows the binding of dopamine to the various MIPs and corresponding NIPs synthesized in this study.

![Fig. 2. Effect of solvent on the % dopamine (employed as the template molecule) bound to various (a) MIPs and (b) corresponding NIPs synthesized in this study.](image)

MIP4, prepared with MBAA as the cross-linking monomer, provided better recognition than the more structurally rigid MIPs prepared using EDMA as the cross-linking monomer. This could be explained by the rigidity of the EDMA polymer preventing the cavity having sufficient flexibility to orientate so that maximum binding occurs within the polymer matrix. Also, the hydrophobic properties of the EDMA-containing polymer may promote a higher non-specific adsorption of the drug to the polymer when placed in an aqueous medium.

MAA and ACM, employed as functional monomers for imprinting in this study contain both amide and carboxylic acid functional groups, which can interact with the hydroxyl group of dopamine via non-covalent bonding. The amide group of ACM is not ionizable, whereas the carboxylic group of MAA monomer can ionize and hence a change in non-specific adsorption of the dopamine to the polymer can occur in aqueous medium as a function of pH. In fact medium pH had a large effect on the binding of dopamine to MIP4 and NIP4 (Fig. 3) with a low pH (pH 3 and 4) to much less binding of dopamine to the polymers than occurring at low pH (pH 5–7). Dopamine (pK_a = 10.6) will be charged positively over the whole pH working range (pH 3–7), whereas at the higher pH values the thermoresponsive polymer will be effectively negatively charged. Thus, non-specific electrostatic interaction between drug and polymer might be expected to occur and indeed the amount bound of dopamine bound to the MIP and NIP was
not significantly different at any of the pH values studied. In addition, the NIP synthesized in this study had a larger specific surface area than the corresponding MIP providing a greater area for a higher non-specific binding of dopamine from solution. Since the selectivity of binding between MIP4 and dopamine was greatest from the methanol/water (4:1, v/v) mixture, it was thought that this solvent would be suitable to be employed in any SPE employing MIP4 as the imprinted polymer.

3.3. Temperature effect on recognition ability

The ability of the thermoresponsive MIP (MIP4) to recognize template molecule after a dynamic change in swelling was evaluated by equilibrium binding analysis. The experiment was performed at temperatures ranging from 25 to 70 °C. Fig. 4a shows effect of temperature on % binding of dopamine to MIP4 and control polymer (NIP4) in methanol/water (4:1, v/v) mixture. The temperature dependent swelling of the thermoresponsive MIP is also shown in Fig. 4b. The adsorption pattern of dopamine to the MIP varies with temperature, with sorption increasing as a function of temperature (see Fig. 4a). A temperature increase from 25 to 35 °C promoted binding of the template molecule to the MIP, whilst binding to the corresponding NIP scarcely changed. The imprinting factor of the MIP was highest at a temperature of 35 °C and this appeared to correspond to the transition temperature of the thermoresponsive MIP. At temperatures beyond 35 °C, it was found that binding of dopamine to both the MIP and NIP gradually increased but that the increase in the binding to the non-selective polymer was greater than that to the MIP (Fig. 4a), resulting in a decrease in the imprinting factor (Fig 4b). This result suggests that the higher binding to the thermoresponsive MIP at high temperatures (≥45 °C) is likely to be primarily the consequence of increasing non-specific adsorption of the template molecules. The % change in binding as a function of temperature also allows the activation energy of dopamine binding to sites within thermosensitive MIP to be determined and the latter was found to be 9.83 Kcal mol⁻¹.

The results show that binding to MIP4 was temperature sensitive and although a degree of molecular selectivity was apparent either in the more swollen or collapsed states, at the transition temperature, 35 °C, the recognition of dopamine by the MIP was maximal.

The size of either MIP or NIP particles was measured before and after 30 min-exposure to dopamine in methanol/water (4:1, v/v) solution at temperatures ranging from 25 to 60 °C. As seen in Fig. 5, the thermoresponsive MIP decreased in size in the presence of template molecule, although this effect was only apparent at temperatures below 35 °C. In contrast, the change in the size of polymer particle of the corresponding NIP exposed to dopamine was relatively little. This result suggests that the shrinkage of the swollen MIP at fixed temperatures below 35 °C, which occurs in the presence of dopamine, is a consequence of the binding of the template molecule at the imprint sites. However, at temperatures over 35 °C the size of polymer exposed to dopamine did not change as a function of temperature. It is apparent that the polymer shrinking/swelling in response to the presence of template was affected by temperature. At higher temperatures the MIP forms a highly compact polymer structure in which binding within the polymer may be hindered. This could account for the decrease in selectivity of the thermoresponsive MIP at higher temperatures (≥40 °C). It would appear that the selectivity of the thermoresponsive MIP is controlled by the size of the cavities in the polymer, and that conformational
changes may be required to open the cavity to enable greater binding to occur. A previous study has demonstrated that ligand binding can affect the geometry of a protein binding site, with significant rearrangements occurring upon ligand binding [21]. An approach towards the handling of ligand-induced domain movements has been reported by Sandak et al. [22]. The flexibility of the thermoresponsive MIP may enable movement of the polymer domains forming the binding cavity, which in turn affects the degree of molecular recognition between the ligand and receptor.

Previous studies have demonstrated that a change in pH, ionic strength, solvent or temperature can alter the conformation of polymer chains within structure of MIPs and that these have a strong effect on the polymer recognition properties [23,24]. Turner et al. [25] showed that any factors that alter the surface potential and conformation of polymer chains will change the size and shape of template-complementary binding pockets thereby disrupting binding. These latter workers also reported that a high buffer concentration can increase the recognition of the MIP such that a compactness in the structure of the polymer is promoted. The results obtained in the present study suggest that thermal-stimuli are capable of changing the binding properties of the thermoresponsive MIP, which is factor that may be exploitable for the selective extraction of the target compound from aqueous media by the polymer.

The influence of temperature on the binding characteristics of the thermoresponsive MIP was examined further. It was found that the adsorption isotherms of the imprinted polymer fitted well to the bi-Langmuir model with a predominance of high-affinity binding sites but with a low fraction of low-affinity binding sites being present. This suggests that the recognition sites of the MIP are heterogeneous. The association constant \( (K_a) \) and binding capacity \( (B_{ma}) \) values of recognition sites at various temperatures (25, 35 and 45 °C) are shown in Table 3. Increasing temperature from 25 to 45 °C greatly increased the \( K_a \) value of high-affinity binding site of the MIP, while the \( K_a \) values for the high and low-affinity binding sites of the corresponding NIP were also increased but to a lesser extent. A significant decrease

![Fig. 5. Effect of temperature on the mean volume diameter of polymer particles of the thermoresponsive imprinted polymer, in the presence of dopamine template in methanol/water (4:1, v/v) (mean \( \pm S.E., n = 3 \)).](image)
in binding capacity with increasing temperature was observed in the case of high-affinity binding site for MIP. The results show that an increase in temperature increases the efficiency of the binding of the template with the polymers. The great increase in the binding affinity of high-affinity binding site of the MIP when temperature increases is most likely due to the increased the strength of the interactions between complementary functionalities in the template and polymer, within imprint cavity. It is possible that the polymer having shrunken dimensions at higher temperatures facilitates a higher order of molecular association. A greater dominance of hydrophobic forces within a more dehydrated polymer matrix might also promote binding affinity.

3.4. The specific binding site of the thermal-responsive polymer

Molecular selectivity in imprinted polymers is often demonstrated by comparing the extent of binding of the template molecule in comparison to the binding of molecules with similar features. This affords an indication of the extent of cross-reactivity between the selected molecules and the polymer. The binding selectivity of the prepared thermoresponsive MIP for its template and a range of structural analogues (serotonin, salbutamol, isoproterenol, epinephrine and methyldopa) as well as non-related compounds (histamine and ascorbic acid) (Fig. 1) was determined. The results suggest that structurally related compounds bound more effectively to the MIP than non-related compounds (histamine and ascorbic acid) (Fig. 1) was determined. The results suggest that structurally related compounds bound more effectively to the MIP than non-related compounds, such as histamine and ascorbic acid (see Fig. 6). This indicated that it was possible to produce a temperature sensitive imprinted polymer with selectivity towards dopamine but with a reasonable cross-reactivity to dopamine analogs, which contained the catecholamine structure.

Subsequent competitive ligand binding tests showed that the relative affinity of the thermoresponsive polymer for molecules related to dopamine was greater than for non-related dopamine compounds, with histamine and ascorbic acid in particular showing very poor dopamine displacement characteristics (Fig. 7).

Competitive binding for the selected probes occurred only at high concentrations. Non-specific probes would be expected to bind to low-affinity sites with a ‘less good’ template complementary only when present at higher concentrations. These results confirm that the thermoresponsive MIP binds the template molecule strongly but that there is partial cross-reactivity to structurally closely related compounds. This specificity of the thermoresponsive MIP might be explained on the basis of the molecular recognition, which relates to binding sites having shape and size selection as well as the correct spatial orientation of the functional groups in the MIP binding sites.

3.5. Application of the thermal-responsive polymer to SPE

The feasibility of using the generated thermoresponsive MIP in an SPE column to recover dopamine and related compounds from a mixture in urine was examined. The influence of temperature on polymer capacity when employed in the SPE protocol was also determined since use of an elevated temperature does potentially offer an elegant approach to promoting specific adsorption, which may increase the selectivity of the polymer.

Table 4 shows % recovery of compounds from a mixture of compounds using the thermoresponsive MIP and corresponding NIP at any of the temperatures studied, indicating that dopamine remained selectively bound through specific interactions with the imprinted binding sites within the polymer. In contrast dopamine was detected in the breakthrough sample of the NIP loaded column particularly at temperature 25°C but also at room temperature (30 ± 1°C), although at higher temperatures (40°C) dopamine remained bound to the NIP. More of the template molecule was retained on the MIP at any of the temperatures studied, indicating that dopamine remained selectively bound through specific interactions with the imprinted binding sites within the polymer. In contrast dopamine was detected in the breakthrough sample of the NIP loaded column particularly at temperature 25°C but also at room temperature (30 ± 1°C), although at higher temperatures (40°C) dopamine remained bound to the NIP. More of the template molecule was retained on the MIP other than NIP and more was eluted by the 9:1 (v/v) methanol-acetic acid solvent. In addition to the template, the MIP displayed selectivity in retaining serotonin, which has structural similarities to that of the template (Fig. 1). The recovery of serotonin from the column was also temperature dependent (see Table 4).
Table 4
Mean recovery (%) of dopamine (0.5 µg ml⁻¹ in urine) and other adrenergic compounds (0.125 µg ml⁻¹ in urine) after solid-phase extraction through MIP and NIP containing cartridges at various temperatures

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Temperature (°C)</th>
<th>Dopamine</th>
<th>Serotonin</th>
<th>Salbutamol</th>
<th>Isoproterenol</th>
<th>Epinephrine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MIP</td>
<td>NIP</td>
<td>MIP</td>
<td>NIP</td>
<td>MIP</td>
</tr>
<tr>
<td>Breakthrough (1 ml)</td>
<td>25</td>
<td>0</td>
<td>13.5</td>
<td>13.4</td>
<td>11.0</td>
<td>27.7</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0</td>
<td>17.2</td>
<td>12.1</td>
<td>34.0</td>
<td>14.4</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0</td>
<td>0</td>
<td>14.4</td>
<td>5.7</td>
<td>45.9</td>
</tr>
<tr>
<td>Methanol:water, 4:1 (v/v) (5 ml)</td>
<td>25</td>
<td>33.3</td>
<td>74.4</td>
<td>14.6</td>
<td>70.4</td>
<td>77.6</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>24.3</td>
<td>72.3</td>
<td>31.6</td>
<td>59.7</td>
<td>63.6</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>14.2</td>
<td>63.7</td>
<td>10.1</td>
<td>77.9</td>
<td>60.1</td>
</tr>
<tr>
<td>Methanol:acetic acid, 9:1 (v/v) (5 ml)</td>
<td>25</td>
<td>36.6</td>
<td>12.1</td>
<td>25.8</td>
<td>22.6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>46.7</td>
<td>10.5</td>
<td>59.6</td>
<td>6.4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>53.3</td>
<td>36.2</td>
<td>21.7</td>
<td>4.4</td>
<td>0</td>
</tr>
<tr>
<td>Total recovery</td>
<td>25</td>
<td>69.9</td>
<td>87.9</td>
<td>53.8</td>
<td>104.0</td>
<td>105.3</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>71.0</td>
<td>89.5</td>
<td>101.3</td>
<td>101.1</td>
<td>100.7</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>65.5</td>
<td>99.9</td>
<td>46.2</td>
<td>88.0</td>
<td>106.0</td>
</tr>
</tbody>
</table>

*The relative standard deviations (RSDs) were 2–10% (n = 3).

The elution of human urine spiked with dopamine and analogues through the MIP columns when employing the SPE protocol at different temperatures (Fig. 8) resulted in different enrichment profiles of the various compounds. At room temperature (30 ± 1 °C) the clean-up and enrichment of the sample in dopamine and serotonin using the thermoresponsive MIP was greater than that achieved at the other temperatures (25 and 40 °C). Marked interference with the absorbance due to the analyte peaks was found to occur due to absorbing compounds within the urine. However, even though some of these interfering compounds still co-eluted with the analytes after the SPE, the feasibility of carrying out an assay based on this method was proven. The pretreatment of human urine using SPE with the thermoresponsive MIP enabled total recoveries of dopamine and other adrenergic compounds to be achieved with the values in this study ranging 70–106%, depending on extraction temperature.

4. Conclusion

The design and synthesis of thermal-responsive materials for separation process was demonstrated in this study. An adsorption phase consisting of molecular recognition and thermoresponsive elements has been developed and evaluated for application in the separation of dopamine and analogues contained in urine samples, using SPE. The results in the present study demonstrated that combining the thermosensitive polymer with molecular imprinting techniques generated a molecular recognition material, which could respond more rapidly to an external temperature change. The material could be employed in aqueous environments and enabled a selective recognition of dopamine and its analogues to be produced. The potential application of this material as a selective sorbent for SPE in the assay of dopamine in human urine has been demonstrated with some degree of success, although a fully validated assay has not been established. Further investigation and development of the system is warranted with a view to developing a thermoresponsive MIP material having high selectivity and suitable properties for applying as a selective sorbent phase of SPE or even as recognition material in other uses, e.g. chromatographic separation, sensor and immunosay.
Acknowledgement

Financial support from the Thailand Research Fund (ID No. RSA4680020) is gratefully acknowledged.

References