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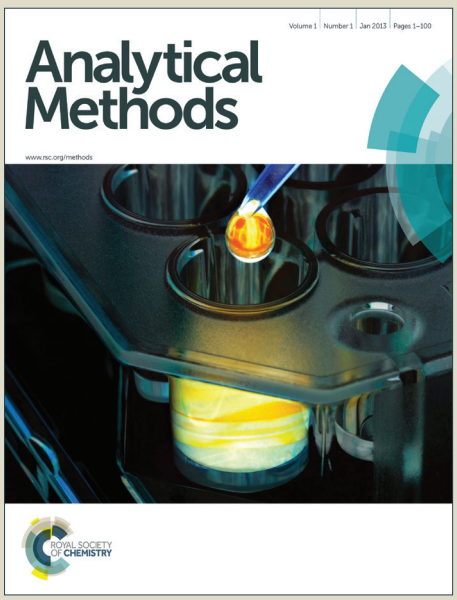

ROYAL SOCIETY OF CHEMISTRY

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Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Molecular modelling and synthesis of polymer for the extraction of amiloride and triamterene from human urine

Iuna Tsyrulneva,*[a] Olga Zaporozhets,[a] Elena Piletska[b] and Sergey Piletsky[b]

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

A new solid phase extraction resin with high affinity towards amiloride and triamterene was developed with the assistance of computational modelling. Itaconic acid was selected as the best functional monomer capable of forming strong complexes with amiloride and triamterene. Conditions for selective solid phase extraction of the diuretics from human urine and for their separation using custom-designed adsorbent were optimised. It was shown that this adsorbent provided high level of purification of the diuretics from human urine, which made possible their quantification using spectrophotometry.

1. Introduction

Amiloride (3,5-diamino-6-chloro-N-(diaminomethylene)-pyrazine-2-carboxamide) and triamterene (6-phenylpteridine-2,4,7-triamine) (Figure 1) are potassium-sparing diuretics, commonly used for the treatment of hypertension and congestive heart failure. The mechanism of these diuretics action involves blocking the epithelial sodium channel and inhibiting sodium reabsorption (1). This promotes increasing of the amount of salt and water removed from kidneys and thus, decreases blood volume. The main characteristic of these diuretics is that even in small doses these drugs cause high-volume urine excretion decreasing further their concentration and making difficult to identify and quantify them. Thus, highly sensitive methods are required for the determination of these diuretics.

Amiloride and triamterene are usually used in conjunction with thiazide or loop diuretics as it helps to correct potassium imbalances caused by these drugs. Due to its potassium-sparing properties amiloride and triamterene can cause hyperkalemia and acidosis. Wrong prescription and excessive abuse of these diuretics can lead to negative side effects. Besides, the presence of amiloride and triamterene in urine is a proof of doping consumption (2). International Olympic Committee and World Anti-Doping Agency (WADA) established the requirement for anti-doping laboratories to control the presence of these compounds in urine at the level less or equal to minimum required performance limit, which is 0.2 μg mL^{-1} (3).

The widely used methods for the determination of amiloride and triamterene are high performance liquid and gas chromatography with mass-spectroscopic detection. These methods provide the necessary selectivity and sensitivity (4-7). The other methods for determination of triamterene and amiloride include spectrophotometry and fluorimetry (8-14). However, direct analysis of urine samples is complicated because complex urine matrix interferes with the determination of analytes. Thus, preliminary liquid-liquid or solid phase extraction (SPE) is necessary. Besides, only few articles propose selective determination of amiloride in the presence of triamterene and their separation (11, 12). Simultaneous determination of amiloride and triamterene is complicated due to their similar molecular structures and chemical properties.

Commercial adsorbents (nylon membranes, C18 discs, etc.) do not always provide necessary selectivity towards the amiloride and triamterene. Besides, in most cases the reduction of the matrix effect has only been achieved by 100x dilution of urine, which also leads to the loss of sensitivity. In recent years, new technique for selective pre-concentration of samples based on the application of molecularly imprinted polymers (MIPs), was developed as an alternative to the use of generic adsorbents. MIPs are widely used for extraction and purification of a number of biological and chemical substances: e.g. drugs, amino acids, pollutants, etc. (15-20). The application of MIPs for extraction of drugs from human and calf urine has already been studied (21, 22). Due to their selectivity and recognition properties MIPs are often called ‘synthetic receptors’. Compared to natural receptors, MIPs are characterised by higher physical stability, strength, resistance to high temperatures and pressure, and inertness towards acids, bases, ion metals and organic solvents. However, the important complication caused by the use of MIPs in analytical application is possible leaching of the template molecule from the cross-linked polymer. One of the common approaches to avoid this problem involves the imprinting of the template analogue (23). Nevertheless, it requires a chemical modification of the target, and might lead to reduced affinity and specificity of the polymeric material. A promising approach, “virtual imprinting”, was proposed by Breton et al. (24). In this case polymer composition is designed using computational methods but polymer is made in the absence of the template.

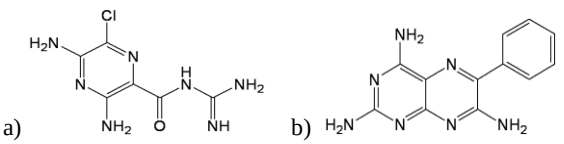

Fig. 1 Structures of amiloride (pK_a=8.7) (a) and triamterene (pK_a=6.2) (b).

The apparent specificity in this case originates from the careful selection of appropriate monomers and from random creation of affinity binding sites in the polymer.

Design of the polymers with predicted properties for the extraction of diuretics from aqueous solutions and biological liquids hasn't attracted much attention. Recently polymers for extraction of furosemide (25, 26), bumetanide (26) and hydrochlorthiazide (27, 28) were proposed.

The aim of the work was to design a new sorbent with predicted high affinity towards amiloride and triamterene and to develop simple, sensitive and inexpensive method of SPE of these diuretics from aqueous solutions and human urine.

2. Materials and methods

2.1. Ethics statement

Research comprising study of urine effect was conducted in Ukraine. Urine samples were provided by 5 laboratory employees, who gave their verbal informed consent to have the samples used for research purposes. All samples were treated anonymously.

2.2. Materials

Standard solutions of 6 diuretic drugs (amiloride, bendroflumethiazide, bumetanide, chlorthiazide, furosemide, triamterene) (SIGMA, St. Louis, MO, USA) were prepared by dissolving 1 mg of the corresponding diuretic in 10 mL of methanol (MeOH). Methanol stock solutions of diuretics were stored in a tightly closed container in a cool and dry place. Working solutions were obtained by taking an appropriate volume of standard solution, evaporating MeOH in a nitrogen stream and diluting it with water. Standard aqueous solution was freshly prepared daily.

Itaconic acid (IA), ethyleneglycol dimethacrylate (EGDMA), 2,2-azobis(isobutyronitrile) (AIBN), dimethylformamide (DMF), HPLC grade MeOH and acetonitrile (MeCN) were purchased from Merck (Darmstadt, Germany). All chemicals were used without further purification. Sodium phosphate dibasic, sodium phosphate monobasic, sodium carbonate, sodium hydrogen carbonate, formic acid (FA) and hydrochloric acid (HCl) (all Merck, Darmstadt, Germany) were of analytical reagent grade.

Negative urine samples were obtained from healthy volunteers who did not consume studied diuretics. Samples were stored in polypropylene bottles at a temperature of -20 ºC. Before conducting the experiment samples were defrozen and centrifuged.

2.3. Instrumentation

High performance liquid chromatography (HPLC) was performed on Agilent1200 liquid chromatograph system equipped with fluorescent detector (FD). The column was kept in column oven at the temperature 25 ºC. The chromatographic separation was performed using a Luna 5 µm C18(2) (150 x 4.6 mm) column (Phenomenex, USA). Instrumental parameters of chromatographic determination were following: flow rate - 0.5 mL min^{-1}, volume of sample injected - 20 µL, mobile phase – 70/30 (v/v) MeOH/water. The detection of amiloride was performed at an excitation wavelength of 285 nm and emission wavelength of 420 nm, of triamterene - at an excitation wavelength of 360 nm and emission wavelength of 440 nm.

Spectrophotometric measurements were performed on spectrophotometer SpectroQuest-2800 UV-vis spectrophotometer (Unico, USA). The calibration curves for amiloride and triamterene were built at an absorbance wavelength of 360 nm and 370 nm, respectively. pH measurements were performed using pH-meter PH150MI (IT, Russia) equipped with a glass electrode. The SPE process was assisted by a peristaltic pump Mityflex 913 (Anko, USA).

2.4. Molecular modelling

Molecular modeling was undertaken using a workstation from Research Machines running the CentOS 5 GNU/Linux operating system, configured with a 3.2GHz core 2 duo processor, 4 GB memory and running the SYBYL 7.3 software suite (Tripos Inc., St. Louis, Missouri, USA). The LEAPFROG algorithm was applied to screen the library of functional monomers for their possible interactions with the template, resulting in a table ranking the monomers according to their binding scores (in kcal mol^{-1}). The library contained 20 functional monomers which are commonly used in molecular imprinting and possessed polymerisable residues and residues able to interact with a template through ionic and hydrogen bonds, van der Waals' and dipole–dipole interactions (29). The charges for each atom were calculated using Powell method in combination with Gasteiger-Huckel charges and Tripos Force Field. The same method was applied to refine the structures of the monomers using the gradient minimisation method (the minimisation is stopped when energy gradient is lower than 0.001 kcal mol^{-1}). The functional monomer which demonstrated the highest binding energy towards the amiloride and triamterene was selected for the polymer preparation.

2.5. Polymer synthesis

General procedure for preparation of custom-designed polymers was following: 6.5 g of monomer IA and 26.0 g of cross-linker EGDMA were dissolved in DMF. The initiator AIBN (0.33 g, 1% of total solid weight of monomer and cross-linker) was then added. The solutions were deoxygenated by purging with nitrogen for 10 min and then thermally polymerised in oil bath at 80 ºC for 20 hours. The resultant polymer monolith was crushed, ground using Ultracentrifuge Mill (Retsch, UK) and sieved. The particle size fraction 45-125 µM was collected. Fine particles were removed by repeated sedimentation in MeOH twice and left to dry for 2 hours. 1-mL SPE tubes were packed with 25, 50 and 100 mg of polymer and used for the optimisation of SPE protocol and for the measurement of the polymer's capacity.

2.6. Evaluation of binding

Aqueous solutions of amiloride and triamterene (1 mL, 2 µg mL^{-1}) were added to 50 mg of polymer particles. Samples were shaken in water bath for 6 hours and centrifuged for 10 min at 3000 rpm. The concentration of free analyte was determined using spectrophotometer and HPLC-FD set-up. The amount of amiloride and triamterene bound to the polymer was calculated by subtracting the equilibrium amount of analyte from the initial amount of analysed diuretic.

2.7. Optimisation of the SPE protocol in aqueous solutions

In order to find the most favorable pH range for the adsorption of amiloride and triamterene, the following experiment was conducted: SPE cartridges were packed with 50 mg of polymer and loaded with 1 mL of amiloride or triamterene (2 μg mL^{-1}) in 0.1 M phosphate buffer, pH 5.0, 6.0 and 7.0, which corresponds to the pH range of normal human urine (5.5-7.0). The adsorption of amiloride and triamterene was evaluated using spectrophotometer.

The elution of amiloride and triamterene from the polymer was optimised. MeCN, MeOH, DMF, MeOH acidified with 0.1 M HCl or 5% FA and MeCN acidified with 5% FA, were tested.

In order to evaluate the effectiveness of a regeneration procedure the cartridge was loaded and washed as it was described above. Regeneration of the cartridge was performed by washing the polymer with MeOH with subsequent treatment with the mixture of MeOH and 0.1 M HCl in order to remove any traces of diuretics. The cartridges were reconditioned using MeOH and water.

50-mg amounts of the polymer were packed into empty 1-mL cartridges between two polyethylene frits. For the determination of amiloride the cartridges were conditioned with 2 mL of MeOH and 2 mL of 0.1 M phosphate buffer solution, pH 6.0. 1-mL aliquots of amiloride solutions in 0.1 M phosphate buffer solution, pH 6.0, of known concentrations (10-500 ng mL^{-1}) were filtered through polymer cartridges.

For the determination of triamterene the cartridges were conditioned with 2 mL of MeOH and 2 mL of 0.1 M phosphate buffer solution, pH 4.5. 1-mL aliquots of triamterene solutions in 0.1 M phosphate buffer solution, pH 4.5, of known concentrations (2-200 ng mL^{-1}) were filtered through polymer cartridges.

The cartridge was washed with 2 mL of MeOH. Amiloride or triamterene were eluted using 1 mL of MeOH/HCl 90/10 (v/v). Fractions were collected, dried under a nitrogen stream, dissolved in 100 μL of mobile phase (MeOH/H_2O 70/30) and analysed using HPLC-FD set-up. Every fraction was analysed by spectrophotometer without any additional pre-treatment and compared to blank solutions (elution solvent without drugs).

2.8. Testing specificity using human urine

Human urine was diluted in 2 times by phosphate buffer with pH 4.5 or 6.0 and spiked with known concentration (10-200 and 25-500 ng mL^{-1}) of triamterene or amiloride, respectively. Each cartridge containing 50 mg of IA-based polymer was treated in a different way for amiloride and triamterene.

For SPE of amiloride polymer cartridge was conditioned with 2 mL of MeOH and 2 mL of 0.1 M phosphate buffer solution pH 6.0. Then, 1 mL of spiked urine was filtered through polymer. Cleaning step included washing with 2 mL of the mixture of MeOH with water (1:1), 1 mL of 0.1 M HCl, 2 mL of MeOH and with 1 mL of 0.05 M carbonate buffer, pH 10.0. Amiloride was eluted by 1-mL aliquot of MeOH acidified with 0.1 M hydrochloric acid (10%, v/v). Every fraction was analysed using spectrophotometer and compared with blank solution.

For SPE of triamterene polymer cartridge was conditioned with 2 mL of MeOH and 2 mL of 0.1 M phosphate buffer solution pH 4.5. Then, 1 mL of spiked urine was filtered through polymer. Cleaning step included washing with 2 mL of 50% MeOH, 1 mL of 0.1 M HCl, 2 mL of MeOH and with 1 mL of phosphate buffer, pH 7.5. Triamterene was eluted by 1-mL aliquot of MeOH acidified with 0.1 M hydrochloric acid (10%, v/v). Every fraction was analysed by UV-vis spectrophotometer and compared with blank solution.

2.9. Testing specificity using different groups of diuretics

Several diuretics, such as furosemide, bumetanide, hydrochlorothiazide, chlorthiazide and bendroflumethiazide, were tested in order to estimate the selectivity of the designed polymer. 1 mL of analyte solution containing 0.5 μg of amiloride or triamterene and 0.5, 5.0 or 50 μg of each functional analogue was filtered through cartridges. The cartridges were washed with 2 mL of MeOH. Amiloride or triamterene were eluted with 1 mL of MeOH acidified with 0.1 M HCl (10%, v/v). Fractions were analysed using UV-vis spectrophotometer.

3. Results and Discussion

3.1. Study of interactions between monomer and templates

The selection of functional monomer is very important because it provides the basis for the strong interactions and therefore, desirable high binding. The computational modelling was performed for protonated amiloride and triamterene. The charged forms of monomers were used in the virtual screening in order to generate the molecular complexes with highest binding. Accordingly to the molecular modelling study, IA (Figure 2) was found as the best monomer for the imprinting of amiloride and triamterene because it demonstrated the highest binding energy, when compared to other monomers (Tables 1, 2). It would be expected that under acidic conditions IA, which possesses two carboxyl groups, will demonstrate strong ionic interactions with positively charged diuretic molecules (Figure 3).

It was found that under acidic conditions carboxyl group of IA is an excellent hydrogen bond donor and acceptor. As it can be seen from Figure 2, at pH≤2.8 IA molecule is neutral and does not form complexes with positively charged amiloride and triamterene. At pH≥5.5 negatively charged dianion of IA interacts with charged amine groups of triamterene and amiloride by electrostatic mechanism of binding and thus, forming stable complexes.

The synthesis of custom-designed polymer was performed by free-radical polymerisation of IA (20%, w/w) in the presence of large excess of cross-linker (80%, w/w), as it was described in the Experimental section.

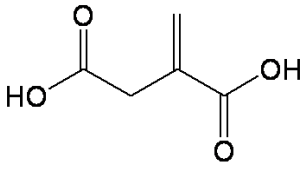

Fig. 2 Structure of IA (pK_a=3.85, 5.45).

Table 1 Binding energies (kcal mol^{-1}) of the molecular complexes between amiloride and functional monomers

Monomer	Binding energy, kcal mol^{-1}
Itaconic acid	-43.1
2-Acrylamido-2-methyl-1-propanesulfonic acid	-41.2
Urocanic acid	-39.7
Trifluoroacetic acid	-37.1
1-vinylimidazole	-32.6

3.2. Optimisation of SPE conditions

Different solvents were tested in order to optimise loading, washing and elution conditions. It was observed that the type of interaction between templates and monomer depended on the nature of the medium. In MeOH electrostatic interactions between amino group of the diuretics and carboxylic groups of IA are reduced by interaction with polar solvent molecules. It is known that electrostatic interactions play the main role in the formation of molecular complexes in aprotic solvent. Hydrophobic interactions in aqueous solutions contribute to the non-specific interactions.

It was found that water influenced the increase of hydrophobic interactions between template and the polymer. As a result the polymer demonstrated high affinity towards amiloride and triamterene with a 99% recovery in aqueous solutions. Thus, water was selected as a loading solvent.

Buffer solutions of amiloride and triamterene at pH 5.0, 6.0 and 7.0 were prepared in order to estimate the adsorption of the corresponding diuretics. It was observed that both analytes were completely adsorbed under the specified conditions. Their retention at pH 5.0 and 6.0 was caused by formation of complexes diuretic-monomer involving electrostatic forces and weak non-specific interactions. At pH 7.0 neutral form of triamterene contributed to non-specific retention by forming hydrophobic bonds.

It was considered that polar properties of MeOH will prevent formation of hydrogen bonds between analyte and polymer matrix. Therefore, MeOH was chosen as a washing solvent due to its ability to decrease effectively the strength of hydrophobic bonds and eliminate non-specific interactions.

Figure 4 demonstrates that MeOH containing 10% hydrochloric acid is an optimal solvent for elution of amiloride and triamterene. It appeared, that in acidic medium negatively charged IA transforms in a neutral molecule. Inability of methanol acidified with 5% formic acid to cause the break of bonds can be the evidence of strong binding between template and monomer. Other tested solvents, like MeCN and DMF, were not suitable as eluents because of low solubility of diuretics.

The amount of the polymer per cartridge was also optimised. The recoveries of diuretics from cartridges packed with 25, 50 and 100 mg of polymer were tested. The highest recovery was observed for cartridges packed with 50-100 mg. The recovery of diuretics at 25 mg of polymer was less than 60%. Quantity of the polymer equal to 50 mg was chosen for the practical extraction of the target analytes from solution. In our experiments 500 mL of solution spiked with 2 μg of amiloride or triamterene were filtered through polymeric adsorbent. Polymer was washed with

Table 2 Binding energies (kcal mol^{-1}) of the molecular complexes between triamterene and functional monomers

Monomer	Binding energy, kcal mol^{-1}
Itaconic acid	-36.0
Acrylamide	-35.2
Methacrylic acid	-31.3
N,N'-methylene-bis-acrylamide	-28.8
Urocanic acid	-26.8

MeOH and target analytes were eluted with 1 mL of MeOH acidified by 0.1 M HCl (10% v/v). High binding capacity of the polymer (up to 40 g kg^{-1}) allowed the 500-times pre-concentrating the diuretics from aqueous solutions. The possibility of re-using cartridges packed with IA polymer was assessed in the experiments in optimising washing and elution protocols. It was observed that no degradation of the polymer was observed after 30 cycles of regeneration and re-use.

So, based on described above experiments, the optimal protocol for selective and accurate SPE of amiloride and triamterene from aqueous solutions was following. The cartridge was conditioned with 0.1 M phosphate buffer solution, pH 6.0 and 4.5 for determination of amiloride or triamterene, respectively. 1-mL aliquots of amiloride or triamterene in 0.1 M phosphate buffer solution pH 6.0 and 4.5, respectively, were filtered through polymer cartridges. The cartridge was washed with 2 mL of MeOH. Amiloride or triamterene were eluted using 1 mL of MeOH acidified with 0.1 M HCl (10% v/v).

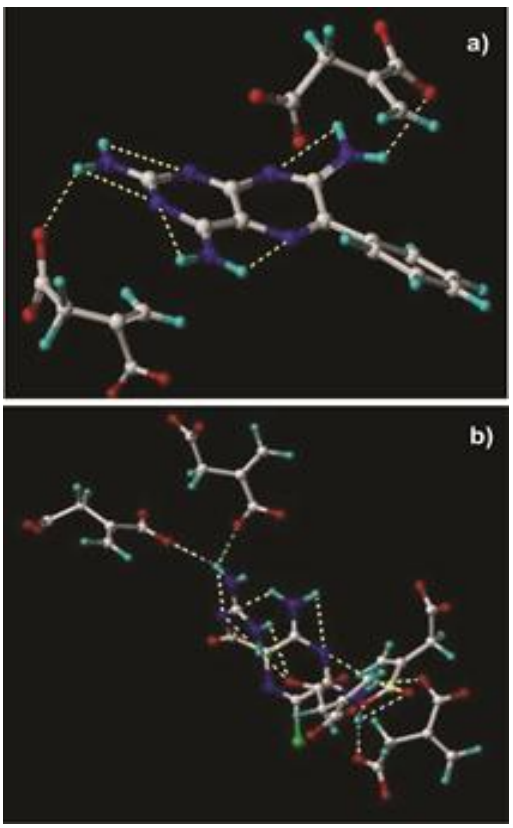

Fig. 3 Molecular complexes between triamterene (a) and amiloride (b) and IA.

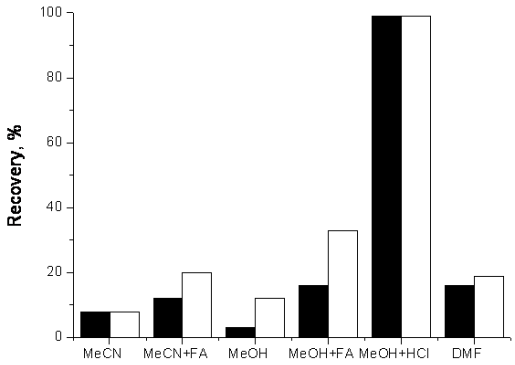

Fig. 4 Selection of solvent for elution of amiloride (■) and triamterene (□).

3.3. Evaluation of SPE for urine samples

The possibility to apply custom-designed adsorbent for the recovery of amiloride and triamterene from human urine samples was tested. These diuretics are supposed to form ion associates with kynurenine, xanthurenic and folic acids (30) which results in disability to detect them without preliminary clean-up of the matrix.

In order to remove the interfering components of the urine matrix, washing of the polymers with acids, MeCN, MeOH and their mixtures with hydrochloric and formic acids was performed. Aqueous MeOH was effective in the removal of salts, very polar components of urine and urobilin. It was found that the strength of 5% formic acid was not sufficient to break the bonds between IA and interfering substances. At the same time, 0.1 M HCl was effective in neutralising all organic acids. It was observed that MeCN demonstrated no recovery of diuretics and slight recovery of urine components, which can be the evidence of its inability to dissolve these substances. MeOH demonstrated high level of recovery of urine components. So, combined treatment with HCl and MeOH gave the possibility to elute components of urine, presumably derivatives of isoindole, which are characterised with strong absorbance intensity in the area where diuretics absorb light and allowed to clean-up urine matrix. Since in the buffer molecules of triamterene and amiloride were uncharged, it allowed them to be bound by IA-based polymer mostly by hydrophobic forces. It was only possible to remove the target analytes by using the mixture of MeOH with HCl.

Table 3 The results of spectrophotometric detection of amiloride and triamterene after SPE from human urine (n=3).

Diuretic	Added, ng mL^{-1}	Found, ng mL^{-1}	Recovery, %
Amiloride	25.0	24.9±0.1	99.4
	50.0	51.0±0.3	102.0
	100.0	98.9±0.1	98.9
	250.0	250.0±0.6	100.0
	500.0	495±1	99.0
Triamterene	10.0	10.10±0.05	101.0
	20.0	19.9±0.1	99.4
	50.0	49.5±0.1	99.0
	100.0	100.2±0.1	100.2
	200.0	198.0±0.2	99.0

Designed polymer was characterised by high recoveries of diuretics (Table 3). Apparently, the weak interactions between the components of urine matrix and diuretics could be destroyed by combined treatment with HCl and MeOH.

The results presented in Table 3 demonstrate that under optimal conditions quantitative recovery and elution of amiloride and triamterene were achieved. It was also established that preliminary clean-up of the matrix was effective as it allowed removing completely the interference with the components of urine, which greatly compromised the determination of amiloride and triamterene.

3.4. Testing the specificity using different groups of diuretics

Acidic diuretics (furosemide and bumetanide) and thiazide diuretics (bendroflumethiazide, chlorthiazide and hydrochlorothiazide) (Figure 5) were chosen for the evaluation of the selectivity of adsorbent in SPE experiments. It was found that the interfering substances were extracted from aqueous solutions during the loading step. It is known that under optimal conditions, which were used in the experiment (pH 4.5 and 6.0), thiazides are neutral and acidic diuretics are negatively charged. Their extraction by negatively charged polymer can be explained by involvement of mostly hydrophobic and, partially, electrostatic forces. It was expected that MeOH will be effective solvent for the removal of non-specifically bound compounds. In contrast, under these conditions the analytes of interest (triamterene and amiloride) are positively charged. They are bound with charged carboxylic groups of IA mostly by electrostatic forces. Thus, target analytes can only be eluted from the polymer using acidified MeOH, which allows their separation from the interfering compounds and, therefore, their purification.

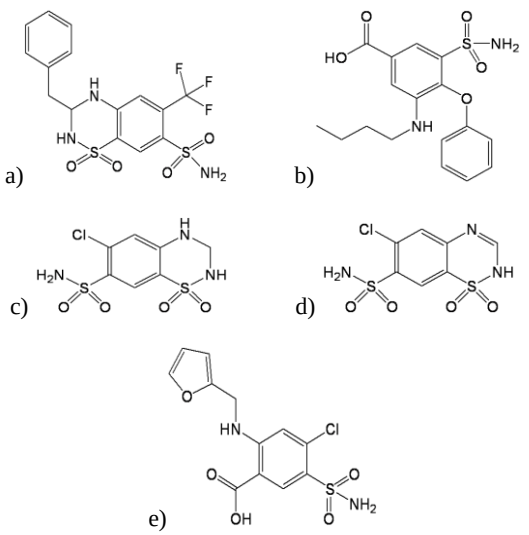

Fig. 5 Structures of bendroflumethiazide (pK_a=8.5) (a), bumetanide (pK_a=3.6) (b), hydrochlorothiazide (pK_a=7.9, 9.2) (c), chlorthiazide (pK_a=6.7, 9.5) (d) and furosemide (pK_a=3.9) (e).

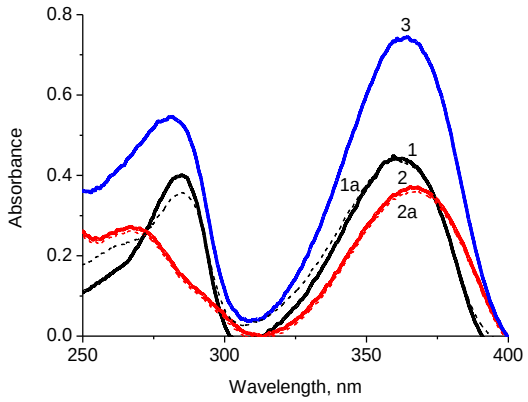

Fig. 6 Absorbance spectra of standard solutions of triamterene (1, black solid line) and amiloride (2, red solid line); their mixture (3, blue solid line); triamterene, which was eluted with MeOH (1a, black dotted line); amiloride, which was eluted with MeOH/HCl (2a, red dotted line).

3.5. Testing of the aqueous solution contained both diuretics

It was shown that selective elution of amiloride and triamterene from polymer surface could be achieved, which is based on the difference in their chemical properties. At pH 7.5 molecule of amiloride is charged, while under the same conditions triamterene is neutral.

The protocol of separation and quantification of each individual diuretic involved conditioning of the polymer with MeOH and 0.1 M phosphate buffer pH 7.5, and filtration of aqueous solution spiked with amiloride and triamterene through adsorbent, followed by washing with 0.1 M phosphate buffer, pH 7.5, in order to obtain required difference in the charges of the tested diuretics and quantitative elution of triamterene with MeOH (Figure 6). Amiloride is eluted by MeOH acidified with 0.1 M HCl (Figure 6).

Conclusions

The results of present study demonstrate that the developed polymeric adsorbent is able to perform a specific recovery of amiloride and triamterene from human urine. Computational approach was applied to identify optimal monomer which provides the highest binding energy and is effective in developing high affinity cavities for the target analytes. Application of the polymer in solid-phase extraction of amiloride and triamterene allows rapid recovery of the analytes from human urine samples, which can be quantified by spectrophotometric detection. It was demonstrated that the designed adsorbent is suitable for rapid, reproducible simultaneous pre-concentration and clean-up of human urine samples. IA-based polymer represents a significant improvement in the extraction of amiloride and triamterene from human urine compared with widely used nylon membranes and octadecyl discs. Besides, a simple method for the separation of amiloride and triameterene was proposed. Proposed simple technique can be a good alternative to the procedures currently used in clinical analysis.

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[a]*Taras Shevchenko National University of Kyiv, 62A Volodymyrska St., Kyiv, Ukraine 01033.* iuna.tsyrulneva@gmail.com. *+380679452988;*
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