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Computational design and synthesis of molecular imprinted polymers for selective extraction of allopurinol from human plasma

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ABSTRACT

The present study was focused on the rational development of polymers for selective extraction of allopurinol (ALP) from human plasma. Therefore, a computational modeling approach was combined with the molecular imprinting technology to obtain the polymers. The computational approach was used in order to screen the functional monomers as well as the polymerization solvents for rational design of molecular imprinted polymers (MIPs). It was based on the comparison of the binding energy (ΔE) of the formed complexes between the template molecule and different functional monomers. In the design, the effect of the polymerization solvent was also included using the polarizable continuum model. The theoretical calculation results showed that among virtual solvents tested, acrylamide (AAM) gave the largest ΔE while acrylonitrile (ACN) gave the smallest ΔE in acetone. Therefore, the MIP prepared using AAM as functional monomer in acetone was desired. To examine the validity of this approach, three MIPs were synthesized with different functional monomers i.e. AAM, acrylic acid (AA), and ACN, and then evaluated using Langmuir–Freundlich (LF) isotherm. The results obtained from this experiment confirmed the computational results that the MIP prepared by AAM was the most appropriate adsorbent. Subsequently, the MIP was used to develop a molecular imprinted solid-phase extraction (MISPE) procedure. Finally, the MISPE procedure followed by HPLC was developed for selective extraction and determination of allopurinol in human plasma. For the proposed MISPE method, the linearity between peak area and concentration was found in the range of 0.100–25.000 μM with a linear regression coefficient (R^2) of 0.995. The limit of detection (LOD) and quantification (LOQ) in plasma were 0.028 and 0.093 μM, respectively. The results of this study indicated the possibility of using computer aided design for rational selection of functional monomers and solvents for preparation of the MIPs capable of extracting allopurinol from human plasma.

1. Introduction

Recently, allopurinol (4-hydroxypyrozolo[3,4-d]pyromidine) has been used in modern treatment of gout and prevention of tumor lysis syndrome. Allopurinol (ALP) (Fig. 1) acts by inhibiting xanthine oxidase that catalyzes the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid [1,2]. Adsorption of ALP is rapid and its half-life range is between 0.7 and 1.5 h [3]. Therefore, in monitoring ALP level, a rapid determination method is required. In order to monitor ALP in bio-fluids, several analytical methods have been developed so far. These methods involve precipitation of the proteins from plasma with various denaturing reagents including trichloroacetic, trifluoroacetic and perchloric acids [4,5], zinc and barium sulfate, and liquid extraction with diethyl ether/isopropanol mixture [6] as well as sample filtration prior to direct injection into the HPLC column [7,8]. In case of the HPLC method, the selectivity of the commercial solid-phase extraction (SPE) adsorbents is low and this would be a setback when a selective extraction from a complex matrix has to be performed.Separation fundamentals of the traditional SPE materials are commonly based on physiochemical retention on the functionalized surface. However, as mentioned above, when a selective extraction from a complex sample has to be performed, the typical SPE adsorbents lack sufficient selectivity are imperfect. Therefore, seeking more selective SPE adsorbents is important. An

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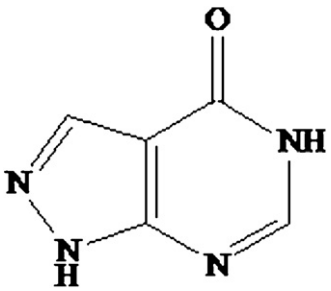

Fig. 1. Allopurinol.

enhancement of the molecular selectivity of SPE can be achieved using molecular imprinted polymers (MIPs). These polymers are a novel group of smart materials with notable recognition properties for their templates. The molecular imprinting technology was first put forward by Wulff and Sarhan in 1972 [9] and has been developed rapidly since 1993 when the MIP with theophylline as template was reported by Mosbach and co-workers [10]. Basically, MIPs are prepared by the polymerization of a suitable monomer and a cross-linker agent in the presence of a template molecule. After polymerization, the template is removed from the polymeric matrix leaving cavities complementary in size and shape to the template, and thus the resulting MIP is able to specifically rebind to this molecule or related compounds from a complex mixture. These polymers could have remarkable recognition properties in a wide range of applications from an analytical point of view, i.e. chromatographic separations [11–14], solid phase extraction [15,16], binding assays [17], chemical sensors [18,19] and in synthetic chemistry [20].

In order to improve the properties of MIPs, computer-aided study of MIPs has been suggested as a rational and fast method to search optimal imprinting conditions. In several papers [21–25], a virtual library of functional monomers was used to assign and screen against the target template molecule, and managed to improve the selectivity of MIP with the computer simulation to a great extent. In some other studies [26,27] computational tools were also successfully applied to achieve an understanding of intermolecular interactions in molecular imprinting of theophylline and chemical warfare agents into complex polymeric and monomeric systems. Moreover, in some investigations [28,29] a methodology based on density functional theory calculations has been developed for the rational design of MIPs.

In the present study, in order to facilitate the determination of ALP in complex matrix such as human plasma by using a novel and selective adsorbent, a rational computational design of ALP-MIP (a selective solid phase for ALP) using ab initio mechanical quantum calculations was developed. Subsequently, the ALP-MIP was synthesized and used to develop a highly selective and economic MISPE procedure for the extraction of ALP from human plasma prior to HPLC analysis. Finally HPLC analysis was performed without any prior sample pretreatments.

2. Material and methods

2.1. Chemicals

Allopurinol, oxypurinol, ethylene glycol dimethacrylate (EGDMA), 2,2′-azobis (isobutyronitrile) (AIBN), acrylamide (AAM), methacrylic acid (MAA), and acrylonitrile (ACN) were purchased from Sigma–Aldrich (Spain). All other chemicals used were of analytical or HPLC grade and used without further purification. Britton-Robinson buffer was prepared by mixing 0.02 M CH_3COOH, 0.02 M H_3PO_4 and 0.02 M H_3BO_3 and then the pH of the solution was adjusted to 7 by adding 0.2 M NaOH. Phosphate buffer was prepared by titrating 1 M H_3PO_4 with 1 M NaOH and the final pH was adjusted at 4.

2.2. Instruments

HPLC was performed on a computer-controlled chromatography system (Waters Assoc., Milford, MA, USA) consisting of a Waters 717auto-sampler, a Waters 501pump, a Waters 486 variable wavelength UV detector, and an automated chromatographic data acquisition system (Millenium32, Version 2.15.01). pH measurements were performed with a model 780 Metrohm (Switzerland) pH meter using a combined glass electrode. A Windaus two-channel peristaltic pump model D-38678 was used to pump solvents during the MISPE experiments. A Honle 100 UV lamp (intensity 0.157 W cm^{-2}) (Honle UV, UK) was used for polymerization process. The variable wavelength UV-visible detector was operated at 254 nm for allopurinol determination.

2.3. Computational design of polymer for selective extraction of allopurinol

In order to comprehend the properties of MIP at molecular level, Gaussian 98 package (Gaussian, Inc., Pittsburgh, PA, US) was used. The software package has several pertinent capabilities including easy software workflow and accurate predictions. The software can predict energies, molecular structures, and vibrational frequencies, and then model them in both their ground state and excited states. Moreover, it can perform many other electronic structure calculations. The computational method used was as follows: first, a virtual library including of seven functional monomers i.e. acrylamide (AAM), acrylic acid (AA), acrylonitrile (ACN), p-vinyl benzoic acid (P-VBA), 4-vinyl pyridine (4-VP), methacrylic acid (MAA) and methylmethacrylate (MMA) was prepared. Then, the conformations of ALP and the functional monomers were optimized and the energy of the molecules in the optimized conformations was calculated. Subsequently, the conformation optimization and energy calculation were applied to the complexes formed between ALP and each the functional monomers. All calculations were carried out using Gaussian 98 software based on the application of Hartree–Fock (HF) method with 6-31G(d) basis set. Finally, the binding energies of ALP with the monomers (ΔE_b) were obtained using Eq. (1):

$$\Delta E_b = E_{t-m} - E_t - \Sigma E_m \quad (1)$$

Where E_{t-m} is the interaction energy between the template and the functional monomer, E_t is the energy level of the template and $\sum E_m$ is the accumulative energy level of the functional monomers.

While the above computations are based on vacuum state, it is well documented that the polymerization process done in presence of a solvent can compete with a functional monomer changing the computational energy and the stability of the template–monomer complexes. Therefore, for a rational and accurate design, the effect of the polymerization solvent must be included as well. Generally, to include the effect of the polymerization solvent, continuous models such as polarizable continuum model are used [30–32]. These models consider the solvent as a uniform polarizable medium using its dielectric constant (ε). Moreover, in these methods, the solute is placed in a suitably shaped cavity in the medium [33]. The solvent energy (ΔE_s) was therefore calculated through Eq. (2) as follows:

$$\Delta E_s = E_{t-m} - \Delta E_{t-m-s} \quad (2)$$

Where E_{t-m-s} is the interaction energy between the template and the monomers in presence of the solvent. Therefore, the corrected binding energy ($C\Delta E_b$) is determined as follows (Eq. (3)):

$$C\Delta E_b = \Delta E_b - \Delta E_s \quad (3)$$

Beside the error caused by the presence of the solvent, the basis set superposition error (BSSE) [34] is also a major problem in obtaining the accurate binding energies of hydrogen-bonded complexes. The error is developed as two molecules approach each other, and the energy of the system falls not only because of the favorable intermolecular interactions but also because of the basis functions on each molecule provide a better description of the electronic structure around the other molecule. The solution to this obstacle is counterpoise (CP) correction [35] which is an approximate way of assessing BSSE for the accurate computation of molecular interaction energies by ab initio methods [36]. The above-mentioned errors i.e. ΔE_s and the BSSE were taken into consideration when the computations were conducted using the Gaussian 98 package. Then the polymer was constructed according to results of the computational design.

2.4. Preparation of molecular imprinted polymers (MIP)

Selective MIPs for the extraction of ALP (ALP-MIP) were synthesized according to the computations results and using the precipitation polymerization method by initially mixing the template molecule (0.8 mmol) with the selected functional monomers (3.2 mmol) in a 20.0 ml screw capped glass vial followed by an addition of 15.0 ml of acetone as the polymerization solvent and the mixture was left in contact at room temperature for 5 min. The cross-linker EGDMA (12 mmol) and the initiator AIBN (0.24 mmol) were then added to the above solution. For the synthesis of the polymers, the oxygen was removed by purging the polymerization mixtures with nitrogen and the polymerization carried out by photochemical irradiation for 20 min using a Honle 100 UV lamp (intensity 0.157 W cm^{-2}) (Honle UV, UK). Finally, the template was removed by Soxhlet extraction with methanol:acetic acid (MeOH:HOAc) mixture (ratio 1:1) for 11 h. The remaining particles were dried at 50 °C and used for the following studies. Corresponding blank polymers were also prepared using the same procedure, but in the absence of the template molecule.

2.5. Characterization of ALP-MIP

In order to characterize the polymer constructed, the ALP adsorption test was conducted by using a batch method. Constructed polymer particles (50 mg) were incubated in 10.0 ml of the same polymerization solvent, i.e. acetone containing different concentrations of ALP and left for 12 h at room temperature. Then, the mixture was centrifuged (5000 rpm) and the amount of ALP bound to the constructed polymer (ALP-MIP) (known as B) was calculated by subtracting the amount of free drug (F) in the medium from its initial value. The binding parameters and characteristic of ALP-MIP were estimated by Langmuir–Freundlich (LF) isotherm.

2.6. MISPE procedure

A 40 mg quantity of each dry constructed polymer was packed into empty 3.0 ml C_{18}-SPE cartridges (Restek U.S., Bellefonte, PA). Prior to the percolation of each sample, cartridges were conditioned with 1.0 ml of acetonitrile. The plasma spiked with a known concentration of ALP was pretreated by 1 ml saturated solution of ammonium sulfate and centrifuged at 5000 rpm for 15 min to precipitate the large proteins. Then, 1.0 ml of the upper-phase was passed through the conditioned cartridges by a flow rate of 1.0 ml min^{-1}. Then, the cartridge was washed by 1.0 ml of Britton–Robinson buffer at pH 7 to remove the undesirable materials which were either chemically similar to the analyte or the analyte molecules adsorbed to nonspecific binding point of MIP. Finally, the analyte was eluted twice each time with 1 ml of 90/10 (v/v) methanol/acetic acid in the flow rate of 0.1 ml min^{-1}. The eluate was then evaporated to dryness at ambient temperature under a stream of nitrogen and dissolved in 100 μl of the HPLC mobile phase (MeOH/phosphate buffer pH 4.0, 50/50). Forty microliters of the solution was analyzed by HPLC to determine ALP.

3. Results and discussion

3.1. Theoretical study of template–monomer interactions

A crucial factor in achieving a successful synthesis of MIPs is rational selection of functional monomers, capable of interacting strongly with a given template. This selection process based on trial and error is basically very time-consuming. Therefore, in this study, molecular modelling based on Hartree–Fock (HF) method with 6-31G (d) basis set was used to identify the best materials for the molecular imprinting of ALP as a rapid, economic, and rational method. However, as mentioned earlier (see Section 2.3.), in order for the computational approach to be carried out accurately, binding energies should be corrected by taking two factors into consideration including the solvent effect and BSSE. The computational selection of functional monomers was carried out as follows: seven functional monomers, i.e. AAM, AA, ACN, P-VBA, 4-VP, MAA and MMA were theoretically selected as available functional monomers. First, the conformation of ALP and the functional monomers at the lowest energy was performed using HF computations with 6-31G(d) basis set and optimized. The optimized conformations are shown in Fig. 2. Then, the possible template–monomer interactions were investigated to obtain optimized geometries (in vacuum) for template–monomer complexes at the molar ratios of 1:1, 1:2 and 1:3.

As an example, Fig. 3 shows the optimized geometries of the complexes between ALP and AAM. According to the calculations of electronic energies, the highest stability was obtained for the template–monomer complexes at the mole ratio of 1:3. As discussed earlier, BSSE can influence the accuracy of binding energy, so conducting BSSE correction to obtain accurate results would be inevitable. Table 1 summarizes the calculated interaction energies (in vacuum) for all the complexes formed between ALP and the functional monomers before and after BSSE correction.

As clearly seen, BSSE changes the binding energy values. The sequence of the interaction energy (in vacuum) as well as the stability obtained for different monomers used was ΔE (AA) > ΔE (AAM) > ΔE (MAA) > ΔE (P-VBA) > ΔE (4-VP) > ΔE (ACN) > ΔE (MMA). However, in presence of a solvent acting as a competitor, this sequence was quite different and all the values decreased significantly (Table 2).

The data listed in Table 2 show that the stability of the complexes depended on the kind of solvent used. Based on these data, acetone was selected as the best polymerization solvent due to both, the high solubility of ALP in acetone and the results concluded from Table 2, that acetone resulted in the highest template–monomer interaction energies. Moreover, the ΔE sequence for different monomers indicated that AAM was the desirable functional monomer. To examine the accuracy of the theoretical calculations, several polymers were prepared by precipitation polymerization method using AAM (MIP1), AA (MIP2) and ACN (MIP3) as the functional monomers with high, medium and low interaction energy, respectively. In the experiments, EGDMA and acetone were used as cross-linker and polymerization solvent, respectively.

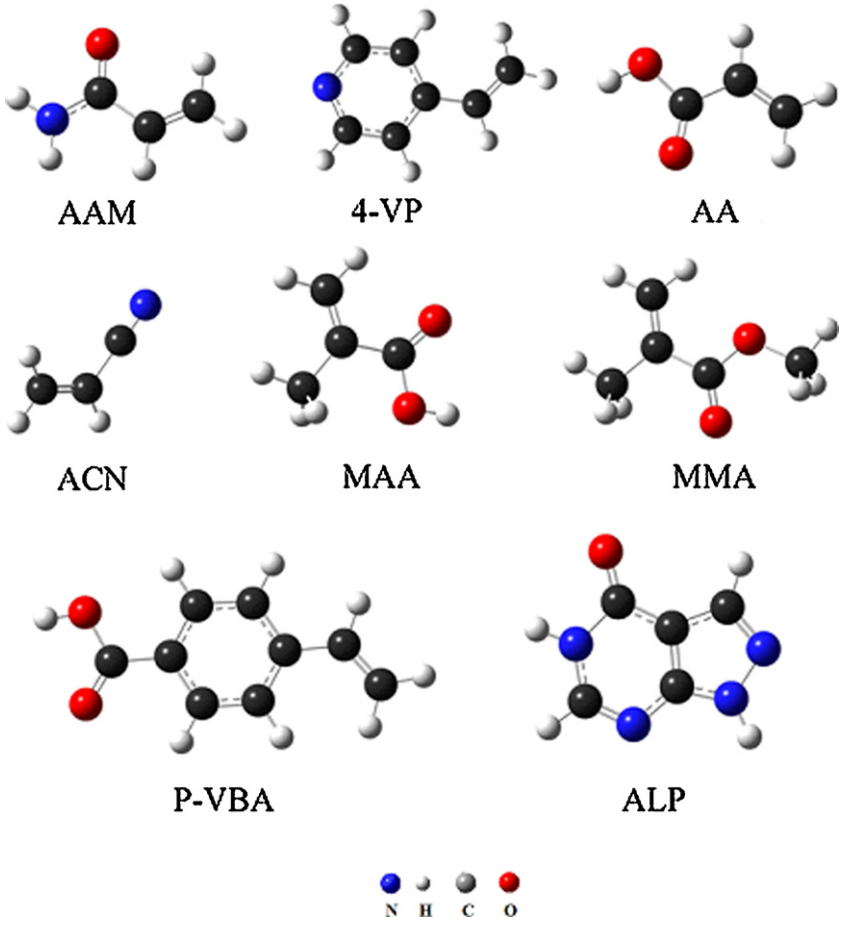

Fig. 2. ALP, AAM, ACN, MAA, MMA, AA, P-VBA and 4-VP in optimized conformations.

3.2. Characterization of MIP

To verify the results of the computational approach for MIP design, three MIPs were synthesized and their binding characteristics were evaluated by a heterogeneous LF isotherm [37]. The LF isotherm was a hybrid model of Langmuir and Freundlich isotherms describing a specific relationship between the equilibrium concentration of the bound (B) and free (F) guest with three fitting coefficients: N_t, a and m, where N_t is the total number of binding sites, a is related to the average binding affinity (K_0) such that

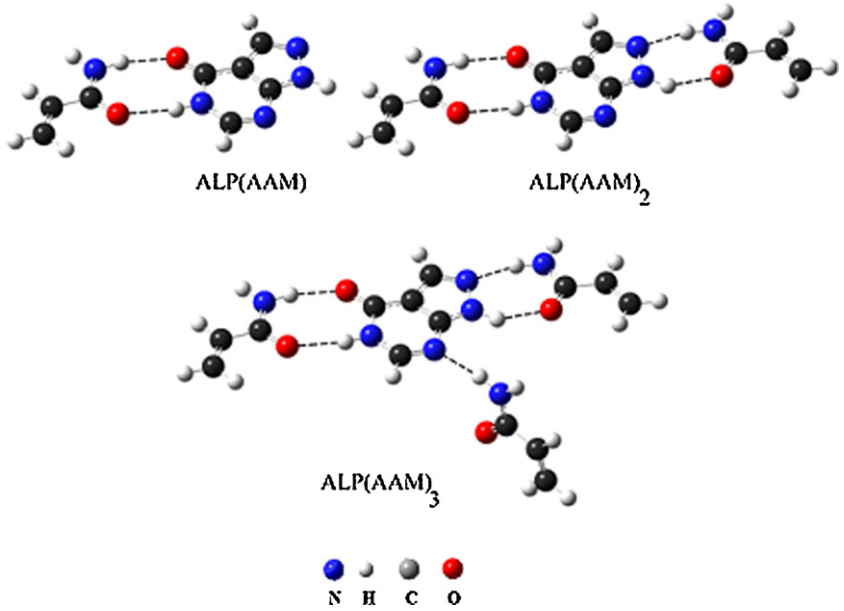

Fig. 3. The most stable 1:1, 1:2 and 1:3 complexes of allopurinol with AAM in optimized conformations originated by HF method with 6-31G(d) basis set.

Table 1
Interaction energies (ΔE, kJ mol^{-1}) of all template–monomer complexes before and after BSSE correction in vacuum.

Complexes	$\Delta E_{non\text{-}corr}$	ΔE_{corr}
ALP(AA)	−76.215	−63.291
ALP(AAM)	−72.356	−61.955
ALP(ACN)	−34.587	−22.359
ALP(MAA)	−71.299	−52.984
ALP(4-VP)	−45.249	−35.721
ALP(P-BA)	−66.225	−45.001
ALP(MMA)	−38.958	−23.691
$ALP(AA)_2$	−139.025	−124.556
$ALP(AAM)_2$	−135.659	−123.950
$ALP(ACN)_2$	−54.598	−48.059
$ALP(MAA)_2$	−126.895	−105.593
$ALP(4\text{-}VP)_2$	−83.564	−58.042
$ALP(P\text{-}BA)_2$	−106.66 8	−89.098
$ALP(MMA)_2$	−52.359	−41.346
$ALP(AA)_3$	−171.998	−146.98
$ALP(AAM)_3$	−167.254	−136.449
$ALP(ACN)_3$	−69.985	−52.521
$ALP(MAA)_3$	−152.398	−122.449
$ALP(4\text{-}VP)_3$	−103.559	−70.012
$ALP(P\text{-}BA)_3$	−129.774	−102.622
$ALP(MMA)_3$	−62.698	−47.546

Table 2
Interaction energies (ΔE, KJ mol^{-1}) of 1:3 template–monomer complexes in different solvents.

Complexes	Acetone	MeCN	MeOH	DMSO
$ALP(AA)_3$	−64.98	−59.65	−52.62	−50.26
$ALP(AAM)_3$	−83.12	−78.52	−69.89	−67.23
$ALP(ACN)_3$	−35.39	−36.23	−33.69	−32.09
$ALP(MAA)_3$	−71.68	−68.03	−63.57	−62.81
$ALP(MMA)_3$	−42.15	−43.11	−40.25	−38.92
$ALP(4\text{-}VP)_3$	−49.07	−45.11	−44.08	−41.09
$ALP(P\text{-}BA)_3$	−58.23	−54.44	−48.36	−43.00

Table 3
Binding parameters obtained for LF fit to the experimental adsorption isotherms.

Polymer	N_t (μmol g^{-1})	a (mM^{-1})	m	K_0 (mM)	R^2
MIP1	98.23	63	0.92	90.32	0.997
MIP2	83.71	42	0.86	77.18	0.995
MIP3	65.98	27	0.89	40.58	0.993

($K_0 = a^{1/m}$), and m is the heterogeneity index which is equal to 1 for a homogeneous material, and ranges between 0 and 1 based on the heterogeneity of the material used.

$$B = \frac{N_t a F^m}{1 + a F^m} \quad (4)$$

In order to evaluate the N_t, K_0, and m values, the experimental isotherm data (F and B) were fitted to the Fig. 4 shows LF isotherm for MIP1. The resulted fitting coefficients at the desired concentration ranges are listed in Table 3. The m value demonstrates the heterogeneity of the MIPs. As concluded, MIP1 showed the highest degree of binding site with the highest heterogeneity index ($m = 0.92$). The comparison of other binding parameters, i.e. N_t and K_0 also confirmed that MIP1 was the most suitable polymer to separate ALP.

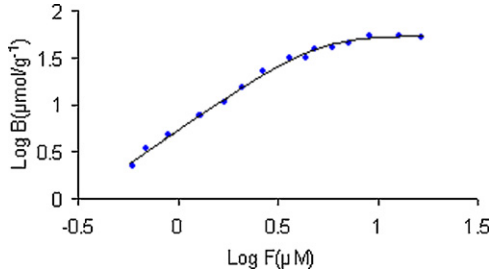

Fig. 4. Experimental isotherm (dot) and LF fit (line) for MIP1.

Table 4
K_d and IF of allopurinol for MIPs and corresponding NIPs.

Polymer	K_d		IF
	MIP	NIP	
MIP1	57.28	20.52	2.79
MIP2	32.98	17.37	1.90
MIP3	25.49	16.91	1.51

3.3. Specific affinity of the polymers for the template; ALP

Generally, two factors are important for an effective recognition of a template by an MIP: the strength and quantity of the interactions between the monomers in the polymer network and the template. The MIP cavities created after the removal of the template are complementary to the imprint molecules in size and coordination geometries. This leads to the MIP's much greater affinity for the template molecules in comparison with non-imprinted adsorbents. Therefore, to prove the existence of the sites (cavities) on a constructed MIP surface or their absence on the corresponding NIP surface, two factors, i.e. distribution ratio and imprinted factor, should be considered. To achieve that, several batch experiments were conducted by separately incubating 10 mg of each MIP and the corresponding NIP particles with 10 ml ALP solution (25 μg ml^{-1}) for 12 h. Then, the distribution ratio (K_d) was calculated based on the Eq. (5):

$$K_d = \frac{(C_i - C_f)V}{C_f m} \quad (5)$$

Where V, C_i, C_f and m represent the volume of the solution (ml), drug concentration before and after adsorption (μg ml^{-1}) and mass of the polymer, respectively. Finally, the imprinting factor (IF) was calculated to evaluate the imprinting effect [38] defined as follows (Eq. (6)):

$$\mathrm{IF} = \frac{K_d(\text{imprinted})}{K_d(\text{non-imprinted})} \quad (6)$$

The obtained results are shown in Table 4. All the synthesized MIPs showed an imprinting effect and were able to rebind to the template and not to the corresponding NIP. Moreover, the results presented in Table 4 indicated that the MIP constructed by using AAM as functional monomer resulted in the maximum IF while the MIP based on ACN resulted in the minimum IF. These findings clearly confirmed the unique shape of the ALP molecule which played an important role its recognition. From the computational and binding experiments data, the MIP1 based on AAM was identified as the desirable imprinted material.

3.4. Optimization of MISPE procedure

3.4.1. Effect of pH

In the SPE method, pH optimization is of great importance to achieve a satisfactory extraction. The occurrence of suitable interactions between the absorbent and target molecules in aqueous media is dependent on the medium's pH. Therefore, the effect of pH on the binding behavior of ALP was investigated using a Britton–Robinson buffer over the pH range of 2.0–11.0 (Fig. 5). The binding behavior of ALP was not greatly affected at pH > 7.0. However, at more acidic pHs, ALP's recovery decreased considerably. This was due to the protonation of the ALP and the consequent break-down of the hydrogen bonds at the pH < 7.0. These charged

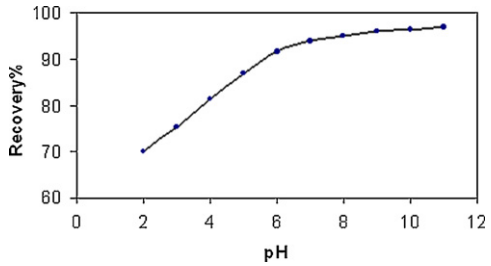

Fig. 5. Effect of pH on the recovery of allopurinol from MIP1 cartridge.

molecules cannot locate themselves in the binding sites of the polymer resulting in their reduced recovery. Finally, as the recovery rate of ALP leveled off at around the pH 9.0, therefore, this pH value was selected for subsequent MISPE experiments.

3.4.2. Washing solvent selection

To develop a successful MISPE procedure, a washing step is necessary to remove non-specific or interfering adsorption on the MIP [36]. So, in order to select the best washing solvent, 1 ml of several solvents such as acetone, methanol, ethanol, acetonitrile and Britton–Robinson buffer, were tested to wash the cartridges. Among the solvents used, the maximum difference between MIP's and NIP's recovery was obtained for Britton–Robinson buffer at pH 7.0. The recovery of ALP from MIP and NIP was 97.8% and 4.6%, respectively (Fig. 6). In other words, because of a good recovery and a high degree of specific retention, Britton–Robinson buffer at pH 7.0 was selected as the washing solvent in this study.

3.4.3. Elution solvent selection

Non-covalent interactions, e.g. hydrogen bonds, are responsible for retaining target molecule on MIP. These bonds can be destroyed by highly polar solvents. To find the most appropriate elution solvent, different solvents and their binary mixtures i.e. MeOH, MeCN, (3% acetic acid + MeOH), (6% acetic acid + MeOH) and (10% acetic acid + MeOH) were tested. The best recoveries were obtained using methanol containing 10% acetic acid (Table 5). Therefore, 2 ml × 1 ml of 90/10 (v/v) methanol/acetic acid was used as the elution solution during the study.

3.5. Selectivity test

In order to evaluate the overall selectivity of the synthesized MIP, several substrates with H-bonding ability such as oxypurinol, acyclovir, acetaminophen, zonisamide, and hydrochlorothiazide were tested in presence of ALP (Fig. 7). As MIP1 was found as the most efficient absorber for ALP, therefore, to assess its selectivity, several batch experiments were conducted and the distribution coefficient (K_D) as well as the selectivity coefficients (α) related to each substrate were calculated and are listed in the Table 6. These higher K_D obtained for ALP strongly confirmed a higher selectivity of MIP1 for this substrate and demonstrated the possibility of utilizing MIP1 as a selective adsorbent for the extraction of ALP from complex matrixes such as biological fluids.

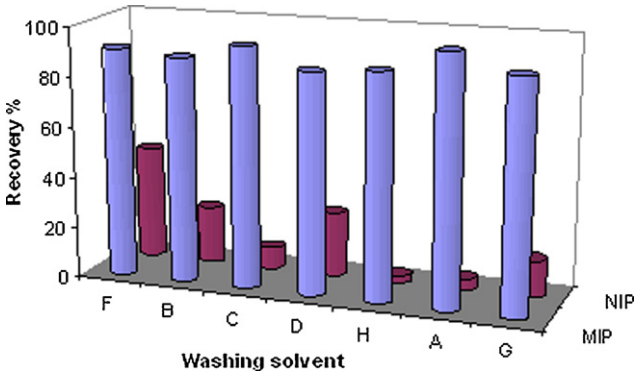

Fig. 6. Allopurinol recoveries percentage on MIP and NIP using 1.0 ml of various washing solvents i.e. (A) acetone, (B) MeCN, (C) MeOH, (D) ethanol, (E) 50/50 (v/v) MeCN/water (F) Britton–Robinson buffer at pH 3, (G) Britton–Robinson buffer at pH 7 (H) Britton–Robinson buffer at pH 9, respectively.

Table 5
Effect of various eluents on recovery of allopurinol from MIP1 cartridge.

Eluent	First time %	Second time %	Total recovery %
MeCN	40.9	25.7	66.6
MeOH	50.5	32.3	82.8
3% acetic acid in MeOH	74.8	18.3	93.1
6% acetic acid in MeOH	93.3	4.9	98.2
10% acetic acid in MeOH	97.2	2.4	99.6

Table 6
K_D and α[a] values for MIP1 and the corresponding NIP.

Substrate	MIP		NIP	
	K_D	α[a]	K_D	α
Allopurinol	81.25		21.28	
Oxypurinol	50.14	1.62	23.91	0.89
Acyclovir	25.78	3.15	22.52	0.94
Acetaminophen	18.95	4.29	19.65	1.08
Zonisamide	22.31	3.64	24.26	0.88
Hydrochlorothiazide	27.56	2.95	26.93	0.79

[a] $\alpha = K_D$ (Allopurinol)/K_D (substrate).

3.6. Method validation

Once the MISPE-HPLC procedure was developed, the applicability of the method was evaluated by determining its performance characteristics regarding linearity, limit of detection (LOD), limit of quantification (LOQ), repeatability, and precision. The chromatograms of the MISPE-HPLC are shown in Fig. 8. The method validation was conducted by establishing ten-point calibration curves for MIP1 and its corresponding NIP. A good linearity was found in the ALP concentration range of 0.100–25.000 μM with a linear regression coefficient (R^2) of 0.995 for the MIP1 while the corresponding NIP did not exhibit the same linear relation and instead, a flat response to the increasing mass of ALP loaded was obtained. As defined by European Pharmacopoeia [39], the LOQ and LOD are the concentrations at which the signal-to-noise ratio is 3 and 10, respectively. The LOD and LOQ values were measured at 0.028 and 0.093 μM, respectively.

Finally, the extraction yield, accuracy and precision of the developed MISPE-HPLC procedure were tested at three different ALP concentration levels within the calibration range (0.2, 5.0 and 20.0 μM) corresponding to the lowest, middle and highest point of the calibration curve. Inter and intra-assay precisions and accuracies are presented in Table 7. It was revealed that the recoveries obtained by this method were satisfactory and the LOD and LOQ values were sufficient for ALP determination in human plasma. Also

Table 7
Precision and accuracy of the developed MISPE-HPLC method ($n = 6$).

Concentration (M)	Accuracy (%)	Precision (RSD %)	
		Intra-day	Inter-day
10^{-7}	91.5	6.02	7.63
10^{-6}	94.8	5.23	6.69
5×10^{-5}	95.6	4.89	6.18

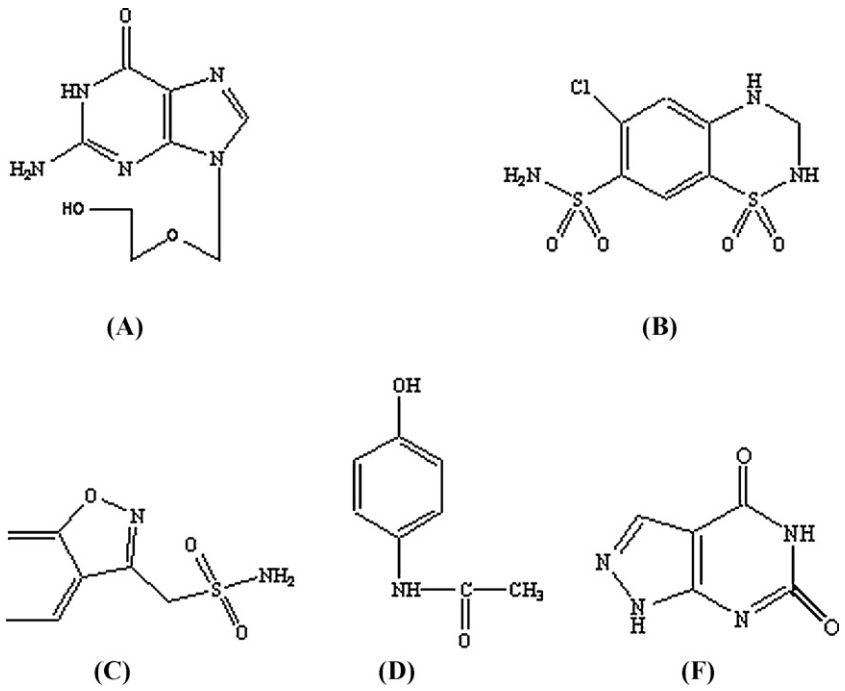

Fig. 7. Chemical structures of: (A) acyclovir (B) hydrochlorothiazide (C) zonisamide, (D) acetaminophen, (F) oxypurinol.

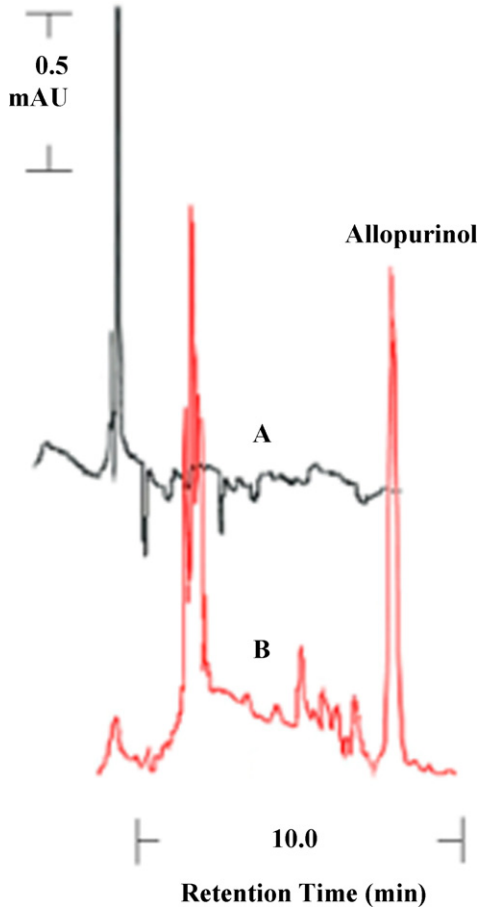

Fig. 8. MISPE-HPLC chromatograms of: (A) blank plasma (B) plasma spiked with allopurinol (10^{-5} M).

the values of inter and intra assay precisions are well within the 10% limits required for the assay to be validated.

4. Conclusion

The results obtained in this study demonstrated the possibility of using computer aided design as an efficient tool to seek suitable functional monomers for a specified template molecule. The computational method was applied to study the intermolecular interactions in the pre-polymerization mixture and to find a suitable functional monomer in MIP preparation. It was found that ALP interacts more strongly with AAM in comparison with the other studied functional monomers. The MIP constructed demonstrated a high selectivity to the ALP rather than the other tested substrates which confirmed the applicability of the constructed MIP for human plasma. Moreover, Langmuir–Freundlich isotherm considerations proved that the MIP prepared by AAM was the best candidate for SPE applications. Finally, when this polymer was utilized in the MISPE approach for selective separation of ALP from human plasma, a good analytical performance in terms of sensitivity, reproducibility and accuracy for quantification of ALP was obtained.

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